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Title: Neonatal Airway Immune Profiles and Asthma and Allergy Endpoints in Childhood

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Abstract (250 words)

Background: The immune system plays a key role in the pathogenesis of asthma and allergy, but the role of the airway cytokine and chemokine composition *in vivo* in early life prior to symptom development has not been described previously. Here, we aimed to examine whether the neonatal airway immune composition associates with development of allergy and asthma in childhood.

Methods: We measured unstimulated levels of 20 immune-mediators related to the Type 1, Type 2, Type 17, or regulatory immune pathways in the airway mucosal lining fluid of 620 one-month-old healthy neonates from the COPSAC₂₀₁₀ birth cohort. Allergy and asthma were diagnosed at our research clinic by predefined algorithms and objective assessments at age 6 years. Principal component analyses were used to describe the airway cytokine and chemokine composition.

Results: A neonatal airway immune profile particularly characterized by enhanced IL-1 β and reduced CCL26 was significantly associated with later development of elevated specific-IgE to inhaled allergens, a positive skin prick test and allergic rhinitis, but not to food sensitization. Conversely, reduced Type 17 immune-associated markers, including IL-1 β and CXCL8, showed trend of association with development of early asthma endpoints.

Conclusions: Development of early asthma endpoints and inhalant allergy during the first 6 years of life seems associated with distinctly perturbed airway immune responses in neonatal life, which is suggestive of an early origin and different pathogenesis of childhood asthma and allergy. These exploratory findings suggest pre- and perinatal life as an important window of opportunity for prevention of asthma and inhalant allergy.

Keywords: Cytokines; chemokines; children; mucosal lining fluid; asthma; allergy; cohort

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

Introduction

Childhood asthma and allergy are thought to arise in a complex interplay between the genetic make-up of the child and early pre- and postnatal environmental exposures. The immune system matures in perinatal life¹ and exposures in this period are important for the trajectories towards respiratory health or disease². Studies of stimulated and unstimulated cord blood have shown that a specific immune mediator composition characterized by enhanced Type 2 immune mediators is evident from birth in children who later develop allergic endpoints^{3,4}, whereas children developing asthma showed enhanced Type 17 immune mediators in stimulated cord blood⁵. However, these studies are based on cord blood analyses, and little is currently known about the immune response in the airways in early life in relation to disease development afflicting the airways, such as allergic sensitization to inhalant allergens and respiratory symptoms such as rhinitis or asthma during childhood.

We have previously shown that presence of pathogenic airway bacteria⁶ and common respiratory viruses⁷ in healthy neonates stimulate the airway immune response. Furthermore, we also showed that older siblings⁸ and maternal asthma and allergy⁹ alter the neonatal airway immune response, suggesting a priming during pregnancy or neonatal life.

The objective of this study was to examine whether the cytokine and chemokine profile *in vivo* in the neonatal airway epithelial lining fluid is associated with development of asthma and allergy later in childhood. To explore this, we measured unstimulated levels of 20 immune mediators related to Type 1, Type 2, Type 17 and regulatory type (Treg) immune responses in airway mucosal lining fluid from one-month-old neonates participating in the Copenhagen Prospective Studies on Asthma in Childhood₂₀₁₀ (COPSAC₂₀₁₀) mother-child cohort^{6,10}, and studied the association between these airway immune mediator profiles and asthma and allergy endpoints by age 6 years.

Methods

The COPSAC₂₀₁₀ cohort

COPSAC₂₀₁₀ is an ongoing population-based clinical prospective mother-child cohort with 700 children enrolled at oneweek of age during 2009-2010¹¹. All clinical assessments of the cohort were performed at the COPSAC research unit according to standard operating procedures¹¹, including sampling of airway mucosal lining fluid^{6,10}. A double-blind randomized controlled trial (DB-RCT) of high-dose vitamin D and long-chain polyunsaturated fatty acids (LCPUFA) in pregnancy was embedded in a factorial design in the COPSAC₂₀₁₀ study^{12,13}. Vitamin D but not LCPUFA influenced the neonatal airway cytokine and chemokine composition¹³.

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). Both parents gave their oral and written informed consent.

Measurements of airway cytokines and chemokines

Airway mucosal lining fluid was sampled at onemonth of age as previously described^{6,10,14}. In brief, strips of filter paper (Accuwik Ultra, Pall Life Sciences, Portsmouth, UK) were inserted bilaterally into the anterior part of the inferior turbinate of the nasal cavity. After 2 minutes of absorption, the filterpapers were removed and immediately frozen at -80°C.

The MesoScale Discovery multiplexed array system (MesoScale Discovery, Gaithersburg, MD, USA) was used for analyzing the samples for IL(interleukin)-12p70, CXCL10 (IP-10), interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), CCL4 (MIP-1 β), 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-1 β , CXCL8 (IL-8), transforming growth factor-beta1 (TGF- β 1), IL-10, and IL-2^{9,10}. The sensitivities for cytokines were ≤ 1 pg/mL and for chemokines 1 to 50 pg/mL depending on the chemokine assay¹⁰.

The 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 Treg responses^{10,15,16}.

Asthma and allergy endpoints

Specific-IgE (sIgE) levels against inhalant (birch, grass, mugwort, horse, dog, cat, D. pteronyssinus, D. farinae, Alternaria, Cladosporium) and food allergens (wheat, egg, milk, beef, rye, soy, pork, cod, peanut) were determined using ImmunoCAP (Thermo Fischer Scientific, Allerod, Denmark), defining sensitization as any sIgE ≥ 0.35 kUa/L¹¹.

Skin prick test (SPT) was conducted with inhalant (birch, grass, mugwort, horse, dog, cat, D. pteronyssinus, D. farinae, Alternaria, Cladosporium) and food allergens (wheat flour, ryeflour, soy, beef, pork, cod, peanut, pasteurized egg and fresh milk) using extracts from ALK-Abello, Horsholm, Denmark. Sensitization was defined as any wheal larger than 3 mm¹⁷.

Allergic rhinitis was diagnosed by the COPSAC pediatricians based on: 1) significant symptoms during the previous year of sneezing or runny or blocked nose in periods when the child did not have a cold, and 2) allergic sensitization towards relevant inhalant allergens^{10,18,19}.

Asthma was diagnosed based on a validated quantitative symptom algorithm requiring all of the following criteria^{20,21}: (1) 5 episodes of troublesome lung symptoms within 6 months, each lasting at least 3 consecutive days; (2) symptoms typical of asthma, including exercise-induced symptoms, prolonged nocturnal cough, and persistent cough outside common cold; (3) need for intermittent rescue use of inhaled β_2 -agonist; and (4) improvement on a 3-month course of inhaled corticosteroids and relapse upon ended treatment²⁰.

Asthma exacerbations were defined from either: 1) acute treatment with inhaled β_2 -agonist in a pediatric emergency room or during hospitalization, or 2) acute treatment with oral or high-dose inhaled corticosteroid prescribed by a pediatrician at the COPSAC research unit or in an emergency room or during hospitalization^{20,21}.

Covariates

Maternal asthma, allergy, or eczema (Yes/No), vitamin D intervention (vitamin D/placebo), siblings (children in the home at birth, Yes/No), pathogenic airway microbes (*Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and/or Picornavirus at age one month, Yes/No)^{6,7,22}.

Statistics

Immune mediator levels below the detection limit (zero values) were set to half of the lowest measured level of the respective cytokine in order to perform log-transformations without loss of data. The mediators were measured in two batches and data were subjected to correction 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. Furthermore, we scaled and centered the individual mediator levels based on the sum of all quantified mediators to obtain a profile of mediators being independent of the individual response level. This strategy was 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 mediators of interest.

We analyzed the airway cytokine and chemokine profile by applying a principal component analysis (PCA) to decompose the signal from the 20 immune 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. The association between the immune profile represented in PC1-4 and asthma endpoints were analyzed using cox proportional hazard regression of time to event. The associations between PC1-4 and the dichotomized endpoints sIgE, SPT and rhinitis by age 6 years were analyzed by logistic regression.

Analyses were done with R version 3.2.4²³, using the packages “survival”, “tidyverse”, “broom” and “ggplot2”.

Role of the funding source

The study sponsors had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Results

Baseline characteristics

A total of 620(89%) of the 700 neonates in COPSAC₂₀₁₀ were included in the current study, excluding 10 neonates enrolled before the method of sampling 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 measured in another laboratory for a pilot study, and 4 samples were lost during transport (**Figure E1**). The absolute levels, the limit of detection of the assay, and the frequency of non-detectable values for each cytokine and chemokine are shown in **Table E1**.

Baseline characteristics of the excluded children without airway immune data and the 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, $p=0.001$) (**Table E2**).

By age 6, 137 of 491 (39%) had any elevated sIgE and 36 of 520 (7%) had any positive SPT and 43 of 591 (7%) were diagnosed with allergic rhinitis. Asthma was diagnosed in 141 of 601 (26%) children before age 6 years and 49 (8%) experienced one or more asthma exacerbation. Among children with asthma and/or elevated sIgE and/or a positive SPT against inhalant allergens by age 6, only 11.3% had both asthma and aeroallergen sensitization (**Figure E2**).

We limited our immune mediator PCA to the first four PCs, which explained a total of 55.9% of the variation in the immune mediator data: PC1 explained 25.8%, PC2 11.5%, PC3 10.8% and PC4 7.8% of the variation. A heatmap of the cytokine and chemokine expression per individual child based on the PC1-4 is shown in **Figure E3**.

Neonatal airway cytokine and chemokine composition and allergy endpoints

Specific-IgE: Elevated sIgE to inhalant and food allergens at age 6 years was associated with the neonatal airway cytokine and chemokine profile represented by PC1: odds ratio adjusted for presence of pathogenic airway microbes, siblings, maternal asthma, allergy or eczema and the vitamin D RCT (aOR) 1.33 (1.04–1.71), $p=0.03$ (**Table 1**). Dividing the positive sIgE into inhalant and food allergens revealed that the association was driven by sIgE against inhalant allergens (aOR 1.33(1.01–1.77), $p=0.04$), whereas sIgE against food allergens had no significant association with the neonatal airway cytokine and chemokine profile (aOR 1.16(0.84–1.61), $p=0.38$).

The PCA biplot of PC1 vs. PC2 in **Figure 1** shows that children with any elevated sIgE against inhalant allergens at age 6 years were characterized by a neonatal airway cytokine and chemokine profile with elevated IL-17A and IL-1 β (Type 17 pathway), the acute phase cytokine TNF- α , and decreased TGF- β 1 (Treg pathway), and the chemokines CCL22, CCL13, CCL17, CCL26 and CCL11, attracting many different cell types, including Th2 cells and Tregs. These findings were supported by univariable analyses, where significantly increased levels of IL-17A, IL-1 β and a trend of increased TNF- α and significantly decreased levels of CCL22 and CCL26 were evident in neonates with later development of elevated sIgE against inhalant allergens by age 6 (**Table E3**).

Further, there was trend of association between the neonatal airway cytokine and chemokine profile represented by PC4 and any elevated sIgE at age 6 years: aOR 1.24 (0.99 – 1.54), $p=0.06$. This was strengthened when restricting the analyses to any elevated sIgE against inhaled allergens: aOR 1.39 (1.09 – 1.78), $p=0.01$. The PCA biplot of PC3 vs. PC4 in **Figure 2** shows that the latter finding was mainly driven by reduced CCL26 and CCL22, and elevated IL-12p70 (Type 1 pathway) and IL-13 (Type 2 pathway).

Skin prick test: A positive SPT at age 6 years was associated with the same airway cytokine and chemokine profiles as elevated sIgE: PC1, aOR 1.63 (1.03 – 2.66), $p=0.05$, and PC4, aOR 1.41 (0.94– 2.10), $p=0.09$ (**Table 1**, **Figures E4-5** and **Table E3**).

Allergic rhinitis: There was no significant association between the neonatal airway cytokine and chemokine profile described in the PCA analysis and allergic rhinitis at age 6 years (**Table 1**).

However, the univariable analyses outlined in **Table E3** showed a significant reduction of CCL26, and an up-regulation of IL-1 β among children developing allergic rhinitis.

Neonatal airway immunecomposition and asthma endpoints

Asthma: The airway cytokine and chemokine profile represented by PC4 showed a trend of association with an increased risk of developing asthma at age 0-6 years: aHR 1.37 (0.96–1.96), $p=0.08$ (**Table 1**). The biplot shows that this trend was driven by reduced IL-17A and IL-1 β (Type 17 pathway), and increased IL-4 (Type 2 pathway), IL-12p70, CXCL10 and IFN- γ (Type 1 pathway) (**Figure 3**).

Asthma exacerbations: The airway cytokine and chemokine profile in PC2 showed a trend of association with development of exacerbations: aHR 0.57 (0.31–1.04), $p=0.07$ (**Table 1**). The PCA biplot shows that this trend was mainly driven by a reduction of TNF- α , CXCL10, CCL4 (Type 1 pathway) and CXCL8, IL-1 β , IL-17A (Type 17 pathway) (**Figure 4**). The univariable analysis supported the findings by showing significantly reduced levels of TNF- α , IL-1 β and CXCL8 (**Table E4**), which are all involved in recruitment of neutrophils.

Post-hoc analyses: Stratifying the asthma cases into early transient asthma with debut at age 0-3 years and remission before age 6 vs. persistent asthma by age 6 years showed that the airway cytokine and chemokine profile represented by PC2 was significantly associated with development of early transient asthma (aHR 0.75 (0.58–0.96), $p=0.02$) and PC4 showed a trend of association with early transient asthma (aHR 1.23 (0.97–1.56), $p=0.09$). In contrast, there was no association between the airway cytokine and chemokine profile and persistent asthma (**Table E5**). Further, stratifying the asthma cases by presence of allergic sensitization showed trends of association between PC2 (aHR 0.63 (0.39–1.04), $p=0.07$) and PC4 (aHR 1.58 (0.98–2.54), $p=0.06$) and asthma without sensitization, whereas this was not apparent for asthma with sensitization, which was associated with the immune profile represented by PC3 (aHR 0.46 (0.23–0.92), $p=0.03$) (**Table E5**).

Discussion

Principle findings

This exploratory study showed that a distinct immune profile of reduced neonatal airway production of especially IL-1 β was associated with development of early transient asthma and showed a trend of association with asthma exacerbations. In contrast, a neonatal airway immune profile characterized by enhanced IL-1 β and reduced CCL26 was a common denominator for development of allergic sensitization to inhalant allergens and allergic rhinitis by age 6 years but showed no association with food sensitization. These exploratory findings of divergent trajectories towards development of allergy and asthma endpoints in early childhood are suggestive of different pathogeneses of asthma and allergy.

Strengths and limitations

It is a strength of the study that the cytokine and chemokine profile in the airway mucosal lining fluid was assessed *in vivo* without stimulation, and by a method which we^{6,10,14} and others^{24,25} have validated for collecting and measuring cytokines and chemokines. 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 rhinitis and asthma as well as first line of defense against airway exposures. It serves as an internal validation, that we found the local airway cytokine and chemokine response to be associated with inhalant allergens but not food allergens.

A priori we decided on a panel of 20 cytokines and chemokines to represent major pathways of the immune system; representing both innate and adaptive mediators involving activation of Type 1, Type 2, Type 17 and Treg responses. This provided a representation of mediators produced by the different airway epithelial and immune cells.

We performed a normalization of the cytokine and chemokine mediator levels by sample centering to account for high within sample collinearity, likely due to individual differences in fluidic excretion dynamics (i.e. amount of nasal mucus collected on the filter paper). This normalization procedure is likely more efficient in enhancing the likelihood of finding statistical significance on a population level but is also more prone for finding relative minimal likely biologically non-relevant findings on an individual level. After the normalization procedure, a multivariate PCA model was applied to the mediator data. Such model can be used for unraveling

the mediator to mediator covariance structure. Furthermore, the multivariate model reduces issues related to multiple testing with a consequently low discovery power raised by the number of immune mediators. Still, despite we found biologically plausible associations between the neonatal airway cytokine and chemokine composition represented by PC1-4 and the asthma and allergy endpoints, neither of these would survive multiple testing correction. Therefore, we cannot rule out the possibility that some associations were detected due to chance, which suggests the need for replication of these exploratory study findings in a separate cohort. Asthma was defined from an algorithm building on daily diary cards and acute visits to the research unit at the onset of respiratory symptoms as well as at half-yearly scheduled visits^{12,13}. All diagnosis and treatments were made by the research medical doctor with pediatric training to avoid the heterogeneity in the medical community. 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 assessment of allergic sensitization was done by using both sIgE levels and SPT as they are often at variance with each other¹⁷. In this study, the cytokine and chemokine responses associated with these endpoints were similar, again serving as an internal validation.

It is not possible to infer a causal relation from the observed correlations between early life airway cytokine and chemokine responses and later respiratory health. However, the prospective nature of the study implies either a shared origin of the early immune phenotype and the later disease, or a direct mechanism, but not reverse causality due to behavioral changes as a consequence of disease. Thus, it is most likely that our findings represent a causal link between early airway immune perturbations and later inhalant allergy and asthma endpoints, which by nature cannot be studied in a randomized trial.

Interpretation

This exploratory study is the first to show suggestive evidence of a different airway cytokine and chemokine profile in healthy neonates preceding development of inhalant allergy and asthma endpoints in early childhood. Children developing elevated sIgE against inhaled allergens, a positive SPT and allergic rhinitis by age 6 were all characterized by a neonatal airway cytokine and chemokine profile with enhanced production of the pro-inflammatory cytokine IL-1 β which is

involved in the Type 17 immune response and recruitment of neutrophils²⁶, and by reduced CCL26. The immune mediator CCL26 is a chemoattractant for CCR3-expressing cells, such as eosinophils, basophils, Th2 cells, and Th1 cells. Combined this may suggest an early involvement of airway neutrophils in programming of aeroallergen sensitization and rhinitis, which aligns with other studies showing neutrophils to participate in allergic sensitization pathways^{27,28}.

We observed opposing trends of airway cytokine and chemokine profiles in neonates, who developed early transient asthma and asthma exacerbations during childhood. Where development of inhalant allergy was associated with an early enhanced Type 17 airway immune response based on IL-17A and IL-1 β , a downregulation of the Type 17 pathway with reduced IL-17A, IL-1 β and CXCL8, which are all involved in neutrophilic recruitment²⁶, was associated with development of early transient asthma and showed a trend of association with asthma exacerbations. Moreover, the acute phase cytokine TNF- α was significantly reduced in children with later asthma exacerbations, while trending towards being increased in children with later allergy. The differences between the immune profiles associated with inhalant allergy vs. asthma endpoints concur with the fact that most asthma/wheeze during preschool age is non-atopic²⁹. The suggested difference in immune profiles between asthma and allergy endpoints in our study is supported by the stratified analyses showing that the association between the neonatal airway immune profile and asthma was driven by asthma cases without concurrent sensitization and by the small overlap between asthma and sensitization present in only 11.3%. Further, it is notable that we observed a trend of a reduced neutrophilic based cytokine and chemokine profile in neonates with increased propensity for asthma exacerbations, as neutrophilic inflammation seems to dominate in active asthma in pre-school children as opposed to eosinophilic inflammation later in life³⁰. However, this fits well with the stratified analyses showing association with early transient asthma but not with persistent asthma by age 6.

We have previously shown that early-life exposure to pathogenic airway bacteria and picornaviruses is associated with a general upregulation of the Type 17, 1 and 2 cytokine and chemokine responses at 4 weeks of age^{6,7}. Those findings are likely carried by the majority of children in the cohort not developing asthma in later life and may represent a “normal” or beneficial response. We also found that perturbations of bacterial colonization in the neonatal

airways are associated with later development of childhood asthma^{31,32} as well as a distinct topical airway immune mediator profile including reduced levels of IL-1 β and TNF- α , which was also independently associated with risk of asthma^{31,32}. Analyzing the same dataset, we now show suggestive evidence that reduced Type 17 immune mediators, including IL-1 β and CXCL8 relates to early transient asthma and asthma exacerbations. Together these findings build the hypothesis, that an intrinsic inability to mount an effective Type 17 immuneresponse, hampering clearance of bacterial infections, is a trace in neonatal life that associates with asthma endpoints in early childhood.

We have previously shown that pre- and perinatal exposures, such as vitamin D supplementation in pregnancy, siblings, pathogenic airway microbes, and maternal atopy are reflected in the neonatal airway cytokine and chemokine profile^{6-9,13}; i.e. the airway immune profile is a snapshot reflecting environmental exposures. Still, the current study suggests that even when adjusting for these exposures, the early life airway immune profile is differently associated with early asthma endpoints and allergic sensitization to aeroallergens and rhinitis later in childhood with plausible altered immune mediators suggestive of a pathophysiological role in the inception of disease. These findings suggest a stable immunophenotype in the neonatal airway and lend support to the hypothesis that childhood asthma and inhalant allergy origin in perinatal life³³, thereby suggesting pre- and perinatal life as an important window of opportunity for prevention of disease.

Conclusion

This exploratory study shows suggestive evidence of separate airway immune compositions in healthy neonates associated with development of early asthma endpoints and allergic sensitization to inhalant allergens and allergic rhinitis later in life. These findings suggest that the immune perturbation characterizing inhalant allergy and asthma are different and have their origin in perinatal life.

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Tables

Table 1: Associations between allergy and asthma endpoints and the neonatal airway immune composition analyzed by principal component 1-4 (PC1-4) from a principle component analysis of 20 immune mediator levels.

All analyses are adjusted for pathogenic airway microbes, siblings, maternal asthma, allergy or eczema and the vitamin D randomized controlled trial. Any elevated specific IgE (sIgE), positive skin prick test (SPT) and allergic rhinitis by age 6 years were analyzed using a logistic regression model generating odds ratios (OR), whereas asthma symptoms and asthma exacerbations were analyzed by cox proportional hazard regression of time-to-event generating hazard ratios (HR). P<0.05 is shown in bold.

End-point	PC1	PC2	PC3	PC4
sIgE ≥0.35 kUa/L	1.33 (1.04 – 1.71) p = 0.03	1.08 (0.87 – 1.40) p = 0.51	0.89 (0.73 – 1.10) p = 0.29	1.24 (0.99 – 1.54) p = 0.06
Positive SPT	1.63 (1.03 – 2.66) p = 0.05	1.04 (0.79 – 1.54) p = 0.85	0.87 (0.61 – 1.27) p = 0.45	1.41 (0.94 – 2.10) p = 0.09
Allergic rhinitis	1.27 (0.87 – 1.90) p = 0.23	1.13 (0.80 – 1.61) p = 0.48	0.79 (0.58 – 1.11) p = 0.16	1.28 (0.91 – 1.79) p = 0.16
Asthma	0.96 (0.65 – 1.43) p = 0.86	0.88 (0.62 – 1.26) p = 0.49	0.84 (0.59 – 1.18) p = 0.32	1.37 (0.96 – 1.96) p = 0.08
Asthma exacerbations	0.74 (0.38 – 1.42) p = 0.36	0.57 (0.31 – 1.04) p = 0.07	1.24 (0.67 – 2.31) p = 0.50	1.42 (0.79 – 2.54) p = 0.24

Figures

Figure 1: Biplot of the principal component analysis (PC1 vs. PC2) showing the distribution of the neonatal airway immune profiles of children with (blue circle) and without (orange circle) elevated specific-IgE ≥ 0.35 kUa/L against inhalant allergens at age 6 years. A significant difference is observed for PC1: $p=0.04$.

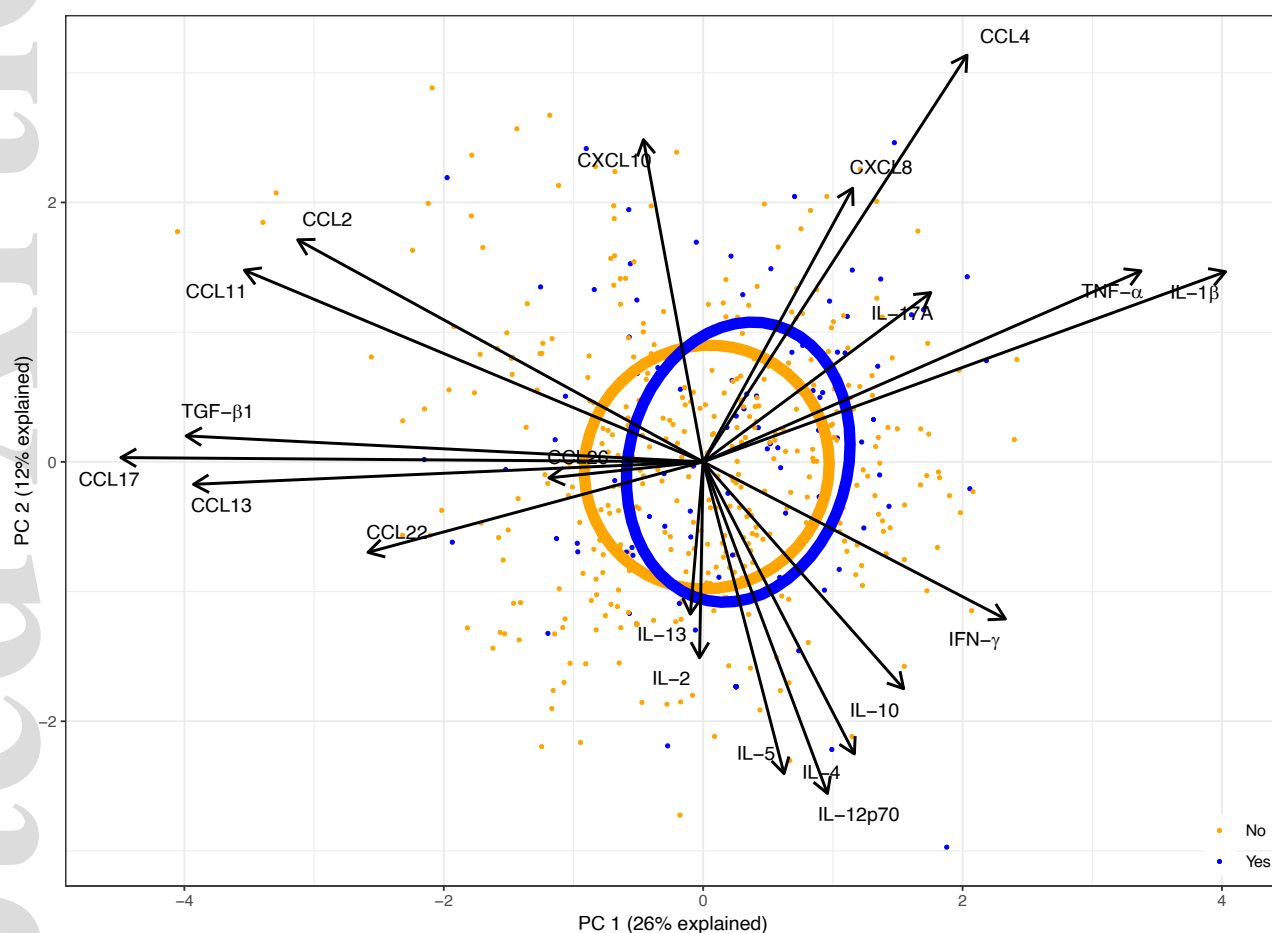


Figure 2: Biplot of the principal component analysis (PC3 vs. PC4) showing the distribution of the neonatal airway immune profiles of children with (blue circle) and without (orange circle) elevated specific-IgE ≥ 0.35 kUa/L against inhalant allergens at age 6 years. A significant difference is observed for PC4: $p=0.01$.

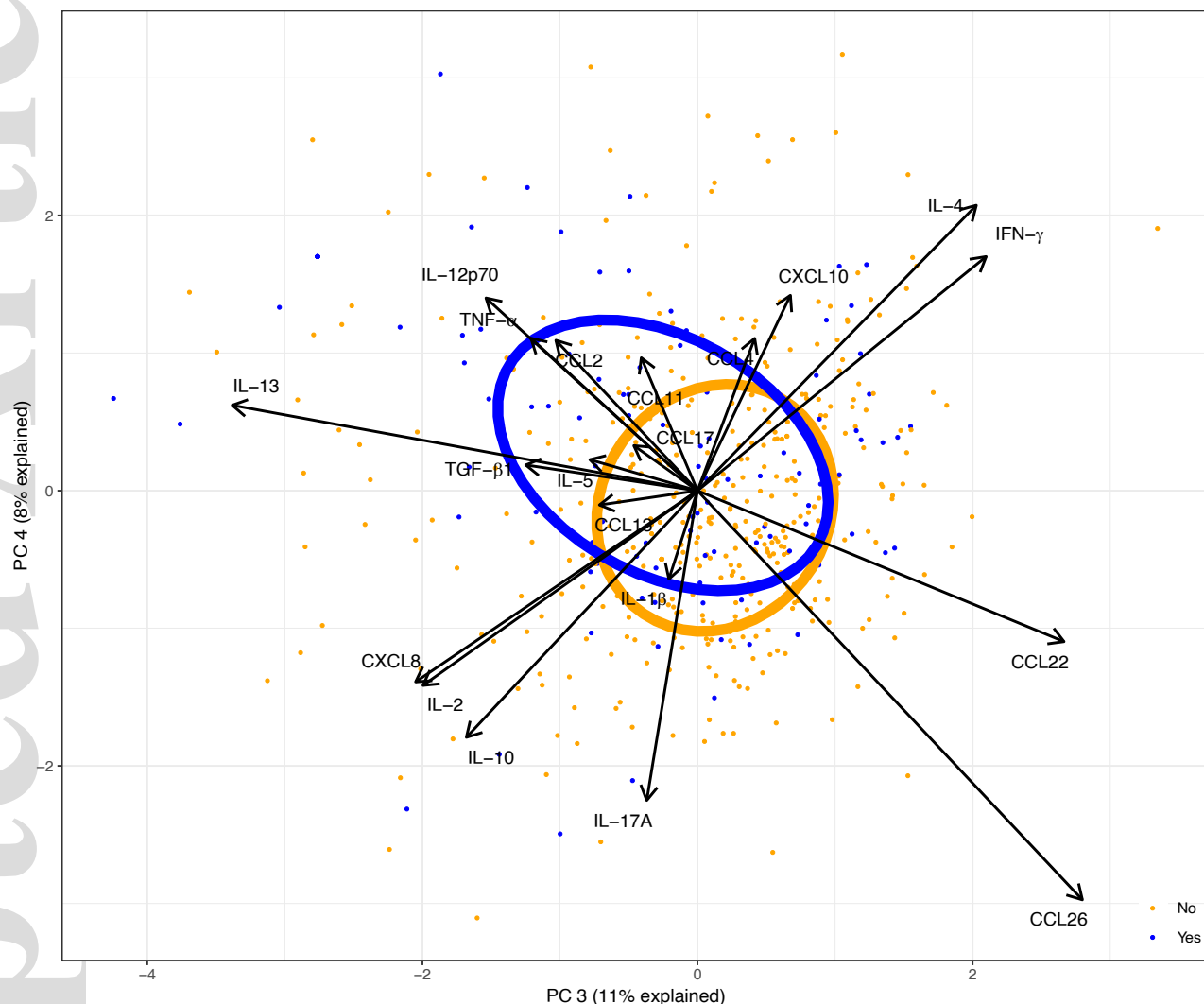


Figure 3: Biplot of the principal component analysis (PC3 vs. PC4) showing the distribution of the neonatal airway immune profiles of children with (blue circle) and without (orange circle) development of asthma through age 6 years. A trend of difference was observed for PC4: $p=0.08$.

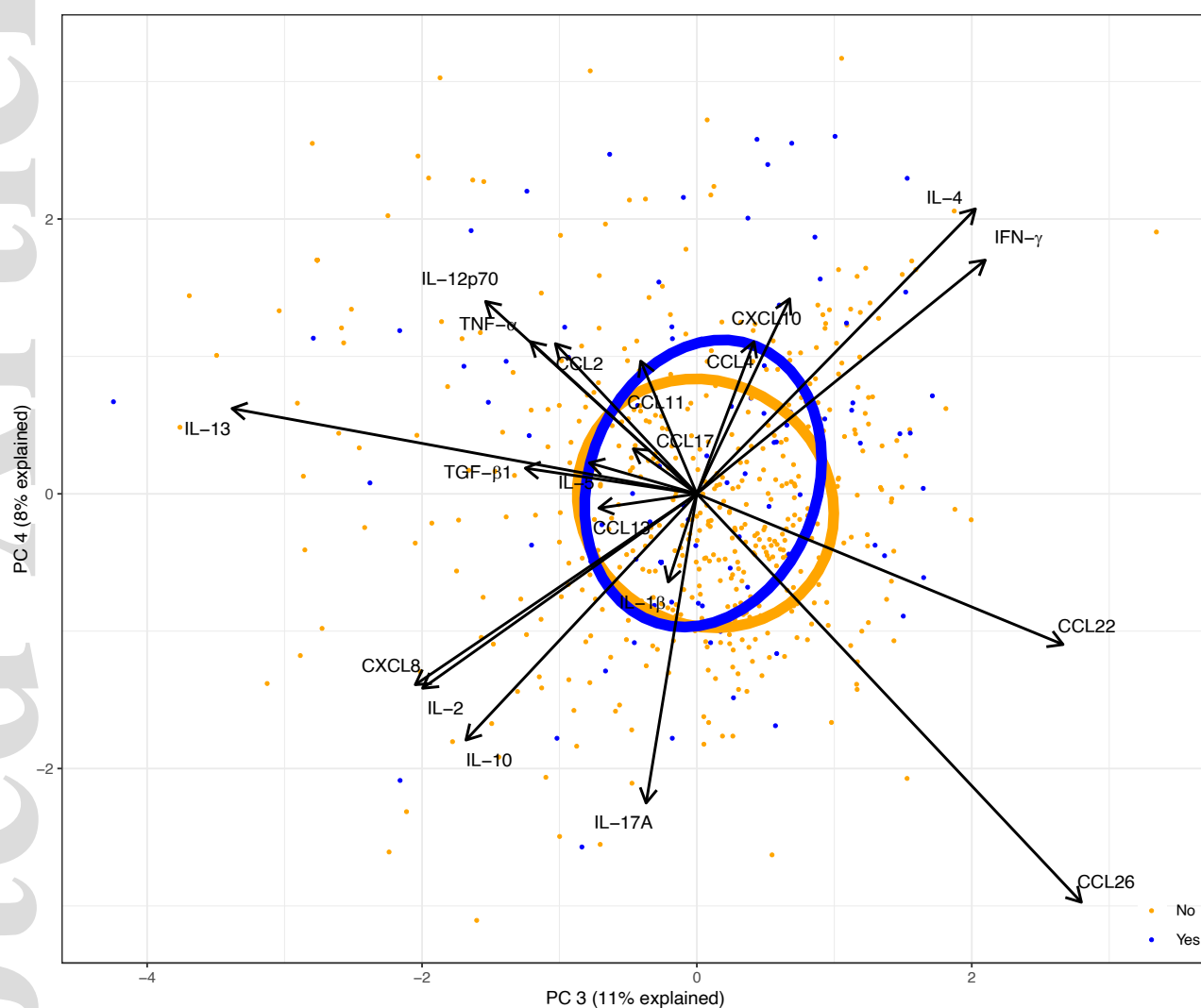
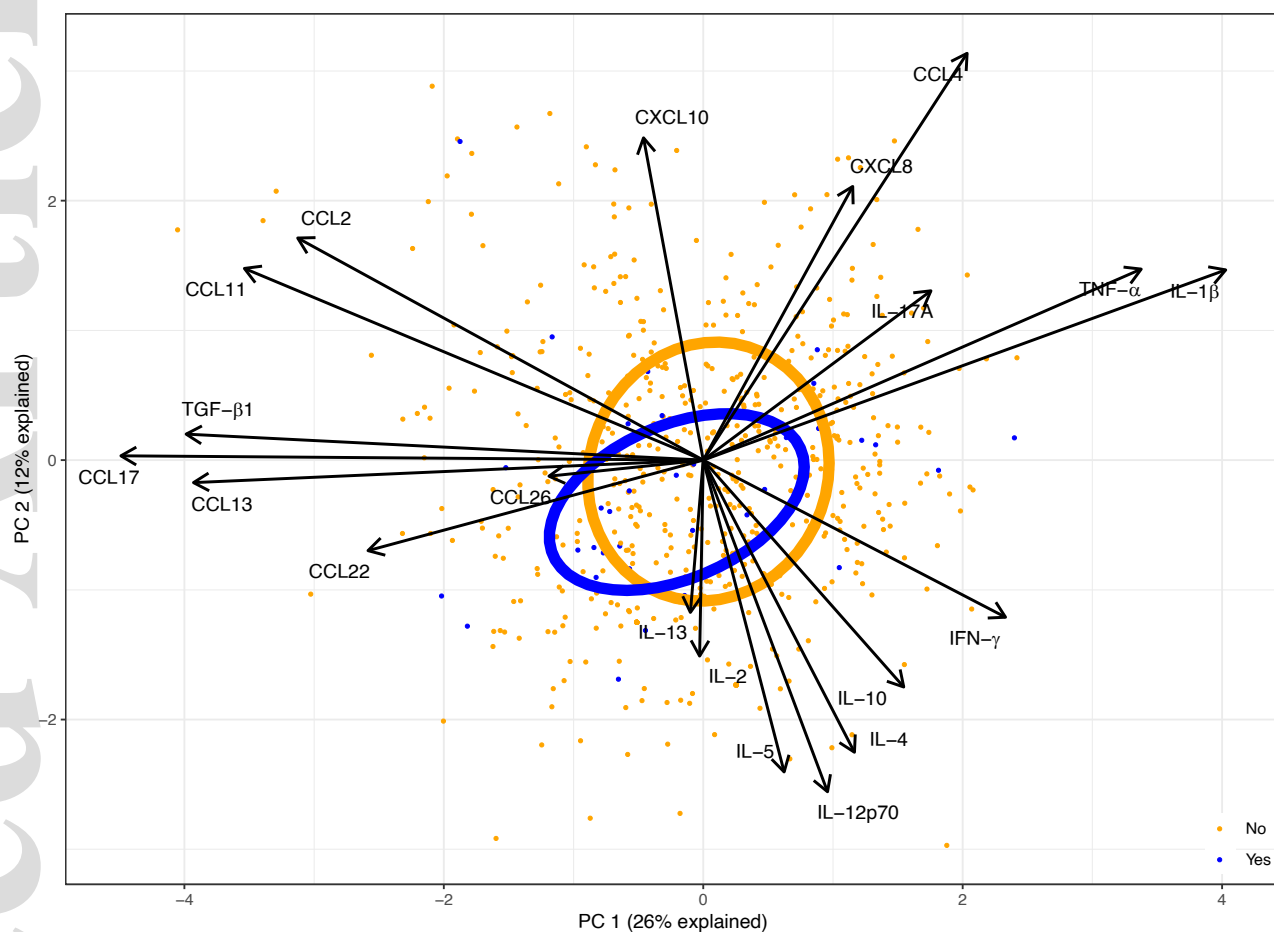


Figure 4: Biplot of the principal component analysis (PC1 vs. PC2) showing the distribution of the neonatal airway immune profiles of children with (blue circle) and without (orange circle) asthma exacerbations. A trend of difference was observed for PC2: $p=0.07$.



ONLINE SUPPLEMENT

Table E1: Absolute levels, limits of detection of the assay, and the frequency of non-detectable values for each cytokine and chemokine.

Mediator	Detection limit pg/ml	< Detection limit N (%)	Median pg/ml	IQR pg/m
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 β	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- β 1	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

Table E2: Drop-out table of the 620 included vs 80 excluded

	Included N (%)	Excluded N (%)	p-value
Maternal asthma, allergy or eczema	282 (46%)	42 (53%)	0.31
Caucasian	29 (5%)	2 (3%)	0.55
Caesarian section	134 (22%)	17 (21%)	1.00
Male	301 (49%)	39 (49%)	1.00
Maternal antibiotics in 3rd trimester	109 (18%)	14 (18%)	1.00
Siblings at birth	358 (58%)	37 (46%)	0.07
Cat and/or dog at birth	401 (65%)	56 (70%)	0.45
Breast feeding at 1 month	46 (7%)	4 (5%)	0.66
Maternal smoking in 3rd trimester	597 (96%)	78 (98%)	0.82
Maternal alcohol consumption in 3rd trim.	591 (96%)	73 (91%)	0.15
Social circumstances (high)	312 (50%)	37 (46%)	0.57
Gestational age (< 37w)	601 (97%)	71 (89%)	<0.001
Apgar score	591 (95%)	76 (95%)	1.00

Table E3: Odds ratios and 95% confidence intervals (CI) of any elevated specific-IgE (sIgE), allergic rhinitis and any positive skin prick test (SPT) at age 6 years adjusted for presence of pathogenic airway microbes, siblings, maternal asthma, allergy or eczema and the vitamin D RCT (aOR). P≤0.05 is shown in bold.

Immune mediator	sIgE, inhalant allergens		Allergic rhinitis		SPT	
	aOR (CI)	p-value	aOR (CI)	p-value	aOR (CI)	p-value
IL-12p70	1.02 (0.81 – 1.31)	0.87	1.15 (0.82 – 1.74)	0.47	1.35 (0.98 – 1.98)	0.09
CXCL10	1.09 (0.86 – 1.38)	0.49	1.00 (0.70 – 1.41)	1.00	0.94 (0.71 – 1.24)	0.67
IFN- γ	1.19 (0.92 – 1.79)	0.21	0.94 (0.70 – 1.32)	0.69	0.88 (0.70 – 1.14)	0.32
TNF- α	1.27 (0.99 – 1.64)	0.06	1.35 (0.94 – 1.96)	0.11	1.15 (0.86 – 1.55)	0.34
CCL4	1.25 (0.96 – 1.63)	0.10	1.19 (0.81 – 1.73)	0.37	0.99 (0.73 – 1.33)	0.93
CCL2	1.04 (0.81 – 1.33)	0.77	0.89 (0.62 – 1.26)	0.52	1.11 (0.84 – 1.47)	0.45
CCL13	0.81 (0.62 – 1.05)	0.11	0.93 (0.64 – 1.33)	0.69	1.02 (0.76 – 1.36)	0.89
IL-4	1.07 (0.84 – 1.39)	0.59	1.07 (0.77 – 1.56)	0.72	0.97(0.75 – 1.28)	0.80
IL-5	1.00 (0.79 – 1.27)	0.99	1.00 (0.72 – 1.42)	0.98	1.11 (0.85 – 1.46)	0.46
IL-13	1.12 (0.88 – 1.43)	0.37	1.20 (0.85 – 1.71)	0.32	1.31 (0.99 – 1.76)	0.06
CCL11	1.07 (0.83 – 1.37)	0.61	1.11 (0.79 – 1.60)	0.56	0.94 (0.71 – 1.25)	0.66
CCL26	0.70 (0.56 – 0.88)	0.003	0.69 (0.51 – 0.94)	0.02	0.69 (0.52 – 0.88)	0.003
CCL17	0.99 (0.76 – 1.29)	0.92	0.92 (0.63 – 1.35)	0.66	1.07 (0.78 – 1.48)	0.66
CCL22	0.62 (0.49 – 0.79)	<0.001	0.78 (0.57 – 1.08)	0.12	0.77 (0.60 – 1.00)	0.04
IL-1 β	1.32 (1.01 – 1.74)	0.04	1.54 (1.04 – 2.31)	0.03	1.09 (0.81 – 1.48)	0.56
IL-17A	1.36 (1.05 – 1.78)	0.02	1.23 (0.85 – 1.81)	0.29	1.32 (0.99 – 1.80)	0.07
CXCL8	0.97 (0.76 – 1.24)	0.81	1.01 (0.72 – 1.45)	0.95	0.91 (0.70 – 1.20)	0.51
TGF- β 1	0.80 (0.60 – 1.04)	0.10	0.85 (0.57 – 1.24)	0.42	1.00 (0.73 – 1.34)	0.99
IL-10	0.88 (0.69 – 1.12)	0.32	0.87 (0.63 – 1.20)	0.39	1.03 (0.79 – 1.35)	0.83
IL-2	1.02 (0.81 – 1.30)	0.85	1.03 (0.74 – 1.44)	0.85	1.10 (0.84 – 1.43)	0.49

Table E4: Hazard ratios and 95% confidence intervals (CI) for asthma and asthma exacerbations adjusted for presence of pathogenic airway microbes, siblings, maternal asthma, allergy or eczema and the vitamin D RCT (aHR). P≤0.05 is shown in bold.

Immune mediator	Asthma	p-value	Asthma exacerbations	p-value
	aHR (CI)		aHR (CI)	
IL-12p70	1.42 (0.98 – 2.05)	0.06	1.34 (0.73 – 2.45)	0.35
CXCL10	0.89 (0.64 – 1.25)	0.51	1.29 (0.75 – 2.20)	0.36
IFN- γ	1.09 (0.78 – 1.50)	0.62	1.13 (0.63 – 2.01)	0.68
TNF- α	0.88 (0.55 – 1.38)	0.57	0.48 (0.23 – 1.01)	0.05
CCL4	1.02 (0.67 – 1.56)	0.92	0.58 (0.28 – 1.19)	0.14
CCL2	1.25 (0.80 – 1.96)	0.32	1.42 (0.68 – 2.97)	0.35
CCL13	1.07 (0.62 – 1.85)	0.81	0.85 (0.34 – 2.13)	0.73
IL-4	1.11 (0.87 – 1.43)	0.39	1.37 (0.87 – 2.16)	0.17
IL-5	1.06 (0.71 – 1.60)	0.77	1.13 (0.58 – 2.19)	0.73
IL-13	1.02 (0.63 – 1.67)	0.93	0.92 (0.41 – 2.06)	0.83
CCL11	1.15 (0.70 – 1.88)	0.57	1.04 (0.45 – 2.42)	0.92
CCL26	0.83 (0.65 – 1.05)	0.13	0.93 (0.62 – 1.41)	0.75
CCL17	1.15 (0.73 – 1.83)	0.54	1.01 (0.48 – 2.12)	0.98
CCL22	0.80 (0.58 – 1.10)	0.18	1.22 (0.67 – 2.21)	0.52
IL-1 β	0.82 (0.59 – 1.14)	0.24	0.53 (0.31 – 0.91)	0.02
IL-17A	0.90 (0.68 – 1.19)	0.46	0.74 (0.47 – 1.16)	0.19
CXCL8	1.33 (0.67 – 2.65)	0.42	0.38 (0.14 – 1.02)	0.05
TGF- β 1	0.93 (0.55 – 1.57)	0.79	0.99 (0.41 – 2.41)	0.99
IL-10	0.93 (0.62 – 1.40)	0.59	1.16 (0.58 – 2.30)	0.68
IL-2	1.09 (0.67 – 1.78)	0.73	1.40 (0.64 – 3.04)	0.40

Table E5: Associations different asthma phenotypes and the neonatal airway immune composition analyzed by principal component 1-4 (PC1-4) from a principle component analysis of 20 immune mediator levels.

All analyses are adjusted for pathogenic airway microbes, siblings, maternal asthma, allergy or eczema and the vitamin D randomized controlled trial.

End-point	PC1	PC2	PC3	PC4
Asthma, 0-6 yrs	0.96 (0.65 – 1.43) p = 0.86	0.88 (0.62 – 1.26) p = 0.49	0.84 (0.59 – 1.18) p = 0.32	1.37 (0.96 – 1.96) p = 0.08
Asthma exacerbations	0.74 (0.38 – 1.42) p = 0.36	0.57 (0.31 – 1.04) p = 0.07	1.24 (0.67 – 2.31) p = 0.50	1.42 (0.79 – 2.54) p = 0.24
Early transient asthma, 0-3 yrs	0.96 (0.74 – 1.24) p = 0.74	0.75 (0.58 – 0.96) p = 0.02	0.96 (0.75 – 1.23) p = 0.76	1.23 (0.97 – 1.56) p = 0.09
Persistent asthma by age 6	0.97 (0.68 – 1.41) p = 0.89	1.16 (0.83 – 1.61) p = 0.37	0.78 (0.57 – 1.10) p = 0.15	0.97 (0.68 – 1.37) p = 0.87
Allergic asthma	0.74 (0.26 – 2.13) p = 0.58	1.36 (0.53 – 3.51) p = 0.52	0.46 (0.23 – 0.92) p = 0.03	1.62 (0.66 – 3.97) p = 0.30
Non-allergic asthma	0.74 (0.44 – 1.23) p = 0.25	0.63 (0.39 – 1.04) p = 0.07	0.92 (0.58 – 1.45) p = 0.72	1.58 (0.98 – 2.54) p = 0.06

eFigures

Figure E1: Flowchart

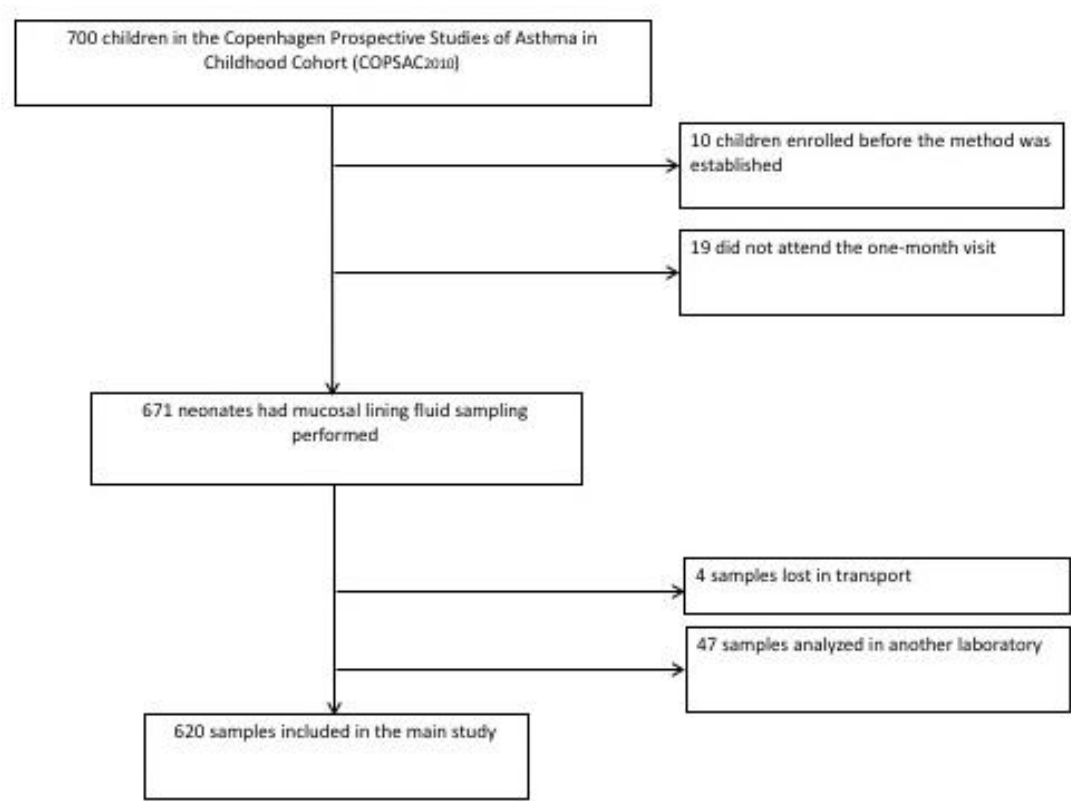


Figure E2: Overlap between asthma (blue circle) and aeroallergen sensitization (yellow circle).

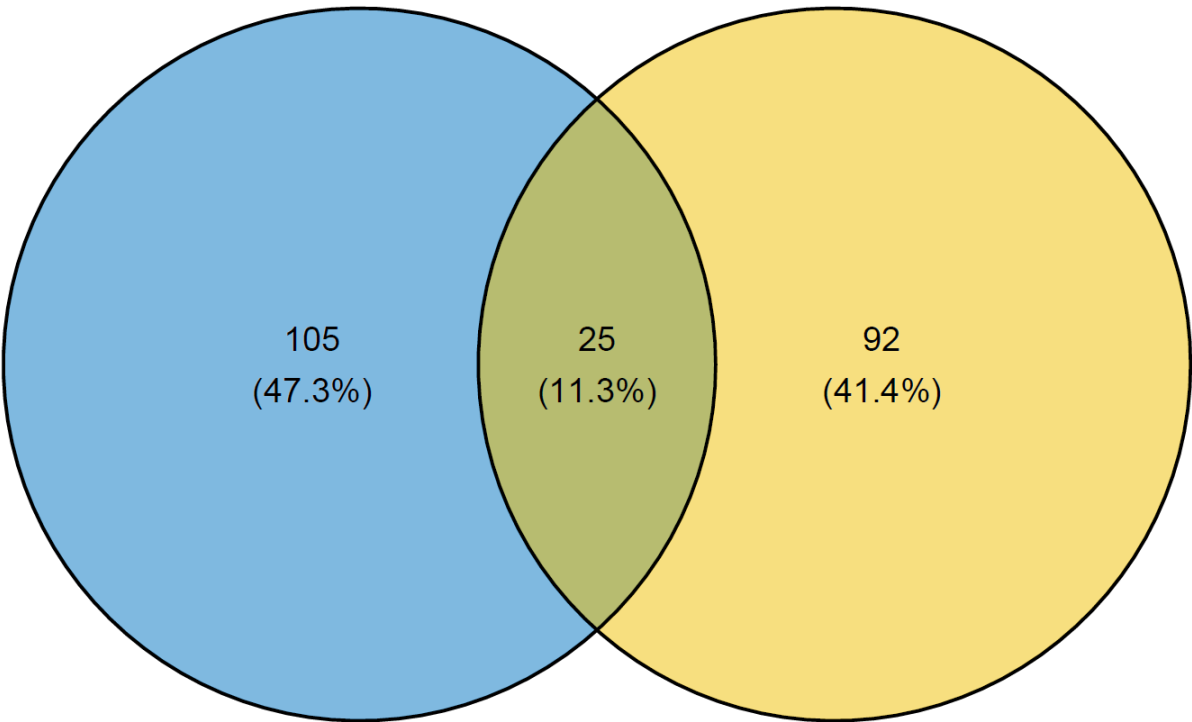


Figure E3: Heatmap of the cytokine and chemokine expression per individual child based on the PC1-4.

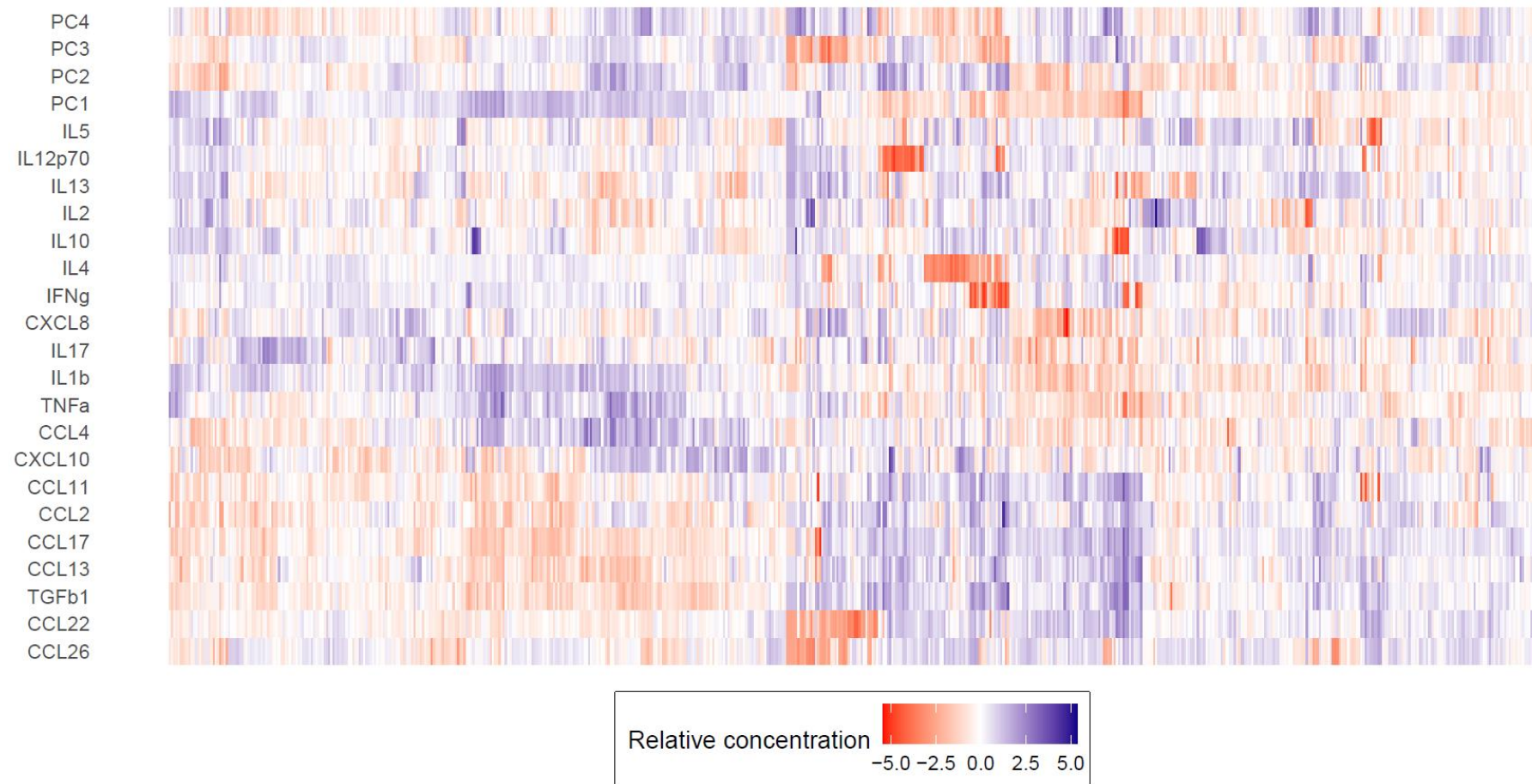


Figure E4: Biplot of the principal component analysis (PC1 vs. PC2) showing the distribution of the neonatal airway immune profiles of children with (blue circle) and without (orange circle) any positive skin prick test at age 6 years. A significant difference is observed for PC1: $p=0.05$.

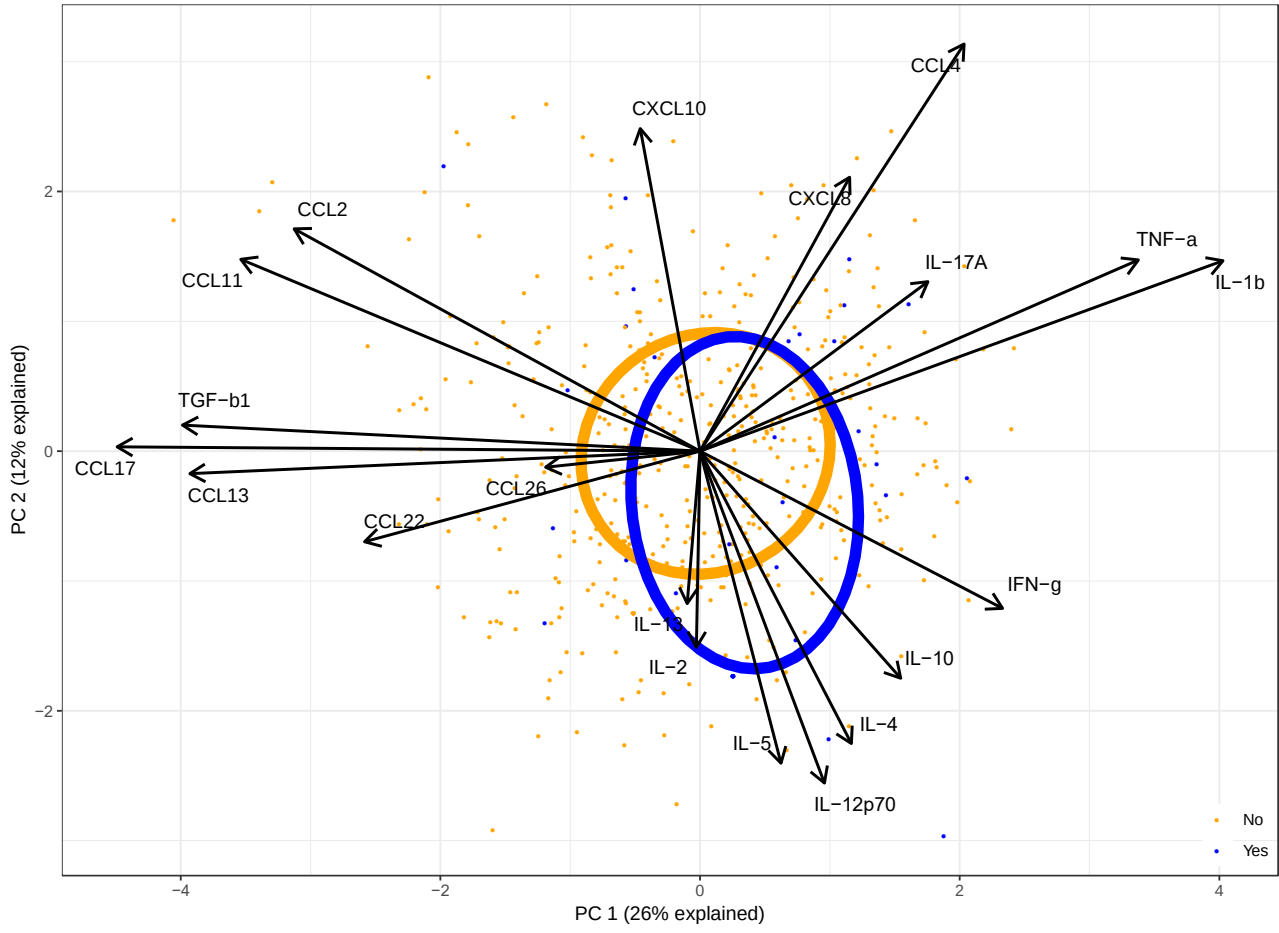


Figure E5: Biplot of the principal component analysis (PC3 vs. PC4) showing the distribution of the neonatal airway immune profiles of children with (blue circle) and without (orange circle) any positive skin prick test at age 6 years. A trend of difference is observed for PC4: $p=0.09$.

