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

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Abstract

Background: Siblings have been shown to reduce the risk of later 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. We hypothesized that siblings exert immune modulatory effects on neonates mirrored in the airway mucosa.

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 Childhood₂₀₁₀ birth-cohort (COPSAC₂₀₁₀). The association between airway mediator levels and presence of siblings was investigated using conventional statistics and principle component analyses (PCA).

Results: Neonates with siblings had an up-regulated 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 presence of unidentified microbes, but further studies are needed to confirm our findings.

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

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

Introduction

The immune system matures in infancy and early childhood. During this period the composition of the exposome is important for the trajectory towards 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, though 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 elder siblings convey immune modulation through an increased risk of virus and bacterial exposure in very early childhood. In this regard we have previously shown that the airway immune response is up-

regulated 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 one-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 Childhood₂₀₁₀ (COPSAC₂₀₁₀) 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 COPSAC₂₀₁₀ birth cohort

COPSAC₂₀₁₀ 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(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 one-month of age as previously described(8,11). 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 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 16,000g.

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 beta1 (TGF-β1), IL-10, and IL-2(11,13) 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, 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 that 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 one-week of age. *Siblings* included biological siblings as well as half-siblings living in the home. Data was analyzed as 1) any siblings (biological and half-siblings), 2) any biological siblings (excluding half-siblings), 3) quantitatively as 0 vs. 1 vs. >1 sibling, and for multiparous women 4) 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 minute” (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 pneumoniae* and *Moraxella catarrhalis*, as previously described (8,9,17).

Statistics

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

In order to obtain normally distributed residuals of the mediator levels, data was 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 non-zero 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) in order 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) and the PCA were conducted in MATLAB R2013a v. 8.1.0.604 (MathWorks Inc, Natick, MA) utilizing the PLStoolbox 7.8.2 (Eigenvector Research Inc. Wenatchee, WA) 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 **Online Figure E1**).

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 1 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%, $p=0.004$) (**Online Table E1**).

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 number of samples below the detection limit are shown in **Table 2**.

Neonates with siblings had a significant up-regulation 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-1 β) associated mediators which were 2 to 3.5-fold increased (**Figure 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 one-month of age, high income and alcohol consumption in 3rd trimester did not significantly modify the results (**Figure 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 (**Figure 1B**).

The same up-regulation of immune mediators was seen when the analyses were restricted to neonates without pathogenic airway bacteria and/or picornavirus (**Online Figure E2**), 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) (**Online Figure E3**) a significant up-regulation was found in all immune-mediators (all p-values < 0.05) except for CCL17 and TGF β 1 (p > 0.07). Finally, we studied the birth-order effect of being second-born (N=216) versus first-born (**Online Figure E4A and E5**) and correspondingly the effect of being third-born (N =108) versus first-born (**Online Figure E4B and E5**) without finding any additional immune-stimulatory or –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 inter-correlation between the 20 different immune mediators in the first component of the loading

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plot (**Figure 2A**). In the first component of the score plot (**Figure 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 one-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 (**Figure 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 towards 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 (**Online Figure E4**). Testing the first three principal components with respect to birth order revealed no significant effect, underlining that there 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 up-regulated 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 suggests 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.

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 was 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, though 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 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), whilst 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 paper, 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, 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 up-regulated 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.

Especially 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 up-regulation 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 also dependent on birth-order. A correlation between levels of specific cytokines in maternal blood and cord blood have 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 have 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* 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 up-regulation 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.

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References

1. Illi S, Weber J, Zutavern A, Genuneit J, Schierl R, Strunz-Lehner C et al. Perinatal influences on the development of asthma and atopy in childhood. *Ann Allergy Asthma Immunol* 2014;**112**:132–139.e1.
2. Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989;**299**:1259–1260.
3. Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. *N Engl J Med* 2000;**343**:538–543.
4. Karmaus W, Botezan C. Does a higher number of siblings protect against the development of allergy and asthma? A review. *J Epidemiol Community Health* 2002;**56**:209–217.
5. Karmaus W, Arshad H, Mattes J. Does the Sibling Effect Have Its Origin In Utero? Investigating Birth Order, Cord Blood Immunoglobulin E Concentration, and Allergic Sensitization at Age 4 Years. *Am J Epidemiol* 2001;**154**:909–915.
6. Butler, NR., Golding, J. *From Birth to Five. A Study of the Health and Behaviour of Britain's Five Year Olds*. Pergamon Press 1986
7. Strachan DP, Ait-Khaled N, Foliaki S, Mallol J, Odhiambo J, Pearce N et al. Siblings, asthma,

rhinoconjunctivitis and eczema: a worldwide perspective from the International Study of Asthma and Allergies in Childhood. *Clin Exp Allergy* 2015;**45**:126–136.

8. Følsgaard NV, Schjørring S, Chawes BL, Rasmussen MA, Krogfelt KA, Brix S et al. Pathogenic bacteria colonizing the airways in asymptomatic neonates stimulates topical inflammatory mediator release. *Am J Respir Crit Care Med* 2013;**187**:589–595.
9. Wolsk HM, Følsgaard NV, Birch S, Brix S, Hansel TT, Johnston SL et al. Picornavirus-induced Airway Mucosa Immune Profile in Asymptomatic Neonates. *J Infect Dis* 2015;;jiv594.
10. Kragh M, Larsen JM, Thyssen AH, Rasmussen MA, Wolsk HM, Bisgaard H et al. Divergent Response Profile in Activated Cord Blood T cells from First-born Child Implies Birth-order-associated in Utero Immune Programming. *Allergy* Published Online First: 1 October 2015. doi:10.1111/all.12799
11. Chawes BLK, Edwards MJ, Shamji B, Walker C, Nicholson GC, Tan AJ et al. A novel method for assessing unchallenged levels of mediators in nasal epithelial lining fluid. *J Allergy Clin Immunol* 2010;**125**:1387–1389.e3.
12. Bisgaard H, Vissing NH, Carson CG, Bischoff AL, Følsgaard NV, Kreiner-Møller E et al. Deep phenotyping of the unselected COPSAC2010 birth cohort study. *Clin Exp Allergy* 2013;**43**:1384–1394.
13. Følsgaard NV, Chawes BL, Rasmussen MA, Bischoff AL, Carson CG, Stokholm J et al. Neonatal Cytokine Profile in the Airway Mucosal Lining Fluid Is Skewed by Maternal Atopy. *Am J Respir Crit Care Med* 2012;**185**:275–280.
14. Prescott SL. New concepts of cytokines in asthma: is the Th2/Th1 paradigm out the window? *J Paediatr Child Health* 2003;**39**:575–579.
15. Ngoc PL, Ngoc LP, Gold DR, Tzianabos AO, Weiss ST, Celedón JC. Cytokines, allergy, and asthma. *Curr Opin Allergy Clin Immunol* 2005;**5**:161–166.
16. Papadopoulos NG, Hunter J, Sanderson G, Meyer J, Johnston SL. Rhinovirus identification by BglI digestion of picornavirus RT-PCR amplicons. *Journal of Virological Methods* 1999;**80**:179–185.
17. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bønnelykke K et al. Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007;**357**:1487–1495.
18. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N et al. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* 2015;**17**:704–715.
19. Miyake Y, Tanaka K, Arakawa M. Sibling number and prevalence of allergic disorders in pregnant Japanese women: baseline data from the Kyushu Okinawa Maternal and Child Health Study. *BMC Public Health* 2011;**11**:561.
20. Kramer MS, Matush L, Bogdanovich N, Dahhou M, Platt RW, Mazer B. The low prevalence of allergic disease in Eastern Europe. *Clinical & Experimental Allergy* 2009;**39**:708–716.
21. Goldberg S, Israeli E, Schwartz S, Shochat T, Izbicki G, Toker-Maimon O et al. Asthma prevalence, family size, and birth order*. *Chest* 2007;**131**:1747–1752.

22. Grabenhenrich LB, Gough H, Reich A, Eckers N, Zepp F, Nitsche O et al. Early-life determinants of asthma from birth to age 20 years: A German birth cohort study. *Journal of Allergy and Clinical Immunology* 2014;**133**:979–988.e3.
23. Jain S, Self WH, Wunderink RG, Fakhran S, Balk R, Bramley AM et al. Community-Acquired Pneumonia Requiring Hospitalization among U.S. Adults. *New England Journal of Medicine* 2015;**373**:415–427.
24. Mueller A, Strange PG. The chemokine receptor, CCR5. *Int J Biochem Cell Biol* 2004;**36**:35–38.
25. Gern JE, Brooks GD, Meyer P, Chang A, Shen K, Evans MD et al. Bidirectional interactions between viral respiratory illnesses and cytokine responses in the first year of life. *Journal of Allergy and Clinical Immunology* 2006;**117**:72–78.
26. Santner-Nanan B, Straubinger K, Hsu P, Parnell G, Tang B, Xu B et al. Fetal–Maternal Alignment of Regulatory T Cells Correlates with IL-10 and Bcl-2 Upregulation in Pregnancy. *J Immunol* 2013;**191**:145–153.
27. Weigl JAI, Puppe W, Meyer CU, Berner R, Forster J, Schmitt HJ et al. Ten years' experience with year-round active surveillance of up to 19 respiratory pathogens in children. *European Journal of Pediatrics* 2007;**166**:957–966.

Tables

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 3 rd trimester	22 (70)	17 (42)	0.17
Maternal smoking in 3 rd trimester	3 (11)	4 (10)	0.68
Maternal alcohol consumption in 3 rd 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

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

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

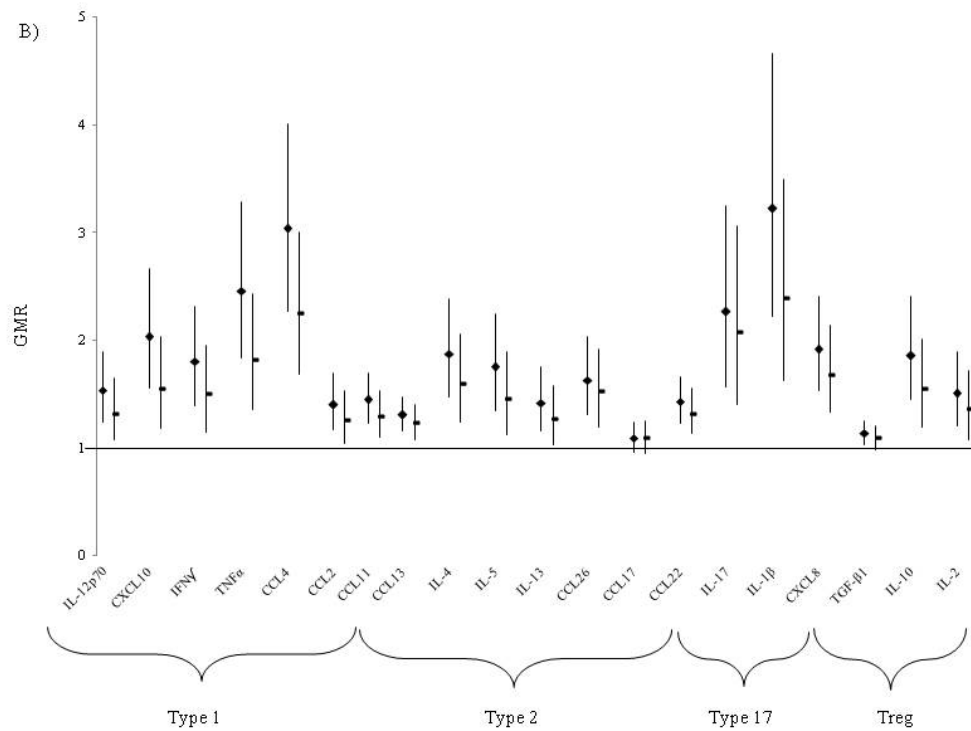
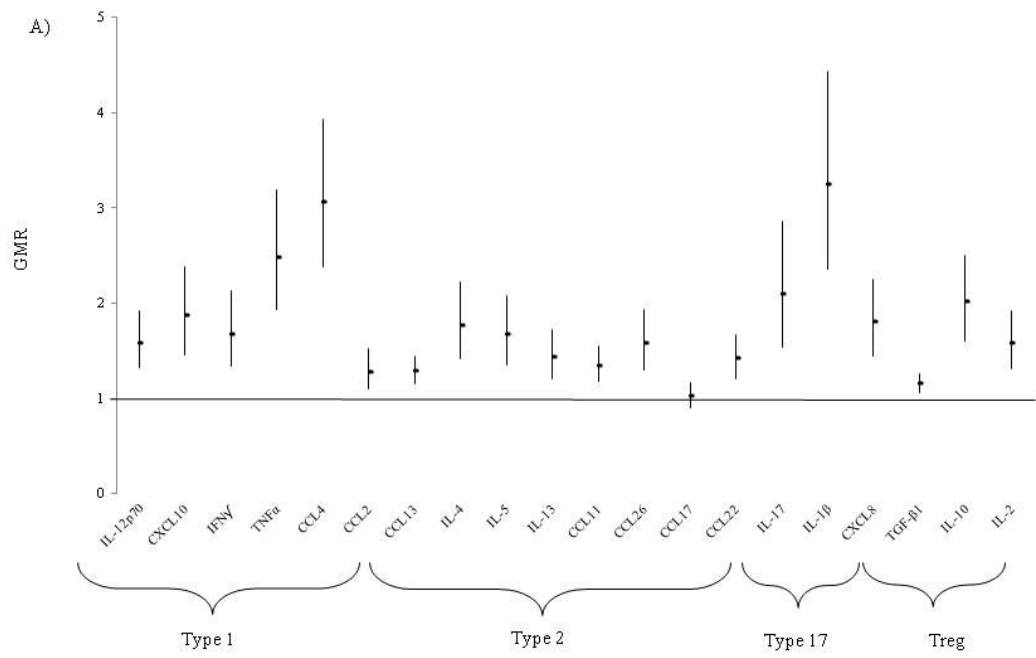
Mediator	<u>Siblings</u>	<u>No siblings</u>	<u>Below detection limit</u>
	Median (lower - upper quartile) pg/mL	Median (lower - upper quartile) pg/mL	
IL-12p70	5.81 (2.56 - 10.31)	3.58 (1.60 - 6.57)	120
CXCL10	683.3 (245.1 - 2424)	372.7 (159.9 - 919.3)	8
IFN- γ	6.04 (2.13 - 17.17)	2.98 (1.32 - 7.16)	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-1 β	163.48 (35.79 - 738.09)	37.60 (14.61 - 150.51)	4
CXCL8	4967 (1983 - 9335)	2535 (901 - 5292)	4
TGF- β 1	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 legends:

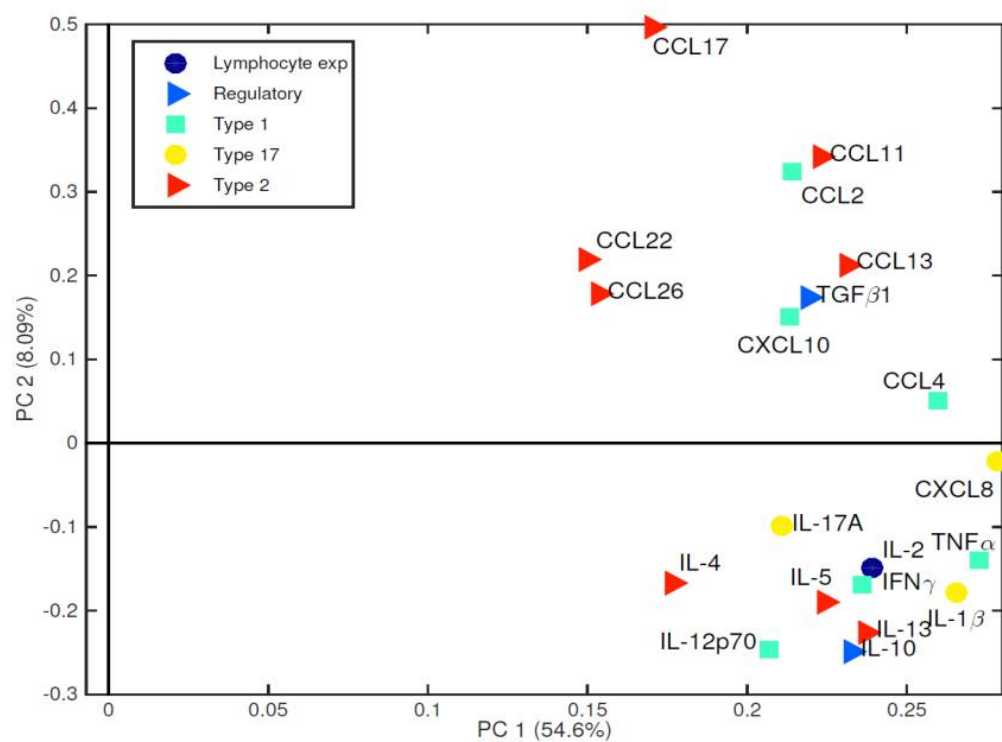
Figure 1: Geometric mean ratios (GMR) with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one-month-old neonates with/without siblings in the home. A) Crude levels. In B) each mediator is shown as the GMR adjusted (bar with a rhombus shape) for maternal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

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 versus 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 ($p < 10^{-10}$).

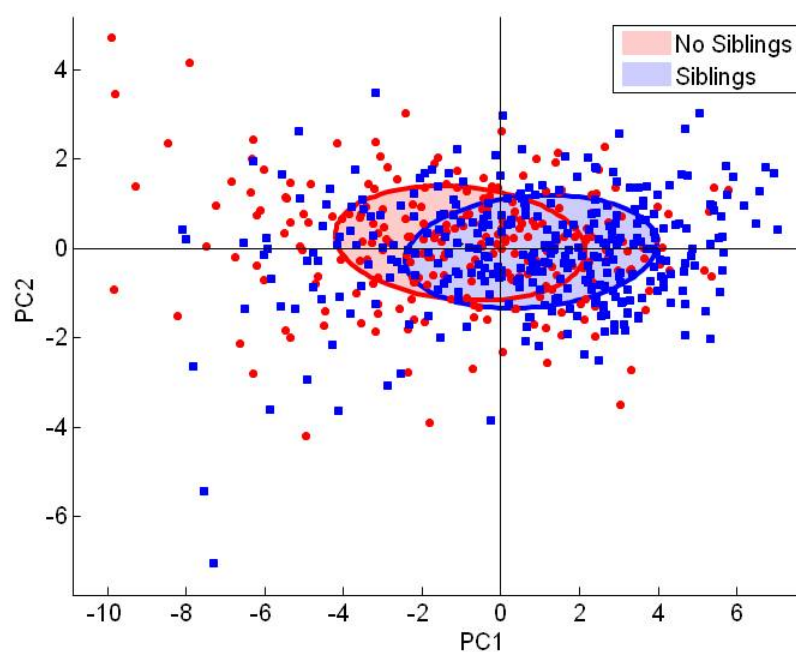
Figure 3: Principal component analysis of the distribution of the time since last childbirth(x-axis) in relation to level of immune mediators (y-axis). Significantly higher immune mediator levels are seen with short time since the last pregnancy (unadjusted $p = 0.0016$, adjusted $p = 0.02$).

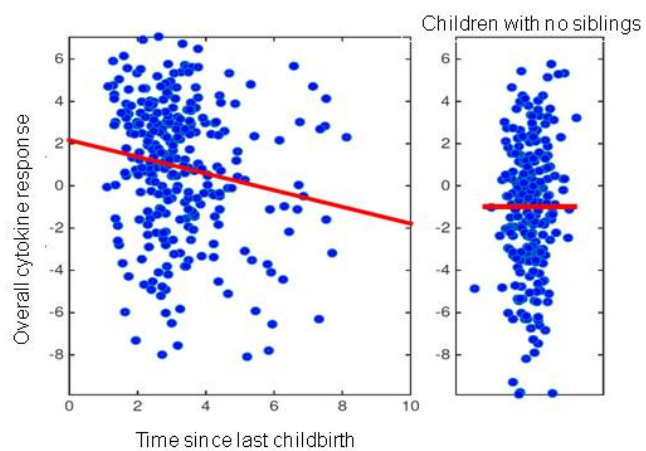


A)



B)





1 **Online data supplement- Figures**

2

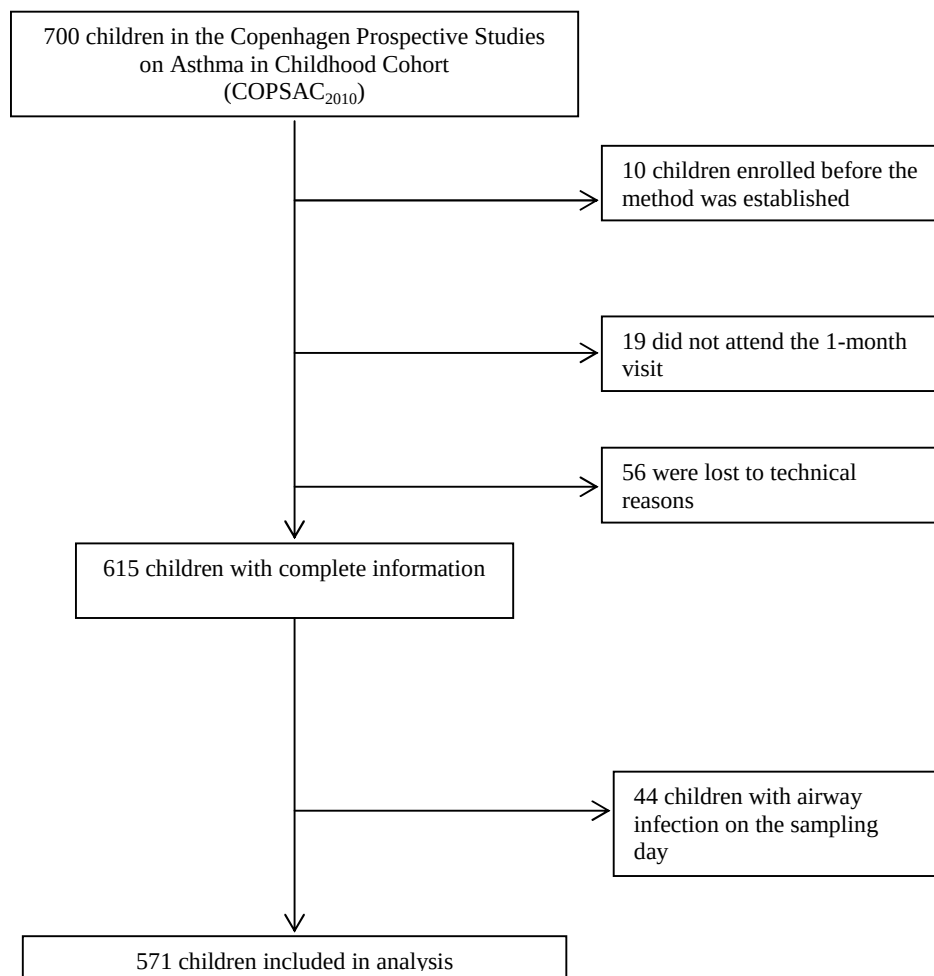
3 **Siblings Promote a Type 1/Type 17-oriented immune response in the airways of**

4 **asymptomatic neonates**

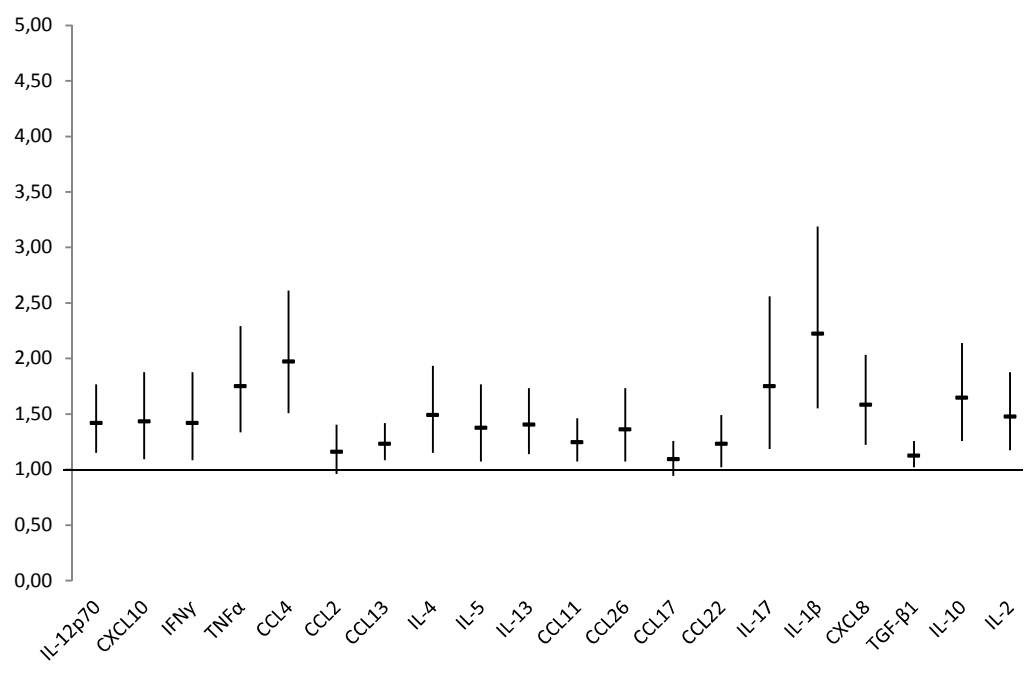
5 **Authors:** Helene Mygind Wolsk, MD; Bo L Chawes, MD PhD ; Nilofar V Følsgaard, MD PhD; Morten A

6 Rasmussen, MSci PhD; Susanne Brix, MSci PhD; Hans Bisgaard, MD DMsc

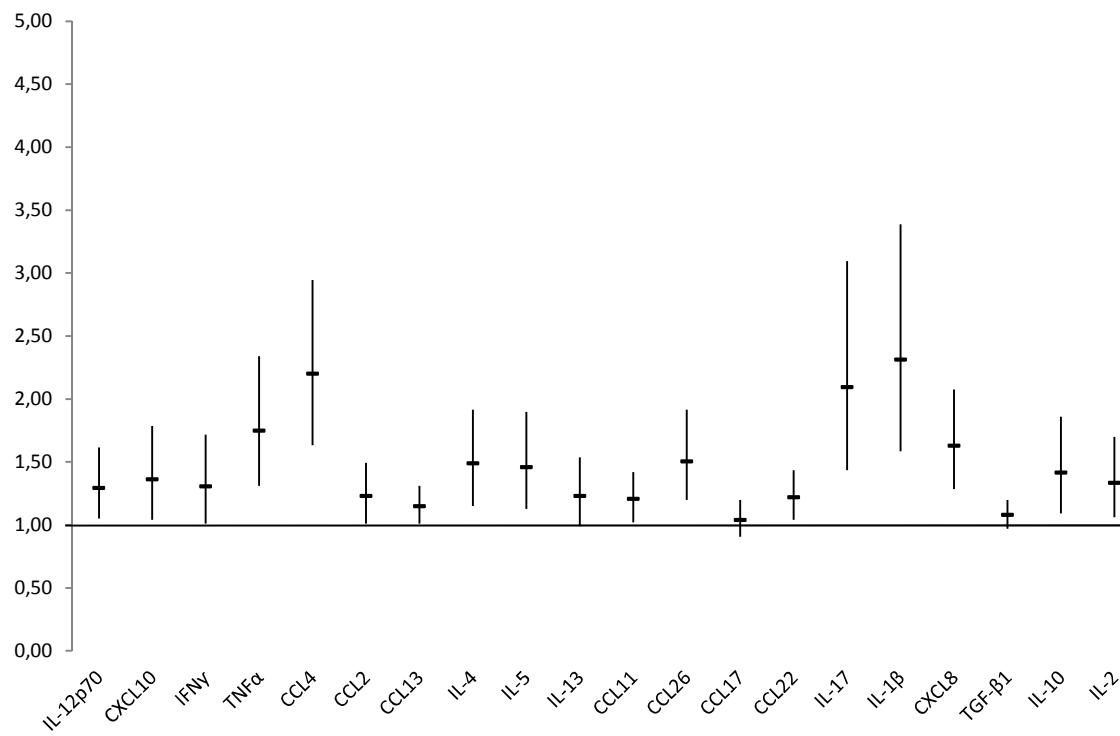
Online figure E1: Study group flow-chart



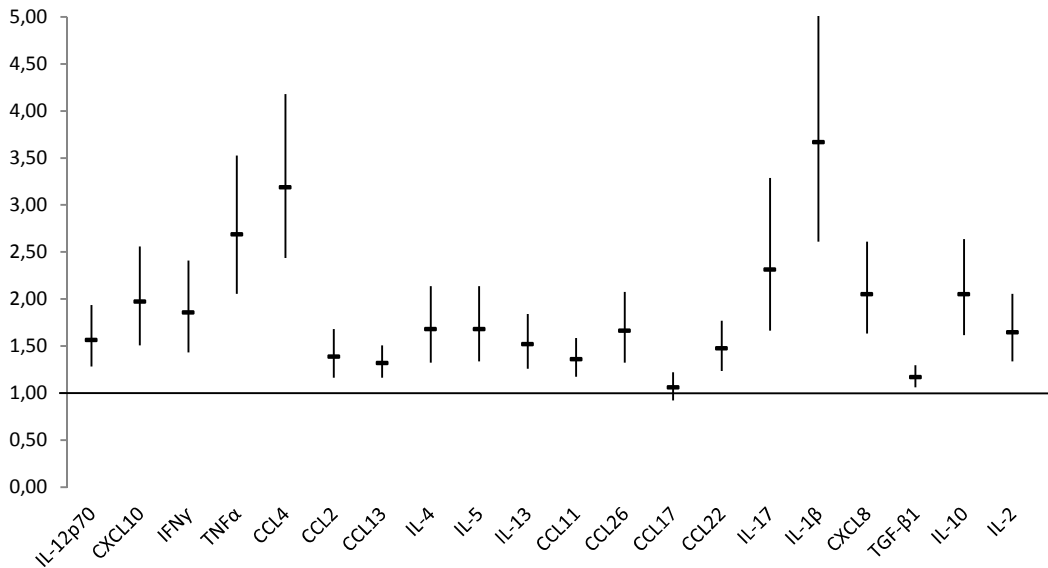
22 **Online Figure E2:**
23 Geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of
24 healthy one-month-old neonates with/without siblings. Only the neonates without concurrent pathogenic airway bacteria
25 and/or picornavirus are included (n=381).



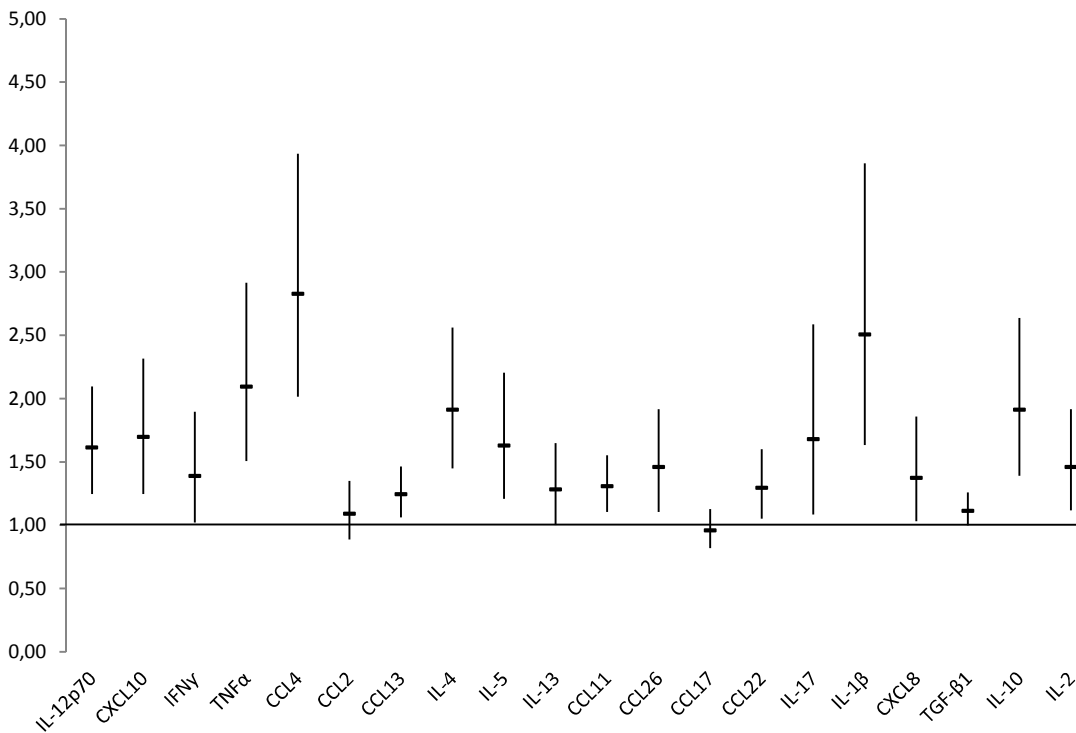
28 **Online Figure E3:**
 29 Geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of
 30 healthy one-month-old neonates with/without biological siblings in the home at birth (n = 310). Adjusted for maternal
 31 asthma, allergy, or eczema, sampling clinic, season of sampling, batch of immune mediator analyses, maternal smoking
 32 in 3rd trimester, breastfeeding at one-month of age, high income, maternal alcohol consumption in 3rd trimester, bacteria
 33 and picornavirus.



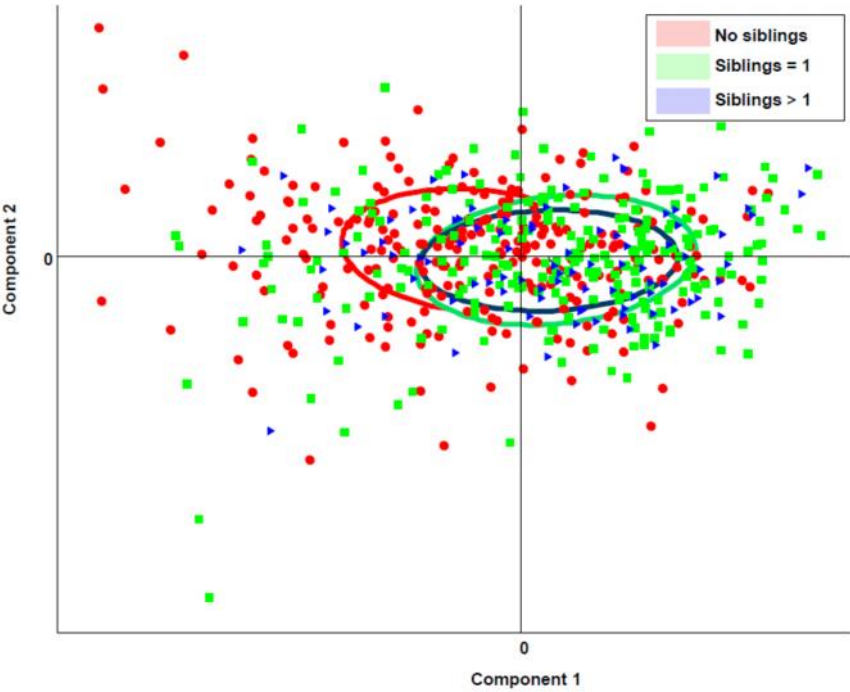
Online Figure E4: Immune response in second-born and third-born compared to first-born
 Geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one-month-old neonates with/without siblings in the home at birth. A) Second-born (biological 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.



B)



48 **Online Figure E5:**
49 Principal component analysis of the distribution of the neonates with no siblings (red circle) versus one sibling (green
50 circle) versus more than one sibling (blue circle).



51

1 **Online data supplement**

2

3 **Siblings Promote a Type 1/Type 17-oriented immune response in the**

4 **airways of asymptomatic neonates**

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

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

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

	<u>Included</u> % (n=571)	<u>Excluded</u> % (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 3 rd trimester	27 (35)	20 (112)	0.06
Maternal smoking in 3 rd trimester*	4 (21)	3 (4)	0.71
Maternal alcohol consumption in 3 rd 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

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