



## Airway Mucosal Immune-suppression in Neonates of Mothers Receiving A(H1N1)pnd09 Vaccination During Pregnancy

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2 **Airway Mucosal Immune-Suppression in Neonates of Mothers Receiving**  
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4 **A(H1N1)pnd09 Vaccination During Pregnancy**  
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23 High TGF- $\beta$ 1 in Neonates of Vaccinated Mothers  
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27 This paper has a Supplemental Digital Content.  
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## Structured Abstract

Word count abstract: 248

### *Background:*

It is recommended to vaccinate pregnant women against influenza. A possibly impact on the immune expression of the fetus has never been studied.

We aim to study the immune signature in the upper airways and the incidence of infections in neonates born to mothers receiving Influenza A(H1N1)pnd09 vaccination during pregnancy.

### *Methods:*

156 women from the unselected Copenhagen Prospective Study on Asthma in Childhood (COPSAC<sub>2010</sub>) received Influenza A(H1N1)pnd09-vaccination during the 2009 pandemic. 51 mothers received the vaccine during pregnancy and 105 after pregnancy. 332 neonates of non-vaccinated mothers were included as secondary controls. Nasal mucosal lining fluid was sampled in 488 neonates and assessed for IL-12p70, IP-10, IFN- $\gamma$ , TNF- $\alpha$ , MIP-1 $\beta$ , MCP-1, MCP-4, IL-4, IL-5, IL-13, eotaxin-1, eotaxin-3, TARC, MDC, IL-17, IL-1 $\beta$ , IL-8, TGF- $\beta$ 1, IL-10 and IL-2. Infections were monitored the first year of life by daily diary cards and clinical controls.

### *Results:*

Neonates of mothers vaccinated during pregnancy had significant up-regulation of TGF- $\beta$ 1 (ratio=1.52 [1.22-1.90], p=0.0002), and corresponding down regulation (p<0.05) of IL-12p70, IFN- $\gamma$ , IL-5, eotaxin-1, TARC, MDC, IL-8 in comparison to those vaccinated after pregnancy. The lag-time from vaccination during pregnancy to assessment of the immune signature showed significant and positive association to up-regulation of TGF- $\beta$ 1 levels (P= 0.0003) and significant negative association to other mediators. The incidence of infections in early infancy did not differ between the study groups.

### *Conclusion:*

Influenza A(H1N1)pnd09 vaccination during pregnancy up-regulates TGF- $\beta$ 1 and down-regulates key mediators of the protective immunity, but the rate of clinical infections was not detectably affected

1 **Unstructured Abstract**

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3 In this paper we aim to study the immune signature of the upper airways and the incidence of  
4 infections in neonates born to mothers receiving Influenza A(H1N1)pnd09 vaccination during  
5 pregnancy. We find that vaccination during pregnancy up-regulates TGF- $\beta$ 1 and down-regulates  
6 key mediators of the protective immunity, we find no influence on clinical infections.  
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## Introduction

Influenza vaccination of pregnant women is generally recommended for the protection of both women and children,<sup>1</sup> in particular during the recent Influenza A(H1N1)pnd09 (H1N1pnd09) pandemic in 2009.<sup>2,3</sup>

The outcome of influenza infection during pregnancy is more severe during seasonal influenza epidemics,<sup>4-6</sup> and even graver during influenza pandemics.<sup>7-9</sup> Accordingly, the hospital admission and mortality rates were higher in pregnant women with influenza than in the general population during the 2009 pandemic,<sup>10-12</sup> with increased rates of stillbirth, perinatal and neonatal mortality in infected pregnant women.<sup>13</sup>

Influenza is also a major cause of morbidity and hospitalization in infants younger than six months.<sup>14-16</sup> In particular the H1N1pnd09 pandemic caused more severe disease in the neonates and young infants than in older children, all unprotected from passive immunity to this novel virus.<sup>17</sup>

No vaccination is approved for infants younger than six months.<sup>18,19</sup> Therefore influenza vaccination during pregnancy has been proposed as a safe way to protect infants,<sup>1,5,20</sup> presumably by cocooning<sup>21,22</sup> and from antibodies transmitted from the mother.<sup>1,15,23</sup> Yet the evidence is ambiguous as to the protective efficacy in the infants of vaccines given during pregnancy.<sup>24</sup>

It is well known that the maternal milieu during pregnancy affects the fetal immune competence.<sup>25-27</sup> While there is no published evidence on the possible effect of vaccination during pregnancy on the neonatal immune status, influenza vaccination is generally considered safe for the pregnant mother and the fetus.

Therefore, to examine the *in vivo* immune signature at the upper airways, we analyzed the cytokine and chemokine signature of the upper airway mucosa in neonates 4 weeks after birth<sup>26,28,29</sup> and monitored the incidence of infections in the first year of life in infants of mothers receiving H1N1pnd09-vaccination during pregnancy versus neonates of mothers not vaccinated during pregnancy.<sup>30</sup> Furthermore we examined whether the time of vaccination during pregnancy was of importance to the impact of the vaccination on the cytokine and chemokine signature.

## 1 **Methods**

### 2 3 4 *Design of the COPSAC<sub>2010</sub> Cohort Study*

5 The novel COPSAC<sub>2010</sub> cohort is an ongoing unselected prospective clinical study of 743 pregnant  
6 women and their 700 children. The pregnant women were recruited during gestational week 20-24  
7 in 2009-2010.<sup>26</sup> Key exclusion criteria were; chronic endocrine-, nephro- or cardiac diseases. The  
8 neonates were enrolled at one week of age excluding infants with severe congenital abnormality.<sup>26</sup>

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13 Information on confounders and baseline data were collected by structured clinical interviews at the  
14 parents' first visit to the COPSAC research clinic during gestational week 24 and 36, and again at  
15 one and four weeks after birth.  
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19 The families were seen at the COPSAC research clinic at age one week, one month, three months,  
20 six months and at six monthly intervals hereafter for close clinical follow-up.  
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Daily diary cards were used to monitor infections in the infants in the first year of life. Infections  
were categorized as common cold, pharyngitis, otitis, pneumonia, fever and gastro-intestinal  
infection. Parents were instructed how to fill in the diaries at study inclusion, and the diary cards  
were reviewed with the research doctor at each visit to clarify symptom definitions. Additionally,  
the research doctor interviewed the parents about other illness and any health care contacts outside  
the research clinic.

The database was double-checked against source data by and was subsequently locked. Data  
validity was assured by compliance with Good Clinical Practice guidelines and quality control  
procedures. An audit trail was done routinely.

All bio-sampling, clinical assessments and data collection were performed by a medical doctor with  
pediatric training and captured in a dedicated database on a novel SQS server.

### 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 *Design of the Nested Influenza Vaccination Study*

Women participating in the COPSAC<sub>2010</sub> cohort study were invited into a nested randomized trial of  
the safety and immunogenicity of a standard dose of 7.5µg of the monovalent influenza  
A/California/2009 (H1N1pnd09) surface-antigen MF59-adjuvanted vaccine (Focetria®, Novartis).  
Details of the study was recently reported.<sup>30</sup>

The study was initiated November 2009 early in the H1N1pnd09 pandemic, i.e. in the middle of the  
cohort recruitment period allowing comparison of the vaccine effects in pregnant and post-partum  
women. The recruitment was hampered by two factors; primarily the Danish Health Authorities

1 were ambiguous in their recommendations for vaccination of pregnant women changing their  
2 recommendations during the pandemic. This ambivalence caused the women to doubt the necessity  
3 and safety of the offered vaccination. Secondly the clinical symptoms of the pandemic proved less  
4 serious than expected causing the women to reject the offer of receiving the vaccine.<sup>30</sup>  
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#### 8 9 *Measurements of Airway Inflammatory Mediators in Upper Airway Mucosal Lining Fluid of* 10 *Neonates*

11 Airway mucosal lining fluid was collected at four weeks of age with a pair of 3x15mm strips of  
12 filter paper (Accuwik Ultra, fibrous hydroxylated polyester sheets, cat no.SPR0730, Pall Life  
13 Sciences, UK) as previously described in detail.<sup>28</sup>  
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17 The mucosal lining fluid was analyzed for levels of Interleukin (IL)-12p70, CXCL10 (IP-10),  
18 Interferon-gamma (IFN- $\gamma$ ), Tumor necrosis factor-alpha (TNF- $\alpha$ ), CCL4 (MIP-1 $\beta$ ), CCL2(MCP-1),  
19 CCL13 (MCP-4), IL-4, IL-5, IL-13, CCL11 (eotaxin-1), CCL26 (eotaxin-3), CCL17 (TARC ),  
20 CCL22 (MDC), IL-17, IL-1 $\beta$ , CXCL8 (IL-8), Transforming growth factor beta (TGF- $\beta$ 1), IL-10  
21 and IL-2 as previously described in detail.<sup>26,28</sup>  
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#### 28 *Covariates*

29 Covariates were decided *a priori* based on our previous analyses showing association between the  
30 airway immune signature of the neonates and maternal atopic disease<sup>26</sup>, older siblings in the house  
31 and airway colonization with pathogenic bacteria.<sup>29</sup> Furthermore we adjusted for vaccination month  
32 because there is a seasonal bias in the data collection since vaccination of participants peaked  
33 around the start of the pandemic in the winter of 2009.  
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#### 40 *Statistical analyses*

41 All mediator levels were log-transformed prior to analysis. The associations between  
42 cytokines/chemokines levels in the two vaccine groups and the associations to the time of  
43 vaccination were tested using generalized linear models (GLM), for unadjusted as well as adjusted  
44 analysis. Results are reported as ratios between mean values of cytokines and chemokines , in the  
45 upper airway mucosal lining fluid in neonates of mothers vaccinated during pregnancy compared to  
46 the control group(vaccinated after pregnancy) with 95% confidence intervals in squared brackets.  
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53 Baseline characteristics in the three groups were compared using Fisher's exact test, Wilcoxon  
54 Rank sum test and one-way analysis of variance (Anova).  
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57 The association between maternal vaccination and cumulative incidence of infections in the  
58 offspring throughout the first year of life was assessed by Quasi-Poisson regression estimating  
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1 Incidence Risk Ratios (IRR), adjusting for season of birth, older siblings, and maternal atopic  
2 disease. In the secondary analysis we further adjusted for participation in the randomized study.  
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5 P-value $\leq$ 0.05 is considered significant.  
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7 The data processing was conducted using SAS version 9.2 (SAS Institute, Cary, NC).  
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## 10 11 12 13 14 15 16 17 **Results**

### 18 19 20 *Baseline Characteristics: Vaccines and Mucosal Lining Fluid*

21 The study included 156 neonates of mothers participating in the randomized controlled trial of  
22 standard dose (7.5 $\mu$ g) of the H1N1pd09 , MF59-adjuvanted vaccine (Focetria®, Novartis) 51 of  
23 the neonates were born to mothers vaccinated during pregnancy and 105 were born to mothers  
24 vaccinated after pregnancy (Figure E1 in SDC).  
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29 A secondary analysis compared children of mothers vaccinated during pregnancy with the children  
30 of 332 non-vaccinated mothers (Figure E1 in SDC).  
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33 The median time from vaccination of the pregnant women to sampling of mucosal lining fluid was  
34 130 days (interquartile range, 95-146 days). The median age at mucosal lining fluid sampling in all  
35 neonates was 31 days (SD 5).  
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39 The 51 women vaccinated during pregnancy and their children did not differ from the 105 women  
40 vaccinated after pregnancy and their children or the 332 non-vaccinated and their children with  
41 respect to gender; older siblings; gestational age at birth; birth weight; Apgar score one min after  
42 birth; baseline antibody titers; mode of delivery; mother's age at birth; exposure to antibiotics,  
43 alcohol, and smoking during third trimester of pregnancy; paternal atopy; and household  
44 income (Table E1 in SDC).  
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### 50 51 *Baseline Characteristics: Diary Cards*

52 152 out of 156 families (97%) included in the randomized study returned their diary cards for the  
53 first year of life. For the secondary analysis, 284 out of 332 non-vaccinated families (86%), returned  
54 their diary cards, yielding a total clinical follow up on 436 children (Figure E1 in SDC). The mean  
55 number of infectious episodes pr. child was 8.2 (SD 4, range 0-24). Incidence of infections was  
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1 significantly associated to older siblings (IRR=1.11 [1.01-1.21], P=0.03) and birth season  
2 (IRR=0.88 [0.78-1.00], P=0.047 for children born in the Autumn compared to Summer, no  
3 significant difference between other seasons), but was not associated to maternal atopic disease or  
4 participation in the randomized study (Table 3).  
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#### 9 *Mucosal Immune Profiles in Neonates of Mothers Vaccinated During versus After Pregnancy*

10 Neonates of mothers vaccinated during pregnancy showed a significant up-regulation of TGF- $\beta$ 1  
11 (ratio=1.52 [1.22-1.90], P=0.0002) compared to neonates of mothers vaccinated after pregnancy.  
12 Additionally, we found a significant (P $\leq$ 0.05) down-regulation of IL-12p70, IFN- $\gamma$ , IL-5, eotaxin-1,  
13 TARC, MDC, IL-8, and a non-significant trend of down-regulation of IL-1 $\beta$  (P=0.06) and IP-  
14 10(P=0.10). Results are adjusted for season of birth, maternal atopy, older siblings, and airway  
15 colonization with pathogenic bacteria strains at sampling time. The adjustment induces greater  
16 effect size (estimates) and enhances the precision of the results. (Table 1 and Figure 1).  
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#### 24 *Effect of Lag-Time from Vaccination to Sampling of Mucosal Lining Fluid*

25 The lag-time from vaccination to the assessment of the immune signature showed a significant and  
26 positive association to the up-regulation of TGF- $\beta$ 1 levels (P= 0.0003) (Figure 2), and a significant  
27 (P $\leq$ 0.05) and negative association with IP-10, IFN- $\gamma$ , IL-5, Eotaxin-3, TARC, MDC, IL-8 and IL-10  
28 in the mucosal lining fluid (Figure E2 and Table E2 in SDC).  
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#### 34 *Mucosal Immune Profiles in Neonates of Mothers Vaccinated During Pregnancy versus non-* 35 *vaccinated Controls.*

36 Neonates of vaccinated mothers showed up-regulated TGF- $\beta$ 1 (ratio=1.32[1.11- 1.57], P=0.002)  
37 compared to neonates of non-vaccinated controls, a significant down-regulation of IFN- $\gamma$ , IL-5,  
38 TARC, MDC (P $\leq$ 0.05) and a non-significant trend of down-regulation of IL-12p70 (P=0.07), IL-4  
39 (P=0.08) and IL-13(P=0.07). Results are adjusted for participation in the randomized study, season  
40 of birth, maternal atopy, older siblings, and airway colonization with pathogenic bacteria strains at  
41 sampling time. (Table 2 and Figure 3).  
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#### 49 *Clinical Infections in Children of Mothers Vaccinated During Pregnancy Versus After Pregnancy* 50 *and non-vaccinated Controls*

51 There was no significant difference in the incidence of infections during the first year of life in  
52 children of mothers vaccinated during pregnancy versus children of mothers vaccinated after  
53 pregnancy (aIRR=1.10 [0.89-1.36], P=0.37). Also there was no significant difference in incidence  
54 of infections in children of mothers vaccinated during pregnancy and non-vaccinated controls  
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1 (aIRR=0.99[0.84-1.18], P=0.95) (Table 3).  
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## 6 **Discussion**

### 7 *Principle findings*

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10 Vaccination with H1N1pnd09 vaccine during pregnancy induces alternations in the primary  
11 mucosal line of defense in the upper airways of newborn children. TGF- $\beta$ 1 is up-regulated and  
12 other key-mediators linked to protective immunity are down-regulated suggesting a compromised  
13 local immune defense. This effect is enhanced the earlier in the pregnancy the woman receives  
14 vaccination.  
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20 We found no differences in the incidence of infections in the first year of life in the infants.  
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### 23 *Strengths and Limitations of the Study*

24 The H1N1pnd09 pandemic emerged in Denmark when the recruitment to our birth cohort was half  
25 completed. Therefore this “Experiment by Nature” allowed us to compare the effects of  
26 H1N1pnd09 vaccination in H1N1pnd09 -naïve women during pregnancy and post-partum.  
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30 Furthermore the design of the study enables us to assess the effect of the vaccine given at different  
31 time points during pregnancy allowing us to demonstrate that the immune suppression, as  
32 determined by TGF- $\beta$ 1 levels, was significantly associated with the lag-time from vaccination to  
33 birth.  
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38 It is a strength of this study that we see strong and similar response between all tested groups.  
39 Children of mothers vaccinated during pregnancy had up-regulated TGF- $\beta$ 1 compared to children  
40 of mothers receiving vaccine after pregnancy and children of non-vaccinated women. This was  
41 further confirmed by the significant association between the lag-time from vaccination in pregnancy  
42 to birth (sampling) and the TGF- $\beta$ 1 signal. Hereby the same conclusions are similar in 3 different  
43 models.  
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49 It is a further strength of this study that it is nested in the well-established ongoing COPSAC birth  
50 cohort clinical research center. The setup of trained research doctors following standard operating  
51 procedures ensures consistency in the data material. The birth cohort study is designed as a clinical  
52 study with deep phenotyping of mother and child including comprehensive information’s obtained  
53 at repeated visits to the research unit. This allowed an extensive comparison of the case and control  
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1 cohorts of women together with detailed clinical follow-up of the children thereby increasing data  
2 validity, reducing the data variation and strengthening the effect estimates.  
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5 The *in vivo* sampling of mucosal lining fluid has become a well validated method for non-invasive  
6 assessment of cytokines and chemokines in the airways of children<sup>28,26,29,31</sup> allowing us to measure  
7 undiluted samples directly from a target organ for respiratory disease, avoiding the dilemma of  
8 dilution accompanying nasal lavage models and intensified response found in challenge models.<sup>32,33</sup>  
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11 It is a limitation to our study that we do not know the exact agent the children were infected with  
12 during their infections recoded in the daily diaries. One could speculate that we would find fewer  
13 cases of influenza in the children of vaccinated mothers and thereby comparably more cases of  
14 other infections.  
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17 It is a limitation of this design that cases and controls did not receive the vaccinations at the same  
18 time of year. Most women were vaccinated before the pandemic reached Denmark by the end of  
19 2009, but we continued vaccinating the pregnant women into early 2010 and therefore these (few)  
20 pregnant women in the study had a higher risk of contracting the natural infection. This we  
21 controlled for by the baseline IgG specific for H1N1pnd09, which did not differ between groups.  
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24 Our findings cannot be uncritically extrapolated to other vaccines but must be limited to the  
25 H1N1pnd09 with MF59-adjuvanted vaccine we used. It is a limitation to the study that only 51  
26 women received the H1N1pnd09 vaccine during pregnancy. This limits the power to detect clinical  
27 effects on immune deviation from vaccination in pregnancy.  
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### 30 *Interpretation*

31 The children born of mothers receiving vaccination during pregnancy had significantly up-regulated  
32 TGF- $\beta$ 1 and down-regulation of Th1, Th2 and Th17 related mediators. TGF- $\beta$ 1 has specific effects  
33 associated with down-regulation of the transcription factors T-bet and GATA-3<sup>34</sup>, necessary for  
34 commitment of naïve CD4+ T-cells into Th1 and Th2 lineages, respectively. Moreover, both T-bet  
35 and GATA-3 activation are needed for maturation of cytotoxic NK cells, and NK functionality is  
36 thus also inhibited by TGF- $\beta$ 1<sup>34</sup>. Since Th1 and NK cells are both sources of IFN- $\gamma$ , and Th2 of IL-  
37 5, IL-4 and IL-13, the lower levels of IFN- $\gamma$  and IL-5 in neonates of vaccinated mothers could  
38 possibly be mediated via TGF- $\beta$ 1-induced suppression, but other mechanisms may also be in play.  
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41 The pattern we found is similar to the general pattern seen during pregnancy, where it serves to  
42 protect the fetus from rejection by the mother's immune response.<sup>35</sup> Activation of the mother's  
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1 adaptive immune reactions during pregnancy may in principle increase the risk of rejection of the  
2 fetus as reflected in the increased risk of abortion and preterm birth during Influenza infections .<sup>9,13</sup>

3 Hence, it may be hypothesized that the boosted adaptive immune response in the vaccinated  
4 mothers is responsible for the down-regulation of the fetal immune system seen in our study.  
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9 Down regulation of Th1 and Th17 response could be speculated to be counterproductive to  
10 clearance of infections but our study shows no difference in incidence of infections in the children.  
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12 The study size limits the strength of this conclusion and these findings must be subject to further  
13 scrutiny in larger randomized control studies.  
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17 Earlier studies on the protection of neonates by antenatal vaccination show that vaccination of the  
18 mother during pregnancy protects the infants against respiratory infections.<sup>36-38</sup> Some studies  
19 propose a protection against Influenza virus due to passively transmitted antibodies<sup>38</sup> while other  
20 studies propose a protection against Influenza morbidity caused by cocooning<sup>36-38</sup>. Yet other studies  
21 show the opposite and advocate an increased risk of respiratory infections in children of mothers  
22 vaccinated during pregnancy<sup>24</sup> the latter may be in line with our immunological findings.  
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25  
26 A study of a population based cohort suggested that maternal influenza infection during pregnancy  
27 was associated with a twofold increased risk of infantile autism.<sup>39</sup> Another study finds decreased  
28 neonatal levels of both Th-1 cytokines (i.e. IFN- $\gamma$ ) and Th-2 cytokines (i.e. IL-4, IL-10) in children  
29 later developing Autism Spectrum Disorders.<sup>40</sup> Our results link these observations by showing that  
30 maternal influenza down-regulates the neonatal level of cytokines and chemokines.  
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### 37 38 *Conclusion*

39 This is the first study to assess airway immunology in neonates of mothers vaccinated during  
40 pregnancy, thereby presenting an unprecedented opportunity to explore the influence of antenatal  
41 vaccination on neonatal mucosal cytokine and chemokine immunology. Our findings suggest that  
42 H1N1pnd09 vaccination during pregnancy affects the mucosal immune competence of the unborn  
43 child and that this affect is dependent of time from birth of the vaccination.  
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49 We observe a 1.6-fold increase in TGF- $\beta$ 1, along with decreased levels of the remaining immune  
50 mediators in upper airway of neonates from vaccinated mothers demonstrating that the fetal  
51 immune response is indeed affected by the maternal immune activation propagated by the  
52 vaccination protocol.  
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57 This is a notable finding, underscoring the necessity for research into vaccination programs in  
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1 pregnant women and their influence on the neonatal immunity, not only focusing on neonatal  
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3 immunity against the specific disease and the vaccine antigens, but the general immune status of the  
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5 newborn child.  
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1 **Abbreviations**

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4 Th – T helper cells

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6 IFN- $\gamma$  – Interferon-gamma

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8 IL – Interleukin

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10 TNF- $\alpha$  – Tumor necrosis factor-alpha

11  
12 Eotaxin-1 (CCL11)

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14 Eotaxin-3 (CCL26 )

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16 IP-10 – Interferon gamma-induced protein 10 kDa (CXCL10)

17  
18 MCP-1 – Monocyte chemotactic protein 1 (CCL2)

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20 MCP-4 – Chemokine (CC-motif) ligand13 (CCL13)

21  
22 MDC – Macrophage-Derived Chemokine (CCL22)

23  
24 MIP-1 $\beta$  – Macrophage Inflammatory Protein-1 $\beta$  (CCL4)

25  
26 TARC – Thymus and Activation Regulated Chemokine (CCL17)

27  
28 TGF- $\beta$ 1 – Transforming Growth Factor beta 1

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## Tables and Figures

### Tables

**Table 1:** Primary analysis of mediator levels in neonates born to mothers vaccinated during pregnancy versus mediator levels in neonates born to mothers vaccinated after pregnancy.

\*Adjusted for season of birth, maternal atopy, older siblings, and bacterial colonization.

° False discovery rate <0.05 (Benjamini-Hochberg procedure for multiple testing correction)

• Th-1      • Th-2      • Th17      • Treg      • Lymphocyte expansion

Mediator	Unadjusted	Adjusted
	Ratio[CI-low, CI-high] <i>P</i> -value	* Ratio[CI-low, CI-high] <i>P</i> -value
IL-12p70	0.74[0.52, 1.04] <i>P</i> =0.08	0.62[0.40, 0.93] <b><i>P</i>=0.02</b>
IP-10	0.92[0.54, 1.58] <i>P</i> =0.77	0.58[0.30, 1.11] <i>P</i> =0.10
IFN- $\gamma$	0.62[0.40, 0.97] <b><i>P</i>=0.04</b>	0.33[0.19, 0.57] <b><i>P</i>=0.0001'</b>
TNF- $\alpha$	1.04[0.60, 1.80] <i>P</i> =0.88	0.78[0.41, 1.46] <i>P</i> =0.43
MIP-1 $\beta$	1.54[0.86, 2.73] <i>P</i> =0.14	0.95[0.50, 1.82] <i>P</i> =0.88
MCP-1	1.16[0.83, 1.62] <i>P</i> =0.39	0.83[0.54, 1.27] <i>P</i> =0.39
MCP-4	1.35[1.09, 1.67] <b><i>P</i>=0.006</b>	1.27[0.97, 1.66] <i>P</i> =0.08
IL-4	0.75[0.49, 1.16] <i>P</i> =0.19	0.79[0.47, 1.34] <i>P</i> =0.38
IL-5	0.81[0.52, 1.26] <i>P</i> =0.35	0.49[0.30, 0.82] <b><i>P</i>=0.0073'</b>
IL-13	0.72[0.51, 1.00] <i>P</i> =0.05	0.77[0.51, 1.14] <i>P</i> =0.19
Eotaxin-1	0.97[0.72, 1.31] <i>P</i> =0.86	0.66[0.45, 0.97] <b><i>P</i>=0.04</b>
Eotaxin-3	0.99[0.68, 1.46] <i>P</i> =0.98	0.72[0.44, 1.18] <i>P</i> =0.19
TARC	0.76[0.60, 0.97] <b><i>P</i>=0.03</b>	0.54[0.40, 0.72] <b><i>P</i>=7.0·10<sup>-5</sup>,</b>
MDC	0.63[0.46, 0.85] <b><i>P</i>=0.003</b>	0.38[0.26, 0.54] <b><i>P</i>=4.8·10<sup>-7</sup>,</b>
IL-17	1.48[0.75, 2.94] <i>P</i> =0.26	1.29[0.54, 3.10] <i>P</i> =0.56
IL-1 $\beta$	0.81[0.41, 1.61] <i>P</i> =0.54	0.48[0.22, 1.04] <i>P</i> =0.06
IL-8	0.94[0.59, 1.52] <i>P</i> =0.81	0.56[0.32, 1.00] <b><i>P</i>=0.05</b>
TGF- $\beta$ 1	1.38[1.16, 1.64] <b><i>P</i>=0.0004</b>	1.52[1.22, 1.90] <b><i>P</i>=0.0002'</b>
IL-10	0.82[0.49, 1.36] <i>P</i> =0.44	0.71[0.40, 1.26] <i>P</i> =0.24
IL-2	1.09[0.71, 1.67] <i>P</i> =0.69	0.99[0.60, 1.64] <i>P</i> =0.97

**Table 2:** Secondary analysis of mediator levels in neonates born to mothers vaccinated during pregnancy versus mediator levels in neonates of non-vaccinated controls.

\*Adjusted for participation in the randomized study, season of birth, maternal atopy, older siblings, and bacterial colonization.

’ False discovery rate <0.05 (Benjamini-Hochberg procedure for multiple testing correction)

• Th-1      • Th-2      • Th17      • Treg      • Lymphocyte expansion

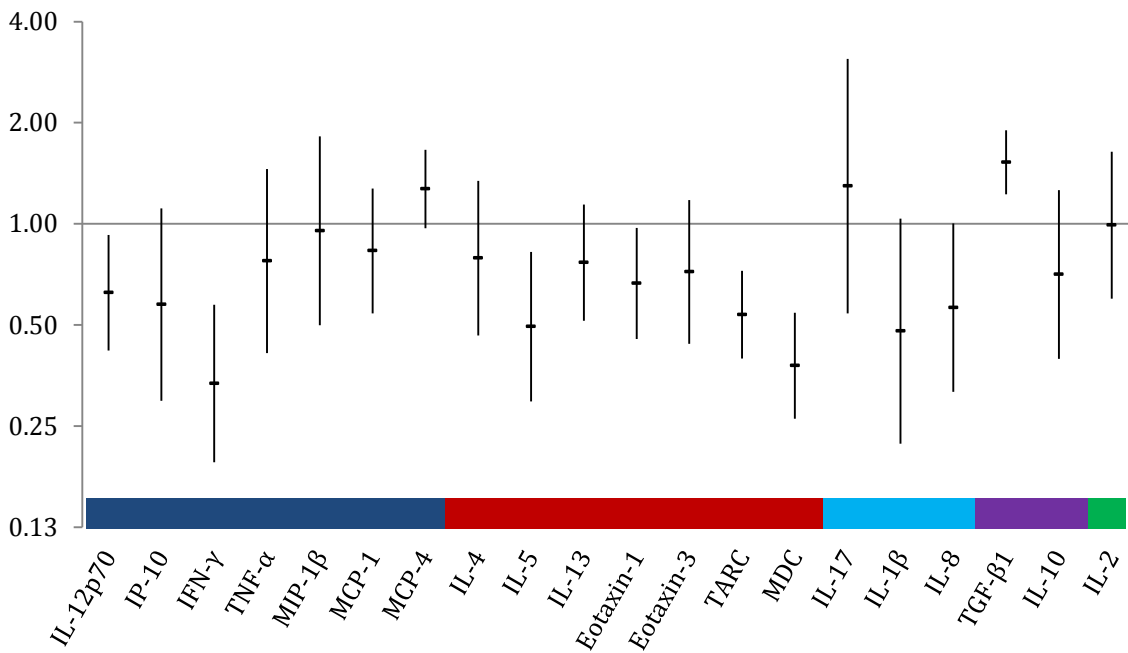
Mediator	Unadjusted		Adjusted	
	Ratio	[CI-low, CI-high] <i>P</i> -value	* Ratio	[CI-low, CI-high] <i>P</i> -value
IL-12p70	0.95	[0.68, 1.33] <i>P</i> =0.78	0.71	[0.48, 1.03] <i>P</i> =0.07
IP-10	1.27	[0.82, 1.96] <i>P</i> =0.28	0.88	[0.52, 1.48] <i>P</i> =0.63
IFN- $\gamma$	1.13	[0.73, 1.73] <i>P</i> =0.59	0.49	[0.30, 0.81] <b><i>P</i>=0.005’</b>
TNF- $\alpha$	1.10	[0.70, 1.75] <i>P</i> =0.68	0.93	[0.55, 1.58] <i>P</i> =0.80
MIP-1 $\beta$	1.24	[0.76, 2.01] <i>P</i> =0.39	1.14	[0.66, 1.94] <i>P</i> =0.64
MCP-1	1.16	[0.87, 1.55] <i>P</i> =0.31	1.07	[0.75, 1.52] <i>P</i> =0.73
MCP-4	1.24	[1.01, 1.51] <b><i>P</i>=0.04</b>	1.19	[0.94, 1.52] <i>P</i> =0.15
IL-4	0.70	[0.47, 1.05] <i>P</i> =0.09	0.65	[0.40, 1.05] <i>P</i> =0.08
IL-5	0.80	[0.54, 1.18] <i>P</i> =0.26	0.56	[0.36, 0.88] <b><i>P</i>=0.01</b>
IL-13	0.82	[0.60, 1.12] <i>P</i> =0.22	0.71	[0.49, 1.03] <i>P</i> =0.07
Eotaxin-1	1.02	[0.80, 1.30] <i>P</i> =0.86	0.90	[0.67, 1.21] <i>P</i> =0.48
Eotaxin-3	1.34	[0.93, 1.95] <i>P</i> =0.12	0.99	[0.63, 1.55] <i>P</i> =0.97
TARC	1.02	[0.81, 1.27] <i>P</i> =0.88	0.72	[0.55, 0.94] <b><i>P</i>=0.02</b>
MDC	1.14	[0.87, 1.48] <i>P</i> =0.34	0.60	[0.44, 0.82] <b><i>P</i>=0.001’</b>
IL-17	1.34	[0.77, 2.35] <i>P</i> =0.30	1.22	[0.63, 2.38] <i>P</i> =0.55
IL-1 $\beta$	1.03	[0.57, 1.87] <i>P</i> =0.92	0.63	[0.32, 1.23] <i>P</i> =0.17
IL-8	1.34	[0.91, 1.97] <i>P</i> =0.14	0.81	[0.51, 1.28] <i>P</i> =0.36
TGF- $\beta$ 1	1.07	[0.92, 1.25] <i>P</i> =0.35	1.32	[1.11, 1.57] <b><i>P</i>=0.002’</b>
IL-10	0.93	[0.63, 1.39] <i>P</i> =0.73	0.85	[0.53, 1.35] <i>P</i> =0.49
IL-2	1.09	[0.77, 1.54] <i>P</i> =0.63	1.10	[0.73, 1.65] <i>P</i> =0.65

**Table 3:** Risk of infections in offspring during the first year of life

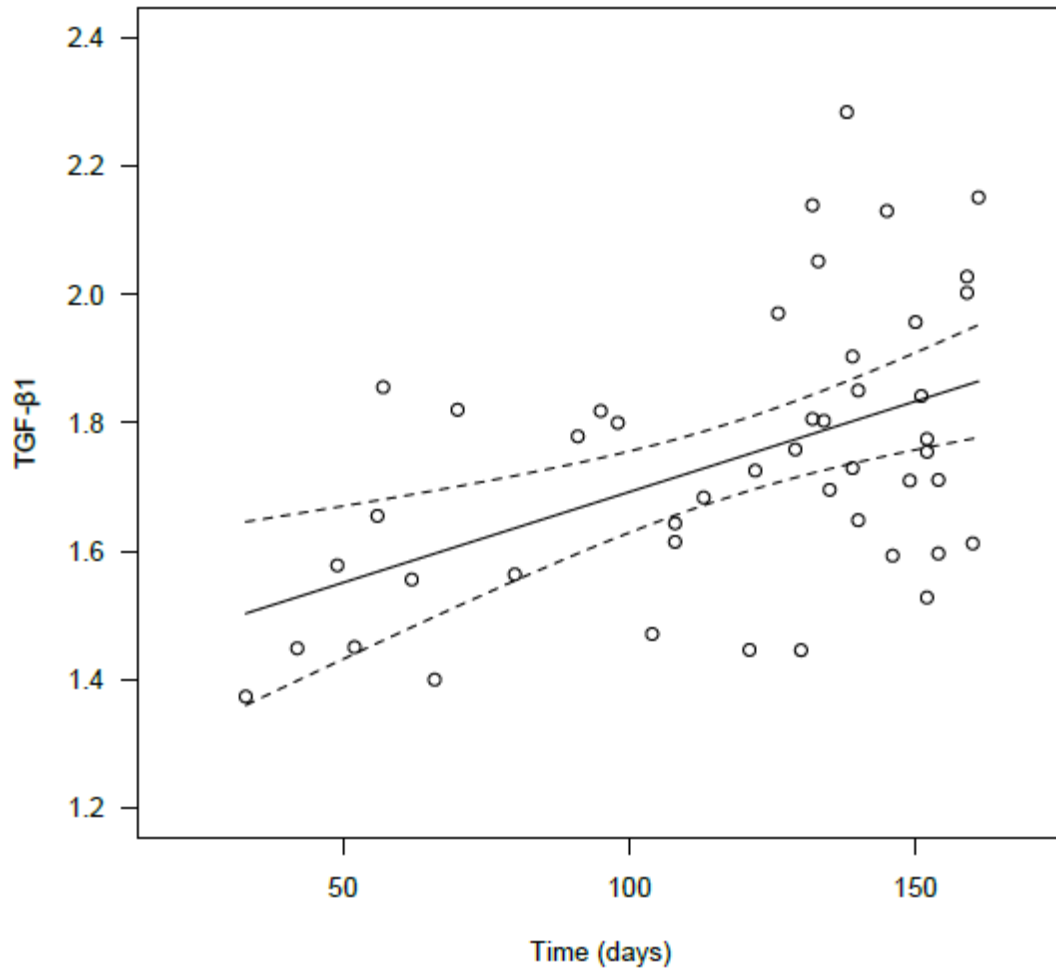
<b>Risk of infections</b>	crude IRR[CI-low, CI-high] <i>P</i> -value	aIRR[CI-low, CI-high] <i>P</i> -value
Vaccinated during pregnancy vs. after birth	0.98[0.83;1.16] <i>P</i> =0.81	1.10[0.89;1.36]* <i>P</i> =0.37
Vaccinated during pregnancy vs. non-vaccinated controls	1.00[0.86;1.15] <i>P</i> =0.95	0.99[0.84;1.18]‡ <i>P</i> =0.95
*Adjusted for season of birth, maternal atopy, and older siblings.		
‡ Adjusted for season of birth, maternal atopy, older siblings, and participation in the randomized study.		
<b>Confounding variables</b>	crude IRR[CI-low, CI-high] <i>P</i> -value	
Older siblings	1.11[1.01;1.21] <b><i>P</i>=0.03</b>	
Birth season (ref=Summer)		
Autumn	0.88[0.78;1.00] <b><i>P</i>=0.047</b>	
Spring	1.02[0.89;1.17] <i>P</i> =0.77	
Winter	0.99[0.88;1.11] <i>P</i> =0.81	
Maternal Atopic Disease (yes/no)	1.02[0.93;1.11] <i>P</i> =0.70	
Participation in RCT	1.04[0.92;1.12] <i>P</i> =0.75	

**Figure 1:** Ratios [95% confidence interval] between mean values of cytokine and chemokine, in the upper airway mucosal lining fluid in healthy neonates of mothers vaccinated during pregnancy *versus* neonates born to mothers vaccinated after pregnancy. Data are adjusted for season of birth, maternal atopy, older siblings, and bacterial colonization.

• Th-1     
 • Th-2     
 • Th17     
 • Treg     
 • Lymphocyte expansion

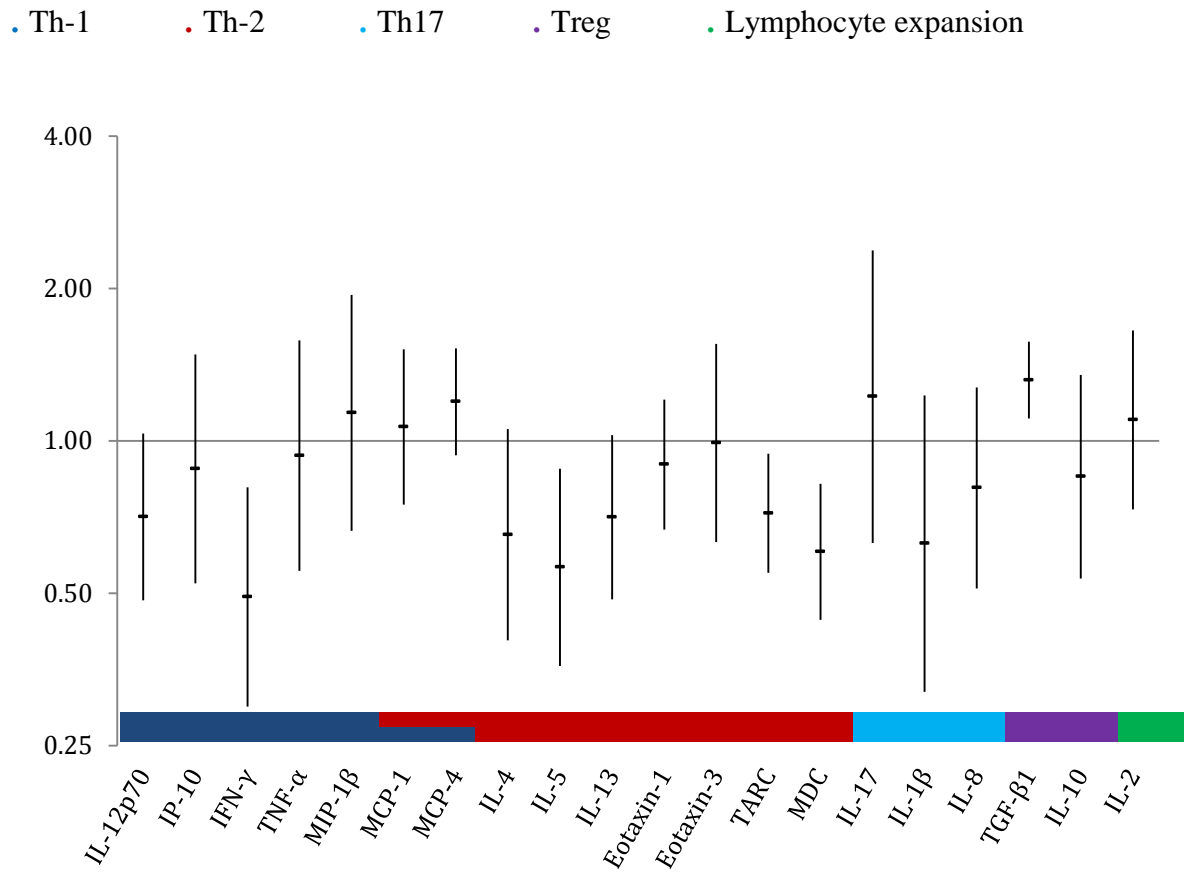


**Figure 2:** TGF- $\beta$ 1 concentration vs. days from H1N1pnd09-vaccination during pregnancy to sampling of mucosal lining fluid from the neonatal offspring.



**Figure 3** Ratios [95% confidence interval] between mean values of cytokine and chemokine, in the upper airway mucosal lining fluid in healthy neonates of mothers vaccinated during pregnancy versus neonates of non-vaccinated controls.

All data is adjusted for participation in the randomized study, season of birth, maternal atopy, older siblings, and bacterial colonization.





## Supplemental Digital Content

### **Airway Mucosal Immune-Suppression in Neonates of Mothers Receiving A(H1N1)pnd09 Vaccination During Pregnancy**

Anne Louise Bischoff \*, MD; Nilofar V Følsgaard\*, MD, PhD; Nadja H Vissing, MD; Sune Birch, MSE; Susanne Brix, MSci, PhD; Hans Bisgaard, MD, DMSci

#### **Methods**

Time from vaccination to sampling of mucosal lining fluid: The time in days from vaccination to sampling of MLF was used as continuous variable in the children born to mothers vaccinated during pregnancy.

Season of birth: Date of birth was grouped into 4 seasons: winter (December-February), spring (March-May), summer (June-August) and fall (September-November).

Older siblings at birth (yes/no): Children living in the home at the time of birth were considered as siblings.

Bacterial colonization of the airway (yes/no): Aspiration from the hypopharyngeal area was done intermittently assuring no suction was applied during retraction through the oropharynx and nasopharynx. The catheter was flushed with 1 ml of saline into a vessel for remaining secretions from the tube. Samples were transported at room temperature to the microbiology laboratories within 24 hours at the Department of Microbiological Surveillance and Research, Statens Serum Institute in Copenhagen, Denmark. Colonization with *Streptococcus pneumoniae*, *Hemophilus influenzae* and/or *Moraxella catarrhalis* was considered as bacterial colonization with airway pathogenic bacteria.

Maternal atopy (yes/no): Maternal atopic status was based on the history of doctor diagnosed asthma, hay fever and/or atopic dermatitis independent of sensitization.

Exposures during 3<sup>rd</sup> trimester of pregnancy included smoking (yes/no), alcohol consumption (yes/no), and antibiotic usage (yes/no).

Paternal atopy was defined from a history of doctor diagnosed asthma, hay fever and/or atopic dermatitis. (yes/no).

Birth details comprised way of delivery (normal birth, planned or acute section); birth weight; gestational age; and Apgar score below or above four one minute after birth.

Baseline antibody titers: Serum was sampled in the mothers prior to vaccination (baseline). Serum plasma levels of specific H1N1pnd09 antibodies were determined in twofold dilutions in a conventional hemagglutination-inhibition assay<sup>39</sup>. Geometric mean titers were computed from the log<sub>10</sub>-transformed mean.

Socioeconomic status was evaluated from yearly household income at birth of the infant classified into five groups as <54·000 €; 54·000-81·000 €; 81·000-108·000 €; 108·000-135·000 €; and >135·000 €.

## Online Tables

**Table E1:** Baseline characteristic of the study subjects

	Vaccinated during pregnancy	Vaccinated after birth	Non-vaccinated Controls	P-value
N	51	105	332	
Sex (Male) %(n)	57(29)	53(56)	49(163)	0.52 <sup>F</sup>
Birth weight, median(IQR)	3.6(3.3-3.9)	3.6(3.1-3.8)	3.5(3.2-4.0)	0.18 <sup>A</sup>
Gestational age, median(IQR)	40.0(39.4-40.9)	40.0(39.1-41.0)	40.1(39.0-41.1)	0.83 <sup>W</sup>
Apgar (1 min) ≤4 %(n)	0	2(2)	2(8)	0.88 <sup>F</sup>
Mode of delivery				0.73 <sup>F</sup>
Normal	80(41)	73(77)	78(259)	
Planned sectio	8(4)	11(12)	11(37)	
Acute sectio	12(6)	15(16)	11(36)	
<b>Season of birth</b>				<b>3.2*10<sup>-24</sup></b> <sup>F</sup>
Spring	14(7)	10(11)	20(65)	
Summer	12(6)	59(62)	21(69)	
Fall	29(15)	30(32)	20(66)	
Winter	45(23)	0	40(132)	
Airway bacteria* (4 week)%(n)	28(14)	21(22)	32(106)	0.09 <sup>F</sup>
Older siblings %(n)	51(26)	65(68)	61(201)	0.26 <sup>F</sup>
Baseline antibody titer, GMT(CI)	11(8-15)	13(11-17)	-	0.29 <sup>A</sup>
Antibiotics (3 <sup>rd</sup> trim) %(n)	24(12)	16(17)	21(69)	0.47 <sup>F</sup>
Smoking (3 <sup>rd</sup> trim) %(n)	8(4)	4(4)	4(13)	0.44 <sup>F</sup>
Alcohol (3 <sup>rd</sup> trim) %(n)	6(3)	7(7)	3(11)	0.23 <sup>F</sup>
Income (pr. year) %(n)				0.57 <sup>F</sup>
<54.000 €	8(4)	13(14)	9(29)	
54.000-134.000 €	80(41)	79(83)	80(263)	
>134.000 €	12(6)	8(8)	11(37)	
Mothers age, median(IQR)	32.5(29.5-35.5)	32.0(29.4-35.6)	31.7(29.0-35.0)	0.45 <sup>A</sup>
<b>Maternal atopy %(n)</b>	<b>39(20)</b>	<b>48(50)</b>	<b>55(181)</b>	<b>0.07<sup>F</sup></b>
Paternal atopy %(n)	38(18)	46(47)	46(147)	0.52 <sup>F</sup>
IQR: Inter Quartile Range				
GMT: Geometric Mean Titer				
<sup>F</sup> Fisher's exact test				
<sup>W</sup> Wilcoxon Rank sum test				
<sup>A</sup> Anova				
*Airway colonization with at least one of: <i>M. Catharalis</i> , <i>H. Influenza</i> , <i>S. Pneumonia</i>				

**Table E2:** Univariate analysis of association between mucosal lining fluid mediator levels and time in days from H1N1pnd09-vaccination with to sampling of mucosal lining fluid.

\*Adjusted for season of birth, maternal atopy, older siblings, and bacterial colonization.

’ False discovery rate <0.05 (Benjamini-Hochberg procedure for multiple testing correction)

• Th-1 • Th-2 • Th17 • Treg • Lymphocyte expansion

Mediator	Unadjusted	Adjusted
	[CI-low, CI-high] <i>P</i> -value	*[CI-low, CI-high] <i>P</i> -value
IL-12p70	[0.98, 1.00] <i>P</i> =0.07	[0.98, 1.00] <i>P</i> =0.16
IP-10	[0.98, 1.00] <i>P</i> =0.03	[0.97, 1.00] <i>P</i> =0.08
IFN- $\gamma$	[0.98, 1.00] <i>P</i> =0.05	[0.98, 1.00] <i>P</i> =0.17
TNF- $\alpha$	[0.98, 1.00] <i>P</i> =0.15	[0.98, 1.01] <i>P</i> =0.37
MIP-1 $\beta$	[0.97, 1.00] <i>P</i> =0.07	[0.98, 1.01] <i>P</i> =0.36
MCP-1	[0.99, 1.00] <i>P</i> =0.24	[0.99, 1.01] <i>P</i> =0.84
MCP-4	[1.00, 1.01] <i>P</i> =0.72	[1.00, 1.01] <i>P</i> =0.58
IL-4	[0.99, 1.01] <i>P</i> =0.63	[0.99, 1.01] <i>P</i> =0.89
IL-5	[0.97, 0.99] <i>P</i> =0.001’	[0.97, 1.00] <i>P</i> =0.01
IL-13	[0.99, 1.00] <i>P</i> =0.18	[0.98, 1.00] <i>P</i> =0.09
Eotaxin-1	[0.99, 1.00] <i>P</i> =0.10	[0.99, 1.01] <i>P</i> =0.64
Eotaxin-3	[0.98, 1.00] <i>P</i> =0.01’	[0.98, 0.99] <i>P</i> =0.003’
TARC	[0.99, 1.00] <i>P</i> =0.03	[0.99, 1.00] <i>P</i> =0.05
MDC	[0.98, 1.00] <i>P</i> =0.01’	[0.98, 1.00] <i>P</i> =0.02
IL-17	[0.98, 1.01] <i>P</i> =0.25	[0.98, 1.01] <i>P</i> =0.62
IL-1 $\beta$	[0.98, 1.01] <i>P</i> =0.21	[0.98, 1.01] <i>P</i> =0.67
IL-8	[0.98, 1.00] <i>P</i> =0.03	[0.98, 1.01] <i>P</i> =0.35
TGF- $\beta$ 1	[1.00, 1.01] <i>P</i> =0.0004’	[1.00, 1.01] <i>P</i> =0.0003’
IL-10	[0.98, 1.00] <i>P</i> =0.03	[0.97, 1.00] <i>P</i> =0.01
IL-2	[0.99, 1.00] <i>P</i> =0.30	[0.99, 1.01] <i>P</i> =0.76

## Online Figures

**Figure E1:** Flowchart of study participants



**Figure E2:** IL-5 concentration vs. days from H1N1pnd09-vaccination during pregnancy to sampling of mucosal lining fluid from the neonatal offspring.

