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Title	Effects of prenatal exposure to perfluoroalkyl acids on prevalence of allergic diseases among 4-year-old children
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Citation	Environment international, 94, 124-132 https://doi.org/10.1016/j.envint.2016.05.020
Issue Date	2016-09
Doc URL	https://hdl.handle.net/2115/71412
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Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	EnvironInt94_124.pdf

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Effects of prenatal exposure to perfluoroalkyl acids on prevalence of allergic diseases among 4-year-old children

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Abstract

Perfluoroalkyl acids (PFAAs) are ubiquitous chemicals extremely resistant and widespread throughout the environment, frequently being detected in human blood samples. Animal studies have revealed that exposure to PFAAs results in immunotoxicity. However, the association between PFAAs, especially long-chain PFAAs, and allergies in humans is not well established. We examined whether prenatal exposure to PFAAs is associated with allergic diseases among 4-year-old children in a large-scale prospective birth cohort in Hokkaido, Japan. 1558 Mother-child pairs were included in this study and prenatal levels of eleven PFAAs were measured in maternal plasma samples obtained between 28 and 32 weeks of pregnancy by using ultra-performance liquid chromatography-tandem mass spectrometry. Participant demographic and characteristic information were obtained from self-administered pre- and postnatal questionnaires and medical birth records. Infant allergies were assessed using the Japanese version of the International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three questionnaire, which was administered 4 years post-delivery including eczema, wheezing and rhinoconjunctivitis with prevalence of 19.0%, 18.7%, and 5.4%, respectively. Associations of PFAA quartiles with allergic outcomes were examined using logistic models. Adjusted odds ratios (ORs) in the 4^{th} quartile vs. 1^{st} quartile (Q4 vs. Q1) for total allergic diseases (including at least one allergic outcome) significantly decreased for perfluorododecanoic acid (PFDoDa) (Q4 vs. Q1 OR: 0.621; 95% confidence interval (CI):

0.454, 0.847) and perfluorotridecanoic acid (PFTrDA) (Q4 vs. Q1 OR: 0.712; 95% CI: 0.524, 0.966) in all children. We found similar results when examining the association between PFAAs and eczema. The adjusted OR (Q4 vs. Q1) for wheezing in relation to higher maternal PFHxS levels was 0.728 (95% CI: 0.497, 1.06) in all children. In conclusion, prenatal exposure to long-chain PFAAs, such as PFDoDa and PFTrDA, may have immunosuppressive effects on allergic diseases in 4-year-old children.

58 1. Introduction

59 Perfluoroalkyl acids (PFAAs) are ubiquitous chemicals with widespread occurrence in the
60 environment, animals, and humans. PFAAs are synthetic chemicals with varying carbon
61 lengths (4 to 14 carbons) that exhibit high thermal and chemical stability owing to fluorine-
62 carbon covalent bonding. PFAAs have extensive industrial applications, such as in textile
63 impregnation, furnishings, non-stick housewares, and food packaging (Kannan et al., 2004;
64 Butenhoff et al., 2006; Fromme et al., 2009). The major routes of exposure to PFAAs are
65 through contaminated food, water, and house dust (Kato et al., 2009; Vestergren et al., 2012).
66 The most commonly used PFAAs are perfluorooctane sulfonic acid (PFOS) and
67 perfluorooctanoic acid (PFOA). While PFOS and PFOA are being actively phased out by
68 several industries, they are still present in older products. PFAAs are resistant to metabolism,
69 with long elimination half-lives of 3.8, 5.4, and 8.5 years in humans for PFOA, PFOS, and
70 perfluorohexane sulfonate (PFHxS), respectively (Olsen et al., 2007). The stability and long
71 half-lives of PFAAs contribute to their continued presence in the environment and to human
72 exposure.

73 Animal studies revealed the resulting endocrine disruption, growth, and neuro- and
74 hepatotoxic properties of PFOS and PFOA (Lau et al., 2003; Leubker et al., 2005; Seacat et al.,
75 2003). Exposure to PFOS and PFOA in animals decreased lymphoid organ weights, and
76 reduced the number of lymphoid cells and antibody production (Yang et al. 2001, 2002; Peden-

77 Adams et al., 2007). In animals, PFOS and PFOA inhibit the T-cell-dependent immunoglobulin
78 M (IgM) antibody response (TDAR), which is an essential mediator of immune system
79 function. For appropriate TDAR, proper function of B cells, T cells, and antigen presenting
80 cells is necessary to produce antibodies. Immune system development starts during the fetal
81 period, and in utero exposure of fetuses to chemicals can alter the development of immune
82 cells, contributing to a modified risk of developing allergic diseases in postnatal life (Luster et
83 al., 1992; DeWitt et al., 2012). Keil et al. (2008) examined the effects of gestational exposure
84 to PFOS and PFOA on the immune function of mice, 4 and 8 weeks following birth and found
85 that functional immune system deficits were not evident until 8 weeks of age when natural
86 killer cell function and IgM production were significantly reduced. This suggests an age-related
87 immune response effect during postnatal life following in utero exposure to PFAAs.
88 PFAAs can pass through the placental barrier during pregnancy and infants are exposed to
89 these chemicals through lactation and indoor house dust (Inoue et al., 2004; Vestergren et al.,
90 2012; Cariou et al., 2015). Exposure to PFAAs during this critical window of susceptibility
91 may affect several aspects of health later in life, including immune function. Previous
92 epidemiological studies proposing immunomodulatory effects of PFAAs indicated that prenatal
93 exposure to PFOS and PFOA was associated with levels of IgE in cord blood with conflicting
94 results (Wang et al., 2011; Okada et al., 2012). Additionally, pre- and post-natal exposure to
95 PFOS and PFOA are related to reduced antibody levels of tetanus and diphtheria (Grandjean et

96 al., 2012), and rubella (Granum et al., 2013) in children, and influenza in adults (Looker et al.,
97 2014). In addition, Fletcher et al. (2009) reported an inverse association between immunologic
98 biomarkers such as IgA, IgE, and C-reactive protein with high PFOA exposure levels in mid-
99 Ohio Valley residents living near a plant that contaminated the water supply with PFOA.
100 Recent studies revealed a trend of decreasing PFOA and PFOS levels in the general
101 population of the United States (Kato et al., 2009; Olsen et al., 2012) and European countries
102 such as Sweden and Germany (Glyn et al., 2012; Schroder-Kermani et al., 2012). Although we
103 previously reported a declining trend of PFOS and PFOA, we observed an increasing trend for
104 perfluorononanoic acid (PFNA, C9) and perfluorodecanoic acid (PFDA, C10) levels among
105 pregnant women between 2003 and 2011 in Hokkaido, Japan (Okada et al. 2013). We
106 previously reported negative association of PFTrDA and eczema in infancy in the same cohort
107 (Okada et al. 2014). Although some animal experiments suggest prenatal PFAA exposure
108 modifies the postnatal immune response throughout the period of early childhood (Keil et al.
109 2008), the long-term effects of PFAAs, including long-chain PFAAs, on allergic diseases in
110 childhood are not well understood thus far. In this study, we examined the association of 11
111 prenatal PFAA levels with allergic diseases among 4-year-old children assessed by ISAAC
112 questionnaires in a prospective birth cohort.
113
114 2. Methods

115 2.1. Study population

116 The current work is a part of a large ongoing birth cohort of over 20,000 mother-infant pairs
117 that were recruited through the Hokkaido Study on Environment and Children's health. The
118 details of this study have previously been described (Kishi et al. 2011 and 2013). This study
119 was initiated in February 2003 with native Japanese mother-child pairs. Pregnant women who
120 attended prenatal visits in early pregnancy (>13 weeks of gestational age) at any of the 37
121 participating hospitals and clinics in the Hokkaido prefecture were eligible for this study.
122 Health care personnel approached the pregnant women during these visits to explain the study.
123 Among 33,500 eligible women from 2003 to 2009, 17,869 mothers agreed to participate in this
124 study. Of these, we selected 12,847 who had submitted a baseline questionnaire with available
125 third trimester blood samples and hospital birth records. After exclusion of cases with
126 miscarriage and stillbirth (n = 19), congenital malformation (n = 143), and multiple births (n =
127 162), we selected 6335 participants who had completed all three postnatal questionnaires at 4,
128 12, and 24 months after birth. Among these, we randomly selected 300 participants per year
129 from 2003 to 2008 and 295 participants in 2009 (n=2095) for PFAA measurement in maternal
130 plasma samples (Okada et al., 2014). After exclusion of cases with congenital malformations
131 that became apparent following completion of the follow-up questionnaire at 12 months (n=17),
132 maternal blood samples obtained before 26 weeks of gestation (n=15), withdrawal (n=6), and
133 an extremely high PFOS level (n=1); among remaining 2056 mother child pairs, 1558 mother-

child pairs sent us questionnaires pertaining to their 4-year-old children for inclusion in the current study (Supplementary data, Figure S1).

This study was conducted after obtaining all of the participants' written informed consent from the time of pregnancy up to two years following delivery. Informed consent was also obtained when the children reached 4 years of age. The institutional ethics board for epidemiological studies at Hokkaido University Center for Environmental and Health Sciences and Hokkaido University Graduate School of Medicine approved the study protocol.

2.2. Data collection

Mothers completed a self-administered baseline questionnaire during the first trimester of pregnancy, which included parental information related to age, prepregnancy BMI, previous medical history, educational level, annual household income, parity, alcohol consumption, medication, and smoking during pregnancy. We extracted information on gestational age, infant gender, and birth weight, as well as miscarriage, stillbirth, multiple births, and congenital anomalies from medical birth records. We collected a self-administered questionnaire at 4 months following delivery, including information about birth size, maternal complications during pregnancy, and maternal smoking status in the third trimester, as reported by mothers. At 4 years post-delivery, participants completed another self-administered questionnaire collecting information related to infant size, breast feeding, smoking status of parents, parental history of allergic diseases, pets in the home, cooling/heating system at homes, environmental

153 tobacco smoke (ETS) exposure and day care attendance. ETS exposure was defined as a self-
154 reported positive response to the presence of a smoker in the environment where children lived
155 daily life at 4 years of age. At this time point, mothers reported any previous or current medical
156 history of infant allergic diseases including eczema, wheezing, and rhinoconjunctivitis
157 symptoms.

158 2.3. Exposure assessment

159 Detailed sample preparation and PFAAs measurement methods have been previously described
160 (Okada et al., 2013). In brief, we collected a 10-mL blood sample from the maternal peripheral
161 vein between 28 and 32 weeks of pregnancy with samples being stored at -80ºc until analysis.
162 We used maternal plasma for exposure assessment using ultra-performance liquid
163 chromatography, coupled with triple quadrupole tandem mass spectrometry instrumentation
164 (UPLC-MS/MS) (Waters, USA). We measured concentrations of 11 PFAAs: PFSAs
165 (perfluoroalkane sulfonates) including PFHxS, PFOS; and PFCAs (perfluorinated carboxylic
166 acids) including perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA,
167 PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, perfluorotetradecanoic acid (PFTeDA) in
168 maternal plasma samples obtained during the third trimester of pregnancy.

169 2.4. Outcome assessment

170 12 months prevalence of three allergic diseases were assessed based on the mothers' self-
171 administered questionnaires, obtained 4 years post-delivery. Allergic diseases were defined

using a modified section of the Japanese version of the International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three questionnaire (Asher et al., 2006). Eczema was defined based on positive answers to all three of the following questions: “Has your child had this itchy rash at any time in the past 12 months?”, “Has your child ever had a recurrent skin rash for at least 6 months?”, and “Has this itchy rash at any time affected any of the following places: the folds of the elbows; behind the knees; in front of the ankles; under the buttocks; or around the neck, ears, or eyes?”. Wheezing was defined as a positive answer to the question: “Has your child had wheezing or whistling in the chest in the past 12 months?” Current rhinoconjunctivitis symptoms were assessed based on positive answers to both of the following questions: “In the past 12 months, has your child had a problem sneezing or a runny or blocked nose when he or she did not have a cold or the flu?” and if the answer is positive, “In the past 12 months, has this nose problem been accompanied by itchy watery eyes?” (Asher et al., 2006). We also defined total allergic diseases as cases with at least one of the symptoms associated with eczema, wheezing, or rhinoconjunctivitis.

2.5. Data analysis

We performed all of the statistical analyses using JMP pro 10 (SAS Institute Inc., NC, USA). The results were considered statistically significant if $p < 0.05$. For participants with PFAA levels less than MDL, a value equal to half of the MDL was substituted. We divided participants into four groups according to quartiles (Q) of prenatal PFAA levels. In crude and adjusted

191 logistic regression analyses, we examined associations between prenatal PFAA concentrations
192 and prevalence of allergic diseases. Odds ratios (ORs) for the risk of allergic diseases were
193 evaluated for PFAA levels in the second through fourth quartiles and compared to those in the
194 lowest quartiles. For calculation of p for trend, we used the linear contrast coefficients -3, -1,
195 +1, +3 assigned to quartiles 1, 2, 3, and 4, respectively (Kishi et al., 2015; Goudarzi et al.,
196 2015). We examined the effects on total allergic diseases and on each allergic symptom
197 separately. We selected study confounders according to a review of the literature and based on
198 the change in estimate criteria, which was set to a value of greater than 10%. Potential
199 confounding variables considered in the analysis were maternal age (continuous), number of
200 older siblings (0, ≥1), maternal education (≤12, >13 years), parental allergic history (yes/no),
201 infant gender, breast-feeding period (<6, ≥6 months), day care attendance (yes/no), and
202 environmental tobacco smoke (ETS) exposure at the age of 4 years (yes/no). The number of
203 older siblings was obtained from parity information. Due to potential sex differences of PFAA
204 health effects, we also stratified the results by sex.
205
206 3. Results.
207 In total, 1,558 mother-child pairs were included in this study. The average maternal age
208 (SD) was 31.1 (4.4), and prepregnancy BMI (SD) was 20.9 (2.9). 45.7 % of mothers were
209 nulliparous and 6.2% were smoking during pregnancy. 50.9% of infants were male (Table 1).

Due to a low detection rate, we excluded PFHxA, PFHpA and PFTeDA prior to data analysis. Among the eight remaining PFAAs, PFHxS and PFDoDA had detection rates of 82.6 and 90.6%, respectively. Other PFAAs had detection rates greater than 97%. PFOS had the highest median exposure levels (4.92 ng/mL) followed by PFOA (2.01 ng/mL), PFUnDA (1.43 ng/mL), and PFNA (1.18 ng/mL).

The number and percentage of children who developed allergic diseases in the preceding 12 months were as follows: wheezing 291 (18.7%), eczema 296 (19.0%), and rhinoconjunctivitis 84 (5.4%). In total, 536 (34.4%) had at least one of the indicated allergic symptoms. Incidence of allergic symptoms was higher among boys than among girls, but this trend was not found to be statistically significant (Table 3).

We analyzed the association of PFAAs with total allergic diseases (Table 4, Figure 1), eczema (Figure 2, Supplementary Table S1), wheezing (Table 5), and rhinoconjunctivitis (Supplementary data, Table S2) using logistic regression models. Although not statistically significant (p-for trend= 0.085), we observed a negative association between total allergic diseases across PFUnDA quartiles (Q4 vs. Q1 adjusted OR: 0.736, 95% CI: 0.538, 1.00) in all children. In addition, adjusted ORs in the highest quartile versus that in the lowest quartile for total allergic diseases significantly decreased for PFDoDA (Q4 vs. Q1 OR: 0.621; 95% CI: 0.454, 0.847; p for trend= 0.008) and PFTrDA (Q4 vs. Q1 OR: 0.712; 95% CI: 0.524, 0.966; p for trend= 0.013). Following sex stratification, we observed a significant association between

229 total allergic diseases and PFDoDA or PFTrDA only within the male population (Table 4, figure
230 2).

231 As shown in Figure 2 and Supplemental Table S1, among males, the adjusted ORs for
232 eczema and PFOA decreased significantly for the three highest quartiles when compared with
233 that in the lowest quartile (Q4 vs. Q1 OR: 0.592; 95% CI: 0.319, 1.08, p for trend= 0.022) .
234 The adjusted ORs for the highest vs. lowest quartiles were 0.566 (95% CI: 0.383, 0.831) for
235 PFDoDA, and 0.672 (95% CI: 0.465, 0.968) for PFTrDA in all children. Effects of these long
236 chain PFAAs were highly prominent among boys. This is further supported by the adjusted
237 ORs of eczema in boys across the second to forth quartiles compared with the lowest quartile
238 of PFDoDA; the adjusted ORs were 0.877 (95% CI: 0.536, 1.43), 0.828 (95% CI: 0.500, 1.36),
239 and 0.451 (95% CI: 0.253, 0.785), respectively, indicating a dose-response relationship (p for
240 trend= 0.008).

241 Among PFAAs, PFHxS was found to be significantly associated with prevalence of
242 wheezing (Table 5); the adjusted OR of PFHxS in the fourth quartile versus the first quartile
243 was 0.728 (95% CI: 0.497, 1.06, p for trend= 0.038) in all children. Following sex stratification,
244 this association was more pronounced among boys (Q4 vs. Q1 OR: 0.650; 95% CI: 0.391, 1.07;
245 p for trend= 0.063).

246 PFNA exhibited a significant association with monotonic reduced prevalence of
247 rhinoconjunctivitis (Q4 vs. Q1 OR: 0.409; 95% CI: 0.192, 0.825; p for trend= 0.019). After sex

stratification, the adjusted OR of the forth quartile compared to the first quartile was reduced for PFNA across both sexes. However, the p value for the trend was not statistically significant (Supplementary data, Table S2). In addition, adjusted ORs for rhinoconjunctivitis decreased among the three highest quartiles of PFUnDA (Q4 vs. Q1 OR: 0.285; 95% CI: 0.099, 0.714; p for trend= 0.030) and PFDoDA (Q4 vs. Q1 OR: 0.430; 95% CI: 0.176, 0.985; p for trend= 0.045) when compared with that in the lowest quartile in a male population. Although we found some associations between PFAAs and rhinoconjunctivitis, these results should be interpreted cautiously due to the small number of participants with rhinoconjunctivitis in our study.

4. Discussion.

In this prospective birth cohort study, we focused on the effects of prenatal exposure to eleven PFAAs, including long-chain molecules, on the prevalenceof allergic diseases in children at 4 years of age. We found that prenatal exposure to long-chain PFAAs, including PFDoDA and PFTrDA, was inversely associated with prevalence of total allergic diseases in 4-year-old children. We observed that PFDoDA and PFTrDA were associated with a reduction in prevalence of eczema. Additionally, exposure to PFHxS was negatively associated with prevalence of wheezing. Within the female population, almost all adjusted ORs of allergic diseases across second to fourth quartiles of PFAAs were less than one when compared with

267 the first quartile values as a reference. However, we observed the association between PFAAs
268 and allergic diseases in 4-year-old children to be statistically significant only in boys.
269 In this study, median values of PFAAs with C6-C8 chains, including PFHxS, PFOS, and
270 PFOA, were low during pregnancy compared to those in the U.S. (Stein et al., 2012), Denmark
271 (Halldorsson et al., 2012), Korea (Lee et al., 2013), and China (Jiang et al., 2014). However,
272 longer chain PFAA concentrations (C≥9) were higher than those reported in western countries
273 such as Spain, Denmark, Sweden, and the U.S. (Harada et al. 2011).
274 We developed several adjusted models using different potential confounding factors to
275 examine the consistency of the results. In addition to the confounders mentioned in the methods
276 section, we included annual household income in the adjusted models, but this had no effect
277 on the results. We also included smoking, alcohol consumption during pregnancy and maternal
278 prepregnancy BMI in the adjusted models and found that the results remained consistent.
279 Furthermore, owing to the importance of home environment in relation to allergic diseases
280 (Araki et al., 2012; Cong et al. 2014), we collected information on owning pets and having
281 carpets, heating/cooling systems (electrical systems vs. fuel systems), and the presence of mold
282 and dew condensation in homes in 4 years post-delivery questionnaires. We included these
283 covariates one by one and collectively in the adjusted model, with no significant changes in the
284 results. This suggests that our results regarding the association between prenatal exposure to
285 PFAAs and allergic diseases at 4 years of age may not be confounded by the noted covariates.

One of sources of exposure to environmental chemicals including PFAAs is sea food, however sea foods contain omega 3 fatty acids which are anti-inflammatory nutrients and linked with better respiratory function (Miata and Arita2015). We have not assessed sources of the mother's intake of PFAAs such as diet in this study, and it might be a confounder in the current analysis.

Several previous animal studies suggest that PFAAs have immunotoxic effects, including suppression of cytokine production affecting TNF-α, IL4, and IFN-γ (Qazi et al., 2010), and reduced IgM production and humoral immunity (Dewitt et al., 2009; Peden-Adams et al., 2007). In animal studies, PFAAs changed T-helper (Th)-1 and -2 cell cytokine balance and shift toward a more Th2 cytokine pathway, lead to suppression of their cellular response and enhancement of their humoral response (Dong et al. 2011; Zheng et al. 2011). However, epidemiological studies have reported the suppression of antibody production in individuals exposed to higher PFAA levels. Prenatal exposure to PFAAs was negatively associated with the presence of the anti-rubella antibody among 3-year-old children (Granum et al., 2013). They examined four specific PFAAs and the strength of the inverse association between PFAA and antibody levels were ranked as follows: PFNA>PFOA>PFHxS>PFOS, indicating that PFCAs have a stronger influence on antibody production than PFSAs. Similarly, we also observed a stronger association between PFCAs with longer carbon chains and allergic outcomes. In another study, higher pre- and postnatal exposure to PFOS and PFOA were inversely associated with tetanus and diphtheria antibody concentrations at 5 and 7 years of

age (Grandjean et al., 2012). Results of these birth cohorts are consistent with our results. In contrast, a Taiwanese case-control study reported a positive association between serum levels of PFHxS, PFOS, PFOA, PFNA, and PFDA and juvenile asthma and IgE levels among 10- to 15-year-old children. In a cross sectional, National Health and Nutrition Examination Survey (NHANES), Humblet et al. (2014) reported an association between PFOA levels and an increased likelihood of asthma diagnosis. An inverse association between PFOS levels and both asthma and wheezing among children aged 12-19 years was noted. In both studies, prenatal exposures were not assessed and serum childhood PFAAs levels were several times higher than the levels in our study. This lack of consistency may be attributable to several factors such as different timing of blood sampling, PFAA exposure levels, age of examined children, and different study designs.

The most likely target of PFAAs has been shown to be the peroxisome proliferator-activated receptor (PPAR)-α (Vanden Heuvel et al., 2006; Takacs and Abbott 2007). Experimental studies suggested that PFOA is a stronger agonist than PFOS for the transactivation of PPAR-α and PPAR-γ in mouse and human cells (Vanden Heuvel et al., 2006). PFOA and PFNA (PFCAs) also have higher transplacental passage efficiencies than PFOS (Gustzkow et al., 2012; Lee et al., 2013). Moreover, laboratory studies suggest that PFAAs with longer carbon chains have a greater toxic potential and lower EC50 values (Kleszczynski et al. 2007; Buhrke et al., 2013). Therefore, these conclusions may partially explain why PFCAs, especially those

with longer carbon chains such as PFTrDA and PFDoDA, display a strong association with allergic outcomes in this study.

We previously reported the relation of PFAAs to infant allergic diseases at 12 and 24 months of age using ISAAC questionnaires (Okada et al., 2014), illustrating a link between prenatal exposure to PFTrDA and a reduced risk of eczema among female infants (n=2,062). In the current study, we followed those infants to 4 years of age and found that other PFAAs that contain shorter carbon chains, including PFHxS, PFNA, PFUnDA, PFDoDA, are also negatively associated with prevalence of allergic outcomes. Taken together, although we observed some differences in characteristics between the two studies, these two reports suggest consistent dyshomeostasis of immune function in infant and early childhood after prenatal exposure to PFAAs.

Some previous animal studies showed sex differences of PFAA effects on immune system functions. Gestational exposure to PFOS induced suppression of innate and humoral immunity in next generation male, but not female offspring, in a mouse model (Keil et al., 2008). Another study that only examined female pups concluded that in utero exposure to PFOA did not suppress IgM production (Hu, Strynar, and DeWitt 2010). Most of the previously conducted epidemiological studies concerning the association between PFAAs and allergic outcomes included sex as a confounder in the adjusted models (Grandjean et al., 2012; Dong et al. 2013; Humblet et al., 2014) but they did not stratify the results according to sex. As explained, the

adjusted OR values of allergic diseases across the second to fourth quartiles of prenatal PFAAs were less than those in the first quartile, which was used as a reference for both sexes. In current study, we found p for trend in the association between PFAAs and prevalence of allergic diseases were significant among boys but not girls; however, p values for exposure-sex interaction did not show significant interaction.

We applied logistic regression to estimate association of PFAAs and allergic outcomes in current study. However, some previous studies suggest that applying logistic regression when outcome is not rare may result in overestimation of odds ratios and using Poisson regression and log-binomial regression may provide more correct estimates (Barros and Hirakata, 2003). Therefore, we performed data analysis using log-binominal regression and Poisson regression. However, the results did not change.

In this study, we examined several types of PFAAs with varying carbon chain lengths. We assessed PFAA exposure levels using UPLC-MS-MS, which is a very sensitive method that involves a standard protocol. Owing to changing blood volume plasma concentration of environmental chemicals during pregnancy, plasma samples were only collected within a narrow blood sampling period (between 28 and 32 weeks of pregnancy) to maintain the standardization and consistency of results. In this study, information was collected relating to a wide variety of demographic characteristics and possible confounding variables through questionnaires completed during and following pregnancy. The prospective design of this study

362 permits the suggestion of a strong causal relationship between exposure and outcome
363 assessment when compared with case-control or cross-sectional studies. However, this study
364 has some limitations. We collected information on allergic diseases based on maternal reports,
365 which is prone to recall bias. We measured PFAA levels once, however due to long half-life
366 and moderate-to-high reliability of PFAA measurements in maternal samples during pregnancy,
367 single measurement of PFAAs is probably enough and has minimal influence on the results.
368 We did not measure postnatal exposure levels of PFAAs and immunological biomarkers in
369 children including IgE, and Th1/Th2 cytokines. In addition, when compared to the original
370 cohort (n>20,000) participants in the current study have higher socioeconomic status as
371 indicated by higher maternal education levels, lower maternal smoking rates during pregnancy
372 and lower postnatal ETS exposure. In addition, we missed around 25% of participants in the
373 current study compare with our previous study regarding the effects of PFAAs on allergic
374 diseases in first 24 months of life (Okada et al. 2014). These suggest the possibility of selection
375 bias. In report of Okada et al. (2014), mothers had higher smoking rate during pregnancy, but
376 other demographic characteristics of these two studies such as maternal age, parity, child
377 gender and parental allergy history were similar (Supplementary data, Table S3). In current
378 study, we examined 12 months prevalence of three allergic diseases using ISAAC
379 questionnaires. Allergic diseases have turnover such as onset, remission and relapse during
380 infancy and childhood. Therefore, further studies with longer observations are needed for

finding remission, relapse and persistence of allergic diseases associated with prenatal chemical exposure in later life.

Conclusion.

We found an inverse association between prenatal exposure to long-chain PFAAs and prevalence of allergic diseases in early childhood. This provides new evidence that PFAAs in humans may disrupt immune system balance consistent with animal studies. However, more studies with longer observation periods need to be conducted to further elucidate longer effects and the underlying mechanisms.

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Abbreviations:

PFAAs, perfluoroalkyl acids; PFSAs, perfluoroalkane sulfonates; PFCAs, perfluorinated carboxylic acids; PFHxA, perfluorohexanoic acid; PFHpA, perfluoroheptanoic acid; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFDA, perfluorodecanoic acid; PFUnDA, perfluoroundecanoic acid; PFDoDA, perfluorododecanoic acid; PFTrDA, perfluorotridecanoic acid; PFTeDA, perfluorotetradecanoic acid; PFHxS, perfluorohexane sulfonate; PFOS, perfluorooctane sulfonate; MDL, method detection limits; CI, confidence

interval; OR, odds ratio; Ig, immunoglobulin; ETS, environmental tobacco smoke; ISAAC, International Study of Asthma and Allergies in Childhood.

Acknowledgements

We are grateful to all of the participants for taking part in this study and the staff of the Hokkaido study on Environment and Children's Health. This study was funded by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; and the Japan Society for the Promotion of Science. The authors declare they have no actual or potential competing financial interests.

Table 1. Characteristics of the Hokkaido Study on Environment and Children's Health Study Population, Japan (n=1558).

Characteristics		4-year postpartum assessment (n=1558), mean±SD or No. (%)
Parental characteristics		
Maternal age (years) (mean ± SD)		31.1±4.4
Prepregnancy BMI		20.9±2.9
Maternal educational level (years)	≤12	660 (42.4)
	>12	898 (57.6)
Parity (times)[a]	0	702 (45.7)
	≥1	835 (54.3)
Maternal smoking status during pregnancy	Nonsmoker	1461 (93.8)
	Smoker	97 (6.2)
Maternal allergic history	Yes	484 (31.0)
Paternal allergic history	Yes	307 (19.7)
Annual household income (million yen)[a]	<5	880 (64.0)
	≥5	495 (36.0)
Children characteristics		
Gender	Male	793 (50.9)
	Female	765 (49.1)
Breast feeding (months)	<6	289 (18.6)
	≥6	1269 (81.4)
Older siblings (numbers)	0	702 (45.7)
	≥1	835 (54.3)
Day care attendance at 4-year-old[a]	Yes	1373 (90.3)
	No	148 (9.7)
ETS exposure at 4-year-old[a, b]	Yes	724 (48.0)
	No	782 (52.0)

[a]Missing data: parity (n=21), annual household income (n=183), day care attendance (N=37), and ETS exposure (n=52).

[b]ETS: environmental tobacco smoke.

619 Table 2. Concentrations of PFAAs in maternal plasma samples from the Hokkaido Study on

620 Environment and Children's Health, Japan, 2003–2013 (n=1558).

Compound	Detection			Concentration (ng/mL)							
	MDL[a]	No.	%	GM	Mean	SD	Minimum	25th	50th	75th	Maximum
PFHxS (C6)	0.2	1287	82.6	0.275	0.322	0.202	<0.2	0.221	0.296	0.395	3.386
PFHxA (C6)	0.1	721	46.2	0.085	0.103	0.071	<0.1	<0.1	<0.1	0.145	0.694
PFHpA (C7)	0.1	549	35.2	0.076	0.095	0.080	<0.1	<0.1	<0.1	0.125	0.757
PFOS (C8)	0.3	1558	100	4.932	5.456	2.61	1.003	3.667	4.925	6.654	30.283
PFOA (C8)	0.2	1557	99.9	2.105	2.713	2.30	<0.2	1.314	2.013	3.346	24.88
PFNA (C9)	0.3	1556	99.8	1.23	1.402	0.94	<0.3	0.908	1.183	1.589	13.189
PFDA (C10)	0.1	1551	99.5	0.514	0.575	0.282	<0.1	0.393	0.522	0.694	2.434
PFUnDA (C11)	0.1	1555	99.8	1.368	1.534	0.722	<0.1	1.037	1.431	1.895	5.89
PFDoDA (C12)	0.1	1413	90.6	0.172	0.191	0.081	<0.1	0.14	0.186	0.233	0.729
PFTrDA (C13)	0.1	1524	97.8	0.316	0.350	0.154	<0.1	0.247	0.332	0.424	1.325
PFTeDA (C14)	0.1	238	15.2	0.057	0.061	0.029	<0.1	<0.1	<0.1	<0.1	0.303

621 [a]MDL: method detection limit , SD: standard deviation

Table 3. Prevalence of allergic diseases in 4 years of age in the Hokkaido Study on Environment and Children's Health, Japan, 2003–2013 (n = 1558).

Symptoms	Total (n=1558)		Male children (n=793)		Female children (n=765)		p[a]
	n	(%)	n	(%)	n	(%)	
Total allergic diseases [b]	536	(34.4)	285	(35.9)	251	(32.8)	0.194
Wheezing	291	(18.7)	162	(20.4)	129	(16.8)	0.071
Eczema	296	(19.0)	153	(19.2)	143	(18.6)	0.762
Rhinoconjunctivitis symptoms	84	(5.4)	46	(5.8)	38	(4.9)	0.467

[a] Chi-square test.

[b] "Total allergic diseases" indicates cases with at least one of the listed symptoms.

Table 4. Prenatal PFAA concentrations and prevalence of total allergic diseases at 4 years of age in the Hokkaido Study on Environment and Children's Health, Japan, 2003–2013 (n= 1558).

Compound	Total (n = 1558)					Male children (n = 793)					Female children (n = 765)				
	n*	Crude		Adjusted [a]		n*	Crude		Adjusted [a]		n*	Crude		Adjusted [a]	
		OR	(95% CI)	OR	(95% CI)		OR	(95% CI)	OR	(95% CI)		OR	(95% CI)	OR	(95% CI)
PFHxS															
Quartile 1	140	1		1		76	1		1		64	1		1	
Quartile 2	143	0.981	(0.732, 1.31)	0.917	(0.675, 1.24)	75	0.995	(0.663, 1.49)	0.886	(0.577, 1.35)	68	0.973	(0.638, 1.48)	0.953	(0.608, 1.49)
Quartile 3	123	0.799	(0.593, 1.07)	0.771	(0.563, 1.05)	65	0.791	(0.524, 1.19)	0.739	(0.479, 1.13)	58	0.811	(0.526, 1.25)	0.836	(0.525, 1.32)
Quartile 4	130	0.858	(0.638, 1.15)	0.841	(0.615, 1.15)	69	0.859	(0.571, 1.29)	0.800	(0.519, 1.23)	61	0.859	(0.559, 1.32)	0.910	(0.572, 1.44)
p for trend		0.163		0.177			0.300		0.223			0.357		0.588	
P for Sex interaction		0.999		0.999											
PFOS															
Quartile 1	148	1		1		78	1		1		70	1		1	
Quartile 2	121	0.727	(0.54, 0.978)	0.658	(0.481, 0.898)	61	0.73	(0.483, 1.10)	0.674	(0.433, 1.04)	60	0.724	(0.473, 1.108)	0.655	(0.416, 1.02)
Quartile 3	131	0.823	(0.614, 1.10)	0.787	(0.577, 1.07)	71	0.948	(0.631, 1.42)	0.884	(0.572, 1.36)	60	0.713	(0.466, 1.091)	0.717	(0.457, 1.12)
Quartile 4	136	0.868	(0.648, 1.16)	0.815	(0.596, 1.11)	75	0.962	(0.643, 1.43)	0.910	(0.590, 1.40)	61	0.776	(0.507, 1.188)	0.750	(0.474, 1.18)
p for trend		0.513		0.391			0.852		0.975			0.257		0.308	

P for Sex interaction		0.708		0.700										
PFOA														
Quartile 1	133	1		1		66	1		1		67	1		1
Quartile 2	144	1.11	(0.833, 1.49)	1.07	(0.791, 1.47)	87	1.362	(0.907, 2.04)	1.24	(0.810, 1.92)	57	0.878	(0.571, 1.34)	0.950 (0.602, 1.49)
Quartile 3	138	1.05	(0.785, 1.41)	0.954	(0.695, 1.31)	73	1.089	(0.719, 1.64)	0.936	(0.601, 1.45)	65	1.017	(0.668, 1.54)	1.02 (0.646, 1.62)
Quartile 4	121	0.862	(0.639, 1.16)	0.830	(0.591, 1.16)	59	0.828	(0.54, 1.26)	0.746	(0.458, 1.20)	62	0.898	(0.589, 1.36)	0.989 (0.612, 1.59)
p for trend		0.300		0.208			0.236		0.125			0.785		0.955
P for Sex interaction		0.335		0.469										
PFNA														
Quartile 1	139	1		1		73	1		1		66	1		1
Quartile 2	143	1.05	(0.784, 1.40)	1.36	(0.891, 2.08)	83	1.278	(0.853, 1.91)	1.36	(0.891, 2.08)	60	0.847	(0.553, 1.29)	0.919 (0.589, 1.43)
Quartile 3	127	0.869	(0.646, 1.16)	0.694	(0.442, 1.08)	58	0.759	(0.498, 1.15)	0.694	(0.442, 1.08)	69	0.989	(0.651, 1.50)	1.00 (0.644, 1.56)
Quartile 4	127	0.865	(0.643, 1.16)	0.873	(0.562, 1.35)	71	0.915	(0.609, 1.37)	0.873	(0.562, 1.35)	56	0.809	(0.525, 1.24)	0.900 (0.562, 1.44)
p for trend		0.192		0.137			0.242		0.143			0.501		0.769
P for Sex interaction		0.159		0.224										
PFDA														
Quartile 1	140	1		1		74	1		1		66	1		1
Quartile 2	132	0.899	(0.669, 1.20)	0.886	(0.652, 1.20)	71	0.93	(0.619, 1.398)	0.867	(0.564, 1.33)	61	0.866	(0.565, 1.32)	0.930 (0.595, 1.45)

Quartile 3	133	0.913	(0.68, 1.22)	0.880	(0.646, 1.19)	76	1.08	(0.724, 1.633)	1.08	(0.709, 1.67)	57	0.756	(0.492, 1.16)	0.746 (0.473, 1.17)
Quartile 4	131	0.879	(0.654, 1.18)	0.906	(0.663, 1.23)	64	0.819	(0.542, 1.239)	0.812	(0.522, 1.25)	67	0.944	(0.62, 1.43)	1.06 (0.680, 1.67)
p for trend		0.433		0.544			0.516		0.590			0.667		0.978
P for Sex interaction		0.399		0.280										
PFUnDA														
Quartile 1	145	1		1		75	1		1		70	1		1
Quartile 2	130	0.838	(0.624, 1.12)	0.859	(0.631, 1.16)	82	0.986	(0.662, 1.47)	0.971	(0.637, 1.48)	48	0.664	(0.426, 1.03)	0.731 (0.459, 1.15)
Quartile 3	137	0.897	(0.669, 1.20)	0.905	(0.667, 1.22)	67	0.908	(0.599, 1.37)	0.919	(0.591, 1.42)	70	0.891	(0.590, 1.34)	0.928 (0.604, 1.42)
Quartile 4	124	0.778	(0.579, 1.04)	0.736	(0.538, 1.00)	61	0.712	(0.469, 1.08)	0.715	(0.460, 1.10)	63	0.851	(0.559, 1.29)	0.777 (0.496, 1.21)
p for trend		0.151		**0.085**			0.100		0.134			0.735		0.461
P for Sex interaction		0.306		0.646										
PFDoDA														
Quartile 1	151	1		1		81	1		1		70	1		1
Quartile 2	129	0.753	(0.561, 1.01)	0.735	(0.540, 0.999)	82	0.854	(0.573, 1.27)	0.810	(0.532, 1.23)	47	0.616	(0.396, 0.960)	0.630 (0.395, 0.998)
Quartile 3	140	0.853	(0.638, 1.14)	0.810	(0.597, 1.09)	68	0.714	(0.473, 1.07)	0.683	(0.443, 1.05)	72	1.02	(0.675, 1.54)	0.976 (0.629, 1.51)
Quartile 4	116	0.638	(0.474, 0.860)	0.621	(0.454, 0.847)	54	0.497	(0.325, 0.760)	0.492	(0.314, 0.766)	62	0.819	(0.538, 1.24)	0.805 (0.516, 1.25)
p for trend		**0.011**		**0.008**			**<0.001**		**0.001**			0.840		0.750
P for Sex interaction		0.033		0.084										

PFTrDA														
Quartile 1	155	1		1		82	1		1		73	1		1
Quartile 2	132	0.772	(0.576, 1.03)	0.784	(0.577, 1.06)	79	0.799	(0.535, 1.194)	0.807	(0.529, 1.23)	53	0.715	(0.464, 1.10)	0.742 (0.472, 1.16)
Quartile 3	118	0.657	(0.488, 0.884)	0.634	(0.464, 0.864)	61	0.565	(0.372, 0.856)	0.557	(0.358, 0.862)	57	0.763	(0.498, 1.16)	0.739 (0.472, 1.15)
Quartile 4	131	0.740	(0.553, 0.991)	0.712	(0.524, 0.966)	63	0.628	(0.414, 0.951)	0.647	(0.416, 1.00)	68	0.869	(0.576, 1.31)	0.806 (0.522, 1.24)
p for trend		**0.024**		**0.013**			**0.008**		**0.017**			0.575		0.359
P for Sex interaction		0.381		0.547										

[a] Adjusted for maternal age, maternal educational level, parental allergic history, number of older siblings, breast feeding, day care attendance, and ETS exposure at 4 years of age. In addition to the aforementioned confounders, we included child gender in adjusted models for total children prior to sex stratification.

*Indicates number of cases with allergic symptoms.

Figure 1. The association between quartiles of PFDoDA (A), PFTrDA (B) and prevalence of total allergic diseases among 4-year old children. The total allergic diseases were defined as cases with at least one of the following symptoms: eczema, wheezing, or rhinoconjunctivitis. Data was adjusted for maternal age, maternal educational level, parental allergic history, number of older siblings, breast feeding, day care attendance, and ETS exposure at 4 years of age. In addition to the mentioned confounders, we included child gender in adjusted models for total children prior to sex stratification. Q: quartile.

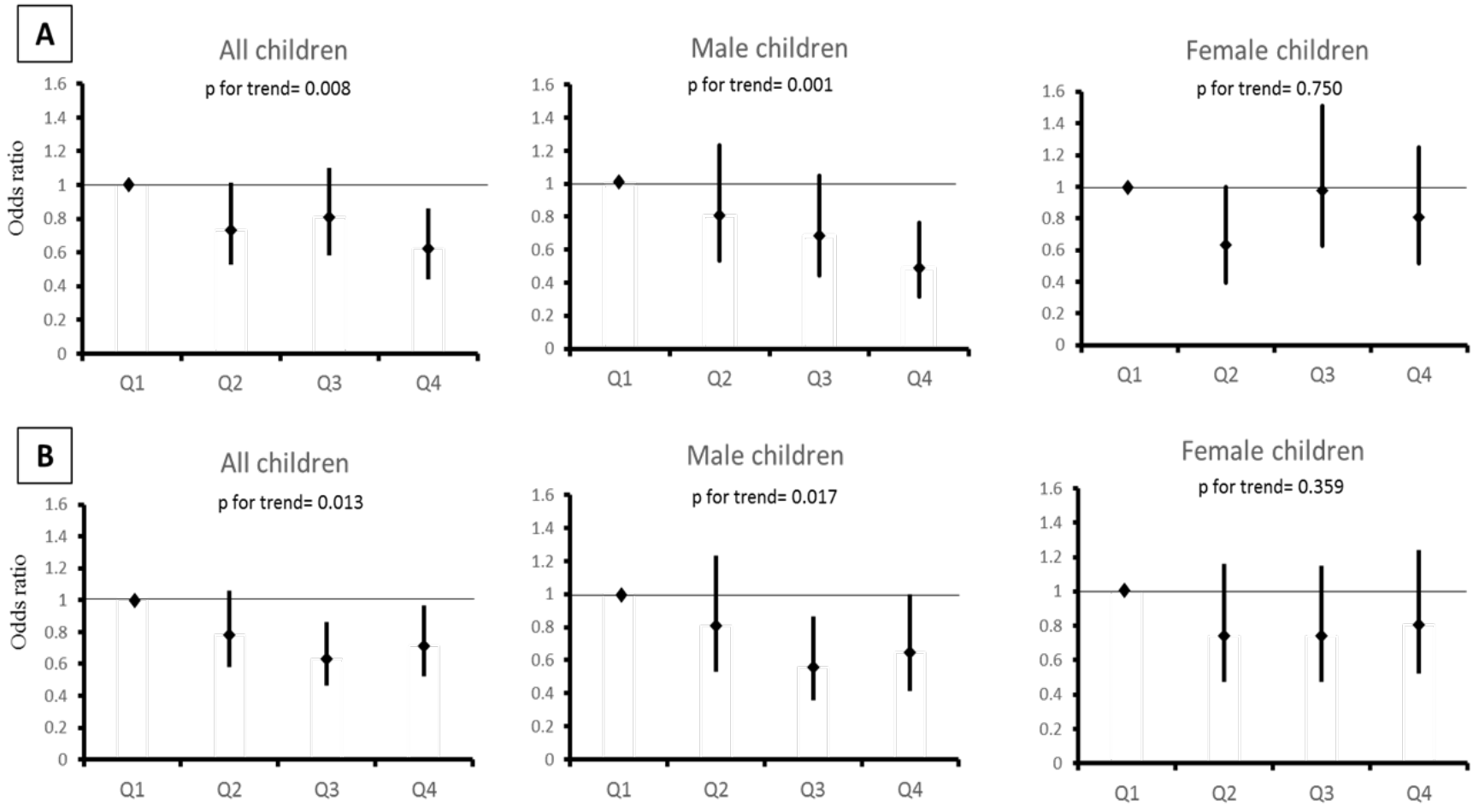


Figure 2. The association between quartiles of PFDoDA (A) and PFTrDA (B) and prevalence of eczema among 4-year old children. Data was adjusted for maternal age, maternal educational level, parental allergic history, number of older siblings, breast feeding, day care attendance, and ETS exposure at 4 years of age. In addition to the mentioned confounders, we included child gender in adjusted models for total children prior to sex stratification. Q: quartile.

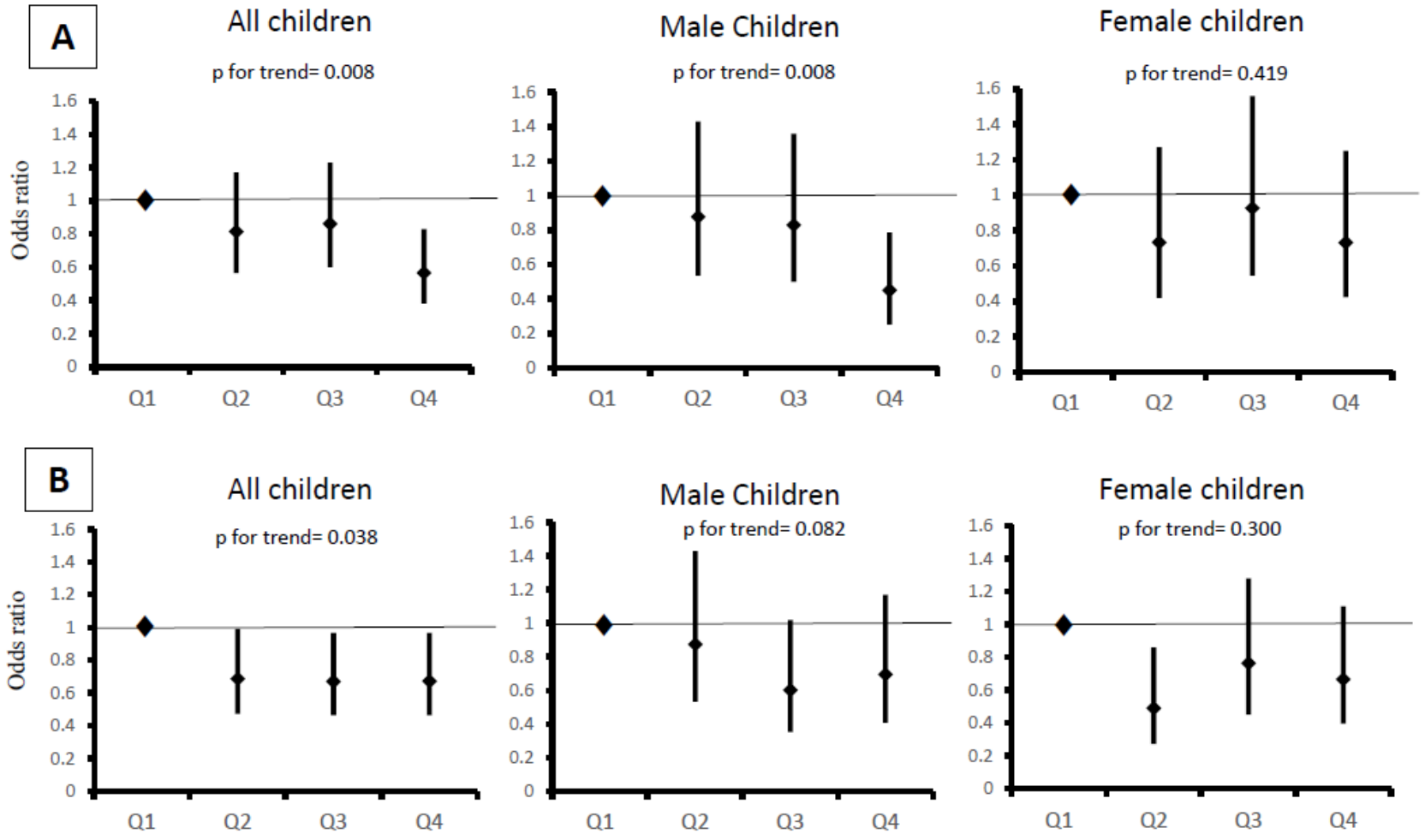


Table 5. Prenatal PFAA concentrations and prevalence of wheezing in 4 years old in the Hokkaido Study on Environment and Children's Health, Japan, 2003–2013 (n= 1558).

Compound	Total (n = 1558)					Male children (n = 793)					Female children (n = 765)				
	n*	Crude		Adjusted [a]		n*	Crude		Adjusted [a]		n*	Crude		Adjusted [a]	
		OR	(95% CI)	OR	(95% CI)		OR	(95% CI)	OR	(95% CI)		OR	(95% CI)	OR	(95% CI)
PFHxS															
Quartile 1	80	1		1		49	1		1		31	1		1	
Quartile 2	83	1.00	(0.711, 1.41)	0.895	(0.624, 1.28)	42	0.829	(0.519, 1.32)	0.705	(0.430, 1.15)	41	1.28	(0.764, 2.14)	1.21	(0.706, 2.10)
Quartile 3	61	0.702	(0.486, 1.01)	0.652	(0.443, 0.954)	33	0.612	(0.374, 1.00)	0.582	(0.346, 0.966)	28	0.842	(0.483, 1.47)	0.811	(0.448, 1.46)
Quartile 4	67	0.778	(0.543, 1.11)	0.728	(0.497, 1.06)	38	0.722	(0.448, 1.16)	0.650	(0.391, 1.07)	29	0.867	(0.499, 1.50)	0.889	(0.494, 1.59)
p for trend		0.056		**0.038**			0.097		0.063			0.320		0.398	
P for Sex interaction		0.654		0.670											
PFOS															
Quartile 1	78	1		1		43	1		1		35	1		1	
Quartile 2	67	0.822	(0.572, 1.18)	0.753	(0.514, 1.09)	33	0.758	(0.458, 1.25)	0.751	(0.439, 1.27)	34	0.899	(0.533, 1.51)	0.753	(0.433, 1.30)
Quartile 3	79	1.01	(0.714, 1.43)	0.980	(0.680, 1.41)	47	1.21	(0.758, 1.94)	1.18	(0.718, 1.94)	32	0.826	(0.487, 1.40)	0.809	(0.467, 1.39)
Quartile 4	67	0.824	(0.574, 1.18)	0.770	(0.526, 1.12)	39	0.901	(0.555, 1.46)	0.889	(0.530, 1.48)	28	0.740	(0.429, 1.27)	0.676	(0.379, 1.19)
p for trend		0.527		0.398			0.855		0.921			0.259		0.238	
P for Sex interaction		0.459		0.582											
PFOA															
Quartile 1	66	1		1		32	1		1		34	1		1	
Quartile 2	74	1.13	(0.79, 1.64)	1.09	(0.743, 1.60)	44	1.33	(0.805, 2.20)	1.22	(0.722, 2.09)	30	0.94	(0.549, 1.61)	0.982	(0.557, 1.72)
Quartile 3	76	1.18	(0.823, 1.70)	1.10	(0.749, 1.62)	44	1.40	(0.845, 2.32)	1.29	(0.762, 2.22)	32	0.977	(0.575, 1.66)	0.969	(0.544, 1.72)
Quartile 4	75	1.16	(0.806, 1.67)	1.09	(0.729, 1.65)	42	1.37	(0.824, 2.28)	1.25	(0.711, 2.22)	33	0.971	(0.574, 1.64)	1.00	(0.555, 1.82)

p for trend	0.411		0.699			0.235		0.427			0.948		0.992	
P for Sex interaction	0.727		0.741											
PFNA														
Quartile 1	70	1		1		36	1		1		34	1		1	
Quartile 2	78	1.14	(0.801, 1.64)	1.16	(0.803, 1.67)	47	1.43	(0.883, 2.34)	1.49	(0.908, 2.49)	31	0.878	(0.514, 1.49)	0.860	(0.493, 1.49)
Quartile 3	67	0.945	(0.654, 1.36)	0.910	(0.617, 1.33)	34	0.987	(0.588, 1.65)	0.911	(0.526, 1.57)	33	0.906	(0.535, 1.53)	0.918	(0.528, 1.59)
Quartile 4	76	1.10	(0.767, 1.57)	1.11	(0.760, 1.63)	45	1.27	(0.781, 2.07)	1.23	(0.732, 2.09)	31	0.918	(0.537, 1.56)	1.04	(0.587, 1.85)
p for trend		0.872		0.875			0.658		0.852			0.788		0.820	
P for Sex interaction	0.523		0.463											
PFDA														
Quartile 1	76	1		1		40	1		1		36	1		1	
Quartile 2	65	0.816	(0.566, 1.17)	0.785	(0.537, 1.14)	36	0.873	(0.529, 1.43)	0.794	(0.468, 1.34)	29	0.755	(0.441, 1.29)	0.785	(0.451, 1.35)
Quartile 3	82	1.09	(0.768, 1.54)	1.08	(0.756, 1.56)	52	1.45	(0.909, 2.32)	1.53	(0.943, 2.51)	30	0.762	(0.447, 1.29)	0.728	(0.415, 1.26)
Quartile 4	68	0.853	(0.594, 1.22)	0.879	(0.602, 1.28)	34	0.834	(0.503, 1.38)	0.859	(0.503, 1.45)	34	0.874	(0.521, 1.46)	0.918	(0.532, 1.58)
p for trend		0.755		0.917			0.966		0.743			0.637		0.702	
P for Sex interaction	0.203		0.119											
PFUnDA														
Quartile 1	72	1		1		37	1		1		35	1		1	
Quartile 2	70	0.96	(0.667, 1.38)	0.994	(0.682, 1.44)	45	1.13	(0.695, 1.83)	1.20	(0.725, 2.01)	25	0.753	(0.43, 1.31)	0.793	(0.444, 1.40)
Quartile 3	77	1.06	(0.748, 1.52)	1.10	(0.762, 1.60)	41	1.216	(0.739, 2.00)	1.32	(0.783, 2.25)	36	0.944	(0.565, 1.57)	0.918	(0.541, 1.56)
Quartile 4	72	0.991	(0.69, 1.42)	1.04	(0.714, 1.51)	39	1.047	(0.635, 1.72)	1.19	(0.709, 2.03)	33	0.931	(0.551, 1.57)	0.906	(0.522, 1.56)

p for trend	0.889		0.706			0.803		0.462			0.980		0.843	
P for Sex interaction	0.735		0.762											
PFDoDA														
Quartile 1	71	1		1		34	1		1		37	1		1	
Quartile 2	71	0.972	(0.675, 1.39)	0.962	(0.659, 1.40)	50	1.42	(0.874, 2.32)	1.41	(0.851, 2.36)	21	0.553	(0.31, 0.987)	0.556	(0.303, 1.00)
Quartile 3	79	1.109	(0.776, 1.58)	1.12	(0.778, 1.63)	41	1.21	(0.731, 2.01)	1.22	(0.728, 2.08)	38	1.01	(0.613, 1.67)	1.02	(0.604, 1.73)
Quartile 4	70	0.946	(0.657, 1.36)	0.999	(0.684, 1.45)	37	1.04	(0.622, 1.74)	1.14	(0.668, 1.95)	33	0.859	(0.512, 1.44)	0.864	(0.502, 1.48)
p for trend		0.960		0.794			0.903		0.781			0.950		0.533	
P for Sex interaction	0.066		0.119											
PFTrDA														
Quartile 1	78	1		1		44	1		1		34	1		1	
Quartile 2	73	0.918	(0.643, 1.31)	0.966	(0.669, 1.39)	38	0.737	(0.452, 1.199)	0.810	(0.487, 1.34)	35	1.16	(0.691, 1.96)	1.19	(0.696, 2.04)
Quartile 3	65	0.800	(0.556, 1.15)	0.805	(0.550, 1.17)	39	0.788	(0.485, 1.281)	0.813	(0.486, 1.35)	26	0.789	(0.453, 1.37)	0.801	(0.449, 1.41)
Quartile 4	75	0.926	(0.650, 1.31)	0.944	(0.653, 1.36)	41	0.883	(0.545, 1.43)	0.978	(0.590, 1.61)	34	0.976	(0.579, 1.64)	0.919	(0.531, 1.58)
p for trend		0.526		0.565			0.694		0.931			0.614		0.474	
P for Sex interaction	0.558		0.597											

[a] Adjusted for maternal age, maternal educational level, parental allergic history, number of older siblings, breast feeding, day care attendance, and ETS exposure at 4 years of age. In addition to the aforementioned confounders, we included child gender in adjusted models for total children prior to sex stratification.

*Indicates number of cases with wheezing.