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Prenatal exposure to perfluoroalkyl substances, immune-related outcomes, and lung function in children from a Spanish birth cohort study

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ABSTRACT

Background: Prenatal exposure to perfluoroalkyl substances (PFASs) has been associated with impaired immune and respiratory health during childhood but the evidence is inconsistent and limited for lung function. We studied the association between prenatal PFASs exposure and immune and respiratory health, including lung function, up to age 7 years in the Spanish INMA birth cohort study.

Methods: We assessed four PFASs in maternal plasma samples collected during the 1st trimester of pregnancy (years: 2003–2008): perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), and perfluorononanoate (PFNA). Mothers reported the occurrence (yes/no) of lower respiratory tract infections, wheezing, asthma, and eczema in the previous 12 months at 1.5 and 4 years of the child (n = 1188) and at 7 years (n = 1071). At ages 4 (n = 503) and 7 (n = 992) years lung function was assessed using spirometry tests.

Results: The most abundant PFASs were PFOS and PFOA (geometric means: 5.80 and 2.31 ng/mL, respectively). The relative risk of asthma during childhood per each doubling in PFNA concentration was 0.74 (95 CI%: 0.57, 0.96). The relative risk of eczema during childhood per every doubling in PFOS concentration was 0.86 (95 CI%: 0.75, 0.98). Higher PFOA concentrations were associated with lower forced vital capacity and lower forced expiratory volume in 1 s z-scores at 4 years [β (95 CI %): −0.17 (−0.34, −0.01) and −0.13 (−0.29, 0.03), respectively], but not at 7 years.

Conclusion: This longitudinal study suggests that different PFASs may affect the developing immune and respiratory systems differently. Prenatal exposure to PFNA and PFOS may be associated with reduced risk of respiratory and immune outcomes, particularly asthma and eczema whereas exposure to PFOA may be associated with reduced lung function in young children. These mixed results need to be replicated in follow-up studies at later ages.

1. Introduction

Perfluoroalkyl substances (PFASs) are synthetic chemicals that have been widely used in a variety of industrial and commercial applications such as the coating of paper and packaging, textiles and leather, fire-fighting foam, photography industry, cleaning products, and pesticides (Casals-Casas and Desvergne, 2011). Previous literature suggests that PFASs may be associated with impaired immune and respiratory health

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Abbreviations

CI	Confidence interval
DAGs	Directed acyclic graphs
$FEF_{25–75}$	Forced expiratory flow between 25% and 75% of forced vital capacity
FEV_1	Forced expiratory volume in 1 s
FEV_1%	Forced expiratory volume in 1 s percent predicted values
FEV_1/FVC	Forced expiratory ratio
FVC	Forced vital capacity
GAM	Generalized additive model
GEE	Generalized estimating equations
GM	Geometric mean
HPLC-MS/MS	High performance liquid chromatography–tandem mass spectrometry
INMA	Environment and Childhood Project (*INfancia y Medio Ambiente)*
LOQ	Limit of quantification
LRTIs	Lower respiratory tract infections
OR	Odds ratio
PFASs	Perfluoroalkyl substances
PFHxS	Perfluorohexane sulfonate
PFOS	Perfluorooctane sulfonate
PFOA	Perfluorooctanoate
PFNA	Perfluorononanoate
PPAR	Peroxisome proliferator-activated receptor
SD	Standard deviation

during childhood (Corsini et al., 2014; DeWitt et al., 2009; Grandjean et al., 2012; Granum et al., 2013; Qin et al., 2017). The PFASs most studied are perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) because of their widespread use, environmental persistence, and long biological half-lives in humans (3–5 years) (Olsen et al., 2007). However, other PFASs such as perfluorohexane sulfonate (PFHxS) and perfluorononanoate (PFNA) are assessed less frequently and can be of concern to human health.

Experimental studies in rodents suggest that early-life exposure to PFASs, especially PFOS and PFOA, may alter the immune system even at low doses (reviewed by DeWitt et al., 2012). In humans, few studies have assessed the association between prenatal PFASs exposure and the risk of allergy, asthma, or other immune-related outcomes during childhood; however, the results from these studies have been inconsistent (Vrijheid et al., 2016). Higher prenatal exposure to PFASs has been associated with reduced immune response to childhood routine vaccination (Grandjean et al., 2012; Granum et al., 2013) and higher number of common colds and episodes of gastroenteritis (Granum et al., 2013; Impinen et al., 2018). Similarly, higher prenatal exposure to PFOA and PFOS was associated with higher immunoglobulin E at 2 years (Wang et al., 2011). The results from these studies suggest that prenatal PFASs exposure may be associated with immune-suppression during childhood. However, these and other studies have reported either no associations (Granum et al., 2013; Lin et al., 2011; Okada et al., 2012; Timmermann et al., 2017) or even a reduced risk of occurrence of child immune and respiratory-related outcomes (Fei et al., 2010; Goudarzi et al., 2016; Okada et al., 2014; Smit et al., 2015).

Prenatal exposure to PFOS may also alter the development and function of the lung as seen in rat models (Grasty et al., 2005). In humans, two prospective studies have evaluated the association between prenatal exposure to PFASs and lung function during childhood; one of them observed a reduction in forced expiratory volume in 1 s percent predicted values (FEV_1%) in relation to prenatal PFOA and PFNA exposure (Agier et al., 2019); the other study did not find any association (Impinen et al., 2018). A case-control study assessed this association and found that postnatal PFASs concentrations were associated with lower lung function parameters at 11–16 years, but only among adolescents with asthma (Qin et al., 2017).

In the present study, we evaluated the association between prenatal PFASs exposure and immune and respiratory outcomes, including lung function, up to age 7 years among participants in a Spanish birth cohort.

2. Materials and methods

2.1. Study population

In this study, we used data from the Spanish INMA (Environment and Childhood - *INfancia y Medio Ambiente*) birth cohort (Guxens et al., 2012). From 2003 to 2008, pregnant women from the regions of Gipuzkoa, Sabadell, and Valencia were recruited during their 1st-trimester of pregnancy (N = 2150). The inclusion criteria were being at least 16 years old, singleton pregnancy, no communication barrier, no reproductive assistance, and giving birth in the reference hospital. Of the 2150 pregnant women, we selected those with available plasma samples during pregnancy and follow-up of their children during childhood. We had 1243 mother-child pairs with data on maternal PFASs blood concentrations (1st trimester) and at least one immune and respiratory outcome in children at each follow-up. From these, 29 mother-child pairs did not have complete information on the covariates of interest (i.e. 2% of the sample) and they were not included in the analysis (Fig. 1). The number of child-pairs was 309 in Guipuzkoa, 403 in Sabadell, and 502 in Valencia (total n = 1214), the mean gestational age at blood collection was 39 weeks for all three regions and the recruitment years were 2006–2008 in Guipuzkoa, 2003–2006 in Sabadell, and 2004–2007 in Valencia.

2.2. Perfluoroalkyl substances determination

We collected maternal blood samples during the first trimester of pregnancy (mean 12.3 weeks; standard deviation (SD): 5.6 weeks). We aliquoted plasma samples in 1.5 mL cryotubes and stored at −80 °C until their analysis at the Institute for Occupational Medicine, RWTH Aachen University, (Aachen, Germany), as previously described (Manzano-Salgado et al., 2015). Briefly, we measured plasma concentrations of PFHxS, PFOS, PFOA, and PFNA using column-switching liquid chromatography (Agilent 1100 Series HPLC apparatus) coupled with tandem mass spectrometry (Sciex API 3000 LC/MS/MS system in ESI-negative mode) according to a modified protocol described by Kato et al. (2011). The limit of quantification (LOQ) was 0.20 ng/mL for PFHxS, PFOS and PFOA and 0.10 ng/mL for PFNA (Manzano-Salgado et al., 2015).

2.3. Immune and respiratory outcomes

Mothers completed interviewer-led questionnaires using the Spanish or Catalan version of the validated International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire (Asher et al., 1995; Carvajal-Urueña et al., 2005; Mata Fernández et al., 2005). Information on bronchitis, bronchiolitis, pneumonia was obtained at 1.5 and 4 years; information on chest infection was obtained at 4 and 7 years. We defined the occurrence of low respiratory tract infections (LRTIs) at 1.5, 4, and 7 years by a positive answer to the questions: "*In the last* 6 months *(or* 12 months *if asked at age 4 years), has the doctor told you that your child has had bronchiolitis (only at 1.5 years) or bronchitis or pneumonia?" or "In the last* 6 months *(or* 12 months *if asked at ages 4 or 7 years), has the doctor told you that your child has had a chest infection?*". Wheeze at 1.5 and 4 years was defined by a positive answer

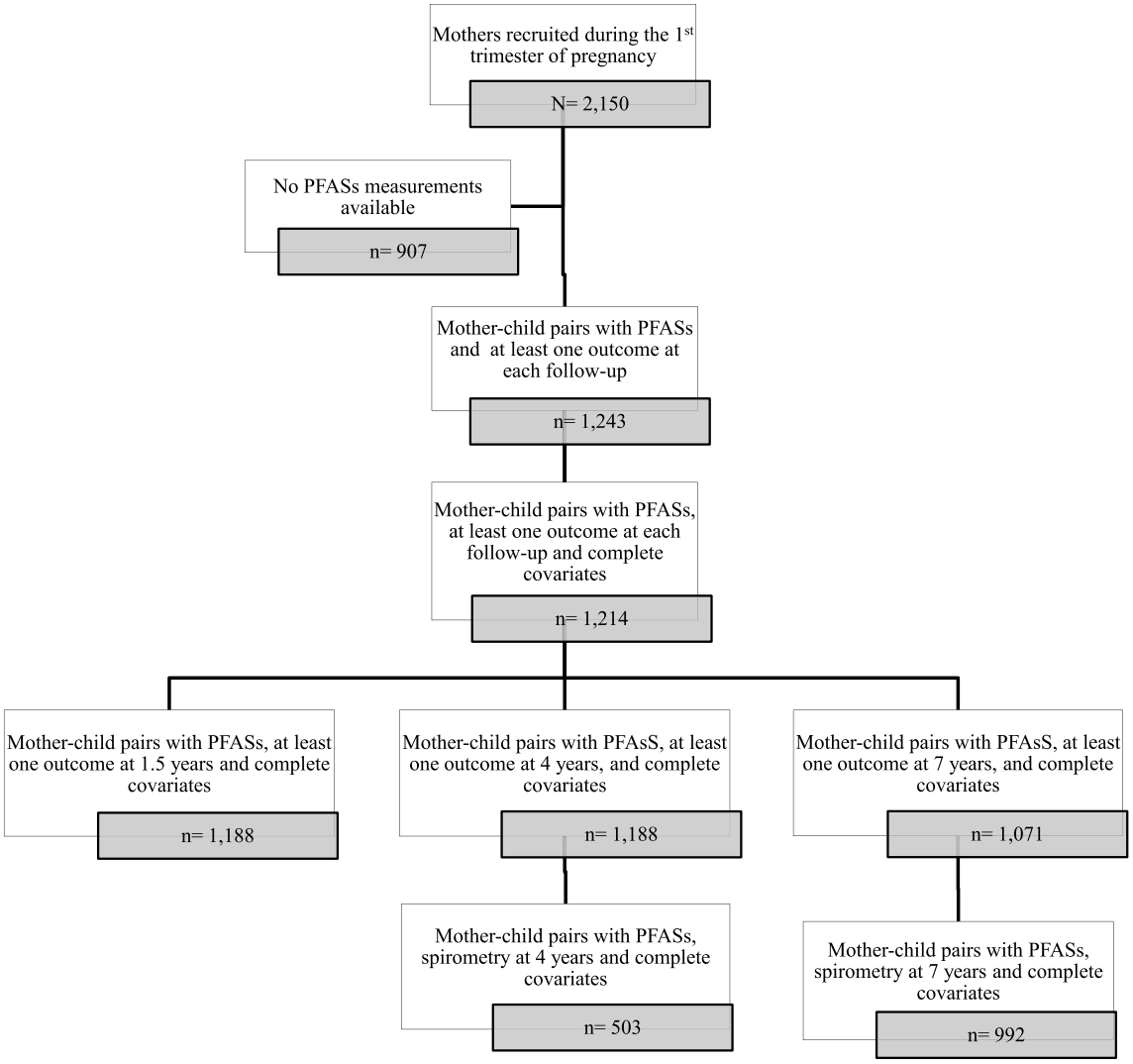

Fig. 1. Flowchart of the mother-child pairs included in this study.

to: "*Has your child ever experienced whistling or wheeze from the chest, but not noisy breathing from the nose in the last* 12 months?". At age 7 years, wheeze was defined as a positive answer to the following question: "*Has your child ever experienced whistling or wheeze from the chest in the last* 12 months?". Asthma at 4 years was defined by a positive answer to the question: "*In the last* 12 months*, has your child ever suffered asthma?"; whereas* at 7 years asthma was defined by a positive answer to: "*Has your child ever been diagnosed by a doctor as having asthma?*". Finally, eczema at 1.5 and 4 years was defined as a positive answer to the question: "*In the last* 12 months*, did your child have atopic eczema?*" and at 7 years as a positive answer to the question: "*Has your child ever had an itchy rash which was intermittently coming and going at any time in the past* 12 months?"

2.4. Lung function assessment

Lung function at 4 and 7 years was measured by trained nurses using spirometry and following the guidelines of the American Thoracic Society and European Respiratory Society (Miller et al., 2005; Morales et al., 2015). For the purpose of this study, we included the children with at least one acceptable maneuver (503 children at 4 years and 992 children at 7 years). We assessed the following lung function parameters: forced vital capacity (FVC, L), FEV_1 (L), forced expiratory ratio (FEV_1/FVC, %), and forced expiratory flow between 25% and 75% of FVC ($FEF_{25–75}$, L/s). We recorded the best FVC and best FEV_1 and derived $FEF_{25–75}$ from the best curve, defined as the greatest sum of FVC and FEV_1. With these parameters, we calculated the age-, sex-, height-, and ethnicity-adjusted z-scores using the Global Lung Function Initiative 2012 prediction equations (Quanjer et al., 2012).

2.5. Covariates

Information on maternal age at delivery, parity, country of birth, region of residence, maternal smoking during pregnancy, and socio economic status, was collected from interview-led questionnaires administered to the mothers during the 1st trimester of pregnancy (Guxens et al., 2012). Information on previous breastfeeding duration was also recorded because PFASs concentrations tend to decrease in women with longer periods of previous breastfeeding (Manzano-Salgado et al., 2016; Sagiv et al., 2015) and it can also be a predictor of breastfeeding duration of the index child. These questionnaires also provided data regarding the maternal history of asthma/allergy symptoms. We obtained information about the maternal diet from a semi-quantitative food frequency questionnaire (FFQ) of 101 items (Vioque et al., 2013) administered to the mothers at 12th weeks of pregnancy. Maternal pre-pregnancy body mass index (BMI, kg/m^2) was calculated from measured height and reported weight by the mother in the 1st trimester of pregnancy. Further, we abstracted from medical records

Table 1
Maternal characteristics in subjects included and excluded in the study.

Maternal characteristics	Included N = 1214	Excluded N = 936	p-value differences[a]
Age at delivery (years) - mean (SD)	31.9 (4.0)	31.4 (4.5)	0.012
Pre-pregnancy BMI (kg/m^2) - mean (SD)	23.6 (4.3)	23.3 (4.3)	0.135
Parity - n (%)			
None	682 (56.2)	486 (53.8)	0.117
One	454 (37.4)	339 (37.5)	
Two or more	78 (6.4)	79 (8.7)	
Education - n (%)			
Primary or without education	277 (22.9)	286 (31.6)	< 0.001
Secondary	508 (41.9)	354 (39.2)	
University	427 (35.2)	264 (29.2)	
Region of residence - n (%)			
Gipuzkoa	309 (25.5)	329 (35.2)	< 0.001
Sabadell	403 (33.2)	254 (27.1)	
Valencia	502 (41.3)	353 (37.7)	
Country of birth (Spain) - n (%)	1131 (93.2)	784 (86.8)	< 0.001
Previous breastfeeding - n (%)			
Never	736 (60.6)	546 (60.4)	0.428
4 months	115 (9.5)	96 (10.6)	
4–6 months	123 (10.1)	75 (8.3)	
>6 months	240 (19.8)	187 (20.7)	
Maternal history of asthma or allergy (yes) – n (%)	315 (26.0)	203 (22.4)	0.059
Smoking during pregnancy (cigarettes/day) – n (%)			
None smoker	833 (68.9)	516 (65.5)	0.169
<10 cigarettes/day	133 (11.0)	86 (10.9)	
≥10 cigarettes/day	243 (20.1)	186 (23.6)	
Fish intake (servings per week) - mean (SD)	5.0 (2.2)	5.0 (2.3)	0.734
Breastfeeding duration of the index child – n (%)			
Never	137 (11.4)	78 (12.0)	0.124
<4 months	274 (22.8)	177 (27.2)	
4–6 months	198 (16.5)	90 (13.8)	
>6 months	592 (49.3)	307 (47.1)	

Abbreviations: SD: standard deviation; BMI: body mass index.
[a] P-values were obtained from the analysis of variance (ANOVA) for continuous variables and chi-squared test for categorical variables.

information about the sex and birth weight of the newborn and type of delivery. Postnatal questionnaires administered to the mothers provided information on breastfeeding duration of the index child, diet, physical activity, passive smoking, and age when we assessed the respiratory outcomes. Trained personnel measured the child weight and height at 4 and 7 years.

2.6. Statistical analysis

We replaced PFASs values < LOQ with LOQ/2. We used the $\log_2$-transformed PFASs concentrations to normalize the distributions of PFASs. We assessed the linearity of the relationship between PFASs and each outcome using generalized additive models (GAMs). Given that our GAMs models did not deviate from linearity, we used PFASs concentrations as continuous variables. We used directed acyclic graphs (DAGs) for selection of the potential confounders and effect modifiers of the association of interest (Supplementary Material Fig. S1). Based on our DAGs we adjusted the models for maternal age at delivery (years), parity (number of pregnancies), previous breastfeeding (weeks), pre-pregnancy BMI (kg/m^2), region of residence (Gipuzkoa, Sabadell, and Valencia), and country of birth (Spain or other).

In order to assess the association between PFASs and immune- and respiratory-related outcomes during the complete study period, i.e. from age 1.5 until 7 years, we used generalized estimating equations (GEE) with an unstructured correlation matrix. GEEs allow for assessing outcomes with unknown correlation at different time-points, and the inclusion of subjects with incomplete data at any given follow-up (Liang and Zeger, 1986). For the individual follow-ups, we used logistic regression models to assess the association between PFASs and categorical outcomes (i.e. LRTIs, wheeze, asthma, and eczema) and linear regression models to assess the association between PFASs and lung function parameters.

We conducted different sensitivity analysis to assess the robustness of our results. First, because fish can be a source of exposure to PFASs but also to other environmental pollutants and nutrients which can interfere with PFASs metabolism, we additionally adjusted our models for maternal fish consumption during pregnancy. We additionally

Table 2
Distribution of maternal PFASs concentrations during 2003–2008 (n = 1243).

PFASs	n < LOQ (%)	PFASs concentrations in ng/mL								
		Mean	SD	Minimum	p10	p25	p50	p75	p90	Maximum
Total (n = 1214)										
PFHxS	46 (3.7)	0.67	0.49	0.05	0.29	0.41	0.58	0.82	1.14	11.01
PFOS	0 (0)	6.41	2.95	0.28	3.27	4.52	6.06	7.82	9.93	38.58
PFOA	0 (0)	2.67	1.68	0.28	1.17	1.63	2.35	3.30	4.36	31.64
PFNA	8 (0.64)	0.74	0.41	0.03	0.34	0.49	0.65	0.90	1.20	5.51
Gipuzkoa (n = 309)										
PFHxS	21 (6.8)	0.45	0.23	0.05	0.25	0.32	0.42	0.55	0.69	1.90
PFOS	0 (0)	5.90	2.62	1.24	2.91	4.15	5.31	7.27	9.30	20.12
PFOA	0 (0)	1.92	1.20	0.28	0.91	1.23	1.66	2.29	3.16	12.27
PFNA	1 (0.3)	0.67	0.37	0.03	0.34	0.46	0.59	0.76	1.05	3.23
Sabadell (n = 403)										
PFHxS	9 (2.2)	0.93	0.68	0.05	0.45	0.63	0.85	1.13	1.45	11.01
PFOS	0 (0)	6.85	3.46	0.28	3.54	4.74	6.47	8.12	10.35	38.58
PFOA	0 (0)	3.22	2.01	0.30	1.69	2.24	2.89	3.84	4.69	31.64
PFNA	23 (0.7)	0.91	0.43	0.03	0.54	0.65	0.83	1.09	1.36	5.51
Valencia (n = 502)										
PFHxS	13 (2.6)	0.59	0.29	0.05	0.30	0.40	0.53	0.72	0.92	2.64
PFOS	0 (0)	6.38	2.64	0.77	3.29	4.60	6.11	7.81	9.97	18.53
PFOA	0 (0)	2.69	1.47	0.29	1.26	1.67	2.36	3.31	4.41	10.10
PFNA	4 (0.8)	0.64	0.38	0.03	0.31	0.42	0.57	0.76	1.01	3.18

Abbreviations: PFAS: perfluoroalkyl substances; PFHxS: perfluorohexanesulfonic acid; PFOS: perfluorooctanesulfonic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid, LOQ: limit of quantification; SD: standard deviation; p: percentile.

adjusted our models for maternal smoking and education because some studies have reported that these variables can be associated with PFASs levels (Ode et al., 2013; Sagiv et al., 2015; Shu et al., 2018). Since PFASs can have sex-specific effects we tested whether sex modified the associations between PFASs and our outcomes by including the interaction terms in our models and by stratified analysis. PFASs levels in the INMA cohort differ by region of residence; therefore, we stratified our analysis by region of residence. We also tested whether breastfeeding duration of the index child could potentially modify the association because breastfeeding can be a predictor of postnatal exposure to PFASs but also can prevent the development of immune-related outcomes. To assess the robustness of the lung function results we repeated all models by excluding those children who were unable to perform reproducible spirometry tests. At 4 years, a test was considered reproducible if FVC and FEV_1 showed an agreement of at least 100 mL between the best two blows (n = 304 (60%)); at 7 years, a reproducible test was defined as FVC and FEV_1 agreeing within 150 mL between the best two blows (n = 641 (65%)). We also repeated the lung function analysis excluding children with asthma at 4 (n = 13) and 7 years (n = 50). Given that PFASs concentrations are moderately correlated in INMA (ranging from Spearman rho = 0.43 to 0.68; p-values < 0.001) (Manzano-Salgado et al., 2015) we performed a multipollutant model including all PFASs in a single model as well a structural equation model by creating a latent variable for PFASs. We interpreted the estimates in our models as the change in the outcome per doubling of maternal PFASs concentrations. We considered a p-value < 0.05 to be statistically significant. We used the STATA 14.1 statistical software (Stata Corporation, College Station, Texas) for our regression analysis. We drew our DAGs using the DAGitty version 3.0 (Textor, 2011).

3. Results

Women included in this study were on average 32 years old, nulliparous, with secondary or higher studies, and born in Spain (Table 1). No major differences in maternal characteristics were observed between the three INMA regions of residence (Supplementary Material Table S1). In the overall population, the subjects excluded in this study had a higher proportion of younger mothers, with lower education, and born outside of Spain than the subjects included in this study (Table 1). PFASs concentrations were detected in every maternal sample, with PFOS and PFOA concentrations being the most abundant (means: 6.41 and 2.67 ng/mL, respectively) (Table 2). PFASs concentrations were similar in the three regions of residence, although the highest levels were observed in the Sabadell region (Table 2). PFOA and PFNA were the most correlated (Spearman's rho = 0.68) PFASs and this was similar across regions of residence (Supplementary Material Table S2). The prevalence of childhood immune and respiratory outcomes ranged from 3% for asthma at 4 years to 35% for LRTIs at 1.5 years (Table 3). We observed a general decrease in the prevalence of LRTIs and wheeze from 1.5 to 7 years old, whereas the prevalence of eczema seemed to increase during childhood (Table 3). The Sabadell region presented the lowest prevalence of asthma but the highest of eczema (Table 3).

In the GEE models, we observed that the relative risk of asthma during childhood decreased per each doubling of PFNA concentration [relative risk (95% CI): 0.74 (0.57, 0.96)] (Table 4). In addition, every doubling of PFOS concentration was associated with a lower risk of eczema during childhood [relative risk (95 CI %): 0.86 (0.75, 0.98)]. We observed similar results between the other PFASs and asthma and eczema but associations did not reach statistical significance. In the analysis by ages, we observed that with higher concentrations of PFASs there was a pattern of lower odds of LRTIs, wheezing, and eczema at older ages (Fig. 2 and Supplementary Material Table S3). Besides the associations observed in the GEE models of PFNA and PFOS with asthma and eczema, we observed that higher PFNA concentrations were associated with less odds of LRTIs [Odds ratio (OR) (95 CI %): 0.85 (0.71, 1.01)] and eczema [OR (95 CI %): 0.79 (0.66, 0.96)] at 4 years and wheezing at 7 years [OR (95 CI %): 0.69 (0.54, 0.88)] (Fig. 2). Moreover, higher PFOA and PFOS concentrations were associated with lower odds of LRTIs [OR (95 CI %): 0.69 (0.47, 1.01)] and wheezing [OR (95 CI %): 0.70 (0.53, 0.95)] at 7 years, respectively (Fig. 2).

Regarding lung function, higher prenatal PFOA concentrations were associated with a lower FVC and FEV_1 z-scores at 4 years [β (95% CI): −0.17 (−0.34, −0.01) and −0.13 (−0.29, 0.03), respectively, per each doubling of PFOA concentration] (Table 5 and Supplementary Material Table S4). These changes in lung function parameters are equivalent to 3.6% and 2.2% change of FVC and FEV_1 means, respectively. These associations were not observed at 7 years of age. We did not observe associations between the other PFASs and lung function.

Results did not change after adjusting our models for maternal fish consumption, maternal smoking during pregnancy, or maternal education (data not shown). We did not see any statistically significant sex-specific difference in our results (Supplementary Material Table S5). The interaction term between the region of residence and PFASs was statistically significant for some of the associations (Supplementary Material Table S6). Thus, we stratified the GEE models by region of

Table 3
Prevalence of child immune and respiratory outcomes.

Outcomes during childhood	Age at follow-up		
	1.5 years	4 years	7 years
Total (n = 1214)			
Child age (years) - mean (SD)	1.1 (0.1)	4.4 (0.2)	7.4 (0.5)
LRTIs (yes) - n (%)	411 (34.6)	297 (25.1)	61 (5.7)
Wheeze (yes) - n (%)	381 (32.1)	216 (18.2)	121 (11.3)
Asthma (yes) - n (%)	-	34 (2.9)	56 (5.2)
Eczema (yes) - n (%)	228 (19.2)	230 (19.4)	358 (33.6)
Spirometry tests – mean (SD)		503	992
FVC (L)	-	1.0 (0.2)	1.7 (0.3)
FEV_1 (L)	-	0.9 (0.2)	1.5 (0.2)
FEV_1/FVC (%)	-	92.8 (0.1)	87.0 (0.6)
FEF_{25-75} (L/s)	-	1.3 (0.4)	1.8 (0.5)
Gipuzkoa (n = 309)			
Child age (years) - mean (SD)	1.2 (0.1)	4.4 (0.2)	7.9 (0.1)
LRTIs (yes) - n (%)	118 (39.9)	53 (18.1)	21 (7.9)
Wheeze (yes) - n (%)	114 (38.5)	44 (14.8)	22 (8.3)
Asthma (yes) - n (%)	-	14 (4.8)	23 (8.7)
Eczema (yes) - n (%)	49 (16.6)	51 (17.4)	72 (27.0)
Spirometry tests – mean (SD)		210	255
FVC (L)	-	1.0 (0.2)	1.9 (0.3)
FEV_1 (L)	-	0.9 (0.2)	1.6 (0.2)
FEV_1/FVC (%)	-	93.0 (0.6)	85.0 (0.6)
FEF_{25-75} (L/s)	-	1.3 (0.4)	1.9 (0.5)
Sabadell (n = 403)			
Child age (years) - mean (SD)	1.2 (0.1)	4.4 (0.2)	6.9 (0.4)
LRTIs (yes) - n (%)	146 (37.4)	117 (29.1)	32 (8.3)
Wheeze (yes) - n (%)	132 (33.9)	86 (21.4)	41 (10.7)
Asthma (yes) - n (%)	-	4 (1.0)	10 (2.6)
Eczema (yes) - n (%)	90 (23.1)	97 (24.1)	131 (34.6)
Spirometry tests – mean (SD)		293	323
FVC (L)	-	1.0 (0.2)	1.6 (0.3)
FEV_1 (L)	-	0.9 (0.2)	1.4 (0.2)
FEV_1/FVC (%)	-	92.7 (0.8)	86.2 (0.6)
FEF_{25-75} (L/s)	-	1.3 (0.4)	1.7 (0.4)
Valencia (n = 502)			
Child age (years) - mean (SD)	1.1 (0.2)	4.3 (0.1)	7.6 (0.3)
LRTIs (yes) - n (%)	147 (29.3)	127 (26.0)	8 (1.9)
Wheeze (yes) - n (%)	135 (26.9)	86 (17.6)	58 (13.8)
Asthma (yes) - n (%)	-	16 (3.3)	23 (5.5)
Eczema (yes) - n (%)	89 (17.7)	82 (16.8)	155 (36.9)
Spirometry tests – mean (SD)			414
FVC (L)	-	-	1.7 (0.3)
FEV_1 (L)	-	-	1.6 (0.2)
FEV_1/FVC (%)	-	-	89.0 (0.6)
FEF_{25-75} (L/s)	-	-	1.9 (0.4)

Abbreviations: SD: standard deviation; LRTIs: lower respiratory tract infections; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; $FEF_{25\%–75\%}$: forced expiratory flow between 25% and 75% of FVC.

Table 4
Generalized estimating equations models – unadjusted and fully adjusted[a] associations between maternal PFASs concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes from age 1.5–7 years in the INMA birth cohort study (n = 1188 at 1.5 and 4 years and n = 1071 at 7 years).

Outcomes during childhood	Relative risk (95% CI)			
	PFHxS	PFOS	PFOA	PFNA
Unadjusted model				
LRTIs	1.07 (0.98, 1.17)	0.94 (0.84, 1.06)	0.97 (0.87, 1.07)	0.98 (0.89, 1.09)
Wheeze	1.00 (0.92, 1.10)	0.89 (0.79, 1.01)	0.94 (0.85, 1.05)	0.92 (0.83, 1.03)
Asthma	0.79 (0.65, 0.96)[c]	0.77 (0.57, 1.04)	0.70 (0.53, 0.91)[d]	0.69 (0.55, 0.86)[d]
Eczema	1.05 (0.96, 1.15)	0.90 (0.80, 1.02)	1.04 (0.94, 1.16)	1.01 (0.91, 1.12)
Fully adjusted model [a]				
LRTIs	1.07 (0.96, 1.18)	0.96 (0.85, 1.09)	0.96 (0.85, 1.08)	0.95 (0.85, 1.05)
Wheeze	0.99 (0.89, 1.10)	0.90 (0.78, 1.03)	0.94 (0.83, 1.06)	0.90 (0.81, 1.01)
Asthma[b]	0.96 (0.74, 1.24)	0.83 (0.59, 1.17)	0.83 (0.61, 1.14)	0.74 (0.57, 0.96)*
Eczema	0.95 (0.86, 1.05)	0.86 (0.75, 0.98)*	0.96 (0.85, 1.08)	0.95 (0.85, 1.06)

Abbreviations: CI: confidence interval; PFHxS: perfluorohexanesulfonic acid; PFOS: perfluorooctanesulfonic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; LRTIs: lower respiratory tract infections.

[a] Models were adjusted for maternal age at delivery, parity, previous breastfeeding, pre-pregnancy BMI, region of residence, and country of birth.

[b] Only available at 4 and 7 years.

[c] p-value < 0.05.

[d] p-values < 0.01.

residence and observed that the association between PFNA and asthma was mainly driven by the region of Sabadell (Supplementary Material Table S6). Regarding breastfeeding of the index child, we observed that higher prenatal PFNA levels were associated with a higher risk of asthma in those children that breastfed less than 4 months [relative risk (95% CI): 2.73 (1.13, 6.57)] (Supplementary Material Table S7). After excluding children with non-reproducible spirometry tests, the associations between PFOA concentrations and FVC and FEV_1 were closer to the null (Supplementary Material Table S8). Repeating the lung function analysis only in non-asthmatic children strengthened the association between higher PFOA concentrations and reduced FVC and FEV_1 z-scores at 4 years [β (95% CI): −0.19 (−0.36,-0.02) and −0.15 (−0.31, 0.02), respectively] (data not shown). The inclusion of all PFASs in one multi-pollutant model did not change the associations between PFNA and PFOS and reduced risk of asthma and eczema, respectively, at any age (Supplemental Material Table S9). In this multipollutant model, however, higher PFHxS concentration was statistically significantly associated with higher occurrence of LRTIs at any age [relative risk (95%CI): 1.14 (1.00, 1.29)]. Results were similar when using the latent variable for PFASs concentrations (Supplemental Material Table S9).

4. Discussion

In the present study, we observed associations between higher prenatal exposure to PFNA and PFOS and reduced risk of asthma and eczema during childhood. These results were less consistent in the analysis by ages. We observed an overall pattern of higher PFASs concentrations and lower odds of LRTIs, wheezing, and eczema at older ages. Further, prenatal PFOA concentrations were associated with lower lung function parameters at 4 years, but not at 7 years.

PFASs concentrations in our study population (samples collected between 2003 and 2008) were lower than the concentrations observed in other studies using maternal blood samples collected before the PFOS phase-out period in the year 2002 (Fei et al., 2007; Midasch et al., 2007). However, studies using maternal samples collected more recently have detected lower PFASs concentrations than ours (Ashley-Martin et al., 2017; Fromme et al., 2010; Hanssen et al., 2010; Porpora et al., 2013). Thus, differences in PFASs may explain the conflicting results with other studies.

In our study, prenatal PFNA and PFOS exposure were associated with reduced risk of immune and respiratory outcomes, especially asthma and eczema. Our results are in line with some previous studies but not with others. In a study from the INUENDO birth cohort, higher prenatal exposure to PFASs, mainly PFOA, was associated with less recurrent wheeze in children 5–9 years of age (Smit et al., 2015). In the Hokkaido birth cohort, higher prenatal PFASs exposure, particularly to the long chain PFASs such as perfluorododecanoic (PFDoDa) and perfluorotridecanoic (PFTrDA) acids, was associated with a lower occurrence of allergic diseases at 1, 2, and 4 years of age (Goudarzi et al., 2016; Okada et al., 2014). In the Danish birth cohort, prenatal PFOA exposure was associated with reduced risk of hospitalizations for infectious diseases in childhood (Fei et al., 2010). However, findings of other studies suggest that prenatal exposure to PFASs may be associated with immune-suppression leading to an increased risk of immune and respiratory outcomes during childhood (Grandjean et al., 2012; Granum et al., 2013; Wang et al., 2011). Other studies did not find any association (Okada et al., 2012; Timmermann et al., 2017; Wang et al., 2011). Differences in exposure levels, outcome assessment, age at follow-up, and population setting might explain the conflicting results between studies. We also need to consider that in our study the association of PFNA with childhood asthma was mainly driven by the Sabadell region. In this region, the prevalence of asthma was the lowest across regions (only 4 and 10 children had asthma at 4 and 7 years, respectively) and these children had lower PFASs levels than children without asthma, a pattern that was not observed in Gipuzkoa and Valencia children. There may be a characteristic that makes asthmatic children in Sabadell different than the rest of children in INMA but we are not able to conclude on anything in specific.

The associations observed in ours and other previous studies between higher prenatal PFASs exposure and lower risk for some of the immune and respiratory outcomes may be attributable to different reasons. Prenatal PFASs exposure has been associated with immune-suppression during childhood (Grandjean et al., 2012; Granum et al., 2013). Thus, prenatal PFASs exposure may indirectly reduce the risk of developing immune hyperactivity and hypersensitivity diseases, such as eczema and wheezing. This, however, cannot explain the decreased risk of LRTIs observed in this study. Postnatal breastfeeding, which can prevent the development of immune-related outcomes, can counteract the adverse effects of prenatal exposure to PFASs. Indeed, in our study we observed that those children who breatsfeed less than 4 months higher prenatal PFNA levels increased the risk of childhood asthma whereas in those children who breastfed more than 4 months we observed a reduced risk of asthma onset associated with PFNA exposure.

The potential mechanisms by which PFASs could affect the immune and respiratory systems are not well understood and may occur via multiple pathways (Corsini et al., 2014). Contrary to our results,

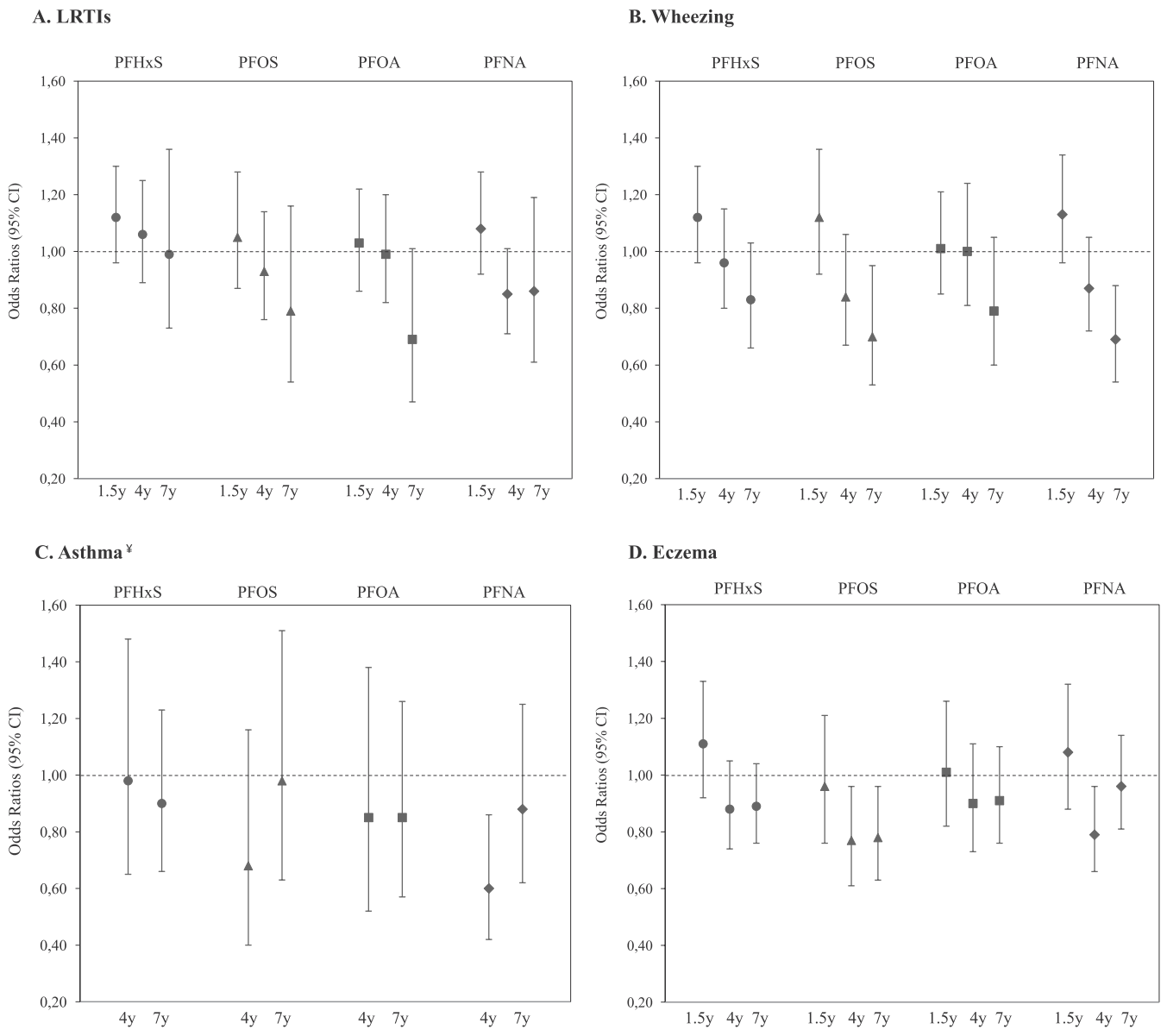

Fig. 2. Logistic regression models - fully adjusted associations [a] between maternal PFASs concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes by age in the INMA birth cohort study (n = 1188 at 1.5 and 4 years and n = 1071 at 7 years). AbbreviationsOR: odds ratio; CI: confidence interval; PFHxS: perfluorohexanesulfonic acid; PFOS: perfluorooctanesulfonic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid. [a] Models were adjusted for age-at-follow-up of the child, maternal age at delivery, parity, previous breastfeeding, pre-pregnancy BMI, region of residence, and country of birth.¥ Only available at 4 and 7 years.

previous animal studies have linked PFOA with higher allergic inflammation and pro-inflammatory cytokines both *in vitro* and *in vivo* (Singh et al., 2012), and with airway hyperreactivity in mice (Fairley et al., 2007). PFOS has been linked with a Th2 immune response characterized by higher interleukin (IL)-4 and IL-10 and lower IL-2 and interferon (IFN)-γ (Dong et al., 2011; Zheng et al., 2011). However, in a murine model, PFOA and PFOS were associated with higher INF-γ mRNA (messenger RNA) in the lungs but not with allergic hyper-responsiveness (Ryu et al., 2014). Further, experimental studies suggest that some of the biological effects of PFASs on respiratory health can be mediated through peroxisome proliferator-activated receptor (PPAR)-alpha and gamma (Corsini et al., 2014; Pennings et al., 2016), which regulate processes involved in the immune system and can directly modulate lipid levels that can lead to hepatoxicity and stress effects (Qazi et al., 2009). If the studied association between PFASs exposure and immune and respiratory health exists then further studies will be needed to understand the corresponding underlying mechanisms.

Studies in rodents have shown that prenatal exposure to PFASs may be associated with impaired lung development at birth (Grasty et al., 2005). In the present study, prenatal exposure to PFOA was associated with a lower FVC and FEV_1 at 4 years. However, these estimates disappeared after restricting the analysis to the subjects with reproducible spirometry tests, suggesting that the initial associations observed would probably reflect the inability to perform a good test rather than to PFASs exposure. These results are in line with those reported by Impinen et al. (2018), who reported no association between prenatal PFASs and lung function at birth and at 10 years in Norway. Recently, Agier et al. (2019) reported a reduction in FEV_1% at school age associated with PFOA and PFNA exposure during pregnancy but none of these associations passed the significance threshold when corrected for multiple testing in exposome-wide association study (ExWAS), and none was selected with the deletion-substitution-addition (DSA) algorithm. We need more prospective studies that evaluate the association between PFASs and lung function during childhood.

Table 5
Linear regression models - fully adjusted associations[a] between maternal PFASs concentrations (log_2-transformed, ng/mL) and lung function z-scores at 4[b] and 7 years in the INMA birth cohort study.

Age at follow-up	n	β (95% CI)			
		PFHxS	PFOS	PFOA	PFNA
FVC					
4 years	503	−0.04 (−0.17, 0.09)	−0.08 (−0.25, 0.09)	−0.17 (−0.34, −0.01)	0.04 (−0.12, 0.20)
7 years	992	−0.03 (−0.10, 0.05)	−0.04 (−0.13, 0.06)	−0.05 (−0.14, 0.04)	−0.02 (−0.10, 0.07)
FEV_1					
4 years	503	−0.04 (−0.17, 0.09)	−0.05 (−0.21, 0.12)	−0.13 (−0.29, 0.03)	0.05 (−0.10, 0.21)
7 years	992	−0.04 (−0.11, 0.04)	−0.02 (−0.12, 0.07)	−0.01 (−0.10, 0.08)	−0.01 (−0.09, 0.07)
FEV_1/FVC					
4 years	503	0.03 (−0.07, 0.13)	0.04 (−0.09, 0.17)	0.09 (−0.04, 0.21)	0.00 (−0.12, 0.13)
7 years	992	−0.02 (−0.10, 0.05)	0.01 (−0.09, 0.11)	0.06 (−0.03, 0.15)	0.01 (−0.08, 0.09)
FEF_{25-75}					
4 years	503	−0.02 (−0.12, 0.08)	0.05 (−0.09, 0.18)	0.02 (−0.11, 0.15)	0.07 (−0.06, 0.19)
7 years	990	−0.01 (−0.09, 0.06)	0.01 (−0.09, 0.10)	0.04 (−0.05, 0.13)	0.03 (−0.06, 0.11)

abrAbbreviationsCI: confidence interval; PFHxS: perfluorohexanesulfonic acid; PFOS: perfluorooctanesulfonic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; FEF25%-75%: forced expiratory flow between 25% and 75% of FVC.
[a] Models adjusted for maternal age at delivery, parity, previous breastfeeding, pre-pregnancy BMI, region of residence, and country of birth.
[b] Spirometry at 4 years was only available in Sabadell and Gipuzkoa.

The main strengths of this study are its prospective design and large sample size, and the objective assessment of lung function. However, we should consider the following methodological limitations. First, we used maternal PFASs concentrations from samples collected early in prenancy as a proxy of fetal exposure. Glynn et al. (2012) collected serial maternal serum samples during pregnancy and after delivery as well as cord blood (n = 16) and observed that the strongest correlations between maternal PFASs levels and cord blood levels were observed for maternal serum sampled shortly before or after the delivery (r = 0.70–0.89 for PFOS and PFOA). These correlations are close to those observed in a previous study conducted in INMA (n = 66) where maternal samples were collected at the beginning of pregnancy (r = 0.70–0.71 for PFOS and PFOA) (Manzano-Salgado et al., 2015). This suggests that PFASs fetal body burden can be assessed using as proxy maternal samples collected early in pregnancy. Second, we have no information on postnatal PFASs exposure, which has been associated with immune-related outcomes or lung function in other studies (Dong et al., 2013; Qin et al., 2017). In a recent exposome paper Agier et al. (2019) observed that prenatal but not postnatal exposure to PFASs was associated with reduced FEV_1% at school age, suggesting that the prenatal life is the most critical period for the impact of PFASs exposure. However, the postnatal PFASs levels were determined at the same time as the outcome which limits the interpretation of results. More studies with information on PFASs concentrations during childhood are needed to corroborate these results. Third, self-reported questionnaires for outcome assessment may introduce recall and/or misclassification bias. Misclassification bias may be most important for asthma at 4 years as cases can be confounded with the occurrence of wheeze due to LRTIs. However, at 7 years, we asked for a doctor diagnosis and our results remained similar to the 4 years ones. Fourth, the effect estimates of PFASs with lung function were small, and need to be carefully interpreted. However, a z-score change of ± 0.05 is considered clinically relevant on a population level. Finally, chance findings are plausible due to multiple comparisons in our study; however, we preferred not to apply statistical correction for multiple comparisons as this can increase false negative findings (type 2 errors) (Perneger, 1998; Rothman, 1990) and instead, we stated the general patterns of associations observed in the present study.

5. Conclusion

The results from this Spanish birth cohort study suggest that different PFASs may affect the developing immune and respiratory systems differently. Prenatal exposure to PFNA and PFOS may be associated with reduced risk of expression of respiratory and immune outcomes, particularly asthma and eczema whereas exposure to PFOA may be associated with reduced lung function in young children. These mixed results need to be replicated in follow-up studies at later ages.

Conflicts of interest

There is no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2019.06.005.

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