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Persistent organic pollutants and children's respiratory health: The role of cytokines and inflammatory biomarkers

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ABSTRACT

Evidence of adverse effects of persistent organic pollutants (POPs) on the developmental respiratory and immune systems in children is still limited, and the biological mechanisms behind such effects are not fully understood. The aim of the present study is to evaluate the effects of prenatal DDE, HCB and ΣPCB exposure on children's respiratory health from birth to 14 years and to evaluate the role of immune biomarkers in these associations.

We measured prenatal DDE, HCB and ΣPCB levels in 405 participants of the INMA-Menorca birth cohort (Spain) and collected information on wheeze, chest infections, atopy and asthma from birth until the age of 14 years. At age 4 years, 275 children provided serum samples and IL6, IL8, IL10, TNFα and C-reactive protein were measured. We applied linear and logistic regression models and generalized estimating equations.

Prenatal DDE was associated with wheeze at age 4 years [RR (95% CI) per doubling of concentration = 1.35 (1.07, 1.71)], but not thereafter. Prenatal HCB was associated with wheeze [1.58 (1.04, 2.41)] and chest infections [1.89 (1.10, 3.25)] at age 10 years. No associations were found with ΣPCBs. IL10 levels increased with increasing POP concentration, with HCB showing the strongest association [β (95% CI) = 0.22 (0.02, 0.41)]. IL8, IL10 and TNFα were associated with wheeze and/or chest infections and IL10 was associated with asthma.

Prenatal DDE and HCB exposure was associated with respiratory health of children at different ages. This study further suggests a possible role of IL10, but not of the other immune biomarkers examined, as an early marker of chronic immune-related health effects of POPs.

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1. Introduction

Even though the production of many persistent organic pollutants (POPs), such as dichlorodiphenyltrichloroethane (DDT),

hexachlorobenzene (HCB) or polychlorinated biphenyls (PCBs), was banned in many countries since the 70s, these compounds can still be detected in the human population because of their capacity to bioaccumulate. Early-life exposure to POPs may adversely influence the development of the respiratory and immune systems in children (Gascon et al., 2013). A recent study including more than 4000 children from eight European birth cohort studies found increasing prenatal DDE levels to increase the risk of wheeze and/or bronchitis under the age of 18 months (Gascon et al., in press). Similar associations were also observed in a Canadian birth cohort in relation to low respiratory tract infections (Dallaire et al., 2004) and acute otitis media (Dewailly et al., 2000). This Canadian cohort also observed associations between prenatal PCB153 (Dallaire et al., 2004, 2006) and HCB (Dewailly et al., 2000) and respiratory infections. Furthermore, a recent birth cohort study including almost 900 mother–child pairs observed that prenatal exposure to HCB and PCB-118 increased the risk of suffering from asthma at the age of 20 years. Several studies have assessed the impact of early life

exposure to POPs on immune cell counts (i.e. T-cells or B-cells), with the aim to evaluate potential biological mechanisms of the association between POP exposure and immune and respiratory health in children. Immune cell counts are useful as general indicators of general immune status (Gascon et al., 2013), however, cytokine assays have the potential to provide more specific mechanistic insights into the effect of environmental exposures (Duramad et al., 2007; Tryphonas, 2001). Increased levels of certain cytokines and biomarkers of inflammation, including interleukin (IL) 4, IL5, IL8, and IL10, soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1) and C-reactive protein (CRP), have been associated to asthma and related symptoms in children (Deraz et al., 2012; Figueiredo et al., 2012; Heaton et al., 2005; van de Kant et al., 2012; Robroeks et al., 2010; Tang et al., 2002). However, only four small studies in children (N < 83) assessed cytokine response in relation to prenatal exposure to POPs (Bilrha et al., 2003; Brooks et al., 2007; Noakes et al., 2006; Tsuji et al., 2012), and three of these measured in cord blood, a matrix where cytokine response has been shown to be very low (Holt and Jones, 2000; Krampera et al., 2000). Therefore, larger studies measuring cytokines later in childhood are required. In the INMA (Infancia y Medio Ambiente) birth cohort study of Menorca, including more than 400 children, increasing prenatal exposure to DDE during pregnancy was found to be associated with children's wheeze at age 4 years and asthma at the age of 6.5 years (Sunyer et al., 2005, 2006). No associations with prenatal HCB and PCBs were observed. Looking for potential mechanisms, no associations were found between prenatal DDE and total cell and eosinophil counts or specific IgE.

Because the long-term respiratory health effects of prenatal exposure to POPs has only been assessed in one study (Hansen et al., 2013) and because there is lack of information on the mechanisms behind the respiratory health of POPs, the present study aims to evaluate the effects of prenatal DDE, HCB and ΣPCB exposure on children's respiratory health, including chest infections and asthma related symptoms, from birth to 14 years of life and to evaluate the role of cytokines and biomarkers of inflammation in these associations.

2. Methods

2.1. Study population

The INMA-Menorca birth cohort (Spain) recruited women presenting for antenatal care between 1997 and 1998 (Guxens et al., 2012). A total of 482 mothers (94% of those eligible) were enrolled into the cohort. Of these, 405 provided information on the respiratory health of their children in the 1st year of life and had information on POP levels in cord blood (study population A). At the age of 4 years, blood samples were drawn from 360 children and stored at −20 °C. Due to budget limitations, cytokines and biomarkers of inflammation were measured in 275 serum samples, which were selected randomly from individuals with complete information on prenatal POP exposure and wheeze and chest infections at age 4 years (study population B).

2.2. Exposure assessment

Cord blood samples were collected and analyzed for DDE, HCB and PCB101, 118, 138, 153 and 180. Analyses were carried out in the Department of Environmental Chemistry of the Institute of Environmental Assessment and Water Research (IDAEA-CSIC) in Barcelona, Spain, using gas chromatography (GC) with electron capture detection (Hewlett–Packard 6890N GC-ECD; Hewlett–Packard, Avondale, PA, USA) and GC coupled to chemical ionization negative-ion mass spectrometry (Hewlett–Packard 5973 MSD) (Carrizo et al., 2007). PCB congeners 101, 118, 138, 153 and 180 were summed to create one single variable (ΣPCBs).

2.3. Respiratory health

The occurrence of wheeze, chest infections and asthma was evaluated via interviewer-led questionnaires with the mother. Wheezing was reported in questionnaires at years 1, 2, 3, 4, 6.5, 10 and 14, and was described as, "whistling or wheezing from the chest, but not noisy breathing from the nose". At the age of 2, 3, 4, 6.5 and 10 years parents were asked about chest infections: "In the last 12 months, did your [child] have a chest infection?". At the age of 6.5 years both the question on wheeze and chest infection referred to the last 24 months. At ages 10 and 14 years, parents were asked if their child had ever been diagnosed of having asthma by a doctor. Also, at the age of 6.5 years atopy status of the children was tested using skin prick test (SPT). A positive skin test to at least one allergen (Der p 1, Der f 1, cat, dog, grass pollen, mixed tree, mixed graminae, parietaria) was considered indicative of atopy. A weal of 2 mm or greater in the presence of a positive histamine control and a negative uncoated control constituted a positive skin test (Polk et al., 2004).

2.4. Immune biomarkers: cytokines and biomarkers of inflammation

Multiplex assays provide multiple advantages in front of older techniques, such as individual enzyme linked immunosorbent assays (ELISA), because they allow to measure multiple analytes from the same sample simultaneously, which results into the use of less sample and analyses at a lower cost (Loo et al., 2011). However, this technique has also some limitations. For instance, the performance of the multiplex assay decreases with increasing number of analytes (Chaturvedi et al., 2011). Since standard cytokine panels can include a lot of analytes, and since detection of these analytes can be very low and with a high coefficient of variation (CV) between duplicates, we performed a first pilot study including 35 samples (in duplicates) in order to select the best analytes in terms of detectability and CV. These analyses were performed at Merck Millipore's laboratory in Abingdon, UK. Interferon gamma (INFγ), IL1β, IL2, IL4, IL5, IL6, IL8, IL10, IL13 and tumor necrosis factor alpha (TNFα) were analyzed in the standard MPXHCYTO-60K Millipore's panel. We chose this panel because it includes a wide spectrum of interleukins and biomarkers related to inflammation and alteration of the immune system. After the pilot study, we decided to analyze IL2, IL8, IL10, and TNF-α because the other analytes were detected in less than 10% of the samples. Further, we performed an ELISA test (R&D systems HS600b panel), with better detection rates than the multiplex technique, to measure IL6. sICAM-1 and sVCAM-1 were measured with Millipore's panel HCVD2MAG-67K. Finally, CRP was measured by immunoturbidimetry at the laboratory of the groups EGEC and CARIN of IMIM Foundation, Barcelona, Spain. All analyses were performed in duplicates except for IL6 ELISA, for which only 35 samples were tested in duplicates spread along the different plates. For CRP, those samples with values over 1 mg/dL were repeated to ensure that results were correct. IL2 was detected in less than 80% of the samples, so it was excluded from the analyses.

2.5. Other variables

Questionnaires administered to mothers during pregnancy and the subsequent follow-ups collected information on maternal and paternal asthma, rhinitis, eczema, smoking during pregnancy and during postnatal life of the child, education and social class (using the UK Registrar General's 1990 classification according to parental occupation by ISCO88 code), maternal age at birth, parity (first child or more), number of siblings (none or one or more), age of the child at the time of starting daycare attendance, duration of breastfeeding and age of the child at the time of outcome assessment. Gender, gestational age and birth weight were extracted from clinical records and maternal body mass index (BMI) during pregnancy (first trimester) and child BMI at different ages were calculated from the weight and height measurements with

the same instrument in all subjects using a standardized protocol. Child's BMI was standardized by age to obtain z-scores (Kuczmarski et al., 2002; de Onis et al., 2009).

2.6. Statistical methods

For each study population (A and B) missing values in co-variables (between 1.1% and 54.6% of missing values) and samples of POPs or biomarkers below the LOD were imputed by multiple imputation (Royston, 2005). Analysis limited to only complete-cases may suffer more from chance variation, and, under the missing at random assumption, multiple imputation increases efficiency and reduces biases that may arise in complete-case analysis (Sterne et al., 2009) (see Supplemental material, p. 2 for further information). The analytical method measuring IL6 could not establish a value for concentrations over 10 pg/mL, the maximum concentration the method was able to detect (2.9% of the samples). We assigned 10 pg/mL to those samples.

Because original distributions were skewed, levels of POPs and immune biomarkers were log_2-transformed. This means that the relative risk is expressed as the percentage increase for each doubling of the exposure (i.e. a RR of 1.35 means an increase of 35% of the risk per doubling of exposure). Levels of sICAM-1 and sVCAM-1 already followed a normal distribution, thus, these were not log-transformed. Correlation between log_2-transformed biomarker levels was tested with Pearson correlations, whereas the correlation between two binary outcomes was tested with tetrachoric correlations.

Analyses of the relationship between POP exposure (DDE, HCB and ΣPCBs) or immune biomarker levels and dichotomous outcome variables (wheeze, asthma, chest infections, atopy) were conducted using logistic regression models. For the association between POPs and immune biomarker levels, linear regression models were performed. To assess the role of POPs or immune biomarker levels on the risk of wheeze and chest infections in each time point of assessment, adjusted for wheeze/chest infection status in all previous years, we used generalized estimating equations (GEE) with an unstructured correlation matrix and an interaction term between the time point and the exposure variable. Because immune biomarkers were measured at age 4 years, only outcomes from this point onwards were included.

For covariate selection, we first identified a set of potential confounding variables based on the previous literature. In order to simplify the analysis, three different models were defined for each combination of explanatory and explained variables: POPs and respiratory outcomes, POPs and immune biomarkers and immune biomarkers and respiratory outcomes. For each combination, if a covariate was associated ($p < 0.05$) to the explained and the explanatory variables, it was included in the first model. Additionally, variables with a p-value between >0.05 and <0.1 in the bivariate analysis, were entered one by one in this first model; if the coefficient was modified by more than 10%, the variable remained in the final model. Gender, plate of biomarker's analysis and age of the child at the time of blood extraction for biomarkers' analysis or outcome assessment were directly included in the respective models.

Finally, the influence of multipollutants on the relationship between DDE, HCB or ΣPCBs and respiratory health outcomes and cytokines and biomarkers of inflammation was examined by including these compounds together in one model. All the analyses were done with STATA 12.

3. Results

3.1. Description of the study populations

Study populations A and B were similar regarding general characteristics, occurrence of respiratory symptoms and exposure to POPs (Table 1). DDE in cord blood was detected in all samples and showed the highest concentrations [median (25th, 75th) = 1.03 (0.57, 1.94) ng/mL], compared to HCB and ΣPCBs [0.68 (0.45, 1.03) and 0.58 (0.44,

0.82), respectively] (study population A — Table 1). The correlation between these compounds varied from 0.25 to 0.38. The prevalence of wheeze and chest infections decreased with years, whereas asthma prevalence, low in this population, increased between the 10th and the 14th year of life (Table 1). The prevalence of atopy at the age of 6.5 years was 15.7%. The correlation between occurrence of wheeze at different ages and atopy at age 6.5 years increased with age (r = −0.13 at age 1, 0.07 at age 2, 0.06 at age 3, 0.20 at age 4, 0.59 at age 6, 0.70 at age 10 and 0.83 at age 14), whereas correlations between wheeze and chest infections at different ages showed a less clear pattern (0.81 at age 2, 0.68 at age 3, 0.67 at age 4, 0.82 at age 6 and 0.49 at age 10). Biomarkers were detectable in practically all 4 year serum samples, except IL8, IL10 and CRP (Table 2). CRP and IL6, and sICAM-1 and sVCAM-1, were the most strongly correlated biomarkers (r = 0.60 and r = 0.49, respectively), whereas the rest were poorly, but always positively, correlated (see Supplemental material Table A). sICAM-1 and sVCAM-1 did not show any association with respiratory outcomes or POP exposure; therefore, results for these cytokines will not be further presented, only in the descriptive tables.

3.2. Prenatal POP exposure and respiratory outcomes

Results of this section are based on study population A (Table 3 and Fig. 1); similar results were obtained for study population B (see Supplemental material Table B), as well as in the complete-case analysis (see Supplemental material Table C). An increased risk of wheeze at age 4 years was observed in relation to prenatal DDE [RR (95% CI) per doubling of concentrations = 1.35 (1.07, 1.71)], however, no associations were found with wheeze at age 6.5, 10 or 14 years, neither with chest infections or asthma at any age or atopy at age 6.5 years (Table 3 and Fig. 1). Prenatal HCB was associated with wheeze and chest infections at age 10 years [1.58 (1.04, 2.41) and 1.89 (1.10, 3.25), respectively] but no further associations were observed (Table 3 and Fig. 1). No associations were found between prenatal ΣPCB concentrations and any of the outcomes assessed (Table 3 and Fig. 1). The inclusion of all pollutants in the model did not modify the associations found in the one pollutant models. For instance, once all pollutants were included in the model, the RR (95% CI) for prenatal DDE and wheeze was 1.33 (1.04, 1.69) and that for prenatal HCB and wheeze was 1.53 (1.00, 2.35).

3.3. Immune biomarkers and respiratory outcomes

The risk of wheeze at age 4 years increased with increasing levels of IL8, IL10 and TNFα assessed at this same age (Table 4). IL10 remained associated with wheeze at age 6.5 years [RR (95% CI) per doubling of levels = 1.41 (1.01, 1.95)]. At older ages there was no association between IL10 and wheeze (Table 4). IL8, and especially TNFα, were associated with chest infections at age 4 and/or 6.5 years (Table 4). The risk of chest infections was always not significantly increased with increasing IL10 levels (Table 4). Increasing levels of IL10 were associated with asthma ever at 10 and 14 years [1.64 (1.05, 2.59) and 1.60 (1.05, 2.44), respectively]. No associations were observed between immune biomarkers and atopy at age 6.5 (Table 4). In the complete-case analysis the direction of the associations remained but some confidence intervals became wider and significance was lost; for instance, the associations between IL10 levels and asthma at ages 10 and 14 [1.56 (0.88, 2.76) and 1.37 (0.69, 2.71), respectively] (see Supplemental material Table D).

3.4. Prenatal POP exposure and immune biomarkers

None of the immune biomarkers assessed at age 4 years were associated to prenatal concentrations of DDE, HCB or ΣPCBs, except IL10, which increased with increasing concentrations of all three compounds [β (95% CI) per doubling levels = 0.11 (−0.01, 0.24), 0.22

Table 1
Characteristics of study populations A and B.

Characteristics	Study population A (N = 405)		Study population B (N = 275)	
	N[a]		N[a]	
Mother				
BMI (%)	391		268	
<=20		17.6		17.2
>20 to <=25		62.2		62.1
>25		20.2		20.7
Education (%)	392		264	
No studies		6.9		6.7
Primary school		52.5		50.6
Secondary		26.6		25.7
University		14.0		17.0
Smoking pregnancy (%)	405	37.5	275	36.4
Asthma (%)	404	7.2	275	6.6
Rhinitis (%)	405	20.5	275	20.7
Father				
Education (%)	395		268	
No studies		11.1		12.1
Primary school		55.7		54.5
Secondary		25.4		24.6
University		7.8		8.9
Social class (%)	394		268	
Professional		15.7		17.1
Skilled manual & non-manual		71.1		69.8
Partially skilled & unskilled		13.2		13.1
Asthma (%)	401	6.5	272	6.9
Eczema (%)	399	13.8	271	12.9
Child				
Gender (male, %)	405	50.9	275	48.0
Breastfeeding weeks [mean (min, max)]	405	18.1 (0.0, 49.0)	275	18.9 (0.0, 49.0)
Z-score BMI 4 years[b] [mean (min, max)]	240	0.4 (−3.1, 3.5)	179	0.4 (−3.2, 3.5)
Age starting daycare attendance (months)	397	17.4 (3.0–62.0)	275	17.1 (3.0–62.0)
Concentrations of prenatal POP exposure [median (25th, 75th) — ng/mL]				
DDE	398	1.03 (0.57, 1.94)	275	1.07 (0.61, 1.99)
HCB	398	0.68 (0.45, 1.03)	275	0.67 (0.46, 0.99)
ΣPCBs	398	0.58 (0.44, 0.82)	275	0.59 (0.47, 0.82)
Respiratory health				
Wheeze				
1 year (last 12 m, %)	405	21.1	275	22.6
2 years (last 12 m, %)	403	29.7	275	31.3
3 years (last 12 m, %)	403	19.1	275	18.6
4 years (last 12 m, %)	398	11.6	275	12.4
6.5 years (last 24 m, %)	389	8.0	269	8.2
10 years (last 12 m, %)	357	7.8	249	8.4
14 years (last 12 m, %)	255	8.2	192	7.3
Chest infections				
2 years (last 12 m, %)	403	62.8	275	65.1
3 years (last 12 m, %)	403	45.2	275	46.2
4 years (last 12 m, %)	398	28.6	275	27.3
6.5 years (last 24 m, %)	389	21.3	268	20.9
10 years (last 12 m, %)	357	4.2	249	4.0
Asthma ever				
10 years (%)	359	4.5	251	5.2
14 years (%)	255	7.5	192	7.3
Atopy (SPT) 6.5 years (%)	356	15.7	261	16.5

BMI: body mass index, DDE: dichlorodiphenyldichloroethylene, HCB: hexachlorobenzene, ΣPCBs: sum of polychlorinated biphenyl congeners 101, 118, 138, 153 and 180, SPT: skin prick test. Study population A includes children with information on POP exposure and respiratory outcomes. Study population B includes children that also have information on cytokines and biomarkers of inflammation.

[a] Number of children with information for each variable before imputation.

[b] In some models z-BMI measured at other ages were also included, but only z-BMI measured at age 4 is shown in the present table.

(0.02, 0.41) and 0.04 (0.00, 0.09)] (Table 5). After inclusion of all pollutants in the model associations were no longer statistically significant: β (95% CI) for prenatal DDE and IL10 was 0.05 (−0.08, 0.19), that for prenatal HCB was 0.16 (−0.05, 0.37) and that for ΣPCBs was 0.03 (−0.03, 0.08). The complete-case analysis resulted into similar estimates but with slightly wider confidence intervals (see Supplemental material Table E).

3.5. IL10 as mediator of the effect

Because IL10 was found to be associated to both POPs and respiratory outcomes, we included this interleukin in the models assessing the association between POPs and respiratory outcomes to study its role as a mediator of the effect; associations, though, did not change by more than 10% (data not shown).

Table 2
Levels of immune biomarkers measured in children's serum at 4 years of age [study population B (N = 275)].

Biomarker	GM (25th, 75th)	%<LOD[a]	CV[b] [mean (min, max)]
IL6 (pg/mL)	1.4 (0.8, 2.2)	0.0[c]	5.9 (0.0, 23.8)
IL8 (pg/mL)	9.1 (6.4, 11.6)	2.2	7.6 (0.0, 72.0)
IL10 (pg/mL)	5.4 (3.5, 7.7)	22.2	10.0 (0.0, 116.9)
TNFα (pg/mL)	14.0 (11.6, 17.0)	0	6.3 (0.0, 37.7)
sICAM-1 (ng/mL)	96.9 (84.4, 112.4)	0	2.9 (0.0, 108.2)
sVCAM-1 (ng/mL)	713.9 (628.8, 811.0)	0	2.8 (0.0, 71.2)
CRP (mg/dL)	0.05 (0.01, 0.2)	8.7	NA

GM: geometric mean, LOD: limit of detection, IL: interleukin, TNFα: tumor necrosis factor alpha, sICAM-1: soluble intercellular adhesion molecule-1, sVCAM-1: soluble vascular cell adhesion molecule-1, CRP: c-reactive protein.

[a] Limit of detection is 3.2 pg/mL for IL8 and IL10 and 0.01 mg/dL for CRP.

[b] Coefficient of variance between duplicates. For IL6 the CV is only based on 35 duplicates.

[c] IL6 did not have values <LOD, but 2.9% of the samples were not detectable above a certain concentration (10 pg/mL).

4. Discussion

This is the first birth cohort study to evaluate the long-term effects of prenatal exposure to POPs on children's respiratory and immune health and to explore the role of cytokines and biomarkers of inflammation in such association. Our results show that early life effects of DDE previously found at the age of 4 years were not observed at later ages, and that HCB was associated to wheeze and chest infections at the age of 10 years. No associations with ΣPCBs were observed. All POPs were associated with increasing levels of IL10 measured at the age of 4 years, with the strongest associations found for HCB. The risk of wheeze and/or chest infections at ages 4 and 6.5 years increased with increasing levels of IL8, IL10 and TNFα measured at the age of 4 years, and the risk of ever suffering from asthma at the ages of 10 and 14 years was increased with increasing IL10 levels.

Table 3
Associations between log$_2$-transformed cord blood concentrations of POPs and respiratory and immune outcomes at different ages [study population A (N = 405)].

	N cases/N total	DDE	HCB	ΣPCBs
		RR (95% CI)	RR (95% CI)	RR (95% CI)
Wheeze[a]				
4 years	46/398	1.35 (1.07, 1.71)	1.18 (0.84, 1.65)	1.06 (0.98, 1.15)
6.5 years	31/389	1.04 (0.79, 1.37)	1.06 (0.71, 1.58)	0.99 (0.90, 1.09)
10 years	28/357	1.22 (0.91, 1.63)	1.58 (1.04, 2.41)	0.97 (0.87, 1.07)
14 years	21/255	0.92 (0.64, 1.31)	1.30 (0.77, 2.19)	0.96 (0.85, 1.08)
Chest infections[a]				
4 years	114/398	1.03 (0.88, 1.22)	0.85 (0.66, 1.09)	1.01 (0.95, 1.07)
6.5 years	83/389	0.98 (0.81, 1.18)	1.06 (0.81, 1.40)	1.03 (0.96, 1.10)
10 years	15/357	1.27 (0.86, 1.86)	1.89 (1.10, 3.25)	1.11 (0.97, 1.29)
Asthma ever				
10 years	16/359	1.03 (0.71, 1.50)	1.21 (0.67, 2.18)	0.94 (0.82, 1.08)
14 years	19/255	0.89 (0.61, 1.31)	1.08 (0.61, 1.90)	0.93 (0.82, 1.06)
Atopy				
6.5 years	56/356	0.97 (0.80, 1.20)	1.10 (0.81, 1.51)	0.99 (0.92, 1.07)

RR: relative risk, DDE: dichlorodiphenyldichloroethylene, HCB: hexachlorobenzene, ΣPCBs: sum of polychlorinated biphenyl congeners 101, 118, 138, 153 and 180.
Adjusted models include gender, breastfeeding weeks, child's z-BMI for each time period (for asthma ever at age 10/14 years z-BMI measured at age 10/14 was used and for atopy 6.5 years z-BMI measured at age 6.5 years was used), age of the child when starting daycare attendance, maternal BMI, maternal asthma and maternal and paternal education (and age of the child at the time of outcome assessment for asthma ever 10/14 years).

[a] Generalized estimating equation models include wheeze since the 1st year of life or chest infections from the 2nd year of life, but only results at the ages of 4, 6.5, 10 and 14 years are shown in the present table.

4.1. POPs and respiratory outcomes

Prenatal exposure to DDE has been found to be related to increased risks of respiratory infections and/or wheeze in a few birth cohort studies in children under the age of 24 months (Dallaire et al., 2004; Dewailly et al., 2000; Gascon et al., 2012; Sunyer et al., 2005, 2006, 2010; Gascon et al., in press) and one at the age of 5–7 years (Dallaire et al., 2006). However, at the age of 20 years Hansen et al. did no find associations between prenatal DDE exposure and asthma occurrence (Hansen et al., 2013). In the present study, prenatal exposure to DDE did not associate with chest infections at any age, and the association with wheeze at 4 years, previously reported (Sunyer et al., 2005, 2006), was not observed at further ages. Regarding HCB, no effects have been observed between prenatal exposure to this compound and wheeze or respiratory infections in children of 14 months of age (Gascon et al., 2012). However, a recent study on prenatal exposure to POPs and asthma occurrence at the age of 20 years described an increased risk of asthma in relation to prenatal HCB exposure in this population (Hansen et al., 2013). These results are consistent with the present study, where wheeze and chest infections at the age of 10 years, but not earlier, were associated to prenatal HCB exposure. Effects of different compounds at different ages may be explained by different mechanisms of action or, alternatively, to chance effects due to the small sample size of most of the studies available so far. Further research should now focus on the follow-up of these effects at later ages in order to confirm the results obtained by Hansen et al. and in the present study. Further, the fact that we did not find associations between prenatal DDE or HCB and atopy at age 6.5 years suggests that the atopic pathway does not explain the potential associations between prenatal DDE or HCB exposure and respiratory outcomes, and that other mechanisms more related to immune response to pathogens may be involved. This is supported by the fact that Sunyer et al. (2005) found no associations with eosinophil counts or specific IgE in this population in relation to prenatal DDE. Asthma can be classified into eosinophilic, neutrophilic or paucigranulocytic asthma (Simpson et al., 2007); if, as discussed above, we hypothesize that the atopic pathway is not the one explaining the potential associations between prenatal DDE or HCB and wheeze/chest infections, and that other mechanisms more related to immune response to pathogens are related, this kind of classifications might be helpful in future studies.

4.2. The role of cytokines

To promote the destruction of the pathogen once an infection occurs, IL8 mediates the initiation and amplification of the inflammatory process (Berry et al., 2007; Puthothu et al., 2006), whereas TNFα seems to be a key factor in the perpetuation of innate immune activation in the airways (Simpson et al., 2007). In subjects with neutrophilic asthma, higher levels of IL8 and TNFα have been described (Simpson et al., 2007). Furthermore, IL8 seems to play an important role in lung diseases such as bronchial asthma or respiratory syncytial virus (Puthothu et al., 2006). IL10, a pleiotropic cytokine with several functions in several tissues, is released to contribute to the destruction of the pathogen but also to limit the inflammatory processes that could cause tissue damage (Mocellin et al., 2003). Also, IL10 has been found to facilitate viral persistence after infection (Wilson and Brooks, 2011). This whole picture might explain why in the present study increasing IL8, IL10 and TNFα levels were associated to wheeze and/or chest infections. The fact that the associations for IL8 and TNFα relapsed or even reversed after the age of 6.5 years might be explained by the fact that these biomarkers, with a relatively short half-life (Oliver et al., 1993) and measured at age 4, are not able to explain events so far in time. However, IL10, with also a very short half-life (Rachmawati et al., 2011), remained increased at all ages, and is even associated to asthma at 10 and 14 years. Therefore, IL10 measured at age 4 years may be an early indicator of other processes of the immune system
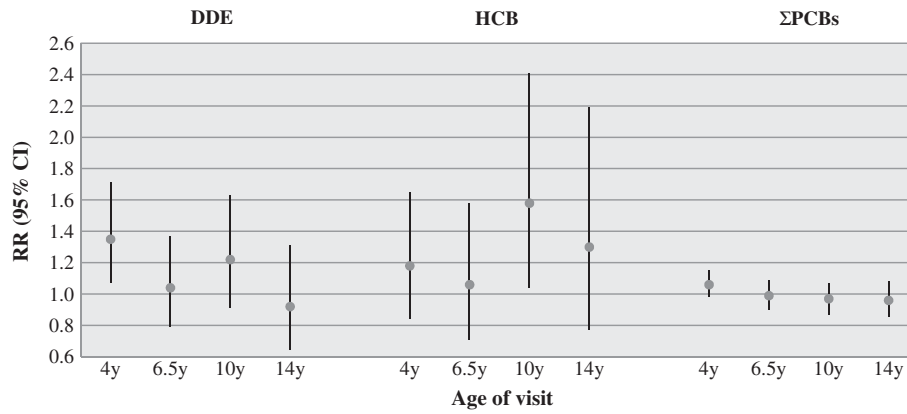
Fig. 1. Association between prenatal DDE, HCB and ΣPCBs with wheeze at different ages.

involved in calming proinflammatory events occurring in children prone to suffer from asthma.

In the present study, IL10, but not IL8 or TNFα, increased with increasing prenatal concentrations of POPs, mostly, HCB. Previous studies are not conclusive about the specific immunotoxic pathways of DDE, however, several of them suggest its role in the depression of T helper cell type 1 after cell stimulation, which is responsible for response to viral infections (Sunyer et al., 2010). In a cross-sectional study with children, DDE was found to induce unregulated apoptosis in human peripheral blood mononuclear cells (PBMC) (Perez-Maldonado et al., 2006). Phagocytosis of apoptotic cells by macrophages seems to activate the production of IL10 and therefore suppress the secretion of proinflammatory cytokines (Byrne and Reen, 2002; Fadok et al., 2000; Voll et al., 1997). Regarding HCB, in animal models, it has been shown to activate macrophages to produce proinflammatory mediators, leading to a systemic inflammatory response (Ezendam et al., 2005a). All these events might in turn induce lung eosinophilia (Ezendam et al., 2005b). Therefore, both DDE and HCB seem to alter the normal functioning of cell-signaling and, in both cases, cytokines seem to be involved. IL10 might be increased by these compounds through different pathways, such as phagocytosis of apoptotic cells by macrophages, or as a response to the general inflammation milieu. However, in the present study IL10 increased in relation to these compounds, but not the proinflammatory cytokines

(TNFα, IL6 and IL8), which might be indicating that pathways other than inflammation are involved in the relationship between prenatal POP exposure and IL10. Additionally, when IL10 was included in the models assessing the association between prenatal POP exposure and respiratory outcomes, it did not significantly modify such associations. This might be indicating that IL10 is not acting as a mediator in the association between prenatal POPs and respiratory health of children. However, IL10 may also be an early marker of other chronic immune-related health effects of exposure to prenatal POPs.

4.3. Strengths and limitations

This is a novel study integrating information on prenatal POP exposure, respiratory health of children from birth until adolescence and cytokines and biomarkers of inflammation. The limitations of using questionnaires to assess occurrence of respiratory symptoms or diseases are well known and, additionally, we did not have information on specific respiratory infections (e.g. bronchitis or pneumonia) or asthma types. However, the high correlations between wheeze and chest infections in the first years of life and its decrease towards adolescence, the increasing association of wheeze with atopy at older ages, and the high correlation between wheeze and asthma (0.69 at age 10 years and 0.89 at age 14 years) are in accordance with what is expected regarding changes in etiology of wheeze with increasing age

Table 4

Association between log$_2$-transformed levels of immune biomarkers measured at age 4 years and respiratory and immune outcomes at different ages [study population B (N = 275)].

	N case/N total	IL6 (pg/mL) RR (95% CI)	IL8 (pg/mL) RR (95% CI)	IL10 (pg/mL) RR (95% CI)	TNFα (pg/mL) RR (95% CI)	CRP (mg/dL) RR (95% CI)
Wheeze[a]						
4 years	34/275	1.20 (0.86, 1.66)	1.67 (1.07, 2.61)	1.31 (0.99, 1.74)	2.80 (1.20, 6.53)	1.11 (0.96, 1.29)
6.5 years	22/269	1.15 (0.79, 1.70)	1.19 (0.69, 2.04)	1.41 (1.01, 1.95)	2.26 (0.94, 5.46)	1.09 (0.91, 1.31)
10 years	21/249	1.02 (0.68, 1.52)	0.79 (0.42, 1.45)	1.03 (0.69, 1.53)	0.83 (0.36, 1.90)	1.00 (0.82, 1.21)
14 years	14/192	1.26 (0.79, 2.00)	0.55 (0.25, 1.22)	1.21 (0.80, 1.84)	0.67 (0.24, 1.86)	1.13 (0.91, 1.42)
Chest infections[a]						
4 years	75/275	1.04 (0.81, 1.32)	1.37 (0.96, 1.94)	1.14 (0.91, 1.42)	2.32 (1.22, 4.42)	1.09 (0.97, 1.22)
6.5 years	56/268	1.10 (0.85, 1.43)	1.47 (1.01, 2.14)	1.15 (0.91, 1.46)	2.44 (1.21, 4.93)	1.03 (0.91, 1.17)
10 years	10/249	0.89 (0.50, 1.59)	0.68 (0.29, 1.64)	1.35 (0.89, 2.06)	0.14 (0.04, 0.51)	0.97 (0.74, 1.26)
Asthma ever						
10 years	13/251	1.05 (0.61, 1.80)	1.62 (0.77, 3.45)	1.64 (1.05, 2.59)	0.99 (0.26, 3.85)	1.03 (0.79, 1.33)
14 years	14/192	1.14 (0.69, 1.87)	1.56 (0.71, 3.41)	1.60 (1.05, 2.44)	0.92 (0.28, 3.01)	1.03 (0.79, 1.35)
Atopy						
6.5 years	43/261	1.08 (0.83, 1.41)	1.07 (0.74, 1.56)	1.05 (0.84, 1.31)	1.31 (0.74, 2.31)	1.04 (0.92, 1.19)

IL: interleukin, TNFα: tumor necrosis factor alpha, CRP: c-reactive protein.
Adjusted models include plate of cytokine's analysis, age of the child at the time of blood extraction for cytokine's analysis, gender (and age of the child at the time of outcome assessment for asthma ever 10/14 years and atopy at age 6.5 years), breastfeeding weeks, child's z-BMI for each time period (for asthma ever and atopy at age 6.5 years z-BMI measured at age 4 years was used), age of the child when starting daycare attendance, maternal BMI, maternal education, maternal smoking during pregnancy and paternal asthma and eczema.
[a] Generalized estimating equation models include wheeze or chest infections since the 4th year of life.

Table 5
Association between log$_2$-transformed cord blood concentrations of POPs and immune biomarker levels measured at age 4 years [study population B (N = 275)].

	IL6 (pg/mL)	IL8 (pg/mL)	IL10 (pg/mL)	TNFα (pg/mL)	CRP (mg/dL)
	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
DDE	0.01 (−0.10, 0.12)	0.00 (−0.08, 0.07)	0.11 (−0.01, 0.24)	0.02 (−0.02, 0.07)	0.01 (−0.23, 0.24)
HCB	−0.03 (−0.20, 0.15)	0.06 (−0.06, 0.18)	0.22 (0.02, 0.41)	−0.02 (−0.10, 0.05)	0.22 (−0.14, 0.59)
ΣPCBs	0.00 (−0.04, 0.04)	0.00 (−0.02, 0.03)	0.04 (0.00, 0.09)	0.01 (−0.01, 0.03)	0.07 (−0.02, 0.15)

β: coefficient, IL: interleukin, TNFα: tumor necrosis factor alpha, CRP: c-reactive protein, DDE: dichlorodiphenyldichloroethylene, HCB: hexachlorobenzene, ΣPCBs: sum of polychlorinated biphenyl congeners 101, 118, 138, 153 and 180.
Adjusted models include plate of cytokine's analysis, age of the child at the time of blood extraction for cytokine's analysis, gender, breastfeeding weeks, child's z-BMI at age 4 years, age of the child when starting daycare attendance, maternal BMI, maternal smoking during pregnancy, maternal education, maternal rhinitis and paternal social class.

(Nair et al., 2011; Stein and Martinez, 2004). The multiplex technique for measuring cytokines and other biomarkers has many advantages because many biomarkers can be measured in one same panel reducing the amount of sample and money needed. However, in the present study we could not detect all cytokines we were interested in (e.g. INFγ, IL4, IL5 and IL13). Additionally, infections can influence cytokines' levels. In the present study we did not have information to exclude children who had infections in the four weeks before blood extraction, a "safe" period left by other studies when working with cytokines (Simpson et al., 2007). In the present study we expressed POP levels on wet-weight basis because we did not have information on lipid levels in our cord blood samples. Thus, we could not evaluate whether the associations found for wet-weight basis would have changed using lipid adjusted exposure information (Schisterman et al., 2005). However, a previous study, in which POP levels in maternal serum were determined in the same laboratory and using the same technique as ours, found that the correlation between lipid adjusted and non-lipid adjusted POP levels was very high (>0.95) and that associations between DDE and lower respiratory tract infections were not influenced by lipid adjustment (Sunyer et al., 2010). Two hundred and forty-five children of the main study population (A) also had information on postnatal POP exposure at the age of 4 years. In this subpopulation, the associations between prenatal DDE and HCB and respiratory health outcomes were not influenced by postnatal exposures (data not shown). Furthermore, no associations with postnatal exposure were observed for any of the outcomes evaluated (data not shown). This suggests that probably prenatal life is the critical period for the respiratory health effects of POP exposure, something already observed for other outcomes such as neurodevelopment (Gascon et al., 2013). The strength of the present study is that this is the first prospective birth cohort study that combines information of in-utero and postnatal exposures (POPs), early immune biomarkers (cytokines and biomarkers of inflammation) and respiratory and allergy outcomes collected from the first year of life until adolescence. Multipollutant models showed little influence of the other pollutants on the associations between DDE and HCB and the respiratory health outcomes, but associations with IL10 were attenuated. In any case, the association found between prenatal POP exposure and IL10 needs replication in future studies. In order to better understand the mechanisms, it would be also interesting in future studies to evaluate whether the health effects observed are modified by atopic status or gender; in the present study the sample size was too small for this. The small sample size and the number of analyses performed in the present study could have led to chance results. However, in the multiple sensitivity analyses performed (study population B, complete-case analyses and inclusion of postnatal and the three compounds in the same model), exposure–response estimates were similar, although in some cases the confidence intervals widened and significance was lost.

5. Conclusions

Prenatal DDE and HCB exposure was associated with respiratory health of children at different ages. This study further suggests a possible role of IL10, but not of the other immune biomarkers examined, as an early marker of chronic immune-related health effects of POPs. These findings require follow-up in larger studies with a longer follow-up and a wider range of immune biomarkers.

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