PERSISTENT ORGANIC POLLUTANTS, BISPHENOL A, PHTHALATES AND RESPIRATORY AND IMMUNE HEALTH IN CHILDHOOD

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A la meva petita família, la base de tot

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Els ecosistemes, a través de l'acció i la interacció de les diferents espècies vegetals i animals i dels elements inanimats que els conformen, proporcionen una gran quantitat de recursos i processos beneficiosos pels humans. En conjunt, aquests beneficis es coneixen com a serveis dels ecosistemes, i inclouen productes com els aliments, la purificació de l'aire o de l'aigua, la producció d'energia o simplement l'entreteniment. És essencialment la gran varietat d'espècies i la interacció entre elles i amb l'entorn la que permet l'existència d'aquestes serveis que ens són beneficiosos; cada element per separat no tindria ni sentit ni possibilitat d'èxit. Per a mi, el CREAL funciona exactament igual que un ecosistema; sense l'activitat de cada un dels seus membres i sense la interacció entre ells, seria impossible acabar proporcionant informació beneficiosa per a la nostra societat. És per això que aquesta tesis no és només meva sinó que també és, en menor o major grau, de la resta de companys que m'han acompanyat durant tot aquest temps; sense ells ara mateix no tindríeu la oportunitat de llegir les 300 pàgines que resten. Gràcies, doncs, a tots els CREALIANS.

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No em puc deixar tampoc a la Mamibel (o Maribel); des del primer dia m'ha cuidat com si fos una filla (bé, a mi i a mig CREAL). Gràcies per les converses al tren, compartir inquietuds i dubtes, i especialment els dinars a Talamanca, juntament amb en Miqueló. No perdis mai la fortalesa que et caracteritza. I què dir dels companys de la Sala B?. Santa paciència han tingut amb mi! Gràcies Donaireman (o alfalfa) per les converses i els bons moments, també per animar-me (i sovint rallar-me fins a la sacietat) a anar més enllà. Ets un "crack". Martínez: ja t'he dit sempre que la meitat d'aquesta tesis és teva. Gràcies per la teva GRAN paciència (sense ella no ens haguéssim entès!) i per dedicar temps a explicar-me conceptes d'estadística (alguns més d'un cop) i solucionar els meus problemes amb l'STATA. Gràcies també per la teva companyonia i les converses a primera hora del matí, on sovint m'has hagut de calmar perquè arribava indignada per alguna cosa o altra. Esther: tres quarts del mateix. Una altra part de la tesis és també teva. Gràcies per la teva perseverança i a dedicar el teu temps a buscar solucions als meus problemes existencials amb l'estadística. Gràcies també per la teva alegria i vitalitat, no perdis mai el riure, diuen que allarga la vida! Lucas i Raquel, gràcies també per donar-me un cop de mà amb l'estadística (es nota que no és el meu fort, eh?). Gràcies també Raquel per ser tant eficient a l'hora d'entregar les dades i respondre dubtes sobre INMA; sempre m'ha impressionat. I ja que vaig sortint de la Sala B, gràcies Eva per la teva visió crítica, l'ajuda a l'hora d'escriure articles i el teu humor sarcàstic (sempre m'ha fet riure). Xoanixxxx, agraïda per la teva companyonia, les teves reflexions sobre la vida i per compartir

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"Si esperes les condicions ideals, aquestes mai es donaran"

Nelson Mandela

Abstract

The hypothesis of the present thesis is that prenatal exposure to persistent organic pollutants (POPs), bisphenol A (BPA) and pthalates may increase the risk of respiratory infections and allergic diseases during childhood, even if exposures are low, and that these effects can persistent until, at least, adolescence. A second hypothesis is that cytokines and biomarkers of inflammation can provide information of the mechanisms behind such associations. Data from the "Infancia y Medioambiente" (INMA) population-based birth cohort project and from six other existing European birth cohort studies have been used in the present thesis, which also includes a systematic review. Results of the present work suggest that prenatal exposure to POPs affects the immune and respiratory health of children, that the effects are observed even at low levels of exposure and that these may last until adolescence. Biological mechanisms behind such effects were not possible to describe in the present thesis, however, it provided information of a potential biomarker (interleukin 10) of chronic immunotoxic effects of POPs. Results also indicate potential effects of prenatal exposure to BPA and phthalates on the development and functioning of the immune and respiratory systems of infants and children. In the present thesis we highlight the limitations of existing studies in the field and provide recommendations for future research. In the meanwhile, and advocating the precautionary principle, legislations to reduce the use of those compounds that are still in the market and are extensively used should be considered.

Resum

La hipòtesis de la present tesis és que l'exposició prenatal a compostos orgànics persistents (COPs), bisfenol A (BPA) i ftalats incrementa el risc de patir infeccions respiratòries i símptomes relacionats amb l'al·lèrgia en infants i nens, inclús a nivells baixos d'exposició, i que aquests efectes poden perdurar fins, com a mínim, l'adolescència. Una segona hipòtesis és que les citoquines i els marcadors d'inflamació poden aportar informació dels mecanismes que hi ha darrera d'aquestes associacions. En la present tesis s'han utilitzat dades de la cohort de naixement "Infancia y Medioambiente" (INMA) i de sis cohorts de naixement Europees. La tesis també inclou una revisió sistemàtica. Els resultats d'aquest treball suggereixen que l'exposició prenatal a COPs afecta els sistemes immunitari i respiratori dels infants i nens, que aquests efectes es donen inclús a exposicions relativament baixes i que aquests efectes poden perdurar fins a l'adolescència. Els mecanismes biològics que podrien explicar els efectes observats no s'han pogut descriure en el present treball, tot i així, hem aportat informació d'un possible biomarcador (interleuquina 10) dels efectes immunotòxics crònics dels COPs. Els resultats també mostren efectes potencials de l'exposició prenatal a BPA i ftalats sobre el desenvolupament i funcionament dels sistemes immunitari i respiratori dels infants i nens. A la present tesis es remarquen les principals limitacions dels estudis existents en aquest camp i es proposen recomanacions de millora per a futurs estudis. Mentrestant, es recomana revisar la legislació actual per tal de reduir l'ús d'aquells compostos que encara estan al mercat i que s'utilitzen àmpliament.

Preface

This thesis was written at the Centre for Research in Environmental Epidemiology (CREAL) between 2011 and 2014 and supervised by Prof. Martine Vrijheid. The work consists of a compilation of five scientific publications co-authored by the PhD candidate according to the procedures of the Biomedicine PhD program of the Department of Experimental and Health Sciences of Universitat Pompeu Fabra.

The present thesis contributed to: 1) the understanding of early and long-term immune and respiratory health effects of prenatal exposure to POPs, 2) the understanding of the mechanism behind such effects, 3) the understanding of the early and mid-term immune and respiratory health effects of prenatal exposure to BPA and phthalates, and 4) highlight the limitations of existing studies in the field and to provide recommendations for future research.

Apart from the original papers included in the present thesis, in which the PhD candidate was responsible for all the statistical analyses and writing of the articles, she has also published three articles as first author on pre and postnatal exposure to POPs and children's neurodevelopment, has co-authored other seven papers related to child health, has participated/collaborated in the European ENRIECO, CHICOS and HELIX projects, and has been the coordinator of the Ribera d'Ebre birth cohort follow-up at the age of 13-15 years. This later work included the preparation of protocols and questionnaires, performance of field work and communication with the personnel of the CAP and the high school of Flix.

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1. INTRODUCTION

Acute respiratory infections are the leading cause of mortality in the world in children less than five years old, accounting for about 20% of all annual deaths in this age group (Rudan et al. 2004). Although deaths related to acute respiratory infections mostly occur in low/middle-income countries, infectious diseases, and in particular lower respiratory tract infections (LRTIs), also remain among the top five causes of death in high income countries (Nair et al. 2011; Rudan et al. 2004; Winans et al. 2011). Additionally, LRTIs occurring during childhood are one of the major risk factors to develop asthma and its symptoms (dyspnoea, wheezing, and others) later in life (Bisgaard and Bonnelykke 2010; Busse et al. 2010; Sigurs et al. 2000). Prevalence of asthma and allergic diseases have rapidly increased over a relatively short period of time in the last 30-40 years (Bousquet et al. 2011; Von Hertzen and Haahtela 2004; Miller and Marty 2010; Williams et al. 1999). In some countries, mainly low/middle-income countries, the prevalence of asthma and allergic related symptoms is still increasing while in others it seems to have stabilized (Bousquet et al. 2011; Spergel 2010). Although genetics play a role in the occurrence of asthma and allergic diseases, the rapid increase in the prevalence of such diseases have put environmental pollutants in the spotlight (Miller and Marty 2010; Winans et al. 2011).

Under the “developmental origin of health and disease” (DOHAD) concept, there is the hypothesis that prenatal and early life

exposures to common environmental contaminants can have an underappreciated but critical impact on children and adult's health (Gluckman and Hanson 2004; Winans et al. 2011). Especially during prenatal life, but also during early postnatal life, the human body rapidly evolves under very regulated processes that, if truncated, can lead to impairment of the development and functioning of the different body systems, including the immune and the respiratory. Thus, slight changes in the immune development can decrease resistance to infectious disease, reduce vaccine efficacy, diminish capacity to fight tumoral cells or enhance autoimmune disease and hypersensitivity reactions (Winans et al. 2011), whereas changes in lung structure and function may predispose individuals to chronic obstructive lung disease and other related disorders later in life (Miller and Marty 2010).

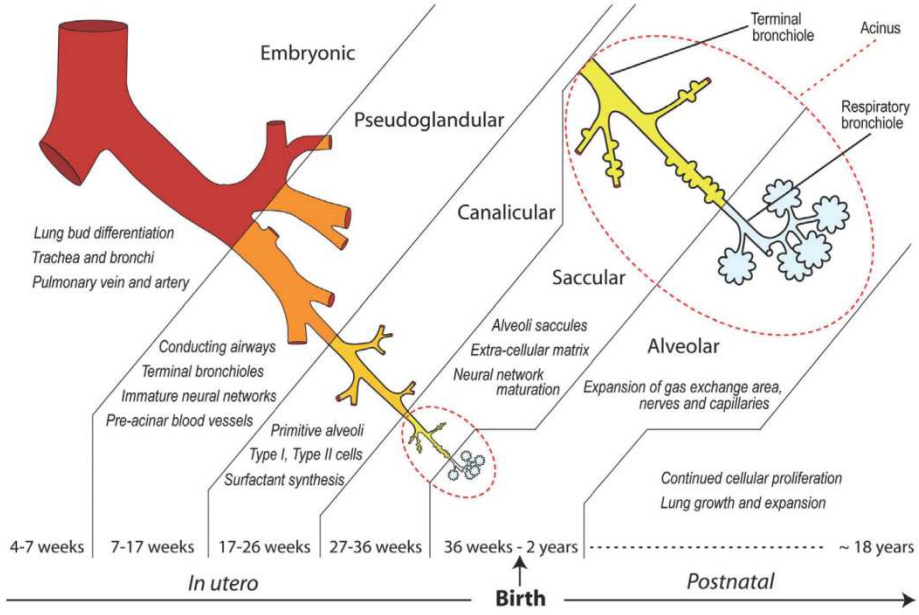
Many environmental pollutants, including synthetic chemicals, have been suggested to disrupt the development and functioning of the immune and respiratory systems in humans and therefore in children (Miller and Marty 2010; Winans et al. 2011). In the present thesis we focused on the immune and respiratory health effects of persistent organic pollutants (POPs), bisphenol A (BPA) and phthalates because exposure to these compounds occurs worldwide and because animal and in-vitro studies suggest potential immune and respiratory health effects of prenatal exposure to these compounds. However, the evidence in humans is still very limited (Braun et al. 2013; Michałowicz 2014; Wigle et al. 2008; Winans et al. 2011).

1.1 The developing respiratory and immune systems

1.1.1 The respiratory system

Development of the human respiratory system involves the differentiation and proliferation of over 40 different cell types and the generation of more than 300 million alveoli. It is a multi-event process that is not restricted to prenatal life, since the majority of changes to the lungs continue postnatally until 18-20 years of life (Figure 1.1) (Dietert et al., 2000; Pinkerton and Joad 2000).

Figure 1.1 Principal stages of lung development in humans: diagrammatic representations of the timeline and development organization of trachea, primary bronchi, intrapulmonary bronchi, and acinus in the mammalian respiratory system. Reprinted from (Kajekar 2007), page 130.



Already during **embryogenesis** transcription factors play an important role in gene expression to regulate the proper temporal

and spatial patterning of lung development. In this period, lung development is also highly dependent on interactions between the epithelium and mesenchyme. These interactions regulate branching and vascularization of the lungs (Dietert et al., 2000; Pinkerton and Joad 2000). During fetal life, lungs develop in three different stages:

- **Pseudoglandular phase:** it is the most critical for the formation of all conducting airways. In this phase, tubular branching continues until full segmentation of the bronchi. A variety of extracellular matrix molecules as well as growth factors and their receptors also play an important role in directing further branching morphogenesis and lung development by influencing the rates of cellular proliferation and differentiation. Epithelial differentiation of ciliated, goblet, and basal cells first appears in the most central airways during this stage of development. Pulmonary arteries follow the branching pattern of the airways.

- **Canalicular phase:** lung morphology changes dramatically and the formation of the future air-blood tissue barrier occurs. Surfactant synthesis and the canalization of the lung parenchyma by capillaries begin.

- **Saccular phase:** widened airspaces, termed saccules, are formed from the peripherial airways. Also, the future gas exchange region expands significantly and populations of fibroblastic cells, responsible for the production of the

extracellular matrix, collagen, and elastin, undergo differentiation. Fibroblastic cells also play an important role in epithelial differentiation and control of surfactant secretion in connection with the growth of the gas exchange region. The vascular tree also grows in length and diameter during this time.

After birth, enlargement of the airways and gas exchange regions by repeated branching and outgrowth of tissues continue. Organized interaction between the epithelial, interstitial, and vascular compartments forming the gas exchange portion of the lungs is critical to the overall growth, development, and formation of alveolar structures. The process of **alveolarization** and cell differentiation, which already starts before birth, continues postnatally (Dietert et al., 2000; Pinkerton and Joad 2000).

1.1.2 The immune system

Development of the immune system involves a coordinated series of events beginning early in gestation and continuing into the postnatal period (Good 1995; Winans et al. 2011). After birth, the ability of the body to produce a normal immune response following antigen exposure (immunocompetence) starts developing and continuous during the first years of life (Dietert et al., 2000). This involves the development of the innate system [natural killer cells (NK-cells), eosinophils, basophils, neutrophils, macrophages or dendritic cells], which provides immediate defense against infection, and the adaptive immune system (antibodies produced by

B-cells and cell-mediate response regulated by cytokines released by T cells), which provides long-lasting or protective immunity through humoral response. Both systems need to be well coordinated and balanced to recognize and fight infectious pathogens, to detect and destroy cancerous cells and prevent tumor growth, and to avoid an overreaction leading to autoimmune or allergic diseases (Luebke et al. 2004; Winans et al. 2011).

The **innate immune response** is based on mechanisms such as phagocytosis, fever and cytokine liberation. In the respiratory system, external mechanisms (secretion of mucus, movement of the mucus by the cilia and presence of alveolar macrophages), which are the first barrier against pathogens, are helped by this innate immunity:

- **Phagocytosis**: there are different types of cells that mediate phagocytosis, which are neutrophils (the first to reach infected sites), monocytes and macrophages, and organ specific phagocytic cells. They kill and ingest bacteria, remainders of cells, proteins and toxins.

- **Endogenous pyrogens**: body temperature is regulated by the hypothalamus, where there is a "thermostat" which maintains the temperature at 37ºC. When endogenous pyrogens [IL1, IL6, TNFα, TNFβ and INFα (Dinarello 1999)] are released, the "thermostat" increases its threshold of activation and then fever appears. There are also

exogenous pyrogens, such as lipopolysaccharids of gram negative. The functions of these pyrogens are to inhibit bacteria's growth, by reducing plasmatic iron, and to estimulate neutrophils' activity and interferon's production, which in turn provides short term unspecific resistance against viruses by inhibiting their replication.

- **Complement system**: involved in bacterial destruction and inflammatory response through cytokine's release.

On the other hand, any individual can acquire the capacity of defending against specific pathogens by being exposed previously to them. This is known as **adaptive immunity** and it is mediated by lymphocytes. The immune system can discriminate between its own molecules and those coming from external organisms. These external molecules are named antigens and they are usually complex and big. Once these antigens are detected by the immune system, it activates in order to counteract the infection. Lymphocytes are the cells responsible for the adaptive immune response and they originally can be found in the thymus, spleen and lymphatic ganglia. There are two types of lymphocytes involved in the adaptive immune response:

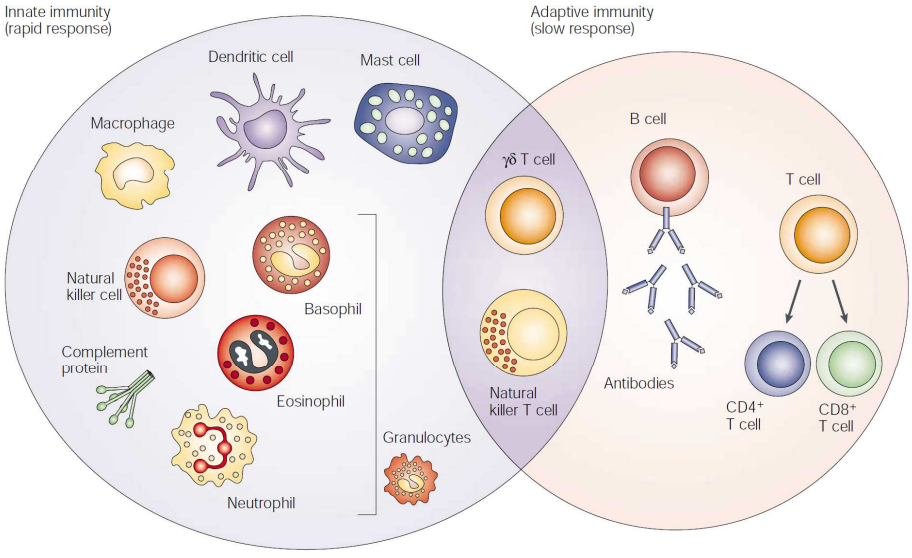
- **T-cells**: these cells, with a long half-life, colonize the thyme and represent the 75-80% of the total lymphocytes amount in blood. Although there are other types of T-cells, these can mainly be divided into CD8+ (Killer T-cells), with a

citotoxic function by secreting perforins and granzymes, and CD4+ (Helper T-cells). When the antigen presenting cells (APC) present the antigens (coming from bacteria, virus or a foreign cell) to CD4+ cells, these activate and divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response. CD4+ cells can differentiate into Th_1 cells, responsible for the activation of macrophages and secretion of interferon-γ (INF-γ) once an infection occurs, and Th_2 cells, important in allergic reactions and parasite infections. Both Th_1 and Th_2 activate B cells.

- **B-cells**: these cells have a short half-life and represent the 10-15% of the total lymphocytes amount in blood. They are processed in the bone marrow and they act against bacterial and some viral infections with the secretion of antibodies in the blood. Once a pathogen is ingested by an APC, like a macrophage or dendrite cell, the pathogen's proteins are digested to peptides and attached to a major histocompatibility complex (MHC) class II protein. This complex is then moved to the outside of the APC cell membrane and presented to Th-cells, which proliferate and differentiate to effector and memory Th-cells. These Th-cells then activate specific B cells and these produce antibodies which assist in inhibiting pathogens.

The use of biomarkers related to a particular immunotoxic end point is valuable in the identification of the presence of a health effect, but also as a predictive biomarker of such health effect later in life (Tryphonas 2001). Most commonly immune function biomarkers used include cell counts, cell surface activation markers, immunoglobulin levels (in particular specific IgE for atopy diagnosis), responses to mitogens, and expression and secretion of cytokines. Counting absolute and relative lymphocyte's subsets serve as a general indicator of immune status, however, intracellular cytokine profile have the potential to provide more mechanistic insights into the effect of environmental exposures (Duramad and Holland 2011; Duramad et al. 2007; Tryphonas 2001). Cytokines are small molecular weight proteins of relatively short half-life

Figure 1.2 The innate and adaptive immune system. Reprinted from (Dranoff 2004), page 13.



secreted by immune cells and regulate the duration and intensity of the immune response. Each cytokine can be secreted by and target different cell types, leading to different activities and effects (Duramad and Holland 2011; Oliver et al. 1993). Despite the potentiality of cytokines to study the immunotoxic effects of environmental exposure, studies in children are still trying to define the key cytokines involved in allergic and asthma related processes or that can be used as early markers of such processes (Heaton et al. 2005; Machura et al. 2007; Robroeks et al. 2010; Tang et al. 2002).

1.2 Respiratory infections, asthma and allergy

1.2.1 Respiratory infections

Respiratory tract infections are divided into those occurring in the upper respiratory tract, which include acute otitis media, laryngitis, pharyngitis, sinusitis or tonsillitis, and those occurring in the lower respiratory tract, which include bronchiolitis, bronchitis and pneumonia. As acute otitis media and LRTIs are the main types of infections analyzed in the present thesis, we limited a further explanation to these respiratory infections. We focused on acute otitis media and LRTIs because these are the most common infections in the first years of life. Additionally, and as mentioned above, LRTIs occurring during childhood are one of the major risk factors to develop asthma and its symptoms later in life.

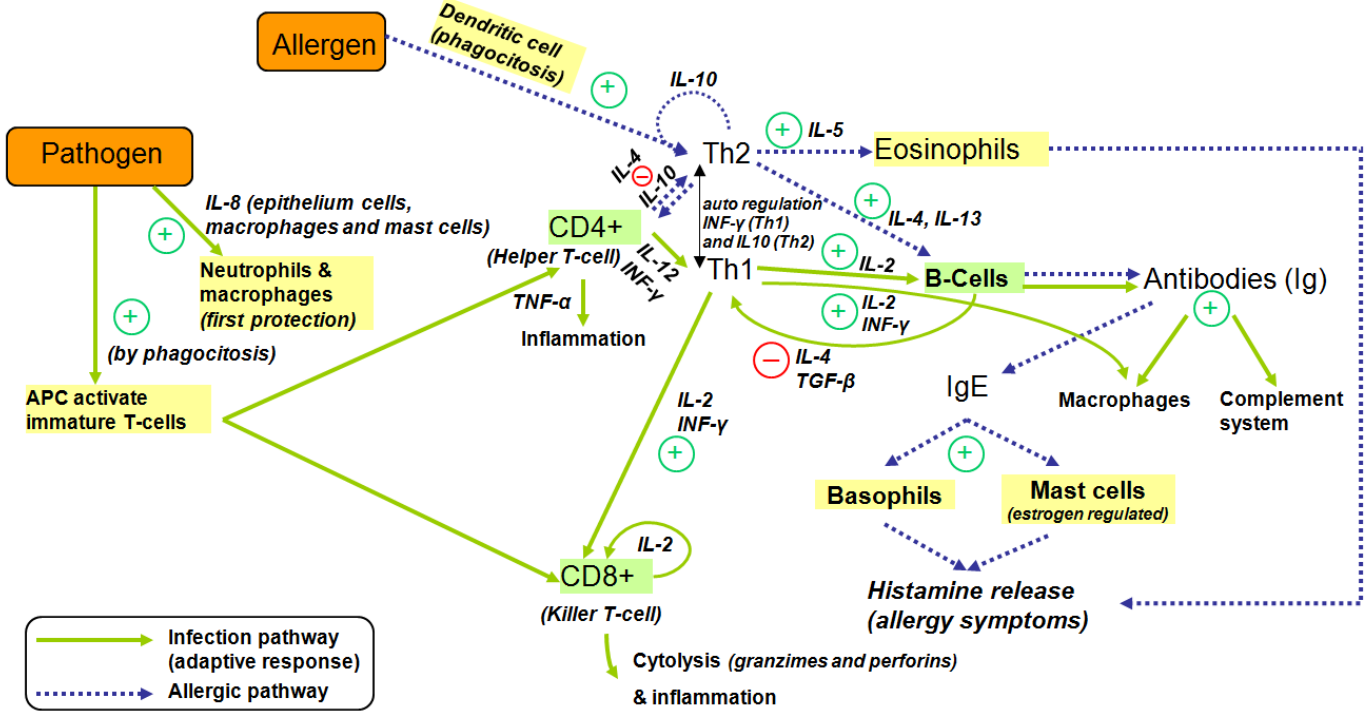
- **Acute otitis media:** it is an inflammation of the middle ear caused by the obstruction of the Eustachian tubes. The origin can be an infection or an allergy reaction. It mostly occurs in infants and children.

- **Bronchiolitis**: it is swelling and mucus build-up in the smallest air passages in the lungs (bronchioles), usually due to a viral infection. Bronchiolitis usually affects children under the age of 2, with a peak age of 3-6 months. It is a common and sometimes severe illness.

- **Bronchitis**: it is an inflammation of the main air passages to the lung. Acute bronchitis generally follows a viral respiratory infection, but sometimes it can be followed by another (secondary) bacterial infection in the airways. At first, it affects the nose, sinuses, and throat and then spreads to the lungs. Infants and young children are among those people at higher risk to contract bronchitis.

- **Pneumonia**: it is an inflammatory condition of the lung, caused by bacteria, viruses, and fungi. In infants and young children, virus is the most common cause of pneumonia, but bacterial infections tend to be the most serious.

Figure 1.3 The complexity of the immune system and the reactions after an infection (pathogen) or the interaction with an allergen.



APC: antigen presenting cell [macrophages and B-cells (activate only memory T-cells) and dendritic cells (activate memory and naïve T-cells]); IL: interleukin; TNF-α: tumor necrosis factor α; INF-γ: interferon- γ; CD4+, CD8+, Th_1 and Th_2: T-cells.

1.2.2 Asthma and allergy

Asthma is an heterogeneous condition with different phenotypes and clinical expressions that depend on age, gender, genetic background and environmental exposures (Antó et al. 2012; Miller and Marty 2010; Stein and Martinez 2004; Winans et al. 2011). The role of the innate immune system in the pathogenesis of asthma is unclear, but it is known that it can have different origins (eosinophilic, neutrophilic or paucigranulocytic) that can explain the heterogenic response of this condition (Simpson et al. 2007). Traditionally, asthma has been classified as an allergic disease because of its co-existence with atopy in many asthmatics. Nowadays it is known that asthma and atopy do not always go together and therefore different asthma phenotypes have been defined according to this co-occurrence, but also according to the persistence of asthma symptoms along an individual's life (Antó et al. 2012; Bousquet et al. 2004; Henderson et al. 2008; Spycher et al. 2010). Wheezing, its major clinical expression, is a nonspecific sign associated with airflow restriction that occurs with the narrowing of central airways. Wheezing can have different causes, which are strongly related to age; in general, at very early ages wheezing is highly associated to the occurrence of LRTIs, and as the child grows, it starts to become more associated to allergic reactions in the airways (Bisgaard and Bonnelykke 2010; Busse et al. 2010). Most cases of chronic asthma begin in preschool age; adults with chronic asthma have a median age of symptom onset of 3 years, and 80% to 90% of cases have onset before the age of 5 years (Bisgaard and Bonnelykke 2010).

1.3 Persistent organic pollutants, bisphenol A and phthalates

1.3.1 Persistent organic pollutants

POPs are chemical substances with a particular combination of physical and chemical properties such that, once released into the environment, they remain intact for exceptionally long periods of time (order of years). They are widely distributed throughout the environment as a result of natural processes involving soil, water and air, and they can be detected even in areas where they have not been produced or used. They are toxic to living organisms, where they bioaccumulate in the fatty tissue, with higher concentrations at higher levels in the food chain (they can magnify by up to 70.000 times the background levels) (Stockholm Convention 2008). Although few of them are naturally generated, human activity has been the major cause of POPs release into the environment. In the past years, levels of some POPs have decreased because they are not produced or used anymore in most countries [i.e. polychlorinated biphenyls (PCBs) or hexachlorobenzene (HCB)] (Jaward et al. 2004; LaKind et al. 2004; Needham et al. 2005). Others, instead, were forbidden in the past but are being used again. This is the case, among a lot of controversy, of dichlorodiphenyltrichloroethane (DDT), a pesticide used in areas with endemic malaria to control its vector, mosquito *Anopheles* (van den Berg 2009; Eskenazi et al. 2009).

POPs have been mainly used as pesticides and as industrial chemicals, but they can also be by-products unintentionally generated in a wide range of processes involving combustion. In 2004, 160 countries signed the Stockholm Convention (Stockholm Convention 2008) with a list of twelve POPs, called the "Dirty Dozen", banned. The aim was to eliminate or reduce its presence in the environment. Later, in May 2009, nine new compounds were added to this list:

- **Pesticides:** aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, HCB, mirex, toxaphene, chlordecone, α-hexachlorocyclohexane (α-HCH), β-hexachlorocyclohexane (β-HCH), lindane, pentachlorobenzene (PeCB).

- **Industrial chemicals:** HCB, PCBs, polychlorinated biphenyls (PBBs), PeCB, perfluorinated compounds (PFCs), polybrominated diphenyl ethers (PBDEs).

- **By-products:** HCB, polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/PCDF), PCBs, α-HCH, β-HCH and PeCB.

Currently, diet is the most important source of exposure to POPs in humans (Llop et al. 2010; Vrijheid et al. 2010). Due to their lipophilic properties, mothers can transfer a certain amount of these compounds during pregnancy across the placenta, but also through breastfeeding during the first months or years of life (LaKind et al.

2004; Rogan et al. 1986). Exposure during infancy and childhood can also take place through other ways, for instance, by toddlers getting in touch with floor dust when crawling, as it has been suggested for PBDEs (Fischer et al. 2006).

According to the Stockholm Convention, POPs can damage the immune system of exposed individuals as well as their offspring (Stockholm Convention 2008). Poisoning incidents in the 70's already provided evidence of these effects in children prenatally exposed to high levels of PCBs and dioxins (Aoki 2001; Guo et al. 2004; Nakanishi et al. 1985). Birth cohort studies have related prenatal and early postnatal exposure to current levels of a variety of POPs with respiratory and other infections, asthma related symptoms and reduced vaccinations response in children (Dallaire et al. 2004; Dewailly et al. 2000; Glynn et al. 2008; Grandjean et al. 2010; Heilmann et al. 2006, 2010; Karmaus et al. 2001; Weisglas-Kuperus et al. 2000). Furthermore, three previous birth cohort studies conducted in Spain already showed an association between prenatal exposure to POPs, particularly DDE, and respiratory infections, wheezing or asthma occurrence between the age of 6 months and 6.5 years (Sunyer et al. 2005, 2006, 2010). Despite a number of studies on the topic, two general reviews on the early effects of POP exposure on the developing immune and respiratory systems concluded that there is limited or inadequate epidemiological evidence (Wigle et al. 2008; Winans et al. 2011). However, none has made a comprehensive and systematic evaluation. Thus, further research on the association between current prenatal and early postnatal exposure to POPs and immune

and respiratory health is needed, as well as a systematic review on the topic. Additionally, most birth cohort studies available have limited their research in evaluating early life effects of POPs in infants and children, but few have assessed long-term effects until adolescence or adulthood.

Regarding the mechanisms behind such potential associations, several animal, in-vitro and human studies, including birth cohort studies, have described altered levels of immune markers in individuals prenatally and postnatally exposed to current levels of POPs. However, mechanisms are not completely understood. The main mechanism described by which DDT, and especially DDE, could affect the immunity system is the induction of apoptosis and necrosis of immune cells (Banerjee et al. 1996; Cooper et al. 2004; Daniel et al. 2002; Dutta et al. 2008; Karmaus et al. 2005; Misumi et al. 2005; Noakes et al. 2006; Perez-Maldonado et al. 2005, 2006; Rehana and Rao 1992; Vine et al. 2001). In one of these studies, DDT was found to increase the production of tumor necrosis factor-α (TNF-α) and nitric oxide (NO) in macrophages of human serum, which contributed to inflammatory reactions, cytokine imbalance and immune-dysregulation (Dutta et al. 2008). The immutoxicity of PCBs has also been described in many animal and in-vitro studies in which PCBs has been shown to inhibit lymphocyte proliferation and reduce macrophage phagocytic activity (Fonnum et al. 2006; Van Den Heuvel et al. 2002; Levin et al. 2005; Lyche et al. 2004; Mori et al. 2008; Sormo et al. 2009; Weisglas-Kuperus et al. 1995, 2000). Furthermore, in a birth cohort study increasing prenatal PCB

exposure was found to reduce thymus size at birth (Park et al. 2008). Similar immunological effects have been found for HCB, which seems to modulate the T cell response and macrophage activity (Ezendam et al. 2004; Michielsen et al. 1999b, 1999a). Presumably, HCB exposure induces macrophage activation, thereby generating adjuvant signals that stimulate T cells (Ezendam et al. 2005a).

1.3.2 Bisphenol A

BPA was first synthesized in 1891, but it was not widely used until the 40's. Currently, it is one of the highest volume chemicals produced worldwide, with over 6 billion pounds produced each year and over 100 tons released into the atmosphere by yearly production (Vandenberg et al. 2009). It is used as an antioxidant, polymerization inhibitor and to strengthen products in polycarbonates, epoxy resins and polyvinyl chloride plastics (PVCs). Polycarbonates are used to produce plastic food containers and plastic films used to protect food, whereas inner parts of metallic food cans are protected by applying epoxy resins as inner coatings. Both polycarbonates and epoxy resins are also used in building materials, CD-ROMs, medical and dental devices, or thermal paper (Rudel et al. 2011; Vandenberg et al. 2009). Also, it has been used in cosmetics as an antioxidant, fungicide, and antimicrobial (Kwak et al. 2009). Overall, diet seems to be the most important route (Rudel et al. 2011). Contrary to POPs, BPA has a short half life and is rapidly metabolized and excreted through urine (Stahlhut et al. 2009).

BPA is estrogenic and binds both to estrogen receptor (ER) ERα and ERβ, with approximately 10-fold higher affinity to ERβ (Vandenberg et al. 2009). It also binds the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor present in almost every tissue. In fact, some studies have observed that BPA can stimulate cellular responses at concentrations below the levels where it is expected to bind to the classical nuclear ERs (Vandenberg et al. 2009; Welshons et al. 2006). This endocrine disruption activity is suggested to explain the immunomodulatory properties of BPA observed in animal and in vitro models (Kwak et al. 2009; Midoro-Horiuti et al. 2010) as well as in general human population (Clayton et al. 2011). So far, only two studies evaluated the effects of prenatal BPA exposure on the occurrence of wheeze in children, obtaining contradictive results (Donohue et al. 2013; Spanier et al. 2012).

1.3.3 Phthalates

It is estimated that total global consumption of phthalate esters is of 11 billion pounds per year. Phthalates family include a large number of congeners with different physico-chemical properties such as di-(2-ethylhexyl) phthalate (DEHP), butylbenzyl phthalate (BBzP), di-n-octyl phthalate (DnOP), dibutyl phthalate (DBP), diisononyl phthalate (DiNP) and diisodecyl phthalate (DiDP), which are high molecular weight phthalates (>250 Da) and dibutyl phthalate (DnBP), diisobutyl phthalate (DiBP) and diethyl phthalate (DEP), which are low molecular weight phthalates (<250 Da) (Bornehag et al. 2004; Hauser and Calafat 2005; Kwak et al. 2009).

These different congeners are present in all kind of products. In general, high molecular weight phthalates are primarily used in flooring and wall coverings, toys, food contact applications and medical devices, whereas low molecular weight phthalates are mainly used in personal-care products (e.g. perfumes, lotions, cosmetics) and in some pharmaceuticals (Bornehag et al. 2004; Hauser and Calafat 2005; Kwak et al. 2009). In general population the main routes of exposure to high molecular weight phthalates are diet and inhalation of residential indoor air, whereas exposure to low molecular weight phthalates is mainly through skin contact (Bornehag et al. 2004; Hauser and Calafat 2005). As with BPA, phthalates have short biological half-lives, metabolize quickly, do not accumulate, and are primarily excreted in the urine (Hauser and Calafat 2005).

Phthalates have been reported to have estrogenic, antiestrogenic and antiandrogenic effects depending on the congener and metabolite analyzed. For instance, DEHP seems to have estrogenic activity, whereas MBzP seems to have antiestrogenic activity. In any case, further studies are needed to clarify the different activities of the different compounds (Christen et al. 2010; Okubo et al. 2003). Because of this potential endocrine disrupting activity, DEHP has been reported to bind the peroxisome proliferator-activated receptor γ (PPARγ), altering airway cell differentiation and surfactant protein production in the lungs and explaining the potential association of DEHP with asthma (Miller and Marty 2010). The evidence that phthalates increase inflammation responses is not very

clear (Bornehag and Nanberg 2010). What seems to be more consistent, according to animal and in-vitro studies, is that several phthalates can have adjuvant effects on Th_2 differentiation and influence antibody response, thus affecting the adaptive immune system (Bornehag and Nanberg 2010; Kimber and Dearman 2010). Most of the studies available up to now studying phthalates compounds suggest that these chemicals, mainly high molecular weight phthalates, might have an effect on the respiratory system of children (Bertelsen et al. 2012; Bornehag et al. 2004; Callesen et al. 2013, 2014; Hoppin et al. 2013; Hsu et al. 2012; Just et al. 2012a, 2012b; Kolarik et al. 2008; Larsson et al. 2010). However, these studies did not assess children's exposure based on biomarkers. Rather, exposure assessment was based on measuring home dust phthalates levels or by counting the number of rooms with PCV flooring in their houses. Only one of these studies assessed prenatal phthalate exposure and did it in relation to eczema, itchy rash and IgE counts in children of 3 months to 5 years of age (Just et al. 2012b). The study reported an increased risk of early onset eczema (before 24 months of age) but not late onset eczema. No other associations were reported.

2. RATIONALE

The rapid increase in the prevalence of asthma and allergic diseases in the last 30-40 years has put environmental pollutants in the spotlight. Fetal life is a critical period in which environmental contaminants can have an underappreciated but critical impact on children and adult's immune and respiratory health. Although the effects of prenatal exposure to high levels of persistent organic pollutants (POPs) on these two systems were clearly observed in the Yusho and Yucheng accidents, the evidence that current low levels of POPs exposure are affecting children's immune and respiratory health is still inadequate or limited. In the last years, new chemicals have become a concern because of their endocrine disrupting activity and because they are produced in large quantities worldwide. Although animal and in-vitro studies show a potential immunomodulatory activity of bisphenol A (BPA) and phthalates, currently there are very few studies evaluating the effects of prenatal exposure to these two chemicals on children's respiratory and immune health. Additionally, birth cohort studies available have mainly focused on the early life effects of POPs, BPA and phthalates, but there is scarce information on whether there are long-term health effects of these exposures occurring during pregnancy. Furthermore, the mechanisms by which these compounds might be affecting these two systems in children are not completed understood. Because of potential prenatal exposure to POPs, BPA and phthalates worldwide and the potential long term immune and respiratory health effects of these compounds, further

research is needed to elucidate their role on the occurrence of immune and respiratory related diseases, the long-term effects and the mechanisms behind such effects.

3. OBJECTIVES

General objective

- To explore the early and long-term effects and the mechanisms behind such effects of prenatal exposure to persistent organic pollutants (POPs), bisphenol A (BPA) and phthalates on the immune and respiratory systems.

Specific objectives

- To systematically review the literature on the effects of POPs on immunotoxicity and respiratory health in infants, children and adolescents.

- To study the associations between prenatal DDE, PCBs and HCB exposure and respiratory infections, wheeze and asthma from infancy to adolescence (ages 1 to 14 years).

- To study the biological mechanisms explaining the associations between prenatal DDE, PCBs and HCB exposure and respiratory infections, wheeze and asthma from infancy to adolescence (ages 1 to 14 years).

- To study the effects of prenatal exposure to BPA and phthalates on respiratory infections, wheeze and atopic eczema during infancy and childhood (ages 1, 4 and 7 years).

4. METHODS

Given their prospective nature, birth cohorts are the best approach to properly assess the relationship between pre and postnatal environmental exposures and the long-term effects associated (Luo et al. 2010). Thus, to achieve the objectives of the present thesis, data from the existing INfancia y Medio Ambiente - INMA (Environment and Childhood - www.proyectoinma.org) birth cohorts will be used, as well as data obtained from other six European birth cohorts participating in the ENRIECO (Environmental Health Risks in European Birth Cohorts - www.enrieco.org) and CHICOS (Developing a Child Cohort Research Strategy for Europe - www.chicosproject.eu) projects. The reason to use information from different birth cohort studies is to increase statistical power of our analyses.

4.1 The "INfancia y Medio Ambiente" (INMA) project

The INMA project is a network of 7 prospective birth cohort studies in Spain following more than 3000 mother-child pairs in total. The aim is to study the role of environmental pollutants in air, water and diet during pregnancy and early in life and their effects on child growth and development (Ribas-Fito et al. 2006). Genetic factors and nutritional, environmental and psychosocial exposures in the prenatal and postnatal periods are being evaluated. Outcomes include prenatal and birth health events, growth, neurodevelopment, behavioural functioning, immunity, respiratory health and endocrine

disruption. The 7 cohorts are divided into the "old" and the "new" cohorts. The "old" cohorts are Menorca (N=482), Ribera d'Ebre (N=102) and Granada (N=668) and were started between 1997 and 2000. The "new" cohorts are Sabadell (N=657), Valencia (N=855), Gipuzkoa (N=638) and Asturias (N=485) and were started between 2004 and 2006 (Figure 4.1). In all this cohorts, follow-ups have been carried out at different ages (Figure 4.2).

Figure 4.1 Localization of the seven INMA birth cohorts in Spain.

4.1.1 The Menorca birth cohort

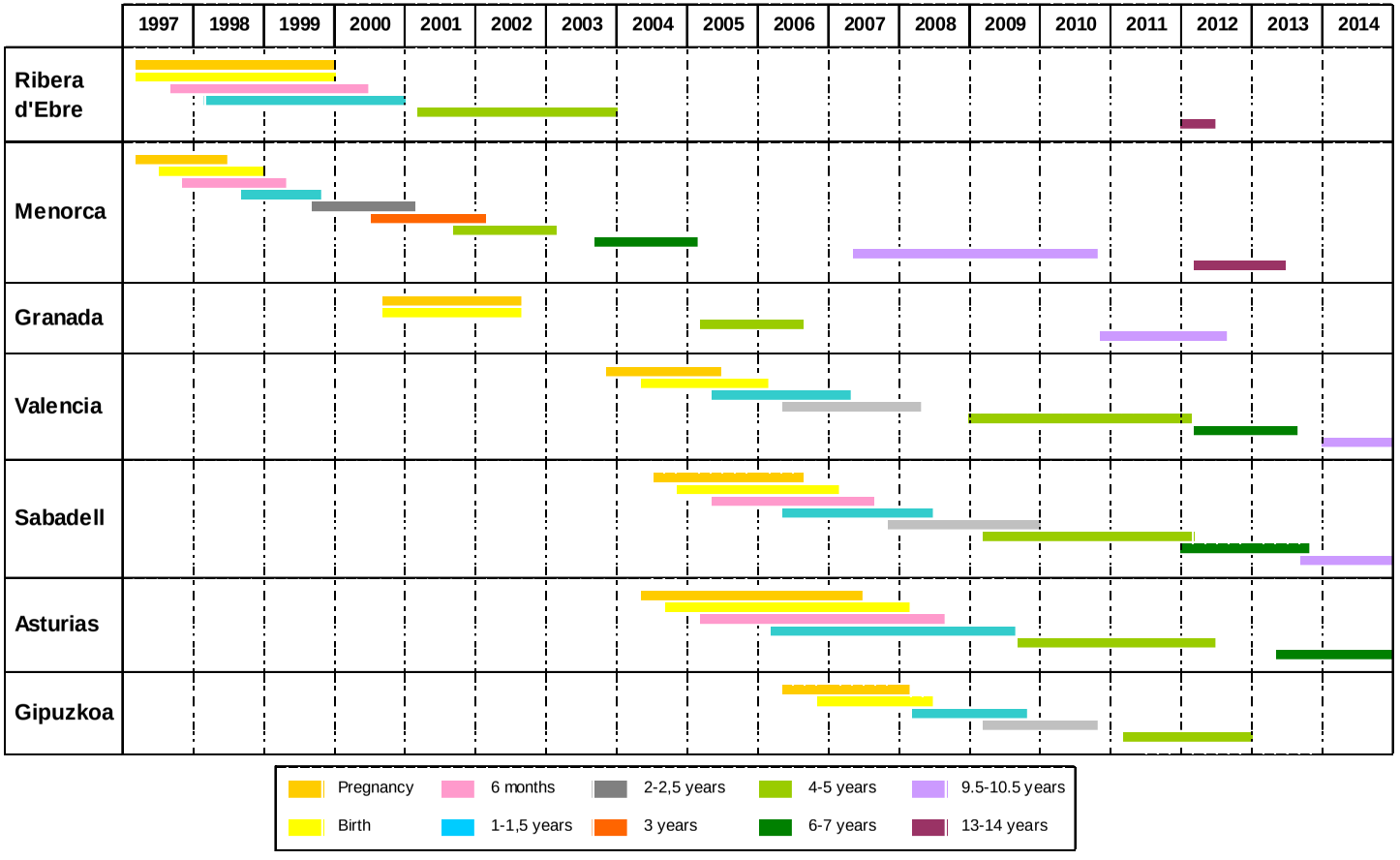
The Menorca birth cohort started in 1997 with 482 born children. The main aim of this cohort is to study the relationship between environmental factors and incidence of allergy and asthma. In this cohort, prenatal exposure to persistent organic pollutants has been assessed in cord blood samples collected at birth. Also, information

on chest infections, wheezing, allergy and asthma occurrence has been collected at ages 1, 2, 3, 4, 6.5,10 and 14 years. At the age of 4 years of the child, serum samples were collected and stored. These samples were used to measure cytokines and biomarkers of inflammation with the aim to explore the mechanisms behind the association found between prenatal exposure to POPs and respiratory health at the age of 4 (Sunyer et al. 2005) and 6.5 years (Sunyer et al. 2006) in this study population.

4.1.2 The "new" INMA birth cohorts

These cohorts were started in Sabadell, Valencia, Gipuzkoa and Asturias between 2004 and 2006. All of them except Asturias, which is not included in the present thesis, collected maternal serum samples during pregnancy and measured prenatal POP exposure. Gipuzkoa and Valencia also collected cord blood samples for some of their children and measured prenatal POP exposure in these samples as well. In these cohorts, information on LRTIs and wheezing occurrence has been collected at the age of 14 months. In the cohort of Sabadell respiratory health information was also collected at the age of 6 months, and 4 and 7 years. Sabadell mothers also provided two urine samples during the 1st and the 3rd trimesters of pregnancy. These samples were used to assess prenatal exposure to BPA and phthalates.

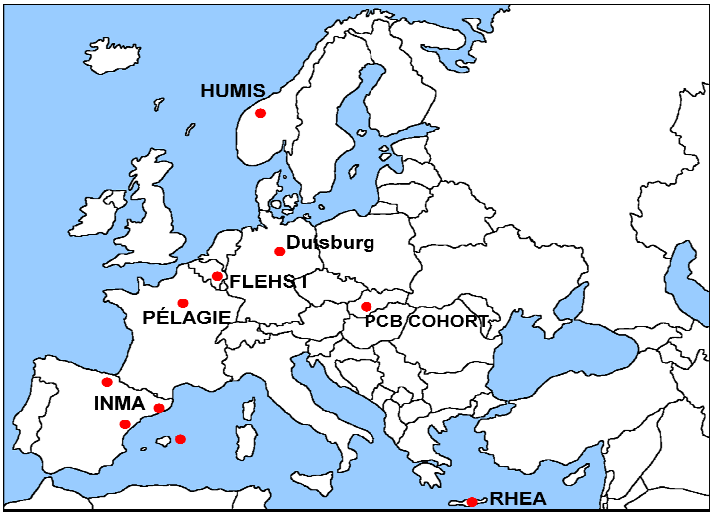
Figure 4.2 Chronogram of the different follow-ups in each INMA birth cohort.



4.2 The ENRIECO and CHICOS projects

ENRIECO and CHICOS are projects conducted within the European Union's 7th Framework Programme. These projects aimed to provide a working platform to exploit data of existing and future European birth cohort studies, to identify gaps in knowledge and to develop recommendations for targeted research action at the European level. In Europe, a number of prospective, population-based birth cohort studies participating in the ENRIECO and CHICOS projects have data available on prenatal exposure to POPs and respiratory health and symptoms in early childhood. The cohorts included in the present thesis are DUISBURG (Germany), FLEHS I (Belgium), HUMIS (Norway), PCB COHORT (Slovakia), RHEA (Greece), PÉLAGIE (France), and INMA-Menorca, INMA-Sabadell, INMA-Gipuzkoa and INMA-Valencia (Spain) (Figure 4.3). These cohorts started between 1997 and 2008.

Figure 4.3 Localization of the ten European birth cohort studies participating in the CHICOS case-study on prenatal exposure to DDE and PCB153 and respiratory health in early childhood.



5. RESULTS

5.1 Paper I

Pre-natal exposure to dichlorodiphenyldichloroethylene and infant lower respiratory tract infections and wheeze.

5.2 Paper II

Effects of persistent organic pollutants on the developing respiratory and immune systems: A systematic review.

5.3 Paper III

Prenatal exposure to DDE and PCB153 and respiratory health in early childhood: A meta-analysis.

5.4 Paper IV

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

5.5 Paper V

Prenatal exposure to bisphenol A, phthalates and respiratory and allergy outcomes during childhood.

**References of all papers are included in the reference's section of this thesis.*

Table 5.1 Summary of the prenatal exposures and outcomes evaluated in each study of the present thesis and the study populations involved.

Paper (study populations)	PRENATAL EXPOSURES			OUTCOMES						
	POPs[a]	BPA	Phthalates	Wheeze	Respiratory infections[b]	Asthma	Eczema	Cytokines and biomarkers of inflammation	Others[c]	Age of children at time of outcome assessment
Paper I (Sabadell, Gipuzkoa, Valencia)	X			X	X					12-14 months
Paper II (Systematic review of 41 studies)	X			X	X	X	X	X	X	From birth up to adolescence
Paper III (10 European birth cohorts)	X			X	X					From birth up to 4 years
Paper IV (Menorca)	X			X	X	X		X		1, 2, 3, 4, 6.5, 10 and 14 years
Paper V (Sabadell)		X	X	X	X		X			6 and 14 months and 4 and 7 years

POPs: persistent organic pollutants, BPA: bisphenol A.
[a]POPs include DDE, HCB and PCBs, but in Paper II (systematic review) it includes many other POPs.
[b]Can include chest infections, low respiratory tract infections (LRTIs) and upper respiratory tract infections (URTIs).
[c]In Paper II (systematic review) many other outcomes related to respiratory and immune health were assessed.

5.1 Paper I

Pre-natal exposure to dichlorodiphenyldichloroethylene and infant lower respiratory tract infections and wheeze

Gascon M[a,b], Vrijheid M[a,b,c], Martínez D[a,b], Ballester F[c,d,e], Basterrechea M[c,f,g], Blarduni E[h], Esplugues A[c,d], Vizcaino E[i,j], Grimalt JO[i], Morales E[a,b,c] and Sunyer J[a,b,c,k] on behalf of the Infancia y Medio Ambiente (Environment and Childhood) (INMA) project. **Pre-natal exposure to dichlorodiphenyldichloroethylene and infant lower respiratory tract infections and wheeze**. *Eur Respir J 2012; 39: 1188–1196.*

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Abstract

The aim of our study was to examine whether pre-natal exposure to dichlorodiphenyldichloroethylene (DDE) increases the risk of lower respiratory tract infections (LRTIs) and wheeze in infants. The study is based on a birth cohort of 1,455 mother–child pairs. Maternal serum concentrations of DDE, polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB) were measured during pregnancy. Parental reports on LRTI and wheeze were obtained when children were 12–14 months old. 35.4% of children developed at least one LRTI episode and 33.6% at least one wheezing episode during their first 12–14 months of life. Median DDE, PCBs and HCB concentrations were 116.3, 113.7 and 46.4 $ng \cdot g^{-1}$ lipid, respectively. DDE concentrations were associated with LRTI risk [relative risk (RR) per 10% increase 1.11, 95% CI 1.00–1.22], also after adjustment for PCBs and HCB. In all quartiles of DDE exposure, the risk of LRTI was increased compared with the lowest quartile, but the increase was statistically significant only in the third quartile (RR 1.33, 95% CI 1.08–1.62). No association was observed for PCBs and HCB. Results were similar for wheeze. This study suggests that pre-natal DDE exposure is associated with a higher risk of LRTI and wheeze in infants independently of exposure to other organochlorine compounds.

1. INTRODUCTION

Acute respiratory infections (ARIs) are a worldwide cause of morbidity and mortality in children aged <5 yrs (Rudan et al. 2004). Lower respiratory tract infections (LRTIs), mainly pneumonia and bronchiolitis, are considered to be the major components that account for the global burden of disease from ARI among young children. Several risk factors have been reported to increase vulnerability to LRTI during infancy and childhood, such as tobacco exposure, type and duration of breastfeeding, and familiar history of atopy or allergic asthma (Busse et al. 2010; Puig et al. 2008). Moreover, growing evidence suggests that pre-natal exposure to organochlorine compounds (OCs), mainly polychlorinated biphenyl (PCB)-153 and dichlorodiphenyldichloroethylene (DDE), may increase the risk of respiratory symptoms during the first years of life, even at low exposure levels (Dallaire et al. 2004, 2006; Glynn et al. 2008; Nakanishi et al. 1985; Sunyer et al. 2010; Weisglas-Kuperus et al. 2000). In addition, LRTIs are one of the major risk factors for developing asthma later in life (Bisgaard and Bonnelykke 2010; Busse et al. 2010) and pre-natal DDE exposure has also been associated with asthma and wheezing in children aged 4 (Sunyer et al. 2005) and 6 yrs (Sunyer et al. 2006).

OCs are synthetic persistent organic pollutants used worldwide and distributed throughout the environment, food and human tissues. Immunological effects of OCs have been reported in studies conducted both in animals (Dutta et al. 2008; Ezendam et al. 2004;

Lyche et al. 2004; Misumi et al. 2005) and humans (Daniel et al. 2002; Karmaus et al. 2005; Noakes et al. 2006; Vine et al. 2001). However, previous epidemiological studies on the association between OCs and LRTI have not been able to clearly determine which compound (PCBs, DDE or other OCs) was responsible for these effects due to the high correlation between concentrations of individual compounds (Dallaire et al. 2004, 2006; Glynn et al. 2008). In a birth cohort study in Sabadell, Spain, Sunyer et al. were the first to indentify DDE as the main responsible compound, but the study was too small to draw strong conclusions or to examine the role of other risk factors as possible effect modifiers (Sunyer et al. 2010).

In our study, we use a larger Spanish birth cohort, including the previous study (Sunyer et al. 2010) in Sabadell, Spain to: 1) provide more precise estimates for the effect of pre-natal DDE exposure on occurrence of LRTI and wheeze in infants; 2) isolate these effects from those of other OCs, including hexachlorobenzene (HCB) and PCBs; and 3) explore the role of other risk factors in this association, including maternal smoking, maternal history of atopy and allergic asthma, and breastfeeding practices. In addition, since recent studies suggest that a high level of adherence to the Mediterranean diet during pregnancy protects against the development of asthma and atopy in children (Chatzi and Kogevinas 2009), maternal diet during pregnancy is also explored as a possible effect modifier.

2. METHODS

2.1 Study population

This study is based in three Spanish regions (Gipuzkoa, Sabadell and Valencia) belonging to the INMA (Infancia y Medio Ambiente (Environment and Childhood)) Project in Spain (Guxens et al. 2012). All regions followed the same protocol and started recruiting pregnant females into the cohort between 2004 and 2008 (Sabadell, n=657; Valencia, n=855; and Gipuzkoa, n=638). Pregnant females coming to the first trimester routine antenatal care visit in the main public hospital or health centre of reference and who fulfilled the inclusion criteria (aged >16 yrs, intention to deliver in the city and no problems of communication) were recruited. Protocol details are described elsewhere (Guxens et al. 2012). This study was conducted with the approval of the hospital ethics committees in the participating regions and written informed consent was obtained from the parents of all children.

2.2 Outcomes

Information about physician-confirmed diagnosis of LRTI was obtained from parents through questionnaires when children were 1 yr old (mean±SD in Valencia was 12.4±1.1 months, in Gipuzkoa was 14.3±1.2 months and in Sabadell was 14.5±0.7 months). Occurrence of an LRTI episode was defined as a positive answer to both a general question (‘‘Since the last interview, has the doctor told you that your child has had a chest infection?’’) and a specific question on the type of infection (bronchiolitis, bronchitis or pneumonia) determined by the doctor. Children with negative

answers to both questions were defined as not having LRTI, and those reporting positive answers to both questions were defined as having LRTI. Those whose answers to both questions did not match (n=58) were excluded from the study. Wheezing was defined as a positive answer to the question ‘‘Has your child ever experienced whistling or wheezing from the chest, but not noisy breathing from the nose from birth to 12–14 months of age?’’. All these questions were based on the validated International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire (Beasley 1998).

2.3 Exposure assessment

Concentrations of OCs (HCB, p,p’-DDE, and PCB congeners 28, 118, 138, 153 and 180) in maternal serum extracted between the 7th week and the 26th week of pregnancy (median 12.9 weeks) from peripheral veins, were stored in crystal tubes at 20ºC and analysed with a gas chromatograph using methods described elsewhere (Goni et al. 2007). The limits of detection (LOD) were 0.071 ng·mL^{-1} in Sabadell and Gipuzkoa and between 0.01 and 0.071 ng·mL^{-1} in Valencia. International intercalibration exercises showed that differences of levels between regions were not due to laboratory differences. For comparison purposes, values in Valencia <0.071 ng·mL^{-1} were set as nondetectable. Samples with nondetectable levels were then set at a value of half the LOD. The sum of PCBs (ΣPCBs) was calculated by summing the concentrations of all individual congeners except PCB-28, which was detectable in <1% of samples. PCB-138, -153 and -180 were the predominant congeners. All exposures are expressed on a lipid basis in ng·g^{-1} of

lipid using the method described elsewhere (Phillips et al. 1989). Correlations between lipid adjusted and not adjusted values were high (0.97 for p,p'-DDE and 0.95 for ΣPCBs).

2.4 Other variables

Information on covariables was extracted from the questionnaires answered by the females during the 3rd trimester of pregnancy and at child age 12–14 months. Covariables of interest for the current study included: maternal age, social class (based on the International Standard Classification of Occupations), education and country of origin of the mother, maternal smoking during pregnancy, maternal smoking during the year after birth, parity (first child or not), daycare attendance, duration of predominant breastfeeding (never breastfeeding, breastfeeding 1–16 weeks, 17–24 weeks, >24 weeks), maternal history of atopy and/or allergic asthma, and maternal consumption of meat, fish and vegetables during pregnancy (divided into tertiles). As maternal atopy and allergic asthma were highly correlated ($p<0.001$), we combined them into a new single variable: ''atopic–asthmatic mother''. Pre-pregnancy weight of the mother, gestational age and weight at birth were collected from clinical records or reported by mothers.

2.5 Statistical methods

Out of the initial population of recruited mother–child pairs (n=2,150), 279 were lost to follow-up at the time of the age 12–14-month visit and 416 had missing information regarding exposure to OCs, regarding one of the outcomes or regarding country of origin,

resulting in 1,455 mother–child pairs with complete exposure–outcome information. Because of their very different exposure profiles, analyses were performed separately for Spanish mothers (n=1,342) and Latin-American mothers (n=79); mothers of other origin were not included (n=34). Some of the covariables of interest for our analysis had missing information (between 0.1% and 3.4%). These missing values were imputed by multiple imputation (Spratt et al. 2010). This method is based on conditioning the missing variables' density to given predictor variables, which in our case were country of origin, parity, gestational age, maternal age, maternal pre-pregnancy weight, maternal social class and maternal education, sex and birthweight, duration of predominant breastfeeding, daycare attendance, smoking during pregnancy or 1 yr after birth, being an atopic–asthmatic mother, maternal consumption of meat, fish and vegetables during pregnancy, and lipid and OC levels in maternal blood. These imputations were performed separately by region of study. A log-binomial regression model was used to analyse the relationship between concentrations of DDE, HCB and ΣPCBs with LRTI and wheezing. Generalised additive models were used to graphically examine the shape of relationships between OC exposure and outcome variables. These did not show statistically significant evidence for a departure from (log)linear relationships (p-values for gain in linearity were between 0.12 and 0.23) (fig. 1).

However, since the evidence was graphically not very strong, especially for PCBs, we performed analyses using OC

concentrations both as (log-transformed) continuous exposure variables and as exposure categories using quartiles as cut-offs. Potential confounder variables were included one by one in the model.

Variables were retained in the final model if they were related to the outcome ($p<0.2$), or changed the b-coefficient for the relationship between exposure and outcome by >10%. Variables that did not meet these criteria, but which were considered important risk factors for LRTI or wheezing were also included in the final model (maternal smoking during pregnancy, maternal smoking during the year after birth, social class and duration of predominant breastfeeding). Covariables included in the final models for each outcome are indicated in the results section. The influence of multi-pollutants on the relationship between DDE, HCB or ΣPCBs and LRTI was examined by including these compounds together in one model. Given that some characteristics of the participants and the mixture of OCs varied by region, sensitivity analyses were carried out stratifying by region. Analyses with the one pollutant model were further stratified by potential effect modifiers, such as atopic–asthmatic mother, maternal smoking during pregnancy, maternal smoking 1 yr after pregnancy, duration of predominant breastfeeding, consumption of vegetables and fruit, and fish consumption during pregnancy. Wald tests were used to test the statistical significance of interaction terms. Since a similar analysis of the Sabadell cohort has been published (Sunyer et al. 2010), we performed a sensitivity analysis excluding subjects from Sabadell.

Separate analyses for the Latin-American population followed the same methodology as those for the Spanish population. Tertiles of DDE concentrations were created instead of quartiles, because of the small population. HCB and the ΣPCBs were not analysed due to the very low concentrations detected within this population (below the LOD in 54.4% and 78.5% of samples). All analyses were conducted using STATA 10 (StataCorp, College Station, TX, USA).

3. RESULTS

3.1 Spanish population: main analysis

There were significant differences between Spanish mother–child pairs included in the main analyses and those excluded (table S1); mothers included were older, had a higher education level, a higher pre-pregnancy weight and smoked less. They also ate more fruit, vegetables and fish, and breastfed their children for longer. Among included subjects, there were fewer pre-term and low birthweight children and a higher percentage of wheezing cases. Maternal pre-natal concentrations of HCB and ΣPCBs were significantly higher among included participants, but DDE concentrations and prevalence of LRTI did not significantly differ from excluded subjects (p=0.21 and p=0.32, respectively).

A total of 35.4% and 33.6% of the children had at least one episode of LRTI or wheezing, respectively, during their first year of life (table 1). LRTI and wheeze were highly correlated, with 76% of children with LRTI also reporting wheezing symptoms. Males,

children attending daycare, those with maternal atopic asthma and smoking, those with multiparous mothers and those who were breastfed for a shorter period of time had a higher risk of ever having LRTI and/or wheezing symptoms (table 1).

Maternal levels of DDE were higher among pre-term, low-birthweight children and those not attending daycare services (table 2). Mothers with higher levels of DDE were older, had a higher pre-pregnancy weight and had lower education levels. A higher consumption of meat was also related to higher maternal DDE levels. In general, concentrations of HCB were much lower (46.4 $ng \cdot g^{-1}$ of lipid) than DDE (116.3 $ng \cdot g^{-1}$ of lipid) or ΣPCBs (113.7 $ng \cdot g^{-1}$ of lipid) (table 3). Correlation coefficients were 0.43 (DDE and ΣPCBs), 0.49 (DDE and HCB) and 0.40 (ΣPCBs and HCB) (all $p<0.001$).

Associations between the covariables in the final regression models and the outcomes are presented in table S2. Risk of LRTI increased with increasing DDE exposure and was statistically significant after adjustment for potential confounders (crude relative risk (RR) for 10% increase in DDE concentration 1.04, 95% CI 0.95–1.14 and adjusted RR 1.11, 95% CI 1.00–1.22). Adjustment for other pollutants gave a RR of borderline statistical significance (RR 1.11, 95% CI 0.99–1.24). In all quartiles of DDE, the risk of LRTI was increased compared with the lowest quartile, but the increase was statistically significant only in the 3rd quartile (adjusted RR 1.33, 95% CI 1.08–1.62). This increase remained after adjustment for

other OCs (RR for 3rd quartile DDE 1.40, 95% CI 1.13–1.73) (table 4). Pre-natal HCB levels did not increase the RR of LRTI. In the multi-pollutant model, the risk of LRTI in the highest quartile of PCB exposure was statistically significantly lower than in the lowest quartile (table 4). Risk estimates for wheezing were very similar to those found for LRTI (table 5).

The association between DDE and LRTI did not differ between strata defined by region (table S3), duration of predominant breastfeeding, maternal smoking during pregnancy or the first year of life, atopic–asthmatic mother or by maternal consumption of vegetables and fruit, meat or fish (data not shown). Sensitivity analysis adjusting for pre-term births or excluding these children (n=51) from the model did not modify the results. Analysis without imputed data also provided similar results.

3.2 Latin-American population

Among mothers of Latin-American origin, DDE was detected in all samples, with a median concentration of 385.0 $ng \cdot g^{-1}$ of lipid (table 3). The risk estimate for continuous DDE exposure in Latin-American mothers was very similar to those in Spanish mothers, but did not reach statistically significance (adjusted RR 1.14, 95% CI 0.92–1.42). When analysing by tertiles, estimates became statistically significant after adjustment (second tertile of DDE RR 2.59, 95% CI 1.00–6.66; and the third tertile RR 2.89, 95% CI 1.10–7.55). Results were similar for wheezing (table 6).

4. DISCUSSION

The present study suggests that pre-natal exposure to DDE is associated with a higher risk of LRTI and wheeze in infants. The DDE effect was independent of HCB or PCB exposure and was not clearly modified by other risk factors, including maternal smoking, maternal medical history of atopy/asthma, maternal dietary habits in pregnancy or breastfeeding practices. These results confirm our previous findings in a subsample of the present study (using a different definition of the outcome) (Sunyer et al. 2010). Only three other cohort studies, in Canada (Dallaire et al. 2004, 2006), Menorca (Spain) (Sunyer et al. 2005, 2006) and Sweden (Glynn et al. 2008), assessed respiratory infections or wheezing in young children in relation to pre-natal DDE exposure, and results are inconsistent. In the Canadian cohort (Dallaire et al. 2004, 2006), with higher levels of DDE (geometric mean 294 $ng \cdot g^{-1}$ of lipid), there was no clear increase in risk of LRTI with DDE concentrations at age 6–12 months (Dallaire et al. 2004), but at age 5 yrs, exposure to OCs (DDE and others assessed using PCB-153 concentrations as surrogate) did increase risk of LRTI (Dallaire et al. 2006). The same study did find increased risks of upper respiratory tract infections and/or otitis in both age groups. The Swedish study (Glynn et al. 2008) found that DDE exposure was related to a nonstatistically significant decrease in LRTI risk, but DDE levels were lower than those in our study (median 88 $ng \cdot g^{-1}$ of lipid) and children were assessed at age 3 months, providing little time to develop infections (Glynn et al. 2008). In the Menorcan birth cohort (Sunyer et al. 2005, 2006), with pre-natal DDE median

levels of ~170 $ng \cdot g^{-1}$ of lipid, DDE was associated with a higher risk of wheeze and asthma at ages 4 and 6.5 yrs, but not to earlier wheezing or LRTI during the first year of life (Sunyer et al. 2005, 2006). This somewhat conflicts with our results, but the number of children in the Menorca cohort (n<400) may have been too small to detect early effects. It will be important to assess the DDE effects in our present cohort at older ages. Most of the previous studies could not separate the effects of DDE on LRTI from those of other OCs (Dallaire et al. 2004, 2006; Glynn et al. 2008), whereas our study clearly identifies DDE as the main responsible compound. This could be partly explained by the different correlations between DDE and other OCs across studies; in the Swedish (Glynn et al. 2008) and Canadian cohorts (Dallaire et al. 2004, 2006), correlations between DDE and other OCs were between 0.66 and 0.89, whereas correlations among the nonmigrant population of the present Spanish cohort were <0.49. In our study, risk of LRTI and wheezing decreased in the highest quartile of PCBs exposure; this decrease was statistically significant only after adjustment for DDE and HCB. We do not have a real explanation for these results, but this may be a chance result, unexplained by confounding or a problem of multicollinearity between OCs. Our quartile results (with significant increase only in the third quartile) indicate that the association between DDE and LRTIs may not be strictly monotonic, even though we observed a linear trend with continuous exposure, and additional linear spline analyses showed no statistical evidence for differences between spline slopes (table S4). Nonmonotonic

functions have also been reported by others (Dallaire et al. 2004, 2006).

In our cohort, mothers from Latin-American origin showed very different patterns of OCs exposure from Spanish mothers, with very high DDE levels, low levels of PCBs and HCB, and low correlations between DDE and other OCs. Diet, other lifestyle factors and differences in industrial development may explain part of these differences (Vrijheid et al. 2010), together with the fact that in Latin-America the use of dichlorodiphenyltrichloroethane (DDT), the parent compound of DDE, lasted until the late 1990s for agricultural and malaria vector control purposes (Roberts et al. 1997). The similarity of our DDE effect in both Spanish and Latin-American children indicates that this effect may apply widely to populations with different OCs exposure patterns. This is of especial interest, as nowadays the practice of using DDT for malaria vector control is still present or planned to be introduced in many developing countries with endemic malaria (van den Berg 2009). However, our results are limited by the small size of our Latin-American population, and further studies in areas with high levels of DDE and low levels of other OCs are indicated.

The mechanisms by which DDE and other OCs may produce LRTI and wheeze are not fully understood. However, some studies have shown an association between DDE exposure levels and the uncontrolled production of cytokines and the increase of nitric oxide production in macrophages, contributing to inflammatory

reactions, cytokine imbalance and immune dysregulation (Daniel et al. 2002; Lyche et al. 2004; Noakes et al. 2006). DDE has also been associated with altered levels and a reduced viability and proliferation capacity of immune system cells (macrophages, lymphocytes and monocytes) (Glynn et al. 2008; Noakes et al. 2006; Vine et al. 2001), mainly through apoptosis (programmed cell death) (Misumi et al. 2005; Perez-Maldonado et al. 2005), which seems to be caused by oxidative stress (Perez-Maldonado et al. 2005). Although apoptosis plays a very important role under normal physiological conditions, when not regulated, apoptosis can contribute to immune dysregulation and immunodeficiency (Perez-Maldonado et al. 2006). Moreover, recent studies suggest that apoptotic cells actively regulate the immune response by releasing immunosuppressive cytokines (e.g. transforming growth factor-β1) and by suppressing the secretion of pro-inflammatory cytokines (e.g. tumour necrosis factor-α), indicating an immunosuppressive response (Duramad et al. 2007; Perez-Maldonado et al. 2005) that could lead to an increased risk of contracting infections. Further studies are needed to understand better the mechanisms by which DDE interferes with the immune system (Perez-Maldonado et al. 2006); although cell counts serve as a general indicator of immune status, future research should focus on the performance of cytokine assays, as they can provide a more mechanistic examination of the effect of exposure (Duramad et al. 2007).

A limitation of our study is the lack of serology or culture to confirm the LRTI diagnosis. However, we used repeated

questionnaire items to define LRTI and our results were consistent, whichever definition criteria was selected (data not shown). The fact that we found similar results for wheezing, a related outcome for LRTI at this age, and that covariables in the final model were associated with the outcomes as expected, also provide strength to our results. Our cohort is somewhat selective, as loss to follow-up and incompleteness of the questionnaires occurred more often in younger mothers with lower educational levels (Vrijheid et al. 2010); this is also reflected in the differences between our included study population and the excluded mothers. It is unlikely that this has led to spurious associations, but it means that these groups of the population are under-represented in our sample. We observed somewhat inconsistent results between the regions of our cohort (table S3), but there was no evidence of heterogeneity between regions (p-value for interaction 0.30–0.82) and inconsistencies may have resulted from small comparison groups, especially in the quartile analyses. Meta-analyses of the estimates of each region were performed and results were similar to those of the pooled analysis (table S4). Any small differences between regions might be due to the percentage of mothers reporting LRTI or wheezing during the first year of life of their children, which was lower in Valencia than in Gipuzkoa or Sabadell. This is probably because, in Valencia, respiratory questionnaires were administered at ~12 months of age, instead of at 14 months in the other regions. Strengths of the current study are its prospective study design and large population size. Also, in a sensitivity analysis, we were able to show that the DDE effect was not only due to the influence of the

one region for which data had already been published (data not shown) (Sunyer et al. 2010).

5. CONCLUSIONS

The present study reinforces the hypothesis that pre-natal exposure to DDE is associated with a higher risk of LRTI and wheeze in infants. As LRTIs cause substantial morbidity in infancy, and LRTI and wheeze are possible risk factors for subsequent childhood asthma, particular attention should be paid to these in countries where DDT is currently used for malaria control.

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TABLES AND FIGURES

Figure 1. Generalised additive models to examine the shape of relationships between exposure to organochlorine compounds and lower respiratory tract infections (LRTIs): a) dichlorodiphenyldichloroethylene (DDE) (p-value for gain in linearity=0.23), b) hexachlorobenzene (HCB) (p=0.12), and c) sum of polychlorinated biphenyls (ΣPCBs) (p=0.13).

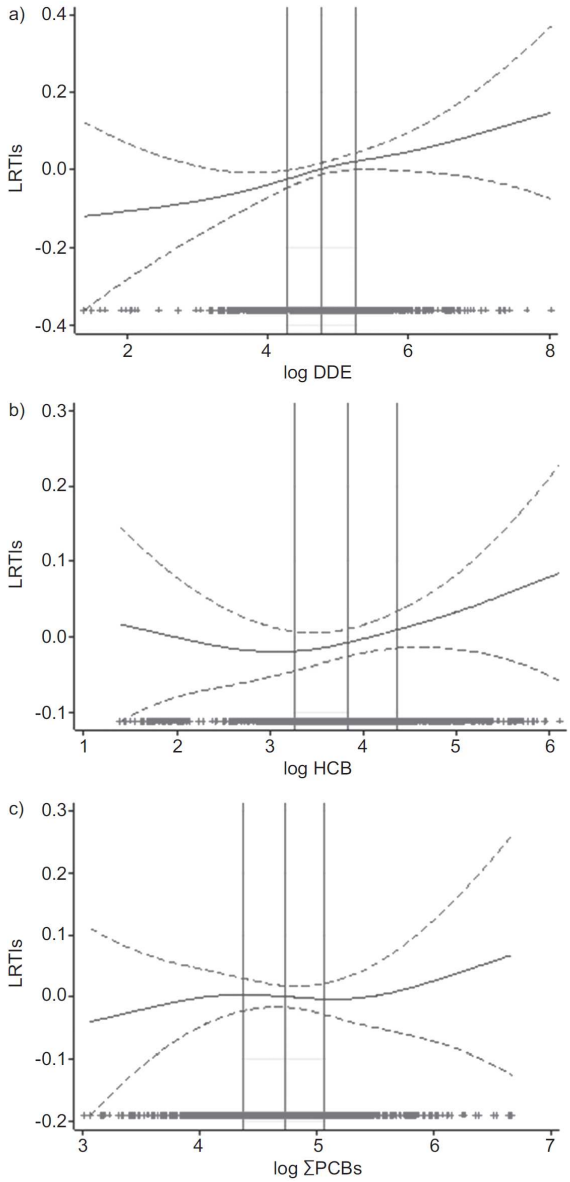

Table 1. Characteristics of the Spanish study population by low respiratory tract infection (LRTI) and by wheezing status during the 12-14 months of life (N=1342).

Characteristics[#]	LRTIs			Wheeze		
	Never (N=867)	**Ever (N=475)**	**p-value**	**Never (N=891)**	**Ever (N=451)**	**p-value**
Characteristics of the children						
Male Sex (%)	48.3	58.2	0.00	48.1	58.4	0.00
Preterm (<37 weeks, %)	3.5	4.4	0.38	4.0	3.6	0.73
Low birth-weight (<2500g, %)	5.7	4.7	0.42	5.8	4.5	0.32
Predominant breastfeeding (%)			0.56			0.06
0 weeks	18.4	20.4		17.8	21.7	
1-16 weeks	33.1	32.1		31.5	35.4	
17-24 weeks	36.8	34.4		38.1	31.6	
>24 weeks	11.7	13.1		12.6	11.4	
Day-care attendance	30.3	41.6	<0.001	30.3	42.2	<0.001
Region (%)			0.01			0.00
Gipuzkoa (N=500)	36.7	38.3		35.9	39.9	
Sabadell (N=455)	31.7	37.9		32.2	37.3	
Valencia (N=387)	31.6	23.8		31.9	22.8	
Characteristics of the mother						
Age, years (mean, SD)	30.7 (4.1)	31.1 (3.7)	0.13	30.8 (4.1)	30.9 (3.7)	0.74
Pre-pregnancy weight, kg (mean, SD)	62.3 (11.3)	63.4 (12.2)	0.12	62.6 (11.7)	62.9 (11.6)	0.34

Characteristics[#]	LRTIs			Wheeze		
	Never (N=867)	**Ever (N=475)**	**p-value**	**Never (N=891)**	**Ever (N=451)**	**p-value**
Social class (%)			0.92			0.08
Non-manual jobs	76.6	75.6		77.7	73.4	
Manuals jobs	22.6	23.6		21.3	26.2	
Unknown or unclassifiable	0.8	0.8		1.0	0.4	
Education (%)			0.87			0.41
Primary school	23.3	24.3		22.6	25.8	
Secondary	39.7	39.9		40.4	38.5	
University	37.0	35.8		37.0	35.7	
Smoking during pregnancy (%)	16.5	17.4	0.70	14.6	21.2	0.00
Smoking during the first year (%)	25.9	28.1	0.43	24.6	30.8	0.02
Maternal allergy and/or asthma (%)	24.5	32.3	0.00	25.6	30.4	0.07
Parity (First child, %)	61.3	43.8	0.00	60.6	44.4	0.00
Diet of the mother (g/day, mean, SD)						
Meat	113.2 (42.3)	113.1 (41.3)	0.96	112.5 (41.9)	114.4 (42.0)	0.44
Fish	66.1 (29.4)	68.0 (31.0)	0.27	66.1 (29.9)	68.0 (30.2)	0.27
Vegetables & fruit	516.7 (211.2)	515.6 (216.0)	0.93	520.1 (213.3)	508.7 (211.8)	0.36

[#]Percentages or mean and standard deviation (sd) are presented based on imputed data. Missing data in this population (N=1342) varied between 0 and 0.7% for most of the co-variables, except for smoking during pregnancy (1.4%) and during the first year of life (1.3%) and breastfeeding duration (3.5%).

Table 2. Geometric mean (GM) and Geometric standard deviation (GSD) of the concentrations[#] of DDE, HCB and ΣPCBs (ng/g lipids) by characteristics of the Spanish study population (N=1342[*†]).

	%[ǂ]	DDE GM (GSD)	HCB[*] GM (GSD)	ΣPCBs[†] GM (GSD)
Characteristics of the children				
LRTIs				
Never	64.6	117.9 (2.1)	44.2 (2.3)	113.4 (1.7)
Ever	35.4	122.1 (2.2)	44.5 (2.3)	110.6 (1.8)
Wheezing				
Never	66.4	117.9 (2.2)	44.5 (2.3)	113.2 (1.8)
Ever	33.6	122.2 (2.2)	43.9 (2.3)	110.7 (1.7)
Sex				
Male	51.8	121.0 (2.2)	44.3 (2.3)	112.8 (1.8)
Female	48.3	117.6 (2.1)	44.3 (2.3)	112.0 (1.7)
Preterm (<37 weeks)				
Yes	3.8	156.1 (2.2)**	48.6 (2.5)	141.2 (1.7)***
No	96.2	118.1 (2.2)	44.1 (2.3)	111.4 (1.7)
Low birth-weight (<2500g)				
Yes	5.3	142.7 (2.2)**	44.9 (2.6)	115.0 (1.8)
No	94.7	118.2 (2.6)	44.3 (2.3)	112.2 (1.7)
Predominant breastfeeding				
0 weeks	18.6	122.9 (2.1)	51.3 (2.3)***	111.3 (1.7)
1-16 weeks	33.5	118.4 (2.3)	46.0 (2.4)	109.4 (1.7)
17-24 weeks	35.7	117.4 (2.1)	40.3 (2.2)	112.5 (4.7)
>24 weeks	12.2	122.8 (2.0)	42.1 (2.2)	122.3 (1.8)
Day-care attendance				
Yes	34.3	109.8 (2.1)***	42.0 (2.2)*	121.4 (1.7)***
No	65.7	124.6 (2.2)	45.5 (2.4)	107.9 (1.8)
Region				
Gipuzkoa (N=500)	37.3	92.8 (2.1)***	34.6 (2.1)***	130.2 (1.6)***
Sabadell (N=455)	33.9	112.6 (2.0)	41.9 (2.0)	82.5 (1.6)
Valencia (N=387)	28.8	176.8 (2.1)	65.0 (2.6)	134.6 (1.8)
Characteristics of the mother				
Age, years				
<29	26.5	98.7 (2.1)***	33.2 (2.2)***	79.8 (1.8)***
29-31	31.7	115.2 (2.0)	42.6 (2.2)	109.1 (1.6)
32-33	17.4	123.6 (2.2)	50.8 (2.3)	128.8 (1.6)
>34	24.4	149.7 (2.3)	57.6 (2.3)	153.5 (1.6)
Pre-pregnancy weight, kg				
<57	36.1	110.4 (2.2)**	37.2 (2.4)***	120.0 (1.7)***
57-65	33.3	123.3 (2.1)	42.5 (2.3)	115.1 (1.7)
>65	30.6	126.3 (2.2)	56.8 (2.2)	101.3 (1.8)

	%‡	DDE GM (GSD)	HCB* GM (GSD)	ΣPCBs† GM (GSD)
Social class				
Non-manual occupation	76.2	117.7 (2.1)	44.3 (2.3)	113.8 (1.7)
Manuals occupation	23.0	126.5 (2.2)	44.3 (2.4)	107.5 (1.8)
Unknown or unclassifiable	0.8	87.6 (4.1)	46.7 (3.2)	124.0 (2.3)
Education				
Primary school	23.7	132.5 (2.3)**	44.1 (2.6)	104.0 (1.8)***
Secondary	39.7	117.1 (2.2)	45.0 (2.3)	105.6 (1.8)
University	36.6	113.9 (2.1)	43.6 (2.2)	126.4 (1.6)
Smoking during pregnancy				
Yes	16.8	125.4 (2.1)	44.5 (2.6)	113.8 (1.7)
No	83.2	118.3 (2.2)	44.2 (2.3)	112.1 (1.7)
Smoking during the first year				
Yes	26.7	124.6 (2.2)	45.0 (2.4)	107.1 (1.8)*
No	73.3	117.4 (2.1)	44.0 (2.3)	114.4 (1.7)
Maternal allergy and/or asthma				
Yes	27.2	122.0 (2.1)	45.1 (2.2)	112.4 (1.8)
No	72.8	118.4 (2.2)	44.0 (2.3)	112.4 (1.7)
Parity‡				
Nulliparous	55.1	122.4 (1.0)	47.2 (1.0)***	116.6 (1.0)***
Multiparous	44.9	115.7 (1.0)	40.9 (1.0)	107.4 (1.0)
Diet of the mother (g/day)				
Meat				
<94.14	33.4	110.9 (2.1)**	39.9 (2.3)***	119.5 (1.7)***
94.14-128.8	33.3	118.6 (2.2)	46.5 (2.2)	114.3 (1.8)
>128.8	33.3	129.2 (2.2)	46.8 (2.4)	103.9 (1.7)
Fish				
<51.6	33.4	116.3 (2.2)	44.7 (2.4)	104.5 (1.8)***
51.6-75.5	33.3	118.9 (2.1)	45.2 (2.2)	115.9 (1.7)
>75.5	33.3	123.0 (2.2)	43.0 (2.3)	117.2 (1.7)
Vegetables & fruit				
<411.0	33.4	113.9 (2.2)	42.2 (2.4)	104.2 (1.7)***
411.0-582.6	33.3	118.0 (2.0)	46.1 (2.2)	117.6 (1.7)
>582.6	33.3	126.5 (2.3)	44.6 (2.3)	115.8 (1.8)

DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls.

‡Percentages are presented based on imputed data. #As concentrations of all compounds were not normally distributed, these were log-transformed before calculating differences of exposure between groups of each characteristic. *One child was excluded from the analysis with HCB because it was an outlier (N=1341). †Two children had no information for PCBs within the Spanish population and one was excluded from the analysis with ΣPCBs because it was an outlier (N=1339). ‡ Adjusted for maternal age at delivery. *p-value<0.10, **p-value<0.05, ***p-value<0.01.

Table 3. Percentage of samples below de limit of detection (LOD) and concentrations ($ng \cdot g^{-1}$ lipid) of DDE, HCB and PCBs in the Spanish (N=1342[*†]) and the Latin-American (N=79) populations.

	Spanish population[†]		Latin-American population	
	% < LOD	**Median (25^{th}, 75^{th})**	**% < LOD**	**Median (25^{th}, 75^{th})**
DDE	0.8	116.3 (72.6, 191.7)	0.0	385.0 (146.2, 953.8)
HCB[*]	5.8	46.4 (26.4, 79.0)	60.8	6.9 (5.8, 18.8)
PCB180	5.2	32.5 (21.7, 47.7)	65.8	6.3 (5.3, 9.3)
PCB153	2.2	45.1 (31.5, 63.2)	54.4	7.0 (5.8, 15.2)
PCB138	10.0	27.1 (17.7, 39.2)	72.2	6.3 (5.3, 12.2)
PCB118	76.5	6.4 (5.6, 7.8)	78.5	6.0 (5.3, 7.2)
Σ PCBs[†]	NA	113.7 (79.4, 158.6)	NA	27.9 (23.4, 45.2)

DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls, NA= not applicable.

[*]One child was excluded from the analysis with HCB because it was an outlier (N=1341).

[†]Two children had no information for PCBs within the Spanish population and one was excluded from the analysis with ΣPCBs because it was an outlier (N=1339).

Table 4. Number of total children (N), cases of LRTI during the first 12-14 months of life (%) and crude and adjusted relative risk [RR (95% Confidence Interval)] for continuous exposure and for each quartile (Q) of DDE, HCB and ΣPCBs exposure within the Spanish study population (N=1342[*†]).

	Exposure level ($ng \cdot g^{-1}$ lipid)	N (% LRTI cases)	Crude RR (95%CI)	p-value	Adjusted RR[#] (95%CI)	p-value	Multipollutant adjusted RR[‡](95%CI)	p-value
DDE								
Continuous		1342 (35.4)	1.04 (0.95, 1.14)	0.43	1.11 (1.00, 1.22)	0.05	1.11 (0.99, 1.24)	0.07
Q1	<72.6	336 (32.4)	1		1		1	
Q2	72.6-115.9	335 (35.5)	1.10 (0.89, 1.35)	0.40	1.16 (0.94, 1.43)	0.16	1.20 (0.97, 1.48)	0.09
Q3	115.5-191.7	336 (39.9)	1.23 (1.00, 1.51)	0.05	1.33 (1.08, 1.62)	0.01	1.40 (1.13, 1.73)	<0.01
Q4	>191.7	335 (33.7)	1.04 (0.84, 1.29)	0.72	1.20 (0.96, 1.51)	0.11	1.28 (1.00, 1.64)	0.04
HCB[*]								
Continuous		1341 (35.4)	1.00 (0.92, 1.10)	0.87	1.06 (0.95, 1.17)	0.30	1.03 (0.92, 1.15)	0.63
Q1	<26.4	336 (35.4)	1		1		1	
Q2	26.4-46.4	335 (34.9)	0.98 (0.80, 1.21)	0.89	0.96 (0.79, 1.18)	0.72	0.95 (0.78, 1.17)	0.65
Q3	46.4-79.0	335 (37.0)	1.04 (0.85, 1.28)	0.67	1.08 (0.88, 1.32)	0.47	1.03 (0.84, 1.29)	0.74
Q4	>79.0	335 (34.3)	0.97 (0.79, 1.19)	0.77	1.06 (0.84, 1.33)	0.63	1.03 (0.80, 1.33)	0.80
ΣPCBs[†]								
Continuous		1339 (35.3)	0.95 (0.83, 1.08)	0.44	1.00 (0.86, 1.18)	0.93	0.92 (0.77, 1.11)	0.39
Q1	<79.4	335 (37.9)	1		1		1	
Q2	79.4-113.7	335 (36.1)	0.95 (0.78, 1.16)	0.63	0.98 (0.80, 1.20)	0.83	0.90 (0.73, 1.11)	0.34
Q3	113.7-158.6	335 (35.5)	0.94 (0.77, 1.14)	0.52	0.94 (0.75, 1.17)	0.56	0.83 (0.66, 1.05)	0.13
Q4	>158.6	334 (31.7)	0.84 (0.70, 1.03)	0.10	0.84 (0.65, 1.08)	0.17	0.73 (0.56, 0.95)	0.02

DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls.

[#]Adjustment: region, sex of the child, age and pre-pregnancy weight of the mother, allergic or asthmatic mother, parity (first child) and social class, predominant breastfeeding, maternal smoking status (during pregnancy and during the 1st year of life of the child) and day-care attendance during the first year of life. [‡]Also adjusted for DDE, HCB and ΣPCBs (N=1338). [*]One child was excluded from the analysis with HCB because it was an outlier (N=1341). [†]Two children had no information for PCBs within the Spanish population and one was excluded from the analysis with ΣPCBs because it was an outlier (N=1339).

Table 5. Number of total children (N), cases of wheezing during the first 12-14 months of life (%) and crude and adjusted relative risk [RR (95% Confidence Interval)] for continuous exposure and for each quartile (Q) of DDE, HCB and ΣPCBs exposure within the Spanish study population (N=1342[†]).

Exposure quartile	Exposure level (ng·g^{-1} lipid)	N (% Wheezing cases)	Crude RR (95%CI)	p-value	Adjusted RR[#] (95%CI)	p-value	Multipollutant adjusted RR[‡](95%CI)	p-value
DDE								
Continuous		1342 (33.6)	1.04 (0.94, 1.15)	0.42	1.09 (0.99, 1.21)	0.08	1.14 (1.01, 1.28)	0.03
Q1	<72.6	336 (31.9)	1					
Q2	72.6-115.9	335 (32.5)	1.02 (0.82, 1.27)	0.85	1.10 (0.88, 1.36)	0.41	1.12 (0.90, 1.40)	0.31
Q3	115.5-191.7	336 (38.4)	1.21 (0.98, 1.48)	0.08	1.30 (1.06, 1.59)	0.01	1.37 (1.10, 1.70)	<0.01
Q4	>191.7	335 (31.6)	0.99 (0.80, 1.24)	0.96	1.11 (0.89, 1.40)	0.35	1.20 (0.94, 1.55)	0.15
HCB[*]								
Continuous		1341 (33.6)	0.99 (0.90, 1.08)	0.78	1.02 (0.93, 1.12)	0.69	1.00 (0.90, 1.13)	0.91
Q1	<26.4	336 (33.3)	1		1		1	
Q2	26.4-46.4	335 (34.0)	1.02 (0.83, 1.26)	0.85	0.98 (0.80, 1.21)	0.86	0.98 (0.79, 1.21)	0.84
Q3	46.4-79.0	335 (34.3)	1.03 (0.83, 1.27)	0.79	1.05 (0.86, 1.30)	062	1.03 (0.83, 1.29)	0.77
Q4	>79.0	335 (32.8)	0.99 (0.79, 1.22)	0.89	1.05 (0.85, 1.31)	0.64	1.06 (0.83, 1.35)	0.65
ΣPCBs[†]								
Continuous		1339 (33.5)	0.95 (0.83, 1.09)	0.49	0.94 (0.81, 1.09)	0.41	0.86 (0.72, 1.02)	0.70
Q1	<79.4	335 (34.3)	1		1		1	
Q2	79.4-113.7	335 (35.2)	1.03 (0.83, 1.26)	0.80	1.02 (0.84, 1.25)	0.83	0.95 (0.77, 1.18)	0.66
Q3	113.7-158.6	335 (34.3)	1.00 (0.81, 1.23)	1.00	0.96 (0.78, 1.19)	0.73	0.86 (0.68, 1.09)	0.21
Q4	>158.6	334 (30.2)	0.88 (0.71, 1.10)	0.26	0.85 (0.67, 1.07)	0.17	0.75 (0.58, 0.97)	0.03

DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls.

[#]Adjustment: region, sex of the child, allergic or asthmatic mother, parity (first child) and social class, predominant breastfeeding, maternal smoking status (during pregnancy and during the 1st year of life of the child) and day-care attendance during the first year of life. [‡] Also adjusted for DDE, HCB and ΣPCBs (N=1338). [*]One child was excluded from the analysis with HCB because it was an outlier (N=1341). [†]Two children had no information for PCBs within the Spanish population and one was excluded from the analysis with ΣPCBs because it was an outlier (N=1339).

Table 6. Number of total children (N), cases of LRTIs and wheezing during the first 12-14 months of life (%) and crude and adjusted relative risk [RR (95% Confidence Interval)] for continuous exposure and for each tertile (T) of DDE within the Latin-American population (N=79).

	DDE	Levels ($ng \cdot g^{-1}$ lipid)	N (%LRTIs)	Crude RR (95%CI)	p-value	Adjusted RR[#†] (95%CI)	p-value
LRTIs							
	Continuous		79 (29.1)	1.10 (0.89, 1.37)	0.37	1.14 (0.92, 1.42)	0.23
	T1	<197.9	27 (22.2)	1		1	
	T2	197.9-595.9	26 (30.8)	1.38 (0.55, 3.47)	0.49	2.59 (1.00, 6.66)	0.05
	T3	> 595.9	26 (34.6)	1.56 (0.64, 3.79)	0.33	2.89 (1.10, 7.55)	0.03
Wheezing							
	Continuous		79 (26.6)	1.16 (0.94, 1.42)	0.17	1.20 (0.98, 1.48)	0.08
	T1	<197.9	27 (14.8)	1		1	
	T2	197.9-595.9	26 (30.8)	2.08 (0.70, 6.12)	0.19	2.34 (0.73, 7.55)	0.15
	T3	> 595.9	26 (34.6)	2.34 (0.81, 6.71)	0.12	3.54 (1.54, 8.12)	<0.01

DDE= dichlorodiphenyldichloroethylene.

[#]LRTIs model adjustment: region, sex of the child, low birth weight, predominant breastfeeding, age of the mother, parity, maternal smoking during pregnancy, being atopic-asthmatic mother and fish and vegetable maternal consumption during pregnancy. [†]Wheezing model adjustment: region, sex of the child, low birth weight, predominant breastfeeding, age and pre-pregnancy weight of the mother, studies of the mother, smoking during the first year of life and maternal consumption of meat during pregnancy.

SUPPLEMENTAL MATERIAL

Table S1. Differences between Spanish children included in the main analyses (N=1342) and Spanish children excluded (N=569)**.**

Characteristics	Inclusion		p-value
	Not included (N=569)	Included (N=1342)	
Characteristics of the children			
Male Sex (%)	48.1	51.8	0.18
N missing	157	3	
Preterm (<37 weeks, %)	6.0	3.8	0.05
N missing	84	2	
Low birth-weight (<2500g, %)	7.9	5.3	0.04
N missing	89	4	
Predominant breastfeeding (%)			<0.001
0 weeks	26.8	19.2	
1-16 weeks	37.5	32.7	
17-24 weeks	27.7	36.1	
>24 weeks	7.9	12.1	
N missing	241	47	
Day-care attendance	22.5	34.3	<0.001
N missing	267	14	
Region (%)			<0.001
Gipuzkoa (N=638)	19.3	37.3	
Sabadell (N=657)	20.7	33.9	
Valencia (N=855)	59.9	28.8	
N missing	0	0	
LRTIs[†] at 12/14 months (%)	32.1	35.4	0.32
N missing	329	0	
Wheeze at 12/14 months (%)	24.6	33.6	<0.001
N missing	228	0	
Characteristics of the mother			
Organochlorine concentrations in maternal serum			
DDE (GM, GSD)	111.6 (2.2)	119.4 (2.2)	0.21
HCB (GM, GSD) [*]	36.8 (2.3)	44.3 (2.3)	<0.001
ΣPCBs (GM, GSD)[‡]	101.3 (1.8)	112.4 (1.8)	<0.001
N missing	314	0 (2)	
Age, years (mean, SD)	29.8 (4.6)	30.9 (4.0)	<0.001
N missing	0	0	
Pre-pregnancy weight, kg (mean, SD)	61.8 (13.2)	62.7 (11.7)	<0.001
N missing	0	0	

Characteristics	Inclusion		p-value
	Not included (N=569)	Included (N=1342)	
Social class (%)			0.08
Non-manual jobs	71.5	76.2	
Manuals jobs	27.2	23.0	
Unknown or unclassifiable	1.2	0.8	
N missing	0	0	
Education (%)			<0.001
Primary school	33.1	23.6	
Secondary	42.3	39.7	
University	24.7	36.7	
N missing	1	3	
Smoking during pregnancy (%)	26.9	16.8	<0.001
N missing	85	19	
Smoking during the first year (%)	35.4	26.6	0.02
N missing	267	17	
Maternal allergy and/or asthma (%)	27.0	27.2	0.92
N missing	2	1	
Parity (First child, %)	57.3	55.1	0.38
N missing	0	1	
Diet of the mother (g/day, mean, SD)			
Meat	116.4 (48.7)	113.2 (42.0)	0.14
Fish	60.7 (32.1)	66.8 (30.0)	<0.001
Vegetables & fruit	484.4 (240.3)	516.3 (212.9)	0.004
N missing	0	0	

DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls, GM= Geometric Mean, SD=standard deviation.
The percentage of missing values within the non-included population is much higher than in the included population because in non-included already a 34.2% of the children either died or withdrew the study at birth or either did not attend the visit at 12/14 months. †This is according to the final definition of having LRTI used in this study. *One child was excluded from the analysis with HCB because it was an outlier (N=1341). ‡Two children had no information for PCBs within the Spanish population and one was excluded from the analysis with ΣPCBs because it was an outlier (N=1339).

Table S2. Associations between confounders and LRTIs and wheezing after adjusting for all confounders.

	DDE (N=1342)			sumPCBs (N=1339)		
	RR	**95%CI**	**p-value**	**RR**	**95%CI**	**p-value**
LRTIs						
Q1	1.16	(0.94, 1.43)	0.16	0.98	(0.80, 1.20)	0.83
Q2	1.33	(1.08, 1.62)	0.01	0.94	(0.75, 1.17)	0.56
Q3	1.20	(0.96, 1.51)	0.11	0.84	(0.65, 1.08)	0.17
Gipuzkoa region	0.93	(0.79, 1.10)	0.40	0.94	(0.79, 1.13)	0.52
Valencia region	0.72	(0.59, 0.88)	0.00	0.78	(0.64, 0.96)	0.02
Male sex	1.29	(1.12, 1.49)	0.00	1.27	(1.10, 1.47)	0.00
Age of the mother	0.98	(0.96, 1.00)	0.06	0.99	(0.97, 1.01)	0.51
Pre-pregnancy weight of the mother	1.00	(1.00, 1.01)	0.10	1.00	(1.00, 1.01)	0.19
Allergic or asthmatic mother	1.25	(1.08, 1.45)	0.00	1.26	(1.09, 1.46)	0.00
Multiparous	1.69	(1.45, 1.97)	0.00	1.66	(1.42, 1.94)	0.00
Maternal smoking during the 1st year of life	1.08	(0.88, 1.32)	0.48	1.08	(0.88, 1.32)	0.48
Maternal smoking during pregnancy	1.05	(0.83, 1.34)	0.69	1.05	(0.82, 1.33)	0.72
Manual jobs	1.09	(0.92, 1.29)	0.33	1.07	(0.90, 1.28)	0.42
Unclassifiable	1.06	(0.46, 2.42)	0.89	1.03	(0.46, 2.35)	0.93
Breastfeeding 1-16 weeks	0.99	(0.81, 1.21)	0.91	0.97	(0.80, 1.19)	0.80
Breastfeeding 17-24 weeks	0.89	(0.73, 1.09)	0.28	0.88	(0.72, 1.07)	0.21
Breastfeeding >24 weeks	1.04	(0.81, 1.33)	0.76	1.03	(0.81, 1.32)	0.79
Day care attendance	1.43	(1.24, 1.67)	0.00	1.45	(1.25, 1.69)	0.00

	DDE (N=1342)			sumPCBs (N=1339)		
	RR	**95%CI**	**p-value**	**RR**	**95%CI**	**p-value**
Wheezing						
Q1	1.10	(0.88, 1.36)	0.41	1.02	(0.84, 1.25)	0.83
Q2	1.30	(1.06, 1.59)	0.01	0.96	(0.78, 1.19)	0.73
Q3	1.11	(0.89, 1.40)	0.35	0.85	(0.67, 1.07)	0.17
Gipuzkoa region	0.97	(0.81, 1.15)	0.70	0.99	(0.82, 1.18)	0.89
Valencia region	0.69	(0.56, 0.85)	0.00	0.73	(0.59, 0.90)	0.00
Male sex	1.31	(1.13, 1.52)	0.00	1.29	(1.11, 1.50)	0.00
Allergic or asthmatic mother	1.16	(0.99, 1.35)	0.07	1.17	(1.00, 1.38)	0.05
Multiparous	1.59	(1.37, 1.84)	0.00	1.61	(1.39, 1.88)	0.00
Maternal smoking during the 1st year of life	1.08	(0.87, 1.33)	0.48	1.07	(0.86, 1.32)	0.55
Maternal smoking during pregnancy	1.30	(1.03, 1.65)	0.03	1.30	(1.02, 1.65)	0.03
Manual jobs	1.23	(1.04, 1.46)	0.02	1.22	(1.03, 1.44)	0.02
Unclassifiable	0.55	(0.15, 2.09)	0.38	0.57	(0.15, 2.15)	0.40
Breastfeeding 1-16 weeks	1.05	(0.86, 1.28)	0.66	1.02	(0.83, 1.24)	0.86
Breastfeeding 17-24 weeks	0.78	(0.63, 0.97)	0.02	0.77	(0.62, 0.94)	0.01
Breastfeeding >24 weeks	0.88	(0.67, 1.15)	0.34	0.87	(0.66, 1.14)	0.31
Day care attendance	1.50	(1.28, 1.75)	0.00	1.52	(1.30, 1.78)	0.00

DDE= dichlorodiphenyldichloroethylene, PCBs= polychlorinated biphenyls.

Table S3. Number of children (N), cases of LRTIs during the first 12-14 months of life (%) and adjusted relative risk (RR (95% Confidence Interval)) for continuous exposure and for each quartile of DDE exposure within the Spanish study population by region (N=1342[*†]).

	Gipuzkoa (N=500)			Sabadell (N=455)			Valencia (N=387)		
	N (% LRTI cases)	**Adjusted RR (95%CI)**	**p-value**	**N (% LRTI cases)**	**Adjusted RR (95%CI)**	**p-value**	**N (% LRTI cases)**	**Adjusted RR (95%CI)**	**p-value**
DDE									
Continuous	500 (36.4)	1.16 (0.98, 1.37)	0.08	455 (39.6)	1.08 (0.92, 1.27)	0.35	387 (29.2)	1.01 (0.82, 1.26)	0.9
<72.6	172 (31.4)	1		129 (34.9)	1		35 (28.6)	1	
72.6-115.9	149 (38.3)	1.25 (0.93, 1.69)	0.14	109 (38.5)	1.09 (0.80, 1.49)	0.57	77 (26.0)	0.99 (0.52, 1.87)	0.97
115.5-191.7	107 (40.2)	1.35 (0.98, 1.86)	0.07	127 (43.3)	1.15 (0.85, 1.56)	0.37	102 (35.3)	1.34 (0.76, 2.35)	0.31
>191.7	72 (38.9)	1.45 (1.01, 2.08)	0.04	90 (42.2)	1.17 (0.83, 1.66)	0.36	173 (27.2)	0.94 (0.53, 1.66)	0.83
HCB[*]									
Continuous	500 (36.4)	1.10 (0.93, 1.31)	0.28	455 (39.6)	1.02 (0.85, 1.23)	0.82	386 (29.3)	1.02 (0.85, 1.22)	0.84
<26.4	166 (36.1)	1		114 (39.5)	1		56 (25.0)	1	
26.4-46.4	152 (34.2)	0.98 (0.73, 1.33)	0.92	137 (37.3)	0.94 (0.70, 1.26)	0.66	46 (30.4)	1.09 (0.57, 2.07)	0.79
46.4-79.0	118 (42.4)	1.26 (0.93, 1.71)	0.13	122 (36.9)	0.91 (0.65, 1.26)	0.57	95 (30.5)	1.13 (0.65, 1.94)	0.66
>79.0	64 (31.3)	1.05 (0.68, 1.26)	0.82	82 (47.6)	1.01 (0.70, 1.46)	0.94	189 (29.6)	1.02 (0.60, 1.74)	0.93
ΣPCBs[†]									
Continuous	498 (36.4)	0.86 (0.65, 1.14)	0.31	455 (39.6)	0.99 (0.74, 1.33)	0.97	386 (29.3)	1.13 (0.86, 1.48)	0.38
<79.4	69 (42.0)	1		204 (37.3)	1		62 (35.5)	1	
79.4-113.7	126 (36.5)	0.96 (0.67, 1.38)	0.83	129 (41.9)	0.95 (0.72, 1.27)	0.74	80 (26.3)	0.70 (0.43, 1.15)	0.16
113.7-158.6	134 (41.0)	1.03 (0.71, 1.50)	0.86	86 (41.9)	0.95 (0.67, 1.33)	0.75	115 (24.4)	0.63 (0.38, 1.04)	0.07
>158.6	169 (30.2)	0.78 (0.51, 1.20)	0.26	36 (38.9)	0.91 (0.58, 1.43)	0.69	129 (31.8)	0.77 (0.49, 1.23)	0.27

DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls.

LRTIs model adjustment: sex of the child, age and pre-pregnancy weight of the mother, allergic or asthmatic mother, parity (first child) and social class, predominant breastfeeding, maternal smoking status (during pregnancy and during the 1st year of life of the child) and day-care attendance during the first year of life.

[*]One child within the Valencia region was excluded from the analysis with HCB because it was an outlier. [†]Two children had no information for PCBs within the Gipuzkoa region and one from the Valencia region was excluded from the analysis with ΣPCBs because it was an outlier.

Table S4. Results of the linear splines using log-transformed exposures and comparison between pooled-analysis and meta-analysis results for the association between DDE exposure (continuous and by quartiles) and LRTIs.

DDE			HCB			PCB		
Knots	**(N)**	**RR (95%CI)**	**Knots**	**(N)**	**RR (95%CI)**	**Knots**	**(N)**	**RR (95%CI)**
< 3.61	55	0.91 (0.40, 2.08)	< 3.75	617	0.93 (0.71, 1.23)	< 3.92	105	2.95 (0.66, 13.18)
3.61-5.82	1174	1.37 (0.55, 3.40)	>= 3.75	724	1.38 (0.84, 2.27)	3.92-4.84	679	0.27 (0.05, 1.6)
> 5.82	113	1.10 (0.50, 2.42)				>4.84-5.75	502	1.05 (0.4, 2.77)
						> 5.75	53	6.77 (0.72, 63.8)

	DDE (N=1342)		HCB[#] (N=1341)		ΣPCBs[†] (N=1339)	
	RR (95%CI)	**p-val**	**RR (95%CI)**	**p-val**	**RR (95%CI)**	**p-val**
			Continuous			
Pooled-analysis	1.11 (1.00, 1.22)		1.06 (0.95, 1.17)		1.00 (0.86, 1.18)	
Meta-analysis	1.09 (0.98, 1.2)	0.66	1.05 (0.94, 1.15)	0.81	0.97 (0.81, 1.13)	0.39
			Quartiles			
Pooled-analysis						
Q2	1.16 (0.94, 1.43)		0.96 (0.79, 1.18)		0.98 (0.80, 1.20)	
Q3	1.33 (1.08, 1.62)		1.08 (0.88, 1.32)		0.94 (0.75, 1.17)	
Q4	1.20 (0.96, 1.51)		1.06 (0.84, 1.33)		0.84 (0.65, 1.08)	
Meta-analysis						
Q2	1.14 (0.9, 1.37)	0.78	0.97 (0.77, 1.16)	0.92	0.88 (0.70, 1.06)	0.34
Q3	1.24 (0.98, 1.49)	0.84	1.04 (0.82, 1.26)	0.39	0.83 (0.63, 1.02)	0.09
Q4	1.17 (0.90, 1.45)	0.52	1.03 (0.77, 1.28)	0.99	0.81 (0.60, 1.02)	0.81

Adjustment of all models: region, sex of the child, allergic or asthmatic mother, parity (first child) and social class, predominant breastfeeding, maternal smoking status (during pregnancy and during the 1st year of life of the child) and day-care attendance during the first year of life.
p-val: p-value for heterogeneity (meta-analysis). [#]One child was excluded from the analysis with HCB because it was an outlier (N=1341). [†]Two children had no information for PCBs within the Spanish population and one was excluded from the analysis with ΣPCBs because it was an outlier (N=1339).

5.2 Paper II

Effects of persistent organic pollutants on the developing respiratory and immune systems: A systematic review

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Abstract

Background: Disruption of developing immune and respiratory systems by early-life exposure to persistent organic pollutants (POPs) could result into reduced capacity to fight infections and increased risk to develop allergic manifestations later in life.

Objectives: To systematically review the epidemiologic literature on the adverse effects of early-life exposure to POPs on respiratory health, allergy and the immune system in infancy, childhood and adolescence.

Methods: Based on published guidelines for systematic reviews, two independent researchers searched for published articles in MEDLINE and SCOPUS using defined keywords on POPs and respiratory health, immune function and allergy. Study eligibility criteria were defined to select the articles.

Results: This review of 41 studies finds limited evidence for prenatal exposure to DDE, PCBs and dioxins and risk of respiratory infections. Evidence was limited also for postnatal exposure to PCBs, specifically ndl-PCBs, and reduced immune response after vaccination in childhood. The review indicates lack of association between postnatal exposure to PCBs/ndl-PCBs and risk of asthma-related symptoms. For the other exposure-outcome associations reviewed evidence was inadequate.

Discussion and Conclusion: Current epidemiological evidence suggests that early-life exposure to POPs can adversely influence immune and respiratory systems development. Heterogeneity between studies in exposure and outcome assessment and the small number of studies for any given exposure-outcome relationship

currently make comparisons difficult and meta-analyses impossible. Also, mechanisms remain largely unexplored. Recommendations for significantly improving our understanding thus include harmonization of exposure and outcome assessment between studies, conduct of larger studies, long-term assessment of respiratory infections and asthma symptoms in order to identify critical periods of susceptibility, integration of the potential immunotoxic mechanisms of POPs, and use of new statistical tools to detangle the role of multiple exposures on multiple outcomes.

1. INTRODUCTION

Acute respiratory infections in under 5-year olds are a worldwide cause of morbidity and mortality, accounting for about 20% of all childhood deaths, mostly in developing countries (Nair et al. 2011; Rudan et al. 2004). Further, the prevalence of asthma and allergy in children is more than 20% in some countries and still rising (Bousquet et al. 2011; Burr et al. 2006; Chad 2001). Diverse studies have pointed environmental pollutants, including persistent organic pollutants (POPs), as important factors that can impact susceptibility to infections and the development of allergy and asthma during the first years of life (Winans et al. 2011). POPs are lipophilic synthetic compounds that persist in the environment and bioaccumulate through the food chain in human and animal fatty tissues and include a wide range of compounds, such as polychlorinated biphenyls (PCBs), dioxins, dichlorodiphenyldichloroethylene (DDE), hexachlorobenzene (HCB), and many other chemicals. Human exposure to POPs begins in the uterus, since most POPs cross the placental barrier (Covaci et al. 2002; Lackmann et al. 1999; Sala et al. 2001). After birth, newborns are exposed to POPs through breastfeeding, diet or by direct contact with materials containing these compounds (Aliyu et al. 2010; Fischer et al. 2006; LaKind et al. 2004). Poisoning incidents in the 1970s provided the first evidence of potential adverse health effects of exposure to high concentrations of PCBs on human respiratory and immune systems (Aoki 2001; Guo et al. 2004); these studies revealed altered levels of immune markers, as

well as higher frequencies of respiratory infections among both children and adults.

Because immune and respiratory systems develop and mature during pre and postnatal early life, these periods may be particularly susceptible to the effects of POPs. All major respiratory elements, including the bronchioles and alveolar ducts, appear before birth, alveoli proliferation continues after birth, and full maturation of the respiratory system is not complete until late adolescence (Dietert et al. 2000; Pinkerton and Joad 2000). The ability of the body to produce a normal immune response following antigen exposure (immunocompetence) starts developing after birth and continuous during the first years of life (Dietert et al. 2000). This involves the development of the innate system (natural killer cells (NK-cells), eosinophils, basophils, neutrophils, macrophages or dendritic cells) that provides immediate defense against infection, and the adaptive immune system (antibodies produced by B-cells and cell-mediate response regulated by cytokines released by T cells) that provides long-lasting or protective immunity through humoral response. Disruption of these systems during development by exposure to POPs may result into reduced capacity to fight infections and increased risk to develop allergic manifestations later in life (Bisgaard and Bonnelykke 2010; Busse et al. 2010; Dietert et al. 2000; Puig et al. 2008).

Although some general reviews are available (Wigle et al. 2008; Winans et al. 2011), none has made a comprehensive and

systematic evaluation of available epidemiological evidence on the effects of early-life POP exposure on the developing immune and respiratory systems. Our aim is thus to systematically review the epidemiologic literature on adverse effects of early-life exposure to POPs on respiratory health, allergy and the immune system in infancy, childhood and adolescence.

2. MATERIALS AND METHODS

2.1 Search of studies

A protocol was written based on the PRISMA statement guidelines for reporting systematic reviews and meta-analysis (Moher et al. 2010). The bibliographic search was carried out by two independent reviewers in the MEDLINE engine search (National Library of Medicine) and SCOPUS engine search using the following keywords: *outcome keywords* ("allergic rhinitis" or "allergic conjunctivitis" or asthma or "shortness of breath" or wheeze or wheezing or cough or "lung function" or otitis or bronchiolitis or bronchitis or pneumonia or "ear infection" or atopy or "atopic eczema" or eczema or "atopic dermatitis" or "food allergy" or "sensitization" or "eosinophilic gastroenteritis" or "immune system" or "upper respiratory tract infection" or "lower respiratory tract infection" or "immunoglobulin" or cytokines or lymphocytes or "B-cell" or "T-cell" or Macrophage or "Mast cell" or "NK cell" or interleukin or vaccination) combined with the following *keywords for POPs* ("aldrin" or "chlordane" or "dichlorodiphenyltrichloroethane" or "DDT" or "dichlorodiphenyldichloroethylene" or "DDE" or "dieldrin" or

"endrin" or "heptachlor" or "hexachlorobenzene" or "HCB" or "Mirex" or "toxaphene" or "chlordecone" or "α-hexachlorocyclohexane" or "α-HCH" or "β-hexachlorocyclohexane" or "β-HCH" or "lindane" or "γ-HCH" or "pentachlorobenzene" or "PeCB" or "polychlorinated biphenyls" or PCBs or "polybrominated biphenyls" or PBBs or "perfluorooctane sulfonic acid" or PFOS or "perfluorooctane sulfonyl fluoride" or "polybrominated diphenyl ether" or "polybromodiphenyl ethers" or PBDEs or "polychlorinated dibenzo-p-dioxins" or "polychlorinated dibenzofurans" or "PCDDs" or "PCDFs" or "dioxins" or "persistent organic pollutants" or "POPs"). *Limits*: Human, English, All Child 0-18 years. Last search on September 18th 2012. Identification and first screening of the articles were performed using the information available in the title and the abstract. Doubts regarding the inclusion or exclusion of studies were resolved by discussion between the two independent researchers, and if no agreement was reached then a third researcher helped to decide. After the first selection, both reviewers read through the articles to decide whether they were eligible or not.

2.2 Study eligibility criteria

The selection criteria were that the article had to: a) include newborns, children or adolescents as study subjects, b) measure individual exposure to POPs in human tissues or matrixes, or estimate individual exposure during pregnancy (prenatal exposure)-, around birth (perinatal exposure), or during childhood (postnatal exposure), c) include health outcomes related to respiratory health,

allergy and immune function, d) be an original research article: abstracts, case reports, ecological studies and letters to the editor were excluded, e) report on an epidemiologic study with a prospective, cross-sectional or case-control design, and f) be written in the English language.

2.3 Evaluation of evidence

In order to evaluate evidence, we first classified as good quality the studies that had prospective study designs, relatively large study size (>100 participants), good use of exposure information and multivariate confounder adjustment (Table C, supplemental material).

We classified strength of evidence for associations between each combination of specific pollutant exposure and specific outcome (separately by pre and postnatal exposure period) based on the levels of evidence used by the International Agency for Research on Cancer (IARC 2006). Two independent reviewers classified the evidence; in case of disagreement a third reviewer gave the decision. Evidence for causal relationships for each chemical compound-outcome was classified as: *sufficient* - if most of the studies, including good quality studies, report an association, but evidence is not yet conclusive enough to conclude that there is an association or a causal relationship, *limited* - several good quality, independent, studies report an association, but evidence is not yet conclusive enough, *inadequate* - if associations are reported in one or more studies, but insufficient quality, insufficient number of

studies, lack of consistency, and/or lack of statistical power preclude a conclusion regarding the presence or absence of an association, *evidence for lack of association* - several good quality studies are consistent in showing no association.

We organized the review by outcome and then by chemical compound, age of the participants (<two years, referred as "younger" and >two years, referred as "older"), and by time of exposure (pre or postnatal).

3. RESULTS

A total of 147 articles were identified in MEDLINE, of which 42 were selected as potentially eligible by both reviewers, who discussed the inclusion of other ten articles; four were finally included (one of them after discussing with a third researcher). In total, 46 articles initially appeared to meet the eligibility criteria, but after reading, seven articles were excluded: four were ecological studies (Belles-Isles et al. 2002; Lan et al. 1990; Landrigan et al. 1979; Milosevic-Djordjevic et al. 2005), one study used breastfeeding as proxy of exposure to POPs (Karmaus et al. 2003), and two studies were carried out on adults (Daniel et al. 2002; Vine et al. 2001). Finally, one study was also found in MEDLINE but not through the keyword search (Jusko et al. 2012), and another one was found via SCOPUS (Grandjean et al. 2012). All the selection process is detailed in the supplemental material (Figure A). Table A of the supplemental material shows the 41 final selected articles, which are based on 28 separate study populations from 13

countries. It also shows the study design of the articles (mostly longitudinal birth cohorts: N=35), the age of the participants at the time of outcome assessment (ranging from birth up to 19 years old), information on exposure assessment methods, and exposure levels.

3.1 Exposure assessment

Practically all articles (N=35) evaluated PCB and/or dioxin compounds, whereas other POPs were assessed to a lesser extent: seventeen articles assessed DDE, ten assessed HCB and ten assessed a diversity of other POPs such as hexachlorohexane congeners (HCHs), dichlorodiphenyltrichloroethane (DDT), dieldrin, chlordane, perfluorinated chemicals or polybromodiphenyl ethers (PBDEs). Biological matrixes used to measure exposure to these compounds differed between studies; most used maternal blood obtained during pregnancy (N=20), followed by breast milk (N=16), children's serum (N=17) and cord blood (N=11). Three articles used placenta and/or adipose tissue. Articles variously considered breast milk as a marker of pre, peri or postnatal exposure; however, postnatal exposure was often the product of concentrations of POPs measured in breast milk and the number of weeks of breastfeeding or the amount of milk consumed by the child. In one study the intake of dioxins and PCBs during pregnancy was estimated from dietary questionnaires and validated with maternal serum concentrations in a subsample ($r=0.34$-0.37, $p<0.05$) (Stolevik et al. 2011). Units of expression also differed; approximately half of the articles adjusted levels by lipids, whereas the other half did not. When measuring dioxins and/or dl-PCBs, the

unit of expression was Toxic Equivalent Factor (TEQ). The specific compounds and congeners used within each study are indicated in Table A of the supplemental material. It is important to note that in the present review the sum of PCBs can indicate either "unknown congeners" or a mix of non dioxin-like (ndl-) and dioxin-like (dl-) PCBs (ndl-PCBs were predominant in the sum of most of the studies). If only non dioxin-like or dioxin-like PCBs are included in the sum, then it is indicated as ndl-PCBs or dl-PCBs, respectively. When the exposures assessed are PCDDs or PCDFs it is indicated as dioxins, unless specifically indicated in the original study.

3.2 Infections

Overall, sixteen studies evaluated the effects of POPs on infections (Table 1 and Table B.1), mostly lower respiratory tract infections (LRTIs), upper respiratory tract infections (URTIs) and acute otitis media (AOM). Other infections included gastrointestinal infections (GIs), chicken pox, pertussis, measles, and others. Information on infections was most often collected through parental questionnaires asking about doctor-diagnosed infections (Dewailly et al. 2000; Gascon et al. 2012; Karmaus et al. 2001; Miyashita et al. 2011; Okada et al. 2012; Sunyer et al. 2005, 2010; Weisglas-Kuperus et al. 2000, 2004) or from the medical chart (Dallaire et al. 2004, 2006). Three studies asked specific questions on the number of infections but not specifically diagnosed by a doctor (Stolevik et al. 2011; ten Tusscher et al. 2001, 2003), one used open-ended questions (Glynn et al. 2008), and in one study it is not clearly specified how the information was obtained (Yu et al. 1998).

3.2.1 Respiratory infections

PCBs and dioxins. Overall, seven studies reported a higher risk of respiratory infections, including AOM, at younger (Dallaire et al. 2004; Glynn et al. 2008; Miyashita et al. 2011; Stolevik et al. 2011) and older ages (Dallaire et al. 2006; ten Tusscher et al. 2001; Weisglas-Kuperus et al. 2000) in relation to prenatal exposure to PCBs and/or dioxins. However, these associations were not always for the same congeners (ndl-PCBs, dl-PCBs or dioxins) or consistent across quartiles of exposure (Dallaire et al. 2004; Glynn et al. 2008). Also, in one study increased or decreased risks were found depending on the congeners assessed (Glynn et al. 2008). On the other hand, several other studies reported no association with exposure to PCBs at younger (Dewailly et al. 2000; Gascon et al. 2012; Sunyer et al. 2010) or at older ages (Sunyer et al. 2005; ten Tusscher et al. 2003; Weisglas-Kuperus et al. 2004). Fewer studies assessed the effects of postnatal exposure to PCBs and dioxins; at young ages, one study reported reduced odds of respiratory infections with increasing exposure to only some PCB congeners (Glynn et al. 2008), whereas a Canadian birth cohort study could not find any association (Dallaire et al. 2004). At older ages, two studies assessing the same study population at different ages found increased odds of recurrent AOM in relation to increasing ΣPCB levels (Weisglas-Kuperus et al. 2000, 2004), whereas a cross-sectional study could only find associations with AOM when high ΣPCB levels were combined with high levels of DDE (Karmaus et al. 2001). In a study with Yucheng children of 8-16 years prenatally exposed to dioxins, influenza frequency was increased in this

population compared to controls (Yu et al. 1998). Another small birth cohort did not observe an association between dioxins and AOM in children aged 7-12 years (ten Tusscher et al. 2001, 2003).

DDE. Higher prenatal DDE levels were associated with an increased risk of developing respiratory infections at young ages in all birth cohort studies evaluated (Dallaire et al. 2004; Dewailly et al. 2000; Gascon et al. 2012; Sunyer et al. 2010), except one (Glynn et al. 2008). However, Dallaire *et al.* found associations only for URTIs (and not LRTIs) showing a non-linear association (Dallaire et al. 2004). At older ages, Dallaire *et al*. mentioned that, although not presented, results for prenatal exposure to DDE where similar to those found for prenatal exposure to PCB-153 (increased risk of LRTIs and AOM) (Dallaire et al. 2006). On the other hand, another birth cohort study could not find any association between prenatal exposure to DDE and respiratory infections at a similar age (Sunyer et al. 2005). Postnatal DDE exposure was not associated to respiratory infections in young children of a Canadian study (Dallaire et al. 2004), whereas in a Swedish cohort a reduced risk of infection was found within the second quartile of exposure but not in the third or the fourth (Glynn et al. 2008). At older ages there is only one study available; the odds of developing AOM increased only when high postnatal levels of DDE were combined with high levels of PCBs or HCB, otherwise the odds were reduced (Karmaus et al. 2001).

HCB, HCHs and other POPs. There are very few studies assessing the effects of POPs other than PCBs or DDE. In a Canadian cohort, prenatal exposure to Mirex was not associated with any type of respiratory infection, whereas increasing levels of dieldrin increased the risk of AOM between the 4th and 7th month of life and increasing levels of HCB increased the risk of AOM during the first year of life (Dewailly et al. 2000). However, in three larger Spanish studies, where levels of HCB were less highly correlated to levels of other OCs, no association between increasing prenatal HCB exposure and LRTIs was found, either at young (Gascon et al. 2012; Sunyer et al. 2010) or at older ages (Sunyer et al. 2005). Adverse respiratory health effects were found regarding postnatal exposure to high levels of HCB when combined with high levels of DDE (Karmaus et al. 2001). A recent study could not find an association between prenatal perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) exposure and AOM in children at age 18 months (Okada et al. 2012).

3.2.2 Other infections

Very few studies assessed infections other than respiratory infections (Table 1). Exposure to DDE was found to increase risk of GIs at age 12 months, but only within the second quartile (Dallaire et al. 2004). Overall, five studies assessing exposure to dioxin and/or PCB compounds reported inconsistent results for exanthema subitum and GIs (Dallaire et al. 2004; Stolevik et al. 2011; Weisglas-Kuperus et al. 2000, 2004; Yu et al. 1998) or no

associations for chickenpox (Stolevik et al. 2011; ten Tusscher et al. 2003).

On the basis of this, we classified the evidence as limited for an association of prenatal exposure to PCBs and dioxins with the risk of respiratory infections and AOM in childhood. The evidence for an association is also limited for prenatal DDE exposure and respiratory infections but inadequate for AOM. For other exposures, including postnatal PCBs, dioxins and DDE, or infections other than respiratory evidence is inadequate (Table 3).

3.3 Allergic manifestations

We distinguish between asthma symptoms (wheeze, asthma, shortness of breath, lung function (fourteen articles)) and symptoms and markers of atopy [eczema, allergy symptoms and reaction, atopic dermatitis, allergic rhinitis, positive skin prick test (SPT) and total and specific IgE]. In the present review IgE was not included as a humoral immune marker but as a marker of atopy because IgE in the mucosal mast cells reflects immediate allergic reactions (Gould et al. 2003). In fact, in most of the studies it was treated specifically as a marker of atopy (nineteen articles included) (Table 1 and Table B.2). Most of studies assessed asthma and wheezing through questionnaires answered by parents, in which most of the questions were based on doctor diagnosed symptoms. One study assessed asthma through direct doctor diagnosis (Grandjean et al. 2010) and another one assessed lung function by spirometry (ten Tusscher et al. 2001). Atopy was assessed by either measuring

immunoglobulin E (IgE) levels or by self-reported symptoms (self-reported allergy, eczema, rhinitis) obtained through questionnaire.

3.3.1 Asthma symptoms

PCBs and dioxins. At young ages higher prenatal levels of different dl- and ndl-PCB congeners and dioxins were associated with an increased odds of wheeze, but not asthma (Stolevik et al. 2011). Other studies assessing wheeze or asthma at a similar age could not find any association (Gascon et al. 2012; Miyashita et al. 2011). At older ages a small study found lung function at age 7-12 years to be reduced after prenatal and postnatal exposure to dioxins (ten Tusscher et al. 2001): however, no association with asthma symptoms was found (ten Tusscher et al. 2003). Other studies at similar ages could not find any association between asthma symptoms and prenatal exposure to ndl-PCBs or ΣPCBs, respectively (Grandjean et al. 2010; Sunyer et al. 2005). In two studies, lower odds of suffering shortness of breath with wheezing after prenatal exposure to PCBs, but not to dioxins, was reported (Weisglas-Kuperus et al. 2000, 2004). A cross-sectional study including 200 individuals aged 7-18 years found an association between increasing postnatal ndl-PCBs levels and risk of ever suffering from asthma, but no associations were found for dl-PCBs (Van Den Heuvel et al. 2002). Other studies including children aged 3 to 8 years did not report an association between postnatal PCB or dioxin exposure and risk of asthma and/or wheeze (Grandjean et al. 2010; Karmaus et al. 2001; ten Tusscher et al. 2003; Weisglas-Kuperus et al. 2000, 2004; Yu et al. 1998).

DDE, HCB and other POPs. At young ages only one study assessed the effects of DDE and HCB exposure on allergic manifestations in the respiratory system; no significant associations were found in children at 14 months of age, but increasing prenatal DDE levels were associated with risk of wheezing (Gascon et al. 2012). At older ages, prenatal DDE exposure was associated with a higher risk of wheeze and/or asthma at different ages in a birth cohort study (Sunyer et al. 2005, 2006), whereas a cross-sectional study found that increasing postnatal DDE levels increased the odds of suffering of asthma (Karmaus et al. 2001). These same studies did not find any association of pre or postnatal exposure to HCB and/or β-HCH and risk of wheeze or asthma (Karmaus et al. 2001; Sunyer et al. 2005). Finally, another study on prenatal exposure to PFOA and PFOS could not find associations with wheeze in children aged 18 months (Okada et al. 2012).

3.3.2 Atopy

PCBs and dioxins. Among studies evaluating the association between prenatal PCBs and/or dioxins and total or specific IgE levels (Jusko et al. 2011; Kaneko et al. 2006; Reichrtova et al. 1999) or eczema or allergy (Miyashita et al. 2011; Stolevik et al. 2011) in young children, only one study reported a positive correlation between prenatal PCB-118 levels (but not other PCBs) and total IgE levels (Reichrtova et al. 1999). Also, in one of these studies the odds of food allergies was increased for dl-PCBs (Miyashita et al. 2011), although associations were not linear across quartiles. Also, authors could not rule out potential misclassification

between food allergies and food intolerance. At older ages, among studies available (Grandjean et al. 2010; Sunyer et al. 2005; ten Tusscher et al. 2001, 2003; Weisglas-Kuperus et al. 2000, 2004; Yu et al. 1998) one small study (N=27) found increasing prenatal dioxin levels to be associated with reduced odds of suffering from allergy, but no associations with eczema were found (ten Tusscher et al. 2003), whereas Grandjean *et al*. reported lower levels of ndl-PCBs among children with atopic dermatitis compared to those with no allergy (Grandjean et al. 2010). Two studies assessed postnatal exposure to PCBs in young children; one found no associations with IgE levels at age 6 months and increasing ndl-PCBs (Jusko et al. 2011). The other study found that increasing ΣPCBs was associated to increasing levels of milk specific IgE at age 23 months (Tsuji et al. 2012). At older ages results are contradictory; a cross-sectional study found no association between ΣPCBs and total or specific IgE levels (Karmaus et al. 2001, 2005). However, another cross-sectional study found increasing dl-PCBs, but not ndl-PCBs, to be associated with a reduced risk of positive radioallergosorbent test (RAST), as well as reduced total IgE levels (Van Den Heuvel et al. 2002). On the other hand, a birth cohort study reported higher concentrations of ndl-PCBs in children with higher total IgE levels, but lower ndl-PCB levels in those with atopic dermatitis compare to those with no allergy symptoms (Grandjean et al. 2010). Van Den Heuvel *et al*. also found contrasted results regarding atopy symptoms: increased odds of suffering hay fever with increasing ndl-PCB levels, but reduced risk of upper airway disease with increasing dl-PCBs exposure (Van Den Heuvel et al. 2002). Two

birth cohort studies also found that increasing dioxin or PCB exposure reduced the odds of being atopic (ten Tusscher et al. 2003; Weisglas-Kuperus et al. 2000); however, results of Ten Tusscher *et al.* are inconsistent, since in a previous study, where practically the same study population was used (N=27 vs N=29), no association was found (ten Tusscher et al. 2001). Two other birth cohorts did not observe an association between PCB or dioxin exposure and atopic symptoms (Weisglas-Kuperus et al. 2004; Yu et al. 1998).

DDE, HCB and other POPs. Prenatal DDE exposure was positively and correlated to cord blood total IgE levels in a Slovakian birth cohort (Reichrtova et al. 1999), however, in a Spanish cohort with children aged 4 to 6.5 years, no association was found with specific IgE levels or positive SPT (Sunyer et al. 2005, 2006), neither with postnatal exposure (Sunyer et al. 2006). Postnatal DDE exposure was found to increase the odds of having higher total but not specific IgE levels in a cross-sectional study among children aged 7-10 years (Karmaus et al. 2001, 2005). Effects of pre or postnatal HCB, HCH or other POPs exposure on eczema and IgE levels have been evaluated in very few studies, and none of them observed associations (Karmaus et al. 2001, 2005; Reichrtova et al. 1999; Sunyer et al. 2005), except two studies evaluating the effects of prenatal exposure to PFOA and PFOS. One study found total IgE levels measured in cord blood, but not in serum at age 2 years, to be increased in relation to increasing PFOA and PFOS levels (Wang et al. 2011), whereas the other study found total, but not specific IgE levels, to be reduced with PFOA exposure (the association was not

linear) (Okada et al. 2012). In the first study, no associations with atopic dermatitis were found (Wang et al. 2011).

In summary, we classified the evidence of an association between prenatal exposure to DDE and risk of asthma-related symptoms as limited, whereas lack of association between postnatal exposure to PCBs, mainly ndl-PCBs, and risk of asthma-related symptoms seems to be evident. For other exposures the evidence is inadequate (Table 3).

3.4 Humoral immune response

Humoral response studies (Table 2 and Table B.3) were subdivided into those studies measuring general antibody concentrations (IgA, IgG and IgM – six articles) and those studies measuring specific antibody concentrations after vaccinations (seven articles).

3.4.1 General antibody concentrations

PCBs and dioxins. Studies measuring IgA, IgG and IgM levels in young children reported no association with prenatal exposure (Dewailly et al. 2000; Jusko et al. 2011; Kaneko et al. 2006) or postnatal exposure (Jusko et al. 2011) to PCBs or dioxins. At older ages, only effects of postnatal exposure were assessed; one cross-sectional study reported a negative correlation between postnatal ndl-PCBs and IgG, a positive correlation between dl-PCBs and IgA, and no correlations for IgM (Van Den Heuvel et al. 2002), whereas another study observed, among children in the highest PCB quartile of exposure, higher IgM levels but no associations with IgG or IgA

(Karmaus et al. 2005). A small birth cohort study found no correlation between exposure to dioxins and antibody concentrations in children aged 8 to 16 years (Yu et al. 1998).

DDE, HCB and other POPs. A Canadian birth cohort study reported no correlation between prenatal exposure to DDE and other OCs and immunoglobulin concentrations measured during the first year of life (Dewailly et al. 2000). However, a cross-sectional German study observed increased IgA concentrations at older ages (7-10 years) in relation to increasing postnatal DDE exposure, reduced IgM concentrations in relation to increasing HCB exposure and no effects of γ-HCH (Karmaus et al. 2005).

3.4.2 Specific antibody counts after vaccination

PCBs and dioxins. A birth cohort study reported decreases in diphtheria and/or tetanus Ab concentrations in relation to prenatal dl- and ndl-PCBs exposure in children under 2 years of age (Heilmann et al. 2006). However, two other studies, one with a larger study population, did not find any association with exposure to PCBs and/or dioxins (Jusko et al. 2010; Weisglas-Kuperus et al. 1995). At older ages, a Dutch study presenting partial correlations found that increasing prenatal ΣPCB levels were associated with reduced mumps and rubella Ab concentrations at age 42 months (Weisglas-Kuperus et al. 2000). Similar results on tetanus antibodies, but not diphtheria, were obtained by another study with children of 7.5 years of age in the Faroe Islands in relation to ndl-PCB exposure (Heilmann et al. 2006). However, in another birth

cohort in the same study area, no association was observed between prenatal exposure to ndl-PCBs and Ab concentrations at a similar age (Heilmann et al. 2010). Regarding postnatal exposure, at young ages Heilmann *et al*. found a decrease of diphtheria Ab concentrations in relation to both dl- and ndl-PCBs (Heilmann et al. 2006), however other two studies could not find an association (Jusko et al. 2010; Weisglas-Kuperus et al. 1995). At older ages, postnatal ndl-PCB exposure reduced tetanus and/or diphtheria Ab concentrations in children aged 5 and 7, mainly when exposure was measured at age 18 months rather than at older ages (5 to 7 years) (Heilmann et al. 2010). It is important to note that associations were mainly found for ndl-PCBs, whereas only one study found associations with dl-PCBs (Heilmann et al. 2006).

PFCs (perfluorinated compounds). In a birth cohort study in the Faroe Islands the effects of different PFC congeners (PFOA, PFOS, PFNA (perfluorononanoic acid), PFHxS (perfluorohexane sulfonic acid) and PFDA (perfluorodecanoate)) on antibody concentrations were also assessed at age 5 and 7 years (Grandjean et al. 2012). Associations were found between prenatal exposure to PFOS, PFOA and PFDA and diphtheria concentrations measured at age age 5 and 7 years. Associations, though, were not always found for all congeners and Ab measurements. The effects of different PFC congeners (PFOS, PFOA, PFHxS and PFDA) on Ab concentrations were stronger for postnatal exposure (Grandjean et al. 2012).

The evidence for an association between postnatal exposure to PCBs, mainly ndl-PCBs, and specific antibody counts is limited, whereas for prenatal exposure to PCBs and pre or postnatal exposure to other POPs the evidence is inadequate (Table 3).

3.5 Cell-mediated immune response and immune cell counts

There are four studies evaluating the effects of POPs on cell-mediated response (cytokines) and sixteen measuring immune cell counts such as lymphocyte (T-cells, B-cells, NK-cells) and monocytes or granulocytes (Table 2 and Table B.4). Many of these studies have small study populations (N≈30) (Brooks et al. 2007; Leijs et al. 2009; Noakes et al. 2006; Tsuji et al. 2012; ten Tusscher et al. 2003; Yu et al. 1998) or do not adjust for potential confounders (Bilrha et al. 2003; Dewailly et al. 2000; Glynn et al. 2008; Van Den Heuvel et al. 2002; Kaneko et al. 2006; Leijs et al. 2009; Nagayama et al. 1998, 2007; Noakes et al. 2006; Tsuji et al. 2012; Weisglas-Kuperus et al. 1995, 2000; Yu et al. 1998). The largest studies (N=362) available and adjusting for potential confounders compared cell counts between a highly polluted area and a less polluted area, but did not introduce measured PCBs exposure in the model (Horvathova et al. 2011a, 2011b). Also, the multiple subtypes of T-cells (i.e. HLA-DR^+, $CD8^+$, $CD3^+$ or $CD16^+$) or cytokines measured differ between studies, making comparisons difficult.

3.5.1 Cell-mediated response

Three studies measured levels of various cytokines in cord blood at birth; in one study TNF-α was negatively correlated to prenatal ΣPCBs, DDE and HCB concentrations (Bilrha et al. 2003). In the other two studies one found interleukin levels and ratios to be positively correlated to DDE levels (Brooks et al. 2007), but the other study did not find any correlation (Noakes et al. 2006). Finally, one study measuring postnatal levels of PCBs found that increasing ΣPCBs and ndl-PCBs levels were associated with increasing levels of COX-2 (cyclooxygenase-2) and/or IL-8. No associations were found for dl-PCBs (Tsuji et al. 2012).

3.5.2 Cell counts

PCBs and dioxins. Most of the studies did not find an association between prenatal (Bilrha et al. 2003; Dewailly et al. 2000; Kaneko et al. 2006; Leijs et al. 2009; Sunyer et al. 2005; ten Tusscher et al. 2003) or postnatal (Leijs et al. 2009; Weisglas-Kuperus et al. 2000; Yu et al. 1998) exposure to PCBs or dioxins and different lymphocyte counts at different ages. Some studies observed increased white blood-cell counts (WBC) and/or different T-cell counts (not B or NK-cell counts) in relation to prenatal exposure to PCBs and/or dioxins (Glynn et al. 2008; Nagayama et al. 2007; Weisglas-Kuperus et al. 1995, 2000). A recent study which measured cell levels at different time points (cord blood, 6 and 16 months of life) also reported increased T-cell and B-cell, but reduced NK-cell levels, in children from a highly polluted area with ΣPCBs compared to children from a less polluted area in Slovakia

(Horvathova et al. 2011a). In this same study population, different T-cell and dendritic cell receptors were found to be reduced, and other T-cell receptors increased, among children from the highly polluted area (Horvathova et al. 2011b). Regarding postnatal exposure to PCBs and/or dioxins, some studies found increased and others found decreased levels of different lymphocyte types (Glynn et al. 2008; Van Den Heuvel et al. 2002; Karmaus et al. 2005; Nagayama et al. 1998; ten Tusscher et al. 2003; Weisglas-Kuperus et al. 1995). Fewer studies evaluated the effects of prenatal and postnatal exposure on granulocyte and monocyte counts and results were inconsistent between studies (Glynn et al. 2008; Van Den Heuvel et al. 2002; Karmaus et al. 2005; Leijs et al. 2009; Sunyer et al. 2005; Weisglas-Kuperus et al. 1995, 2000; Yu et al. 1998).

DDE, HCB and other POPs. Pre or perinatal DDE or DDT exposure was not associated with cell counts in any of the available studies (Bilrha et al. 2003; Dewailly et al. 2000; Nagayama et al. 2007; Sunyer et al. 2005), only one found a decrease in the percentage, but not the number, of eosinophil counts (Glynn et al. 2008). Karmaus *et al.* found postnatal exposure to DDE to increase WBC counts in children aged 7 to 10 years when comparing the highest quartile of exposure with the lowest (Karmaus et al. 2005), but another study could not detect any association at younger ages (Glynn et al. 2008). No associations between prenatal HCB, Mirex or dieldrin exposure and cell counts were found in most of the studies (Bilrha et al. 2003; Dewailly et al. 2000; Sunyer et al. 2005). Increasing perinatal HCH and HCE (heptachlor epoxide) exposures

increased counts of different T-cell subtypes in Japanese children aged 10 months (Nagayama et al. 2007). No, or at least not consistent, effects of postnatal exposure to HCB (Karmaus et al. 2005; Sunyer et al. 2005) or γ-HCH (Karmaus et al. 2005; Leijs et al. 2009) were described, only PBDE levels were negatively correlated to lymphocyte counts in adolescents aged 14 to 19 years (Leijs et al. 2009).

We summarize the evidence for an association between immune response and any pre or postnatal exposure to POPs as inadequate (Table 3).

3.6 Other outcomes

A Slovakian study including 982 newborns found that the size of the thymus, the organ where T-cells are 'educated', was reduced at birth due to prenatal exposure to PCBs (β (SE, pval)=-0.036 (0.018, p=0.047)), even after selecting those children (N=187) assessed by the most experienced radiologist (-0.102 (0.047, p=0.033)). No associations were found for DDT or DDE (Park et al. 2008). In the same study population no associations were found between prenatal exposure to ndl-PCBs and the size of the thymus at age 6 (change in thymus index SD (95%CI)=-0.09 (-0.29, -0.11)) or 16 months (-0.02 (-0.21, 0.17)). However, results showed an association between postnatal exposure to ndl-PCBs measured at 6 months of age and the size of thymus at the same age (-0.40 (-0.76,-0.04)), whereas no associations between ndl-PCBs measured at age 16 months and the

thymus size at that time were found (0.17 (-0.04, 0.38)) (Jusko et al. 2012).

4. DISCUSSION

This review finds limited evidence for prenatal DDE, PCBs and dioxins exposure to increase the risk of respiratory infections (with the exception of DDE and AOM, for which evidence is inadequate) and for postnatal exposure to PCBs, specifically ndl-PCBs, to reduce immune response after vaccination in childhood. Results of available studies also indicate lack of association between postnatal exposure to PCBs/ndl-PCBs and risk of asthma-related symptoms. For the other combinations of exposures and outcomes evidence is inadequate, mainly due to the reduced number of studies available within each combination. Heterogeneity between studies in exposure and outcome assessment, as well as the small number of studies for any given exposure-outcome relationship, currently make the combination of studies for meta-analysis impossible, but this review raises important issues that may guide both the design and presentation of future studies.

4.1 Exposure assessment

In the reviewed studies, chemical compounds were measured in different matrixes, reported in different units, and postnatal estimates were sometimes derived by multiplying breastmilk concentrations and breastfeeding duration. These differences make it difficult to compare exposure levels or results, let alone perform meta-analyses. However, a recent European study on POPs and

birthweight has shown that it is possible to perform a meta-analyses of cohort studies that measured POPs in different matrices by going back to the original data and using conversion factors to compare between matrices (Govarts et al. 2012). For the estimation of postnatal levels physiologically based pharmacokinetic (PBPK) models are now being developed for studies on environmental health effects in children (Verner et al. 2010, 2013). These may provide improved estimates of postnatal exposure as they take into account information on exposure determinants such as time of breastfeeding, maternal age or pre-pregnancy weight. These models could also help researchers in identifying critical windows of susceptibility, a field poorly explored so far (Dietert et al. 2000; Verner et al. 2010, 2013).

Different PCB and dioxin congeners have been measured in different studies and the sums of congeners calculated are thus not always based on the same congeners. This may affect risk estimation since congeners may interact differently with elements of the immune system; for instance, some PCB congeners interact more strongly with the Ah receptor than others (Holladay and Smialowicz 2000). Also, correlations between different POP exposures, including different congeners, can be very high in some populations (Dallaire et al. 2004; Karmaus et al. 2001); in this sense, effects seen for one compound (i.e. PCB) could be explained by another highly correlated (i.e. DDE). Synergistic effects of mixtures of POPs, together with other pollutants like heavy metals, for instance, is another important issue that coming studies should

address (Carpenter et al. 1998); some in-vitro and animal models have shown that different POPs can enhance their immunotoxic effects when cells or animals are coexposed to them (Ferrante et al. 2011; Mori et al. 2008). Some of the reviewed studies applied multipollutant models to detangle the effects of multiple exposures. This approach may sometimes be helpful, as shown, for example, by Sunyer et al. (2010) who found that the association between prenatal DDE and LRTIs was not modified by exposure to other POPs (Sunyer et al. 2010). However, most of the epidemiological studies currently available generally have little power to study complex interactions between multiple exposures and multiple effects because of the small study populations included. On the other hand, more sophisticated statistical tools, such as the use of latent class analysis, to identify clusters of exposures (Sánchez et al. 2012), combined with the use of Structural Equation Models (SEM) (Budtz-Jorgensen et al. 2002; Davis 2012; Sanchez et al. 2005) and/or Bayesian Profile Regression techniques (Molitor et al. 2010), may help to study increasingly complex exposure-outcome associations (Davis 2012; Sanchez et al. 2005; Sohn et al. 2004). In fact, SEMs have already been applied in a couple of studies of the present review, with the aim to disentangle the effects of pre and postnatal exposure to PBCs or to determine the joint effects of different PFCs (Grandjean et al. 2012; Heilmann et al. 2006).

Finally, animal and in vitro models have shown potential immunotoxic capacity of PBDEs (Gill et al. 2004) and PFCs (Dewitt et al. 2012), relatively new POPs. However, as also shown

in the present review, there is still very scarce information on their effects on humans (Grandjean et al. 2012; Leijs et al. 2009; Okada et al. 2012; Wang et al. 2011). There is also concern on other non-persistent compounds, such as phthalates, for which studies seem to indicate that they increase the risk of allergy and asthma in children (Jurewicz and Hanke 2011; Winans et al. 2011), and bisphenol A (BPA), for which data on animal models suggests its capacity to modulate the developing immune system (Winans et al. 2011). Further epidemiological studies on such compounds suspected of being immunotoxic are also required.

4.2 Outcome assessment

Most studies assessed respiratory and allergy symptoms by standardized questionnaires and definitions (i.e. The ISAAC questionnaire (Beasley 1998)), thus resulting in fairly comparable outcome assessment for these outcomes. However, an important difference between the reviewed studies is age of the children at outcome assessment: this may be particularly important for respiratory infections and allergy manifestations since the behavior of these diseases differs by age (Nair et al. 2011; Rudan et al. 2004; Stein and Martinez 2004). In order to take this factor into account, in the present review we described results according to the age of the children among younger (<two years) and older ages (>two years). Conclusions were similar when considering these age groups separately; we therefore presented our summary table (Table 3) for all ages together.

Previous studies have reported early life respiratory infections, basically LRTIs, to be one of the major risk factors to develop asthma later in life (Bisgaard and Bonnelykke 2010; Busse et al. 2010). However, very few of the reviewed longitudinal studies have made continued assessments of respiratory and asthma symptoms at different ages (Karmaus et al. 2005; Sunyer et al. 2005, 2006; Weisglas-Kuperus et al. 2000). More studies should use continued assessments of children (from birth up to adolescence) in order to identify critical stages of the effects of POPs and to see if the effects seen at early ages (i.e. increased respiratory infections due to prenatal exposure to DDE) persist later in life. Additionally, recent studies focusing on other risk factors have tried to identify groups of individuals with similar patterns of related respiratory and allergic symptoms using latent class analysis techniques (Hepworth et al. 2010; Siroux et al. 2011), rather than analyzing each symptom as a separate outcome. Employing such methods in the field of immunotoxicants would facilitate our understanding on how such exposures may affect interrelated symptoms. Regarding immune response, a very large variety of cell subtypes, cell-markers and cytokine was investigated, covering many aspects of the innate and adaptive immune responses. There was little correspondence between studies in the indicators used making comparisons difficult. Also, an explanation of why specific markers were chosen instead of others, and of the exact meaning of increases or decreases in the biomarker levels, is often lacking for some of these biomarkers. In a novel and big European project, MeDALL (Mechanisms of the Development of ALLergy), current aims are to

generate novel knowledge on the mechanisms of initiation of allergy and to propose early diagnosis, prevention, and targets for therapy (Bousquet et al. 2011). Within this project harmonized questionnaires and clinical examinations are being developed. Also, new biomarkers, such as cytokines and inflammation markers, are being evaluated and implemented. Such tools and methods could serve as a basis for harmonizing outcome assessment in future studies.

4.3 Risk of bias

As seen in the supplementary tables B.1-B.4, in a great number of studies when no association was found between a compound and an outcome, results were not reported, and if so, only a brief comment was sometimes given stating "no association (data not shown)". This leads to reporting bias which makes impossible to evaluate with more exactitude the evidence of an association. Also, the possibility of publication bias cannot be discarded, since probably many studies with no significant results have not been published. This is certainly a limitation of the present systematic review. On the contrary, the possibility of recall and differential information bias is minor or inexistent in most of the studies, given their prospective nature (cohort studies, except a few cross-sectional studies) and because participants were blind to exposure levels to POPs.

4.4 Mechanisms

The biological mechanisms by which POPs may be immunotoxic are not fully understood; animal and in vitro studies have reported immunotoxicity of POPs and have found that these compounds reduce cell proliferation (Misumi et al. 2005; Mori et al. 2008; Rehana and Rao 1992; Sormo et al. 2009), increase inflammatory biomarkers, such as TNF-α and NO (Dewitt et al. 2012; Dutta et al. 2008; Ezendam et al. 2004, 2005a), and induce oxidative stress (Perez-Maldonado et al. 2005) which increases apoptosis and necrosis of leukocyte cells (Dutta et al. 2008; Misumi et al. 2005; Perez-Maldonado et al. 2006). However, results between animal studies can differ, in part due to differences in the species or strains used, leading to difficulties to interpret the results (Dewitt et al. 2012; Grandjean et al. 2010). In epidemiological studies cell counts can serve as a general indicator of the immune status, but cytokine assays can provide a more mechanistic examination of the effect of exposure (Duramad et al. 2007). In the present review only four studies assessed cytokine response, three of them in cord blood, where cytokine response has been shown to be very low (Holt and Jones 2000; Krampera et al. 2000). Some other studies assessed humoral cell response, but very few assessed specific antibody counts. Improvements in the state of the art can thus be made by larger studies that include mechanistic markers of specific antibody responses [i.e. T-cell-dependent IgM antibody responses (TDAR)], markers of specific cell-receptor expression [i.e. peroxisome proliferator-activated receptor (PPARα)] and markers of inflammation (i.e. TNFα and IL-6) and patterns of cytokine

alterations, including alterations in the Th_1/Th_2 cytokine profile, which if shifted toward one subset over another, the risk of immune dysfunction increases (Dewitt et al. 2012; Dietert and Piepenbrink 2006). Measurement of key components of the immune system, such as collectins, important in the innate immunity, or serum lysozyme, which is an indicator of antibacterial responses of the innate immune system, might be another interesting new field to explore (Dewitt et al. 2012; Dietert and Piepenbrink 2006). Additionally, animal and human studies have shown differences in immunotoxicity according to sex (Dewitt et al. 2012; Dietert and Piepenbrink 2006); thus, future studies should assess effects by performing stratified analyses according to sex.

4.5 Limitations of the classification

In the present review, the IARC classification for evidence of an association was used as a guide. However, such classifications can be subject to a degree of subjectivity. In the present review, in order to avoid subjectivity, two independent reviewers classified the evidence; in case of disagreement a third reviewer gave the decision. Only in the case of the evidence for prenatal dioxins and dl-PCBs exposure and respiratory infections, including AOM, the first two reviewers did not agree.

5. CONCLUSIONS AND RECOMMENDATIONS

The present review suggests that POP compounds can adversely influence immune and respiratory systems development. However, the scientific evidence for many of the exposure-outcome

associations evaluated in this review is inadequate, mainly due to the reduced number of studies and heterogeneity between studies in exposure and outcome assessment. Also, mechanisms remain largely unexplored. Recommendations for significantly improving our understanding of how POPs may affect the developing human respiratory and immune systems thus include:

Congeners, mixtures of chemicals and exposures of emerging concern: application of conversion factors of levels measured in different matrixes would facilitate the performance of meta-analysis including different birth cohort studies. PBPK models should also be applied when possible in order to explore critical windows of exposure. Potential immunotoxicity effects of the mixtures of chemicals and exposures of emerging concern should be assessed, as should the effects of co-exposures.

Related outcomes and persistent effects: studies should focus on long-term assessment of respiratory infections and asthma-related symptoms in order to identify critical stages and persistency of effects. Also, further understanding is needed on how exposures may affect interrelated respiratory and allergic symptoms by applying latent class analysis techniques. The methods and tools under development as part of MeDALL and other asthma and allergy projects could serve as a basis for outcome harmonization in future studies.

Mechanisms: Improved knowledge on the potential mechanisms of immunotoxic action of POPs is needed; this may be achieved by the integration of the measurement of specific immunoglobulin and cytokine response, as well as specific cell-

receptor expression and other important components of the immune system. Sex differences should also be considered.

Larger studies and meta-analyses: this review reveals that there are many small-sized studies with often inconsistent results. Larger studies are needed to improve statistical power and causal inference. Combining data from different ongoing birth cohorts may be a promising way to improve statistical power (Govarts et al. 2012; Vrijheid et al. 2012); for the study of respiratory and immune system outcomes this would require a substantial effort on both exposure and outcome harmonization.

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TABLES AND FIGURES

Table 1. Studies on persistent organic pollutants, respiratory and other infections and asthma symptoms and atopy (ordered by age group, year and author).

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[d]		Statistically significant main findings[e]
				Respiratory & other Infections[c]	Asthma symptoms and atopy	Timing	Compound	
<2 years								
Rogan (1987)	USA Cohort (I)	802	3, 6, 12, 18m & 5y	LRTIs & URTIs (NS), AOM, GIs	Asthma, reported eczema and allergy symptoms (only prevalences described)	Postnatal	ΣPCBs:	↓ prevalence of LRTIs, URTIs & AOM in higher categories of exposure
							DDE:	↓ prevalence of LRTIs, URTIs & AOM and of having allergy, ↑ prevalence of GIs in higher categories of exposure
Reichertová (1999)	Slovakia Cross-sectional	120	At birth (CB)		Total IgE	Prenatal	PCB118:	↑ total IgE levels
							DDE:	↑ total IgE levels
							HCB, other PCBs or POPs:	-
Dewailly (2000)	Canada Cohort (I)	65-83	3, 7 & 12m	AOM		Prenatal	Σ(ndl-)PCBs, Mirex:	-
							Dieldrin:	↑ AOM from 4 to 7m
							HCB, DDE[f]:	↑ AOM from 0 to 12m
Dallaire (2004)	Canada Cohort (II)	199	6 & 12m	Bronchitis, bronchiolitis, pneumonia, AOM, diverse URTIs[g], GIs & all infections together		Prenatal	PCB153[fh]:	↑ LRTIs at 6m & GIs at 12m
							DDE[f]:	↑ AOM, URTIs & all infections at 6m and URTIs & GIs at 12m
						Postnatal	ΣPCBs, PCB153[h], DDE	-

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[d]		Statistically significant main findings[e]
				Respiratory & other Infections[c]	Asthma symptoms and atopy	Timing	Compound	
Kaneko (2006)	Japan Cohort (II)	281	12m		Total & specific IgE to HDM, milk egg	Perinatal	PCDDs, PCDFs & dl-PCBs:	-
Glynn (2008)	Sweden Cohort (I)	190	3m	Open general questionnaire on infections		Prenatal	ΣPCBs:	↑ respiratory infections
							PCB153[f], ndl-PCBs, dl-PCBs:	↓ respiratory infections
							DDE:	-
						Postnatal	ndl-PCBs, dl-PCBs[f], DDE[f]:	↓ respiratory infections
							ΣPCBs, PCB153:	-
Sunyer (2010)	Spanish Cohort (II)	520	6 & 14m	Bronchitis, bronchiolitis, pneumonia		Prenatal	DDE:	↑ LRTIs at age 6,14m & recurrent LRTIs
							ΣPCBs, HCB, β-HCH:	-
Jusko (2011)	Slovakia Cohort (I)	350	6m		Total IgE	Prenatal	ΣPCBs:	-
						Postnatal	Σ(ndl-)PCBs:	-
Miyashita (2011)	Japan Cohort (IV)	364	18m	AOM	Asthma, reported allergic diseases & eczema	Prenatal	ΣPCDFs:	↑ AOM
							ΣPCDDs:	-
							dl-PCBs (non-*ortho*)[f]:	↑ AOM, food allergies
							dl-PCBs (mono-*ortho*)[f]:	↑ food allergies
							Total Dioxins:	-

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[d]		Statistically significant main findings[e]
				Respiratory & other Infections[c]	Asthma symptoms and atopy	Timing	Compound	
Stolevik (2011)	Norway Cohort (I)	195	12m	Pneumonia, URTIs (NS), AOM, GIs, urinary infections, chickenpox, exanthema subitum	Asthma, wheeze, reported eczema	Prenatal	ndl-ΣPCBs:	↑ URTIs, exanthema subitum & wheeze
							Dioxins & dl-ΣPCBs:	↑ URTIs, exanthema subitum & wheeze
Wang (2011)	Taiwan Cohort (II)	244	At birth (CB), 2y		Doctor diagnosed atopic dermatitis, total IgE	Prenatal	PFOA, PFOS:	↑ total IgE levels in CB
							PFNA, PFHxS:	-
Gascon (2012)	Spanish Cohort (II extended)	1342 (Spanish population)	12-14m	Bronchitis, bronchiolitis, pneumonia	Wheeze	Prenatal	DDE:	↑ LRTIs (& wheeze[i])
							ΣPCBs, HCB:	-
Okada (2012)[j]	Japan Cohort (IV)	231-343	18m	AOM	Whezee, food allergy, eczema, total IgE	Prenatal	PFOS:	-
							PFOA:	↓Total IgE
Tsuji (2012)[k]	Japan Case-control	30	23m		Specific IgE to egg, milk, wheat and HDM	Postnatal	ΣPCBs:	↑ Milk IgE
							ndl-PCB congeners:	not reported
							dl-PCB congeners:	not reported

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[d]		Statistically significant main findings[e]
				Respiratory & other Infections[c]	Asthma symptoms and atopy	Timing	Compound	
>2 years								
Yu (1998)	Taiwan Cohort (I)	31	8-16y	Influenza, AOM, GIs	Asthma, allergic rhinitis	Postnatal	Dioxins:	↑ influenza frequencies in Yucheng children vs controls in the previous 6m
Weisglas-Kuperus (2000)	The NL Cohort (I)	175	42m	Bronchitis, pneumonia, cough, chest congestion & phlegm, AOM, other diverse infections[l]	Asthma, SOB with wheeze, reported eczema & allergic reaction	Prenatal	ΣPCBs:	↓ SOB with wheeze (only maternal PCB levels)
							Dioxins:	↑ cough, chest congestion & phlegm
							dl-PCBs (planar & mono-*ortho*):	↑ AOM
						Postnatal	ΣPCBs:	↑ AOM and chicken pox, ↓ allergic reaction
Karmaus (2001)	Germany Cross-sectional (I)	343	7-10y	Pneumonia, whooping cough (pertussis), AOM	Asthma, total IgE	Postnatal	ΣPCBs, HCB, β-HCH:	-
							DDE:	↑ asthma, ↑ total IgE levels, ↓ AOM, ↓ whooping cough
							Combinations □	
							High levels DDE + ΣPCBs:	↑ AOM
							High levels DDE + HCB:	↑ AOM
Ten Tusscher (2001)	The NL Cohort (II+III)	29	7-12y	Bronchitis, pneumonia, coughing spells, chest congestion, AOM	Lung function, reported atopy	Prenatal	Dioxins:	↑ chest congestion, ↓ FEV_1/FVC (reduced lung function)
						Postnatal	Dioxins:	↓ FEV_1/FVC (reduced lung function)

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[d]		Statistically significant main findings[e]
				Respiratory & other Infections[c]	Asthma symptoms and atopy	Timing	Compound	
Van den Heuvel (2002)	Belgium Cross-sectional	200	17-18y		Asthma, bronchial wheezing, rhinitis, reported atopy, hay fever, total IgE & RAST to HDM, cat, grass pollen	Postnatal	Σ(ndl-)PCBs: dl-PCBs:	↑ reporting ever asthma, hay fever ↓ total IgE levels, positive RAST to HDM, cat & grass pollen, history of rhinitis
Ten Tusscher (2003)	The NL Cohort (III)	27	8y	Bronchitis, pneumonia, coughing spells, AOM, chickenpox, measles	Asthma, dyspnoea, reported eczema & allergy	Prenatal Postnatal	Dioxins & furans: Dioxins & furans:	↓ allergy ↓ allergy
Weisglas-Kuperus (2004)	The NL Cohort (I)	167	3-7y	AOM, chickenpox	SOB with wheeze, reported allergic reaction	Prenatal Postnatal	ΣPCBs: ΣPCBs: Dioxins, ndl-PCBs:	↓ chicken pox from 3 to 7 years of age, SOB with wheeze ↑ AOM -
Karmaus (2005)	Germany Cross-sectional (I)	331	7-10y		Total & specific IgE to aeroallergens	Postnatal	DDE ΣPCBs, HCB, γ-HCH:	↑ total IgE levels -
Sunyer (2005)	Spain Cohort (I)	306-405	4y	Defined as chest infection	Asthma, (persistent) wheeze, specific IgE to cat dander, HDM, grass pollen	Prenatal	DDE: ΣPCBs, HCB:	↑ (persistent) wheeze -

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[d]		Statistically significant main findings[e]
				Respiratory & other Infections[c]	Asthma symptoms and atopy	Timing	Compound	
Dallaire (2006)	Canada Cohort (III)	343	5-7y	Bronchitis, bronchiolitis, pneumonia, AOM, other diverse infections[m]		Prenatal	PCB153[h]:	↑ LRTIs and AOM.
							DDE:	Not presented but similar as for PCB-153
							Other OCs:	-
Sunyer (2006)	Spain Cohort (I)	402 (pre) 285 (post)	1-6.5y		Asthma (only at 6.5y), (persistent) wheeze & skin prick test (only at 6.5y)	Prenatal	DDE:	↑ wheeze at age 4 and asthma (& persistent wheeze at age 6.5y[n])
							DDT:	-
						Postnatal	DDE, DDT:	-
Grandjean (2010)	Faroe Islands Cohort (II)	464	7y		Asthma, reported atopic dermatitis, total & specific IgE to grass	Prenatal	Σ(ndl-)PCBs:	↓ atopic dermatitis
						Postnatal	Σ(ndl-)PCBs:	↑ total IgE levels (only 7y PCB levels), ↓ atopic dermatitis

LRTI=low respiratory tract infection (bronchitis, bronchiolitis, pneumonia); URTI=upper respiratory tract infection (tonsillitis, pharyngitis, laryngitis, sinusitis, acute otitis media (AOM), rhinitis, nasopharyngitis, epiglottitis, laryngotracheitis, tracheitis, common cold); GIs=gastrointestinal infections; NS=the type of LRTI or URTI was not specified; CB=measured in cord blood; SOB=shortness of breath; IgE=immunoglobulin E; RAST=radioallergosorbent; HDM=house dust mite.

[a]Different study populations coming from a same country are indicated with (I), (II), (III) or (IV).

[b]At the time of outcome assessment (m=months, y=years).

[c]Although AOM is an upper respiratory tract infection, it is usually analyzed separately in the studies.

[d]Regarding PCB and dioxin exposure, ΣPCBs can indicate either "unknown congeners" or a mix of ndl- and dl-PCBs (ndl-PCBs were predominant in the sum of most of the studies). If only dioxin-like or non dioxin-like PCBs are included in the sum, then it is indicated as dl-PCBs or ndl-PCBs, respectively. When the exposures assessed are PCDDs or PCDFs it is indicated as dioxins, unless specifically indicated.

[e](↑) increasing risk with increasing exposure level, (↓) decreasing risk with decreasing exposure level, (-) association was not statistically significant.

[f]Associations are not significant for the highest tertile/quartile, but are significant among other tertiles/quartiles of exposure, showing a non-linear association.
[g]Pharyngitis, tonsillitis, rhinitis, common cold.
[h]Due to the high correlations between compounds (r=0.94-0.99), PCB-153 was used in the models as a surrogate of the ΣPCBs exposure [Dallaire (2004)], and as a surrogate of the ΣPCBs and DDE exposure [Dallaire (2006)]. These two studies were based on two different populations in Canada.
[i]Wheeze was borderline significantly associated to DDE (RR=1.09, 95%CI=0.99, 1.21) in the Spanish population. Results for the Latin American population (N=79) went towards the same direction, but these were not significant for LRTIs (1.14 (0.92,1.42)) or wheeze (1.20 (0.98,1.48)).
[j]As the association between PFOA/S and IgE was not linear, cubic polynomial regression analyses were performed. After stratification by gender, associations were significant for females (quadratic=-1.43 (-2.42,-0.42), cubic=-3.08 (-5.43,-0.73)), but not for males.
[k]The study compares exposure levels between children with positive (≥0.35 UA IgE/ml) and negative (<0.35 UA IgE/ml) test for specific IgE.
[l]Scarlatina, chicken pox, rubella, measles, mumps, pertussis, hepatitis, meningitis or other infectious diseases.
[m]Pharyngitis, laryngitis, tonsillitis, sinusitis tracheitis, rhinitis, common cold.
[n]Persistent wheeze was bordeline signficantly associated to DDE (RR=1.13, 95%CI=0.98,1.30).

Table 2. Studies on persistent organic pollutants, immune cell-mediated response and immune humoral response (ordered by age group, year and author).

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[c]		Statistically significant main findings[d]
				Cell-mediated response (cell counts and interleukin levels)	Humoral response (general and specific Ab counts)	Timing	Compound	
<2 years								
Weisglas-Kuperus (1995)	The NL Cohort (I)	12-205	CB, 3 & 18m	WBC, T-cells, B-cells, NK cells, monocytes, granulocytes (NS)	Ab counts for mumps, measles, rubella (only at 18m)	Prenatal	ΣPCBs:	↑ T-cells at 18m ($CD3^+CD8^+$)
							Dioxins & dl-PCBs:	↑ T-cells in CB ($TcR\gamma\delta^+$), ↓ monocytes & granulocytes at 3m, ↑ T-cells at 18m ($CD3^+CD8^+$, $TcR\gamma\beta^+$)
						Postnatal	Dioxins & dl-PCBs:	↓ monocytes, granulocytes & B-cells at 3m ($CD19^+CD20^+$)
Nagayama (1998)	Japan Cohort (I)	36	12m	T-cells, B-cells		Postnatal	Dioxins & dl-PCBs:	↑ T-cells ratio ($CD4^+/CD8^+$ ratio)
Dewailly (2000)	Canada Cohort (I)	94-109	3, 7 & 12m	WBC, T-cells, B-cells	IgA, IgG, IgM	Prenatal	Σ(ndl-)PCBs, DDE, HCB, mirex, dieldrin:	-
Bilrha (2003)	Canada Cross-sectional	83	At birth (CB)	T-cells, *In vitro* cytokine production (IL-10 & TNF-α).		Prenatal	ΣPCBs:	↓ TNF-α
							DDE:	↓ TNF-α
							HCB:	↓ TNF-α
Heilmann[e] (2006)	Faroe Islands Cohort (II)	119	18m		Ab concentrations after tetanus and diphtheria vaccination	Prenatal	Σndl-PCBs:	↓ diphtheria Ab concentrations
							dl-PCB (mono-*ortho*):	↓ diphtheria Ab concentrations
						Postnatal	Σndl-PCBs:	↓ diphtheria (& tetanus[f]) Ab concentrations
							dl-PCB (mono-*ortho*):	↓ diphtheria Ab concentrations

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[c]		Statistically significant main findings[d]
				Cell-mediated response (cell counts and interleukin levels)	Humoral response (general and specific Ab counts)	Timing	Compound	
Kaneko (2006)	Japan Cohort (II)	281	12m	T-cells, B-cells, NK cells	IgA, IgG, IgM	Perinatal	PCDDs, PCDFs & dl-PCBs:	-
Noakes (2006)	Australia Cohort (I)	26	At birth (CB)	*In vitro* cytokine production (IL-13, IL-16, IL-10, INF-γ), lymphoproliferation response		Prenatal	DDE, HCB, dieldrin, or others:	-
						Perinatal	DDE, HCB, dieldrin:	-
Brooks (2007)	USA Cross-sectional	19	At birth (CB)	IL-13, IL-4, INF-γ		Prenatal	DDE:	↑ IL-13, IL-4/IFN-γ ratio, IL-13/INF-γ ratio
Nagayama (2007)	Japan Cohort (III)	92	10m	T-cells, B-cells, NK cells		Perinatal	HCH:	↑ T-cells ($HLA\text{-}DR^+$)
							HCE:	↑ T-cells ($CD8^+$)
							Chlordane:	↑ T-cells ($CD3^+$)
							Dioxins & dl-PCBs:	↑ T-cells ratio ($CD4^+/CD8^+$ ratio)
							ΣPCBs, DDE & DDT:	-
							Combinations □	
							Dioxins & dl-PCBs + ΣPCBs:	↓ T-cells ($CD16^+$)
							DDE & DDT + ΣPCBs:	↓ T-cells ($CD16^+$)
							HCE + Chlordane	↑ T-cells ($CD8^+$)

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[c]		Statistically significant main findings[d]
				Cell-mediated response (cell counts and interleukin levels)	Humoral response (general and specific Ab counts)	Timing	Compound	
Glynn (2008)	Sweden Cohort (I)	52 -86	3m	WBC, T-cells, B-cells, NK cells, monocytes, eosinophils, neutrophils		Prenatal	ΣPCBs:	↑number of WBC, total lymphocytes & monocytes
							DDE:	↓ % eosinophils
							PCB-153, ndl-PCBs, dl-PCBs:	-
						Postnatal	PCB-153:	↑ number & % NK cells ($CD56^{+}$)
							ΣPCBs, ndl-PCBs, dl-PCBs, DDE:	-
Jusko (2010)	Slovakia Cohort (I)	329	6m		Ab counts for influenza type b, tetanus, diphteria	Prenatal	ΣPCBs:	-
						Postnatal	Σ(ndl-)PCBs:	-
Horváthová (2011a)[g]	Slovakia Cohort (I)	313-362	CB, 6m, 16m	T-cells, B-cells, NK-cells		Prenatal	ΣPCBs:	↑ B-cells (CD19, HLADRCD19) at all ages, T-cell (CD3) at 16m, T-cell (CD8) in CB, ↓ NK-cells (CD3CD56/16)
Horváthová (2011b)[g]	Slovakia Cohort (I)	313-362	CB, 6m, 16m	T-cells recepector expression		Prenatal	ΣPCBs:	↓ CD4CD62, $CD4CD62^{+}CD45RA^{+}$, $CD19CD11c^{+}CD9b^{+}$ at all ages, $CD4CD45RO^{-}CD45RA^{+}$, CD4CD25, CD83CD19 at 6 and 16m and $CD19CD11c^{-}CD11b^{+}$ at 6m, ↑ $CD4CD62^{-}CD45RA^{+}$ at all ages and $CD4CD45RO^{+}CD45RA^{-}$ at 6 and 16m

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[c]		Statistically significant main findings[d]
				Cell-mediated response (cell counts and interleukin levels)	Humoral response (general and specific Ab counts)	Timing	Compound	
Jusko (2011)	Slovakia Cohort (I)	350	6m		IgA, IgG, IgM	Prenatal	ΣPCBs:	-
						Postnatal	Σ(ndl-)PCBs:	-
Tsuji (2012)[h]	Japan Case-control	30	23m	IL-8, COX-2		Postnatal	ΣPCBs:	↑COX-2
							ndl-PCB congeners:	↑IL-8, COX-2
							dl-PCB congeners:	-
>2 years								
Yu (1998)	Taiwan Cohort (I)	31	8-16y	T-cells, B-cells, NK cells, monocytes, eosinophils, basophils, neutrophils	IgA, IgG, IgM	Postnatal	Dioxins:	-
Weisglas-Kuperus (2000)	The NL Cohort (I)	85-150	42m	T-cells, B-cells, NK cells, monocytes, granulocytes (NS)	Ab counts for mumps, measles, rubella	Prenatal	ΣPCBs:	↑ T-cells ($CD3^+$, $CD3^+CD8^+$, $CD4^+CD45RO^+$, $CD3^+HLA\text{-}DR^+$)[i] & lymphocyte counts, ↓ mumps and rubella Ab counts.
							Dioxins, dl-PCBs (planar & mono-*ortho*):	-
						Postnatal	ΣPCBs:	-
Van Den Heuvel (2002)	Belgium Cross-sectional	200	17-18y	WBC, T-cells, B-cells, NK cells, eosinophils	IgA, IgG, IgM	Postnatal	Σ(ndl-)PCBs:	↓ IgG levels
							dl-PCBs:	↓ eosinophils & NK cells ($CD16^+CD56^+$), ↑ IgA levels

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[c]		Statistically significant main findings[d]
				Cell-mediated response (cell counts and interleukin levels)	Humoral response (general and specific Ab counts)	Timing	Compound	
Ten Tusscher (2003)	The NL Cohort (III)	27	8y	T-cells, B-cells, NK cells		Prenatal	Dioxins & furans:	-
						Postnatal	Dioxins & furans:	↑ T-cells ($CD4^+$, $CD45RA^+$)
Karmaus (2005)	Germany Cross-sectional (I)	331	7-10y	WBC, T-cells, B-cells, NK cells, eosinophils (granula content), basophils (IgE count)	IgA, IgG, IgM	Postnatal	ΣPCBs:	↓ WBC[j], IgM
							DDE:	↑ WBC[j], IgG, IgA & IgE count in basophils[j], ↓ eosinophilic granula[j]
							HCB:	↓ IgA, IgM
							γ-HCH:	Associated to NK-cells ($CD16^+CD56^+$, $CD16^+CD56^+CD57^+$), but unclear dose-response (use of quartiles)
Sunyer (2005)	Spain Cohort (I)	306	4y	WBC, T-cells, B-cells, NK cells, eosinophils		Prenatal	ΣPCBs, DDE, HCB:	-
Heilmann[e] (2006)	Faroe Islands Cohort (I)	129	7.5y		Ab concentrations after tetanus and diphtheria vaccination	Prenatal	Σndl-PCBs:	↓ tetanus Ab concentrations
							dl-PCBs (mono-ortho):	-
						Postnatal	Σndl-PCBs:	-
							dl-PCBs (mono-ortho):	-

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[c]		Statistically significant main findings[d]
				Cell-mediated response (cell counts and interleukin levels)	Humoral response (general and specific Ab counts)	Timing	Compound	
Leijs (2009)	The NL Cohort (IV)	18-33	14-19y	WBC, lymphocytes (NS), monocytes, eosinophils, basophils, (polymorphic) neutrophils		Prenatal	Dioxins:	↓ polymorphic neutrophils
						Postnatal	dl-ΣPCBs:	↓ polymorphic neutrophils
							PBDEs:	↓ lymphocytes
							Dioxins:	-
Heilmann (2010)	Faroe Islands Cohort (II)	106-587	5 & 7y		Ab concentrations for tetanus and diphteria	Prenatal	Σndl-PCBs:	-
						Postnatal[k]	Σndl-PCBs:	↓ diphtheria Ab concentrations at age 5y, ↓ diphtheria & tetanus Ab concentrations at age 7y, ↑ risk of an Ab concentration below a clinically protective level at age 5y
Heilmann (2010)	Faroe Islands Cohort (II)	106-587	5 & 7y		Ab concentrations for tetanus and diphteria	Prenatal	Σndl-PCBs:	-
						Postnatal[k]	Σndl-PCBs:	↓ diphtheria Ab concentrations at age 5y, ↓ diphtheria & tetanus Ab concentrations at age 7y, ↑ risk of an Ab concentration below a clinically protective level at age 5y

Author (Year)	Country & Study design[a]	N	Child age[b]	Outcomes assessed		Exposure assessment[c]		Statistically significant main findings[d]
				Cell-mediated response (cell counts and interleukin levels)	Humoral response (general and specific Ab counts)	Timing	Compound	
Grandjean (2012)	Faroe Islands Cohort (II)	380-510	5 & 7y		Ab concentrations for tetanus and diphteria	Prenatal	PFOS:	↓ diphtheria prebooster Ab at 5y
							PFOA:	↓ diphtheria at 7y
							PFDA:	↓ diphtheria pre and postbooster at 5y
							PFHxS, PFNA:	-
						Postnatal	PFOS, PFOA[l]:	↓ diphtheria at 7y
							PFNA:	↓ diphtheria prebooster Ab at 5y
							PFHxS[l], PFNA:	-

WBC=White blood cell counts (total leukocytes), which include lymphocytes (T-cells, B-cells and Natural Killer (NK) cells), monocytes (derive into macrophages and dendritic cells) and granulocytes (basophils, eosinophils and neutrophils); IL=interleukin; TNF-α=tumor necrosis factor-α; INF-γ=interferon-γ; NS=subtypes of leukocytes are not specified in the text of the paper; IgA=immunoglobulin A; IgG=immunoglobulin G; IgM=immunoglobulin M, IgG=immunoglobulin G; Ab=antibody; COX-2=cyclooxygenase-2.

[a]Different study populations coming from a same country are indicated with (I), (II), (III) or (IV).

[b]At the time of outcome assessment (m=months, y=years).

[c]Regarding PCB and dioxin exposure, ΣPCBs can indicate either "unknown congeners" or a mix of ndl- and dl-PCBs (ndl-PCBs were predominant in the sum of most of the studies). If only dioxin-like or non dioxin-like PCBs are included in the sum, then it is indicated as dl-PCBs or ndl-PCBs, respectively. When the exposures assessed are PCDDs or PCDFs it is indicated as dioxins, unless specifically indicated.

[d](↑) increasing risk with increasing exposure level, (↓) decreasing risk with decreasing exposure level, (-) association was not statistically significant.

[e]This is a same article including two different birth cohorts of different ages each.

[f]Significant associations only when performing structural equation models.

[g]Exposure is not introduced in the models as continous PCB levels measured in maternal serum but as a dichotomous variable (highly exposed children (polluted area of Michalovce) vs less exposed children (area of Svidnik/Stropkvov)). In the original papers results are also shown for blood measurements at age 6m, but in the present tables these are not shown (results were similar to those obtained in both CB and at age 16m).

[h]The estimations for those congeners for which no significant results were found (all dl-PCBs and some ndl-PCBs) are not shown in the manuscript. In the present table and the supplementary tables we only show the results for the sum of PCBs, but not the results for the different congeners for which significant associations were found; they are available in the original manuscript.

[i]Only significant among non breastfeeding children and for PCBs measured in maternal and cord blood.
[j]In the highest quartile of PCBs exposure the WBC are significantly reduced compared to the lowest quartile, but the reduction is not linear across quartiles. There is a similar situation with the increase of WBC or IgE BC within the 4th and the 3rd quartiles of DDE exposure, respectively. Although the F-test is not significant, eosinophilic granula levels within the highest quartile were significantly lower than in the lowest quartile of DDE exposure (T-test). For γ-HCH, there are significant differences between quartiles of exposure, however, in some quartiles NK cells levels are increased and in others these are decreased.
[k]In all cases, the associations found between Ab counts and postnatal exposure measured at age 18 months was only significant after imputation of 88.5% of the 18m serum samples (based on maternal blood and breastmilk, serum at age 5 and 7y levels).
[l]The association between postnatal PFOA and PFHxS and tetanus and diphtheria Ab counts at age 7 years were signficiant when models were adjusted for Ab counts at age 5y.

Table 3. Summary of the evidence[a].

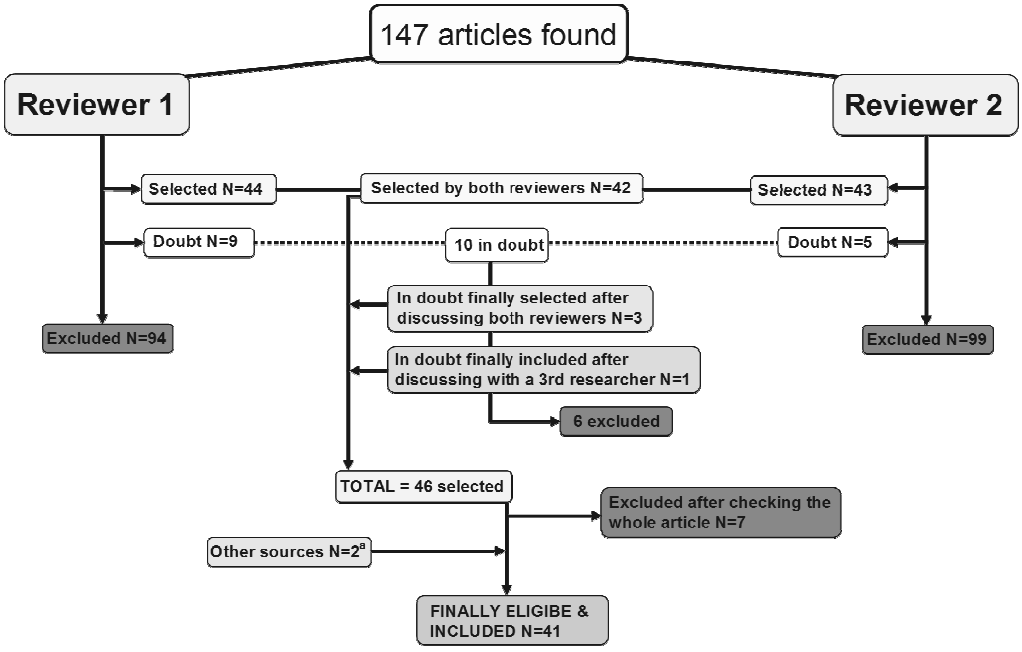
	PCBs and ndl-PCBs		Dioxins and dl-PCBs		DDE/DDT		HCB		Other POPs[c]	
	Prenatal[b]	**Postnatal**	**Prenatal**	**Postnatal**	**Prenatal**	**Postnatal**	**Prenatal**	**Postnatal**	**Prenatal**	**Postnatal**
Infections										
Acute Otitis Media (AOM)	**limited**	inadequate	**limited**	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate
Respiratory tract infections	**limited**	inadequate	**limited**	inadequate	**limited**	inadequate	inadequate	inadequate	inadequate	inadequate
Other infections	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate
Allergic manifestations										
Asthma symptoms	inadequate	**Evidence of lack of association**	inadequate	inadequate	**limited**	inadequate	inadequate	inadequate	inadequate	inadequate
Atopy	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate
Immune humoral response										
General antibody concentrations	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate
Specific antibody counts after vaccination	inadequate	**limited**	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate
Immune cell-mediated response & cell counts										
Cell-mediated response	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate
Cell counts	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate	inadequate
Other outcomes										
Thymus size	inadequate	inadequate	-	-	inadequate	-	-	-	-	-

[a]*Sufficient* - if most of the studies, including good quality studies, report an association, but evidence is not yet conclusive enough to conclude that there is an association or a causal relationship, *limited* - several good quality, independent, studies report an association, but evidence is not yet conclusive enough, *inadequate* - if associations are reported in one or more studies, but insufficient quality, insufficient number of studies, lack of consistency, and/or lack of statistical power preclude a conclusion regarding the presence or absence of an association, *evidence for lack of association* - several good quality studies are consistent in showing no association. [b]Prenatal exposure can also include perinatal. [c]HCHs, mirex, dieldrin, chlordane, PBDEs, PFCs.

SUPPLEMENTAL MATERIAL

**Note: due to the large tables of the supplemental material, only Figure A has been included in this document. The rest of the tables can be found at http://www.sciencedirect.com/science/article/pii/S0160412012002425.*

Figure A. Articles selection process.



[a]After checking references there were 5 possible new articles found by reviewer 1, but after discussing with reviewer 2, the articles were not finally included. One study was also found through MEDLINE but not through the standard search, and the other one was found through the second search engine, SCOPUS.

5.3 Paper III

Prenatal exposure to DDE and PCB153 and respiratory health in early childhood: A meta-analysis

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Abstract

Background: Persistent organic pollutants may affect the immune and respiratory systems, but available evidence is based on small study populations. We studied the association between prenatal exposure to dichlorodiphenyldichloroethylene (DDE) and polychlorinated biphenyl 153 (PCB153) and children's respiratory health in European birth cohorts.

Methods: We included 4608 mothers and children enrolled in ten birth-cohort studies from seven European countries. Outcomes were parent-reported bronchitis and wheeze in the first four years of life. For each cohort, we performed Poisson regression analyses, modeling occurrences of the outcomes on estimates of cord serum concentrations of PCB153 and DDE as continuous variables (per doubling exposure) and as cohort-specific tertiles. Summary estimates were obtained through random- effects meta-analyses.

Results: Risk of bronchitis or wheeze (combined variable) assessed before 18 months of age increased with increasing DDE exposure (relative risk [RR] per doubling exposure=1.03 [95% confidence interval = 1.00 - 1.07]). When these outcomes were analyzed separately, associations appeared stronger for bronchitis. We also found an association between increasing PCB153 exposure and bronchitis in this period (RR per doubling exposure=1.06 [1.01 - 1.12]), but not between PCB153 and wheeze. No associations were found between either DDE or PCB153 and ever-wheeze assessed after 18 months. Inclusion of both compounds in the models attenuated risk estimates for PCB153 tertiles of exposure, whereas DDE associations were more robust.

Conclusions: This large meta-analysis suggests that prenatal DDE exposure may be associated with respiratory health symptoms in young children (below 18 months), whereas prenatal PCB153 levels were not associated with such symptoms.

1. INTRODUCTION

Persistent organic pollutants (POPs), which include dichloro diphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE) and polychlorinated biphenyls (PCBs), are synthetic compounds distributed worldwide and present for long periods of time in the environment and human tissues, being food the main source of exposure. Immunologic effects of these compounds have been reported in studies conducted in animals (Dutta et al. 2008; Ezendam et al. 2004; Lyche et al. 2004; Misumi et al. 2005), adult humans (Daniel et al. 2002; Karmaus et al. 2005; Noakes et al. 2006; Vine et al. 2001) and children (Gascon et al. 2013a). Although in most countries the use of these compounds was banned in the 70's, some of them are still in use. For instance, DDT, the parent compound of DDE, is still applied in many developing countries for the control of the malaria vector mosquito (van den Berg 2009) or for agricultural purposes (Polder et al. 2010). Because the immune and respiratory systems develop and mature during pre and postnatal early life, these periods may be particularly susceptible to the effects of POPs (Dietert et al. 2000; Pinkerton and Joad 2000), which could lead to the disruption of these systems and to a reduced capacity to fight infections and an increased risk to develop allergic manifestations later in life (Bisgaard and Bonnelykke 2010; Busse et al. 2010; Dietert et al. 2000; Puig et al. 2008). A recent systematic review on POPs and respiratory and immune health in children concluded that current epidemiological evidence suggests that early-life exposure to POPs, mainly DDE, PCBs and dioxins, can adversely influence the development of the

immune and respiratory systems (Gascon et al. 2013a). However, the current number of studies is scarce and most of them include small study populations.

In Europe, a number of prospective, population-based birth cohort studies participating in the ENRIECO (Gehring et al. 2013) and CHICOS (Larsen et al. 2013) projects, have data available on exposure to POPs during pregnancy and respiratory health and symptoms in children. However, published studies so far are restricted to Spanish birth cohorts (Gascon et al. 2012; Sunyer et al. 2005, 2006, 2010). The ENRIECO project made major efforts to harmonize prenatal POPs exposure between the cohorts in order to facilitate pooled and meta-analyses; these were used in recent meta (Govarts et al. 2012) and pooled (M Casas, unpublished data, 2013) analysis of PCB153, DDE and birth weight. The present study, the largest so far, uses harmonized PCB153 and DDE exposure data from ten European birth cohort studies to evaluate associations between these exposures and the occurrence of bronchitis and wheeze in young children.

2. METHODS

2.1 Description of the cohorts

Among the European birth cohorts included in the ENRIECO database (www.birthcohortsenrieco.net), we identified nine population-based cohorts that had measured biomarkers of DDE and PCB153 during pregnancy, at birth, or right after birth and had collected information on respiratory health of children in the first

four years of life. Two invited cohorts declined participation. Seven cohorts participated, of which one (INMA) consisted of four subcohorts in different regions that were counted as separate cohorts in the present analyses. . Thus, ten cohorts in seven countries were included: DUISBURG (Germany), FLEHS I (Belgium), HUMIS (Norway), PCB COHORT (Slovakia), RHEA (Greece), PÉLAGIE (France), and INMA-Menorca, INMA-Sabadell, INMA-Gipuzkoa and INMA-Valencia (Spain) (Table 1). Each cohort included births from 1997 to 2008. The HUMIS cohort was restricted to mothers who breastfed their child for at least one month. In total, 4608 live-newborns with information on DDE and PCB153 exposure and at least one respiratory health outcome as defined below, were included. All studies were conducted with the approval of the corresponding ethics committees in the participating countries and written informed consent was obtained from the parents of all children.

2.2 Exposure assessment

Concentrations of DDE and PCB153 were analyzed in maternal serum/whole blood collected during pregnancy, cord serum/plasma, or breast milk (Table 1). As described in the previous ENRIECO meta-analysis (Govarts et al. 2012), PCB153 was used as a bio-marker of PCB exposure because concentrations of this PCB congener are relatively high and highly correlated with the total molar concentration of PCBs (Hagmar et al. 2006). The elimination half-life of PCB-153 is >10 years (Ritter et al. 2011) and that of DDE is approximately 5 years (Ferreira et al. 2011). To facilitate

comparisons of results from cohorts using different matrices for exposure assessment, we expressed all contaminant levels as wet weight cord serum levels (ng/L), which directly reflects fetal exposure at the time of delivery (some compounds do not cross the placenta efficiently). For populations that did not have cord blood analyses, we estimated concentrations in cord serum from the concentrations measured in maternal serum/whole blood or breast milk using cohort specific conversion factors detailed in Table 1, and based on previous ENRIECO (Govarts et al. 2012) and CHICOS (M Casas, unpublished data, 2013) work.

2.3 Outcomes

Participating cohorts collected information on low respiratory tract infections and symptoms between the ages of 6 months and 4 years: bronchitis, bronchiolitis, pneumonia, chest infection, and wheezing. This information was collected in questionnaires answered by the mothers administered either as postal questionnaires (FLEHS I, HUMIS and PÉLAGIE), through telephone interview (RHEA) or through face-to-face interview (the rest of the cohorts). After reviewing the outcome information available in each cohort, we decided to focus on those outcomes for which questions referred clearly to the same symptom/disease across cohorts and that were available in at least four cohorts. These outcomes were wheeze and bronchitis. We were not able to use information on bronchiolitis, pneumonia, chest infection, asthma or shortness of breath because questions differed too much between cohorts or not enough cohorts had the information. Most, but not all cohorts, asked for doctor

diagnosed diseases/symptoms or based their questionnaires on previous standardized questionnaires such the ISAAC one (Asher et al. 1995). Detailed information on the exact questions and the mode of data collection within each cohort is available in the supplemental material (eTable 1). Because the behavior of respiratory infections and allergy symptoms changes with age (Bisgaard and Bonnelykke 2010; Busse et al. 2010) we decided to assess occurrence of the different outcomes according to the age of the children at the time of assessment. We analyzed occurrence of ever bronchitis or wheeze assessed before 18 months of age (<18 months), defined as younger ages, and occurrence of ever wheeze assessed above this age until 49 months of life (>18 months), defined as older ages. This 18 months cut-off point is based on the average age of assessment in a cohort is; the range of ages of individuals in a cohort can exceed the cut-off point (Table 2). It is known that at early ages the presence of low respiratory tract infections is highly correlated to the presence of wheeze (Everard 2012). We tested the correlation between bronchitis and wheeze at younger ages applying tetrachoric correlations; as expected, these outcomes were highly correlated (coefficient of 0.75 for all cohorts together – see Table 2 for cohort-specific correlations). In order to minimize misclassification, in those cohorts with information on both bronchitis and wheeze, a new outcome variable combining both outcomes ("bronchitis and/or wheeze") was thus created. In those cohorts where several follow-up visits were performed (eTable 1), a single variable was created for each outcome in order to define occurrence of a symptom/disease at younger and older

ages: if the symptom occurred at least once, then the child was classified as having the symptom during this period; if the child had no symptoms reported at any of the follow-up points, then the child was classified as not having the symptom; if the child had no symptoms reported at one follow-up point and missing information at the others, then the child was classified as having missing information for that particular symptom.

2.4 Other variables

Covariate data was obtained from medical record information for children and their mothers, antenatal health care visits and questionnaires with questions on maternal lifestyle, diet, use of tobacco and alcohol during pregnancy and the first years of life of the child, residence history, health, country of origin, education, occupation and child daycare attendance. In order to harmonize the information for these co-variables between cohorts, we sent a questionnaire to participating cohorts asking which variables were available and if they could be provided in specified categories (i.e. maternal education: primary school, secondary school and university degree or higher – eTable 2).

2.5 Statistical methods

Some covariates of interest for our analysis had missing information (between 0.1% and 48%, depending on the variable and the cohort – eTable 2). These missing values and values of DDE and PCB153 below the limit of detection (LOD), were imputed by multiple imputation (Spratt et al. 2010), a method based on conditioning the

missing variables density to given predictor variables (detailed information on the imputation process and the predictor variables is available in eTable 3). The reason to apply multiple imputation procedures was that 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-cases analysis (Sterne et al. 2009). Imputations were done separately by cohort and outcome.

We introduced DDE and PCB153 concentrations in the models as continuous variables, and as categorical variables based on cohort-specific tertile cut-offs. For the models where exposure was introduced as continuous variables, DDE and PCB153 concentrations were base 2 log-transformed in order to obtain a normal distribution of the exposure as the original distributions were skewed. This means that the relative risk is expressed as the percentage increase for each doubling of the exposure (i.e. a RR of 1.03 means an increase of a 3% of the risk per doubling of exposure). The Spearman correlation coefficient between non-log transformed DDE and PCB153 levels was calculated within each cohort.

Poisson regression models were applied to obtain cohort-specific estimates of the relative risk (RR) for each outcome of interest. Adjusted models for all cohorts included covariates selected according to previous literature (see eTable 2 for more information): gender, age of the child at the time of outcome

assessment (months), duration of breastfeeding (months), gestational age (weeks), number of siblings of the child at the time of birth (none, one, two or more), maternal age (16-25 years, >25-30 years, >30-35 years and >35 years), maternal body mass index (BMI: ≤18.5, >18.5-25, >25-30, >30), maternal smoking during pregnancy (yes/no) and during postnatal life of the child (yes/no), maternal education (primary school, secondary school, university or higher degree), maternal allergy or asthma (yes/no) and time of sample collection for POPs analyses (pregnancy 1st, 2nd or 3rd trimester or after birth). Birth weight was not included in the final model because of its correlation with gestational age (r=0.49).

Because descriptive tables showed important heterogeneity between cohorts regarding exposure, outcome prevalence, and most of the co-variables, we decided to perform meta-analyses (random effects), and not pooled analyses of individual data, to calculate summary risk estimates. To test for heterogeneity among cohorts, we used the Cochran's Q test. The meta-analyses for the outcome variable bronchitis and/or wheeze included those cohorts with information on both outcomes as well as those cohorts with information on only one single outcome. RHEA and INMA-Menorca had information only on wheeze and the PCB cohort had information only on bronchitis. We produced forest plots to show relative risks from each of the individual cohorts included in the meta-analyses and the estimation of the summary RR. The sizes of the square markers of each RR in the plot represent the relative weight each study contributed to the summary estimation.

As daycare attendance and country of origin of the mother were not available in Duisburg and FLEHS I, respectively, these variables were only included in a sensitivity analysis of the cohorts that had this information available. The influence of each pollutant on the outcome associations for the other was examined by including both compounds in the models together. We also performed sensitivity analyses excluding each cohort one by one from the meta-analyses, and stratifying by matrix (cord blood, maternal serum or breast milk), in order to explore the influence of using different matrices. Finally, within each cohort we tested interaction between exposure and gender, months of breastfeeding (<6 months and ≥6 months), maternal history of allergy or asthma, and pre and postnatal maternal smoking. We also reported results for complete-case data. All analyses were performed with STATA version 12.

3. RESULTS

3.1 Characteristics of cohorts

RHEA (N=996) and the PCB COHORT (N=720) were the biggest cohorts. INMA-Menorca was the oldest cohort (enrollment years between 1997 and 1999), whereas RHEA was the most recent (2007-2008). Exposure to DDE and PCB153 during pregnancy differed between cohorts, with cord serum geometric mean concentrations respectively ranging from 52.4 ng/L in HUMIS to 1067.7 ng/L in INMA-Menorca and from 39.2 ng/L in HUMIS to 280.8 ng/L in the PCB COHORT (Table 3). Correlations between DDE and PCB153 also varied substantially between cohorts, from 0.37 in INMA-Gipuzkoa to 0.69 in HUMIS (Table 3). Different

socio-demographic variables were associated with DDE and PCB153 exposure levels in each cohort, with older maternal age and higher maternal education showing the most consistent associations with increasing DDE and PCB153 levels across the cohorts (eTable 4 and 5).

At younger ages, children in RHEA and INMA-Menorca were, on average, the youngest at the time of assessment (9.3 months) and children in the PCB COHORT the oldest (16 months) (Table 2). Eight cohorts had information on wheeze and in these, 27% of 3675 children had at least one occurrence of wheeze. Bronchitis, available in seven cohorts, occurred in 25% of 2990 children, and the combination of bronchitis and wheeze, available in nine cohorts, occurred in 33% of the 4394 children included. Occurrence of all outcomes differed between cohorts (Table 2). At older ages, six cohorts had information on wheeze (N=1754) assessed at ages ranging from 18 months up to 49 months of life. In this period, 36% of children reported ever wheezing since birth, varying from 17% in HUMIS to 52% in INMA-Sabadell (Table 2). There were differences between cohorts for all other characteristics except for gender (eTable 2).

3.2 DDE, PCB153 and respiratory health associations

Below 18 months, increasing prenatal DDE exposure was associated with a 3% increased risk of bronchitis and/or wheeze (RR per doubling of DDE exposure [95%CI]=1.03 [1.00, 1.07]) (Table 4 and eFigure 1). The risk was also increased with increasing

categories of exposure (medium vs lowest: 1.07 [0.97, 1.18] and highest vs lowest: 1.14 [1.03, 1.26]) (Table 4 and Figure 1). When considering both outcomes separately, the risk of bronchitis and wheeze was of 1.05 [1.00, 1.11] and 1.02 [0.96, 1.07], respectively. An increased risk was also observed for increasing categories of exposure (highest vs lowest: 1.23 [0.98, 1.55] for bronchitis and 1.16 [1.01, 1.32] for wheeze) (Table 4 and eFigures 2 to 5). No associations were observed between DDE exposure and wheeze assessed at older ages (Table 4, eFigures 6 and 7). In general, results for DDE exposure were robust to the exclusion of one cohort at the time (eTable 6). However, when comparing categories of exposure, the risk estimates and the confidence intervals for the association between DDE and bronchitis and DDE and wheeze somewhat changed (RR ranging from 1.15 to 1.32 and 1.10 to 1.22, respectively) with the exclusion of some of the cohorts (eTable 6). Stratification by matrix of exposure measurement did not show meaningful differences between the matrices (eTable 8).

PCB153 was not associated with bronchitis and/or wheeze or with wheeze alone assessed below 18 months, and heterogeneity between cohorts was observed (Table 4, Figure 2, eFigure 8, 11 and 12). However, the risk of bronchitis increased with PCB153 exposure as a continuous variable (RR per doubling PCB153 exposure [95%CI]=1.06 [1.01, 1.12]) and when comparing the highest with the lowest exposure categories [1.17 [0.97, 1.41] (Table 4 and eFigures 9 and 10). These RRs moved closer to one with the exclusion of the PCB COHORT and the INMA-Sabadell

cohort (eTable 7). The risk of wheeze assessed above 18 months of age increased with increasing PCB153 exposure (RR per doubling PCB153 exposure [95%]=1.06 [0.98, 1.15]), also when comparing the highest vs the lowest tertile (1.12 [0.95, 1.32]) (Table 4 and eFigures 13 and 14). For tertiles of exposure, the exclusion of INMA-Sabadell cohort from the main analysis reduced the RR [95%] to 1.05 [0.83, 1.33] (eTable 7). Risk estimates for PCB153 showed differences between matrices with increases observed only in those cohorts that measured PCB153 in cord blood (RR for the highest vs the lowest tertile=1.20 [1.03, 1.39]) (eTable 8). This increase was largely due to the PCB COHORT (RR excluding PCB COHORT=1.05 [0.84, 1.31]).

The inclusion of PCB153 and DDE in the models did not substantially modify the associations found between DDE and the outcomes assessed (eTable 9). For instance, the RR [95%CI] for the association between continuous DDE exposure and bronchitis and/or wheeze changed from 1.03 [1.00, 1.07] to 1.03 [0.99, 1.07], and for the highest vs the lowest tertiles of DDE exposure from 1.14 [1.03, 1.26] to 1.15 [1.00, 1.32] (eTable 9). However, risk estimates for PCB153 tertiles of exposure substantially changed and previous increases in risk attenuated. For instance, the increased risk found between tertiles of PCB153 exposure and bronchitis (1.17 [0.97, 1.41]) was no longer observed (0.97 [0.71, 1.34]) (eTable 9). Inclusion or exclusion of daycare attendance or country of origin of the mother as potential confounders did not influence results (data not shown). There was no evidence for interaction between DDE or

PCB153 exposure and gender, breastfeeding duration, maternal asthma or allergy, or pre or postnatal maternal smoking ($p>0.05$).

In non-imputed datasets between 10% and 20% of the children were excluded, most of them from the PCB COHORT, which had a high percentage of missing values for maternal allergy or asthma (28%), maternal smoking during postnatal life of the child (48%) and number of siblings (48%). When complete-case data was used, some risk estimates with DDE exposure attenuated, whereas those with PCB153 changed substantially, in some cases even reversing direction (eTable 10).

4. DISCUSSION

Results from the present study, the largest so far in children, suggest that current prenatal DDE levels may be associated to low respiratory tract infections and related symptoms in the early years of life, particularly below 18 months of age. Results for DDE were relatively robust to the exclusion of one cohort at the time and to the inclusion of PCB153 in the models. The association observed between prenatal PCB153 and bronchitis was mainly influenced by the PCB COHORT and, to a lesser extent, by the INMA-Sabadell cohort. In addition, it was not always robust to the inclusion of DDE in the model. PCB153 was not associated with wheeze or the combination of wheeze and bronchitis.

Only five previous studies published results in relation to prenatal DDE exposure and occurrence of respiratory infections/symptoms

in children in their early-life. These studies, two based on the INMA cohorts, also find DDE exposure to be associated with increased risk of wheezing and respiratory infections (Dallaire et al. 2004; Dewailly et al. 2000; Gascon et al. 2012; Sunyer et al. 2010), except one (Glynn et al. 2008). In the present study, results were similar for exposure expressed as continuous variable and as categories and were robust to the exclusion of one cohort at the time and to the inclusion of PCB153 in the models. This reinforces evidence for an association between DDE and respiratory infections and related symptoms. However, we did not find an association between DDE and wheeze above 18 months. This may be explained by the fact that after the first years of life, wheeze starts to become an indicator of asthma symptoms more than of respiratory infections (Bisgaard and Bonnelykke 2010; Busse et al. 2010). Additionally, analyses above 18 months included a smaller number of children than those below 18 months (only 1754 out of 4608 children). Continued follow-up of the cohorts will be needed to assess the effects of DDE on immune and respiratory outcomes later in childhood. The mechanisms behind the potential immune effects of DDE are not clear: it is known that DDE interferes with hormonal receptors and mimics estrogen and antiandrogen activity, which might modulate immunologic responses (Rogan and Ragan 2007). Also, some studies suggest depression of T helper cell Type 1 after cell stimulation, which is responsible for response to viral infections (Sunyer et al. 2010). For this reason, in order to improve knowledge on mechanisms, future epidemiological studies on children could consider analyzing biomarkers, including general

parameters of the immune system, humoral and cellular immunity, and other potential indicators such as thymus size or cell-surface antigens and cytokines (Tryphonas 2001).

Regarding PCB153, present in much lower levels than DDE, we did not find associations between prenatal exposure and respiratory infections/symptoms. Only a previous study assessed the effects of PCB153 at these early ages, and in this case an increased risk of low respiratory tract and acute otitis media infections was observed. Although immunological effects of PCBs have been described in several studies (Gascon et al. 2013a; Tryphonas 2001), results are inconsistent (Gascon et al. 2013a). The reasons behind these inconsistencies, also seen in the present study, might be due to the congeners assessed; although we used PCB153 as a marker of general PCBs exposure, it could be that the mixtures of PCBs, including hydroxylated congeners (OH-PCBs), are different in each study region, resulting in exposure misclassification. These mixtures can include different amounts of coplanar and non-coplanar congeners as well as dioxin compounds, each of which follows different mechanisms of action or even opposite hormonal activity (Fielden et al. 1997). Therefore, the null associations found in the present study may indicate that current levels of PCB153 are in fact not affecting the immune and the respiratory health of children. However, we cannot extrapolate these results to other PCB congeners.

Although the present study is the largest so far and includes a wide representation of different European populations, there are several limitations. From all the respiratory diseases and related symptoms for which information was collected within each cohort, we could only assess bronchitis and wheeze at younger ages and wheeze at older ages. At younger ages, this forced us to use bronchitis as a marker of low respiratory tract infections and not a wider definition of such infections. The different low respiratory tract infections (bronchitis, bronchiolitis and pneumonia) are sometimes difficult to differentiate or can be misdiagnosed and thus, the possibility that in some study regions the diagnosis of bronchitis was over or under reported in place of other low respiratory tract infections cannot be discarded. In order to reduce potential misclassification of the outcome occurrence and to increase the number of children in the analyses, we combined occurrence of wheeze and bronchitis in a single variable. Despite significant differences among cohorts in the occurrence of bronchitis, wheeze and the combination of both, results obtained for DDE were consistent for all three outcome variables. Another limiting aspect of the present study is that there were important differences on outcome occurrence among cohorts, which are probably due to underlying risk factors we could not control for, as well as country-specific medical practices. This leads to the necessity of harmonizing questionnaires and protocols for the collection of data among studies, as well as the use of more standardized and objective tools at younger and older ages (Bousquet et al. 2011). A strength of the present study is that despite important differences between cohorts for some of the co-

variables (i.e. occurrence of maternal allergy/asthma), we were able to define and harmonize common potential confounders. Although we included information on all important variables thought to potentially act as confounders, residual confounding by unmeasured factors (e.g. breastfeeding of the mothers) cannot be excluded as explanation of the small increases in risk observed. On the other hand, the relationships between co-variables and both outcomes and exposures were as expected, which gives us confidence on the validity of our data. For example, the risk of bronchitis and/or wheeze was consistently increased in male children, in children with one or more siblings and in children whose mothers had a history of asthma and allergy. Further, increasing levels of DDE and PCB153 were higher among older and higher educated mothers, and these are well described determinants of exposure) (Vrijheid et al. 2010). We did not observe evidence for effect modifications by gender, breastfeeding, maternal history of allergy or asthma, or smoking. The performance of a pooled analysis would increase the statistical power to better assess potential interactions as well as provide better estimations of the dose-response relationships, but this would require more standardized outcome data.

A previous systematic review concluded that one of the limitations to performing a meta-analysis of published results was the heterogeneity in exposure assessment between studies (Gascon et al. 2013a). In this sense, in the present study, harmonization of exposure using conversion factors is certainly an improvement. We were not able to calculate specific conversion factors for the

different trimesters of pregnancy (Govarts et al. 2012). However, in those cohorts with maternal samples at different time points (Duisburg, the three INMA cohorts and RHEA) we did not observe differences in DDE and PCB153 levels between trimester of sampling, except for PCB153 in the RHEA and INMA-Valencia cohorts (data not shown). This is in accordance to a previous paper on the variability of PCB levels along pregnancy in which levels (expressed on a fresh weight, and not lipid adjusted) do not seem to significantly vary across pregnancy periods (Glynn et al. 2011). From the data available, it is difficult to know if these differences are due to matrix or country-specific factors, since differences in exposure levels across Europe could also be explained by differences in the intensity of use and production of these compounds, and in diet, the main exposure source nowadays (Vrijheid et al. 2010). In any case, we performed meta-analyses adjusting for sampling time within cohorts, making this issue less problematic than in pooled analyses. Further, results of analyses comparing cohort-specific tertiles of exposure were consistent in direction with those using continuous exposure variables, making it less likely that results were influenced by differences in sampling matrices and time points. On the other hand, previous studies on the topic have reported nonmonotic functions across categories of POPs exposure (Dallaire et al. 2004, 2006; Gascon et al. 2012; Glynn et al. 2008), indicating that the association might not be linear. In the present, results showed that the risk of bronchitis or wheeze increased with increasing categories of DDE exposure, whereas results for PCB153 showed, in some

cases, nonmonotic functions. Models including both DDE and PCB153 at the same time showed that results for DDE were more robust. However, collinearity between DDE and PCB153 exposure occurred in all cohorts (STATA command vif, uncentered, VIF>10), so results of these models should be interpreted cautiously.

5. CONCLUSIONS

This meta-analysis, studying the largest number of children thus far, suggests that prenatal DDE exposure may be associated with respiratory health symptoms in young children (below 18 months), whereas current PCB153 levels were not associated with such symptoms. Continued follow-up, harmonization of outcome assessments (respiratory infections, asthma symptoms and markers of allergy) and mechanistic studies are priorities for future studies.

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TABLES AND FIGURES

Table 1. Characteristics of included cohorts.

Cohort	Country	Enrolment year	N	Matrix for DDE and PCB153 measurement (units)	Time of collection of biological sample	Conversion factors[a]	
						DDE	PCB153
DUISBURG	Germany	2000-2002	204	Maternal whole blood (ng/L)	2nd, 3rd trimester of pregnancy or at birth	0.40	0.40
FLEHS I	Belgium	2002-2004	133	Cord plasma (ng/L)	At birth	-	-
HUMIS	Norway	2002-2006	386	Breast milk (ng/g lipid)	At birth	1.20	1.20
PCB COHORT	Slovakia	2002-2004	720	Cord serum (ng/L)	At birth		
RHEA	Greece	2007-2008	996	Maternal serum (ng/L)	1st, 2nd, 3rd trimester of pregnancy or at birth	0.303	0.405
INMA-Menorca	Spain	1997-1999	395	Cord serum (ng/L)	At birth	-	-
INMA-Gipuzkoa	Spain	2006-2008	540	Cord serum (ng/L) (54.6%[b])	At birth	-	-
				Maternal serum (ng/L) (45.4%[b])	1st or 2nd trimester of pregnancy	0.303	0.405
INMA-Sabadell	Spain	2004-2006	543	Maternal serum (ng/L)	1st, 2nd or 3rd trimester of pregnancy	0.303	0.405
INMA-Valencia	Spain	2004-2005	505	Cord serum (ng/L) (90.7%[b])	At birth	-	-
				Maternal serum (ng/L) (9.3%[b])	1st or 2nd trimester of pregnancy	0.303	0.405
PÉLAGIE	France	2002-2006	186	Cord serum (ng/L)	At birth	-	-

[a]Conversion factors from maternal serum/whole blood and breast milk to cord serum concentrations (M Casas, unpublished data, 2013 and Govarts *et al.* 2012).

[b]Percentage of total samples measured in different matrices

Table 2. Child age and outcome occurrence at the time of assessment by cohort.

	Assessment <18 months[a]					Assessment >18 months[a]	
	Child age (months) at assessment	**Ever bronchitis and/or wheeze**	**Ever bronchitis**	**Ever wheeze**	**Correlation between bronchitis and wheeze**	**Child age (months) at assessment**	**Ever wheeze**
	Mean (min-max)	**N assessed (% occurrence)**	**N assessed (% occurrence)**	**N assessed (% occurrence)**	**N (r^b)**	**Mean (min-max)**	**N assessed (% occurrence)**
DUISBURG	12.0 (12-12)	204 (29)	204 (20)	202 (22)	202 (0.78)	24.0 (24-24)	194 (30)
FLEHS I	12.7 (12-19)	131 (50)	130 (38)	130 (32)	127 (0.54)	24.7 (23-31)	130 (42)
HUMIS	12.3 (10-17)	381 (10)	381 (8)	381 (10)	381 (1.00)	24.8 (18-31)	355 (17)
PCB COHORT	16.0 (16-16)	720 (49)	720 (49)	NA	NA	NA	NA
RHEA	9.3 (6-21)	996 (26)	NA	996 (26)	NA	NA	NA
INMA-Menorca	9.3 (5-21)	395 (21)	NA	395 (21)	NA	25.0 (23-40)	394 (40)
INMA-Gipuzkoa	14.5 (12-18)	540 (37)	540 (11)	540 (36)	540 (0.75)	NA	NA
INMA-Sabadell	14.6 (10-18)	532 (46)	523 (33)	526 (39)	514 (0.81)	35.7 (30-49)	495 (52)
INMA-Valencia	12.3 (11-20)	495 (29)	492 (9)	505 (25)	492 (0.54)	NA	NA
PÉLAGIE	NA	NA	NA	NA	202 (0.78)	26.8 (23-40)	186 (18)

NA (not available)

[a]The 18 months cut-off is not based on individual subjects but on cohorts: the average age of assessment in a cohort is categorized along this cut-off, but the range of ages can exceed it.

[b]Tetrachoric correlations.

Table 3. Estimated[a] cord serum concentrations of DDE and PCB153 and correlation between the compounds.

	DDE (ng/L)			PCB153 (ng/L)			Correlation between PCB153 and DDE concentrations[b]
	GM	25^{th}-75^{th} percentile	% <LOD	GM	25^{th}-75^{th} percentile	% <LOD	
DUISBURG	201.1	(130-295)	0	112.9	(83-180)	0	0.43
FLEHS I	285.0	(161- 450)	0	78.5	(50-132)	3.9	0.60
HUMIS	52.4	(33-78)	0	39.2	(30-51)	0	0.69
PCB COHORT	934.2	(561-1708)	0.6	280.8	(170-451)	0	0.58
RHEA	641.5	(362-1130)	0	48.6	(33-73)	0	0.46
INMA-Menorca	1067.7	(587-1986)	0	188.2	(137-269)	0	0.49
INMA-Gipuzkoa	208.0	(129-332)	4.1	113.9	(86-168)	12.0	0.37
INMA-Sabadell	229.0	(129-349)	0.2	74.9	(57-117)	8.0	0.41
INMA-Valencia	503.5	(291-785.3)	0	104.1	(79-162)	7.3	0.40
PÉLAGIE	165.8	(110-310)	0	116.6	(85-170)	0	0.55

DDE (dichlorodiphenyldichloroethylene); **PCB153** (polychlorinated biphenyl 153); **GM** (Geometric mean); **LOD** (Limit of detection)

[a]These are estimated cord serum concentrations after applying, when appropriate, the respective conversion factors detailed in Table 1.

[b]Spearman correlations of non-$\log_2$ transformed concentrations.

Table 4. Summary relative risk estimates (RR per doubling exposure) for prenatal DDE and PCB exposure and children's respiratory health outcomes assessed before and after 18 months of age.

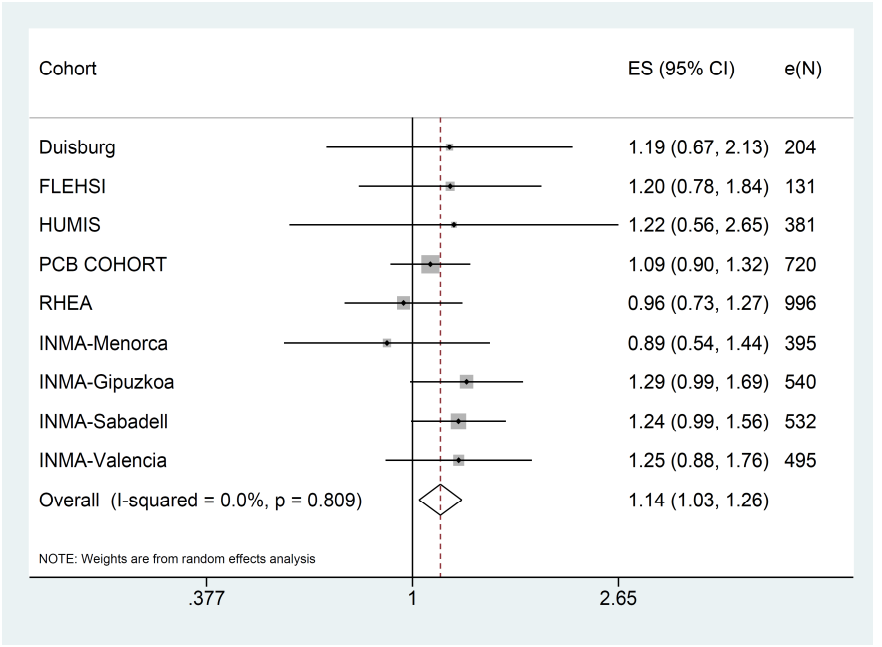
			DDE		PCB153	
	Nc	**Ns**	**RR (95% CI)[a]**	***p*-het**	**RR (95% CI)[a]**	***p*-het**
Assessment <18 months[b]						
Ever bronchitis and/or wheeze	9	4394				
Continuous (per $\log_2$ ng/L)			1.03 (1.00, 1.07)	0.43	1.02 (0.96, 1.08)	0.22
Medium vs lowest tertile			1.07 (0.97, 1.18)	0.66	1.04 (0.92, 1.18)	0.19
Highest vs lowest tertile			1.14 (1.03, 1.26)	0.81	0.95 (0.75, 1.21)	<0.001
Ever bronchitis	7	2990				
Continuous (per $\log_2$ ng/L)			1.05 (1.00, 1.11)	0.38	1.06 (1.01, 1.12)	0.89
Medium vs lowest tertile			1.19 (0.90, 1.56)	0.03	1.13 (0.98, 1.31)	0.90
Highest vs lowest tertile			1.23 (0.98, 1.55)	0.12	1.17 (0.97, 1.41)	0.33
Ever wheeze	8	3675				
Continuous (per $\log_2$ ng/L)			1.02 (0.96, 1.07)	0.16	1.01 (0.94, 1.09)	0.25
Medium vs lowest tertile			1.07 (0.94, 1.22)	0.47	1.06 (0.89, 1.25)	0.15
Highest vs lowest tertile			1.16 (1.01, 1.32)	0.43	0.92 (0.68, 1.25)	0.001
Assessment >18 months[b]						
Ever wheeze	6	1754				
Continuous (per $\log_2$ ng/L)			1.03 (0.98, 1.08)	0.46	1.06 (0.98, 1.15)	0.66
Medium vs lowest tertile			0.93 (0.75, 1.15)	0.18	1.02 (0.87, 1.19)	0.76
Highest vs lowest tertile			1.04 (0.90, 1.20)	0.71	1.12 (0.95, 1.32)	0.82

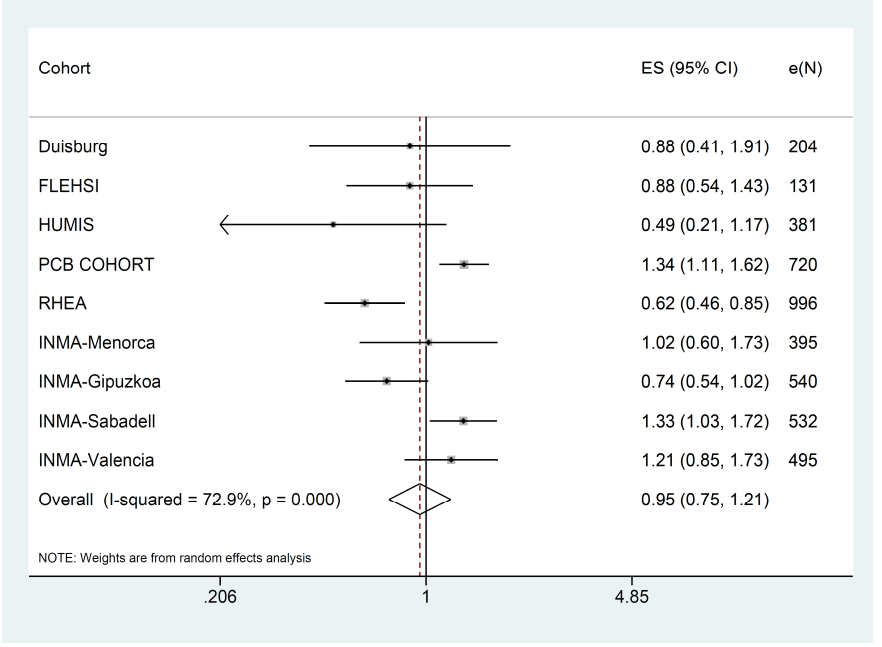
Nc (number of cohorts); Ns (number of subjects); RR (relative risk); CI (confidence interval); *p*-het (*p*-value of Cochran's Q test for heterogeneity).

[a]All models were adjusted for gender, age of the child at the time of outcome assessment, duration of breastfeeding, gestational age, number of siblings of the child at the time of birth, maternal age, maternal body mass index, maternal smoking during pregnancy and during postnatal life of the child, maternal education, maternal allergy or asthma and time of sample collection for POPs analyses.

[b]The 18 months cut-off is not based on individual subjects but on cohorts: the average age of assessment in a cohort is categorized along this cut-off, but the range of ages can exceed it.

Figures 1 and 2. Forest plots showing risk estimates for individual studies and the combined meta-analysis results for the highest versus the lowest DDE (Fig 1) and PCB153 (Fig 2) exposure and the occurrence of bronchitis and/or wheeze.





SUPPLEMENTAL MATERIAL

eTable 1. Outcome definition within each cohort.

Cohort	Age of follow-up	Questionnaire	Bronchitis	Wheeze
DUISBURG	12 months	Trained interviewer (face-to-face)	Has a doctor diagnosed one of the following diseases in your child during the first year of life? - Asthmatic, spastic or obstructive bronchitis (y/n)	Did your child have wheezing or whistling noises in its chest during the first year of life? (y/n)
	24 months		NA	Did your child have wheezing or whistling noises in its chest during the first year of life? (y/n)
FLEHS I	6 months	Self-reported	Did your child ever had bronchitis? (y/n). If yes, was this together with wheezing? (y/n)[a]	Did your child ever wheeze? (y/n). If yes, how often did this occur? (once, several times/ almost constantly)
	12 months		Did your child have in the last 6 months bronchitis? (y/n). If yes, was this together with wheezing? (y/n)[a]	Did your child wheeze in the last 6 months? (y/n). If yes, how often did this occur? (once, several times/ almost constantly)
	18 months		Idem to 12 months	Idem to 12 months
	24 months		NA	Idem to 12 months
HUMIS	6 months	Self-reported	Has your child had any of the following diseases (bronchitis)? (y/n), (confirmed by a doctor diagnose (y/n))	Has your child had any of the following diseases (bronchitis and RS virus)? (y/n), (confirmed by a doctor diagnose (y/n))[b]
	12 months		Has your child had any of the following diseases (bronchitis) during the last half year? (y/n), (confirmed by a doctor diagnose (y/n))	Has your child had any of the following diseases (bronchitis and RS virus) during the last half year? (y/n), (confirmed by a doctor diagnose (y/n))[b]
	24 months		NA	Has your child had any of the following diseases (bronchitis and RS virus) during the last year? (y/n), (confirmed by a doctor diagnose (y/n))[b]
PCB COHORT	6 months	Trained interviewer (face-to-face)	How many of these respiratory infections (*previous question on respiratory infections in the past 6 months*) were bronchitis? 0,1,2,3 or more	NA

Cohort	Age of follow-up	Questionnaire	Bronchitis	Wheeze
	16 months		How many of these respiratory infections (*previous question on respiratory infections since last visit)* were bronchitis? 0,1,2,3 or more	NA
RHEA	9 months	Trained interviewer (via phone call)	NA	Has your child ever had dyspnea – breast whistling? (*since birth*) (y/n)
INMA-Menorca	12 months	Trained interviewer (face-to-face)	NA	Since birth, which of the following describe better your child? 1) Never wheezes, even with infections, 2) Sometimes wheezes, 3) Wheezes most of the time or great part of it. Indicate number of episodes.
	24 months		NA	Idem 12 months but symptoms in the last year asked
INMA-Gipuzkoa, Sabadell and Valencia[a]	14 months	Trained interviewer (face-to-face)	Since birth, has the doctor told you that your child has had a chest infection? Please specify each possible respiratory infection at each months of presentation (bronchitis at each month of life)	Since birth, which of the following describe better your child? 1) Never wheezes, even with infections, 2) Sometimes wheezes, 3) Wheezes most of the time or great part of it. Indicate number of episodes.
	30 months (only Sabadell)		NA	Idem 14 months (symptoms in the last year asked)
PÉLAGIE	24 months	Self-reported	NA	Has your child had whistling in the chest at any time since birth? How many attacks of wheezing/chest whistling did your child have?

(y/n)=answer yes/no, NA=not available or not assessed in the present study.
[a]Last question was not taken into account to define occurrence of bronchitis. [b]In Norwegian there is not an exact word for wheeze, so a combination of low respiratory tract infections associated to wheeze was used to obtain information on wheezing.

eTable 2. Descriptive table with information on child and maternal characteristics by study population.

Cohort (Total N)	**DUISBURG (N=204)**	**FLEHS I (N=133)**	**HUMIS (N=386)**	**PCB COHORT (N=720)**	**RHEA (N=996)**	**INMA – Mnc (N=395)**	**INMA – Gip (N=540)**	**INMA – Sab (N=543)**	**INMA – Val (N=505)**	**PÉLAGIE (N=186)**
Child characteristics										
Gender										
(% males)	50	55	52	51	51	51	51	53	54	53
% missing	0	0	0	0.1	0	0	0.2	0.2	0	0
Gestational age										
(mean, min-max,	39.3	39.4	39.8	39.7	38.2	39.4	39.7	39.7	39.6	39.4
weeks)	(33-43)	(36-41)	(31-44)	(33-43)	(28-43)	(30-42)	(33-43)	(32-43)	(25-43)	(32-42)
% missing	0	0	0.3	1	0.3	0	0.4	0.4	0	0
Breastfeeding										
months	6.1	3.9	10.4	6.9	3.3	4.1	6.6	6.2	5.6	3.4
(mean, min-max)	(0-12)	(0-13)	(1-16)	(0-16)	(0-18)	(0-12)	(0-16)	(0-18)	(0-18)	(0-26)
% missing	0	30	0	0.3	0.2	0	1	1	1	4
Daycare attendance										
(%)	NA	66	23	68	3	32	47	31	23	78
% missing		1	1	48	0.1	2	1	2	1	8
Siblings (%)										
None	53	44	45	36	43	49	57	57	54	32
One	36	38	3	38	38	38	38	37	40	41
Two or more	11	17	17	26	19	13	5	5	6	27
% missing	0	5	0	48	0	0	0	0.4	1	0
Maternal characteristics										
Age (mean, min-	31.1	30.6	29.2	26.2	29.7	29.1	31.4	30.4	30.2	30.4
max, years)	(19-41)	(20-41)	(16-42)	(18-45)	(16-46)	(17-41)	(19-43)	(17-40)	(16-43)	(19-44)
16-25 years (%)	11	10	21	45	20	15	4	12	15	11
>25-30 years (%)	25	27	43	33	37	46	41	39	37	37
>30-35 years (%)	46	52	28	17	31	31	43	36	37	38
>35 years (%)	18	11	9	5	12	8	13	13	11	14
% missing	0	0	0	1	1	1	0	0.2	0	0

Cohort (Total N)	DUISBURG (N=204)	FLEHS I (N=133)	HUMIS (N=386)	PCB COHORT (N=720)	RHEA (N=996)	INMA – Mnc (N=395)	INMA – Gip (N=540)	INMA – Sab (N=543)	INMA – Val (N=505)	PÉLAGIE (N=186)
Education (%)										
Primary school	20	0	14	20	19	59	14	26	33	17
Secondary school	39	78	21	73	49	27	37	43	42	16
University or higher degree	41	22	65	7	32	14	50	31	25	68
% missing	0	3	2	1	2	3	0.2	1	0	0
BMI (mean, min-max)	24 (15-51)	23 (17-36)	24 (16-44)	22 (14-41)	24 (15-48)	23 (16-42)	23 (15-41)	24 (16-54)	24 (15-48)	22 (17-38)
≤18.5 (%)	3	6	4	13	4	5	4	5	3	5
>18.5-25 (%)	67	73	62	66	61	75	75	66	66	78
>25-30 (%)	20	15	22	16	22	17	16	20	21	12
>30 (%)	11	6	12	5	12	4	5	8	9	4
% missing	0	1	2	5	2	4	0	0	0	0
Allergy or asthma (%)	38	32	34	2	27	43	25	33	25	1
% missing	12	2	0	28	4	0.3	0	0.2	0.4	3
Smoke during pregnancy (%)	23	11	13	14	21	37	13	14	22	11
% missing	0	1	0	0	2	0	3	2	0	2
Smoke during the postnatal life of the child (%)	26	17	13	35	29	30	20	26	31	19
% missing	30	3	0.3	48	0.4	0.3	1	2	1	1
European (%)	88	NA	98	79	100	98	98	92	91	99
% missing	0		6	3	1	1	0	2	0	1

Mnc (Menorca); **Gip** (Gipuzkoa); **Sab** (Sabadell); **Val** (Valencia); **BMI** (body mass index).

eTable 3. Description of the imputation procedure.

Software used and key setting: STATA 12 software (Stata Corporation, College Station, Texas) –*ice* command (with 20 cycles)
Number of imputed datasets created: 20 **Variables included in the imputation procedure:** Child's variables: child age, gestational age, birth weight, gender, number of siblings, duration of breastfeeding, presence of pets at home. Mother's variables: age, weight, height, maternal allergy or asthma, smoking during pregnancy and during the first two years of life of the child, education level, levels of DDE and PCB153, moment of blood sampling for persistent organic pollutants analyses. Father's variables: paternal allergy or asthma. *Daycare attendance and country of origin of the mother were not available for Duisburg and FLEHS I, respectively. As imputations were done separately by study population and outcome, for these two study populations these variables were respectively not included. **Treatment of non-normally distributed variables:** $\log_2$-transformed. **Treatment of binary/categorical variables:** logistic, ordinal, and multinomial models. **Statistical interactions included in imputation models:** imputations were done separately by study population and outcome. **The aim of using multiple imputation for DDE and PCB153 values <LOD was to obtain a more normal distribution of these values, which is a more correct approach than performing a simple imputation with the half of the LOD, for instance (see Chen H, Quandt SA, Grzywacz JG, Arcury TA. A distribution-based multiple imputation method for handling bivariate pesticide data with values below the limit of detection. Environmental health perspectives. 2011;119(3):351–6).

eTable 4. Geometric mean prenatal DDE (ng/L) levels by cohort and categories for some of the covariables of adjustment.

Cohort (Total N)	**DUISBURG (N=204)**	**FLEHS I (N=133)**	**HUMIS (N=386)**	**PCB COHORT (N=720)**	**RHEA (N=996)**	**INMA – Mnc (N=395)**	**INMA – Gip (N=540)**	**INMA – Sab (N=543)**	**INMA – Val (N=505)**	**PÉLAGIE (N=186)**
Child characteristics										
Siblings										
None	205	263	55*	1154	585*	1048	215	215*	473	138
One	203	254	54	1206	718	1057	197	243	541	185
Two or more	190	354	43	1042	632	1175	203	305	544	173
Maternal characteristics										
Age										
16-25 years	208*	135*	43*	729*	402*	672	175*	185*	410*	120*
>25-30 years	152	274	52	1030	560	1058	188	198	452	141
>30-35 years	219	306	57	1254	780	1260	221	238	528	183
>35 years	242	385	75	1648	1269	1477	242	404	825	248
Education										
Primary school	199	NA	45	682*	570*	1038	249	229	489	188
Secondary school	191	284	48	1003	607	1056	215	230	487	153
University or higher degree	216	290	56	1069	729	1227	193	232	553	164
BMI										
≤18.5	182	282	38	682*	497	581*	148	239	338*	147
>18.5-25	195	292	53	913	642	1032	202	222	479	164
>25-30	227	269	53	1171	672	1335	246	261	562	192
>30	212	218	60	1273	592	1644	232	217	646	158
Allergy or asthma										
No	206	295	54	958	627	1067	204	233	495	163
Yes	203	247	51	593	673	1068	218	226	526	424
Smoke during pregnancy										
No	206	292	53	955	634	1095	209	234	511	171
Yes	192	194	49	803	645	1022	205	219	478	125
European										
No	330*	NA	30*	605*	338	1053	1135*	778*	936*	Only 1 case
Yes	189	NA	51	1027	642	1067	200	208	473	165

BMI: body mass index. *p-value<0.05 (ANOVA using $\log_2$-transformed exposures).

eTable 5. Geometric mean prenatal PCB153 (ng/L) levels by cohort and categories for some of the covariables of adjustment.

Cohort (Total N)	DUISBURG (N=204)	FLEHSI (N=133)	HUMIS (N=386)	PCB COHORT (N=720)	RHEA (N=996)	INMA – Mnc (N=395)	INMA – Gip (N=540)	INMA – Sab (N=543)	INMA – Val (N=505)	PÉLAGIE (N=186)
Child characteristics										
Siblings										
None	117*	73	40	331	47	190	109	69*	106*	103*
One	121	65	40	340	51	191	112	80	108	129
Two or more	78	88	37	326	48	175	124	91	72	116
Maternal characteristics										
Age										
16-25 years	45*	40*	32*	237*	28*	140*	47*	39*	54*	75*
>25-30 years	85	62	38	295	44	183	90	65	90	97
>30-35 years	140	77	44	364	62	215	130	89	136	134
>35 years	171	148	53	400	91	230	167	117	146	184
Education										
Primary school	79*	NA	39	273	36*	182	91*	68*	96	104*
Secondary school	111	72	36	284	48	191	108	70	102	100
University or higher degree	137	82	40	264	59	217	119	87	112	124
BMI										
≤18.5	83	98*	35*	280	44*	190	113	77	96	129
>18.5-25	123	87	41	274	52	189	113	78	103	119
>25-30	94	57	38	279	46	193	106	69	110	105
>30	98	34	34	345	41	189	90	59	85	93
Allergy or asthma										
No	110	85*	41*	285	47	192	109	73	101	118*
Yes	130	53	37	359	51	184	118	76	110	42
Smoke during pregnancy										
No	119*	72	39	274*	48	183	111	73	100	117
Yes	94	77	40	328	51	198	114	78	111	113
European										
No	50*	NA	55	228*	38	97*	27*	18*	35*	Only 1 case
Yes	127	NA	39	289	49	189	114	83	114	116

BMI: body mass index. *p-value<0.05 (ANOVA using log_2-transformed exposures).

eTable 6. Sensitivity analyses for the association between DDE and respiratory symptoms/diseases when excluding cohorts one by one.

	Continuous (log_2-transformed)[a]				Highest vs lowest (tertiles)			
	RR	95%CI		p- het	RR	95%CI		p- het
Assessment <18 months[b]								
Ever bronchitis and/or wheeze								
All cohorts included	1.03	1.00	1.07	0.43	1.14	1.03	1.26	0.81
Excluding Duisburg	1.03	1.00	1.07	0.35	1.14	1.03	1.26	0.72
Excluding FLEHS I	1.03	1.00	1.07	0.33	1.14	1.03	1.26	0.73
Excluding HUMIS	1.03	1.00	1.07	0.33	1.14	1.03	1.26	0.72
Excluding PCB COHORT	1.03	0.99	1.07	0.33	1.16	1.03	1.31	0.76
Excluding RHEA	1.05	1.01	1.08	0.69	1.17	1.05	1.31	0.91
Excluding INMA-Menorca	1.04	1.01	1.07	0.63	1.15	1.04	1.28	0.84
Excluding INMA-Gipuzkoa	1.03	0.99	1.07	0.38	1.12	1.00	1.25	0.83
Excluding INMA-Sabadell	1.02	0.99	1.06	0.55	1.12	1.00	1.25	0.80
Excluding INMA-Valencia	1.03	0.99	1.07	0.33	1.13	1.02	1.26	0.75
Ever bronchitis								
All cohorts included	1.05	1.00	1.10	0.38	1.23	0.98	1.55	0.12
Excluding Duisburg	1.04	1.00	1.09	0.80	1.15	0.96	1.38	0.31
Excluding FLEHS I	1.06	1.01	1.11	0.39	1.29	0.99	1.68	0.09
Excluding HUMIS	1.06	1.00	1.12	0.327	1.24	0.96	1.60	0.08
Excluding PCB cohort	1.07	0.99	1.16	0.31	1.32	0.96	1.81	0.12
Excluding INMA-Gipuzkoa	1.05	0.99	1.10	0.37	1.15	0.94	1.41	0.25
Excluding INMA-Sabadell	1.06	0.98	1.14	0.28	1.25	0.91	1.71	0.08
Excluding INMA-Valencia	1.06	1.00	1.12	0.28	1.29	1.01	1.63	0.13
Ever wheeze								
All cohorts included	1.02	0.96	1.07	0.16	1.16	1.01	1.32	0.43
Excluding Duisburg	1.02	0.97	1.08	0.16	1.18	1.03	1.35	0.56
Excluding FLEHS I	1.02	0.95	1.08	0.11	1.14	0.99	1.33	0.33
Excluding HUMIS	1.02	0.96	1.08	0.11	1.15	0.99	1.33	0.32
Excluding RHEA	1.04	0.98	1.10	0.28	1.22	1.05	1.42	0.57
Excluding INMA-Menorca	1.03	0.98	1.09	0.26	1.18	1.03	1.35	0.43
Excluding INMA-Gipuzkoa	1.01	0.94	1.08	0.14	1.12	0.96	1.30	0.40
Excluding INMA-Sabadell	1.00	0.95	1.05	0.50	1.10	0.95	1.28	0.48
Excluding INMA-Valencia	1.02	0.95	1.09	0.11	1.13	0.97	1.32	0.35
Assessment >18 months[b]								
Ever wheeze								
All cohorts included	1.03	0.98	1.08	0.46	1.04	0.90	1.20	0.71
Excluding Duisburg	1.03	0.98	1.09	0.46	1.06	0.91	1.24	0.76
Excluding FLEHS I	1.01	0.96	1.07	0.84	1.01	0.87	1.18	0.84
Excluding PÉLAGIE	1.03	0.97	1.09	0.34	1.05	0.90	1.22	0.66
Excluding HUMIS	1.03	0.98	1.09	0.39	1.03	0.89	1.20	0.58
Excluding INMA-Menorca	1.03	0.96	1.11	0.33	1.04	0.88	1.23	0.57
Excluding INMA-Sabadell	1.03	0.95	1.11	0.32	1.04	0.84	1.29	0.59

[a]RR per doubling exposure. [b]The 18 months cut-off is not based on individual subjects but on cohorts: the average age of assessment in a cohort is categorized along this cut-off, but the range of ages can exceed it.

eTable 7. Sensitivity analyses for the association between PCB153 and respiratory symptoms/diseases when excluding cohorts one by one.

	Continuous (log_2-transformed)[a]				Highest vs lowest (tertiles)			
	RR	95%CI		p- het	RR	95%CI		p- het
Assessment <18 months[b]								
Ever bronchitis and/or wheeze								
All cohorts included	1.02	0.96	1.08	0.22	0.95	0.75	1.21	<0.001
Excluding Duisburg	1.02	0.96	1.08	0.15	0.96	0.74	1.23	<0.001
Excluding FLEHS I	1.02	0.96	1.09	0.16	0.96	0.74	1.24	<0.001
Excluding HUMIS	1.02	0.96	1.08	0.16	0.99	0.78	1.26	<0.001
Excluding PCB COHORT	1.00	0.94	1.05	0.42	0.90	0.70	1.15	0.05
Excluding RHEA	1.04	1.00	1.09	0.44	1.04	0.84	1.28	0.02
Excluding INMA-Menorca	1.02	0.96	1.08	0.15	0.94	0.73	1.22	<0.001
Excluding INMA-Gipuzkoa	1.05	1.00	1.10	0.46	0.99	0.77	1.27	0.001
Excluding INMA-Sabadell	1.01	0.94	1.08	0.17	0.90	0.69	1.17	0.001
Excluding INMA-Valencia	1.01	0.95	1.07	0.20	0.92	0.70	1.20	<0.001
Ever bronchitis								
All cohorts included	1.06	1.01	1.12	0.89	1.17	0.97	1.41	0.33
Excluding Duisburg	1.06	1.01	1.12	0.80	1.13	0.90	1.41	0.23
Excluding FLEHS I	1.07	1.01	1.13	0.86	1.12	0.88	1.42	0.23
Excluding HUMIS	1.06	1.01	1.12	0.82	1.24	1.07	1.45	0.59
Excluding PCB cohort	1.02	0.93	1.12	0.93	1.04	0.82	1.33	0.50
Excluding INMA-Gipuzkoa	1.07	1.01	1.13	0.97	1.23	1.04	1.45	0.39
Excluding INMA-Sabadell	1.06	1.00	1.13	0.80	1.07	0.81	1.41	0.23
Excluding INMA-Valencia	1.06	1.00	1.12	0.80	1.20	0.99	1.45	0.33
Ever wheeze								
All cohorts included	1.01	0.94	1.09	0.25	0.92	0.68	1.25	<0.001
Excluding Duisburg	1.01	0.93	1.10	0.18	0.92	0.66	1.27	0.001
Excluding FLEHS I	1.01	0.92	1.09	0.19	0.93	0.67	1.29	0.001
Excluding HUMIS	1.01	0.93	1.10	0.19	0.97	0.71	1.33	0.002
Excluding RHEA	1.03	0.96	1.11	0.42	1.01	0.75	1.36	0.03
Excluding INMA-Menorca	1.01	0.93	1.10	0.17	0.90	0.64	1.28	0.001
Excluding INMA-Gipuzkoa	1.04	0.96	1.13	0.37	0.95	0.67	1.36	0.002
Excluding INMA-Sabadell	0.98	0.91	1.05	0.41	0.84	0.64	1.09	0.06
Excluding INMA-Valencia	0.98	0.91	1.06	0.37	0.86	0.62	1.19	0.003
Assessment >18 months[b]								
Ever wheeze								
All cohorts included	1.06	0.98	1.15	0.66	1.12	0.95	1.32	0.82
Excluding Duisburg	1.08	0.99	1.17	0.79	1.15	0.97	1.36	0.95
Excluding FLEHS I	1.06	0.97	1.15	0.52	1.13	0.95	1.34	0.74
Excluding PÉLAGIE	1.06	0.97	1.14	0.61	1.13	0.95	1.33	0.73
Excluding HUMIS	1.07	0.98	1.16	0.53	1.13	0.95	1.34	0.72
Excluding INMA-Menorca	1.04	0.95	1.14	0.71	1.10	0.90	1.33	0.73
Excluding INMA-Sabadell	1.08	0.96	1.22	0.54	1.05	0.83	1.33	0.79

[a]RR per doubling exposure. [b]The 18 months cut-off is not based on individual subjects but on cohorts: the average age of assessment in a cohort is categorized along this cut-off, but the range of ages can exceed it.

eTable 8. Meta-analyses (random-effects) of the association between DDE and PCB153 exposure and bronchitis and/or wheeze <18 months stratifying by matrix of exposure measurement (cord blood, maternal serum and breastmilk)[a].

			Geometric mean (25^{th}-75^{th})		DDE				PCB153			
	Nc	Ns	DDE	PCB153	Continuous (log_2-transformed) RR (95% CI)[b]	p-het	Highest vs lowest (tertiles) RR (95% CI)	p-het	Continuous (log_2-transformed) RR (95% CI)[b]	p-het	Highest vs lowest (tertiles) RR (95% CI)	p-het
All cohorts	9	4394	397.2 (184, 938)	96.4 (51, 173)	1.03 (0.99, 1.07)	0.41	1.15 (1.00, 1.32)	0.20	1.01 (0.96, 1.08)	0.20	0.88 (0.67, 1.16)	<0.001
Cord blood	5	1979	630.1 (320, 1246)	165.6 (104, 270)	1.04 (0.98, 1.10)	0.19	1.14 (1.00, 1.31)	0.56	1.06 (1.01, 1.12)	0.84[c]	1.20 (1.03, 1.39)	0.39[c]
Maternal serum	5	2034	370.6[d] (182, 707)	67.5[e] (41, 113)	1.02 (0.95, 1.09)	0.20	1.11 (0.95, 1.30)	0.66	0.94 (0.81, 1.09)	0.04	0.81 (0.50, 1.30)	0.001
Breastmilk	1	381	52.4 (33, 78)	39.2 (30, 51)	0.99 (0.73, 1.35)	-	1.22 (0.56, 2.65)	-	0.90 (0.54, 1.51)	-	0.49 (0.21, 1.17)	-

Nc (number of cohorts); **Ns** (number of subjects); **RR** (relative risk); **CI** (confidence interval); ***p*-het** (*p*-value of Cochran's Q test).

[a]All models were adjusted for gender, age of the child at the time of outcome assessment, duration of breastfeeding, gestational age, number of siblings of the child at the time of birth, maternal age, maternal body mass index, maternal smoking during pregnancy and during postnatal life of the child, maternal education, maternal allergy or asthma and time of sample collection for POPs analyses. [b]RR per doubling exposure. [c]RR and 95% once the PCB COHORT is excluded from the analysis: 1.03 (0.95, 1.13) per doubling exposure and 1.05 (0.84, 1.31) for the highest vs the lowest tertile. [d]Geometric mean DDE (ng/L) levels by moment of collection: 565.3 (1^{st} trimester), 310.5 (2^{nd} trimester), 218.9 (3^{rd} trimester), 654.9 (at birth), p-value for differences<0.05. [e]Geometric mean PCB153 (ng/L) levels by moment of collection: 50.2 (1^{st} trimester), 74.4 (2^{nd} trimester), 109.8 (3^{rd} trimester), 55.3 (at birth), p-value for differences<0.05.

Sample size by moment of maternal serum sampling: 708 (1^{st} trimester), 1090 (2^{nd} trimester), 208 (3^{rd} trimester), 15 (at birth).

eTable 9. Meta-analyses (random-effects) for both continuous and high versus low tertiles of exposure including both compounds in the models[a].

			DDE adjusted for PBC153				PCB153 adjusted for DDE			
	Nc	Ns	Continuous (log_2-transformed) RR (95% CI)[b]	p-het	Highest vs lowest (tertiles) RR (95% CI)	p-het	Continuous (log_2-transformed) RR (95% CI)[b]	p-het	Highest vs lowest (tertiles) RR (95% CI)	p-het
Assessment <18 months[c]										
Ever bronchitis and/or wheeze	9	4394	1.03 (0.99, 1.07)	0.41	1.15 (1.00, 1.32)	0.20	1.01 (0.96, 1.08)	0.20	0.88 (0.67, 1.16)	<0.001
Ever bronchitis	7	2990	1.05 (0.98, 1.13)	0.21	1.28 (0.92, 1.77)	0.01	1.06 (1.00, 1.12)	0.80	0.97 (0.71, 1.34)	0.05
Ever wheeze	8	3675	1.02 (0.96, 1.08)	0.17	1.19 (1.02, 1.39)	0.32	1.01 (0.93, 1.10)	0.21	0.86 (0.62, 1.19)	<0.001
Assessment >18 months[c]										
Ever wheeze	6	1754	1.01 (0.95, 1.07)	0.37	1.00 (0.85, 1.18)	0.62	1.06 (0.97, 1.15)	0.56	1.14 (0.95, 1.36)	0.82

Nc (number of cohorts); **Ns** (number of subjects); **RR** (relative risk); **CI** (confidence interval); ***p*-het** (*p*-value of Cochran's Q test).
[a]All models were adjusted for gender, age of the child at the time of outcome assessment, duration of breastfeeding, gestational age, number of siblings of the child at the time of birth, maternal age, maternal body mass index, maternal smoking during pregnancy and during postnatal life of the child, maternal education, maternal allergy or asthma and time of sample collection for POPs analyses. [b]RR per doubling exposure. [c]The 18 months cut-off is not based on individual subjects but on cohorts: the average age of assessment in a cohort is categorized along this cut-off, but the range of ages can exceed it.

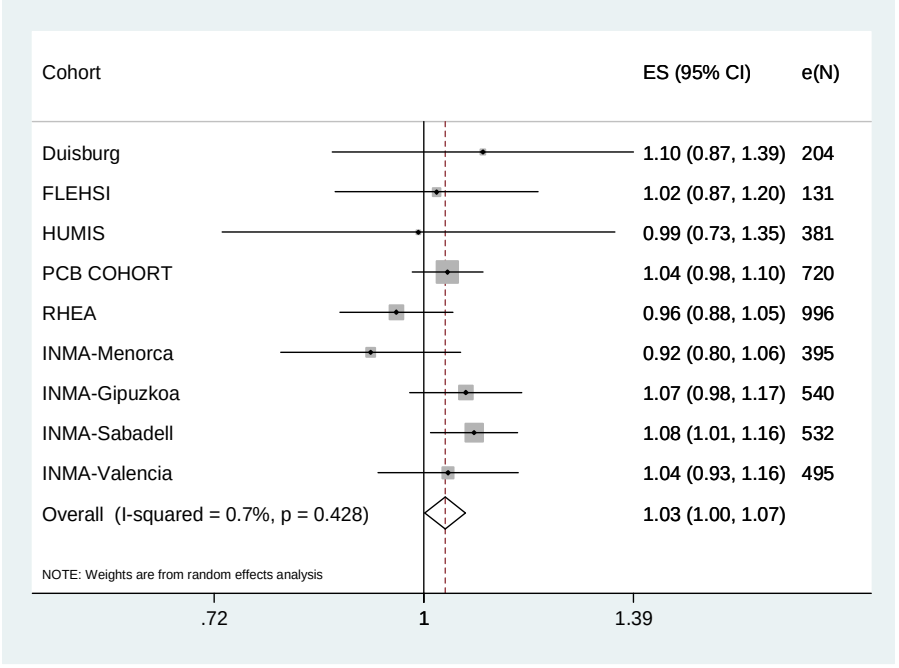
eTable 10. Meta-analyses (random-effects) for both continuous and high versus low tertiles of exposure using complete-case data[a].

			DDE				PCB153			
	Nc	Ns	Continuous (log_2-transformed) RR (95% CI)[b]	p-het	Highest vs lowest (tertiles) RR (95% CI)	p-het	Continuous (log_2-transformed) RR (95% CI)[b]	p-het	Highest vs lowest (tertiles) RR (95% CI)	p-het
Assessment <18 months[c]										
Ever bronchitis and/or wheeze	9	3464	1.02 (0.97, 1.07)	0.19	1.08 (0.94, 1.24)	0.27	0.95 (0.87, 1.04)	0.12	0.83 (0.65, 1.06)	0.005
Ever bronchitis	7	2155	1.04 (0.95, 1.14)	0.10	1.11 (0.82, 1.51)	0.06	0.99 (0.91, 1.08)	0.72	0.98 (0.79, 1.22)	0.39
Ever wheeze	8	3208	1.02 (0.97, 1.08)	0.28	1.11 (0.94, 1.30)	0.45	0.95 (0.84, 1.07)	0.13	0.81 (0.59, 1.11)	0.004
Assessment >18 months[c]										
Ever wheeze	6	1475	1.02 (0.96, 1.08)	0.94	1.03 (0.88, 1.20)	0.89	1.03 (0.89, 1.19)	0.18	1.00 (0.77, 1.30)	0.20

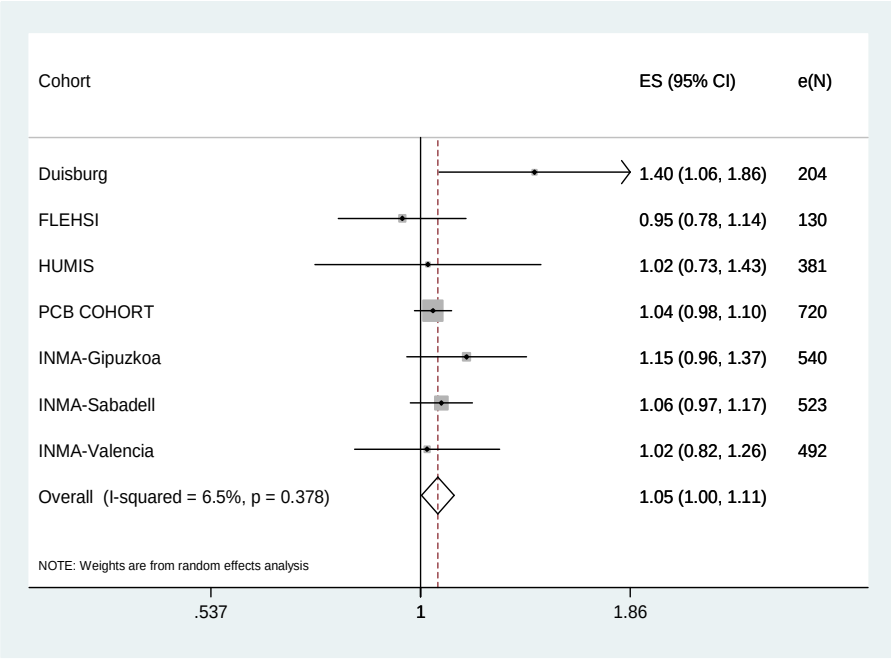
Nc (number of cohorts); **Ns** (number of subjects); **RR** (relative risk); **CI** (confidence interval); ***p*-het** (*p*-value of Cochran's Q test).
[a]All models were adjusted for gender, age of the child at the time of outcome assessment, duration of breastfeeding, gestational age, number of siblings of the child at the time of birth, maternal age, maternal body mass index, maternal smoking during pregnancy and during postnatal life of the child, maternal education, maternal allergy or asthma and time of sample collection for POPs analyses. [b]RR per doubling exposure. [c]The 18 months cut-off is not based on individual subjects but on cohorts: the average age of assessment in a cohort is categorized along this cut-off, but the range of ages can exceed it .

eFigures 1 to 7. Forest plots showing risk estimates for individual studies and the combined meta-analysis results for DDE exposure.

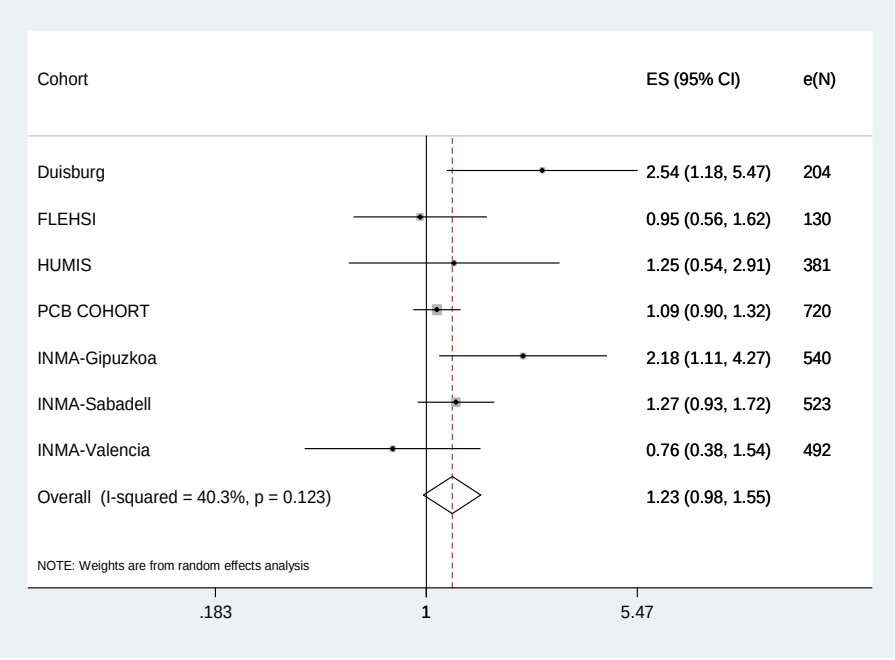
eFigure 1. Bronchitis and/or wheeze assessed <18 months of age and continuous DDE exposure **.**



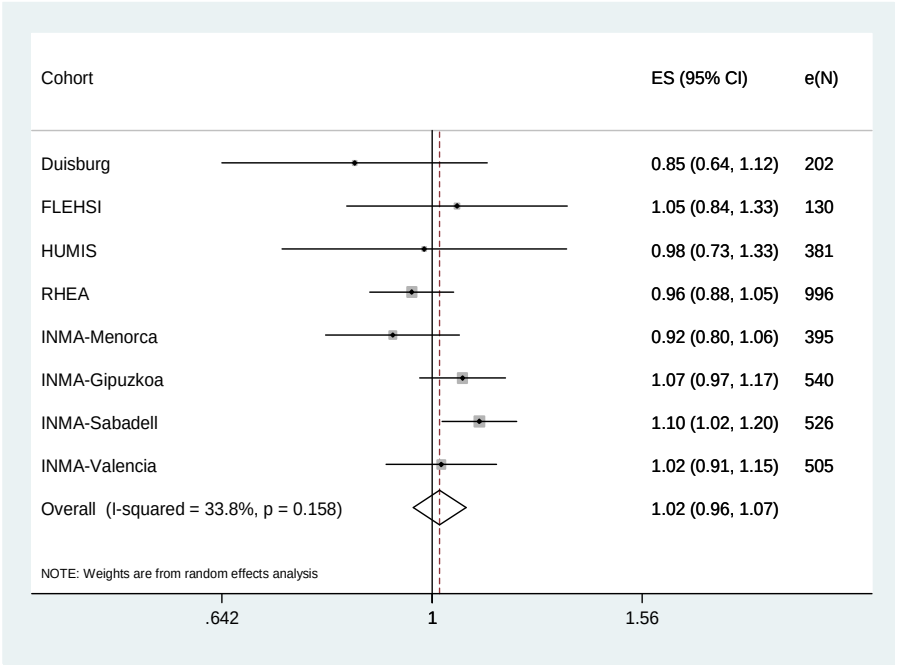
eFigure 2. Bronchitis assessed <18 months of age and continuous DDE exposure.



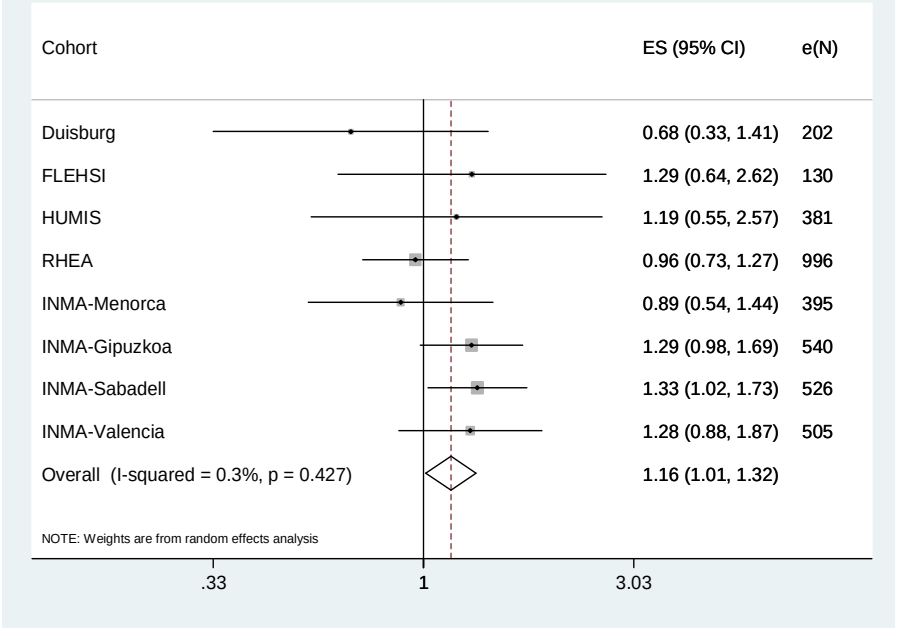
eFigure 3. Bronchitis assessed <18 months of age and the highest vs the lowest tertile of DDE exposure.



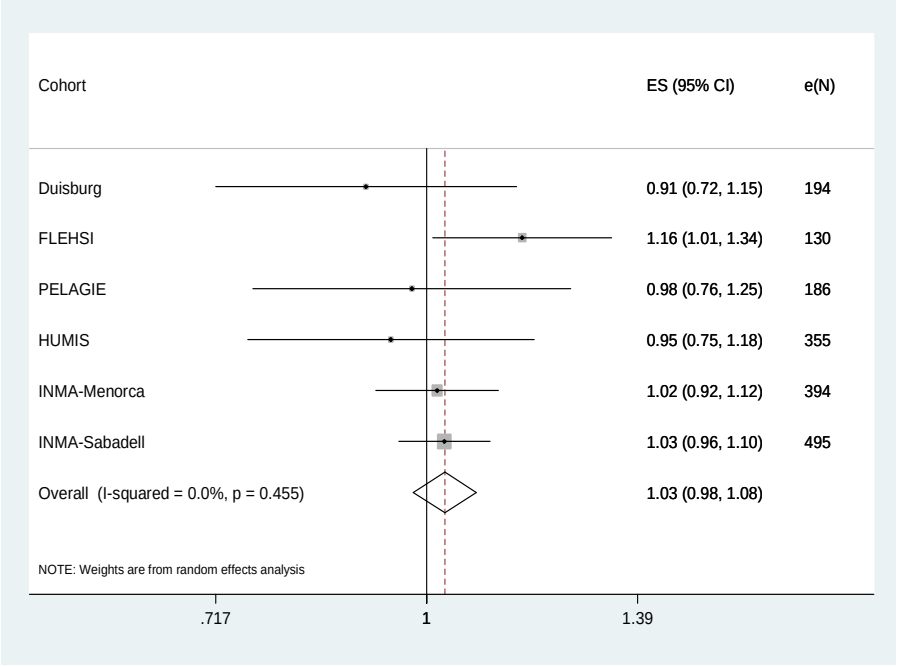
eFigure 4. Wheeze assessed <18 months of age and continuous DDE exposure.



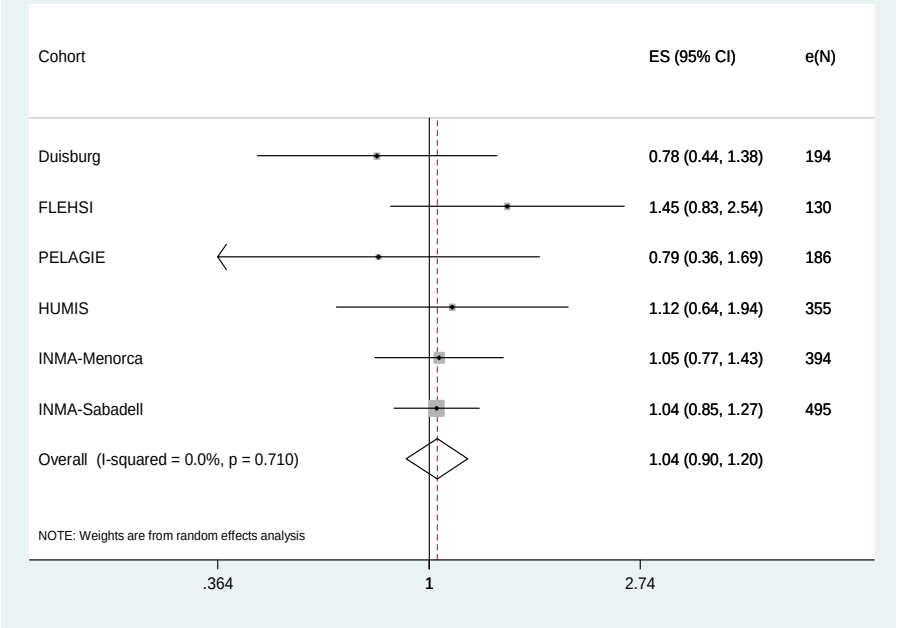
eFigure 5. Wheeze assessed <18 months of age and the highest vs the lowest tertile of DDE exposure.



eFigure 6. Wheeze assessed >18 months of age and continuous DDE exposure.

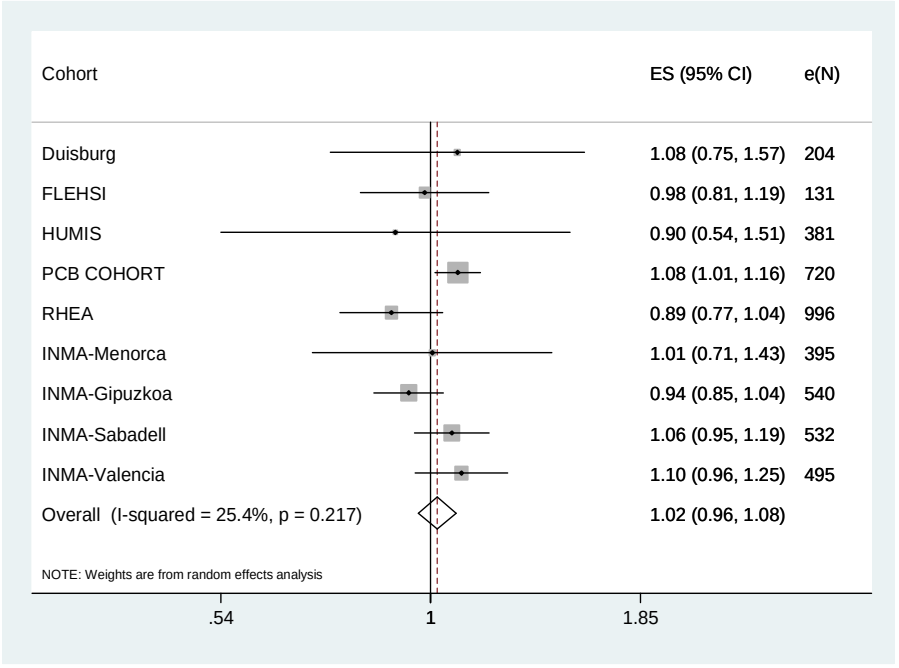


eFigure 7. Wheeze assessed >18 months of age and the highest vs the lowest tertile of DDE exposure.

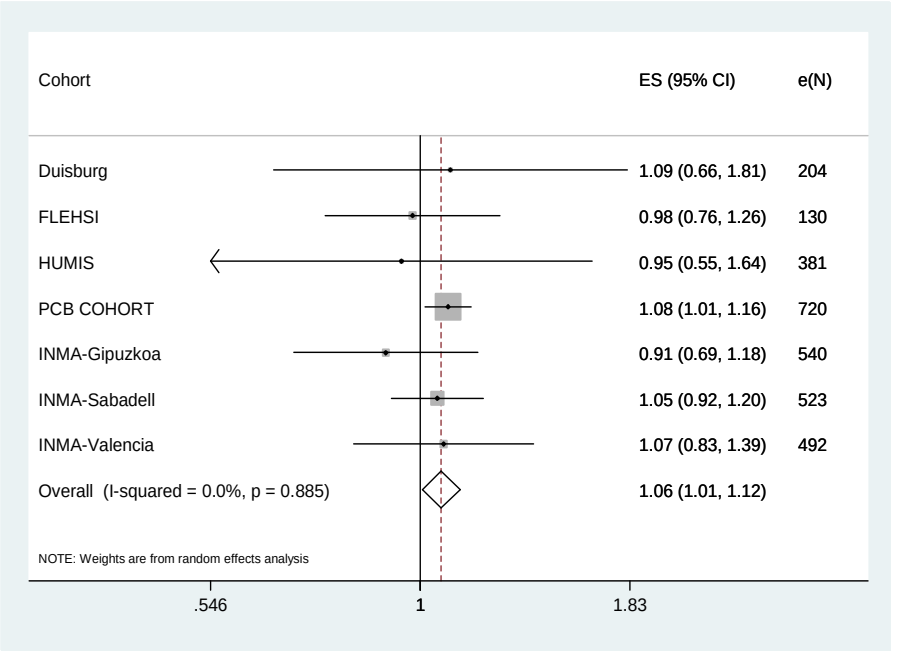


eFigures 8 to 14. Forest plots showing risk estimates for individual studies and the combined meta-analysis results for PCB153 exposure.

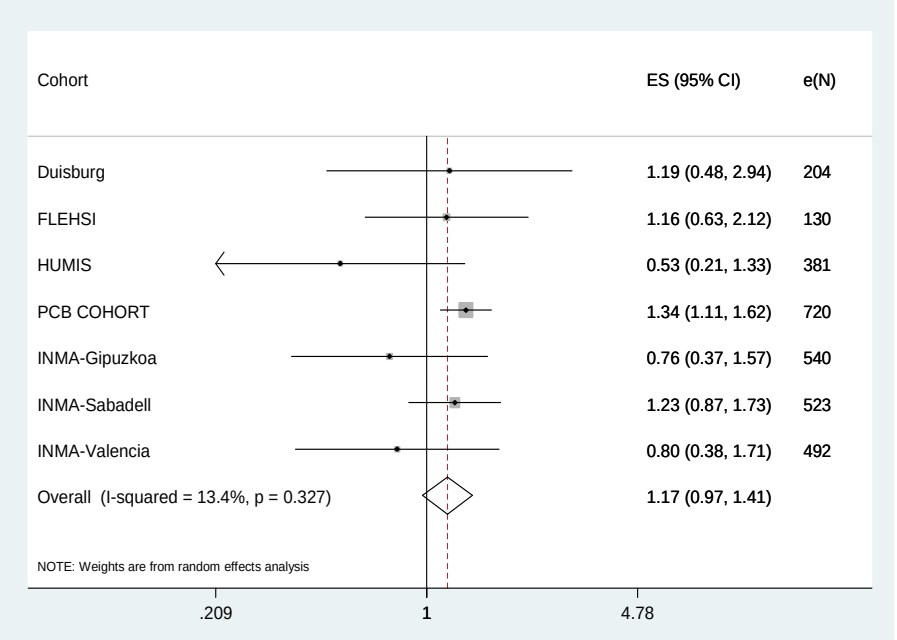
eFigure 8. Bronchitis and/or wheeze assessed <18 months of age and continuous PCB153 exposure.



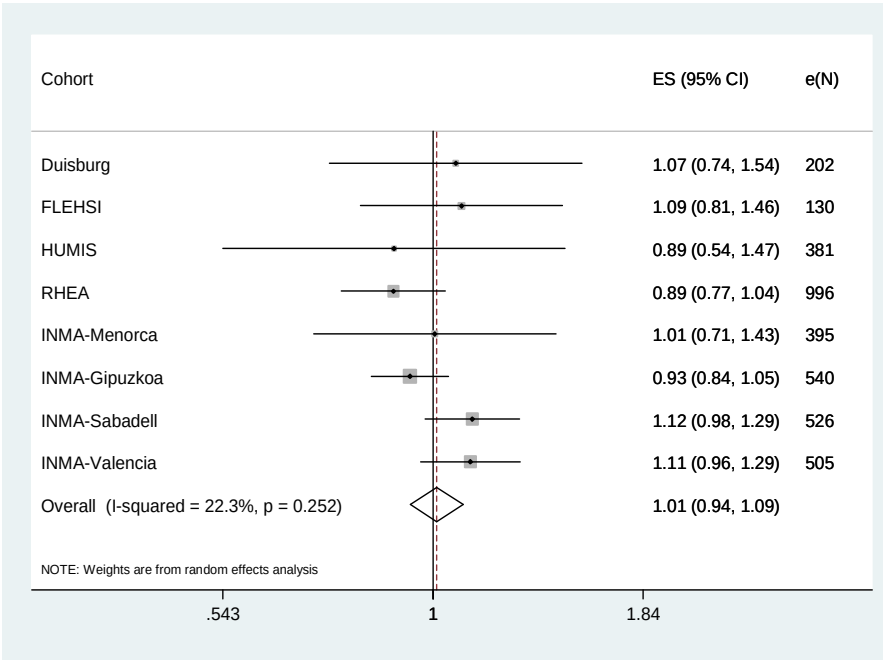
eFigure 9. Bronchitis assessed <18 months of age and continuous PCB153 exposure.



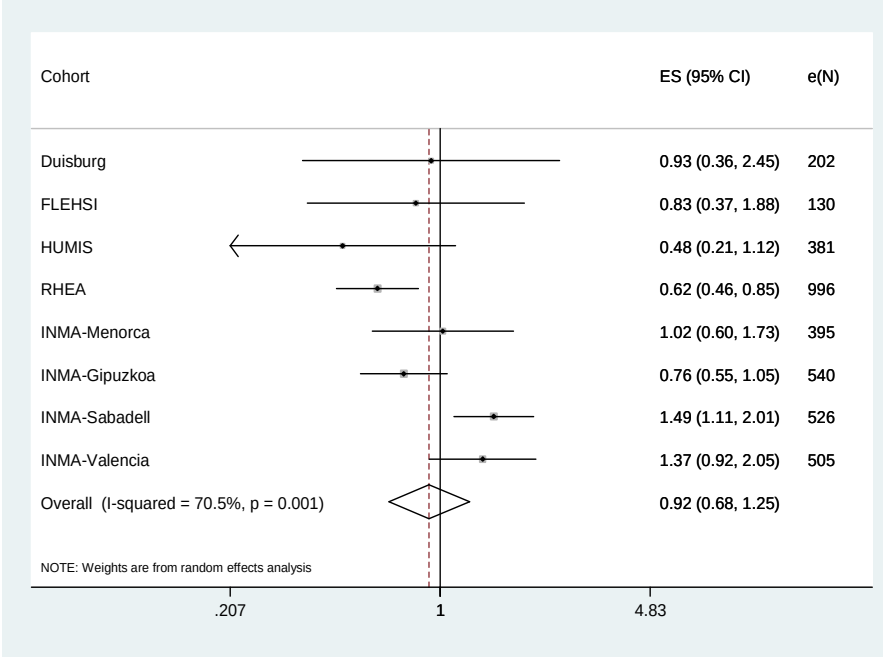
eFigure 10. Bronchitis assessed <18 months of age and the highest vs the lowest tertile of PCB153 exposure.



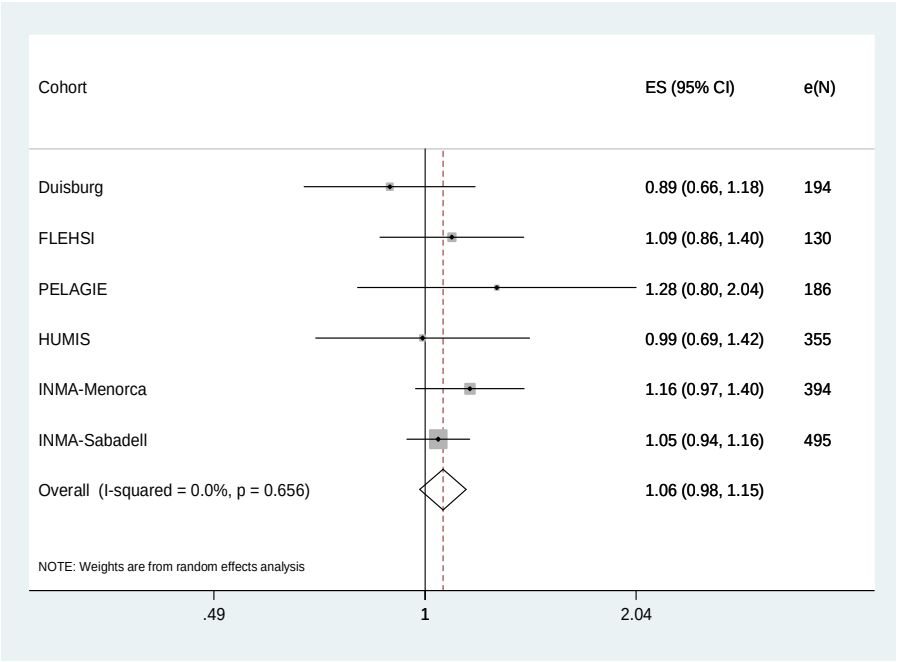
eFigure 11. Wheeze assessed <18 months of age and continuous PCB153 exposure.



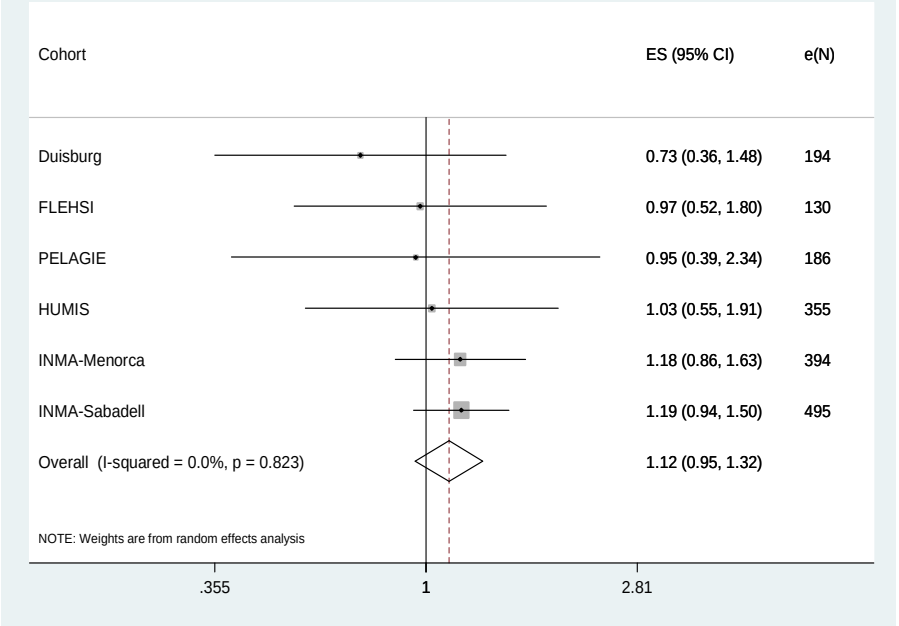
eFigure 12. Wheeze assessed <18 months of age and the highest vs the lowest tertile of PCB153 exposure.



eFigure 13. Wheeze assessed >18 months of age and continuous PCB153 exposure.



eFigure 14. Wheeze assessed >18 months of age and the highest vs the lowest tertile of PCB153 exposure.



5.4 Paper IV

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

Gascon M[a,b,c,d], Sunyer J[a,b,c,d], Martínez D[a,b,c,d], Guerra S[a,b,c,d], Lavi I[a,b,c,d], Torrent M[e] and Vrijheid M[a,b,c,d]. **Persistent organic pollutants and children's respiratory health: the role of cytokines and inflammatory biomarkers.** *Under review in Environ Int.*

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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 ΣPCBs 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 ΣPCBs 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 POPs 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 to 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.

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 70's, these compounds can still be detected in 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. 2013a). 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 M, unpublished data). 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 (Hansen et al. 2014). 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 POPs exposure and immune and respiratory health in children. Immune cell counts are useful as general indicators of general immune status (Gascon et al. 2013a), 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) IL4, IL5, IL8, 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 information on the long-term respiratory health effects of prenatal exposure to POPs is limited 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 ΣPCBs 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 POPs levels in cord blood (Study population A). At the age of 4 years, blood samples were drawn from 360 children and stored at -20ºC. 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 POPs 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 ionisation negative-ion mass spectrometry (Hewlett-Packard 5973 MSD) (Carrizo et al. 2007). PCBs 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] had 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 age 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 2mm 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- and sVCAM-1 were measured with the Millipore's panel HCVD2MAG-67K. Finally, CRP was

measured by immunoturbidimetry at the Laboratory of the groups EGEC and CARIN of IMIM Fundation, Barcelona, Spain. All analyses were performed in duplicates except 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% to 54.6% of missings) 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-cases 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 a 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 biomarkers levels were 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 biomarkers levels and dichotomous

outcome variables (wheeze, asthma, chest infections, atopy) were conducted using logistic regression models. For the association between POPs and immune biomarkers levels, linear regression models were performed. To assess the role of POPs or immune biomarkers levels on the risk of wheeze and chest infections in each time point of assessment, adjusted for wheeze/chest infections 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 a 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 biomaker'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 (25^{th}, 75^{th})=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 10^{th} and the 14^{th} 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 years serum's 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 POPs exposure; therefore, results for these cytokines will not be further presented, only in the descriptive tables.

3.2 Prenatal POPs exposure and respiratory outcomes

Results of this section are based on study population A (Table 3 and Figure 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 Figure 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 Figure 1). No associations were found between prenatal ΣPCBs concentrations and any of the outcomes assessed (Table 3 and Figure 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 of 1.33 (1.04, 1.69) and for prenatal HCB and wheeze was of 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 non-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 in the associations between IL10 levels and asthma at age 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 POPs 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 was increased with increasing concentrations of all three compounds [β (95%CI) per doubling levels=0.11 (-0.01, 0.24), 0.22 (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

of 0.05 (-0.08, 0.19), for prenatal HCB was of 0.16 (-0.05, 0.37) and for ΣPCBs was of 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 mediator of the effect; associations, though, did not change by more than 10% (data not shown).

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 age 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 age of 10 and 14 years was increased with increasing IL10 levels.

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, M, unpublised data) 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. 2014). 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. 2014). These results are consistent with the present study, where wheeze and chest infections at the age of 10 years, but not earlier, 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, suggest 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 eosinophils 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 relatively a 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 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 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 was increased in relation to these compounds, but not proinflammatory cytokines (TNFα, IL6 and IL8), which might be indicating that pathways other than inflammation are involved in the relationship between prenatal POPs exposure and IL10. Additionally, when IL10 was included in the models assessing the association between prenatal POPs 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 POPs 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 (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 POPs levels on wet-weight basis because we did not have information on lipids 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 POPs 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 POPs 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) had also 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 POPs exposure, something already observed for other outcomes such as neurodevelopment (Gascon et al. 2013a). 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 POPs exposure and IL10 need 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 lead 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 statistical 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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TABLES AND FIGURES

Table 1. Characteristics of study populations A and B[a].

Characteristics	Study population A (N=405)		Study population B (N=275)	
	N[b]		N[b]	
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 4y[c] [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)

Characteristics	Study population A (N=405)		Study population B (N=275)	
	N[b]		N[b]	
Concentrations of prenatal POPs exposure [median (25^{th},75^{th}) – 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				
1y (last 12m, %)	405	21.1	275	22.6
2y (last 12m, %)	403	29.7	275	31.3
3y (last 12m, %)	403	19.1	275	18.6
4y (last 12m, %)	398	11.6	275	12.4
6.5y (last 24m, %)	389	8.0	269	8.2
10y (last 12m, %)	357	7.8	249	8.4
14y (last 12m, %)	255	8.2	192	7.3
Chest infections				
2y (last 12m, %)	403	62.8	275	65.1
3y (last 12m, %)	403	45.2	275	46.2
4y (last 12m, %)	398	28.6	275	27.3
6.5y (last 24m, %)	389	21.3	268	20.9
10y (last 12m, %)	357	4.2	249	4.0
Asthma ever				
10y (%)	359	4.5	251	5.2
14y (%)	255	7.5	192	7.3
Atopy (SPT) 6.5y (%)	356	15.7	261	16.5

BMI: body mass index, DDE: dichlorodiphenyldichloroethylene, HCB: hexachlorobenzene, ΣPCBs: sum of polychlorinated biphenyls congeners 101, 118, 138, 153 and 180, SPT: skin prick test.

[a]Study population A include children with information on POPs exposure and respiratory outcomes. Study population B includes children that also have information on cytokines and biomarkers of inflammation.

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

[c]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.

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

Biomarker	GM (25^{th},75^{th})	%<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 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).

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

Figure 1. Association between prenatal DDE, HCB and ΣPCBs with wheeze at different ages.

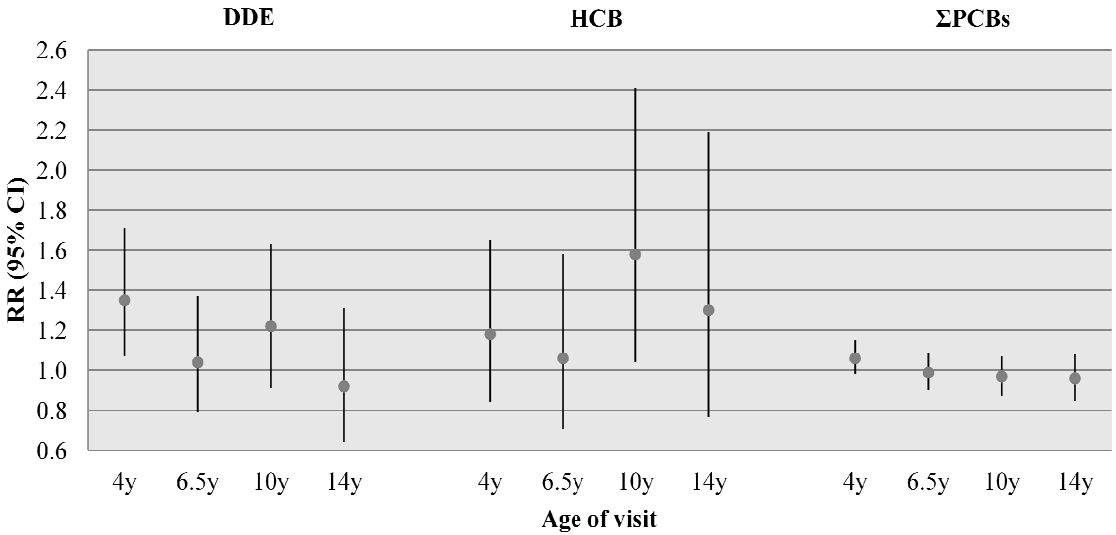

Table 4. Association between log_2-transformed levels of immune biomarkers measured at age 4 years and respiratory and immune outcomes at different ages [study population B (N=275)]

	N cases / 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]						
4y	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)
6.5y	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)
10y	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)
14y	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]						
4y	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)
6.5y	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)
10y	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						
10y	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)
14y	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 6.5y	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 biomarkers 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 biphenyls 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.

SUPPLEMENTAL MATERIAL

Description of the imputation procedure

- **Software used and key setting:** STATA 12 software (Stata Corporation, College Station, Texas) –*ice* command (with 100 cycles).
- **Number of imputed datasets created:** 100.
- **Variables included in the imputation procedure:**
  - Child's variables: sex, gestational age, birth weight, weeks of breastfeeding, parity, siblings, age starting daycare attendance, z-score BMI at age 4, 6, 10 and 14 years, child age at the time of skin prick test.
  - Parental variables: maternal age, pre-pregnancy maternal weight and height, maternal and paternal asthma, rhinitis, eczema and atopy, maternal and paternal education and social class, maternal and paternal smoking during pregnancy and subsequent follow-ups, presence of pets at home during pregnancy and subsequent follow-ups.
  - Other variables: all POPs measured in cord blood and in child's serum collected at age 4 years. For study population B all biomarkers measured in child's serum collected at 4 years were also included.
- **Treatment of non-normally distributed variables:** $\log_2$-transformed.
- **Treatment of binary/categorical variables:** logistic, ordinal, and multinomial models.
- **Statistical interactions included in imputation models:** imputations were done separately for each study population analyzed [A (N=405) and B (N=275)].
- **Percentage of samples under de LOD or LOQ**:
  - For POPs, from 1.5 to 72.0% of cord blood samples and 0.1 to 31.2% of 4 years serum.
  - For biomarkers, from 2.2 to 22.2% of the samples.

Table A. Pearson correlation between immune biomarkers (after log_2–transformation) measured at age 4 years [study population B (N=275)].

	IL6	**IL8**	**IL10**	**TNFα**	**sICAM-1**	**sVCAM-1**	**CRP**
IL6	1						
IL8	0.17	1					
IL10	0.23	0.33	1				
TNFα	0.15	0.29	0.25	1			
sICAM-1	0.12	0.15	0.14	0.24	1		
sVCAM-1	0.06	0.08	0.24	0.18	0.49	1	
CRP	0.60	0.08	0.20	0.13	0.16	0.04	1

IL: interleukin (pg/mL), TNFα: tumor necrosis factor alpha (pg/mL), sICAM-1: soluble intercellular adhesion molecule-1 (ng/mL), sVCAM-1: soluble vascular cell adhesion molecule-1 (ng/mL), CRP: c-reactive protein (mg/dL).
All correlations were significant (p-value <0.05) except the correlation between vCAM and IL6, IL8 and CRP and the correlation between CRP and IL8.

Table B. Associations between log_2–transformed cord blood concentrations of POPs and respiratory and immune outcomes at different ages [study population B (N=275)]

		DDE	HCB	ΣPCBs
	N cases / N total	RR (95%CI)	RR (95%CI)	RR (95%CI)
Wheeze[a]				
4y	34/275	1.33 (1.00, 1.77)	1.25 (0.82, 1.90)	1.02 (0.92, 1.13)
6.5y	22/269	0.91 (0.64, 1.28)	1.07 (0.65, 1.78)	0.94 (0.83, 1.07)
10y	21/249	1.04 (0.73, 1.49)	1.90 (1.13, 3.18)	0.97 (0.85, 1.10)
14y	14/192	0.92 (0.59, 1.45)	1.26 (0.64, 2.48)	0.95 (0.81, 1.11)
Chest infections[a]				
4y	75/275	1.00 (0.81, 1.24)	0.78 (0.56, 1.08)	0.98 (0.91, 1.06)
6.5y	56/268	1.00 (0.79, 1.26)	1.13 (0.80, 1.61)	1.04 (0.95, 1.13)
10y	10/249	1.30 (0.77, 2.12)	2.50 (1.27, 4.91)	1.16 (0.98, 1.39)
Asthma ever				
10y	13/251	0.93 (0.57, 1.50)	1.14 (0.54, 2.41)	0.88 (0.73, 1.07)
14y	14/192	0.80 (0.49, 1.32)	0.85 (0.43, 1.67)	0.86 (0.72, 1.03)
Atopy 6.5y	43/261	0.99 (0.77, 1.26)	1.29 (0.90, 1.84)	1.02 (0.94, 1.20)

RR: relative risk, DDE: dichlorodiphenyldichloroethylene, HCB: hexachlorobenzene, ΣPCBs: sum of polychlorinated biphenyls 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), 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.

Table C. Associations between log_2–transformed cord blood concentrations of POPs and respiratory and immune outcomes at different ages in complete-case study population.

		DDE	HCB	ΣPCBs
	N cases / N total	RR (95%CI)	RR (95%CI)	RR (95%CI)
Wheeze[a]				
4y	41/360	1.45 (1.03, 2.04)	0.99 (0.62, 1.59)	1.07 (0.94, 1.23)
6.5y	30/353	1.10 (0.82, 1.47)	1.20 (0.79, 1.82)	1.00 (0.91, 1.10)
10y	27/327	1.10 (0.81, 1.48)	1.56 (1.02, 2.40)	0.94 (0.85, 1.04)
14y	20/235	0.93 (0.65, 1.34)	1.50 (0.89, 2.51)	0.95 (0.85, 1.06)
Chest infections[a]				
4y	105/360	1.09 (0.87, 1.36)	0.88 (0.65, 1.20)	1.03 (0.95, 1.12)
6.5y	76/353	1.06 (0.86, 1.31)	1.10 (0.82, 1.49)	1.06 (0.99, 1.14)
10y	15/327	1.26 (0.85, 1.86)	1.92 (1.08, 3.40)	1.10 (0.96, 1.26)
Asthma ever				
10y	15/310	1.14 (0.78, 1.69)	1.37 (0.75, 2.50)	0.96 (0.84, 1.10)
14y	18/223	0.95 (0.64, 1.39)	1.19 (0.65, 2.15)	0.92 (0.81, 1.05)
Atopy 6.5y	54/322	0.99 (0.81, 1.23)	1.15 (0.85, 1.57)	0.98 (0.91, 1.06)

RR: relative risk, DDE: dichlorodiphenyldichloroethylene, HCB: hexachlorobenzene, ΣPCBs: sum of polychlorinated biphenyls 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.

Table D. Association between log_2-transformed levels of immune biomarkers measured at age 4 years and respiratory and immune outcomes at different ages in complete-case study population.

	N cases / 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]						
4y	30/249	1.15 (0.71, 1.87)	3.23 (1.56, 6.69)	1.44 (1.00, 2.09)	11.2 (2.06, 61.29)	1.05 (0.84, 1.30)
6.5y	22/245	1.12 (0.75, 1.65)	1.30 (0.72, 2.32)	1.47 (1.07, 2.02)	2.23 (0.85, 5.84)	1.06 (0.87, 1.28)
10y	21/227	0.98 (0.65, 1.46)	0.84 (0.44, 1.59)	1.03 (0.69, 1.53)	0.91 (0.33, 2.55)	0.97 (0.79, 1.19)
14y	14/176	1.22 (0.76, 1.96)	0.60 (0.27, 1.33)	1.18 (0.76, 1.83)	0.68 (0.21, 2.22)	1.14 (0.90, 1.44)
Chest infections[a]						
4y	67/249	1.07 (0.78, 1.47)	1.26 (0.79, 2.00)	1.14 (0.87, 1.48)	2.74 (1.16, 6.51)	1.06 (0.92, 1.22)
6.5y	51/244	0.69 (0.41, 1.17)	1.32 (0.86, 2.01)	0.81 (0.34, 1.95)	3.41 (1.49, 7.80)	1.00 (0.88, 1.15)
10y	10/227	0.10 (0.04, 0.24)	0.72 (0.29, 1.81)	0.10 (0.03, 0.36)	0.10 (0.02, 0.40)	1.03 (0.78, 1.35)
Asthma ever						
10y	9/152	0.96 (0.46, 2.02)	3.38 (0.96, 11.83)	1.56 (0.88, 2.76)	2.04 (0.28, 15.14)	0.97 (0.70, 1.35)
14y	8/117	1.06 (0.46, 2.45)	2.31 (0.63, 8.43)	1.37 (0.69, 2.71)	0.94 (0.10, 9.14)	1.04 (0.72, 1.50)
Atopy 6.5y	26/155	1.24 (0.87, 1.77)	1.37 (0.84, 2.25)	1.23 (0.94, 1.59)	1.23 (0.62, 2.47)	1.16 (0.99, 1.35)

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 E. Association between log2-transformed cord blood concentrations of POPs and immune biomarkers levels measured at age 4 years in complete-case study population (N=166).

	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	0.06 (-0.08, 0.20)	0.07 (-0.02, 0.17)	0.13 (-0.04, 0.30)	0.04 (-0.02, 0.11)	0.15 (-0.15, 0.46)
HCB	-0.07 (-0.30, 0.15)	0.09 (-0.06, 0.24)	0.24 (-0.02, 0.50)	-0.02 (-0.13, 0.08)	0.18 (-0.30, 0.66)
ΣPCBs	0.02 (-0.04, 0.07)	0.01 (-0.03, 0.04)	0.08 (0.02, 0.15)	0.02 (-0.01, 0.04)	0.14 (0.02, 0.26)

β: coefficient, IL: interleukin, TNFα: tumor necrosis factor alpha, CRP: c-reactive protein, DDE: dichlorodiphenyldichloroethylene, HCB: hexachlorobenzene, ΣPCBs: sum of polychlorinated biphenyls 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.

5.5 Paper V

Prenatal exposure to bisphenol A, phthalates and respiratory and allergy outcomes during childhood

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Abstract

Background: There is growing concern that prenatal exposure to bisphenol A (BPA) and phthalates, widely used in consumer products, may impact susceptibility to infections and the development of allergy and asthma in children, but there are currently very few prospective studies.

Objective: To evaluate whether prenatal exposure to BPA and phthalates increases risk of respiratory and allergic outcomes in children at repeated ages from birth to 7 years.

Methods: We measured BPA and metabolites of high molecular weight phthalates, four di-2-ethylhexyl metabolites (Σ_4DEHP) and mono-benzyl (MBzP), and three low molecular weight phthalates metabolites (Σ_3LMWP) in urine samples collected during the 1st and 3rd pregnancy trimester in women participating in the INMA-Sabadell birth cohort study. The occurrence of chest infections, bronchitis, wheeze and eczema in children was assessed at ages 6 and 14 months and 4 and 7 years, through questionnaires with the mothers.

Results: The relative risks of wheeze (RR, 1.20; 95% CI, 1.03, 1.40), chest infections (RR, 1.15; 95% CI, 1.00, 1.32) and bronchitis (RR, 1.18; 95% CI, 1.00, 1.37) at any age increased for each doubling in concentration of maternal urinary BPA. Σ_4DEHP metabolites were associated with the same outcomes (RR; 95% CI for wheeze: 1.25; 1.04, 1.50, chest infections: 1.15; 0.97, 1.35, bronchitis: 1.20; 1.01, 1.44). MBzP was associated with higher risk of wheeze (RR, 1.15; 95% CI, 1.00, 1.33). These associations were

generally consistent over the repeated child ages. There were no other exposure-outcome associations.

Conclusions: Prenatal exposure to BPA and high molecular weight phthalates may increase risk of wheeze and respiratory infections throughout childhood.

1. INTRODUCTION

Increasing prevalence of asthma and allergic diseases over a relatively short period of time (Bousquet et al. 2011) have put environmental pollutants in the spotlight for such increase (Winans et al. 2011). Certain pollutants have been suggested to impact susceptibility to infections and the development of allergy and asthma during the first years of life, including compounds commonly used in plastic manufacture (Bousquet et al. 2011; Winans et al. 2011). In recent years, researchers have focused on bisphenol A (BPA) and phthalates, which are endocrine disrupting chemicals (EDCs), because of their potential immunomodulatory capacities (Bornehag and Nanberg 2010; Kwak et al. 2009) and interaction with the development of the respiratory system during fetal life (Miller and Marty 2010). These compounds are produced and used in large quantities worldwide and are present in all kind of consumer products including cosmetics, plastics, carpets, building materials, toys or cleaning products (Braun et al. 2013; Dodson et al. 2012; Vandenberg et al. 2007). The routes of exposure for the general population are diet (for BPA and high molecular weight phthalates) and personal care products (for low molecular weight phthalates) (Bertelsen et al. 2012; Casas et al. 2013; Hoppin et al. 2002; Vandenberg et al. 2007). Prenatal lifetime is a critical period in the development of the immune and respiratory systems, and potential harmful effects by exposure to toxic pollutants may result into long-lasting impaired capacity to fight infections and increased risk to develop allergic manifestations later in life (Bisgaard and Bonnelykke 2010; Busse et al. 2010; Dietert et al. 2010; Pinkerton

and Joad 2000; Warner 2004). Although there is some evidence of the immunomodulatory properties of both BPA and phthalates in animal and in vitro models (Kimber and Dearman 2010; Konkel 2013; Rogers et al. 2013), there is limited evidence on their health effects in susceptible human populations such as children. Results of the few previous studies have been inconsistent mainly due to cross-sectional or retrospective study design, or the use of environmental rather than biomarker assessed exposure estimates (Bertelsen et al. 2012; Bornehag et al. 2004; Callesen et al. 2014; Donohue et al. 2013; Hoppin et al. 2013; Hsu et al. 2012; Just et al. 2012a; Kolarik et al. 2008; Spanier et al. 2012). The aim of the present study is to evaluate whether urine biomarker measurements of BPA and phthalates during pregnancy are associated with increased risks of respiratory and allergy outcomes in children at repeated ages from birth to 7 years in a longitudinal birth cohort study.

2. METHODS

2.1 Study population

Pregnant women were recruited into the INMA-*INfancia y Medio Ambiente* (Environment and Childhood) birth cohort set up in Sabadell (Catalonia, Spain) between 2004 and 2008 (N=657). Protocol details are described elsewhere (Guxens et al. 2012). Briefly, women were recruited during the 1st trimester routine antenatal care visit in the main public hospital or health centre of reference if they fulfilled the inclusion criteria: age≥16 years, intention to deliver in the reference hospital, singleton births, no

assisted conception, and no problems of communication. The study was conducted with the approval of the hospital ethics committee and written informed consent was obtained from the parents of all children.

2.2 Respiratory and allergy outcomes

Information on occurrence of wheeze, chest infections and eczema in offspring at ages 6 and 14 months and 4 and 7 years was obtained from the mother through interviewer-led questionnaire based on the validated ISAAC questionnaire (Asher et al. 1995), whereas information on bronchitis was obtained at 6 and 14 months and 4 years of age. The occurrence of chest infection/bronchitis, respectively, was defined as a positive answer to the question "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/bronchitis?". Wheeze was defined as a positive answer to "Has your child ever experienced whistling or wheeze from the chest, but not noisy breathing from the nose in the last 6 (or 12) months?". At age 7 years wheeze was defined as a positive answer to "Has your child ever experienced whistling or wheeze from the chest in the last 12 months?". At 6 and 14 months and 4 years of life, occurrence of eczema was defined as a positive answer to "In the last 6 (or 12 months) did your child suffered from atopic eczema?". At age 7 years eczema was defined as a positive answer to "Has your child ever had any itchy rash which was intermittently coming and going at any time in the past 12 months?".

2.3 Exposure variables

Spot urine samples of mothers were collected at 12 and 32 weeks of gestation and stored in 10 mL polypropylene tubes at −20 °C. Creatinine was determined at the Echevarne laboratory of Barcelona (Spain) by the Jaffé method (kinetic with target measurement, compensated method) with Beckman Coulter© reactive in AU5400 (IZASA®).

BPA concentration was determined in the Department of Analytical Chemistry — University of Cordoba (Spain), as previously explained (Casas et al. 2013). Total BPA (free plus conjugated) was quantified by liquid chromatography mass spectrometry with a detection limit (LOD) of 0.1 μg/L. A total of eight phthalate metabolites were quantified in the Bioanalysis Research Group at Hospital del Mar Medical Research Institute (IMIM, Barcelona, Spain): mono-(2-ethyl-5-hydroxyl-hexyl) phthalate (MEHHP), mono-(2-ethyl-hexyl) phthalate (MEHP), mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP), mono-(2-ethyl-5-carboxy-pentyl) phthalate (MECPP), MBzP, mono-ethyl phthalate (MEP), mono-isobutyl phthalate (MiBP) and mono-n-butyl phthalate (MnBP). The concentration of total (free plus glucuronoconjugated) phthalate metabolites determination consisted of a sample preparation including enzymatic hydrolysis with β-glucuronidase enzymes and solid-phase extraction and analysis by ultraperformance liquid chromatography coupled to tandem mass spectrometry. The LOD for the different congeners ranged from 0.5 to 1 μg/L.

Both BPA and phthalates concentrations were creatinine-adjusted (μg/g creatinine) to control for urine dilution. Due to short half-life (i.e. hours) of these compounds, we calculated and analyzed the average concentration determined in 1st and 3rd trimester in order to reduce misclassification and to provide a better estimate of exposure throughout pregnancy (Braun et al. 2013; Casas et al. 2013; Chevrier et al. 2013; Hoppin et al. 2002). Phthalate metabolites were then grouped based on the common parent of the metabolites [the sum of di-2-ethylhexyl phthalate (Σ_4DEHP) metabolites: MEHP, MEHHP, MEOHP, MECPP], MBzP metabolite, or the type of phthalates [the sum of low molecular weight phthalate (Σ_3LMWP) metabolites: MEP, MiBP, MnBP] as these are thought to have different physicochemical properties (Bornehag et al. 2004).

2.4 Covariates

Information on the following covariates was obtained through questionnaires answered by the mothers at the 1st and 3rd trimesters of pregnancy and at age 14 months: maternal age, education and country of origin, maternal smoking during pregnancy, number of older siblings, day care attendance during the 1st year of life, duration of exclusive breastfeeding, maternal consumption of canned tuna, and maternal and paternal history of asthma/allergy symptoms. Parents were classified as allergic if they reported to suffer from allergic asthma, atopic dermatitis, eczema or rhinitis during the health questionnaire in the 3rd trimester of pregnancy. Maternal pre-pregnancy body mass index (BMI), gestational age,

weight at birth, season of birth and child sex were collected from clinical records or reported by mothers.

2.5 Statistical methods

Missing values in covariates (between 0 and 0.8%) were imputed performing multiple imputation (Royston 2005). The same method was applied when BPA and phthalates samples were below the LOD (between 0% and 0.8% of the samples) by defining the range of imputed values between 0 and the LOD value for each compound. Detailed information on the imputation process can be found in Table E1 in the Online Repository.

Concentrations of pollutants were $\log_2$-transformed because of skewed distributions. Here, we report risk estimates per doubling of the concentration of pollutants. To assess the association of BPA and phthalate exposure on the risk of respiratory or immune outcomes in offspring from birth to age 7 years we used generalized estimating equations (GEE) with an unstructured correlation matrix and an interaction term between year of visit and the exposure variable. To determine multivariate models for each exposure variable we applied directed acyclic graphs (DAGs) (Shrier and Platt 2008) using DAGity software (Textor et al. 2011). Covariates were included in the respective DAGs based on previous literature and on bivariate analyses (Casas et al. 2013; Gascon et al. 2012) of our data: variables were included in the respective DAGs if they associated ($p \leq 0.1$) to both the outcomes and the exposure (BPA and each single phthalate metabolite) ("see Table E2 in the Online

Repository"). According to the DAGs obtained, final multivariate models for BPA included maternal education, number of siblings and maternal smoking during pregnancy, and multivariate models for phthalate metabolites additionally included maternal pre-pregnancy BMI and maternal history of asthma and/or allergy. Linearity of the association between the different exposure variables and outcomes was assessed by using Generalized Additive Models (GAM). Since there was no evidence of non-linearity, BPA and phthalate concentrations were treated as continuous variables. Sensitivity analyses were performed for each single phthalate metabolite. In order to differentiate the role and importance of each exposure assessed, we also performed a multipollutant model where the four main exposure variables (BPA, Σ_4DEHP, MBzP and Σ_3LMWP) where included. Finally, because previous studies have suggested that associations may differ by child sex (Bertelsen et al. 2012; Vaidya and Kulkarni 2012), we tested this interaction. Analyses were conducted by using STATA software, version 12.0 (StataCorp, College Station, TX), and R statistical package version 3.0.2.

3. RESULTS

3.1 Study population characteristics

This study included mother-child pairs with information on wheeze, chest infections, bronchitis and eczema in at least one period of follow-up between birth and 7 years. From the 657 pregnant women initially recruited in the INMA-Sabadell cohort, 608 provided the outcome information, and, out of these, 462 had information on

prenatal BPA and 391 on prenatal phthalates. Children not included in the present study (those without outcome or BPA/phthalate data) had lower prevalence of wheeze and chest infections at the age of 14 months compared to included children. Also, their mothers were younger, more likely to be primiparous and had lower education level than those included (results not shown).

In the current study population the prevalence of wheeze and chest infections decreased from birth until 7 years of age, and the prevalence of bronchitis and eczema was lowest at 6 months of age (Table 1). The median BPA concentration (average of two trimesters) was 2.4 μg/g creatinine (Table 2). Among phthalate metabolites, the highest median concentration was found for the low molecular weight phthalate metabolite MEP (405.3 μg/g creatinine), followed by Σ_4DEHP metabolites (101.7 μg/g creatinine). Concentration of MBzP was the lowest (11.9 μg/g creatinine) (Table 2). Moderate correlations were found between the different groups of compounds with Pearson correlation coefficients varying between 0.15 and 0.31. The highest correlations were observed between Σ_4DEHP and MBzP (r=0.31) and Σ_4DEHP and BPA (r=0.21) (results not shown).

3.2 BPA and respiratory and allergy outcomes

Adjustment for confounding factors had little influence on the risk estimates obtained (Table 3). The adjusted relative risks of wheeze (RR, 1.20; 95% CI, 1.03, 1.40), chest infections (RR, 1.15; 95% CI, 1.00, 1.32) and bronchitis (RR, 1.18; 95% CI, 1.00, 1.37) at any age

increased for each doubling in concentration of maternal urinary BPA (Table 3). No significant associations were found between maternal urinary BPA concentrations and risk of eczema (Table 3). When associations between BPA and wheeze were assessed at each age of follow-up the confidence intervals became somewhat wider, but the increased risks were consistent across the different ages (p-value for age interaction=0.80; Figure 1a). For chest infections and bronchitis relative risks results were also consistent across the ages (p-values>0.5; Figures 1b and 1c). Relative risks tended to be higher in girls than in boys but there was no statistical evidence that BPA associations differed between the sexes (p-values for interaction>0.15; data not shown).

3.3 Phthalates and respiratory and allergy outcomes

As with BPA, adjustment for confounding factors had little influence on the risk estimates obtained (Table 3). The adjusted relative risks of wheeze (RR, 1.25; 95% CI, 1.04, 1.50), chest infections (RR, 1.15; 95% CI, 0.97, 1.35) and bronchitis (RR, 1.20; 95% CI, 1.01, 1.44) at any age increased for each doubling in concentration of maternal urinary Σ_4DEHP (Table 3). Secondary (MEHHP, MECPP, MEOHP) metabolites showed the strongest associations, whereas no associations were found for the primary metabolite MEHP ("see Table E3 in the Online Repository"). MBzP was associated with an increased risk of wheeze at any age (RR, 1.15; 95% CI, 1.00, 1.33); smaller and non-significant increases were found for chest infections (RR, 1.08; 95% CI, 0.95, 1.23) and bronchitis (RR, 1.06; 95% CI, 0.92, 1.22). Concentrations of

prenatal Σ_3LMWP metabolites in maternal urine were not associated with any of the outcomes assessed (Table 3). None of the phthalate metabolites were associated with eczema (Table 3). As with BPA, associations between Σ_4DEHP and wheeze were consistent over the repeated age points (p-value for age interaction 0.43; Figure 1a). Associations with chest infections remained until the age of 4 years (p-value for interaction=0.09; Figure 1b), and for bronchitis they were consistent across the ages (p-value for interaction=0.81; Figure 1c). For MBzP increased wheeze risks were mainly seen at age 7 (p-value for age interaction 0.11; Figure 1). RRs for the associations between MBzP and wheeze were greater in girls than boys (girls: RR, 1.28; 95% CI, 1.03, 1.58, boys: RR, 0.96; 95% CI, 0.81, 1.13, interaction p-value=0.02); a similar pattern was observed for chest infections (p-value for interaction=0.06). DEHP associations tended to be higher in girls than in boys but these differences were not statistically significant (p-values for interaction>0.10; data not shown).

Including all four main groups of pollutants (BPA, Σ_4DEHP, MBzP, Σ_3LMWP) in one multipollutant model led to small reductions of between 1 and 8% in the relative risk estimates compared to the single pollutant model ("see Table E4 in the Online Repository").

4. DISCUSSION

This is the first prospective study evaluating the association between urine biomarkers of prenatal exposure to both BPA and phthalates and respiratory outcomes and eczema in children at

repeated ages from birth until the age of 7 years. We found that higher concentrations of BPA and higher molecular weight phthalates (Σ_4DEHP and MBzP) in maternal urine during pregnancy were associated with increased risk of wheeze and respiratory infections (including chest infections and bronchitis) in offspring during childhood. These associations were generally consistent over the repeated child ages. Also, there was little evidence that results were influenced by confounding factors.

Urine BPA concentrations in the present study were of similar magnitude as those reported previously in two birth cohort studies on BPA and respiratory health in children from the United States (Donohue et al. 2013; Spanier et al. 2012). In one of these studies mean prenatal BPA concentrations were associated with higher risk of wheeze at age 6 months, although the association did not last until age 3 years (Spanier et al. 2012). The other study reported that higher urinary BPA concentrations measured during the 3rd trimester of pregnancy were associated with reduced risk of wheeze at age 5 years and, on the contrary, postnatal urinary levels were found to be related to increased risk of wheeze and asthma at 5-7 years of age (Donohue et al. 2013). In the present study we found an increased risk of wheeze and respiratory infections during childhood, and this was consistent over the ages points up to age 7 for wheeze and up to age 4 for respiratory infections. Additionally, associations with BPA were robust to the inclusion of phthalates in multivariate models.

Many studies have reported a potential relationship between phthalates and allergic symptoms including asthma and related symptoms in children (Bertelsen et al. 2012; Bornehag et al. 2004; Callesen et al. 2014; Hoppin et al. 2013; Hsu et al. 2012; Just et al. 2012a; Kolarik et al. 2008). However, most of these studies have a case-control (Bornehag et al. 2004; Callesen et al. 2014; Hsu et al. 2012; Kolarik et al. 2008) or cross-sectional (Bertelsen et al. 2012; Hoppin et al. 2013; Just et al. 2012a) design, or have assessed phthalates' levels in dust as a marker of phthalate exposure (Bornehag et al. 2004; Callesen et al. 2014; Hsu et al. 2012; Kolarik et al. 2008); this limits the conclusions that can be drawn. In the present study the risk of wheeze and respiratory infections increased with increasing concentration of Σ_4DEHP in maternal urine during pregnancy, whereas increasing concentration of MBzP was mainly associated with wheeze. No associations with Σ_3LMWP concentrations and offspring assessed outcomes were observed. Also, we found that secondary rather than primary DEHP metabolites were more likely to be associated with risk of child respiratory outcomes. Differences in results between congeners and metabolites might be due to their different physico-chemical properties (Bornehag et al. 2004) and hormonal activity action (Christen et al. 2010; Okubo et al. 2003). A previous birth cohort study observed an association between maternal urinary MBzP concentrations during pregnancy and eczema in offspring before 24 months of age, but not at older ages (5 years) (Just et al. 2012b). We did not observe an association between prenatal MBzP, or any phthalate metabolite, and eczema at any age during childhood.

Both BPA (Rogers et al. 2013), and in a lesser extent phthalates (Christen et al. 2010; Okubo et al. 2003), mimic estrogen activity, a female hormone which seems to play an active role in immunomodulation in women (Bonds and Midoro-Horiuti 2013). Additionally, incidence and severity of allergic disorders are higher in adult females (Bonds and Midoro-Horiuti 2013). Two studies reported higher risk of allergic asthma in females in relation to BPA (Vaidya and Kulkarni 2012) and DEHP metabolites (Bertelsen et al. 2012) exposure. In the present study girls seemed to be at higher risk of suffering from respiratory infections and wheeze in relation to BPA, Σ_4DEHP, and MBzP exposure. However, statistically significant interactions were only observed for MBzP, and differences between sexes were not always consistent across similar outcomes (i.e. chest infections and bronchitis) for one same exposure. The lack of more consistency among results obtained after stratifying by sex could be partly explained by limitations in the size of our study population. In addition, we cannot rule out the possibility that until 7 years of age, sex differences in allergic symptoms/diseases are not completely manifested. For example, in the present study it was not possible to evaluate the associations, and thus the interaction with sex, between the exposures of interest and asthma because at age 4 and 7 years its prevalence was very low (0.7% and 2.6%, respectively). Thus, larger studies are warranted to confirm the potential role of these compounds in the etiology of asthma in childhood.

Although there are some studies trying to explain the mechanisms by which BPA and phthalates affect the immune and respiratory systems, these are not completely understood. In animal and in vitro models, BPA has been described to increase the production of the proallergic interleukin (IL) 4 and serum immunoglobulin E (IgE) and to promote eosinophilic inflammation in the airways (Kwak et al. 2009; Midoro-Horiuti et al. 2010). For phthalates, the mechanism seems to be their capacity to act as adjuvants which promote Th_2 differentiation and influence antibody response (Bornehag and Nanberg 2010; Kimber and Dearman 2010). Also, DEHP has been described to alter airway cell differentiation and surfactant protein production in the lungs (Miller and Marty 2010). If results of the present study are confirmed in future studies, the inclusion of humoral and cellular immunity markers in child birth cohort studies would help researchers in understanding the mechanistic pathways of the potential effects of BPA and phthalates on the respiratory and immune systems in children (Tryphonas 2001). Although the allergy pathway is related to a bias towards Th2 cells, it has been observed that both a suppressive effect on Th1 and Th2 cells can take place (Warner 2004) and that diseases of both cell types coexist more frequently than might be expected by chance (Simpson et al. 2002). If prenatal exposure to BPA and high molecular weight phthalates affects both Th1 and Th2, this would explain the association observed in the present study between these exposures and both wheeze and respiratory infections.

BPA and phthalates have a very short half-life (of hours) and are rapidly excreted from the body. Thus, one single measurement is

not representative of a long period of time (i.e. the whole pregnancy) (Braun et al. 2013; Casas et al. 2013; Chevrier et al. 2013; Frederiksen et al. 2013; Hoppin et al. 2002). For this reason, in the present study we used the average concentration determined in two times during pregnancy (1st and 3rd trimester) to mitigate prenatal BPA and phthalate exposure misclassification of children. But according to several studies (Braun et al. 2013; Chevrier et al. 2013; Frederiksen et al. 2013; Hoppin et al. 2002), including ours (Casas et al. 2013), the use of two measurements might not be enough to avoid exposure misclassification because of the low correlation among serial urinary BPA and phthalate measurements from the same individual. This non-differential misclassification of the exposure is likely to result in dilution of risk estimates (Pollack et al. 2013), which means that in our study there are more chances of an underestimation of the risks than an overestimation. In any case, future studies need to perform multiple exposure assessments along pregnancy in order to provide more realistic and better exposure and risk estimates. Finally, it was not possible to assess whether postnatal exposure was also associated with these respiratory outcomes because of the unavailability of such information. On the contrary, and for the first time in a birth cohort study, we were able to include in one model the different compounds analyzed; these results showed that the compounds more strongly associated to wheeze and chest infections were BPA and Σ_4DEHP, whereas associations between MBzP and wheeze were considerably weaker.

The present study suggests that prenatal exposure to BPA and high molecular weight phthalates (Σ_4DEHP and MBzP) may increase risk of wheeze and respiratory infections at repeated ages until childhood. Future studies with improved pre and postnatal exposure estimates and with larger study populations to allow stratification by child sex are needed. Also, mechanisms underlying the suggested associations warrant further investigation. In the meanwhile, and where possible, policies to reduce exposure to such compounds should be advocated.

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TABLES AND FIGURES

Table 1. Prevalence of respiratory outcomes and eczema.

	BPA (N=462)		Phthalates (N=391)	
	N	%	N	%
Wheeze				
6m	437	19.7	370	20.8
14m	424	30.4	382	30.1
4y	385	21.8	387	22.2
7y	361	11.1	361	11.4
Chest infections				
6m	445	22.0	377	23.3
14m	423	33.8	381	32.3
4y	385	2.9	387	2.6
7y	361	7.5	361	8.3
Bronchitis				
6m	445	16.0	377	17.2
14m	423	25.3	387	24.4
4y	385	24.4	370	24.8
Eczema				
6m	437	11.7	371	12.9
14m	421	17.8	380	18.7
4y	385	23.6	387	23.8
7y	361	18.3	361	17.7

BPA: Bisphenol A.

Table 2. BPA (N=462) and phthalate (N=391) metabolite levels (μg/g creatinine)[a].

	Median	25^{th}-75^{th} percentile	Min-Max
BPA	2.4	1.7-3.7	0.3-69.4
Phthalates			
Σ_4DEHP metabolites	101.7	69.5-147.9	26.5-1670.0
MEHP	11.0	7.3-17.2	1.8-266.9
MEHHP	28.0	17.9-41.5	5.3-503.4
MEOHP	20.9	14.3-30.3	4.1-378.3
MECPP	39.5	27.2-59.8	7.7-718.9
MBzP	11.9	7.2-20.1	1.5-405.1
Σ_3LMWP metabolites	483.0	265.3-866.6	65.2-10003
MEP	405.3	199.4-804.0	34.0-9379.8
MiBP	31.4	21.7-48.2	5.1-334.2
MnBP	30.7	19.9-47.3	5.8-835.7

BPA: Bisphenol A, MEHP: Mono-(2-ethylhexyl) phthalate, MEHHP: Mono-(2-ethyl-5-hydroxyl-hexyl) phthalate, MEOHP: Mono-(2-ethyl-5-oxo-hexyl) phthalate, MECPP: Mono-(2-ethyl-5-carboxy-pentyl) phthalate, MBzP: Mono-benzyl phthalate, MEP: Mono-ethyl phthalate, MiBP: Mono-iso-butyl phthalate, MnBP: Mono-n-butyl phthalate, DEHP: Di-(2-ethylhexyl) phthalate, LMWP: low molecular weight phthalates.

[a]Average of measurements at two time points in the 1^{st} and 3^{rd} trimester of pregnancy.

Table 3. Associations between maternal urinary BPA and phthalate metabolite levels[a] and occurrence of respiratory outcomes and eczema during childhood[b].

	BPA RR (95%CI)	Σ_4DEHP[c] RR (95%CI)	MBzP RR (95%CI)	Σ_3LMWP[d] RR (95%CI)
N	462	391	391	391
Unadjusted				
Wheeze	1.16 (1.00, 1.35)	1.29 (1.08, 1.54)	1.15 (1.00, 1.32)	0.99 (0.86, 1.14)
Chest infections	1.10 (0.96, 1.26)	1.18 (1.00, 1.39)	1.07 (0.95, 1.22)	1.02 (0.90, 1.16)
Bronchitis	1.12 (0.97, 1.30)	1.22 (1.03, 1.46)	1.06 (0.92, 1.22)	0.96 (0.84, 1.11)
Eczema	0.99 (0.84, 1.16)	1.05 (0.88, 1.26)	1.07 (0.93, 1.23)	0.94 (0.82, 1.08)
Adjusted				
Wheeze	1.20 (1.03, 1.40)	1.25 (1.04, 1.50)	1.15 (1.00, 1.33)	0.95 (0.82, 1.10)
Chest infections	1.15 (1.00, 1.32)	1.15 (0.97, 1.35)	1.08 (0.95, 1.23)	1.00 (0.88, 1.14)
Bronchitis	1.18 (1.00, 1.37)	1.20 (1.01, 1.44)	1.06 (0.92, 1.22)	0.95 (0.82, 1.09)
Eczema	1.00 (0.85, 1.18)	1.00 (0.83, 1.20)	1.05 (0.91, 1.21)	0.91 (0.79, 1.05)

BPA: Bisphenol A, DEHP: Di-(2-ethylhexyl) phthalate, MBzP: Mono-benzyl phthalate, LMWP: low molecular weight phthalates.

BPA models adjusted for maternal education, number of siblings and maternal smoking during pregnancy, and phthalates models additionally adjusted for mother history of asthma/allergy and maternal BMI.

[a]RR per doubling concentration (levels were $\log_2$-transformed).

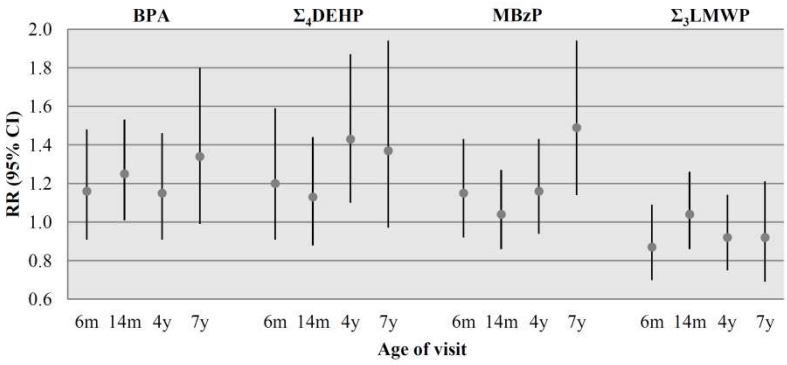
[b]Wheeze, chest infections and eczema assessed at ages 6 and 14 months and 4 and 7 years, bronchitis at ages 6 and 14 months and 4 years.

[c]The Σ_4DEHP metabolites include MEHHP, MEHP, MEOHP, MECPP.

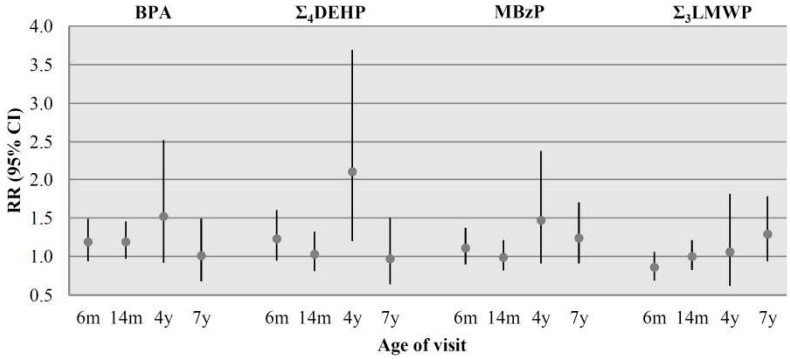
[d]The Σ_3LMWP metabolites include MEP, MiBP, MnBP.

Figure 1. Associations between maternal urinary BPA and phthalate metabolite levels[a] and occurrence of wheeze, chest infections and bronchitis in each follow-up from birth until the age of 7 years.

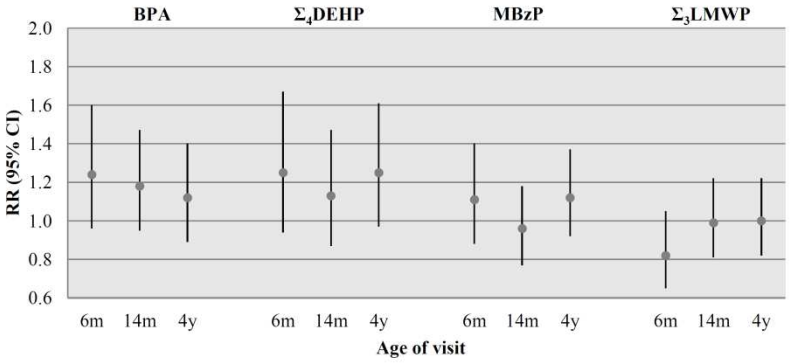
1a. Wheeze



1b. Chest infections



1c. Bronchitis



BPA: Bisphenol A, DEHP: Di-(2-ethylhexyl) phthalate, MBzP: Mono-benzyl phthalate, LMWP: low molecular weight phthalates.
[a]RR per doubling concentration (levels were $\log_2$-transformed).
[b]The Σ_4DEHP metabolites include MEHHP, MEHP, MEOHP, MECPP.
[c]The Σ_3LMWP metabolites include MEP, MiBP, MnBP.

SUPPLEMENTAL MATERIAL

Table E1. Description of the imputation procedure.

Software used and key setting: STATA 12 software (Stata Corporation, College Station, Texas) –*ice* command (with 100 cycles)

Number of imputed datasets created: 100

- **Variables included in the imputation procedure:**
  - Child's variables: sex, gestational age, birth weight, weeks of breastfeeding, season of birth, siblings, daycare attendance. For the imputation of the lung function databases, age and height of the child at the time of performing the lung function test were included.
  - Parental variables: maternal age, pre-pregnancy maternal weight and height, maternal and paternal asthma, rhinitis, eczema and atopy, maternal education, maternal smoking during pregnancy and 1st year of life, maternal total IgE, maternal country of origin, parity.
  - Other variables: time of sample collection (1^{st} and 3^{rd} trimester), BPA and all phthalates measured in maternal urine (1^{st} and 3^{rd} trimester), consumption of canned tuna (1^{st} and 3^{rd} trimester).
- **Treatment of non-normally distributed variables:** $\log_2$-transformed.
- **Treatment of binary/categorical variables:** logistic, ordinal, and multinomial models.
- **Statistical interactions included in imputation models:** imputations were done separately for each study population analyzed [BPA (N=462), phthalates (N=391) and all compounds (N=359)]. The same for the lung function databases [BPA (N=276) and phthalates (N=278)].
- **Percentage of samples under de LOD**:
  - BPA: 0% of the 1^{st} trimester samples and 0.6% of the 3^{rd} trimester samples.
  - Phthalates: from 0 to 0.5% of the 1^{st} trimester samples and from 0 to 0.8% of the 3^{rd} trimester samples, depending on the metabolite.

Table E2. Characteristics of the study population and median levels of BPA and phthalate metabolites (µg/g creatinine) in maternal urine samples.

			Phthalate metabolites								
	% (N=462)	BPA	% (N=391)	MEHHP	MEHP	MEOHP	MECPP	MBzP	MEP	MiBP	MnBP
Child characteristics											
Sex											
Girls	48.4	2.45	48.1	28.02	10.82	20.99	39.70	12.32	443.12	33.20	30.97
Boys	51.6	2.46	51.9	27.92	11.06	20.83	39.48	11.66	350.01	30.54	29.03
Birth season											
Winter	26.5	2.57*	26.3	28.72*	11.16	21.17*	39.35*	12.71	491.87	31.37	31.31*
Spring	26.7	2.14	25.6	23.85	10.26	18.86	34.91	12.12	419.42	27.31	26.51
Summer	25.4	2.99	26.9	28.37	11.81	22.15	43.45	11.51	401.04	37.44	36.47
Autumn	21.5	2.32	21.2	30.00	10.04	21.98	39.96	10.90	306.49	30.57	25.35
Siblings											
None	55.2	2.76*	57.6	26.26	10.27*	20.21	37.51	11.91	393.72	31.83	32.77
One	37.8	2.23	37.1	31.42	11.72	23.15	43.81	11.35	423.86	31.39	27.99
Two or more	7.0	2.00	5.4	26.02	8.50	22.67	41.31	13.03	458.24	27.29	25.35
Maternal characteristics											
Age (years)											
≤25	11.3	3.04*	10.0	30.96	11.06	20.33	45.27	10.20*	317.82	31.65	33.73
>25 to 30	39.9	2.46	40.8	26.26	11.18	20.49	38.47	13.39	408.71	33.51	32.34
>30 to 35	36.3	2.36	36.2	28.36	10.66	21.17	40.30	10.78	437.60	30.21	26.93
>35	12.6	2.08	13.1	26.02	11.34	21.17	39.81	10.80	347.76	30.84	32.77
Education											
Primary school	26.8	2.74*	22.4	29.80	12.59*	21.74	43.90	12.71	536.68*	35.60*	31.04
Secondary school	41.0	2.34	42.7	26.24	9.80	20.21	38.84	10.77	405.28	29.87	29.03
University or higher degree	32.2	2.56	34.9	27.37	11.06	21.17	38.49	12.70	328.51	31.98	32.65

	% (N=462)	BPA	% (N=391)	Phthalate metabolites MEHHP	MEHP	MEOHP	MECPP	MBzP	MEP	MiBP	MnBP
BMI											
≤18.5	5.8	1.94	5.6	27.81	10.02	20.19	36.66*	11.51*	351.56	27.54*	26.21
>18.5 to 25	66.7	2.59	67.8	26.26	11.06	20.53	38.42	11.01	393.72	31.46	30.80
>25 to 30	18.8	2.40	18.2	32.02	11.58	22.67	48.07	13.82	458.24	34.48	36.18
>30	8.7	2.22	8.4	28.36	9.11	20.48	43.50	11.91	543.8	27.93	27.54
Prenatal smoking											
No	84.6*	2.37*	85.1	27.14	10.96*	20.60	39.35	11.89	373.45*	30.57*	30.09
Yes	15.4	3.05	14.9	31.40	12.37	22.49	39.70	11.33	562.73	35.65	38.15
Maternal allergy[a]											
No	68.7	2.63	67.9	26.06*	10.96	20.10*	38.46*	11.99	401.04	31.98	30.78*
Yes	31.3	2.14	32.1	32.02	11.06	23.46	44.62	11.56	474.63	30.79	30.71

BPA: Bisphenol A, MEHHP: Mono-(2-ethyl-5-hydroxyl-hexyl) phthalate, MEHP: Mono-(2-ethyl-hexyl) phthalate, MEOHP: Mono-(2-ethyl-5-oxo-hexyl) phthalate, MECPP: Mono-(2-ethyl-5-carboxy-pentyl) phthalate, MBzP: Mono-benzyl phthalate, MEP: Mono-ethyl phthalate, MiBP: Mono-isobutyl phthalate, MnBP: Mono-n-butyl phthalate.

*p-value ≤0.1.

[a]Mothers were classified as allergic if they answered positively to suffer from allergic asthma, atopic dermatitis, eczema or rhinitis during the health questionnaire of the 3rd trimester of pregnancy.

Table E3. Associations between maternal urinary single DEHP metabolite levels[a] and occurrence of respiratory outcomes and eczema during childhood[b] (N=391).

	MEHP[c] RR (95%CI)	MEHHP[c] RR (95%CI)	MEOHP[d] RR (95%CI)	MECPP[d] RR (95%CI)
Wheeze	1.07 (0.90, 1.26)	1.22 (1.04, 1.45)	1.27 (1.07, 1.51)	1.25 (1.05, 1.49)
Chest infections	0.96 (0.82, 1.12)	1.16 (1.00, 1.34)	1.18 (1.01, 1.38)	1.16 (0.99, 1.37)
Bronchitis	1.02 (0.87, 1.21)	1.22 (1.04, 1.43)	1.22 (1.03, 1.45)	1.20 (1.01, 1.43)
Eczema	0.93 (0.78, 1.10)	1.03 (0.88, 1.21)	1.01 (0.84, 1.20)	1.01 (0.85, 1.20)

Models adjusted for maternal education, number of siblings, maternal smoking during pregnancy, mother history of asthma/allergy and maternal BMI.

[a]RR per doubling concentration (levels were log_2-transformed).

[b]Wheeze, chest infections and eczema assessed at ages 6 and 14 months and 4 and 7 years, bronchitis at ages 6 and 14 months and 4 years.

Primary[c] [MEHP: Mono-(2-ethyl-hexyl) phthalate], secondary[d] [MEHHP: Mono-(2-ethyl-5-hydroxyl-hexyl) phthalate, MEOHP : Mono-(2-ethyl-5-oxo-hexyl) phthalate and MECPP: Mono-(2-ethyl-5-carboxy-pentyl) phthalate] DEHP metabolites.

Table E4. Associations between maternal urinary BPA and phthalate metabolite levels[a] and occurrence of respiratory outcomes and eczema during childhood[b] in a multipollutant model (N=373).

	BPA RR (95%CI)	Σ_4DEHP[c] RR (95%CI)	MBzP RR (95%CI)	Σ_3LMWP[d] RR (95%CI)
One pollutant model				
Wheeze	1.18 (1.00, 1.40)	1.25 (1.03, 1.51)	1.11 (0.95, 1.28)	0.90 (0.78, 1.05)
Chest infections	1.17 (1.00, 1.36)	1.16 (0.98, 1.38)	1.07 (0.94, 1.23)	1.01 (0.89, 1.16)
Bronchitis	1.17 (0.99, 1.38)	1.22 (1.01, 1.47)	1.03 (0.89, 1.20)	0.94 (0.82, 1.09)
Eczema	1.00 (0.84, 1.19)	1.02 (0.84, 1.24)	1.06 (0.92, 1.23)	0.91 (0.79, 1.06)
Four pollutants model				
Wheeze	1.16 (0.97, 1.37)	1.21 (0.99, 1.48)	1.06 (0.91, 1.23)	0.87 (0.74, 1.01)
Chest infections	1.14 (0.98, 1.34)	1.11 (0.93, 1.34)	1.03 (0.89, 1.19)	0.97 (0.84, 1.12)
Bronchitis	1.14 (0.96, 1.36)	1.20 (0.98, 1.47)	0.98 (0.84, 1.15)	0.91 (0.79, 1.06)
Eczema	1.01 (0.84, 1.20)	1.00 (0.82, 1.24)	1.08 (0.92, 1.26)	0.90 (0.78, 1.05)

BPA: Bisphenol A, DEHP: Di-(2-ethylhexyl) phthalate, MBzP: Mono-benzyl phthalate, LMWP: low molecular weight phthalates.

In order to have the same adjusted models, all were adjusted for maternal education, number of siblings, maternal smoking during pregnancy, mother history of asthma/allergy and maternal BMI.

[a]RR per doubling concentration (levels were log_2-transformed).

[b]Wheeze, chest infections and eczema assessed at ages 6 and 14 months and 4 and 7 years, bronchitis at ages 6 and 14 months and 4 years.

[c]The Σ_4DEHP metabolites include MEHHP, MEHP, MEOHP, MECPP.

[d]The Σ_3LMWP metabolites include MEP, MiBP, MnBP.

6. GENERAL DISCUSSION

Exposure to environmental pollutants such as persistent organic pollutants (POPs), bisphenol A (BPA) and phthalates is thought to influence the development and functioning of respiratory and immune systems, even at low levels of exposure. Because fetal life is a critical period in which environmental pollutants can have an underappreciated but critical impact on children and adult's immune and respiratory health, the present thesis focused on the early and long-term immune and respiratory health effects of current prenatal exposure to these compounds. Also, we hypothesized that cytokines and biomarkers of inflammation can provide information on the mechanisms behind such associations. The results of the different studies included in the present thesis have already been presented and discussed in the previous sections. Here, a more general discussion of the general methodological issues, the main contribution to current knowledge, implications for public health and suggestions for further research are provided.

6.1 Methodological considerations

Except for the systematic review, the studies included in the present thesis were based on prospective population-based cohort studies with a follow-up from fetal life onwards. Three of the studies used data from the Infancia y Medioambiente (INMA) birth cohort project, located in Spain, whereas a fourth study included data from six additional European birth cohort projects, providing more statistical power to our analyses. Given their prospective nature, birth cohorts are the best approach to properly assess the relationship between early environmental exposures and the long-term effects associated (Vrijheid et al. 2012). Furthermore, they allow exposure, outcome and covariates assessment at different time points and re-call bias is easier to avoid than in other study designs (Luo et al. 2010). However, the present work also presents some limitations, mainly regarding exposure and outcome assessment.

6.1.1 Exposure assessment

Persistent organic pollutants

POPs have a long half-life (of years) and therefore one single measurement is considered to be representative of fetal exposure along the whole pregnancy (Glynn et al. 2011). In this sense, in our studies the risk of exposure misclassification is very low. However, the different POPs present in human tissues are generally highly correlated, which means that the results found for one compound (i.e. DDE) could be explained by another (i.e. HCB). In most of the

European birth cohort studies included in the present thesis correlations between DDE, PCBs or HCB were lower ($r<0.50$; see Paper I and III) than correlations described in previous studies (Dallaire et al. 2004, 2006; Glynn et al. 2008), which facilitates interpretation of our results. Additionally, to address this issue, we performed multipollutant models (see Paper I, III and IV), and in the meta-analysis we also assessed robustness of the results by excluding the different participating cohorts, which have different exposure profiles, from the main analysis one by one (see Paper III). In all sensitivity analyses associations between DDE and the respiratory outcomes assessed (wheeze and respiratory infections) under the age of ~2 years remained. Furthermore, in the Latin-American population, with high DDE levels and very low HCB and PCBs levels, the risk of wheeze and LRTIs was also increased with increasing prenatal DDE exposure (see Paper I), which reinforces the results obtained in Paper I and Paper III for European populations. Finally, most of the PCB congeners included in the present work were non dioxin-like PCBs, which have different mechanisms of action than those of dioxin-like PCBs and therefore potentially different health effects (Giesy et al. 2000). We did not find associations between the non dioxin-like PCBs and the respiratory and immune health effects assessed; however, it is important to understand that we cannot draw any conclusions for dioxin-like PCB congeners or dioxin compounds, as they were not assessed in the present work.

BPA and phthalates

Unlike POPs, BPA and phthalates have a much shorter half-life (of a few hours to a few days) and therefore a single measurement is not enough to properly assess fetal exposure during the whole pregnancy (Braun et al. 2013; Chevrier et al. 2013; Hoppin et al. 2002). According to several studies, the use of two measurements, which is what we did in the present thesis, is likely not to be enough to avoid exposure misclassification. The correlation among serial urinary BPA and phthalate measurements from the same individual has been described to be low in other studies (Braun et al. 2013; Casas et al. 2013; Chevrier et al. 2013; Frederiksen et al. 2013; Hoppin et al. 2002) and in ours (Casas et al. 2013). Because there are no reasons to think that misclassification of participants regarding exposure would be differential (i.e. related to their outcome status), our results are more likely to suffer from an underestimation of the risks than from an overestimation (Pollack et al. 2013). In any case, further studies addressing this issue and providing better exposure estimates are needed.

The role of postnatal exposures

We could evaluate the role of postnatal exposures in the development of respiratory infections and asthma symptoms in only one of our studies, which included data from the Menorca birth cohort (see Paper IV). In a study assessing the role of pre and postnatal POPs exposure on children's neurodevelopment, estimated postnatal POP exposures through PBPK models were not associated to motor or cognitive function of children, and only

associations with prenatal PCB153 exposure were found (Gascon et al. 2013b), supporting results of previous studies which most of them did not find associations with postnatal exposures, including a study that used data from the Menorca birth cohort (Forns et al. 2012). The information obtained through PBPK models was also used to evaluate the association between estimated postnatal POP exposure and wheeze and LRTIs in the 1st year of life, and no associations were obtained (data not published in Paper I). Furthermore, from the studies included in the systematic review, we could not find consistent results supporting an effect of postnatal exposure to POPs on the immune and respiratory health of children, at least with current levels of exposure (see Paper II). Overall, evidence seems to indicate that prenatal life is a more critical period than postnatal life in relation to POPs exposure; however, further studies are necessary to confirm our results and to better understand how pre and postnatal exposures may interact. For BPA and phthalates the situation is a little bit different, since only one cohort study assessed the health effects of both pre and postnatal exposures and did find an increased risk of wheeze only for the later (Donohue et al. 2013). However, the correlation between prenatal maternal BPA and postnatal child BPA levels at the age of 4 years has been described to be practically inexistent in the cohort of Sabadell (Casas et al. 2013). Thus, the results obtained for prenatal exposure in the present work (see Paper V) are not expected to be confounded by postnatal exposures, independently of the potential associations of these with respiratory and allergy outcomes.

Influence of the metabolism

Referring to a paper on prenatal chlordecone exposure and birth outcomes (Kadhel et al. 2014), Savitz et al. highlighted maternal metabolism as an important factor determining levels of biomarkers of exposure, and that there could be reverse causation if this metabolism was also related to the outcome of interest (Savitz 2014). This is certainly an issue to consider when interpreting results and might be relevant for certain outcomes, but we do not have evidence or reasons to think that mothers with allergy or with a worse immune response to infections, for instance, metabolize POPs, BPA or phthalates in a different manner than mothers not suffering from these immune alterations.

6.1.2 Outcome assessment

Use of questionnaires and objective tools

The limitations of using questionnaires to assess occurrence of respiratory symptoms or diseases are well known. However, in the studies included in the present work, the correlations with the different outcomes assessed, the decrease of prevalence of wheeze and respiratory infections and the increasing association of wheeze with atopy with age, and the high correlation between wheeze and asthma at older ages are in accordance with what is expected regarding changes in etiology of wheeze with increasing age (Nair et al. 2011; Stein and Martinez 2004). Thus, this ensures, somehow, that the data collected is reliable.

Regarding objective tools of outcome measurement, in the present thesis we did not include data on lung function parameters, which are more objective markers of respiratory health status than questionnaires. The reason not to include such data was that the number of children with data available on lung function and with acceptable tests was very limited (Paper IV and V, not mentioned in the papers). The skin prick test (SPT), used to define atopic status, was only used in one study. Thus, there are other tools apart from questionnaires that could be used in further follow-ups of the studied cohorts or in future studies in order to improve health status information.

Phenotypes

It is known that nonallergic mechanisms are intertwined with allergic mechanisms in many diseases (Antó et al. 2012). In the present work we had information on the occurrence of wheeze and respiratory infections, but in most of the studies we did not have information on other outcomes (i.e. atopy), or the prevalence of specific outcomes in children was very low and could not be included in the analyses (i.e. asthma in Paper V). Thus, we had limited information to actually assess the coexistence of different outcomes related to immune and respiratory health, and therefore to classify children into different phenotypes that would have provided more refined information. For instance, one of the questions could be whether the associations found in the present study are explained by an atopic pathway or not. In only one study of the present work atopy was assessed and no associations with prenatal POPs

exposure were described (Paper IV). Additionally, a previous study using the same data suggested that, if there is an association between prenatal POP exposure and respiratory health, it is not mediated by IgE or an atopic pathway (Sunyer et al. 2005, 2006). In any case, more refined information to better classify individuals are needed in future studies.

The influence of age

As already explained above, age is also a key factor because the occurrence and etiology of these outcomes change as children grow up. It is thus important to understand the behavior of such diseases along time, how they coexist and when is the best time to start assessing each of them, as certain respiratory/allergic diseases and markers of respiratory health are better evaluated or more representative of a particular health effect at certain age or ages. One example of this is that at early ages (below 4-7 years) asthma prevalence is quite low, as shown in the study populations of Menorca (Paper IV) and Sabadell (Paper V). Also, and as explained above, in the present thesis we did not include data on lung function parameters because the number of children with data available on lung function and with acceptable tests was very limited, and this was partly due to the early age of children (i.e. 4 and 7 years in Paper V), when acceptable lung function tests are harder to obtain. It is expected that participants will provide better and more acceptable results during adolescence in order to include this information in future studies and to better understand how the studied chemicals affect human respiratory health.

Cytokines and biomarkers of inflammation

In the present thesis we evaluated cytokines and biomarkers of inflammation as potential biomarkers explaining the association between prenatal POPs exposure and respiratory and immune health effects in children. Thanks to the multiplex technique, we could evaluate a few interleukins (IL8, IL10, TNF-α) in a small quantity of serum sample. However, this technique has also limitations, as explained in Paper IV, and other relevant interleukins involved in allergic reactions could not be evaluated (i.e. IL4 or IL5). This reduced our ability to understand the role of a wider range of biomarkers in the occurrence of respiratory infections and allergic events, and whether these biomarkers could explain the associations of the studied outcomes with early exposure to POPs. Another aspect that limits the interpretation of our results is that infections can influence cytokine 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). Thus, we do not know until which extent this issue influenced our results.

6.1.3 Susceptible populations

In the studies included in the present thesis we tried to determine whether certain subpopulations were more susceptible to the exposures evaluated. These were: *females* (Papers I, III and V) - incidence and severity of allergic disorders are higher in adult females (Bonds and Midoro-Horiuti 2013); *children from*

allergic/atopic mothers (Papers I and III) - studies suggest that *in-utero* immunological environment provided by the mother may confer additional susceptibility of children to develop certain allergy-related diseases or other immune diseases (Herberth et al. 2011; Warner 2004); *children with no breastfeeding or under the recommended 6 months* (Papers I and III) - studies have shown that breastfeeding reduces the risk of developing respiratory infections (Duijts et al. 2010; Morales et al. 2012; Puig et al. 2008, 2010); and *children from smoking mothers* (Papers I and III) - especially during pregnancy, maternal smoking has been found to increase the risk of wheeze and respiratory infections in children (Grabenhenrich et al. 2014; Karmaus et al. 2008; Keil et al. 2009; Metzger et al. 2013; Montgomery et al. 2013).

We did not find evidence of the existence of specific subpopulations susceptible to the effects of POPs, BPA or phthalates; however, the size of the study populations was probably not big enough to properly achieve this aim. The biggest study, with over 4000 participants, was a meta-analysis of 10 participant cohorts and not a pooled analysis of individual data, which also limited the capacity to identify interactions between the exposure and the covariates of interest (see Paper III). In relation to sex differences, the age of the children might have also influenced the results; whereas the prevalence of asthma is greater in boys than in girls during childhood, this trend reverses after puberty. If estrogenic activity of the studied compounds is partly explaining the increase of asthma, and if this especially occurs in females, then the interaction between the studied exposures and child sex will not

probably be observed until adolescence (Bonds and Midoro-Horiuti 2013). Finally, the information collected for some of the variables of interest was not as accurate as it might be necessary, which leads to misclassification and to the dilution of potential interactions. For instance, the definition of allergic/atopic status of the mother was based on self-reported questionnaires, but did not include information on atopy based on SPT or other objective tools. Furthermore, and as already pointed out in the previous section, allergic diseases are complex and heterogeneous and are not fully understood (Antó et al. 2012).

6.1.4 Confounding

Because of the prospective nature of birth cohorts, in the present work rich information on potential confounders was collected at different time points, including home, parental and child characteristics. Thus, a lot of variables were tested and the final models were adjusted for many covariables that influenced or could influence the studied associations (maternal education, age, BMI, allergic/asthmatic status and smoking, number of siblings, breastfeeding, etc). However, we cannot discard residual confounding of other variables that we were not aware of or that we did not explore (i.e. specific dietary patterns (Roduit et al. 2014)) and that could be explaining part of the increased risks, some of them quite small, found in our studies (i.e. an increased risk of wheeze or bronchitis of 3% with doubling prenatal DDE exposure in Paper III).

6.2 Contribution to the current knowledge

Overall, and according to the objectives, the present thesis contributed to: 1) the understanding of early and long-term immune and respiratory health effects of prenatal exposure to POPs, 2) the understanding of the mechanism behind such effects, 3) the understanding of the early and mid-term immune and respiratory health effects of prenatal exposure to BPA and phthalates, and 4) highlight the limitations of existing studies in the field and to provide recommendations for future research. More specific contributions are detailed below and results obtained in each study summarized in Table 6.1.

6.2.1 Persistent organic pollutants

Current state of the evidence

The systematic review conducted in the present work comprehensively evaluated the evidence of the effects of prenatal and postnatal POPs exposure on the immune and respiratory health of infants, children and adolescents (see Paper II). Based on the quality of the studies available and on the levels of evidence used by the International Agency for Research on Cancer, the review highlighted that for most of the exposure-outcome associations the evidence was inadequate, mainly due to the small number of studies and heterogeneity between them regarding exposure and outcome assessment. The only exposure-outcome combinations for which there was a little bit more of evidence, but still limited, were: prenatal DDE, PCBs and dioxins and respiratory infections and

Table 6.1 Summary of the main results obtained in the different studies included in the present thesis (except Paper II[a]).

Paper (study populations)	Prenatal exposures assessed	Outcomes assessed	Age of children at time of outcome assessment	Main results
Paper I (Sabadell, Gipuzkoa, Valencia)	DDE, PCBs, HCB	Wheeze, LRTIs	12-14 months	↑ risk of wheeze and LRTIs with increasing DDE
Paper III (10 European birth cohorts)	DDE, PCB153	Wheeze, bronchitis	From birth up to 4 years (<18 months and >18 months)	↑ risk of wheeze and bronchitis with increasing DDE only in children <18 months
Paper IV (Menorca)	DDE, PCBs, HCB	Wheeze, chest infections, atopy, asthma	1, 2, 3, 4, 6.5, 10 and 14 years[b]	↑ risk of wheeze at the age of 4 years with increasing DDE ↑ risk of wheeze and chest infections at the age of 10 years with increasing HCB
		IL6, IL8, IL10, TNF-α, CRP	4 years	↑ IL10 levels with increasing HCB, and in a lesser extent DDE and PCBs
Paper V (Sabadell)	BPA, phthalates	Wheeze, chest infections, bronchitis, eczema	6 and 14 months and 4 and 7 years	↑ risk of wheeze, chest infections and bronchitis with increasing BPA and high molecular weight phthalates[c]

BPA: bisphenol A; LRTIs: low respiratory tract infections.

[a]Paper II will be discussed in main text, as it is a systematic review of the evidence of the adverse effects of early-life exposure to POPs on respiratory helath, allergy and immune system.

[b]Sunyer et al. 2006 already published results including data collected until the age of 6.5 years.

[c]High molecular weight phthalates include DEHP metabolites and MBzP. Low molecular weight phthalates include MEP, MiBP and MnBP.

postnatal PCBs and reduced vaccination response. On the other hand, the review also pointed out that mechanisms remain largely unexplored. Based on the limitations highlighted, we provided recommendations for future research in the field. Moreover, we used these recommendations to conduct the meta-analysis included in the present work (see Paper III).

Early and long-term respiratory and immune health effects

Very few birth cohort studies assessed the association between prenatal DDE exposure and wheeze and respiratory infections occurring in early life of children (before ~2 years of age). Further, results between these studies were inconsistent; two of them found increased risk of respiratory infections (Dallaire et al. 2004; Dewailly et al. 2000) and two of them did not (Glynn et al. 2008; Sunyer et al. 2006). These studies had a study population that ranged between 65 and 199 subjects, except one that included 402 (Sunyer et al. 2006). In the present thesis, a first study including more than 1400 children from three different study regions reported increasing prenatal DDE exposure to increase the risk of wheeze and LRTIs at the age of 12-14 months. Furthermore, these effects were observed in two study populations with very different POP exposure profiles (Spanish and Latin American populations; see Paper I). In a second analysis in which ten European birth cohorts participated (N=4608 participants) - including those of Paper I and the birth cohort included in the paper of Sunyer et al. 2006 (the Menorca cohort) -, increasing prenatal DDE levels increased the risk of wheeze and bronchitis in children below 18 months of age

(see Paper III). In both Paper I and III, the results obtained were not influenced by the inclusion of other POPs (HCB and/or PCBs) in the models. Thus, the present thesis provides further evidence of the potential immune and respiratory health effects of DDE at early ages.

Only two studies assessed the role of prenatal DDE exposure in the respiratory health of children above 2 years of age (Dallaire et al. 2006; Sunyer et al. 2006). In summary, both studies reported increased risk of respiratory infections or asthma related symptoms at ages between 4 and 7 years of age. In the present work, we used the same study population as Sunyer et al. 2006, thus, we also obtained an increased risk of wheeze at the age of 4 years of children (see Paper IV). However, we did not observe associations at age 6.5, 10 or 14 years. In the study of Sunyer et al. 2006, increasing prenatal DDE was also associated with asthma occurrence at the age of 6.5 years, but we did not observe this effects at the age of 10 or 14 years (see Paper IV). Furthermore, in the European meta-analysis, no associations were observed between prenatal DDE exposure and wheeze occurrence from birth until the age of ~4 years (see Paper III). Overall, results suggest that the early-life effects of DDE do not persist at older ages. However, there are few studies available and therefore it would be interesting to keep on assessing the immune and respiratory long-term effects of DDE in existing birth cohorts that already assessed the early life effects, as are the cohorts of INMA-Sabadell, Gipuzkoa and Valencia.

No associations were observed between prenatal HCB exposure and respiratory infections or wheeze at early ages (see Paper I and IV). At older ages, an increased risk of wheeze and chest infections at age 10 years was observed (see Paper IV). However, in this study population we did not observe associations with asthma at age 10 or 14 years, but the number of asthma cases was very small. A recent study reported increased risk of asthma at the age of 20 years with increasing prenatal HCB (Hansen et al. 2014). These recent results demand further studies to confirm the long-term respiratory health effects of HCB.

We did not find associations between non dioxin-like PCBs and the respiratory and immune heath effects assessed both at early and older ages (see Paper I, III and IV); however, and as discussed above, it is important to understand that we cannot draw any conclusions for dioxin-like PCB congeners or dioxin compounds, as they were not assessed in the present work.

In summary, we show that DDE may increase the risk of respiratory infections and wheeze at early ages (until ~2 years); however we could not show long-term effects of prenatal exposure to this compound. We also suggest long-term effects of HCB on late wheeze and respiratory infections, however, our results need to be further explored, as well as the role of dioxin compounds and dioxin-like PCBs on the respiratory and immune health of children.

Biological mechanisms

We were the first to evaluate the effects of prenatal POPs exposure on the expression of cytokines and biomarkers of inflammation in

childhood in relation to respiratory and immune health outcomes (see Paper IV). IL10 measured at the age of 4 years was found to be increased with increasing prenatal POPs exposure, mainly HCB. According to the results, this association did not seem to be related to the association found between prenatal DDE and HCB exposure and respiratory health of children at different ages. Thus, cytokines evaluated in this study did not answer our research question but opened the door to new hypothesis, such as the role of IL10 as an early marker of chronic immunotoxic effects of POPs.

6.2.2 Bisphenol A

Respiratory and immune health effects in infants and children

When the present thesis started, there were no studies evaluating the immune and respiratory health effects of BPA. In the last three years, two birth cohort studies published contradictive results on the respiratory health effects of prenatal BPA exposure; one reported an increased risk of wheeze in infants (Spanier et al. 2012) and the other one a reduced risk in children (Donohue et al. 2013). In the present work, we found an increased risk of wheeze and respiratory infections with increasing prenatal BPA and this increase was generally consistent across ages until the age of 7 years (see Paper V). Further studies are needed to confirm the present results, but our study supports results of previous animal and in-vitro studies that suggest immune and respiratory health effects of prenatal exposure to BPA (Casals-Casas and Desvergne 2011; Kwak et al. 2009; Rogers et al. 2013). Additionally, our results also suggest that

females could be more sensitive to the effects of BPA, but this needs to be confirmed in further studies.

6.2.3 Phthalates

Respiratory and immune health effects in infants and children

Most of the studies available up to now studying phthalates compounds suggest that these chemicals, mainly high molecular weight phthalates, might have an effect on the respiratory system of children (Bertelsen et al. 2012; Bornehag et al. 2004; Callesen et al. 2013, 2014; Hoppin et al. 2013; Hsu et al. 2012; Just et al. 2012a, 2012b; Kolarik et al. 2008; Larsson et al. 2010). However, only one of these studies assessed prenatal phthalate exposure and did it in relation to eczema, itchy rash and IgE counts in children of 3 months to 5 years of age (Just et al. 2012b). Thus, our study was the first birth cohort study assessing the association between prenatal phthalate exposure and respiratory and allergy outcomes in childhood. We found an increased risk of wheeze and respiratory infections with increasing high molecular weight phthalates levels (DEHP and MBzP) and this increase was generally consistent across ages until the age of 7 years (see Paper V). No associations were found with low molecular weight phthalates, mainly used in cosmetics. Further studies are needed to confirm the present results, but our study provided additional evidence of the immune and respiratory health effects of prenatal exposure to high molecular weight phthalates, chemicals found in a number of consumer products. Additionally, and as with BPA, our results also suggest

that females could be more sensitive to the effects of high molecular weight phthalates, but this needs to be confirmed in further studies.

6.3 Implications for public health

The present thesis suggests effects of prenatal exposure to POPs, BPA and phthalate on the immune and respiratory systems of infants, children and adolescents. These effects were observed even at low levels of exposure. Our discussion provides many recommendations regarding the methodological aspects that ongoing and new birth cohort studies should try to address, mainly regarding refinement of exposure and outcome assessment. Here, we pretend to focus on the public health implications of our findings.

6.3.1 Persistent organic pollutants

DDT is probably the most controversial POP, as it is still being used to control mosquito *Anopheles*, responsible for the transmission of malaria. Furthermore, the global use of DDT has not changed substantially since the Stockholm Convention went into effect, leading also to high levels of exposure in inhabitants of affected areas (van den Berg et al. 2012; Carpenter 2011). In fact, in the present thesis we provide an example of these higher exposures in such populations, particularly in Latin American mothers (see Paper I). The use of DDT for malaria vector has been strongly defended by some organizations, by affirming that DDT "remains one of the

safest and most effective methods of saving lives from malaria" (Tren and Roberts 2010). However, evidence show that DDT and its metabolite DDE are not totally safe and that human health effects occur, even at low levels of exposure (Bouwman et al. 2011). Additionally, DDT mosquito resistance has increased in recent years and therefore in many places application of the pesticide is not that effective anymore (van den Berg et al. 2012). For all these reasons, currently there is a strong debate on whether this pesticide should be further used for malaria vector control. The present thesis has contributed to add evidence on the potential immunotoxic activity of DDE, and therefore has provided additional information for policy makers to include in this "hot" and complex debate, in which we will not enter since it is beyond the scope of the present work. Some regulatory bodies have provided recommendations to reduce exposure to POPs that include the reduction or elimination of certain food items, particularly specific type of fishes, from the diet of pregnant women and children because currently diet is the main source of POPs exposure in humans. However, a recent study showed that the effectiveness of such policies is very limited for persistent pollutants (Binnington et al. 2014), which reinforces the fact that the most effective way to reduce exposure to POPs in humans is to ban the use of these compounds. This means that in those countries where DDT is still in use or where POPs are still unintentionally emitted (i.e. dioxins or HCB) efforts to provide dietary recommendations to vulnerable population are not or will not be effective.

6.3.2 Bisphenol A and phthalates

Our results in relation to the immune and respiratory health effects of BPA and high molecular weight phthalates exposure during pregnancy are in accordance with previous studies based on animal and in-vitro models (Braun et al. 2013; Casals-Casas and Desvergne 2011; Kwak et al. 2009; Rogers et al. 2013; Talsness et al. 2009). Although further research addressing the limitations already discussed is needed, in the meanwhile, and advocating the precautionary principle, policies to reduce exposure to these compounds should be considered (The Lancet Diabetes Endocrinology 2013). This conclusion is not only based on the results obtained in the present work, but also on other studies that suggest that these compounds could be involved in many other diseases or health effects (Braun et al. 2013; Michałowicz 2014), and because daily human exposure occurs worldwide. In fact, some European countries, such as France, following the precautionary principle, have already taken some action to reduce BPA exposure from plastic food containers and baby drinking bottles.

6.4 Future research

6.4.1 Exposures

Persistent organic pollutants and non-persistent pesticides

Although most POPs are currently banned, some of them, including dioxins and in a lesser extent HCB, are still unintentionally produced as result of industrial processes and therefore are still released into the environment. In fact, dioxin and dioxin-like

compounds originated in incinerators and installations for the recovery or disposal of hazardous waste are in the spotlight because of their potential immunotoxic activity and suggested associations with cancer (García-Pérez et al. 2013). However, it is considered that after improvement in the burning of waste disposals, high-income countries have tightened the release of dioxins from incinerators, and that burning of household wastes in the backyard, a practice still very common in rural areas, especially in low-income countries, is a major source of dioxin formation (Carpenter 2011). In the present work we could not assess the immune and respiratory health effects of such compounds, but it would be certainly interesting to do it in countries where burning practices of household waste is common. Currently, there is an interesting new, cheap and quick technique that estimates exposure to dioxins and dioxin-like compounds, the Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX) bioassay. This technique is more biological relevant than old techniques because instead of measuring a number of individual congeners in a complex mixture it provides the total toxic equivalence of it (Vafeiadi et al. 2014). Thus, this is of special interest when studying the immune health effects of such mixtures.

Future research should also focus on areas affected by vector-borne diseases, such as malaria or Chagas diseases, in which significant amounts of pesticides – persistent (DDT) but also non-persistent (organophosphates, carbamates and pyrethroids) – are currently used. Usually, in these areas, a significant amount of pesticides is used for agriculture purposes as well, however, there

are limited ongoing birth cohorts or environmental health studies focused in child health (Harari et al. 2010; Suarez-Lopez et al. 2013; van Wendel et al. 2012). In this sense, CREAL and CRESIB, within the ISGlobal Alliance, are working together and are studying the possibility of starting new research lines in platforms that CRESIB has in Mozambique and in Bolivia, two middle/low-income countries with endemic diseases transmitted by insects. In any case, the immune and respiratory health effects of non-persistent pesticides need to be evaluated in westernized countries as well; although exposures to these compounds might not be as high as in low/middle-income countries, daily exposures occur through diet (Boon et al. 2008; Heudorf et al. 2004; Nougadère et al. 2012; Saieva et al. 2004), but there is limited information on the early and long-term immune and respiratory health effects of these compounds at these low levels of exposure in humans (Amaral 2014; Corsini et al. 2013).

Bisphenol A and phthalates

The main limitation of studying the health effects of BPA and phthalates is their short half life and therefore the difficulty is to obtain a good estimate representing exposure over a long period of time. This issue needs to be improved in future studies by obtaining multiple samples during the exposure period of interest (i.e pregnancy). Also, the role of postnatal exposure is not clear and needs to be further explored.

Multiple exposures

There is very little knowledge on the synergistic effects of multiple exposures to synthetic chemicals. Further, statistical tools used until now are somehow limited (see discussion Paper II). As we live in a world where everyday a number of synthetic compounds enter our bodies and therefore multiple exposures interact with our systems, efforts to evaluate the effects of such combined exposures are needed to understand the real health effects of such combinations. This concept of multiple exposures occurring along an individual's life and that can affect human health is nowadays known as the "exposome" (Wild 2012). In order to characterize early-life exposure to a wide range of environmental hazards and integrate and link these with data on major child health outcomes, CREAL is currently leading a FP7 European Project, called HELIX (http://www.projecthelix.eu/), which aims to exploit novel tools and methods to address the health effects of the "exposome" and that hopefully will provide answers to some of the open questions we still have.

6.4.2 Refining phenotypes

Understanding the coexistence of respiratory, allergic and atopic diseases and the association of these coexistences with different environmental exposures would provide key information on the mechanisms behind such associations: is the association between a certain pollutant and respiratory diseases explained by an atopic or allergic pathway, or it is totally independent of such processes?

These are the type of questions that need to be answered in future studies in order to define the mechanisms and the exact health effects associated to each environmental pollutant assessed. For that, and as discussed in section 6.1, future studies should try to collect the maximum information regarding immune and respiratory health outcomes, including biomarkers and outcomes measured with objective tools, in order to define the coexistence of the different outcomes assessed and therefore classify children into more refined phenotypes. In this sense, within the MEDALL project, big efforts are currently being done to understand the different phenotypes and to properly classify individuals (Antó et al. 2012). Additionally, this will help to evaluate the role of the *in-utero* immunological environment in the development of certain allergy-related diseases and its interaction with environmental exposures.

6.4.3 Long-term health effects

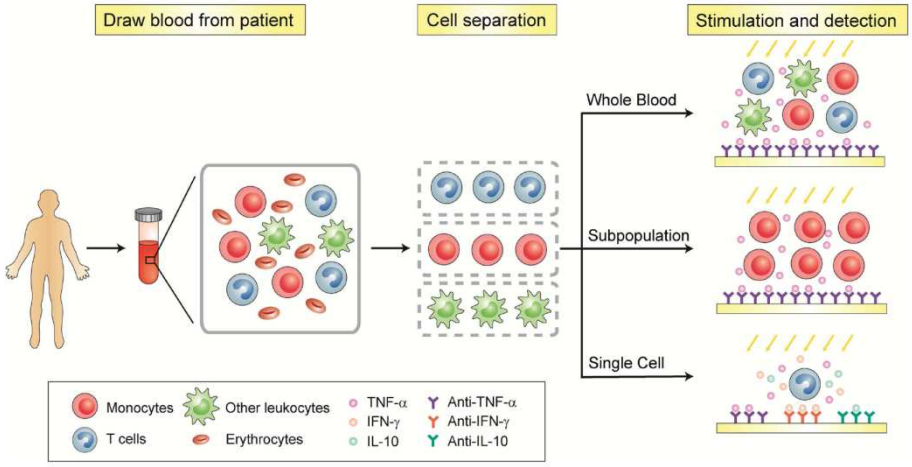
In our work we described long-term respiratory and immune health effects of prenatal HCB at the age of 10 years (see Paper IV). Hansen *et al.* also described long-term effects of this compound in adults of 20 years of age in relation to asthma (Hansen et al. 2014). Also, in the present thesis we described an increased risk of wheeze until the age of 7 years with increasing prenatal BPA and high molecular weight phthalates exposure. These results might already indicate persistent effects of such exposures, however, further evidence from other studies is still needed.

6.4.4 Mechanistic biomarkers

Including the analysis of biomarkers of immonotoxicity in birth cohort studies might represent a big expense. However, the investment is worth in order to understand the role of different actors of the immune system in the occurrence of certain diseases and to explore the short and the long-term effects of environmental exposures. In this sense, an integrative and deep evaluation of different biomarkers of immunotoxicity, such as analysis of cytokines and immunoglobulin levels, cell counts, and other markers (Tryphonas 2001), would help to provide some answers. Currently there are new tools available or being developed, such as multiplexing or microfluidic techniques, that allow or will allow analyzing biological samples at a lower cost than "traditional" techniques and for a much smaller amount of sample (Chen et al. 2013; Chokkalingam et al. 2013; Loo et al. 2011).

Microfluids immunephenotyping techniques are still under development, but are promising tools for environmental epidemiological studies focused on immunotoxicity. Scientists are working to obtained devices to perform rapid, accurate, and sensitive cellular functional assays at a single-cell resolution on different types or subpopulations of immune cells, which will provide information on the distribution of these different immune cell types and their functionalities (Figure 6.1) (Chen et al. 2013; Chokkalingam et al. 2013). Furthermore, new microfluids chips are being developed to measure pesticide residues as well (Duford et al. 2013). Thus, in the near future these techniques might provide a full range of useful data for environmental epidemiological studies.

Figure 6.1 Schematic of functional immunophenotyping of immune cells with microfluids techniques. Reprinted from (Chen et al. 2013), page 2.



7. CONCLUSIONS

Results of the present work suggest that prenatal exposure to persistent organic pollutants (POPs) affects the immune and respiratory health of children, that the effects can occur even at low levels of exposure and that these may last until adolescents. Biological mechanisms behind such effects were not possible to describe in the present thesis, however, it provided information of a potential biomarker (interleukin 10) of chronic immunotoxic effects of POPs. Results also indicate potential effects of prenatal exposure to bisphenol A (BPA) and phthalates on the right functioning of the immune and respiratory systems of infants and children. In the present thesis we highlight the limitations of existing studies in the field and provide recommendations for future research. In the meanwhile, and advocating the precautionary principle, legislations to reduce the use of those compounds that are still in the market and are extensively used should be considered.

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Glossary

Ab	antibody
AhR	aryl hydrocarbon receptor
AOM	acute otitis media
BMI	body mass index
BPA	bisphenol A
COX-2	cyclooxygenase-2
CRP	c-reactive protein
CV	coefficient of variation
DAGs	directed acyclic graphs
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DEHP	di-(2-ethylhexyl) phthalate
EDCs	endocrine disrupting chemicals
ER	estrogen receptor
ELISA	enzyme linked immunosorbent assays
GAM	generalized additive models
GC	gas chromatography
GIs	gastrointestinal infections
HCB	hexachlorobenzene
HCH	hexachlorohexane
HCE	heptachlor epoxide
HDM	house dust mite
Ig	immunoglobulin
IL	interleukin
INF-γ	interferon-γ
LMWP	low molecular weight phthalates

LOD	limit of detection
LOQ	limit of quantification
LRTIs	lower respiratory tract infections
MEHHP	mono-(2-ethyl-5-hydroxyl-hexyl) phthalate
MEHP	mono-(2-ethylhexyl) phthalate
MEOHP	mono-(2-ethyl-5-oxo-hexyl) phthalate
MECPP	mono-(2-ethyl-5-carboxy-pentyl) phthalate
MBzP	mono-benzyl phthalate
MEP	mono-ethyl phthalate
MiBP	mono-isobutyl phthalate
MnBP	mono-n-butyl phthalate
NK-cells	natural killer cells
PBDEs	polybrominated diphenyl ethers
PBMC	peripheral blood mononuclear cells
PBPK	pharmacokinetic model
PCBs	polychlorinated biphenyls
dl-PCBs	dioxin-like polychlorinated biphenyls
ndl-PCBs	non-dioxin-like polychlorinated biphenyls
PCDDs	polychlorinated dibenzo-*p*-dioxins
PCDFs	polychlorinated dibenzofurans
PFCs	perfluorinated compounds
PFDA	perfluorodecanoate
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
PFNA	perfluorononanoic acid
PFHxS	perfluorohexane sulfonic acid
POPs	persistent organic pollutants

PPARα	peroxisome proliferator-activated receptor
RAST	radioallergosorbent test
SEM	structural equation models
sICAM-1	soluble intercellular adhesion molecule-1
SOB	shortness of breath
SPT	skin prick test
sVCAM-1	soluble vascular cell adhesion molecule-1
TDAR	T-cell-dependent IgM antibody responses
TEQ	toxic equivalent factor
TNF-α	tumor necrosis factor α
URTIs	upper respiratory tract infections
WBC	white blood-cell count

Annex

Apart from the original papers included in the present thesis, the PhD candidate has also published other papers, three as first author and seven as co-author, on child health. She has also participated in European projects and has been the coordinator of the Ribera d'Ebre birth cohort follow-up:

Other papers as first author

- **Gascon M**, Fort M, Martinez D, Carsin AE, Forns J, Grimalt JO, Santa Marina L, Lertxundi N, Sunyer J, Vrijheid M. Polybrominated Diphenyl Ethers (PBDEs) in Breast Milk and Neuropsychological Development in Infants. *Environ Health Perspect 2012; 120: 1760-1765.*

- **Gascon M**, Verner MA, Guxens M, Grimalt JO, Forns J, Ibarluzea J, Lertxundi N, Ballester F, Llop S, Haddad S, Sunyer J, Vrijheid M. Evaluating the neurotoxic effects of lactational exposure to persistent organic pollutants (POPs) in Spanish children. *Neurotoxicology 2013; 34: 9-15.*

- **Gascon M**, Vrijheid M, Martinez D, Forns J, Grimalt JO, Torrent M, Sunyer J. Effects of pre and postnatal exposure to low levels of polybromodiphenyl ethers on neurodevelopment and thyroid hormone levels at 4years of age. *Environ Int 2011; 37(3): 605-611.*

Participation and collaboration in European projects

- ENRIECO: participation in work package 4 preparing a review on the work done by European birth cohorts on persistent organic pollutants exposure and child neurodevelopment.

- CHICOS: leader of the case-study on prenatal exposure to persistent organic pollutants and child respiratory health. The work included ten European birth cohorts and resulted in a published article included in the present thesis.

- HELIX: small collaborations providing recommendations in the field of child respiratory health.

Coordinator of the follow-up of the Ribera d'Ebre birth cohort

Ribera d'Ebre was the first birth cohort to be set up in Catalonia and Spain (year 1997). At the age of 13-15 years of children, CREAL performed another follow-up under the project "*Papel de las exposiciones ambientales pre- y postnatal en la salud del adolescente-seguimiento de la cohorte de Ribera d'Ebre*", in which the PhD candidate was the coordinator. The project, which took place between 2010 and 2012, implemented the same core protocol in the Ribera d'Ebre and Menorca cohorts and included the participation of Tecnatox Institute from Universtitat Rovira i Virgili (URV). Two papers have been published with the data collected in this follow-up.

- Torrente M, **Gascon M**, Vrijheid M, Sunyer J, Forns J, Domingo JL, Nadal M. Levels of metals in hair in childhood: Preliminary associations with neuropsychological behaviors. *Toxics 2014; 2(1): 1-16.*

- **Gascon M**, Vrijheid M, Garí M, Fort M, Grimalt JO, Martínez D, Torrent M, Guxens M, Sunyer J. Temporal trends of organochlorine compounds levels from birth until adolescence in two birth cohorts and determinants of exposure. *To be submitted to Environ Int.*