Prenatal exposure to perfluoroalkyl substances and child health

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A Tania,
mi sister, cómplice y amiga

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Pero no cambia mi amor
Por más lejos que me encuentre
Ni el recuerdo ni el dolor
De mi pueblo y de mi gente

Lo que cambió ayer
Tendrá que cambiar mañana
Así como cambio yo
En esta tierra lejana

Cambia, todo cambia

Mercedes Sosa

Summary

Perfluoroalkyl substances (PFAS), such as perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), are synthetic chemicals commonly used in industrial and commercial products including consumer care products, fire-fighting foams, ski wax, and oil- and water-repellents for leather, paper, and textiles. Prenatal PFAS exposure may modulate fetal growth, fat accumulation, metabolic function, and immune response yet evidence coming from birth cohort studies is limited. In this thesis, we first evaluated the transfer of PFAS from mother to fetus and the determinants of maternal PFAS exposure during pregnancy. This led us to the main objective of this thesis, to evaluate the association between prenatal exposure to PFAS and child health, specifically: fetal growth and preterm birth, obesity and cardiometabolic risk, and immune and respiratory health in early and mid-childhood. Data from the "Infancia y Medioambiente" (INMA) population-based Spanish birth cohort was used. The results from the this thesis indicate that PFOA can cross the placental barrier more efficiently than other PFAS, and that mothers were ubiquitously exposed to PFOS and PFOA during the years 2003-2008. Prenatal PFAS concentrations were mainly determined by maternal country of birth, region of residence, previous breastfeeding, parity, and age. We found little and inconsistent evidence for an association between prenatal PFAS exposure and child health outcomes (i.e. fetal growth and preterm birth, obesity and cardiometabolic risk, and immune and respiratory health). Prospective studies with follow-ups beyond mid-childhood are recommended.

Resum

Els compostos perfluorats (PFAS per la seva abreviació en anglès), especialment l'àcid perfluorooctanosulfònic (PFOS) i l'àcid perfluorooctanoic (PFOA), són productes químics sintètics utilitzats habitualment en productes industrials i comercials, per exemple escumes antiincendis, cera d'esquí, i repel·lents d'oli i aigua per a cuir i tèxtils. Durant les primeres etapes de la vida, l'exposició a PFAS pot influenciar el creixement fetal, l'acumulació de greix i la resposta immunitària però l'evidència encara és limitada. En aquesta tesi es va avaluar en primer lloc la transferència de PFAS de la mare al fetus i els determinants de l'exposició materna durant l'embaràs. Tot seguit vam avaluar l'associació entre l'exposició prenatal a PFAS i la salut infantil, específicament: el creixement fetal i el part prematur, l'obesitat i el risc cardiometabòlic, i la salut immunològica i respiratòria a principis i mitjans de la infància. Es van utilitzar dades de la cohort poblacional de naixement espanyola Infància i Medi Ambient (INMA). Els resultats de la present tesi indiquen que els PFOA travessen la placenta més eficientment que els altres PFAS i que les mares van estar exposades a PFOS i PFOA de manera ubiqua durant els anys 2003-2008. Les concentracions prenatals de PFAS estaven principalment determinades pel país de naixement de la mare, la regió de residència, la lactància prèvia, la paritat, i l'edat de la mare. En general, vam trobar poca evidència d'associació entre l'exposició prenatal a PFAS i els efectes en la salut infantil estudiats. Es recomanen estudis prospectius amb un seguiment posterior a la mitjana infància.

Resumen

Los compuestos perfluorados (PFAS por su abreviación en inglés), por ejemplo el ácido perfluorooctanosulfónico (PFOS) y el ácido perfluorooctanoico (PFOA), son productos químicos sintéticos comúnmente utilizados en productos industriales y comerciales, tales como espumas anti-incendios, cera de esquí, y repelentes de aceite y agua para cuero y textiles. La exposición prenatal a PFAS puede modular el crecimiento fetal, la acumulación de grasa y la respuesta inmune sin embargo, la evidencia aún es limitada. En esta tesis se evaluó, en primer lugar, la transferencia de PFAS de la madre al feto y los determinantes de la exposición materna durante el embarazo. A continuación, se evaluó la asociación entre la exposición prenatal a PFAS y la salud infantil, específicamente: el crecimiento fetal y el parto prematuro, la obesidad y el riesgo cardiometabólico, y la salud inmunológica y respiratoria en la primera y mediana infancia. Se utilizaron datos de la cohorte poblacional de nacimiento española "Infancia y Medioambiente" (INMA). Los resultados de esta tesis indican que PFOA atraviesa la barrera placentaria con mayor eficiencia que el resto de PFAS, y que las madres estuvieron expuestas a PFOS y PFOA de manera ubicua durante los años 2003-2008. Las concentraciones prenatales de PFAS estaban principalmente determinadas por el país de nacimiento de la madre, la región de residencia, la lactancia previa, la paridad, y la edad de la madre. En general, encontramos poca evidencia de asociación entre la exposición prenatal a PFAS y los efectos en la salud infantil estudiados. Se recomiendan estudios prospectivos con un seguimiento posterior a la mediana infancia.

"Yo soy yo y mi circunstancia,
y si no la salvo a ella no me salvo yo."

Jose Ortega y Gasset, 1914

Preface

"The womb may be more important than the home."

David J.P. Barker, 1990

This thesis represents a compilation of five scientific articles first-authored by the PhD candidate and supervised by Dr. Martine Vrijheid and Dr. Maribel Casas, according to the procedures of the Biomedicine PhD program of the Department of Experimental and Health Sciences of the Universitat Pompeu Fabra, Barcelona, Spain. The research presented in this thesis was done at the Instituto de Salud Global de Barcelona (ISGlobal)/ Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain during the years 2013-2017.

The rapid increase in the prevalence of obesity and asthma worldwide may be attributable to environmental exposures during sensitive periods in life. The main aim of this thesis is to contribute to the understanding of how environmental chemical exposures, specifically to perfluoroalkyl substances (PFAS), during the prenatal period may influence child health. For this thesis data from the Environment and Childhood (INMA) Spanish birth cohort was used. The first two articles included in this thesis aim to better characterize prenatal PFAS exposure and to identify its socio-demographic, lifestyle, and dietary determinants. The three remaining articles evaluate the association between prenatal PFAS exposure and outcomes related to child health. These articles specifically evaluate fetal growth and preterm birth, obesity and cardiometabolic risk, and immune and respiratory health during early- and mid-childhood.

During these years, the PhD candidate collaborated in the Human Early-life Exposome Project (HELIX), which is a multicenter project involving six different countries (Spain, France, Greece, Lithuania, United Kingdom, and Norway). In HELIX, the PhD candidate coordinated, implemented, and did part of the fieldwork for the pregnancy panel study in Barcelona. This study consisted of recruiting 55 women that were asked to do an intensive and detailed assessment of their environmental exposures during two time

periods in their pregnancy. This data is currently being used for the development of the early-life exposome within HELIX. Currently the PhD candidate is collaborating and coordinating the use of PBPK models for PFAS exposure in WP1 of HELIX.

Besides these research projects, the PhD candidate has coordinated the biomarker and cardiometabolic data collection in the INMA study, has supervised three students' final projects, has coauthored three manuscripts, and has coordinated the ISGlobal-Campus Mar scientific seminars. The PhD candidate also participated in a 3-months research stay at the Norwegian Institute of Public Health in Oslo.

CONTENTS

1. INTRODUCTION

"There is a major paradigm shift taking place in science that while simple is profound."

Jerrold J. Heindel, 2008

The worldwide prevalence of obesity, cardiometabolic diseases, and asthma has been rapidly increasing during the lasts decades. This rapid increase cannot be explained only by genetic changes in the population as a result of adaptation to a changing environment. In order to explain this high prevalence and the rate with which it has appeared, the focus of epidemiological research has shifted to environmental pollutants. Environmental exposure to chemicals during sensitive periods in life has become of particular concern.

The first 1,000 days of life refer to the period between the first day of pregnancy until the end of the second year of life (Taveras 2016). This period is characterized by body growth and organ development at the fastest rate during our lifetime. Given the vulnerability of this period, environmental insults have higher chances of making a long-lasting health impact or even shifting the course of health throughout life. This is the underlying idea of the Developmental Origins of Health and Disease paradigm (DOHaD), an hypothesis proposed in 1990 by the British epidemiologist David Barker (Barker 1990).

The DOHaD paradigm considers that the environmental conditions during the prenatal period signal the fetus about how the postnatal environment will be. The developmental plasticity provides the fetus with the ability to use these environmental cues and make predictive adaptive responses, which may not have immediate advantages but that may be needed in the postnatal environment; a process known as fetal programming (Barouki et al. 2012; Gluckman and Hanson 2004).

From an evolutionary perspective, the main aim of fetal programming is to increase the chances of survival; however if the adaptive response (or programming) mismatches the actual postnatal environment then disease can appear (Barouki et al. 2012;

Gluckman and Hanson 2004). For example, if the mother is undernourished during her pregnancy the fetus may anticipate a postnatal environment where food and nutrients are scarce and thus adapt to cope with the needs of this predicted environment (Barker et al. 1993). A possible programming response is for the fetus to increase its capacity of fat and energy storage. However, if the prediction of the future environment is incorrect then the adopted fetal programming can lead to disease, such as obesity (Barouki et al. 2012). This phenomenon was observed as a consequence of the Dutch wartime famine of 1944-1945: women and men born to mothers that were undernourished during early pregnancy had higher risk of being obese when adults (Roseboom et al. 2006).

Under the DOHaD paradigm, a growing list of environmental chemicals has been suggested to induce functional, structural, and epigenetic changes in the fetus that can result in higher vulnerability to disease or dysfunction later in life (Barouki et al. 2012; Heindel 2007; Heindel and Vandenberg 2015; Schug et al. 2011). Some of these changes may affect the infant growth or the proper functioning of the cardiovascular, metabolic, immune, and/or respiratory systems. In this thesis I will focus on the chemical group of perfluoroalkyl substances and their potential effects on fetal growth and preterm birth, obesity and cardiometabolic risk, and immune and respiratory health during childhood. The main reasons to focus on this group of chemicals is that they are still produced in large quantities, they are ubiquitous in the environment, they can persist in our body for many years, and *in-vitro* and animal studies have shown effects on obesity and immunotoxicity; yet the evidence from epidemiological studies is either scarce or coming from cross-sectional studies (Vrijheid et al. 2016).

1.1. Perfluoroalkyl substances (PFAS)

1.1.1. PFAS historical uses

Perfluoroalkyl substances are synthetic chemicals produced in large quantities since the 1950s (Lau et al. 2004; Prevedouros et al. 2006). Throughout this thesis I will use the term PFAS to refer to all perfluoroalkyl substances. PFAS have been commonly used as surfactants in industrial and commercial products including floor

polishes, cleaning formulations, consumer care products, inks, medical inhalers, fuel additives, air fresheners, textile treatments, cleaning products, coating formulations, fire-fighting foams, polyurethane production, lubricants, ski wax, and oil-, and water-repellents for leather, paper, and textiles (Lau et al. 2004; Prevedouros et al. 2006).

The most common PFAS, specifically perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) have been produced in high volumes (tons per year) during the last decades. The total environmental release of PFAS ranged between 3,200 up to 7,300 tons since the introduction of PFOS in the market until the year 2002 (Prevedouros et al. 2006). Given their high persistence and bio-accumulative properties, PFOS production began a phased out period by its major USA producer (3M Company) in 2002, thus decreasing PFOS emissions from tons to kg per year (Prevedouros et al. 2006). In 2009, PFOS was included as persistent organic pollutants (POPs) in Annex B of the Stockholm Convention (Stockholm Convention, 2009) limiting its use to certain applications (e.g. photographic industry, aircraft technologies, and fire fighting foams). For PFOA, the phase-out period in the USA began in the year 2013 by its major producer (DuPont) and was completed by 2015 as part of the PFOA Stewardship Program (United States Environmental Protection Agency). In Europe, a total restriction on the manufacturing and selling of PFOA will be applicable by 2020 (The European Commission 2017). These regulatory actions are consistent with reported declines in PFOS serum concentrations since the 2000s and to some extent PFOA concentrations (Gebbink et al. 2015; Glynn et al. 2012; Haug et al. 2009; Kato et al. 2011b; Okada et al. 2013; Toms et al. 2014).

The use of other PFAS besides PFOS and PFOA has increased in the last decade, these include perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), and perfluorononanoic acid (PFNA) (Glynn et al. 2012; Renner 2006). Also, PFAS production has shifted from the USA and Europe to Asia, especially China, where PFOS production has scaled up from 30 tons in 2001 up to 250-300 tons in 2006 (Lim et al. 2011; Wang et al. 2014). Even after efforts to cease the production of PFOS, and more recently PFOA, and decrease its emission into the environment, environmental PFOS exposure is still ubiquitous.

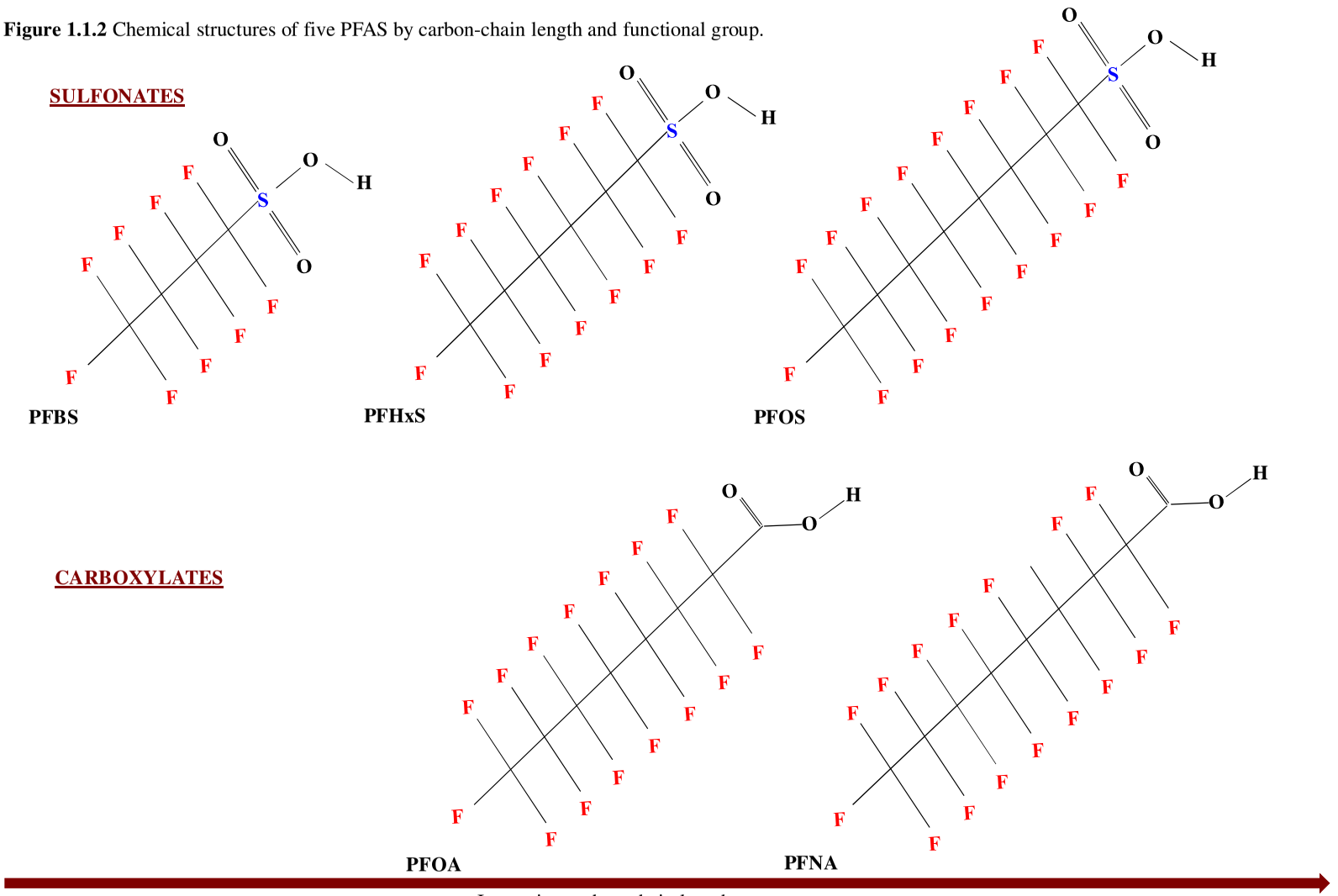
1.1.2. PFAS chemical properties

The chemical structure of PFAS consists of a carbon-chain in which every hydrogen (H) atom bonded to a carbon atom in the chain is replaced by a fluorine (F) atom, as shown in Figure 1. Even when thousands of different isomers of PFAS exist, in this thesis I will focus on the most widely used, specifically PFOS and PFOA, but additionally on, PFHxS and PFNA that are also widely used (Prevedouros et al. 2006) but less assessed in epidemiological studies. PFBS will also be addressed in paper I of this thesis but to a lesser extent than the other mentioned PFAS. Thus, from this point forward, I will only describe the chemical properties of PFHxS, PFOS, PFOA, and PFNA. These four PFAS have carbon-chain lengths ranging from 6 to 9 carbons bonded to a functional group, either a sulfonate or carboxylic group (Figure 1). Both the carbon-chain length and the functional group contribute to some degree to PFAS hydrophobic and lipophobic characteristics (Lau 2015). Moreover, the carbon-fluorine (C-F) bond is highly stable and confers PFAS heat-, oil-, and water-resistant properties making PFAS ideal for many industrial and commercial applications. On the other hand, PFAS high stability makes them non-degradable and persistent in the environment, wildlife and humans (Giesy and Kannan 2001; Lau et al. 2007; Tao et al. 2006; Vestergren and Cousins 2009). Also, as PFAS have a high capacity to bind to albumin, they can accumulate in human blood and have biological half-lives ranging from 3-7 years (Olsen et al. 2007).

1.1.3. Sources of environmental PFAS exposure for the general population and infants

Environmental exposure to PFAS is spread worldwide by atmospheric and/or oceanic transfer, reaching remotes places such as the Arctic and Antarctic regions (Butt et al. 2010; Taniyasu et al. 2013; Yamashita et al. 2005). The detection of PFAS in all source of environmental matrices, including soil, water, air, and house dust (Lau et al. 2004) suggest that PFAS distribution in environmental media is ubiquitous. These chemicals, especially PFOS and PFOA, have been also detected in wildlife with potential of bioaccumulation and biomagnifying in food chains (reviewed by Houde et al. 2006).

Figure 1.1.2 Chemical structures of five PFAS by carbon-chain length and functional group.



PFBS: perfluorobutane sulfonate; PFHxS: perfluorohexane sulfonate, PFOS: perfluorooctane sulfonate, PFOA: perfluorooctanoate; PFNA: perfluorononanoate.

The main pathway of PFAS exposure for the general population is considered to be the diet (D'Hollander et al. 2010; Domingo et al. 2012a; Egeghy and Lorber 2011; Ericson-Jogsten et al. 2008a; Fromme et al. 2007; Tittlemier et al. 2007). The European Food and Safety Agency reported that from 2006 until 2012 the dietary exposure to PFOS and PFOA in the adult population was 3-7% and <0.3-0.5% of the total daily intakes (TDI), respectively. In children, however, dietary exposure can contribute up to 19% of their TDI (European Food Safety Authority 2012). Fish and seafood consumption is suggested to be the main dietary source of PFAS exposure at a global scale, especially in countries with high consumption of fish, such as Spain (Pérez et al. 2014). Other dietary sources include red meat, animal fats, microwavable popcorn, and fast foods (Halldorsson et al. 2008; Tittlemier et al. 2007). Fruits and vegetables have generally presented low levels of PFAS concentrations, except for PFOA concentrations in vegetables, which may account for around 20% of the overall dietary PFOA exposure in Europe (European Food Safety Authority 2012; Herzke et al. 2013). Besides diet, other sources of PFAS exposure include drinking water (Ericson-Jogsten et al. 2008b; Gómez et al. 2011; Vestergren and Cousins 2009), and indoor and house dust (Beesoon et al. 2011; Egeghy and Lorber 2011; Ericson-Jogsten et al. 2012; Fu et al. 2015). In Spain, PFAS have been detected in tap water (Domingo et al. 2012b; Flores et al. 2013), which could contribute to higher PFAS concentrations.

Exposure to these chemicals can begin since early-life as PFAS have been detected in cord blood samples suggesting that they can cross the placental barrier and expose the fetus. Also, newborns are exposed to PFAS through breastfeeding as these chemicals have been detected in maternal breast milk samples (Llorca et al. 2010; Mondal et al. 2014) and infant milk formula (Llorca et al. 2010). Fetuses and newborns have small body volume and their renal clearance is not well developed, making the total body burden of PFAS higher than in adults. Additionally, infant behaviors such as crawling and hand-to-mouth activity can increase their PFAS exposure especially considering that house dust can be a source of exposure. For example, a study in the United States showed that house dust contributes to 36% of PFOS concentrations in two-year old infants and only 6% in adults (Egeghy and Lorber 2011). Thus, summing-up the prenatal (i.e. placental transfer) and postnatal

exposure (i.e. through breastfeeding, food, and house dust) shows that the early-life period is an important peak of human PFAS exposure.

1.1.4. Assessing prenatal PFAS exposure

PFAS can bind to human serum albumin (Salvalaglio et al. 2010) so they are usually measured in blood. Blood samples from adults worldwide have shown detectable levels of PFOS and PFOA. Other PFAS, such as PFHxS and PFNA have also been detected but less frequently and at lower exposure levels that PFOS and PFOA. Similarly, PFAS have been consistently detected in maternal and cord blood samples collected during pregnancy, which indicates that PFAS exposure can start during the prenatal life (see Table 1.1.4.).

Prenatal PFAS exposure may be best estimated in cord blood samples, however maternal blood during pregnancy has been commonly used as a surrogate; probably due to difficulties of cord blood collection or low availability of cord blood samples. Furthermore, many studies alternately use either maternal plasma or serum assuming that PFAS distribute evenly between both blood compartments (Fei et al., 2007; Hanssen et al., 2010; Monroy et al., 2008; Porpora et al., 2013). Only one study has evaluated the distribution of PFHxS, PFOS, and PFOA between plasma and serum samples from the same subject and concluded that they distributed evenly between both compartments, but 78% of their subjects were men (average age: 49 years) working in a fluorochemical factory (Ehresman et al., 2007) and thus direct extrapolation of these findings to pregnant women seems inadequate especially when considering the hematological changes (e.g. total volume expansion) that occur during pregnancy. Understanding if PFAS have a similar distribution between plasma and serum during pregnancy could also ease the comparison between studies, as will be further expanded in paper I of this thesis. Additionally, no information is available on the PFAS concentrations in maternal or cord blood samples in Spain.

Table 1.1.4. Prenatal median PFAS concentrations (ng/mL) in selected studies (N>50) around the world from 1978-2011.

Years	PFOS	PFOA	PFHxS	PFNA	N	Sample Type	Location	Reference
1978-	15.0	2.1	0.2	-	263	MS at delivery	South Sweden	Ode et al. 2013
1978-	6.5	1.7	0.2	-	263	CS at birth	South Sweden	Ode et al. 2013
1988-	21.1	3.6	-	-	343	MS at 30 wks	Denmark	Kristensen et al.
1991-	19.6	3.7	1.6	-	447	MS at 10-28	Great Britain	Maisonet et al.
1996-	35.3*	5.6*	-	-	1,400	MP at 4-14 wks	Denmark	Fei et al. 2008
2002-	4.9**	1.2**	-	-	428	MS at delivery	Hokkaido,	Washino et al. 2009
2003-	13.0	2.3	0.6	0.4	891	MP at 17-20	Norway	Starling et al. 2014
2004-	5.9**	1.8**	-	2.4	439	CP at birth	Taiwan	Chen et al. 2012
2004-	4.9**	1.6**	-	-	299	CS at birth	Maryland,	Apelberg et al.
2004-	14.5	1.8	1.6	0.7	101	MS at delivery	Canada	Monroy et al. 2008
2004-	6.1	1.6	2.1	0.7	101	CS at birth	Canada	Monroy et al. 2008
2005-	1.6	1.3	0.5	0.5	71	MS at delivery	South Africa	Hanssen et al. 2010
2005-	0.7	1.3	0.3	0.2	58	CS at birth	South Africa	Hanssen et al. 2010
2005-	7.8	1.5	1.0	-	252	MS at 15 wks	Alberta,	Hamm et al. 2010
2005-	4.4**	1.5**	0.6**	0.4**	100	CS at birth	Ottawa,	Arbuckle et al.
2007	-	17.0	-	-	108	MS at delivery	Guiyu, China	Wu et al. 2012
2007-	5.0	1.2	0.3	0.3	123	MP at delivery	Norway	Gutzkow et al.
2007-	1.5	0.9	0.1	0.2	123	CP at delivery	Norway	Gutzkow et al.
2009	2.9	1.3	0.1	0.5	50	MS at delivery	Jinhu, China	Liu et al. 2011
2009	1.5	1.1	0.1	0.3	50	CS at birth	Jinhu, China	Liu et al. 2011
2011	9.4	2.6	1.2	-	70	MS at delivery	South Korea	Lee et al. 2013
2011	3.2	2.1	0.6	-	70	CS at birth	South Korea	Lee et al. 2013

*Arithmetic mean; ** Geometric mean; -: data not available or PFAS not measured.; MS: maternal serum; MP:maternal plasma; CS: cord serum: CP: cord plasma; wks: weeks. Table adapted from *Toxicological Effects of Perfluoroalkyl and Polyfluoroalkyl Substances,* 2015.

Further, studies with available PFAS concentrations in maternal and cord blood samples have estimated the placental transfer of PFAS, showing that these chemicals do not transfer at the same rate and that PFOA seems to transfer more efficiently across the placenta than PFOS (Beesoon et al. 2011; Fei et al. 2007; Fromme et al. 2010; Hanssen et al. 2010; Kato et al. 2014; Kim et al. 2011; Midasch et al. 2007; Needham et al. 2011; Ode et al. 2013; Porpora et al. 2013). The transfer efficiency of other PFAS, including PFHxS and PFNA, has been assessed in fewer studies showing inconsistent results (Hanssen et al. 2010; Kato et al. 2014; Kim et al. 2011; Needham et al. 2011; Ode et al. 2013). Estimating the proportion of PFHxS and PFNA that transfers from the mother to fetus is especially relevant for studies that are only using maternal PFAS concentration during pregnancy as a proxy for prenatal PFAS exposure (as we did in this thesis). Further, evidence is limited on the maternal determinants that influence the PFAS transfer efficiency from the mother to the fetus.

An extended contribution and discussion on the assessment of prenatal PFAS exposure can be found in paper I of this thesis.

1.1.5. Factors that influence maternal PFAS concentrations during pregnancy

If maternal PFAS concentrations are used to assess prenatal PFAS exposure then, several factors should be considered in epidemiological studies in order to understand the determinants of exposure during pregnancy. In this thesis, we aimed to identify the determinants of maternal PFAS exposure in the INMA birth cohort, as will be further explored in paper II.

1.1.5.1. Socio-demographic, lifestyle, and dietary determinants

Maternal PFAS concentrations during pregnancy may be determined by socio-demographic, lifestyle, and dietary characteristics. The maternal socio-demographic and lifestyle characteristics that have consistently determined PFAS concentrations in previous studies include: (1) parity, because PFAS can transfer to the fetus during pregnancy contributing to lower maternal PFAS concentrations with increasing number of previous pregnancies (Berg et al. 2014; Brantsæter et al. 2013; Fei et al.

2007, 2010a; Lien et al. 2013; Mørck et al. 2015; Ode et al. 2013; Sagiv et al. 2015), (2) age, because older women accumulate higher PFAS concentrations (Kato et al. 2014; Lien et al. 2013; Sagiv et al. 2015), and (3) previous breastfeeding, given that breastfeeding is a route of PFAS elimination from the body, longer periods of breastfeeding contribute to lower PFAS concentration in the mother (Fei et al. 2010a; Mondal et al. 2014; Ode et al. 2013; Sagiv et al. 2015). Other determinants of PFAS exposure have been identified but less consistently: PFAS concentrations were different by country of birth in studies assessing different ethnical backgrounds, e.g. Swedish (Ode et al. 2013), USA and Peruvian (Calafat et al. 2006); higher PFAS concentrations have been associated with smoking habit in pregnant and non-pregnant USA women (Jain 2013; Sagiv et al. 2015); higher maternal educational level has been associated with lower concentrations in the USA (Sagiv et al. 2015) and Taiwan (Lien et al. 2013); body mass index (BMI) has been positively associated with PFAS blood concentrations in Norway (Brantsæter et al. 2013) but inversely associated in Denmark (Eriksen et al. 2011) and not associated in Japan (Inoue et al. 2004).

As previously mentioned, diet has been suggested as one of the main sources of PFAS exposure in the general population (Haug et al. 2010; Pérez et al. 2014; Domingo et al. 2012a; Vestergren et al. 2009). Fish intake was a major predictor of PFAS concentrations, in adult males and females from France (Yamada et al. 2014) and Norway (Haug et al. 2010; Rylander et al. 2009), and in pregnant women from Norway (Berg et al. 2014). Yet the studies looking at how much is the contribution of fish to PFAS exposure are either of small sample size or in non-pregnant populations, or have assessed PFAS in food items instead of in human biological samples. Other dietary sources, e.g. red meat, snacks, and animal fats, have been positively associated with PFAS blood concentrations (Halldorsson et al. 2008). Drinking water has also been associated with increased levels of PFAS especially with PFOA but the majority of studies were carried out near contaminated settings where PFOA exposure is higher than for the general population (Hölzer et al. 2008; Mondal et al. 2012; Schwanz et al. 2016).

In summary, maternal PFAS concentrations can be influenced by many maternal socio-demographic, lifestyle, and dietary characteristics but consensus is lacking as to which are the major

factors that determine PFAS concentrations during pregnancy (Brantsæter et al. 2013; Halldorsson et al. 2008; Ode et al. 2013; Sagiv et al. 2015). Finally, no study has assessed PFAS concentrations or its determinants during pregnancy in Spain, which is a Mediterranean country with potentially high dietary PFAS exposure (Pérez et al. 2014). More on this topic will be discussed in paper II.

1.1.5.2. Timing of blood collection and physiological changes during pregnancy

Maternal PFAS concentrations during pregnancy may be influenced by the timing of blood sample collection because of physiological changes occurring throughout pregnancy. Maternal PFAS concentrations decline during pregnancy (Fei et al. 2007; Fromme et al. 2010; Glynn et al. 2012), in part, due to a dilution effect caused by increasing plasma volume expansion, which fully occurs after gestational week 12 (Bernstein et al. 2001). Glomerular filtration rate (GFR), which indicates the rate of fluids filtered by the kidneys, also increases by 50% already at 14 weeks of pregnancy (Costantine 2014). Lower GFR has been associated with higher PFAS concentrations in adults (Shankar et al. 2011) and adolescents (Watkins et al. 2013), so it seem plausible that changes in GFR during pregnancy might contribute to different PFAS concentrations. The influence of these physiological changes can be reduced by adjusting regression models for serum albumin (as a proxy for plasma volume expansion) and serum creatinine (to estimate maternal GFR), or by assessing maternal PFAS exposure in blood samples collected early in pregnancy.

When I started this thesis project in 2013, most studies with maternal PFAS concentrations used samples collected after the first trimester of pregnancy (Fromme et al. 2010; Hanssen et al. 2010, 2013; Inoue et al. 2004; Kato et al. 2011a; Kim et al. 2011; Midasch et al. 2007; Monroy et al. 2008; Needham et al. 2011; Ode et al. 2013; Porpora et al. 2013). In addition, only the study of Whitworth et al. (2012) had evaluated confounding by serum albumin levels in the association between prenatal PFAS exposure and birth weight in a Norwegian birth cohort. No other study published before 2013, had evaluated confounding by plasma volume expansion or maternal GFR, or their related proxies. Epidemiological studies using maternal PFAS concentrations from blood samples collected

early in pregnancy (as a proxy for the fetal body burden of PFAS) or studies assessing confounding by physiological changes during pregnancy are needed as these factors may influence the associations between prenatal PFAS exposure and child health outcomes (Verner et al., 2015). This issue will be addressed in papers III and IV of this thesis.

1.2. Prenatal PFAS exposure and child health

In this thesis I have used maternal PFAS concentrations during pregnancy as a proxy for fetal exposure in order to evaluate the association between prenatal exposure to PFAS and child health outcomes, specifically fetal growth and preterm birth, obesity and cardiometabolic risk, and immune and respiratory health. Besides these outcomes, prenatal exposure to PFAS has been associated with childhood neurodevelopment (reviewed by Braun 2016 and Rappazzo et al. 2017) and thyroid function (reviewed by Ballesteros et al. 2017); however, these fall outside the scope of this doctoral thesis as the work is currently being done within the INMA–Valencia research group.

1.2.1. Fetal growth and preterm birth

1.2.1.1. State-of-the-art

Birth outcomes are commonly used as indicators of fetal growth during pregnancy and some of them, for example birth weight, have been associated not only to perinatal mortality and morbidity but also with health outcomes later in life, such as obesity (Parsons et al. 1999), cardiometabolic disorders (Whincup et al. 2008), and asthma (Mu et al. 2014). Throughout pregnancy there is a constant interplay between the internal and external environment leading to better or worse health status of the offspring. This interplay can be influenced by many factors including exposure to environmental chemical pollutants such as PFAS.

The association between prenatal PFAS exposure and birth outcomes has been commonly assessed in previous studies using anthropometric measurements such as weight, length, and head circumference at the time of birth. Most of the evidence available suggests an association between prenatal PFOS and PFOA exposure

and reductions in average birth weight as reviewed by Bach et al. (2015a); Johnson et al. (2014); Verner et al. (2015). The reviews from Johnson et al. (2014) and Verner et al. (2015) also included a meta-analysis of 9 and 7 studies respectively, and concluded that every 1-ng/mL increase of PFOS (only Verner et al.) and PFOA was associated with a reduction of 5g and between 15g and 19g in birth weight, respectively. After the publication of these meta-analyses, other studies have assessed this association and have reported similar, contradictory, or null associations. A study from Canada reported that maternal PFOA concentrations, but not PFOS, was inversely associated with birth weight z-scores, though the null value was included in the confidence intervals (Ashley-Martin et al. 2017). One of the most comprehensive studies to date from Aarhus, Denmark, assessed 16 different PFAS - including PFOS and PFOA - and observed no association between PFOS and PFOA and birth weight (Bach et al. 2015b). In the INUENDO birth cohort (Poland, Greenland, and Ukraine), maternal PFOA concentrations were associated with reduced birth weight in term births (Lenters et al. 2015). In Sweden, maternal PFOS and PFOA concentrations were inversely associated with birth weight (Lauritzen et al. 2017). In Japan, PFOS but not PFOA, was associated with reduced birth weight only in girls (Kishi et al. 2015); however a recent publication from the same birth cohort using a different study population reported that only PFOA was associated with reduced birth weight (Minatoya et al. 2017). In China, high maternal PFOA exposure was inversely associated with weight at birth (Wu et al. 2012); however, another study from China with relatively low prenatal PFOS and PFOA concentrations observed non-significant associations with increased birth weight (Shi et al. 2017). In a small study from Australia both PFOS and PFOA were non-significantly associated with reductions in birth weight (Callan et al. 2016). In these studies the median exposure concentrations ranged from 1.0 up to 17.0 ng/mL for PFOS and from 1.1 up to 17.0 ng/mL for PFOA, with the studies of Lauritzen et al. (2017) and Wu et al. (2012) reporting the highest and the study of Callan et al. (2016) the lowest concentrations. Differences in sample size, exposure levels, sample matrix (maternal vs. cord blood), and location need to be considered when making comparisons between studies.

For other PFAS, such as PFHxS and PFNA, the literature is scarce. In Denmark, maternal PFNA concentration showed a pattern of

inverse association with birth weight only in girls (Bach et al. 2015b). In Canada, PFHxS was positively correlated with birth weight in girls (Ashley-Martin et al. 2017). In China, PFHxS and PFNA were non-significantly associated with increases in birth weight (Shi et al. 2017). In Great Britain using data from the Avon Longitudinal Study of Parents and Children (ALSPAC) birth cohort, the upper tertile of maternal PFHxS concentration was inversely associated with birth weight; however this study only included girls (Maisonet et al. 2012).

The association between PFAS and low birth weight (LBW), defined as birth weight < 2500g, has been assessed in four prospective studies (Chen et al. 2012; Darrow et al. 2013; Fei et al. 2007; Wu et al. 2012) showing no association between PFOS, PFOA, and PFNA and LBW. In addition, the association between PFAS and being small for gestational age (SGA defined as birth weight < 10^{th} percentile for gestational age and sex) is limited to five studies, with two showing that higher prenatal PFOS and PFOA exposure were associated with higher odds of being born SGA in Taiwan (Chen et al. 2012) and Sweden (Lauritzen et al. 2017), respectively, but lower odds in Canada (Hamm et al. 2010). However, no associations between PFAS and SGA were observed in Norway (Whitworth et al. 2012) and Denmark (Fei et al. 2007).

As birth weight is a measurement that summarizes the total contribution of the different body parts at a given gestational age; other anthropometric measurements such as birth length, and head circumference can provide further information (Slama et al. 2014). In this sense, few studies have assessed the association between PFAS and length and head circumference at birth. From these studies, some have reported inverse associations between maternal PFAS concentration and birth length (Fei et al. 2008; Lauritzen et al. 2017; Maisonet et al. 2012; Wu et al. 2012) and head circumference (Apelberg et al. 2007; Chen et al. 2012); whereas the others reported no association with any of these outcomes (Shi et al. 2017; Washino et al. 2009).

Preterm birth, which is being born before 37 weeks of gestation, is an indicator of early birth and is associated with higher neonatal morbidity and mortality and also poorer health during childhood and adolescence (Saigal et al. 2008). The association between PFAS

and the odds of preterm birth is inconsistent, with some studies showing positive (Chen et al. 2012), inverse (Hamm et al. 2010; Whitworth et al. 2012), or no associations (Bach et al. 2015b; Darrow et al. 2013; Fei et al. 2007).

Maternal PFAS concentrations may be confounded by maternal GFR during pregnancy, which may influence the association between PFAS and fetal growth (Verner et al., 2015). Lower GFR has been associated with higher PFAS blood levels (Verner et al. 2015; Watkins et al. 2013) and smaller babies (Gibson 1973; Verner et al. 2015). Indeed, a recent study found that a large proportion of the association between PFOS and PFOA and reduced average birth weight may be attributable to confounding by maternal GFR (Verner et al. 2015). This study used a physiologically based pharmacokinetic (PBPK) model to generate pairs of predictions for maternal PFAS levels and birth weight. Only one epidemiological study has considered the role of GFR on the association between PFOA and birth weight in a sub-analysis of 953 mother-child pairs in Norway concluding that maternal GFR attenuated by 66% the association between PFOA and reduced birth weight (Morken et al. 2014).
In summary, and as recently reviewed by Vrijheid et al. (2016), there seems to be a consistent association between prenatal exposure to PFOA and reduced average birth weight; whereas for PFOS the evidence is still insufficient. However, less evidence is available for other PFAS such as PFHxS and PFNA; and other outcomes such as birth length, head circumference, LBW, SGA, and preterm birth. Finally, confounding by maternal GFR should be explored in prospective studies that assess other PFAS besides PFOA. Contributing to the understating of these knowledge gaps will be the main objective of paper III of this thesis.

1.2.1.2. Potential mechanisms of action for PFAS effects on fetal growth and preterm birth

There is a lack of information regarding the toxicity of most PFAS on fetal growth and preterm birth, with PFOS and PFOA being the most studied. The main mechanism described is the direct interaction with peroxisome proliferator activated receptors (PPAR) – alpha (α) (Figure 1.2.1.2.). In mice, activation of PPARα by PFOA was related to neonatal mortality, delayed eye opening, and reduced birth weight (Abbott et al. 2007; Lau et al. 2004; Wolf et al.

2010). The activation of PPARα by PFNA (which has shown more potent effects than PFOA) was related to neonatal mortality at higher doses and impaired growth and developmental delays at lower doses (Wolf et al. 2010).

Figure 1.2.1.2. Perfluoroalkyl substances and metabolic disruption through PPARα.

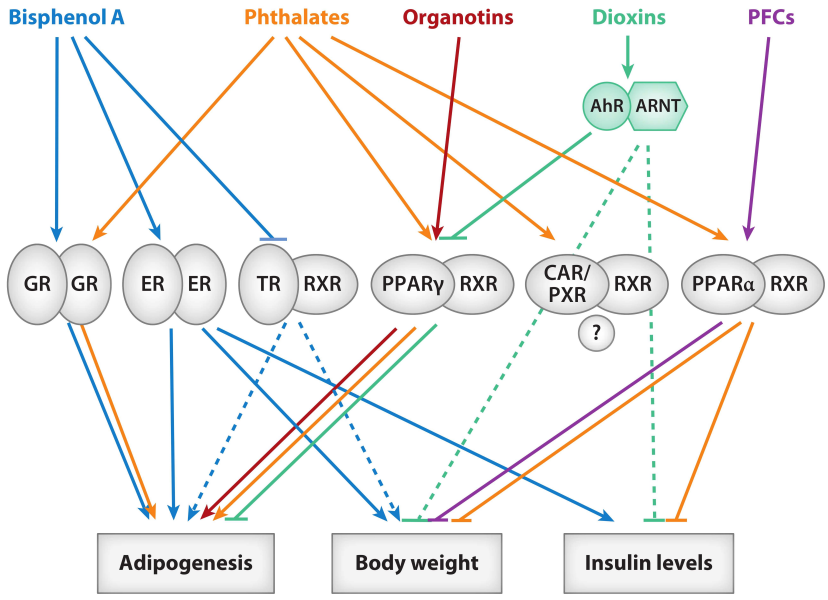


Figure from Cristina Casals-Casas and Béatrice Desvergne, 2011. *Endocrine Disruptors: From Endocrine to Metabolic Disruption*. Please note that PFCs: stands for perfluorinated compounds, which is a former term of perfluoroalkyl substances (PFAS).

1.2.2. Obesity and cardiometabolic risk

1.2.2.1. State-of-the-art

Birth weight has been associated with overweight and obesity later in life. Childhood overweight and obesity have been steadily increasing during the past four decades (de Onis et al. 2010) and has more than doubled in developed countries including Spain, where almost 39% of school-age children are overweight or obese (Sánchez-Cruz et al. 2013). Obese children are more likely to present higher prevalence of cardiometabolic risk factors including obesity, hypertension, dyslipidemia, and cardiovascular diseases in

adulthood than normal-weight children (Deshmukh-Taskar et al. 2006; Janssen et al. 2005; Kumar and Kelly 2017). Childhood overweight is defined by a body mass index (BMI, kg/m^2) above the 85^{th} percentile and below the 95^{th} percentile for the same age and sex, and obesity is defined as a BMI at or above the 95^{th} percentile for the same age and sex.

The term obesity refers to an excess of body fat. Body fat can be measured by several methods, including densitometry, bioelectrical impedance analysis, dual energy X-ray absorptiometry (DXA), and magnetic resonance (Sahoo et al. 2015). However, given their time-consuming techniques or high costs, BMI has been used as the standard measure for overweight and obesity in children (age $\geq$ 2 years old) (Kumar and Kelly 2017). BMI offers an inexpensive, non-invasive, and quick measurement, although indirect, of body adiposity. Given that the height and weight of children vary according to age and sex then age- and sex-specific BMI z-scores should be calculated using internal standardization or an external reference population such as the one suggested by the World Health Organization (WHO) (de Onis 2007; de Onis et al. 2009). Measurements of central adiposity reflecting intra-abdominal fat, which is more metabolically active than fat stored in other regions of the body (Björntorp 1991), include the waist circumference (WC) and waist-to-height ratio (WhTR) (Kumar and Kelly 2017). Adiposity can also be measured by skin-fold thickness, which reflects subcutaneous fat, but its measurement requires trained personnel (Horan et al. 2015).

Obesity is caused by a mismatch between caloric intake and energy expenditure. Nonetheless, multiple environmental, psychological, behavioral, and societal factors can interact with the individual's genetic background, and influence the energy balance (Glass and McAtee 2006; Kumar and Kelly 2017; Trasande et al. 2009). Factors such as maternal nutrition, gestational weight gain, sleep deprivation, stress, the built environment, the gut microbiota, and certain chemical exposures, including PFAS, can contribute to childhood fat accumulation and obesity with some factors starting since the in-utero period (Brisbois et al. 2012; Cameron et al. 2015; El-Behadli et al. 2015; Gangwisch et al. 2005; Harakeh et al. 2016; Holtcamp 2012; Patel and Hu 2008; Rahman et al. 2011; Robinson et al. 2012).

PFAS, especially PFOA, are considered potential obesogens (Holtcamp 2012) that may promote fat accumulation (Grün and Blumberg 2009). Rodents that were prenatally exposed to PFOA showed higher weight gain, body fat accumulation, and cardiometabolic risk in mid-life and adulthood (Hines et al. 2009; Lv et al. 2013; Tan et al. 2013). The few epidemiological studies on children suggest an association between PFAS and BMI and adiposity; however most studies have primarily assessed PFOS and PFOA with inconsistent findings (reviewed by Vrijheid et al. 2016). Prenatal PFOS and PFOA exposure has been associated with higher weight at 20 months in a study of girls from the ALSPAC birth cohort (Maisonet et al. 2012), higher risk of WHtR>0.5 at 5-9 years in children from Greenland and Ukraine (Høyer et al. 2015), higher adiposity at 8 years in USA children (Braun et al. 2016), and higher BMI among Danish women at age 20 (but not among Danish men) (Halldorsson et al. 2012). Prenatal PFAS exposure was associated with higher BMI, skin-fold thickness, and total fat mass — measured using DXA— in 7-year-old girls, but not boys from a USA birth cohort (Mora et al. 2016). In a study of children from the Danish National Birth Cohort (DNBC) study, prenatal exposure to PFOS and PFOA was associated with lower weight at 5-12 months of life (Andersen et al. 2010) but not with BMI and WC in a later follow-up of children from the same cohort at age 7 (Andersen et al. 2013). Estimated early-life PFOA exposure was also not associated with self-reported BMI at 20-40 years among adults who resided near a PFOA manufacturing facility in the USA (Barry et al. 2014).

In addition to overweight and adiposity, cardiometabolic risk factors include elevated blood pressure, lipid abnormalities, and abnormal glucose homeostasis, all of which are considered components of metabolic syndrome (Kassi et al. 2011). The metabolic syndrome has been associated with a higher risk of all-cause mortality, cardiovascular diseases, and diabetes (Ford 2005). A cross-sectional study of adolescent participants (12–19 years of age) in the USA National Health and Nutrition Examination Survey (NHANES) reported inverse associations between PFNA and metabolic syndrome, but positive associations of PFOS with markers of abnormal glucose homeostasis (Lin et al. 2009) while another larger NHANES study reported that the highest quartiles of PFOS and PFOA exposure were inversely associated with hypertension in adolescents (Geiger et al. 2014b). Two additional cross-sectional studies of PFOA and PFOS include a study of Danish children at 8–

10 years of age, which reported no associations with cardiometabolic risk factors (Timmermann et al. 2014), and a study of USA children and adolescents (1–18 years) with potential exposure via contaminated drinking water, which reported positive associations with serum lipid levels (Frisbee et al. 2010). Two longitudinal studies of PFAS and cardiometabolic risk factors other than overweight and adiposity include a study of girls in the ALSPAC cohort, which reported that prenatal PFOA concentrations within the lowest tertile of the distribution (but not in the second or third tertiles) were positively associated with total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) at 7 and 15 years (Maisonet et al. 2015). Finally, a USA study reported no association between prenatal PFAS and insulin resistance at approximately 8 years of age, though concurrent serum PFOA and PFOS at 8 years were inversely associated with insulin resistance (Fleisch et al. 2016).

In 2013 when I started my PhD research, few prospective studies looking at the association between PFAS and obesity were available. Fortunately more research has been done in this area but still most studies have focused only on PFOS and PFOA and their associations with BMI and adiposity, and most studies have shown inconsistent findings. Less is known about the association between PFAS and other cardiometabolic risk factors, including blood pressure and lipids, and most of the literature comes from cross-sectional studies, thus a causal relationship cannot be established. In addition, only one cross-sectional study has assessed the association between PFAS and cardiometabolic risk in adolescence (Lin et al. 2009). No study has prospectively assessed the association between prenatal PFAS exposure and cardiometabolic risk in childhood. These gaps will be covered in paper IV of this thesis.

1.2.2.2. Potential mechanisms of action for PFAS obesogenic and cardiometabolic effects

The main mechanism described for PFAS obesogenic and cardiometabolic effects is the direct interaction with PPARα and gamma (γ) (Figure 1.2.1.). PPARα is a major regulator of lipid metabolism in the liver (other tissues include the kidney, heart, muscle and adipose cells), and its endogenous agonist is a fatty acid. Activation of PPARα regulates several genes responsible for fatty acid metabolism, including acid binding proteins (Latruffe et

al. 2000). On the other hand, PPARγ regulates fatty acid storage and glucose metabolism. PFAS, especially PFOA, can act as agonist inducing PPARα and PPARγ activity (Jiang et al. 2016).

Other less described mechanisms include: (1) interaction with estrogen receptor (ER)-α that disrupts the development or functioning of the ovary and can lead to impaired estrogen production (Hines et al. 2009); (2) impact on the thyroid hormone receptors leading to disrupted metabolism (Wei et al. 2008); and (3) alteration of the leptin signaling pathways or leptin levels and thus increasing body weight (Hines et al. 2009).

1.2.3. Immune and respiratory systems

1.2.3.1. State-of-the-art

In this thesis I assess the following immune and respiratory outcomes during childhood: respiratory tract infections, wheezing, asthma, eczema, and lung function.

Respiratory tract infections can be divided in two main categories, those that occur in the upper respiratory tract (nasal cavity, pharynx, and larynx) including otitis media, pharyngitis, laryngitis, sinusitis, tonsillitis and the common cold; and those that occur in the lower respiratory tract (trachea, bronchia, and lungs) including bronchiolitis, bronchitis, and pneumonia. The occurrence of lower respiratory tract infections (LRTIs) is less common than upper respiratory tract infections and around 6% of children are affected during their first two years of life (Patria et al. 2013). LRTIs are one of the major factors for the development of asthma and its symptoms during childhood.

In Spain, an increase in asthma prevalence from 6% to 10% has been reported among children aged 6-7 years between 1998 and 2003 (Asher et al. 2006). Asthma is a complex disease characterized by chronic airway inflammation with widespread but variable airflow obstruction that is often reversible either naturally or after treatment (Mims 2015). Typical asthma symptoms include recurrent episodes of wheezing (a non-specific sign of airway obstruction), breathlessness, chest tightness, and coughing (van den Wijngaart et al. 2015). Childhood asthma is related to reduced quality of life and

exercise tolerance, and higher risk of school absenteeism and hospitalizations. Asthma often co-occurs with other allergy-related diseases such as rhinitis and eczema increasing its burden (Garcia-Aymerich et al. 2015). Atopic eczema (also known as atopic dermatitis) is a skin disorder that is common in over 20% of young children in developed countries (Flohr and Mann 2014) and that can persist until adulthood (Wallach and Taïeb 2014). Atopic eczema can be measured by an increase in total and/or allergen-specific serum immunoglobulin E (IgE) levels (Eichenfield et al. 2014). Atopic eczema has been associated with asthma and reduced lung function in early childhood (Lowe et al. 2002). In epidemiological studies the occurrence of childhood asthma is often evaluated by parental or self-reported reported questionnaires (Beasley 1998) and to a lesser extent through lung function tests.

Lung function is an indicator of respiratory health and a predictor of respiratory and cardiovascular morbidity (Sin et al. 2005). Spirometry test is considered the recommended technique to assess lung function but it is not easy to conduct in very young and untrained children participating in population-based studies. During a spirometry test, the rate of changing lung volumes after forced breathing maneuvers is measured (van den Wijngaart et al. 2015). The principal parameters of a spirometry test include forced vital capacity (FVC) and forced expiratory volume in the first 1 second of exhalation (FEV_1), as well as FEV_1/FVC ratio. FVC is a marker of airway restriction whereas FEV_1 and FEV_1/FVC are markers of airway obstruction. Lung function impairment can be tracked throughout childhood and into adulthood, and asthmatics usually present lower lung function than non-asthmatics (Guerra and Martinez 2009; Sears et al. 2003).

The underlying causes of childhood asthma are not fully understood (Beasley et al. 2015). Environmental exposures, including PFAS, have been suggested to contribute to the increase in asthma prevalence because they can impair the development of the immune and respiratory systems. Slight changes in the immune system development can decrease resistance to infectious diseases and reduce vaccine efficacy (Winans et al. 2011) whereas changes in lung function and structure during childhood may increase the susceptibility to develop respiratory disorders such as chronic obstructive pulmonary disease later in life (Sears et al. 2003).

Immunotoxicity occurs when an exogenous agent induces an inadequate immune response either directly or indirectly. In fact, experimental studies using rodent models suggest that early-life exposure to PFAS, especially PFOS and PFOA, may alter the immune system even at low doses (reviewed by DeWitt et al., 2012). Moreover, consistent findings for an association between higher prenatal PFAS exposure and reduced response to childhood routine vaccinations was observed in the Faroe Islands and in Norway (Grandjean et al. 2012; Granum et al. 2013).

Few prospective studies have assessed the association between prenatal PFAS exposure and respiratory infections, asthma, and atopic eczema, showing either inverse or no associations. In Japan, prenatal PFOS and PFOA were not associated with self-reported asthma, allergies, and infections at 18 months of life (Okada et al. 2012). In the Hokkaido birth cohort, also from Japan, patterns of inverse associations were observed between prenatal PFAS exposure and total allergic diseases including eczema in the first 24 months of life (Okada et al. 2014). In a later follow-up of the same cohort, prenatal PFHxS exposure was inversely associated with wheezing at 4 years (Goudarzi et al. 2016). In Taiwan, prenatal exposure to PFAS was not associated with atopic eczema at age 2 years (Wang et al. 2011). In Norway, prenatal PFAS exposure was positively associated with the number of common colds and gastroenteritis episodes from 1 until 3 years of age (Granum et al. 2013). Further, in Denmark prenatal PFOA exposure was positively associated with the occurrence of hospitalizations due to infections disease from birth until 8 years in girls whereas inverse associations and no associations were observed for boys and in the overall population, respectively (Fei et al. 2010b). Finally, in the INUENDO birth cohort higher prenatal PFOA exposure was associated with reduced wheezing symptoms at 5-9 years of age (Smit et al. 2015).

Most of these studies have conducted their outcome assessment before age 5 years, a time when the clinical diagnosis of asthma is challenging and thus the number of asthma cases might be low, or misclassification might be high due to the use of self-reported occurrence of symptoms. Only two recent studies from the Faroe Islands and the INUENDO birth cohort have assessed the

association between prenatal PFAS exposure and asthma after age 5 years showing no association between PFAS exposure and asthma at ages 5 to 13 years old (Smit et al. 2015; Timmermann et al. 2017).

Finally, during gestation and infancy the lung is rapidly growing and developing all of its anatomical and functional structures, making this period especially sensible to environmental insults (Kajekar 2007). Only one case-control study assessed the association between PFAS and lung function during childhood showing that higher postnatal PFAS exposure was associated with reduced lung function among asthmatic adolescents aged 11-16 years old (Qin et al. 2017). At the time of starting this thesis project, no prospective study had evaluated the association between prenatal PFAS exposure and lung function during childhood. These knowledge gaps will be further assessed in paper V of this thesis.

1.2.3.2. Potential mechanisms of action for PFAS effects on the immune and respiratory systems

The mechanisms of action for the effects of PFAS on the immune and respiratory systems are far from understood. PFAS can act through the activation of PPARα, which regulates processes involved in the immune system and can indirectly modulate lipid levels that can lead to hepatoxicity and stress effects (Qazi et al. 2009). For example, mice exposed to PFOS and PFOA (at high doses) showed higher levels of proinflammatory markers including TNF-alpha and interleukin (IL)-6; thus reducing their innate immunity response (Qazi et al. 2009). Similarly, exposure to PFOS and PFOA was related to a reduced adaptive response by causing severe atrophy to the thymus (disturbing cell proliferation and differentiation) and the spleen (reducing specific humoral immune responses against foreign antigens) in mice (Yang et al. 2000). In rats, PFOS exposure caused failure of lung development and function that resulted in neonatal mortality (Grasty et al. 2005) and is suggested to be mediated by activation of PPARα. These finding suggest that PPARα plays an important role in PFAS immune and respiratory effects.

Other mechanisms less described include the suppression of antigen-specific immunoglobulin (Ig)-M antibody response (Corsini et al. 2014), and the interaction of PFAS with signaling molecules

such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF_KB) (Corsini et al. 2014), which is a complex of DNA transcription factors that regulate the response to cellular stress and that can act as tumor suppressors (Jat et al. 2013).

2. RATIONALE

The rapid increase in the prevalence of obesity and asthma worldwide may be attributable to environmental exposures during sensitive periods in life. Exposure to environmental chemicals during sensitive periods, such as fetal life, may have long-lasting health consequences. PFAS are contaminants produced in large quantities and distributed globally since the 1950's. In vitro and animal studies suggest that early-life exposure to PFAS may modulate fetal growth, fat accumulation, metabolic function, and immune response, yet until recently evidence on PFAS health effects coming from birth cohort studies was limited. Also, the available studies mainly focused on PFOS and PFOA but little is known about the efficiency and predictors of the placental transfer for other PFAS, including PFHxS and PFNA. Maternal PFAS concentrations during pregnancy may be influenced by socio-demographic and dietary characteristics yet these differ by study population and location setting and, to date, no information is available in Spain. Birth cohort studies have mainly focused on prenatal exposure to PFOS and PFOA and birth weight; however the association between other PFAS and other birth outcomes (such as preterm birth) has been scarcely assessed. Finally, considering that PFAS may have long-lasting effects that go beyond fetal life, large prospective studies are needed to evaluate the association between prenatal PFAS exposure and obesity and cardiometabolic risk, and immune and respiratory health during childhood.

3. OBJECTIVES

The main aim of this thesis was to evaluate the association between prenatal PFAS exposure and health outcomes in children from a Spanish birth cohort. This was evaluated in five specifics objectives:

1. To evaluate the transfer of PFAS concentrations between mother and fetus and determine its predictors in the INMA birth cohort (Paper I).

2. To evaluate the socio-demographic and dietary determinants of maternal PFAS concentrations in the INMA birth cohort (Paper II).

3. To evaluate the association between prenatal exposure to PFAS and birth outcomes including weight, length, head circumference, and gestational age in the INMA birth cohort (Paper III).

4. To evaluate the association between prenatal exposure to PFAS and obesity and cardiometabolic risk in early- and mid-childhood in the INMA birth cohort (Paper IV).

5. To evaluate the association between prenatal exposure to PFAS and immune and respiratory health in children from the INMA birth cohort (Paper V).

4. METHODS

This section provides a general overview of the study population and PFAS determination used in this thesis. A detailed explanation of the study design, sample selection, exposure and outcomes assessment, and statistical analysis is given in each paper included in Section 5.

4.1. The INMA Project

The **IN**fancia y **Me**dio**Am**biente - Childhood and Environment-Project is a network of seven prospective birth cohorts in Spain (Figure 4.1.) following more than 3,000 mother-child pairs from pregnancy until adolescence. The main aim of INMA is to evaluate the influence of environmental pollutants during early life and their effect on child growth and development. Maternal and childhood genetic factors as well as several environmental, dietary, and psychosocial exposures have been evaluated. The outcomes evaluated include pre and postnatal growth, childhood obesity, neurodevelopment, and immunologic and respiratory health (Guxens et al. 2012).

The general inclusion criteria in this birth cohort were: (1) being 16 years old or older, (2) intention of giving birth at the reference hospital, (3) having a singleton pregnancy, (4) not having a communication or language barrier, and (5) not have used assisted reproduction for the current pregnancy (Guxens et al. 2012).

Each cohort has a different period of maternal recruitment and follow–ups. In the older cohorts of Ribera d´Ebre and Granada mothers were recruited at the moment of delivery from March 1997 until December 1999, whereas in Menorca mothers were recruited all throughout pregnancy from October 2000 until July 2002. The newer cohorts of Asturias, Gipuzkoa, Sabadell, and Valencia recruited mothers at the first prenatal visit (i.e. around 10-13 weeks of pregnancy) from February 2003 until January 2008. In these newer cohorts, mother-child pairs have been followed at birth, 6 months, and at 1.5, 4, 7, 9, and 11 years old. Parents completed interview-led questionnaires providing socio-demographic, dietary, health, and exposure information. Maternal and child biological

samples (e.g. blood and urine samples) were collected at different follow-ups.

Figure 4.1. Geographical distribution of the INMA birth cohorts in Spain.

4.2. Description of PFAS study sample

In this thesis we used data from the INMA regions of Gipuzkoa, Sabadell, and Valencia (Figure 4.1). From 2003-2008, a total of 2,150 pregnant women were recruited during their 1st trimester of pregnancy. The cohort of Asturias was not included in this thesis because PFAS measurements were not available. From the 2,150 pregnant women, a total of 1,243 mother-child pairs had data on maternal PFAS concentrations and at least one child health outcome (Figure 4.2.1). Also, in Sabadell and Valencia PFAS were measured in a 66 cord blood samples collected at birth. Figure 4.2.2 provides a general timeline of the exposures and outcomes assessed in this thesis. A more in detailed explanation of the specific study population will be given in each paper in Section 5.

Figure 4.2.1. Flowchart of PFAS study sample in INMA.

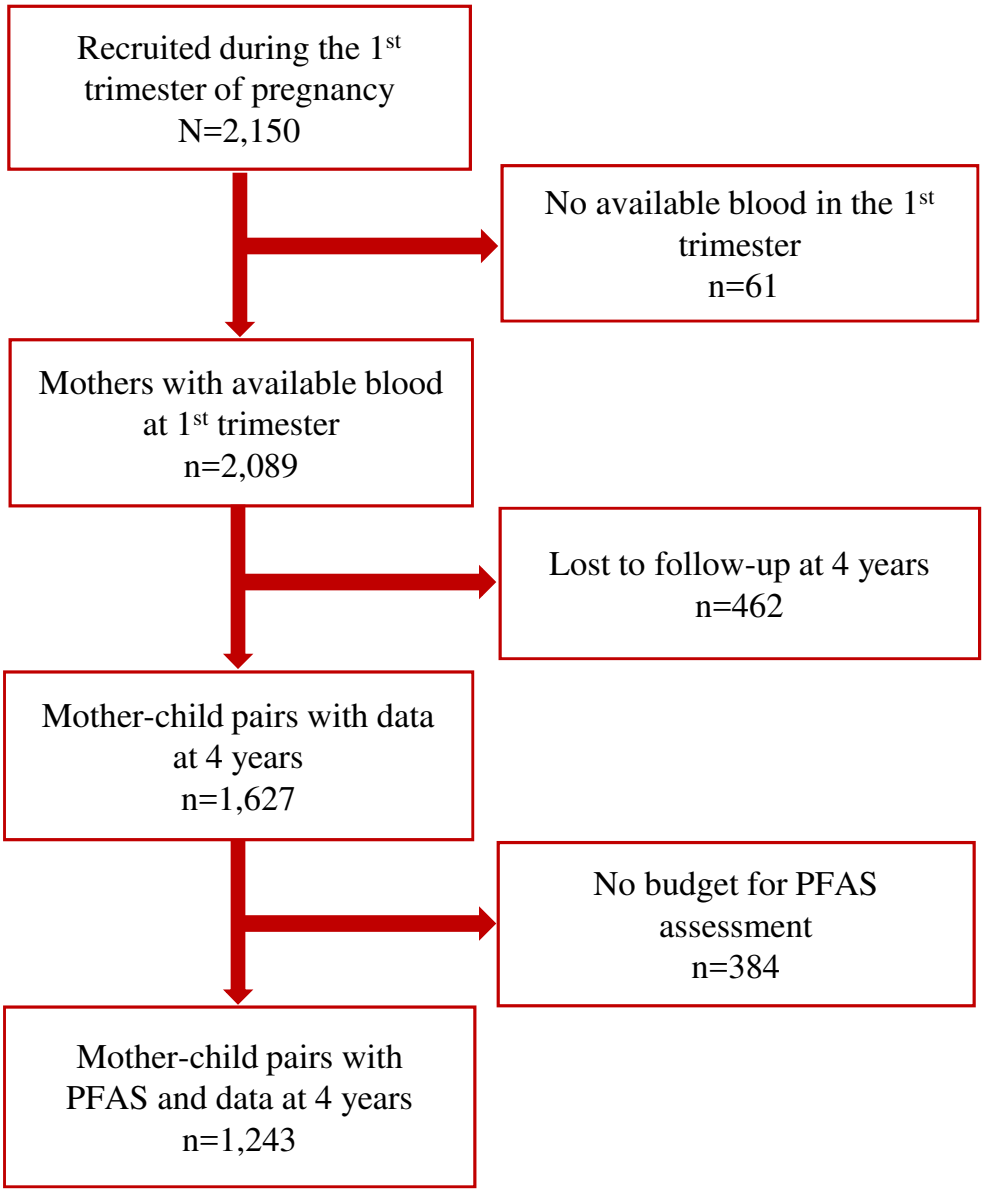


Figure 4.2.2. Timeline of exposure and outcome assessment in this thesis.

Exposure assessment			Outcome assessment				
Pregnancy			Birth	0-6 months	1.5 years	4 years	7 years
Papers 1 & 2	Maternal concentrations of PFHxS, PFOS, PFOA, and PFNA	Paper 3	**Anthropometrics:** Birth weight, length, head circumference, gestational age. Low birth weight, small for gestational age, preterm birth				
		Paper 4		**Anthropometrics:** Weight gain and rapid growth		**Anthropometrics:** BMI z-score and waist circumference **Blood pressure** **Lipids** (total cholesterol, HDL-C, LDL-C, and triglycerides) **CM-risk score**	**Anthropometrics:** BMI z-score and waist circumference **Blood pressure**
		Paper 5			**Occurrence of** Chest infections LRTIs Wheezing Asthma Eczema **Lung function by spirometry test**	**Occurrence of** Chest infections LRTIs Wheezing Asthma Eczema **Lung function by spirometry test**	**Occurrence of** Chest infections Wheezing Asthma Eczema **Lung function by spirometry test**

PFHxS: perfluorohexane sulfonate, PFOS: perfluorooctane sulfonate, PFOA: perfluorooctanoate; PFNA: perfluorononanoate; BMI: body mass index; HDL-C: high density lipoprotein; LDL-C: low density lipoprotein; CM: cardiometabolic; LRTIs: lower respiratory tract infections.

4.3. Determination of PFAS in the INMA Project

A full description of PFAS determination is given in paper I of this thesis.

Briefly, maternal plasma collected during the first trimester of pregnancy was aliquoted in 1.5mL criotubes and stored at -80°C until their analysis at the Institute for Occupational Medicine, RWTH Aachen University, (Aachen, Germany). Plasma concentrations of PFBS, PFHxS, PFOS, PFOA, and PFNA were determined by column-switching liquid chromatography (Agilent 1100 Series HPLC apparatus) coupled with tandem mass spectrometry (Sciex API 3000 LC/MS/MS system in ESI-negative mode) according to a modified protocol described by Kato et al. (2011a). The limits of quantification (LOQ) were 0.20 ng/mL for PFHxS, PFOS and PFOA and 0.10 ng/mL for PFNA. The limits of detection (LOD) were LOQ/2.

5. RESULTS

Paper I: Transfer of perfluoroalkyl substances from mother to fetus in a Spanish birth cohort.

Paper II: Variability of perfluoroalkyl substance concentrations in pregnant women by socio-demographic and dietary factors in a Spanish birth cohort.

Paper III: Prenatal exposure to perfluoroalkyl substances and birth outcomes in a Spanish birth cohort.

Paper IV: Prenatal exposure to perfluoroalkyl substances and cardiometabolic risk in children from the Spanish INMA birth cohort study.

Paper V: Prenatal exposure to perfluoroalkyl substances, immune and respiratory outcomes in children from a Spanish birth cohort study.

5.1. Paper I

Manzano-Salgado CB, Casas M, Lopez-Espinosa M-J, Ballester F, Basterrechea M, Grimalt JO, et al. Transfer of perfluoroalkyl substances from mother to fetus in a Spanish birth cohort. Environ Res. 2015 Oct;142:471–8. DOI: 10.1016/j.envres.2015.07.020

5.2. Paper II

Manzano-Salgado CB, Casas M, Lopez-Espinosa M-J, Ballester F, Martinez D, Ibarluzea J, et al. Variability of perfluoroalkyl substance concentrations in pregnant women by socio-demographic and dietary factors in a Spanish birth cohort. Environ Int. 2016 Jul;92–93:357–65. DOI: 10.1016/j.envint.2016.04.004

5.3. Paper III

Manzano-Salgado CB, Casas M, Lopez-Espinosa M-J, Ballester F, Iñiguez C, Martinez D, et al. Prenatal exposure to perfluoroalkyl substances and birth outcomes in a Spanish birth cohort. Environ Int. 2017 Nov;108:278–84. DOI: 10.1016/j.envint.2017.09.006

5.4. Paper IV

Manzano-Salgado CB, Casas M, Lopez-Espinosa M-J, Ballester F, Iñiguez C, Martinez D, et al. Prenatal Exposure to Perfluoroalkyl Substances and Cardiometabolic Risk in Children from the Spanish INMA Birth Cohort Study. Environ Health Perspect. 2017 Sep 20;125(9):97018. DOI: 10.1289/EHP1330

5.5. Paper V

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Prenatal exposure to perfluoroalkyl substances and immune and respiratory outcomes in children from a Spanish birth cohort study

Manuscript in preparation.

Prenatal exposure to perfluoroalkyl substances and immune and respiratory outcomes in children from a Spanish birth cohort study

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Short running title

Prenatal PFAS exposure and childhood immune and respiratory health

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Conflict of interest statement

There is no conflict of interest to declare.

Abstract

Background: Prenatal exposure to perfluoroalkyl substances (PFAS) may be associated with impaired immune and respiratory health during childhood but the evidence is scarce and inconsistent. We studied the association between prenatal PFAS exposure and immune and respiratory health up to age 7 years in the Spanish INMA birth cohort study.

Methods: We assessed perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), and perfluorononanoate (PFNA) in maternal plasma samples collected during the 1st trimester of pregnancy (years: 2003-2008). Mothers reported the occurrence (yes/no) of chest infections, lower respiratory tract infections (LRTIs), wheezing, asthma, and eczema in the previous 12 months at 1.5 (n=1188), 4 (n=1188) and 7 (n=1071) years of the child. At ages 4 (n=501) and 7 (n=997) years, lung function was assessed using spirometry tests.

Results: The most abundant PFAS were PFOS (mean: 5.80 ng/mL) and PFOA (mean: 2.31 ng/mL). At any age, the relative risks (RR) of asthma [RR (95 CI %): 0.75 (0.57, 0.97)] decreased per each doubling in concentration of PFNA, particularly in boys. The RR of eczema at any age [RR (95 CI %): 0.86 (0.75, 0.98)] decreased per every doubling in PFOS concentration. At 4 years, the odds of chest infections and eczema decreased with increasing prenatal PFNA exposure; and at 7 years, the odds of wheeze also decreased with increasing PFOS and PFNA concentrations. At 4 years, higher PFOA concentrations were associated with a lower forced vital capacity (FVC) and lower forced expiratory volume in 1 second (FEV_1), although associations did not reach statistical significance. No other association was observed.

Conclusion: In this study, prenatal PFAS concentrations were associated with decreased risk of chest infections, wheeze, asthma, and eczema during childhood. Prenatal exposure to PFOA may decrease FEV_1 and FVC at early childhood. These findings require replication.

Abbreviations

CI	Confidence interval
DAG	Directed acyclic graphic
$FEF_{25–75}$	Forced expiratory flow between 25% and 75% of forced vital capacity
FEV_1	Forced expiratory volume in 1 second
FEV_1/ FVC	Forced expiratory ratio
FVC	Forced vital capacity
GAM	Generalized additive model
GEE	Generalized estimating equations
GM	Geometric mean
HPLC-MS/MS	High performance liquid chromatography–tandem mass spectrometry
Ig	Immunoglobulin
IFN-	Interferon
IL	Interleukin
INMA	Environment and Childhood Project (*INfancia y Medio Ambiente)*
LOQ	Limit of quantification
LRTI	Lower respiratory tract infection
OR	Odds ratio
PFAS	Perfluoroalkyl substances
PFHxS	Perfluorohexane sulfonate
PFOS	Perfluorooctane sulfonate
PFOA	Perfluorooctanoate
PFNA	Perfluorononanoate
PPAR	Peroxisome proliferator-activated receptor
RR	Relative risk
SD	Standard deviation

1. Introduction

Perfluoroalkyl substances (PFAS) are synthetic chemicals that may be associated with impaired immune and respiratory health during childhood (Corsini et al. 2014; DeWitt et al. 2009; Grandjean et al. 2012; Granum et al. 2013; Qin et al. 2017). PFAS have been widely used in a variety of industrial and commercial applications such as the coating of paper and packaging, textiles and leather, fire-fighting foam, photography industry, cleaning products and pesticides (Casals-Casas and Desvergne 2011). The PFAS most studied are perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) because of their widespread use, environmental persistence and long biological half-lives (3-5 years) in humans (Olsen et al. 2007). However, there are other less frequently assessed PFAS, such as perfluorohexane sulfonate (PFHxS) and perfluorononanoate (PFNA) that can be of concern to human health.

Experimental studies in rodents suggest that early-life exposure to PFAS, especially PFOS and PFOA, may alter the immune system even at low doses (reviewed by DeWitt et al., 2012). In humans, few studies have assessed the association between prenatal PFAS exposure and the risk of allergy, asthma, or immune-related outcomes during childhood, showing inconsistent results. In the Faroe Islands and Norway, prenatal exposure to PFAS was associated with reduced response to routine vaccination in during childhood (Grandjean et al. 2012; Granum et al. 2013). The study in Norway also reported positive associations between prenatal PFOA and PFNA exposure and the number of common colds and, between PFOA and PFHxS exposure and the number of gastroenteritis episodes (Granum et al. 2013). These results suggest that prenatal PFAS exposure may be associated with immune-suppression during childhood. However, other studies have reported no associations (Okada et al. 2012) or even a reduced risk of occurrence of child immune and respiratory-related outcomes (Fei et al. 2010; Goudarzi et al. 2016; Okada et al. 2014; Smit et al. 2015). For example, in the INUENDO birth cohort for example, higher prenatal PFOA exposure was associated with reduced wheezing symptoms at 5-9 years of age (Smit et al. 2015). Finally, the association between PFAS and lung function has only been assessed in one case-control study showing that higher postnatal PFAS exposure impaired lung function at 11-16 years, but only among adolescents with asthma (Qin et al. 2017). To date, no prospective study has evaluated the association between prenatal PFAS exposure and lung function during childhood.

In this study, we evaluated the association between prenatal PFAS exposure and immune and respiratory outcomes up to age 7 years in the Spanish INMA birth cohort study.

2. Methods

2.1 Study population

In this study, we used data from the Spanish INMA (Environment and Childhood - *INfancia y Medio Ambiente*) birth cohort. From 2003-2008, a total of 2,150 pregnant women from the regions of Gipuzkoa, Sabadell and Valencia were recruited during their 1st-trimester of pregnancy. The inclusion criteria were: being at least 16 years old, singleton pregnancy, no communication barrier, no reproductive assistance and giving birth in the reference hospital (Guxens et al. 2012). We had 1,243 mother-child pairs with data on PFAS concentration and at least one immune and respiratory outcome at each follow-up. From these, 55 mother-child pairs did not have complete information on the covariates of interest (i.e. 4% of the sample). For the purpose of this study, we only included the 1,188 mother-child pairs with data on

prenatal PFAS exposure and at least one immune or respiratory outcome at each follow-up and also complete data on the covariates of interest (Figure 1).

2.2 Exposure assessment

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

2.3 Reported immune and respiratory outcomes

Interviewer-led questionnaires were given to the mothers using the Spanish or Catalan version of the validated International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire. The occurrence of chest infections at 1.5, 4, and 7 years was defined by 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?"*. Lower respiratory tract infections (LRTIs) at 1.5 and 4 years were defined by a positive answer to: *"In the last 6 months (or 12 months if asked at age 4 years), has the doctor told you that your child has had bronchiolitis (only at 1.5 years) or bronchitis or pneumonia?"*. Wheeze at 1.5 and 4 years was defined by 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 12 months?"*. At age 7 years, wheeze was defined as a positive answer to the following question: *"Has your child ever experienced whistling or wheeze from the chest in the last 12 months?"*. Asthma at 4 years was defined by a positive answer to the question: "*In the last 12 months, has your child ever suffered asthma?"* and "*How many times did your child have medical assistance due to asthma?"*; at 7 years it was defined by a positive answer to: *"Has your child ever been diagnosed by a doctor as having asthma?"*. Eczema at 1.5 and 4 years was defined as a positive answer to the question: *"In the last 12 months, did your child have atopic eczema?"*. At 7 years, eczema was defined as a positive answer to the question: *"Has your child ever had any itchy rash which was intermittently coming and going at any time in the past 12 months?"*

2.4 Lung function assessment

Lung function at 4 and 7 years was measured by trained nurses using spirometry and following the guidelines of the American Thoracic Society and European Respiratory Society (Morales et al. 2015). For the purpose of this study, we included those children with at least 1 acceptable maneuver (501 children at 4 years and 997 children at 7 years). We assessed the following lung function parameters: forced vital capacity (FVC, mL), forced expiratory volume in 1 second (FEV_1, mL), forced expiratory ratio (FEV_1/ FVC, %), and forced expiratory flow between 25% and 75% of FVC ($FEF_{25–75}$, mL/s). With these parameters we calculated the age-, sex-, height and ethnicity-adjusted z-scores using the Global Lung Function Initiative 2012 prediction equations (Quanjer et al. 2012). We defined a test as

reproducible if FVC and FEV_1 showed an agreement of at least 100mL between the best two blows (Morales et al. 2015). The number of reproducible tests were 302 (60%) at 4 years and 644 (65%) at 7 years.

2.5 Maternal and child covariates

Information on maternal socio-demographic and dietary characteristics was collected from interview-led questionnaires administered during the 1st trimester of pregnancy (Guxens et al. 2012). These questionnaires also provided data regarding the maternal history of asthma/allergy symptoms. Further, information on the sex and birth weight of the newborn and type of delivery was abstracted from medical records. Other characteristics of the child such as age at the time of the respiratory outcomes, diet, and physical activity were reported by the mother using questionnaires at each follow-up. The child weight and height was measured by trained personnel at 4 and 7 years.

2.6 Statistical Analysis

We replaced PFAS values <LOQ with LOQ/2. Because PFAS distribution was skewed to the right we used the log_2-transformed PFAS concentrations. We assessed the linearity of the relationship between PFAS and each outcome using generalized additive models (GAMs). We used directed acyclic graphics (DAGs) for confounder selection (Supplementary Material Figure S1). We first assessed as confounders the maternal determinants of PFAS exposure previously evaluated in our cohort (Manzano-Salgado et al. 2016). These were maternal age (years), parity (number of pregnancies), previous breastfeeding (weeks), pre-pregnancy body mass index (BMI, kg/m^2), region of residence (Gipuzkoa, Sabadell and Valencia), country of birth (Spain or other), and fish consumption during pregnancy (servings per week). We also included in our DAGs other confounders that are commonly described in the literature: maternal smoking during pregnancy (number of cigarettes), social economic status, number of siblings, birth weight, postnatal breastfeeding, delivery mode at birth, child's physical activity, passive smoking, child's diet, and child's BMI (Supplementary Material Figure S1). All models, except for lung function, were minimally adjusted by the child's sex and age (years) at follow-up. Based on our DAGs the final models were further adjusted by maternal age, parity, previous breastfeeding, pre-pregnancy BMI, region of residence, country of birth, and fish consumption during pregnancy.

In order to assess the association between PFAS and immune and respiratory-related outcomes during the complete study period (i.e. from age 1.5 until 7 years) we used generalized estimating equations (GEE) with an unstructured correlation matrix. GEEs allow for assessing outcomes with unknown correlation at different time-points, and the inclusion of subjects with incomplete data at any given follow-up (Liang and Zeger 1986). For the individual follow-ups we used logistic regression models to assess the association between PFAS and categorical outcomes (i.e. chest infections, LRTIs, wheeze, asthma, and eczema). Finally, we assessed the association between PFAS and lung function parameters at 4 and 7 years using linear regression models.

Sensitivity analysis included assessing the effect modification of sex of the child, region of residence, maternal history of asthma/allergy symptoms, smoking during pregnancy, birth weight, and childhood breastfeeding duration by including the interaction terms in our models and stratified analysis. Given that PFAS concentrations are moderately correlated in INMA (ranging from Spearman rho=0.43 up to Spearman rho=0.68; p-values <0.001) (Manzano-

Salgado et al. 2016) we performed a multipollutant model including all PFAS in a single model only in the GEE analysis. We interpreted the estimates in our models as the change in the outcome per doubling of maternal PFAS concentrations. We considered a p-value<0.05 to be statistically significant. We used the STATA 14.1 statistical software (Stata Corporation, College Station, Texas) for our regression analysis. We drew our DAGs using the DAGitty version 3.0 (Textor 2011).

3. Results

PFAS were detected in every maternal sample, with PFOS (mean: 5.80 ng/mL) and PFOA (mean: 2.31 ng/mL) concentrations being the most abundant (Table 1). Women included in this study were 32 years old on average, were mostly nulliparous, had secondary or higher studies, and were born in Spain (Table 1). The subjects not included in this study (n=907), because lost to follow-up or lack of exposure data (Figure 1), had a higher proportion of younger mothers, with lower education, and born outside of Spain than the subjects included in this study (data not shown). The prevalence of childhood immune and respiratory outcomes ranged from 3% for asthma at 4 years to 35% for chest infections and LRTIs at 1.5 years (Table 1). We observed a general decrease in the prevalence of chest infections and wheeze from 1.5 until 7 years old, whereas the prevalence of eczema seemed to increase (Table 2).

In general, prenatal PFAS exposure was inversely associated with immune and respiratory outcomes, especially at the ages of 4 and 7 years (Table 2). Because we did not observe significant differences between the minimally adjusted and the fully adjusted models, we only report the adjusted results (minimally adjusted and non-adjusted models can be found in the Supplementary Material Table S1 and S2). In our GEE models, we observed that at any age during the study period the risk of asthma decreased per each doubling of PFNA concentration [RR (95 CI %): 0.75 (0.57, 0.97)] (Table 3). Also, every doubling of PFOS concentration was associated with 14% (95 CI %: 0.75, 0.98) lower risk of eczema at any age. We observed similar results between the other PFAS and asthma and eczema but associations were not statistically significant. When associations between PFAS and immune and respiratory outcomes were assessed separately, the association between PFNA and reduced odds of asthma became stronger at 4 than at 7 years and the association between PFOS and eczema was stronger at 4 and 7 years than at 1.5 years (Table 4). In this multivariate logistic models, we also observed that PFNA concentrations were inversely associated with chest infections [OR (95 CI %): 0.74 (0.56, 0.97)] at 4 years, wheeze at 7 years [OR (95 CI %): 0.69 (0.54, 0.88)], and eczema at 4 years [OR (95 CI %): 0.79 (0.66, 0.96)]. PFOS concentrations were associated with reduced odds of wheeze at 7 years [OR (95 CI %): 0.70 (0.52, 0.94)].

Regarding lung function, we did not observe any significant association between maternal PFAS concentrations and lung function parameters during the study period (Table 5). PFHxS, PFOS, and PFOA showed patterns of associations with reduced FVC and FEV_1 at 4 and 7 years (Table 5); but only the associations between PFOA and FVC [β (95% CI): -0.16 (-0.33, 0.00)] and FEV_1 [β (95% CI): -0.12 (-0.29, 0.04)] at 4 years were marginally significant (Table 5).

For the sensitivity analyses, we did not see any statistically significant sex-specific difference in our results (Supplementary Material Table S3); however boys compared to girls had lower risk of developing asthma with higher PFNA exposure at any age of the study period (p-value for sex-interaction = 0.11). The interaction-terms for region of residence were significant only

in the analysis of PFOA and asthma at 4 years (p-value=0.03) so we stratified this analysis by region of residence; and still we did not observe any difference from our main results (Supplementary Material Table S4). Maternal history of asthma/allergy symptoms, smoking during pregnancy, birth weight, or breastfeeding duration of the index child did not modify the associations (data not shown). The inclusion of all PFAS in one multi-pollutant model did not change the associations between PFNA and PFOS and reduced risk of asthma and eczema, respectively, at any age (Supplemental Material Table S5). In this multipollutant model, however, higher PFHxS concentration was associated with higher occurrence of LRTIs at any age [RR (95%CI): 1.15 (1.00, 1.32)].

4. Discussion

In the present study, we observed that at any age, prenatal PFNA exposure was inversely associated with asthma, particularly in boys, and that prenatal PFOS exposure was inversely associated with eczema. These associations did not differ by region of residence and remained after including adjustment for other pollutants. PFNA and PFOS were also associated with lower risk of chest infections, wheeze, asthma, or eczema, especially at 4 and 7 years. No major associations were observed between PFHxS and PFOA and immune and respiratory outcomes. PFHxS, PFOS, and PFOA showed patterns of non-significant associations with reduced FVC and FEV_1, especially at 4 years.

We detected PFOS and PFOA in every maternal sample collected during the years 2003-2008. PFAS concentrations were lower than other studies using maternal blood samples collected before the PFOS phase-out period in the year 2002 (Fei et al. 2007; Midasch et al. 2007). However, studies using maternal samples more recently have detected lower PFAS concentrations than ours (Ashley-Martin et al. 2017; Fromme et al. 2010; Hanssen et al. 2010; Porpora et al. 2013).

Prenatal PFNA and PFOS exposure was associated with reduced risk of immune and respiratory outcomes, especially asthma and eczema. Our results are in line with some studies but not with others. Prenatal exposure to PFOA was inversely associated with recurrent wheeze in children 5-9 years old in a study from the INUENDO birth cohort study (Smit et al. 2015). In the Hokkaido birth cohort study, prenatal PFAS exposure, particularly to the long chain PFAS such as perfluorododecanoic (PFDoDa) and perfluorotridecanoic (PFTrDA) acids, was inversely associated with allergic diseases at 1, 2, and 4 years old (Goudarzi et al. 2016; Okada et al. 2014). In the Danish birth cohort study, prenatal PFOA exposure has been associated with reduced risk of hospitalizations for infectious diseases (Fei et al. 2010). However, other studies in Norway, Taiwan, Japan, and the Faroe Islands found no association between prenatal PFAS exposure and self-reported allergy, asthma, atopic dermatitis, or wheeze (Granum et al. 2013; Okada et al. 2012; Timmermann et al. 2017; Wang et al. 2011). Differences in the exposure levels, outcome assessment, age at follow-up, and population setting might explain the conflicting results in prospective studies, though there are many other causal and non-causal factors that also might contribute to variation among studies.

The inverse associations observed in our and other previous studies may be attributable to numerous reasons. First, prenatal PFAS exposure has been associated with immune-suppression during childhood (Grandjean et al. 2012; Granum et al. 2013). Thus, prenatal PFAS exposure may suppress the developing immune system in infants and indirectly reduced the risk of developing immune hyperactivity and hypersensitivity diseases, such as eczema and wheezing. In addition, we cannot rule out the possibility of some negative

confounding due to covariates that are positively associated with the exposure and inversely associated with the outcome. For example, maternal fish intake has been positively associated with PFAS concentrations (Manzano-Salgado et al. 2016) and fish intake has also been suggested to decrease the risk of allergic diseases due to the high content of n-3 long-chain fatty acids. Although we adjusted our models for maternal fish intake during pregnancy we used self-reported dietary information, and we cannot rule out the possibility of measurement error. A substantial imprecision of the confounder can cause an underestimation of the PFAS concentrations and its beneficial effects on the outcome even after confounder adjustment. Failure to adjust for a negative confounder can result in an underestimation of the toxicity of the exposure on the outcome (Choi et al. 2008).

The mechanisms for which PFAS could affect the immune and respiratory systems are not well understood and may occur via multiple pathways (Corsini et al. 2014). Contrary to our results, previous animal studies have linked PFOA with higher allergic inflammation and pro-inflammatory cytokines both *in vitro* and *in vivo* (Singh et al. 2012), and with airway hyper-reactivity in mice (Fairley et al. 2007). PFOS has been linked with a Th2 immune response characterized by higher interleukin (IL)-4 and IL-10 and lower IL-2 and interferon (IFN)-γ (Dong et al. 2011; Zheng et al. 2011). However, in a murine model, PFOA and PFOS were associated with higher INF-γ mRNA (messenger RNA) in the lungs but not with allergic hyper-responsiveness (Ryu et al. 2014). Further, studies in mice suggest that some of the biological effects of PFAS can be mediated through peroxisome proliferator-activated receptor (PPAR)-alpha, whereas a human study gives indication for a role of PPAR-gamma (Corsini et al. 2014; Pennings et al. 2016). Future studies are needed in order to understand the mechanisms by which prenatal PFAS exposure could reduce the risk of immune and respiratory outcomes during childhood.

Studies on rodents have shown that prenatal exposure to PFAS is associated with impaired lung development at birth (Grasty et al. 2005). Overall, in the present study we observed a pattern of non-significant associations between prenatal higher PFHxS, PFOS, and PFOA exposure and reduced FVC and FEV_1 at 4 and 7 years. The association was strongest between higher PFOA concentration and reduced FVC at 4 years. No other clear association between PFAS exposure and lung function during childhood was observed. Only one case-control study in 132 asthmatic children and 168 non-asthmatic controls from Taiwan has evaluated the association between postnatal PFAS exposure and lung function in adolescents (10-15 years old) showing that PFAS concentrations were significantly associated with lung function reduction among asthmatics (Qin et al. 2017). Given this study`s cross-sectional design their results cannot be directly extrapolated to our study. More studies with a prospective exposure and outcome assessment are needed to evaluate the association between PFAS and lung function during childhood.

The main strengths of this study are its prospective design and large sample size, and the assessment of childhood lung function by spirometry test. However, we should consider the following methodological limitations. First, we have no information on postnatal PFAS exposure, which has been associated with immune-related outcomes or lung function in other studies (Dong et al. 2013; Qin et al. 2017). To overcome this limitation we used the breastfeeding duration of the index child as a proxy for dietary PFAS exposure during childhood and our findings did not change. In the future, studies should also evaluate what is the role of postnatal PFAS exposure on immune and respiratory outcomes. Second, using questionnaires for outcome assessment can introduce recall and/or misclassification bias. Misclassification bias may be most important for asthma at 4 years as cases can be

confounded with the occurrence of wheeze due to chest infections. However, at 7 years we asked for a doctor diagnosis and our results remained similar to the 4 years results. Third, performing a spirometry test before age 5 years is difficult (Turner et al. 2007). Given that children in INMA performed the spirometry test at 4 years the test reliability and thus, the quality of the lung function assessment at this age may be compromised. Nonetheless, the results remained similar when considering spirometry data at 7 years. Finally, chance findings are plausible due to multiple comparisons in our study; however we preferred not to perform Bonferroni p-value corrections as this can increase type 2 error (Perneger 1998; Rothman 1990) and instead, we stated the general patterns of associations observed in the present study.

5. Conclusion

In this Spanish birth cohort, higher prenatal PFAS concentrations were associated with lower risk of self-reported wheezing, chest infections, eczema, and asthma during childhood at 4 and 7 years. To date, this is the first study evaluating the association between prenatal PFAS exposure and lung function during childhood; showing a pattern of associations between higher PFHxS, PFOS, and PFOA exposure and reduced FVC and FEV_1 at 4 and 7 years. Future studies are needed to understand the mechanisms behind the associations observed in the present study.

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Tables and Figures

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

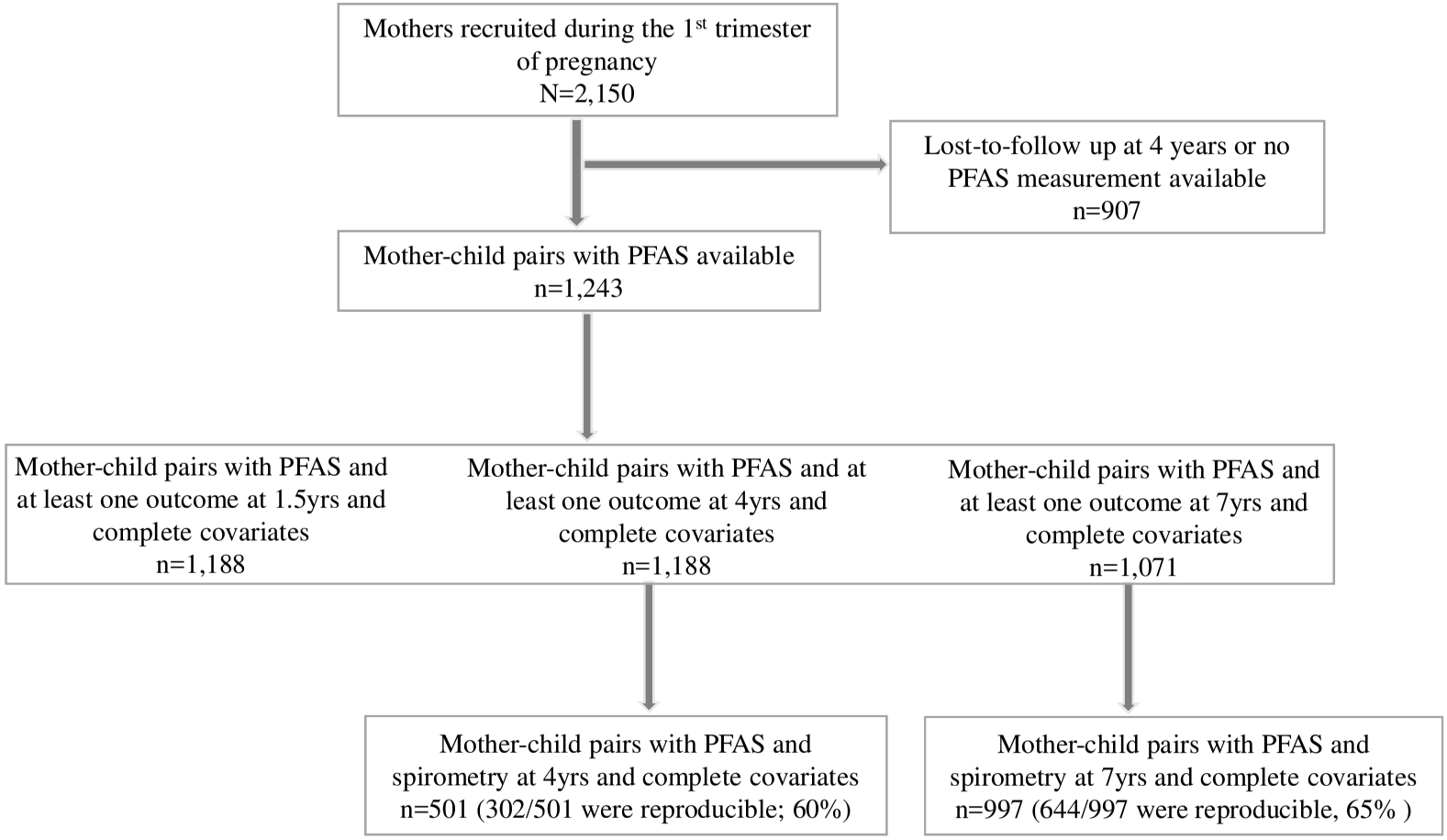


Table 1. Maternal characteristics during pregnancy in the study population.

Maternal Characteristics	**Mean (SD) or n (%)**
PFAS (ng/mL) **-** mean (SD)	
PFHxS	0.55 (0.37)
PFOS	5.80 (2.73)
PFOA	2.31 (1.26)
PFNA	0.64 (0.36)
Age (years) - mean (SD)	31.93 (4.03)
Pre-pregnancy BMI (kg/m^2) - mean (SD)	23.64 (4.32)
Parity (number of children) - n (%)	
None	672 (57)
One	441 (37)
Two or more	75 (6)
Previous breastfeeding (weeks) - n (%)	
Never	725 (61)
Short-term (<16 weeks)	111 (9)
Long-term (16–24 weeks)	122 (10)
Very long-term (>24 weeks)	230 (19)
Education (completed) - n (%)	
None or primary	270 (23)
Secondary	501 (42)
University	415 (35)
Region of residence - n (%)	
Gipuzkoa	296 (25)
Sabadell	390 (33)
Valencia	502 (42)
Country of birth - n (%)	
Spain	1106 (93)
Other	82 (7)

Abbreviations: SD: standard deviation; PFHxS: perfluorohexanesulfonic acid; PFOS: perfluorooctanesulfonic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; BMI: body mass index.

Table 2. Prevalence of immune and respiratory outcomes in the present study.

	Age at follow-up		
Outcome	**1.5 years**	**4 years**	**7 years**
Child age (years) - mean (SD)	13.64 (1.31)	4.39 (0.16)	7.40 (0.52)
Chest infections - n (%)			
No	777 (65)	1106 (93)	1010 (94)
Yes	411 (35)	78 (7)	61 (6)
Total	1188 (100)	1184 (100)	1071(100)
LRTIs - n (%)			
No	777 (65)	887 (75)	-
Yes	411 (35)	297 (25)	-
Total	1188 (100)	1184 (100)	-
Wheeze - n (%)			
No	807 (68)	972 (82)	948 (89)
Yes	381 (32)	216 (18)	121 (11)
Total	1188 (100)	1188 (100)	1069 (100)
Asthma - n (%)			
No	-	1150 (97)	1012 (95)
Yes	-	34 (3)	56 (5)
Total	-	1184 (100)	1068 (100)
Eczema - n (%)			
No	960 (81)	954 (81)	708 (66)
Yes	228 (19)	230 (19)	358 (34)
Total	1188 (100)	1184 (100)	1066 (100)
Spirometry – mean (SD)			
FEV_1 (mL)	-	-0.64 (1.25)	0.18 (0.97)
FVC (mL)	-	-0.58 (1.30)	0.37 (0.96)
FEV_1/FVC (%)	-	-0.01 (0.99)	-0.33 (0.99)
FEF_{25-75}	-	-0.56 (1.02)	-0.21 (0.99)

Abbreviations: SD: standard deviation; LRTIs: lower respiratory tract infections.

Table 3. GEE models - fully-adjusted associations between maternal PFAS concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes during childhood in the INMA birth cohort study.

	RR (95% CI)			
Outcomes during childhood	**PFHxS**	**PFOS**	**PFOA**	**PFNA**
Unadjusted model				
Chest infections	0.98 (0.90, 1.08)	0.90 (0.79, 1.03)	0.89 (0.79, 0.99)*	0.95 (0.85, 1.07)
LRTIs §	1.08 (0.99, 1.19)	0.96 (0.84, 1.10)	0.99 (0.89, 1.11)	0.99 (0.88, 1.10)
Wheeze	1.00 (0.92, 1.10)	0.89 (0.79, 1.01)	0.94 (0.85, 1.05)	0.92 (0.83, 1.03)
Asthma	0.79 (0.65, 0.96)*	0.77 (0.57, 1.04)	0.70 (0.53, 0.91)**	0.69 (0.55, 0.86)**
Eczema	1.05 (0.96, 1.15)	0.90 (0.80, 1.02)	1.04 (0.94, 1.16)	1.01 (0.91, 1.12)
Adjusted model [a]				
Chest infections	1.05 (0.94, 1.18)	0.94 (0.81, 1.08)	0.96 (0.84, 1.09)	0.96 (0.85, 1.09)
LRTIs §	1.09 (0.98, 1.22)	0.98 (0.85, 1.13)	1.01 (0.88, 1.15)	0.95 (0.85, 1.07)
Wheeze	0.99 (0.89, 1.11)	0.89 (0.78, 1.02)	0.95 (0.83, 1.07)	0.90 (0.80, 1.01)
Asthma ¥	0.95 (0.73, 1.24)	0.82 (0.58, 1.16)	0.84 (0.61, 1.15)	0.75 (0.57, 0.97)*
Eczema	0.95 (0.86, 1.05)	0.86 (0.75, 0.98)*	0.96 (0.85, 1.08)	0.94 (0.84, 1.06)

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

[a] Models adjusted for maternal age, parity, previous breastfeeding, pre-pregnancy BMI, region of residence, country of birth, and fish consumption during pregnancy, and sex of the child.

§ Only available at 1.5 and 4 years. ¥ Only available at 4 and 7 years. *p-value<0.05; **p-values<0.01.

Table 4. Fully adjusted associations between maternal PFAS concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes during childhood in the INMA birth cohort study. [a]

			OR (95% CI)			
Age at follow-up	**n**	**n° of cases**	**PFHxS**	**PFOS**	**PFOA**	**PFNA**
Chest infections						
1.5 years	1188	411	1.13 (0.97, 1.32)	1.05 (0.87, 1.28)	1.05 (0.88, 1.25)	1.10 (0.93, 1.29)
4 years	1184	78	0.89 (0.67, 1.18)	0.70 (0.49, 1.02)	0.89 (0.64, 1.24)	0.74 (0.56, 0.97)*
7 years	1071	61	0.99 (0.73, 1.36)	0.78 (0.53, 1.15)	0.68 (0.46, 1.00)	0.86 (0.62, 1.20)
LRTIs §						
1.5 years	1188	411	1.13 (0.97, 1.32)	1.05 (0.87, 1.28)	1.05 (0.88, 1.25)	1.10 (0.93, 1.29)
4 years	1184	297	1.06 (0.90, 1.25)	0.93 (0.75, 1.14)	1.00 (0.83, 1.21)	0.85 (0.72, 1.01)
Wheeze						
1.5 years	1188	381	1.13 (0.96, 1.32)	1.12 (0.91, 1.36)	1.03 (0.86, 1.23)	1.14 (0.96, 1.36)
4 years	1188	216	0.96 (0.80, 1.15)	0.83 (0.66, 1.05)	1.01 (0.82, 1.26)	0.87 (0.72, 1.05)
7 years	1069	121	0.83 (0.66, 1.03)	0.70 (0.52, 0.94)*	0.80 (0.60, 1.06)	0.69 (0.54, 0.88)**
Asthma ¥						
4 years	1184	34	1.00 (0.66, 1.52)	0.70 (0.41, 1.18)	0.86 (0.53, 1.40)	0.62 (0.43, 0.89)**
7 years	1068	56	0.90 (0.66, 1.23)	0.96 (0.62, 1.48)	0.87 (0.58, 1.29)	0.87 (0.61, 1.25)
Eczema						
1.5 years	1188	228	1.11 (0.92, 1.34)	0.95 (0.75, 1.19)	1.03 (0.83, 1.28)	1.09 (0.89, 1.33)
4 years	1184	230	0.88 (0.74, 1.05)	0.77 (0.61, 0.96)*	0.90 (0.73, 1.12)	0.79 (0.66, 0.96)*
7 years	1066	358	0.89 (0.76, 1.04)	0.78 (0.63, 0.96)*	0.91 (0.75, 1.10)	0.96 (0.80, 1.14)

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

[a] Models adjusted maternal age, parity, previous breastfeeding, pre-pregnancy BMI, region of residence, country of birth, and fish consumption during pregnancy, and sex and age-at-follow-up of the child.

§ Only available at 1.5 and 4 years. ¥ Only available at 4 and 7 years. * p-value<0.05; ** p-values<0.01

Table 5. Fully adjusted associations between maternal PFAS concentrations (log_2-transformed, ng/mL) and lung function z-scores during childhood in the INMA birth cohort study. [a]

		β (95% CI)			
Age at follow-up	**n**	**PFHxS**	**PFOS**	**PFOA**	**PFNA**
FEV_1					
4 years §	501	-0.04 (-0.16, 0.09)	-0.05 (-0.22, 0.12)	-0.12 (-0.29, 0.04)	0.06 (-0.10, 0.21)
7 years	997	-0.04 (-0.11, 0.04)	-0.03 (-0.12, 0.07)	-0.01 (-0.10, 0.08)	-0.01 (-0.09, 0.07)
FVC					
4 years §	501	-0.04 (-0.17, 0.09)	-0.08 (-0.25, 0.09)	-0.16 (-0.33, 0.00)	0.05 (-0.11, 0.21)
7 years	997	-0.03 (-0.10, 0.04)	-0.04 (-0.13, 0.06)	-0.05 (-0.14, 0.04)	-0.02 (-0.10, 0.06)
FEV_1/ FVC					
4 years §	501	0.03 (-0.07, 0.13)	0.04 (-0.09, 0.17)	0.08 (-0.05, 0.21)	0.00 (-0.12, 0.12)
7 years	997	-0.02 (-0.10, 0.06)	0.01 (-0.08, 0.11)	0.06 (-0.03, 0.14)	0.01 (-0.08, 0.09)
FEF_{25-75}					
4 years §	501	-0.02 (-0.12, 0.08)	0.04 (-0.09, 0.18)	0.03 (-0.10, 0.16)	0.07 (-0.05, 0.20)
7 years	990	-0.01 (-0.09, 0.06)	0.01 (-0.09, 0.10)	0.04 (-0.05, 0.13)	0.03 (-0.06, 0.11)

Abbreviations: CI: confidence interval; PFHxS: perfluorohexanesulfonic acid; PFOS: perfluorooctanesulfonic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; FEV_1: forced expiratory volume in first second; FVC: forced vital capacity; FEF: forced expiratory flow (i.e. 25%-75%).

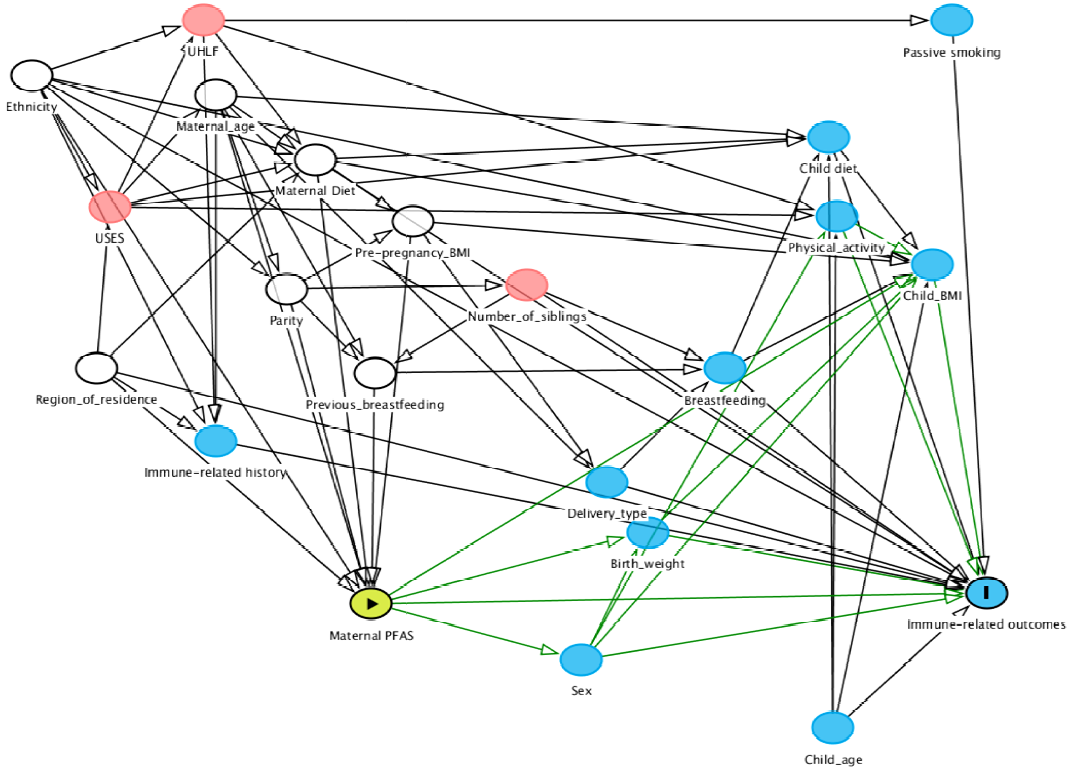
[a] Models adjusted for maternal age, parity, previous breastfeeding, pre-pregnancy BMI, region of residence, country of birth, and fish consumption during pregnancy.

§ Spirometry at 4 years was only available in Sabadell and Gipuzkoa.

Supplementary Material

1. Figure S1. Directed acyclic graph for confounder selection in this study.
2. Table S1. Minimally adjusted associations between maternal PFAS concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes during childhood in the INMA birth cohort study.
3. Table S2. Non-adjusted associations between maternal PFAS concentrations (log_2-transformed, ng/mL) and lung function z-scores during childhood in the INMA birth cohort study.
4. Table S3. Fully adjusted associations between maternal PFAS concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes by sex of the child in the INMA birth cohort study.
5. Table S4. Maternal PFAS concentrations (log_2-transformed, ng/mL) and asthma during childhood by region of residence in the INMA birth cohort study.
6. Table S5. Multipollutant model (GEEs) for maternal PFAS concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes during childhood in the INMA birth cohort study.

Figure S1. Directed acyclic graph for confounder selection in this study.



Color legend: White: maternal confounders included in the fully adjusted models; Red: common ancestors of exposure and ou
ancestors of outcome. USES: unmeasured socio economic status (i.e. household income); UHFL: unmeasured health and lifestyl
smoking).

Table S1. Minimally adjusted associations between maternal PFAS concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes during childhood in the INMA birth cohort study. [a]

			OR (95% CI) [a]			
Age at follow-up	**n**	**n° of cases**	**PFHxS**	**PFOS**	**PFOA**	**PFNA**
Chest infections						
1.5 years	1188	411	1.04 (0.92, 1.18)	0.99 (0.82, 1.18)	0.93 (0.80, 1.09)	1.07 (0.92, 1.25)
4 years	1184	78	0.80 (0.64, 0.99)*	0.72 (0.52, 0.98)*	0.82 (0.61, 1.09)	0.66 (0.52, 0.83)**
7 years	1071	61	1.05 (0.78, 1.42)	0.83 (0.58, 1.20)	0.77 (0.54, 1.09)	1.02 (0.72, 1.43)
LRTIs [§]						
1.5 years	1188	411	1.04 (0.92, 1.18)	0.99 (0.82, 1.18)	0.93 (0.80, 1.09)	1.07 (0.92, 1.25)
4 years	1184	297	1.14 (0.99, 1.32)	0.95 (0.78, 1.16)	1.12 (0.94, 1.32)	0.91 (0.78, 1.07)
Wheeze						
1.5 years	1188	381	1.03 (0.90, 1.17)	1.05 (0.87, 1.25)	0.91 (0.78, 1.06)	1.11 (0.95, 1.30)
4 years	1188	216	1.05 (0.89, 1.23)	0.87 (0.70, 1.07)	1.09 (0.90, 1.31)	0.93 (0.78, 1.11)
7 years	1069	121	0.87 (0.72, 1.06)	0.73 (0.56, 0.95)*	0.86 (0.67, 1.11)	0.68 (0.54, 0.85)**
Asthma [¥]						
4 years	1184	34	0.83 (0.61, 1.14)	0.72 (0.45, 1.13)	0.77 (0.50, 1.17)	0.61 (0.45, 0.83)**
7 years	1068	56	0.83 (0.63, 1.08)	0.84 (0.57, 1.25)	0.77 (0.54, 1.10)	0.83 (0.59, 1.15)
Eczema						
1.5 years	1188	228	1.20 (1.02, 1.41)*	1.02 (0.83, 1.27)	1.11 (0.92, 1.33)	1.16 (0.96, 1.40)
4 years	1184	230	0.99 (0.85, 1.15)	0.80 (0.65, 0.98)*	0.97 (0.81, 1.17)	0.89 (0.75, 1.06)
7 years	1066	358	0.92 (0.80, 1.06)	0.82 (0.68, 0.99)*	0.96 (0.81, 1.13)	0.94 (0.79, 1.11)

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

[a] Models minimally adjusted for the sex and age-at-follow-up of the child.

[§] Only available at 1.5 and 4 years. [¥] Only available at 4 and 7 years. *p-value<0.05; ** p-values<0.01.

Table S2. Non-adjusted associations between maternal PFAS concentrations (log_2-transformed, ng/mL) and lung function z-scores during childhood in the INMA birth cohort study.

		β (95% CI)			
Age at follow-up	**n**	**PFHxS**	**PFOS**	**PFOA**	**PFNA**
FVC					
4 years [§]	501	-0.03 (-0.13, 0.08)	-0.08 (-0.24, 0.08)	-0.11 (-0.25, 0.03)	0.04 (-0.11, 0.19)
7 years	997	-0.08 (-0.14, -0.01)[*]	-0.09 (-0.18, 0.00)	-0.11 (-0.19, -0.03)[**]	-0.03 (-0.11, 0.05)
FEV_1					
4 years [§]	501	-0.04 (-0.15, 0.06)	-0.08 (-0.23, 0.08)	-0.11 (-0.24, 0.02)	0.03 (-0.12, 0.17)
7 years	997	-0.09 (-0.15, -0.02)[**]	-0.06 (-0.16, 0.03)	-0.06 (-0.14, 0.02)	-0.06 (-0.14, 0.02)
FEV_1/ FVC					
4 years [§]	501	0.00 (-0.08, 0.08)	0.02 (-0.11, 0.14)	0.03 (-0.07, 0.14)	-0.01 (-0.13, 0.10)
7 years	997	-0.04 (-0.10, 0.03)	0.03 (-0.07, 0.12)	0.06 (-0.02, 0.14)	-0.07 (-0.15, 0.01)
FEF_{25-75}					
4 years [§]	501	-0.07 (-0.16, 0.01)	-0.01 (-0.14, 0.12)	-0.06 (-0.17, 0.05)	0.01 (-0.11, 0.12)
7 years	990	-0.05 (-0.11, 0.02)	-0.01 (-0.10, 0.09)	0.00 (-0.08, 0.09)	-0.02 (-0.10, 0.07)

Abbreviations: CI: confidence interval; PFHxS: perfluorohexanesulfonic acid; PFOS: perfluorooctanesulfonic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; FVC: forced vital capacity; FEV: forced expiratory volume in first second; FEF: forced expiratory flow (i.e. 25%-75%).

[§] Spirometry at 4 years only available in Sabadell and Gipuzkoa. [*] p-value<0.05; [**] p-values<0.01.

Table S3. Fully adjusted associations (GEE analysis) between maternal PFAS concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes by sex of the child in the INMA birth cohort study.

	RR (95% CI)							
Outcomes during childhood	**PFHxS**	**p-value interaction**	**PFOS**	**p-value interaction**	**PFOA**	**p-value interaction**	**PFNA**	**p-value interaction**
Chest infections								
Girls	1.02 (0.86, 1.21)	0.46	0.98 (0.78, 1.22)	0.71	0.95 (0.78, 1.16)	0.50	0.95 (0.79, 1.15)	0.69
Boys	1.08 (0.93, 1.26)		0.95 (0.79, 1.15)		1.02 (0.85, 1.22)		1.01 (0.86, 1.19)	
LRTIs [§]								
Girls	1.03 (0.87, 1.21)	0.27	1.00 (0.81, 1.24)	0.67	0.99 (0.82, 1.20)	0.60	0.98 (0.82, 1.18)	0.82
Boys	1.14 (0.98, 1.34)		0.96 (0.80, 1.17)		1.03 (0.86, 1.24)		0.93 (0.79, 1.10)	
Wheeze								
Girls	0.96 (0.81, 1.12)	0.67	0.85 (0.69, 1.05)	0.47	0.91 (0.75, 1.10)	0.78	0.82 (0.69, 0.98)*	0.19
Boys	1.00 (0.86, 1.17)		0.92 (0.76, 1.12)		0.95 (0.79, 1.15)		0.96 (0.81, 1.13)	
Asthma [¥]								
Girls	0.78 (0.49, 1.24)	0.81	0.50 (0.27, 0.92)*	0.52	0.83 (0.46, 1.50)	0.19	1.02 (0.57, 1.83)	0.11
Boys	1.03 (0.69, 1.54)		0.88 (0.53, 1.46)		0.67 (0.42, 1.07)		0.62 (0.44, 0.87)**	
Eczema								
Girls	0.89 (0.77, 1.03)	0.71	0.78 (0.64, 0.94)*	0.38	0.90 (0.75, 1.07)	0.90	0.90 (0.76, 1.06)	0.94
Boys	1.01 (0.87, 1.18)		0.91 (0.74, 1.10)		0.98 (0.81, 1.18)		0.93 (0.79, 1.10)	

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

[a] Models adjusted for maternal age, parity, previous breastfeeding, pre-pregnancy BMI, region of residence, country of birth, and fish consumption during pregnancy, and sex of the child.

[§] Only available at 1.5 and 4 years. [¥] Only available at 4 and 7 years. * p-value of sex-interaction<0.05; ** p-value of sex-interaction<0.01.

Table S4. Maternal PFAS concentrations (log_2-transformed, ng/mL) and asthma during childhood by region of residence in the INMA birth cohort study.

			OR (95% CI)			
Age at follow-up	**N**	**N° of cases**	**PFHxS**	**PFOS**	**PFOA**	**PFNA**
4 years						
Overall	1184	34	1.00 (0.66, 1.52)	0.70 (0.41, 1.18)	0.86 (0.53, 1.40)	0.62 (0.43, 0.89)**
Gipuzkoa	296	14	0.98 (0.51, 1.90)	0.82 (0.36, 1.85)	0.79 (0.35, 1.78)	0.52 (0.25, 1.10)
Sabadell	400	4	0.48 (0.20, 1.16)	0.07 (0.01, 0.70)*	0.16 (0.03, 0.80)*	0.22 (0.05, 0.99)*
Valencia	488	16	1.30 (0.57, 2.94)	0.97 (0.38, 2.50)	1.15 (0.54, 2.44)	1.04 (0.51, 2.12)
7 years ¥						
Overall	1068	56	0.90 (0.66, 1.23)	0.96 (0.62, 1.48)	0.87 (0.58, 1.29)	0.87 (0.61, 1.25)
Gipuzkoa	264	23	0.84 (0.53, 1.34)	1.22 (0.60, 2.48)	1.21 (0.66, 2.23)	1.06 (0.60, 1.87)
Sabadell	384	10	0.69 (0.37, 1.29)	0.46 (0.23, 0.91)*	0.40 (0.18, 0.88)*	0.57 (0.34, 0.94)*
Valencia	420	23	1.03 (0.55, 1.93)	1.21 (0.57, 2.57)	0.73 (0.39, 1.37)	1.08 (0.59, 1.99)

Abbreviations: or: odds ratio; CI: confidence interval; PFHxS: perfluorohexanesulfonic acid; PFOS: perfluorooctanesulfonic acid; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid.

[a] Models adjusted for maternal age, parity, previous breastfeeding, pre-pregnancy BMI, country of birth, and fish consumption during pregnancy, and sex and age-at-follow-up of the child.

¥ Only available at 4 and 7 years. * p-value<0.05. All interaction terms had p-values>0.05, except for PFOA and asthma at 4 year.

Table S5. Multipollutant model (GEE analysis) for maternal PFAS concentrations (log_2-transformed, ng/mL) and immune and respiratory outcomes during childhood in the INMA birth cohort study.

	RR (95% CI)			
Outcomes during childhood	**PFHxS**	**PFOS**	**PFOA**	**PFNA**
Chest infections	1.11 (0.96, 1.27)	0.90 (0.74, 1.09)	0.97 (0.80, 1.17)	1.00 (0.84, 1.20)
LRTIs [§]	1.15 (1.00, 1.32)*	0.93 (0.77, 1.12)	1.04 (0.87, 1.25)	0.91 (0.77, 1.08)
Wheeze	1.06 (0.93, 1.22)	0.90 (0.75, 1.09)	1.02 (0.85, 1.23)	0.91 (0.77, 1.07)
Asthma [¥]	1.19 (0.81, 1.76)	0.85 (0.49, 1.47)	0.91 (0.56, 1.49)	0.71 (0.47, 1.06)
Eczema	1.02 (0.90, 1.16)	0.83 (0.70, 0.99)*	1.03 (0.86, 1.22)	1.00 (0.85, 1.18)

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

[a] Models adjusted for all PFAS, maternal age, parity, previous breastfeeding, pre-pregnancy BMI, region of residence, country of birth, and fish consumption during pregnancy, and sex of the child.

[§] Only available at 1.5 and 4 years. [¥] Only available at 4 and 7 years. * p-value<0.05.

6. DISCUSSION

6.1. General Discussion

In this thesis, the association between prenatal PFAS exposure and child health was evaluated in five research articles. Results from these articles, as well as strengths and limitations pertaining to each of these studies individually were discussed in detail in the previous chapter. In this chapter, the more general methodological considerations of my research will be reviewed to complement the discussion of the five research articles. Additionally, I will discuss the contributions and implications for public health of my research, and a proposal of future research ideas within this field.

6.2. Methodological considerations

The articles included in this thesis were based on the INMA birth cohort study that has a prospective follow-up from birth until mid-childhood. The prospective nature of the INMA birth cohort allowed me to assess the association between early-life exposure to PFAS and long-term child health. Birth cohorts are also less prone to memory recall bias and allow for studying exposure and outcomes at different time-points. Further, four of the papers of this thesis (all but paper I) had a large sample size, which strengthens the conclusions of the current work. However, several methodological aspects of this thesis should be considered.

6.2.1. Exposure assessment

6.2.1.1. Underestimation of PFOS concentration

The production of PFOS has been historically done by two main processes electrochemical fluorination (ECF) and telomerization. Before the year 2002, ECF was the major PFOS manufacturing process (Benskin et al. 2009a). In the ECF process approximately 21-35% of PFOS is reorganized from linear to branched PFOS (Benskin et al. 2009b; Chu and Letcher 2009; Lindstrom et al. 2011). The ECF process was largely phased-out in 2002 (by the major USA producer of PFOS in the United States) and since then, telomerization, which exclusively produces linear PFOS isomers, is the main manufacturing process used (Greaves and Letcher 2013).

In this thesis only linear isomers of PFOS were quantified. Thus, PFOS concentrations may have been underestimated in this thesis if the exposure occurred before the year 2002, and the results cannot be directly compared with other studies assessing branched and linear PFOS.

6.2.1.2. Confounding by correlated PFAS exposures

The PFAS assessed in this thesis have biological half-lives of 3 to 7 years and, thus, a single measurement is considered to represent the fetal exposure throughout the entire pregnancy with little risk of exposure misclassification. However, PFAS, as a chemical group, are moderately correlated, with PFOA and PFNA (Spearman rho: 0.68, p-value<0.001) being the most correlated, and PFHxS and PFNA being the less correlated (Spearman rho: 0.43, p-value<0.001). The correlation of PFAS makes it difficult to disentangle their independent effects (if any). To overcome this limitation, we performed multipollutant models including all PFAS as a sensitivity analysis in papers III, IV, and V. Using a model that included all four PFAS (compared with estimates from single-PFAS models) had small changes in some of the associations observed in this thesis. For example, in paper IV, the estimated associations with the CM-risk score differed in the multipollutant model with coefficients suggesting a stronger positive association with PFNA (β = 0.85; 95% CI: 0.01, 1.69), a weaker positive association with PFOS (β = 0.11; 95% CI: –0.73, 0.95), and a stronger negative association with PFHxS (β = –0.36; 95% CI: –1.05, 0.33), while the association with PFOA changed from positive to negative (β = –0.26; 95% CI: –1.20, 0.68). However, in paper IV the estimates from the multipollutant model were imprecise, and, with the exception of the association with PFNA, none were clearly different from the null.

In general, risk estimates in this thesis remained unchanged after including all maternal PFAS concentrations in a single model, thus there was little evidence of confounding of one PFAS by another. Future studies may use a summary risk score or other statistical methods, such as structural equation models (Grandjean et al. 2012; Sánchez et al. 2005), in order to assess the joint effects of PFAS exposure.

6.2.1.3. Confounding by postnatal PFAS exposure

Postnatal PFAS exposure has been associated with cardiometabolic risk (e.g. Domazet et al. 2016; Zeng et al. 2015) and immune and respiratory health (Grandjean et al. 2012; Qin et al. 2017) during childhood. Therefore, postnatal exposure to PFAS may confound the association between prenatal PFAS exposure and child health outcomes. In this thesis we did not have information on postnatal PFAS exposure. However, in papers III, IV, and V, the duration of breastfeeding of the index child was used as a proxy of postnatal PFAS exposure in the sensitivity analyses. For most of the newborns in INMA, breastfeeding is the principal dietary source and, probably, the main route of PFAS exposure during the first months of life; and thus, using the duration of breastfeeding as a proxy of postnatal dietary exposure to PFAS seemed appropriate. Based on the sensitivity analyses, some results suggest that postnatal PFAS exposure through longer breastfeeding duration may confound the associations assessed in this thesis. For example, in paper IV the associations between PFOA and BMI z-scores at 4 and 7 years increased with longer breastfeeding duration of the index child, and these associations were statistically significant in very long-term breastfeeding duration (≥ 6 months), i.e. at 4 years: β=0.14 (0.03, 0.26; p-interaction= 0.09)] and 7 years: β=0.17 (0.02, 0.32; p-interaction= 0.01). Future studies assessing postnatal PFAS exposure at different time periods during childhood would allow understanding the susceptible windows of PFAS exposure.

6.2.1.4. Confounding by maternal GFR during pregnancy

Maternal excretion rates during pregnancy may influence the associations between prenatal exposure to PFOS and PFOA and birth weight (Verner et al. 2015). In paper III and IV, we performed sensitivity analysis adjusting the regression models by maternal GFR and showed that excretion rates are unlikely to have confounded the association between maternal PFAS concentration in plasma and birth and cardiometabolic outcomes. Contrary to the results of paper III, in a sub-analysis done in a Norwegian birth cohort, maternal GFR attenuated by at least 66% the association between maternal PFOA (measured in mid-pregnancy) and birth weight (Morken et al. 2014). For the results of paper IV, two other studies from the VIVA birth cohort reported similar findings as ours. Fleisch et al. (2016) reported that after adjustment for GFR their exposure-outcome (PFAS and mid-childhood glucose

homeostasis) estimates did not change by more than 10%. Mora et al. (2016) reported that adjusting for GFR marginally strengthened the associations between prenatal PFAS exposure and adiposity in mid-childhood, suggesting some confounding by GFR. Both in the VIVA and in our cohort, maternal PFAS concentrations were measured early in pregnancy when changes in GFR might not have a big impact on PFAS concentrations (Verner et al. 2015).

In this thesis, we calculated maternal GFR with a single measurement of serum creatinine which might be imprecise (Aras et al. 2012). Still we adjusted for maternal GFR during pregnancy, which is novel in this type of studies. Future studies should evaluate GFR at different time-points during pregnancy. Finally, we estimated GFR using the Cockcroft-Gault formula, however inulin clearance is the gold standard measurement for estimating GFR (Morken et al. 2014) but this method seems to be impractical in settings like ours because the technique is time-consuming and has a high price.

6.2.2. Outcome assessment

6.2.2.1. Use of markers of fetal growth

In paper III, anthropometric measurements at birth were used as markers of fetal growth but direct ultrasound measurements may be preferable to study the direct effect of PFAS on fetal growth (Zheng et al. 2016). Even though pregnancy ultrasound measurements are available in INMA, these were not included in this thesis because this study work is currently being done by our collaborators from INMA-Valencia.

6.2.2.2. Use of indirect measurements of adiposity

In this thesis, anthropometric data was abstracted from medical registries or measured by trained personnel during the follow-up period. This allowed us to have repeated anthropometric (including weight, height, and waist circumference) at different ages in order to prospectively evaluate the association between PFAS and postnatal growth and cardiometabolic risk throughout childhood (something still scarce in this field). Using measured instead of self-reported weight and height strengthens the results of paper IV given that parents, especially mothers, may inaccurately estimate the

weight of the child especially during mid-childhood and thus lead to outcome misclassification (Dubois and Girad 2007). Childhood weight-gain, BMI, and waist circumference have been shown to correlate with later obesity risk and obesity-related diseases (Druet et al. 2012; Franks et al. 2010; Monteiro and Victora 2005; Vanhala et al. 1998), yet these anthropometric measurements only provide an indirect assessment of total body composition and fat content and other tools such as bioelectrical impedance or DXA may be preferable. A study from the Project Viva birth cohort (United States) assessed adiposity using DXA and observed that among girls, prenatal exposure to PFAS was associated with small but constant increases in total fat mass (Mora et al. 2016). These associations were also observed for BMI and waist circumference but less consistently. In the ALSPAC birth cohort (United Kingdom) that only includes girls, prenatal PFOS and PFOA exposure was positively associated with total percent of body fat at 9 years but only among daughters of middle educational group (Hartman et al. 2017). In the population overall, there was no association between prenatal PFAS exposure and body fat measured by DXA (Hartman et al. 2017). DXA is the gold standard measurement to assess adiposity, and although complex and expensive, its use may increase the precision in the assessment of body content and fat composition in birth cohort studies.

Using direct adiposity measurements may be even more important for overweight and obese children given that BMI z-scores were reported to be a strong predictor of total fat mass but not of relative body fat in these subgroups (Vanderwall et al. 2017). In INMA, and as part of the HELIX project (Vrijheid et al. 2014), adiposity at age 8-10 years was measured using bioelectrical impedance, which may provide a better assessment of body composition. Having a more accurate measurement of body composition may increase the measurement precision and lead to less outcome misclassification. In the future, the correlation between bioelectrical impedance and BMI z-scores should be estimated in order to guide researchers which measurement should be used to properly assess childhood obesity in the INMA birth cohort.

6.2.2.3. Blood pressure assessment

In paper IV, blood pressure was measured twice at 7 years of age, and averaged as recommended (Pickering et al. 2005), but was

measured only once at age 4 years, and only in children from 2 (Valencia and Sabadell) of the 3 study regions. Further, we used the average of systolic and diastolic blood pressure as the blood pressure outcome, consistent with the study of Ahrens et al. (2014) though other studies have evaluated systolic and diastolic blood pressure as separate outcomes, or have used mean arterial pressure (Eisenmann 2008; Geiger et al. 2014b; Sardinha et al. 2016; Shafiee et al. 2013). Thus, we cannot discard that using other methods to estimate blood pressure would result in different conclusions of paper IV.

6.2.2.4. Use of non-fasting blood for lipid assessment

In this thesis, we assessed lipid levels in non-fasting blood samples collected at 4 years. Lipid levels were measured using fasting samples in children from the Valencia region, but non-fasting samples for children from Sabadell and Gipuzkoa, which may influence lipid levels, especially triglycerides. In paper IV, we restricted our analysis to the Valencia region (fasting blood) and the findings did not change. Nevertheless, future studies using fasting blood samples are recommended in order to overcome the limitation in the present thesis.

6.2.2.5. No marker of glucose homeostasis and low sample size in the CM-risk score

In paper IV, a cardiometabolic risk (CM-risk) score was calculated as a proxy for the clinical criteria used for diagnosing metabolic syndrome in adults. The CM-risk score included 3 of 4 individual components (e.g. anthropometric measurements, blood pressure and lipids) that are typically used to define metabolic syndrome (Eisenmann 2008). This score was based on a previous study by the IDEFICS consortium, yet the CM-risk score did not include a marker of glucose homeostasis, which is one of the components that is normally used to define metabolic syndrome (Ahrens et al. 2014; Eisenmann 2008). Therefore, this CM-risk score might not fully characterize the potential impact of PFAS on the prevalence of metabolic syndrome at age 4 or the future risk of cardiometabolic disease. Future follow-ups with available information on glucose homeostasis or insulin resistance at later ages are recommended. Finally, we could only calculate the CM-risk score in 386 children that were generally healthier than the rest, thus limiting the

extrapolation of the conclusions of paper IV to the full sample at 4 years.

6.2.2.6. Use of self-reported immune and respiratory outcomes

In paper V, the immune-related outcomes were assessed using self-reported questionnaires which may be a source of recall and misclassification bias. For example, misclassification bias can be an important issue for the occurrence of asthma at age 4 years as it can be confounded with the occurrence of wheezing due to chest infections instead of asthma. At 7 years we asked if a doctor had given an asthma diagnosis and our results remained similar to the 4 years results. Thus we should consider this as a non-differential misclassification bias that will drive our associations, if any, towards the null (Rothman 2002). Also in paper V, we lacked serology or culture to confirm the presence of LRTIs or bronchitis diagnosis but similar results were observed with wheezing, which is a related outcome of LRTIs especially at 1.5 years.

6.2.2.7. The influence of age

The age at follow-up may influence the occurrence and etiology of the health outcomes assessed in papers IV and V of this thesis and may partially explain some of the null associations observed.

In paper IV, we did not observe any clear association between PFAS and BMI, overweight, WC, and WHtR>0.5 at ages 4 and 7 years. However, a study from Denmark reported that maternal PFOA concentrations were associated with a higher risk of being overweight in women aged 20 years (Halldorsson et al. 2012), suggesting that *in-utero* exposure to PFOA may have long latency periods before health effects are manifested.

Further, in paper IV we assessed the cardiometabolic risk at age 4 years as a proxy for adult metabolic syndrome; however at these young ages, there is no cutoff that defines metabolic syndrome and its prevalence is relatively low because clinical signs may not be evident until later (Eisenmann 2008). Considering the low prevalence of metabolic syndrome in children and that it can be tracked from childhood to adulthood, we used a continuous score (instead of a dichotomous outcome) to assess cardiometabolic risk and increase our statistical power. Yet the ideal assessment of cardiometabolic risk would include repeated measurements of body

fat composition, lipids levels, blood pressure, and glucose homeostasis throughout childhood, puberty, and adulthood. Given that the cardiometabolic risk, as an entity, is not fixed but rather develops progressively according to age and pubertal changes (Kassi et al. 2011), future studies evaluating the association between PFAS and cardiometabolic risk factors at later ages are needed.

The immune and respiratory outcomes assessed in paper V are also highly dependent on the age at follow-up, since their etiology and occurrence changes as the child grows up. For instance, as asthma is rarely diagnosed or prevalent before the age 5 years assessing asthma at 4 years may introduce non-differential misclassification bias that can drive our associations towards the null. Further, performing a spirometry test before age 5 years is not recommended (Turner et al. 2007). Given that children in INMA performed the spirometry test at 4 years, the test reliability and thus, the quality of the lung function assessment at this age may be compromised. Yet one of the strengths of this thesis is that spirometry tests were also performed in a later follow-up at 7 years improving the assessment of lung function in the INMA birth cohort.

6.2.3. The influence of sex

PFAS are endocrine disruptors that may exhibit sex-specific effects. In this thesis, PFAS sex-specific behavior was evaluated by including the sex-interaction term and by stratifying the regression models in papers I, III, IV, and V. After sex-stratification, some associations (even though most of them were not statistically significant) did suggest that PFAS may exhibit sex-specific effects that are worth considering in other, probably larger, populations. This was the case in paper IV in which we observed a pattern of inverse associations between PFHxS and BMI and waist circumference in the overall population and in boys; whereas for girls positive associations were observed (p-values for sex-interaction at 4 years ≥ 0.12 and at 7 years ≥ 0.16). At 4 and 7 years, PFOS, PFOA, and PFNA showed patterns of positive associations with BMI in the overall population and in boys (p-values for sex-interaction > 0.18). Further, at 7 years we observed non-significant

associations between PFOA and blood pressure that were positive in boys but negative in girls (p-values for sex-interaction = 0.11).

6.2.4. Other sources of confounding

One of the main advantages of using data from a prospective birth cohort is the extensive information on potential confounders that was collected at different time points including socio-demographic, behavioral, and dietary characteristics. This provided data to test and adjust our models by many potential confounders, such as maternal previous breastfeeding, parity, region of residence, smoking, pre-pregnancy BMI, and others. Nonetheless, we cannot exclude residual confounding for other characteristics that we are not aware of, did not explore [e.g. exposure to other chemicals or consumption of fast foods (Tittlemier et al. 2007)], or do not have information about in this cohort (e.g. postnatal PFAS exposure). Given the overall small or null associations observed in this thesis, we consider that although residual confounding may be present it did not affect the interpretation of our findings.

6.2.5. Attrition bias

As in any birth cohort there were losses to follow-up during the study period. This can result in bias if attrition is related either to maternal PFAS exposure or the outcomes assessed in this thesis. In INMA, the study sample for PFAS assessment was randomly selected from those mother-child pairs that participated at the 4 years follow-up (n = 1,627 / 2,150, 75% of the initial cohort), which included mothers that were more likely to be older and nulliparous than in the initial cohort. Due to budget restrictions, the final PFAS assessment was done in 1,243 mother-child pairs (n = 1,243 / 1,627, 76 % of subjects at 4 year follow-up). Similarly to the full sample at 4 years, mothers included in this thesis project were more likely to be older and nulliparous than those included in the initial cohort. Considering that older and nulliparous women tend to have higher PFAS levels in INMA (as observed in paper II) then we probably included mothers and children with higher PFAS exposures than in the cohort as a whole.

6.3. Main findings and contributions to current knowledge

Overall, and according to the objectives, this thesis contributed to (1) the understanding of PFAS transfer between mother and fetus and its predictors in the INMA birth cohort; (2) the understanding of the main socio-demographic, lifestyle, and dietary factors that influence maternal PFAS concentrations in INMA; (3) and the understanding of the association between prenatal PFAS exposure and birth outcomes, obesity and cardiometabolic risk, and immune and respiratory health in children from the INMA birth cohort.

6.3.1. Placental transfer of PFAS in a Spanish birth cohort

In paper I, PFAS concentrations were assessed for the first time in matched maternal-cord samples in Spain. In this study, PFOS exposure levels were higher than in previous recent studies (Fromme et al., 2010; Hanssen et al., 2010; Porpora et al., 2013), but lower than in studies conducted before 2003 (Fei et al., 2007; Midasch et al., 2007). As a whole, this tendency is better explained by the voluntary phasing-out of PFOS in the year 2002, showing world-wide reductions in exposure levels to this specific chemical. In this sense, PFOS phased-out shows the quick and extended benefits of eliminating or regulating sources of chemical exposure.

Paper I confirmed, for the first time, that PFAS do distribute proportionally between maternal plasma and serum during pregnancy. This is beneficial for the direct comparison of studies using either plasma or serum to assess PFAS concentrations. This finding may also be important in birth cohort studies, where the lack of available biological samples is a common logistical issue, as studies can increase their statistical power by measuring PFAS in either maternal plasma or serum and directly compare the concentrations.

In paper I, we observed moderate to high correlations between concentrations of PFAS in maternal and cord samples indicating that maternal samples collected early in pregnancy can also be used to assess prenatal exposure to PFAS in epidemiological studies.

This can be an advantage over cord blood given the logistic problems in its collection at the time of delivery (e.g. having onsite personnel) or the lack of available archived cord blood samples that is common in cohort studies.

Paper I also contributed to the current knowledge of the transfer of PFAS across the placenta showing that PFOA was more easily transferred from mother to fetus than the rest of PFAS. Although mothers had higher PFOS concentration the fetus was more exposed to PFOA than PFOS. Our results are in agreement with previous studies (Hanssen et al. 2013; Kato et al. 2014) suggesting that the easiness of transfer of PFAS across the placenta depend on the functional group and carbon-chain length of PFAS. In this sense, carboxylates and shorter-chained PFAS transferred easier than sulfonates and longer-chained PFAS.

6.3.2. Determinants of maternal PFAS exposure

Following the results from paper I, we extended the assessment of PFAS to a larger sample in the INMA birth cohort. Given that PFAS correlated well between maternal and cord blood samples, we decided to measure PFAS using maternal plasma samples collected during the first trimester of pregnancy. This measurement was used for prenatal PFAS exposure in the subsequent papers included in this thesis. In paper II, PFAS were assessed in 1,216 maternal plasma samples and, at least PFOS (median concentration = 6.05 ng/mL) and PFOA (median concentration = 2.35 ng/mL) were detected in every maternal sample showing that mothers from the INMA birth cohort were ubiquitously exposed to these two chemicals during the years 2003-2008.

Paper II also suggested that maternal PFAS exposure in the INMA birth cohort was determined by five main socio-demographic and lifestyle characteristics. These were:

(1) Maternal age: older women had higher PFAS concentrations than younger women;

(2) Maternal region of residence: women from the region of Gipuzkoa had generally lower exposure levels than women from the Sabadell and Valencia regions;

(3) Maternal previous breastfeeding: longer duration of previous breastfeeding contributed to lower PFAS concentration in the index pregnancy;

(4) Maternal country of birth: women born in Spain had higher PFAS concentrations than women born in other countries; and

(5) Maternal parity: a higher number of previous pregnancies contributed to lower PFAS concentrations in the index pregnancy.

Paper II, also suggested that fish and shellfish were the main dietary determinants of PFOS concentration in the INMA birth cohort showing that higher intake was associated with higher PFOS exposure. In general, the findings of this paper indicated that maternal PFAS concentrations in INMA were mostly determined by socio-demographic and lifestyle characteristics, rather than by dietary characteristics. The models included in paper II were able to predict from 26% up to 40% of the variability of PFAS concentrations in this cohort. This implies that other sources of PFAS exposure or better assessment of the determinants is needed in order to fully understand the variability of PFAS exposure in the INMA birth cohort study. Finally, the findings of paper II were used as the main covariates for the rest of analysis included in this thesis.

6.3.3. Prenatal PFAS exposure and child health outcomes

A summary of the main findings of the papers in this thesis that evaluated the associations between prenatal PFAS exposure and child health outcomes (papers III, IV, and V) can be found in Table 6.3.3.

Table 6.3.3. Summary of the main results of the studies included in this thesis (papers I and II are not included). [a]

Paper	Outcomes assessed	Age at outcome assessment	Main results
III	Standardized weight, length, head circumference, and gestational age. LBW, SGA, and preterm birth.	At birth	Overall, PFAS were not significantly associated to birth outcomes. ↓ Birth weights with PFOA, PFHxS and PFNA. ↑ Odds of LBW in boys with PFOS Maternal GFR did not confound the associations.
IV	Weight-gain z-score Age-and sex-specific z-scores for BMI, WC, and BP Age-, sex-, and region specific z-scores for TC, HDL-C, LDL-C, and TG, CM-risk score	Birth - 6 months 4 and 7 years 4 years	Overall, PFAS were not associated with individual outcomes or the combined CM-risk score. Except for: ↑ TGs at age 4 years with PFHxS ↑ CM-risk score with PFNA exposure
V	Wheeze, chest infections, LRTIs, asthma, and eczema Lung function by spirometry test	1.5, 4, and 7 years 4 and 7 years	↓ Odds of chest infections and asthma at 4 years with PFNA ↓ Odds of eczema at 4 years with PFOS and PFNA ↓ Odds of eczema at 7 years with PFNA and PFOS ↓ Odds of wheeze at 7 years with PFOS and PFNA ↓ FEV at 4 years with PFOA (borderline significant)

PFAS: perfluoroalkyl substances, PFHxS: perfluorohexane sulfonate, PFOS: perfluorooctane sulfonate, PFOA: perfluorooctanoate; PFNA: perfluorononanoate; LBW: low birth weight; SGA: small for gestational age; GFR: glomerular filtration rate; BMI: body mass index; WC: waist circunference; BP: blood pressure; TC: total cholesterol; HDL-C: high density lipoprotein; LDL-C: low density lipoprotein; TG. triglycerides; CM: cardiometabolic; LRTIs: lower respiratory tract infections; FEV: forced vital capacity. [a] Papers I and II are not included because the association between prenatal PFAS exposure and child health outcomes was not evaluated.

6.3.3.1. Fetal growth and preterm birth

In paper III, we assessed the association between prenatal PFAS exposure and birth outcomes in the INMA birth cohort. We further evaluated if these associations were confounded by maternal GFR early in pregnancy. The results from this paper suggest that maternal PFAS concentrations are not significantly associated to birth outcomes. However, PFHxS, PFOA, and PFNA showed weak, non-statistically significant associations with reduced birth weights ranging from 8.6 g to 10.3 g per doubling of exposure. These results are in line with the most recent meta-analysis on PFOA including 4,149 births, which concluded that higher prenatal PFOA concentrations are associated with reduced average birth weight (Johnson et al. 2014); this was also the main conclusion from other reviews (Bach et al. 2015a; Olsen et al. 2009a).

In paper III, the association between maternal PFAS and birth weight was not influenced by maternal GFR during pregnancy as previously suggested. This finding does not support one birth cohort study (Morken et al. 2014) and another simulation study (Verner et al. 2015) reporting that the association between maternal PFOA and birth weight may be attributable, in part, to confounding by maternal GFR (Morken et al. 2014; Verner et al. 2015). In paper III, confounding by GFR was also evaluated for the first time in a cohort study assessing more than PFOA, observing that adjusting for GFR did not influence the estimated associations, if any, between maternal PFHxS, PFOS, and PFNA and birth weight. The lack of confounding may be related to having measured maternal PFAS concentrations early in pregnancy when physiological changes, such as plasma volume expansion or changes in renal clearance, have not fully occurred.

In paper III, higher prenatal PFOS exposure was associated with boys having increased odds ratio of being born with low birth weight (< 2500g), whereas girls had decreased odds ratios. Sex-specific associations were observed in other studies (Bach et al. 2015b; Kishi et al. 2015; Washino et al. 2009) however in these studies girls seemed to be more vulnerable to PFAS exposure than boys. Sex-differences have been scarcely looked at in studies assessing the association between PFAS and birth outcomes, and more research is needed in order to identify a susceptible group.

6.3.3.2. Obesity and cardiometabolic risk

In paper IV, we evaluated the association between prenatal PFAS exposure and individual cardiometabolic risk factors (anthropometric measurements, blood pressure, and serum lipids) in early- and mid-childhood in the INMA birth cohort. In addition, a combined cardiometabolic risk score (CM-risk score) was calculated as an alternative predictor of overall cardiometabolic risk (Eisenmann 2008; Pandit et al. 2011).

In this study, overall, prenatal PFAS exposure was little or not associated with cardiometabolic risk components from birth until 7 years. None of the PFAS were significantly associated with anthropometric measurements or blood pressure at ages 4 or 7 years. We did observe, however, that a doubling of prenatal PFHxS was associated with higher triglycerides levels at 4 years, and prenatal PFNA exposure was associated with a higher CM-risk score. No other statistically significant associations was observed between PFAS concentrations and lipid levels or the CM-risk score in the overall population, or when stratified by sex. Furthermore, in this paper we assessed whether maternal GFR had any influence on the association between prenatal exposure to PFAS and weight of the child (Verner et al. 2015), showing that excretion rates are unlikely to confound the association between maternal PFAS concentration in plasma and childhood cardiometabolic outcomes.

In paper IV, higher prenatal PFHxS concentrations were associated with higher triglycerides levels at 4 years, with a higher point estimate for boys, but given the low precision of the estimates and p-interaction = 0.85, there is not clear evidence of a stronger association in boys than in girls. Few studies have assessed PFAS effect on lipids during childhood and adolescence, and even though they are of cross-sectional design they suggest that PFAS, especially PFOS and PFOA, alter the lipid profile in children (Frisbee et al. 2010; Geiger et al. 2014a; Lin et al. 2009; Zeng et al. 2015). Also, in a prospective study prenatal PFOA in the lowest tertile was positively associated with LDL-C but not with total cholesterol, high-density lipoprotein (HDL-C), or triglycerides in girls at 7 and 15 years old (Maisonet et al. 2015).

In this study, we observed higher CM-risk scores with higher PFOS, PFOA and PFNA but the association was only significant for

PFNA. In contrast, Lin et al. (2009) reported that serum PFHxS, PFOA, PFOS, and PFNA concentrations in NHANES participants 12–19 years of age were inversely associated with the prevalence of metabolic syndrome (based on ≥ 3 of the following conditions: high waist circumference, high serum triglycerides, low serum HDL-C, elevated systolic or diastolic blood pressure or medication for hypertension, elevated fasting blood glucose or medication to reduce blood glucose), with a significant negative association for PFNA. However, direct comparisons between our study and Lin et al. (2009) are not possible given differences in the study design, population age, and outcome.

The main contributions of Paper IV were the use of a prospective design and large sample size, and the ability to estimate associations between prenatal PFAS exposures and outcomes that may contribute to future cardiometabolic risk, including weight gain from birth to 6 months; BMI, waist circumference, and blood pressure at 4 and 7 years of age; and blood lipids and a composite CM-risk score (based WC, BP, and lipids) at age 4 years.

6.3.3.3. Immune and respiratory health

In paper V, we evaluated the association between prenatal PFAS exposure and immune and respiratory outcomes up to age 7 years in INMA.

The preliminary results from paper V suggest that prenatal PFNA and PFOS exposure was associated with reduced risk of immune and respiratory outcomes, especially asthma and eczema, at any age. These results are in line with some studies but not with others. Prenatal exposure to PFOA was inversely associated with recurrent wheeze in children 5-9 years old from the INUENDO birth cohort study (Smit et al. 2015). In the Hokkaido birth cohort study, prenatal PFAS exposure, particularly to the longer-chained PFAS such as perfluorododecanoic (PFDoDa) and perfluorotridecanoic (PFTrDA) acids, was inversely associated with allergic diseases at 1, 2, and 4 years old (Goudarzi et al. 2016; Okada et al. 2014). In the DNBC, prenatal PFOA exposure has been associated with reduced risk of hospitalizations for infectious diseases (Fei et al. 2010b). However, other studies in Norway, Taiwan, Japan, and the Faroe Islands found no association between prenatal PFAS exposure and self-reported allergy, asthma, atopic dermatitis, or

wheeze (Granum et al. 2013; Okada et al. 2012; Timmermann et al. 2017; Wang et al. 2011). Differences in exposure levels, outcome assessment, age at follow-up, and population setting may contribute to the conflicting results.

The inverse associations observed in paper V and other previous studies may be attributable to numerous reasons. First, prenatal PFAS exposure has been associated with immune-suppression during childhood (Grandjean et al. 2012; Granum et al. 2013). Thus, prenatal PFAS exposure may suppress the developing immune system in infants and indirectly reduced the risk of developing immune hyperactivity and hypersensitivity diseases, such as eczema and wheezing. In addition, we cannot rule out the possibility of negative confounding due to covariates that are positively associated with the exposure and inversely associated with the outcome. For example, maternal fish intake is positively associated with PFAS concentrations (as observed in paper II) and fish intake has also been suggested to decrease the risk of allergic diseases due to the high content of n-3 long-chain fatty acids (Hageman et al. 2012). Thus, even when we adjusted our models for maternal fish intake during pregnancy we used self-reported dietary information, and we cannot rule out the possibility of measurement error. A substantial imprecision of the confounder can cause an underestimation of the PFAS concentrations and its beneficial effects on the outcome even after confounder adjustment. Failure to adjust for a negative confounder can result in an underestimation of the toxicity of the exposure on the outcome (Choi et al. 2008).

Further in paper V, we observed a general pattern of non-significant associations between prenatal higher PFHxS, PFOS, and PFOA exposure and reduced FVC and FEV_1 at 4 and 7 years. The association was strongest between higher PFOA concentration and reduced FVC at 4 years. Only one case-control study from Taiwan has evaluated the association between postnatal PFAS exposure and lung function in adolescents (10-15 years old) showing that higher PFAS concentrations were associated with impaired lung function among asthmatics (Qin et al. 2017). Given this study`s cross-sectional design their results cannot be directly extrapolated to our study.

Finally, paper V contributed by using a prospective design and large sample size to assess the association between prenatal PFAS exposure and immune and respiratory outcomes during childhood. Also, the assessment of lung function was done using objective measurements (spirometry test) during childhood, something needed in this field.

6.4. Implications for public health

During the years 2003-2008 every mother that participated in this research project was environmentally exposed to PFOS and PFOA with a potential of transferring half of their serum PFOA concentrations to their fetuses. This in itself should be of public health concern.

Further, we observed that shorter-chained and carboxylated PFAS cross the placenta more easily than other PFAS. This is especially relevant in the context of regulation and introduction of newer PFAS in the market. The market of PFAS is shifting towards shorter-chained PFAS because of their reduced half-lives, for example, PFOS is being replaced by PFBS because the latter exhibits a much shorter half-life in humans (26 days vs. 5 years) (Olsen et al. 2009b). On the other hand, shorter-chained PFAS may also transfer more readily to the fetus. Therefore, in terms of reducing fetal PFAS exposure, introducing shorter-chained PFAS (especially carboxylates) may actually contribute to higher fetal exposure during pregnancy. A careful regulation combined with thorough toxicological and risk-assessment studies is needed to make efficient decisions on the use and safety of PFAS.

6.5. Future research

6.5.1. Assessment of shorter-chained PFAS

To date, most of the research efforts on PFAS have focused on the widely used compounds, these are PFOS and PFOA. However, these two chemicals are quickly being substituted in the industry by newer shorter-chained PFAS, for example PFBS is the main substitute of PFOS (Oldham et al., 2012; Renner, 2006). One of the main characteristics of some of these PFAS is that they have shorter

half-lives than PFOS and PFOA. For example, PFOS can last up to 5 years in human blood whereas PFBS has a 26 days half-life in humans (Olsen et al. 2009b). This means that researchers need to find new ways of dealing with exposure assessment. Up to now, the research community has used one blood measurement to assess continuous PFAS exposure for 3-5 years with little risk of exposure misclassification. In the future, studies assessing the health implications of shorter-chained PFAS, may require the use of repeated blood (or urine for the more hydrophilic PFAS) measurements in order to fully characterize human exposure to these chemicals.

6.5.2. Postnatal PFAS exposure

One important question that remains unanswered in the assessment of PFAS exposure is:

> Which is the more relevant window of PFAS exposure to observe human health effects: is it the prenatal or the postnatal period?

In this thesis we only assessed prenatal PFAS exposure but future studies would benefit from assessing PFAS during the postnatal period. The literature available suggests that the weaning (0-3 years old) and pre-puberty periods might be vulnerable windows of exposure for endocrine disruption by chemicals such as PFAS. In paper IV, when postnatal breastfeeding was considered, as a proxy for postnatal PFAS exposure, a pattern of positive associations between maternal PFOA and BMI z–scores at 4 and 7 years was observed (paper IV). This finding suggests that postnatal PFAS exposure may also play a role in the development of obesity during childhood. Regarding immune health, higher PFAS concentrations at 5 years were associated with reduced antibody levels at age 7 years in the Faroe Islands (Grandjean et al. 2012). For lung function, a case-control study in Japan reported that postnatal exposure to PFAS was associated with impaired lung function in asthmatic children (Qin et al. 2017). Understanding the different windows of exposure susceptibility will enhance the preventive strategies to reduce exposure to these chemicals.

6.5.3. Chemical mixtures and multiple exposures

One of the main problems when studying the health effects of chemicals is that exposures usually come in mixtures instead of individually. These mixtures may, at times, produce health effects that are greater than a single chemical would do alone. Results from the INMA birth cohort demonstrate that mothers have been exposed to a multitude of environmental pollutants at the same time, including PFAS, persistent organic pollutants (polychlorinated biphenyl and pesticides), bisphenol A, phthalates, and others. Up to now, most epidemiological and toxicological research regarding environmental pollutants has evaluated the health effects of a single pollutant at a given time-point. Future research should use flexible statistical methods to allow for correlated exposures in order to better characterize and understand the health effects of chemical mixtures.

In addition, during our lifetimes we are exposed not only to chemical mixtures but to a wide range of multiple environmental agents that may impact our health status. This concept is known as the *exposome* (Wild 2012). The HELIX project (http://www.projecthelix.eu/), which aims to characterize the early-life *exposome* and its associated health effects (Vrijheid et al. 2014), has now finished the data collection and is now providing fruitful information to guide some of these unanswered questions.

6.5.4. Mechanisms for PFAS health effects

To date, the mechanisms through which PFAS could affect fetal growth, obesity and cardiometabolic risk, and immune and respiratory health are far from understood. The main mechanism described is through the direct interaction with PPAR-α and -ɣ, at least for PFOA. For the other PFAS the evidence is not clear. Other mechanisms may also exist including endocrine disruption and oxidative stress (see sections 1.2.1., 1.2.2., and 1.2.3.) but little information is known. As of yet, there is no sound evidence of the main mechanisms of PFAS and their effects on human health. Future research in this area would help to establish the appropriate environmental and health guidelines concerning the use of PFAS.

6.5.5. Assessment of cardiometabolic risk, dose-response relationships, and immune response

In this thesis we used a CM-risk score as a proxy for metabolic syndrome however the score did not include a marker of glucose homeostasis, which is one of the components that is normally used to define metabolic syndrome (Ahrens et al. 2014; Eisenmann 2008). Therefore, to fully characterize the potential impact of PFAS on the prevalence of metabolic syndrome or the future risk of cardiometabolic disease; future follow-ups with available information on glucose homeostasis or insulin resistance at later ages are recommended.

Further, epidemiological studies have observed dose-response relationships that are consistent with a threshold effect of PFAS and adiposity (Braun et al. 2016; Maisonet et al. 2015). These findings are supported by in vitro studies in which exposure to PFOA above a certain level triggered obesogenic effects that were undetectable at lower exposure levels (Moreau et al. 2008; Peters and Gonzalez 2011). Thus, future studies evaluating the obesogenic effects of PFAS need to include a longitudinal assessment of patterns of adiposity and dose-response relationships.

Also, prenatal exposure to PFAS has been associated with reduced antibody concentrations following childhood routine vaccinations (Grandjean et al. 2012; Granum et al. 2013). In this thesis, we could not evaluate the response to vaccination because data was not available in INMA. Future research investigating what is the role of PFAS in reducing immunological response to vaccines during childhood or adolescence (for example, human *papillomavirus* vaccine) would be interesting.

7. CONCLUSIONS

In this thesis, the association between prenatal PFAS exposure and child health was evaluated. We first assessed the transfer of PFAS from mother to fetus showing that shorter-chained and carboxylated PFAS, especially PFOA, transferred more efficiently than other PFAS. We evaluated maternal PFAS concentrations during pregnancy for the first time in a Spanish birth cohort, indicating that mothers were ubiquitously exposed to PFOS and PFOA during the years 2003-2008. In this cohort, maternal PFAS exposure was mainly determined by country of birth, region of residence, previous breastfeeding, parity, and age. Fish and shellfish intake during pregnancy was the main dietary factor contributing to maternal PFAS concentrations, especially for PFOS.

Overall, the findings presented in this thesis showed largely null associations between prenatal PFAS exposure and child health. We observed inconsistent findings between prenatal PFAS exposure and non-significant associations with reduced birth weight, no association with obesity, little or no association with cardiometabolic risk, inverse associations with immune and respiratory health, and non-significant inverse associations with lung function up to age 7 years.

Future studies with pre and postnatal PFAS assessment, follow-ups beyond mid-childhood, and larger sample size for some of the outcomes are needed in order to better understand the association between early-life PFAS exposure and child health. In the meantime, the precautionary principal should remain.

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GLOSSARY

ALSPAC	Avon Longitudinal Study of Parents and Children
ANOVA	Analysis of variance
BMI	Body mass index
BP	Blood pressure
CI	Confidence interval
CM	Cardiometabolic
CRL	Crown-rump-length
DNBC	Danish National Birth Cohort
$FEF_{25–75}$	Forced expiratory flow between 25% and 75% of forced vital capacity
FEV_1	Forced expiratory volume in 1 second
FEV_1/ FVC	Forced expiratory ratio
FFQ	Food frequency questionnaire
FVC	Forced vital capacity
GAM	Generalized additive model
GEE	Generalized estimating equations
GFR	Glomerular filtration rate
GM	Geometric mean
HDL-C	High-density lipoprotein
HPLC-MS/MS	High performance liquid chromatography–tandem mass spectrometry
Ig	Immunoglobulin
IFN-	Interferon
IL	Interleukin
INMA	Environment and Childhood Project (*INfancia y Medio Ambiente)*
IQR	Interquartile range
LBW	Low birth weight
LDL-C	Low-density lipoprotein
LOQ	Limit of quantification
LOD	Limit of detection
LOG-KOW	Logarithms of the octanol-water partition coefficients
LRTI	Lower respiratory tract infection
MeOH	Methanol
MetS	Metabolic syndrome

NHANES	National Health and Nutrition Examination Survey
OR	Odds ratio
PBPK	Physiologically-based pharmacokinetic
PFAS	Perfluoroalkyl substances
PFBS	Perfluorobutane sulfonate
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoate
PFOA	Perfluorooctanoate
PFOS	Perfluorooctane sulfonate
PPAR	Peroxisome proliferator-activated receptor
RAM	Restricted access material
RR	Relative risk
SD	Standard deviation
SGA	Small-for-gestational-age
TC	Total cholesterol
TG	Triglycerides
WC	Waist circumference
WHO	World Health Organization
WHtR	Waist-to-Height ratio

ANNEX

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Ithaka

As you set out for Ithaka
hope your road is a long one,
full of adventure, full of discovery.
Laistrygonians, Cyclops,
angry Poseidon—don't be afraid of them:
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
stirs your spirit and your body.
Laistrygonians, Cyclops,
wild Poseidon—you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.

Hope your road is a long one.
May there be many summer mornings when,
with what pleasure, what joy,
you enter harbors you're seeing for the first time;
may you stop at Phoenician trading stations
to buy fine things,
mother of pearl and coral, amber and ebony,
sensual perfume of every kind—
as many sensual perfumes as you can;
and may you visit many Egyptian cities
to learn and go on learning from their scholars.

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithakas mean.