Prenatal Exposure to Environmental Contaminants and Newborn Immune System Biomarkers

by

Jillian M. Ashley-Martin

Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
December 2014

Dedications

I dedicate this thesis to those who inspired and mentored me throughout this journey of learning and exploration.

Table of Contents

List of Tables

List of Figures

Abstract

Allergic diseases are among the most common chronic childhood diseases and a leading cause of hospitalization. Exposure to certain environmental contaminants has been hypothesized to be a risk factor for childhood allergies and asthma. Scientific understanding regarding the effect of prenatal contaminant exposure on fetal immune system development and subsequent susceptibility to allergic disease is limited. Elevated levels of Immunoglobulin E (IgE), interleukin-33 (IL-33), and thymic stromal lymphopoietin (TSLP) are implicated in inflammatory responses and early life manifestations of allergic disease. TSLP and IL-33 have not been measured in neonates. It is not known whether prenatal environmental contaminant exposure is associated with elevated levels of these biomarkers at birth. The specific objectives of this research were to 1) assess the levels of, correlation among and predictors of IgE, IL-33, and TSLP in umbilical cord blood and 2) determine the association between prenatal exposure to environmental contaminants and elevated levels of IgE, TSLP and IL-33. Concentrations of metals, phthalates, bisphenol A, pesticides, and polychlorinated biphenyls were measured in maternal urine, blood or plasma. IgE, IL-33 and TSLP were measured in umbilical cord blood samples. This study utilized prenatal exposure and socio-demographic data collected in the Maternal-Infant Research on Environmental Chemicals (MIREC) Study, a trans-Canada cohort study of 2,001 pregnant women recruited from 10 sites between 2008 and 2011. TSLP and IL33 were highly correlated with each other but neither was strongly correlated with IgE. Elevated levels of TSLP and IL-33 were significantly associated with maternal characteristics indicative of inflammatory responses. None of the environmental contaminants were associated with elevated immune system biomarker levels. These findings support the mechanistic hypothesis that IL-33 and TSLP are both operational in early life inflammatory pathways. Determination of whether the observed absence of environmental exposure-induced immunotoxicity persists into childhood requires follow-up in a cohort of children.

List of Abbreviations Used

IL-33	interleukin-33
TSLP	thymic stromal lymphopoietin
IgE	immunoglobulin E
IgA	immunoglobulin A
LOD	limit of detection
MEP	mono ethyl phthalate
MBP	mono butyl phthalate
MBzP	mono benzyl phthalate
MEHP	mono-2-ethylhexyl phthalate
MCPP	mono-3-carboxypropyl phthalate
MEHHP	mono-(2-ethyl-5-hydroxyhexyl) phthalate
MEOHP	mono-(2-ethyl-5-oxohexyl) phthalate
BPA	bisphenol A
PB	lead
CD	cadmium
AS	arsenic
HG	mercury
DMP	dimethylphosphate
DEP	dimethylthiophosphate
DEHP	di(2-ethylhexyl phthalate)
DMTP	dimethyldithiophosphate
DETP	diethylthiophosphate
PCB	polychlorinated bipheynl
DDE	dichlorodiphenyldichloroethylne
PFOS	perfluorooctane sulfonate
PFOA	perfluorooctanoic acid
PFHxS	perfluorohexane acid
IARC	International Agency for Research on Cancer
EPA	Environmental Protection Agency
DIT	Developmental immunotoxicity
MIREC	Maternal-Infant Research on Environmental Chemicals
SG	specific gravity
OR	odds ratio
CI	confidence interval
aOR	adjusted odds ratio
ELISA	enzyme linked immunoassay

Acknowledgements

I would like to foremost acknowledge my supervisory team. Thank you for your motivation, guidance, unwavering support, and confidence in me. Thank you for the opportunity to grow, learn, and reflect.

I would like to give special thanks to the entire Marshall lab for their tremendous help with the analysis of the cord blood samples. My time in the Marshall lab was an invaluable learning experience that gave me great insight into the basic science world.

Thanks to the biobank committee of the MIREC study for granting access to the biobank data. I would also like to acknowledge the participants in the MIREC study for whom this work would not have been possible.

This work was supported by the Nova Scotia Research Foundation (scholarship award) and the IWK Health Centre (Category A research grant) and the Department of Obstetrics & Gynecology (Atlee Foundation grant).

Thanks to my friends and family who offered their laughs, inspiration, childcare, encouragement, perspective, and time.

Chapter One. Introduction

Allergic disease, including atopic dermatitis, allergies and asthma, are among the most common chronic childhood diseases and leading causes of hospitalization. It is estimated that 11% of girls and 16% of boys living in Canada have been diagnosed with asthma (1). Established risk factors, such as genetics and environmental tobacco smoke exposure, are likely involved in less than one half of childhood allergic disease cases (2,3). Exposure to environmental contaminants has been hypothesized to be responsible for some portion of childhood allergic disease (4,5). There is insufficient evidence to describe the nature and magnitude of the possible association between environmental contaminant exposure and childhood allergic disease. A notable gap is the lack of focus on exposure during periods of vulnerability (6,7).

The intrauterine time period is a critical window of immune system development and vulnerability to the potential adverse effects of environmental exposures. A number of common environmental contaminants have been hypothesized to exhibit developmental immunotoxic properties (8–10). There are few epidemiological investigations of the association between prenatal environmental contaminant exposure and childhood allergic disease. There are also few studies that explore whether the immunotoxic effects of *in utero* environmental contaminant exposure manifest at birth. In addition to strengthening etiological knowledge, determining whether immune system changes manifest at birth may facilitate identification of high risk individuals and implementation of prevention strategies prior to sensitization.

The present research aimed to determine the association between prenatal exposure to multiple individual environmental contaminants and levels of newborn

immune system biomarkers. Environmental contaminants studied in this research include those that are in current use (phthalates, bisphenol A) as well as those that have been phased out (polychlorinated biphenyls, organochlorine pesticides). Chemicals that have been phased out continue to pose health concerns due to their persistence in the environment and potential for human exposure. Many chemicals in current use such as phthalates, bisphenol A and perfluorinated compounds are ubiquitous in our environment (11,12).

This research, which is conducted from exploratory and etiological perspectives, fits within the second step of the research cycle (etiology or causation) illustrated in Figure 1.1 (13). The burden of allergic disease (step one on the research cycle) is discussed in chapter two. Chapter two also reviews literature relevant to the exposure and outcome biomarkers used in this research. Chapter three outlines the objectives. Chapter four discusses the sources of data. Chapters five through seven are individual manuscripts that have been or are in the process of being submitted for publication. The public health context of the present findings is discussed in chapters six and seven. Chapter eight discusses the process of translating etiological knowledge (step 2 of the research cycle) into interventions and recommendations (steps 3-5). Existing evidence regarding the potential health effects of exposure to many currently used environmental contaminants is insufficient to support health based guidelines (14). In light of the lack of guidelines, chapter eight discusses some of the challenges inherent in the regulatory process. Conclusions are offered in chapter nine. Though efforts have been made to minimize repetition and ease readability, there is some overlapping content between chapters due to the manuscript based nature of this thesis.

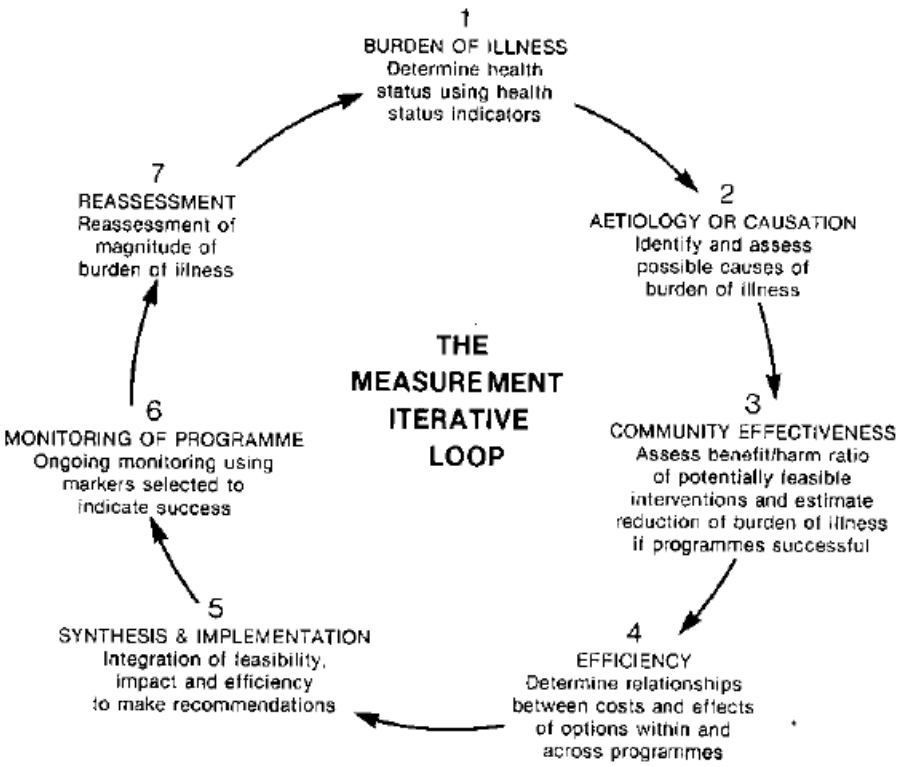


FIG. 1.

Figure 1.1 The Research Cycle. Source (13)**, p. 340.**

Chapter Two. Literature Review

This chapter provides an overview of the epidemiology and fetal origins of childhood allergic disease. The chapter concludes with a discussion of the outcome biomarkers and environmental chemicals examined in this thesis.

Allergic Disease Epidemiology

The umbrella term "allergic disease" will be used in this thesis to encompass diagnoses that are characterized by an imbalance in the production of infection-fighting (T helper 1 (Th1)) and inflammatory (T helper 2 (Th2)) cytokines and are associated with atopy. Typically, individuals with allergic diagnoses have excess production of IgE and Th2 cytokines (15,16) and give positive skin prick tests to environmental allergens. These include conditions such as atopic eczema, asthma, rhinitis, and food allergy. In addition to shared physiologic mechanisms, onset of these conditions often occurs in a temporal manner, known as the allergic march. Allergic individuals commonly develop atopic dermatitis in the first year of life, followed by the development of allergies and/or asthma in childhood as depicted in Figure 2.1 (15,17).

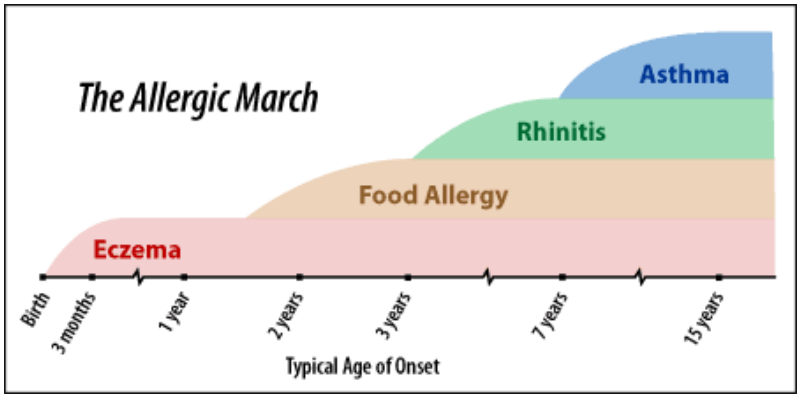


Figure 2.1 The Allergic March. Source (18)

There has been a global rise in the prevalence of allergic disease in recent decades (4,7,19). This trend has occurred at a rate that cannot be explained by alterations in the gene pool (7). Among children in North America, the prevalence of asthma, allergic rhinitis, and atopic dermatitis has been estimated at 13.4% (1), 40.0% (20) and 20.0% (17) respectively. The prevalence of wheezing, allergic rhinitis, and atopic eczema among Canadian adolescents is estimated to range from 13.7 to 33.3%, 14.6 to 22.6%, and 8.2 to 10.4% respectively (21). It is also estimated that 13.4% and 15.5% of children (ages 0 to 11 years) in Canada and the Atlantic Provinces, respectively, were diagnosed with asthma in 2000. Prevalence of asthma is typically higher among boys in childhood (1). The prevalence of food allergy among Canadian children (< 18 years) has been estimated to be 7% (22,23).

The growing public health burden of allergic disease has paralleled the increasing prevalence. The economic burden of allergic disease, including direct and indirect costs of care, poses challenges to patients, caregivers, and government. Costs of uncontrolled asthma in Canada are reported to be $162 million annually (24) and the direct costs of atopic dermatitis in the US range between $364 million and $3.8 billion (25). Uncontrolled asthma places additional strain on health care resources as a result of extra emergency room visits, unscheduled physician visits, and ambulance rides (24).

Though considerable research has examined risk factors for childhood allergy, uncertainty regarding etiology and the recent temporal trends remains (6). Allergic disease results from a complex interplay between preexisting susceptibilities and exposure to risk factors, particularly during windows of vulnerability (6,7). Family history is one of the few well established risk factors for allergic disease (19). Though

no single gene can predict allergic disease, Bisgaard and Bonnelykke (6) state that the most well established genetic risk factor for eczema is a variant in the gene for the skin barrier protein filaggrin. This gene has also been implicated in asthma exacerbations and sensitization (26). Prenatal and postnatal tobacco smoke exposure is another well established risk factor for asthma and wheeze (6,27). A recent systematic review reported that prenatal tobacco smoke exposure increases the risk of childhood wheezing and asthma by at least 20% (28). Moreover, elevated cotinine levels have been associated with a decreased forced expiratory volume and peak expiratory flow (29). Though sometimes examined in combination with environmental tobacco smoke exposure, maternal exposure to air pollutants (e.g. particulate matter, NO2) has been shown to be an independent risk factor for allergies and asthma (19,30).

Sensitization to allergens early in life may also be a risk factor for childhood asthma, particularly when combined with other pre-existing susceptibilities (e.g. genetics, co-exposure to other allergens). Allergens derived from house dust mites, cockroaches, airborne particulates, traffic emissions, pets, and molds are possible sensitization triggers (6). Sensitization to these allergens may occur *in utero* (31). Other prenatal factors that may increase risk of childhood asthma and allergy include maternal stress, antibiotic use, and Cesarean section (4,7). Prenatal nutrient intake, specifically fish oil (4,7) and vitamin D (4), has been shown to protect against risk of childhood allergic disease.

The potential role of early life viral and bacterial exposures in allergic disease has received considerable attention but the exact relationship remains elusive. Respiratory syncytial virus infection among infants has been associated with childhood asthma,

wheezing, and reduced pulmonary function (6,32,33) though this effect may diminish with age (32). It is not clear whether the virus promotes asthma development or if pre-existing asthma exacerbates the viral response. In contrast, studies have shown that early life exposure to infections via presence in day care or a large family may be protective against allergies and asthma (19,34). The role of bacteria as a risk factor is similarly complex. Exposure to endotoxin, a gram negative bacteria, during infancy and childhood, has been shown to be inversely related to risk of childhood asthma (35,36) but positively associated with asthma in later life (5).

One of the gaps in this body of literature is the effect of prenatal exposure to environmental contaminants, other than tobacco smoke, on childhood allergic disease. Given the ubiquity of exposure and hypothesized immunotoxic properties, environmental contaminant exposure are plausible risk factors (37–39).

Fetal Origins of Allergic Disease

There is a growing body of evidence suggesting that allergic disease has fetal origins. This literature is rooted in Barker's investigation into the association between fetal undernutrition and risk of adult cardiovascular disease (40). Recent research has provided further evidence to support the hypothesis that chronic diseases (41–43), including asthma, have fetal origins (43,44). This literature is backed by knowledge that the fetal time period is a critical window of immune system development (45,46). For example, airway development occurs primarily *in utero* (47). Moreover, the fetal time period is a window of enhanced susceptibility to exogenous exposures. In contrast to observed effects in adults, adverse effects of exposures in fetuses may manifest at concentrations lower than expected, differently than expected, or after a temporal delay

(48). This enhanced susceptibility may stem from 1) the physiology of pregnancy and 2) the developmental plasticity of the fetus.

Pregnancy is characterized by a Th2 skewed immune system response to protect the fetus from potentially damaging Th1 immune responses (49). As a result, newborns must realign their immune systems postnatally to achieve an appropriate balance between Th1 and Th2 responses. This realignment is likely regulated by cytokines of the innate immune system. Newborn immune systems may be susceptible to the adverse effects of immunotoxic chemicals prior to and during this critical window of realignment (5,50). For example, *in utero* and early life exposures to developmental immunotoxicants may trigger infant airways to undergo changes that promote allergen sensitivity and airway hyper-responsiveness (5,50).

In response to environmental disruptions (pollutant, infection, undernutrition), fetal organs adapt and establish an altered phenotype that is designed to suit the needs of later life (5). This developmental plasticity (51) may, however, result in subsequent increased dysfunction and disease (51,52). Epigenetic changes are a major underlying mechanism in this process (53). Other potential mechanisms include direct or indirect promotion of cytokine microenvironment, alterations in hematopoiesis or antigen presentation. These changes may occur as a consequence of immunotoxic effects of alarmins or tissue degradation products. Briefly, epigenetic changes are non-heritable changes to DNA that result in altered gene expression via DNA methylation or histone modification. These changes can determine whether a certain gene is activated or silenced. For example, epigenetic changes to genes that regulate differentiation of naïve Th cells into Th1 and Th2 cells may disrupt the Th2/Th1 balance by promoting excess

Th2 cell production. Epigenetic mechanisms have been identified in the relationship between asthma-like immune system changes and exposure to particulate matter, tobacco smoke, dust mites, and endotoxin (5).

Despite some understanding of the particular susceptibility of the fetal time period, there is relatively little known about intra-uterine influences on childhood asthma risk (54), specific windows of vulnerability and key pollutants (45). In contrast to the traditional emphasis on dose as a determinant of risk, there is a need for greater emphasis on exposure during periods of vulnerability, such as the time of fetal development (55). In recognition of the potential fetal origins of childhood allergic disease, the present investigation sought to explore fetal susceptibility to immunotoxic effects of prenatal environmental contaminant exposures by examining biomarkers of 1) maternal environmental contaminant exposure and 2) fetal immune system status. These biomarkers are detailed in the following section.

Immune System and Environmental Exposure Biomarkers

A biomarker is defined as:

'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological response to a therapeutic intervention' (56) (p.91).

or

'a cellular or molecular indicator of exposure, health effects, or susceptibility. Biomarkers can be used to measure internal dose, biologically effective dose, early biological response, altered structure of function and susceptibility' (57) (p. 17).

Well known examples of biomarkers include liver enzymes, blood pressure, cholesterol levels, and blood levels of heavy metals such as lead. Recently, biomarkers have played an increasingly prominent role in molecular, genetic and environmental epidemiology (58). Technology has facilitated the ability to detect increasing numbers of chemicals at increasingly lower levels of detection. Biomonitoring studies, however, face significant challenges regarding the use and interpretation of such data. In the report *Human Biomonitoring of Environmental Contaminants*, the US National Research Council states that 'the ability to detect chemicals has outpaced the ability to interpret health risks' (59) (p.9). Thus, careful consideration of the appropriate use, interpretation and challenges inherent to biomonitoring studies is warranted.

Biomarkers can serve as indicators of exposure, effect, or susceptibility (Figure 2.2). Each of these biomarkers provides a snapshot on the exposure-disease continuum. The maternal concentrations of environmental contaminants in this thesis are biomarkers of exposure which serve to reflect internal dose and/or biologically effective dose. Internal dose indicates the level of the contaminant absorbed whereas biologically effective dose reflects the dose at the site of toxic action or receptor site (60). Interpretation of these biomarkers of exposure is challenged by the degree to which they are accurate reflections not only of biologically effective dose but also of exposure during the target window. Measurements of contaminants with short-half lives (e.g. phthalates) may represent immediate exposure levels and are not necessarily reflective of average levels during the critical window of target organ development. The influence of this issue on internal validity is discussed further in the manuscript chapters.

The immune system biomarkers of interest were examined as indicators of susceptibility to the potential adverse effects of the environmental exposures. The target organ(s) in the present research is the developing fetal immune system. Pinpointing the target window of immune system development is a challenge. The immune system is comprised of multiple different organs (e.g. thymus, lung). Each stage of immune system maturation may be uniquely susceptible to different contaminants. For example, though lead has been shown to be immunotoxic throughout gestation, limited experimental evidence suggests that third trimester exposure may be more likely to promote Th2 responses than exposure earlier in pregnancy (61).

Another overriding challenge relevant to all biomarker analyses are the limitations of expense and practicality. This thesis benefitted from a comparatively rich set of exposure data collected as part of the Maternal-Infant Research on Environmental Chemicals (MIREC) study. Previous large biomonitoring studies (e.g. the US National Health and Nutrition Examination survey (NHANES), Canadian Health Measures Survey) did not focus on pregnant women. Considering the susceptibility of the developing fetus to environmental toxicants, obtaining baseline data on environmental contaminant concentrations and examining potential health effects have notable public health relevance. Cost and practicality limitations, however, precluded the ability to analyze serial measurements of chemicals, such as phthalates, with short half-lives throughout pregnancy. The immune system biomarker data was analyzed specifically for this thesis research. Financial and practical issues precluded the ability to assess a wider panel of immune system cytokines. For example, $10,000 was raised for this thesis research to fund the purchase of ELISA (enzyme linked immunoassay) kits, lab

supplies, and shipping costs. Analyzing more cytokines would increase not only the cost but also the time required to conduct this analysis. Further challenges relevant to the specific immune system biomarkers of interest are discussed below.

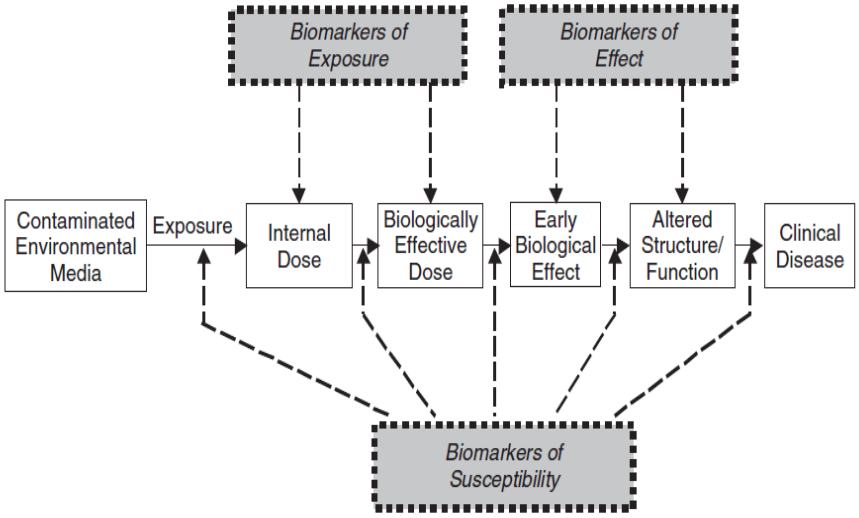


Figure 2.2 Exposure-disease continuum. Source: (62)**, p. 717.**

IMMUNE SYSTEM BIOMARKERS

Biomarkers of immunotoxicity provide information on the structure and/or function of the immune system and potential alterations in normal structure and function (63). The immune system biomarkers of interest in this thesis are immunoglobulin E (IgE), and two cytokines, interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP) (Table 2.1). Briefly, IgE and these cytokines play critical roles in the pathophysiology of allergic disease (64,65). Elevated levels of IgE are a hallmark sign of an allergic reaction. Levels of this antibody are typically higher in atopic than non-atopic individuals. Antigen recognition of IgE on mast cells results in the cardinal features associated with an allergic reaction (ranging from minor pruritus and

inflammation to anaphylactic shock) (16). This research assessed umbilical cord blood levels of IL-33 and TSLP as these cytokines have been the subject of recent attention in the immunological literature and are known to establish a microenvironment favorable for a Th2 response, IL-33 and TSLP (64,66–69). Recent experimental studies have demonstrated that TSLP and IL-33 play a critical role in the development of food allergy (64,65). These cytokines have also been implicated in atopic dermatitis, a form of allergic disease that often presents within the first year of life (70,71).

Efficient production of these cytokines stems primarily from epithelial cells, rather than T-cells, and is not dependent on the presence of a mature acquired immune system (67). Epithelial cells comprise the interface between internal immune system responses and exogenous stimuli. Whereas it was previously thought that this barrier was inert, the dynamic role of epithelial cells in atopic dermatitis (70) and asthma (69) has recently been recognized. Exogenous stimuli, particularly allergens, viruses, pollutants, can induce epithelial cell production of IL-33 and TSLP (72,73). For example, diesel exhaust has been shown to promote epithelial cell production of TSLP (74). IL-33 and TSLP can also be released from epithelial cells in response to infection or tissue damage (75). These cytokines can promote inflammatory responses by stimulating a cascade of events in the immune system. Briefly, IL-33, via production of group 2 innate lymphoid cells (ILC2), can induce production of inflammatory interleukins, such as IL-5 and IL-13, and subsequently promote B-cell production of IgE (75,76). TSLP may enhance production of Th2 cytokines through its effect on dendritic cells, an immune system cell that acts as a messenger between innate and adaptive immune system responses (66). In addition, there is mechanistic data suggesting that

IL-33 and TSLP can operate together to induce mast cell production of IgE (77). Investigation into levels of these biomarkers at birth is a biologically plausible means of assessing the susceptibility of the fetal immune system to the potentially immunotoxic effects of prenatal exposures, particularly given the lack of available clinical data in early life.

Table 2.1 Immune system biomarkers used in this thesis

Biomarker	Description	Relationship with Allergic Disease
Immunoglobulin E (IgE)	Antibody that attaches to mast cells in the skin, respiratory and intestinal tract that plays a major role in allergic reactions. (78)	Elevated levels of serum IgE are hallmark sign of atopy.
Interleukin-33 (IL-33)	Epithelial produced cytokine Member of IL-1 family (67) Released as a result of cell death and may act as an alarmin to the immune system by promoting local inflammation (69,79)	IL-33 induces expression of Th2 cytokines, activates eosinophils and mast cells (80,81) and is important for the development of food allergy (64) Elevated IL-33 levels in patients with: Atopic dermatitis (82) Allergic asthma (83) Experimental study reported critical role of IL-33 in mechanisms underlying house dust mite allergic asthma and peanut allergy (64)
Thymic Stromal Lymphopoietin (TSLP)	Produced by epithelial cells in response to allergens, pollutants, and viruses Member of IL-2 family Structurally related to IL-7. (84) Expressed in skin, gut, lung, and thymus (85)	TSLP stimulates hematopoietic cells and dendritic cells to promote production of Th2 cytokines (IL4,IL-5, IL-9, IL-13). Over -expression in lung leads to airway inflammation Hypothesized to play role in mechanisms underlying both atopic dermatitis and asthma (85) Elevated TSLP levels in patients with: Atopic dermatitis (adults, (86), children (87)) Asthmatics (88) Eosinophilic esophagitis (89) Children treated with anti-TSLP antibodies had less bronchoconstriction and airway inflammation following allergen challenge than controls (68) Experimental study reports role of TSLP in allergen specific IgE production and anaphylaxis after cutaneous allergen sensitization (65)

One of the challenges relevant to interpreting the immune system biomarker data is that elevated levels in umbilical cord blood are not established predictors of childhood allergic disease. There are no prospective data examining TSLP and IL-33 levels at birth and subsequent development of allergic disease.

Literature examining the value of cord blood IgE levels in predicting childhood allergic disease has shown that IgE has fairly low sensitivity (24-38%) but reasonable specificity (71-90%) (90–96). Despite this low sensitivity, elevated levels of IgE at birth have been associated with statistically significantly elevated odds of childhood allergic disease in four studies (Table 2.2). The low sensitivity of cord blood IgE in predicting childhood allergy has raised questions regarding the clinical relevance of this biomarker (97). *In utero* sensitization is biologically feasible. The developing fetus may be exposed to allergens and maternal IgE that are present in amniotic fluid. The primary means of fetal allergen exposure is likely via ingestion of amniotic fluid. The fetal gut has mature immune cells, including the presence of IgE receptors, as early as 16 weeks of gestation (98). Moreover, the fetus produces IgE from the 11^{th} week of gestation onward (99,100). This potential *in utero* sensitization may prime the fetal immune system towards future allergic disease. The lack of clear relationship between cord blood IgE and childhood allergic disease may be explained by the complex trajectory from fetal immune system development to ultimate diagnosis of childhood atopic disease. This trajectory is influenced by changes in maternal immune system physiology during pregnancy (31), early life exposure to allergic disease protective and risk factors, and the intricate interaction between multiple immune system biomarkers including antibodies (IgE), cytokines, and T-regulatory cells (101).

In recognition of the limitations of using these biomarkers as predictors, the present thesis does not aim to predict future allergic disease risk. The focus is on examining the snapshot of the exposure-disease continuum from prenatal exposures to the time of birth.

Table 2.2 Reported sensitivity, specificity, and unadjusted odds ratios in studies examining the association between cord blood levels of IgE and allergic sensitization

Reference	IgE Level (kU/L)	Type of Outcome (Age of Dx)	Sensitivity	Specificity	Odds Ratio (95% CI)
Pesonen 2009 (90)	0.5	Skin prick test (5 y)	38	84	4.6. (1.7-12.7)
Odelram 1995 (91)	0.5	Atopy (18 mon)	25	74	1.0 (0.4-2.4)
Hansen 1993 (92)	0.5	Atopic disease (5 y)	25	87	
Hansen 1993 (92)	0.3	Atopic disease (5 y)	67	71	
Bergmann 1997 (102)	0.35	Atopic dermatitis (2 y)			1.0 (0.8-1.4)
Edenharter 1998 (102)	0.5	Sensitization (12 mon)	44	78	2.4 (1.7-3.6)
Tariq 1999 (95)	0.5	Aeroallergen sensitization (4 y)	24	90	2.6 (1.7-4.1)
Ferguson 2009 (103)	0.5	Allergen sensitization (7 y)	--	--	2.6 (1.4-4.8)
Kaan 2000 (96)	0.5	Skin prick test (12 mon)	--	--	1.3 (0.7-2.4)
Sadeghnejad 2004 (104)	0.5	Allergic sensitisation (4 y)	--	--	2.4 (1.5-3.6)

Abbreviations: Dx=diagnosis, mon=months, y=years, CI = confidence interval

Another potential challenge with cord blood samples is potential contamination with maternal blood. Cord blood IgE measurements are largely representative of fetal rather than maternal IgE production because maternal IgE does not cross the placenta (105). Some maternofetal transfer of IgE during pregnancy and delivery and subsequent contamination of umbilical cord blood samples may still be possible. To address this

challenge, the present study analyzed levels of immunoglobulin A (IgA) in cord blood. This approach is a reliable means of assessing contamination as IgA is typically not produced *in utero* (106). Samples with IgA concentrations greater than 10 μg/L were, therefore, excluded to minimize the potential influence of contamination from maternal blood (106).

Though ELISA is the gold standard for analyzing these molecules, this technique does have limitations. This method does not distinguish reliably between intact, bioactive molecules and molecules that are degraded, aggregated or otherwise inactivated. Standard curves are used in ELISA as a means of calibrating wavelengths to estimated concentrations. Accurate estimation is reliant on the assumption that the molecules to be measured are not bound to other proteins in the plasma or polymerized into a larger molecules that might lead to an over or under estimation by ELISA methods. The use of similar diluents for samples and standard curves can minimize this type of issue. A number of quality assurance practices were employed in this research to minimize bias and inter and intra-assay variation; these are detailed in Appendix A.

BIOMARKERS OF ENVIRONMENTAL EXPOSURES

The environmental exposures of interest in the present research include metals, phthalates, perfluorinated compounds, polychlorinated biphenyls (PCBs), pesticides, and bisphenol A. As described in chapter four, maternal concentrations of these contaminants were analyzed as part of a national birth cohort study. Literature relevant to the association between prenatal environmental contaminant exposure and measures of childhood allergic disease is also discussed in the corresponding manuscript chapters.

Many of these chemicals are ubiquitous in the environment and have been widely detected in humans (12,107,108). Exposure occurs from chemicals that are currently in production (e.g. phthalates, bisphenol A) and from reservoirs of lipophilic, persistent chemicals that are either decreasing in production or being phased out (e.g., perflourinated compounds, organochlorine pesticides, PCBs). Maternal exposure to these chemicals creates direct exposure to the developing fetus as the majority of these chemicals can pass through the placenta into fetal circulation (48,109,110). Chemicals such as cadmium, that do not directly enter fetal circulation, can potentially have adverse effects on the fetus by accumulating in the placenta and altering normal placental processes and function (109).

METALS

Exposure to metals can occur via their natural presence in the environment as well as via by-products of industrial processes. Primary sources of exposure to the metals of interest are outlined in Table 2.3.

Table 2.3 Metals studied in this thesis and common sources of exposure

Metal	Sources of Exposure
Lead	Water pipes, imported ceramics & toys Historical: paint, gasoline, batteries
Mercury	Seafood, dental fillings
Arsenic	Drinking water, rice
Cadmium	Cigarette smoking, shellfish

Results from toxicological studies demonstrate that metals can exert immunosuppressive effects at high concentrations and immune system enhancement effects at lower concentrations (48). Moreover, toxicological evidence has demonstrated that the heavy metal lead exhibits developmental immunotoxic properties (8,111). Intrauterine levels of lead that were not observed to adversely affect adults resulted in

changes to the fetal newborn immune system (increased IgE, altered cytokine production). These changes persisted into adulthood (111). Rabinowitz first hypothesized an association between lead exposure and childhood asthma over twenty years ago (112). Epidemiological evidence has since demonstrated that elevated levels of lead exposure are associated with increased levels of serum IgE (113–115). In one of the few investigations examining prenatal lead exposure, authors of a Polish birth cohort study reported that frequency of atopy diagnosed at five years of age was statistically significantly associated with cord blood but not blood lead levels at age five years; suggesting the critical role of early life exposures in disease etiology (116). Though limited, toxicological and epidemiological studies suggest there may be immunotoxic properties of arsenic (117–119), mercury (55,120) , and cadmium (48).

PHTHALATE METABOLITES

Phthalates are a group of chemicals widely used in the manufacture of plastics and consumer products due to their plasticizing properties. High molecular weight phthalates are commonly used in the manufacture of vinyl products whereas low molecular weight phthalates are frequently used in personal care products (121). The phthalate metabolites of interest are outlined in Table 2.4. Phthalate exposure can result from dermal absorption or inhalation of phthalate-laden products such as cosmetics and fragrances, or ingestion of contaminated foods (122). Phthalate exposure has been hypothesized to exhibit reproductive toxicity (e.g. hypospadias, cryptorchidism, reduced anogenital distance) (121). The potential allergy-related role of phthalate exposure is less well established. Experimental studies have shown that phthalates may induce IgE production and enhance sensitization by acting as adjuvants in predisposed

individuals (123,124). The relationship between phthalate exposure and allergy has been examined in cross-sectional (125,126) and case-control studies (127). Though results were suggestive of a positive association, the cross-sectional study design and/or lack of objective exposure measures hinders interpretation of the results. More recently, the potential role of prenatal phthalate exposure in childhood allergy has been examined in birth cohort studies (Table 6.5). Due to the number of phthalate metabolites measured and diversity in outcome measures, summarizing this literature is not straightforward. However, prenatal exposure to MBzP was significantly associated with an increased risk of both eczema (128) and asthma (129) among a population of African American and Dominican women residing in New York city. Replication of these findings in other populations with differing ethnicities and risk profiles for asthma are warranted.

Table 2.4. Phthalate parent compound and metabolites studied in this thesis and common sources of exposure (121)

Phthalate Type	Parent Compound	Metabolites	Exposures Sources
Low Molecular Weight Compounds	di-n-butyl phthalate (DnBP)	Mono butyl phthalate (MBP)	Personal care products
	Diethyl phthalate (DEP)	Mono ethyl phthalate (MEP)	Personal care products
	Benzyl butyl phthalate (BBzP)	Mono benzyl phthalate (MBzP)	Vinyl Flooring Personal Care Products
High Molecular Weight Compounds	Non-specific HMW	Mono-3-carboxypropyl phthalate (MCPP)	Plastics (Food packaging) Medical tubing
	Di(2-ethylhexyl) phthalate (DEHP)	Mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)	PVC plastics in household products
		Mono-2-ethylhexyl phthalate (MEHP)	(Toys, flooring, walls)
		Mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP)	

BISPHENOL A (BPA)

BPA is a chemical widely used in the production of plastics, dental sealants, food and beverage packaging. Individuals are primarily exposed to BPA through diet though dermal absorption from paper products is also thought to influence urinary BPA concentrations (130,131). Toxicological evidence suggests that BPA exposure can induce asthmatic symptoms in mice (132,133). The limited epidemiological evidence of the association between prenatal BPA exposure and childhood allergy has reported both positive (134) and inverse associations (135).

PERFLUORINATED COMPOUNDS

Also known as perfluoroalkyl acids, these compounds are widely used in the production of carpet, clothing, and cookware for their water and oil repellant properties (136). Perfluorinated compound exposure can result from ingestion of contaminated food (e.g. seafood) and water (137). Degradation of perfluorinated compounds applied to household products (carpet, upholstery) can result in the accumulation of perfluorinated compounds in household dust, another source of exposure (138). Production of PFOA and PFOAS has been curtailed within the last decade due to recognition of potential health effects to wildlife and humans and persistence in the environment. Table 2.5 lists the perfluorinated compounds of interest to this research.

Table 2.5 Perfluorinated compounds studied in this thesis and common sources of exposure (136)

Compound	Exposure Sources
Perfluorohexane sulfonate(PFHxS)	Surfactants; water/stain protective coating
Perfluorooctanoic acid (PFOA)	Material used in transformers
Perfluorooctane sulfonate (PFOS)	Surfactant, emulsifier

Toxicological evidence has suggested that exposure to perfluorinated compounds enhances IgE responses (139). These chemicals have been observed to alter

inflammatory responses and cytokine production in animal models at levels consistent with those experienced by exposed humans (10).

A study of the association between perfluorinated compounds and immune system responses in a Japanese birth cohort reported that prenatal exposure to PFOA was associated with a significantly decreased level of cord blood IgE levels in girls though no significant associations were observed between prenatal exposures and maternal-reported atopic disease at 18 months (140). In a birth cohort study of Taiwanese children, Wang reported that elevated cord blood levels of IgE were correlated with cord blood levels of perfluorinated compounds in boys but that the compounds were not associated with atopic dermatitis at age two years (141).

POLYCHLORINATED BIPHENYLS (PCBS)

PCBs were used widely in North America prior to the 1970s in the transformers and capacitors of electrical equipment for their insulating and heat resistant properties. Manufacture of PCBs ceased in the late 1970s after recognition of their persistence in the environment and health effects. As there are no natural sources of PCBs, current exposure occurs from existing reservoirs of PCBs in the environment, particularly waste disposal sites. The International Agency for Cancer (IARC) has declared PCBs as probably carcinogenic to humans though uncertainty remains regarding other health effects of PCBs in adults and children (142). PCBs are hypothesized to alter immune system function by shifting T helper cell 2 responses (143). Epidemiologic literature suggests both positive (144,145) and inverse (55,146) associations between prenatal PCB exposure and measures of childhood allergy. The congeners studied in this thesis are listed in Table 2.6.

Table 2.6 PCBs congeners studied in this thesis and common sources of exposure

PCB 118 PCB 138 PCB 153 PCB 180	Historical use from industrial settings, waste disposal sites (for all congeners) No current production in North America

(142)

ORGANOCHLORINE AND ORGANOPHOSPHATE PESTICIDES

Organochlorine pesticides are persistent chemicals that were widely used as insecticides in the post World War II era. In the late 1970s and early 1980s, use was banned in the US and Canada due to persistence in the environment and potential carcinogenic effects (147,148). Despite the lack of current production and use within Canada, individuals may be exposed through direct use or ingestion of contaminated food. Due to their lipophilic nature and persistence, these contaminants bioaccumulate and can remain in the environment for decades (149). Although the potential carcinogenic effects of organochlorines have received a considerable amount of attention, this class of pesticides is associated with a range of other health outcomes including immunotoxicity (149). The underlying immunotoxic mechanism may be an enhanced production of Th2 cytokines (150). Epidemiologic literature is limited and inconsistent and has shown both positive (151–153) and null findings (144). Table 2.7 lists the organochlorine pesticides examined in the present research.

Table 2.7 Organochlorine pesticides studied in this thesis and common sources of exposure

Contaminants	Exposures Sources
Trans-nonachlor Dichlorodiphenyldic-hloroethylene (DDE) Hexachlorobenzene Oxychlordane	Historical use of pesticides in agricultural settings

Organphophate pesticides are still widely used as insecticides in the agriculture industry. Individuals can be exposed via ingestion of food or in household dust (154,155). Examples of currently used organophosphate pesticides include azinphos and dichlorvos. In contrast to the organochlorine pesticides, these pesticides are metabolized relatively quickly and are not persistent in the environment (156). Organophosphate metabolites, rather than the parent compound, are used as a biomarker of exposure as these chemicals are metabolized and excreted in urine (Table 2.8). One of the primary health effects of concern from exposure to these pesticides is neurotoxicity because these chemicals irrerversibly inactivate acetylcholinesterase, a critical enzyme in neural transmission (157). Limited experimental and epidemiologic evidence has demonstrated that there may be an association between exposure to this class of chemicals and allergic disease (158–160).

Table 2.8 Organophosphate pesticides metabolites studied in this thesis and common sources of exposure

Parent compound	Non-Specific Metabolites	Exposures Sources
Chlorpyrifos, Diazinon	Dimethylphosphate (DMP) Dimethylthiophosphate (DMTP) Dimethyldithiophosphate (DMDTP) Diethyldithiophosphate (DEDTP) Diethylthiophosphate (DETP) Diethylphosphate (DEP)	Ingestion of food treated with pesticides

Chapter Three. Study Objectives

The specific objectives of this research are as follows:

1) Determine the levels of and correlation among IL-33, TSLP and IgE in the plasma of umbilical cord blood samples.

2) Determine the association between maternal and neonatal characteristics and elevated umbilical cord blood concentrations of IL-33, TSLP and IgE (Manuscript #1).

3) Determine the association between prenatal exposure to environmental contaminants and elevated umbilical cord blood concentrations of IL-33, TSLP and IgE. The specific environmental classes of interest include; phthalates, BPA, perfluorinated compounds, polychlorinated biphenyls, metals, organochlorine pesticides, and organophosphorous pesticide metabolites (Manuscripts #2-3).

As levels of TSLP and IL-33 had not been previously measured in umbilical cord blood, one manuscript was dedicated to this component of the thesis. The existing experimental and epidemiological literature regarding the potential immunotoxic properties of the environmental contaminants of interest provides rationale for the third objective. Due to the limited literature specific to the association between the environmental chemicals and the immune system biomarkers of interest, this research was conducted from an exploratory perspective.

Chemical classes that exhibited a high degree of correlation were analyzed in manuscript #2 using a Bayesian hierarchical model. The remaining chemical classes were analyzed in manuscript #3 using a maximum likelihood estimation method to account for those samples with values below the limit of detection.

Chapter Four. Sources of Data

This research has used data from the Maternal-Infant Research on Environmental Chemicals Study (MIREC), a trans-Canadian birth cohort study of 2,001 pregnant women that were recruited during their first trimester between 2008 and 2011. Briefly, the primary objectives of the MIREC study were to determine whether non-occupational exposure to metals, such as lead, was associated with gestational hypertension or fetal growth restriction and to measure contemporary biomarkers of exposure (*in utero* and lactational) to priority chemicals (161). Study sites included Vancouver, Edmonton, Winnipeg, Sudbury, Toronto, Hamilton, Ottawa, Kingston, Montreal and Halifax. Each of these sites had established clinical research infrastructure.

Women were eligible for inclusion in the study if they were at least18 years of age, less than 14 weeks of gestation, planning on delivering at a local hospital, and able to consent and communicate in French or English. The exclusion criteria included a history of medical complications (renal disease, epilepsy, collagen disease, systemic lupus erythematosus, scleroderma, liver disease, heart disease, pulmonary disease, cancer, haematological disorders), threatened spontaneous abortion, and illicit drug use (161).

Questionnaires were administrated by trained research staff during first and third trimester to obtain demographic, life style, and medical history. Clinical maternal and neonatal data were obtained from chart review. Participants were seen during each trimester, at delivery, and postpartum to collect biospecimens (blood, urine, cord blood, meconium, breast milk, hair) for laboratory analysis of environmental chemicals, nutrients, genetic polymorphisms, and oxidative stress biomarkers. All chemical

analyses were conducted at the Toxicology Laboratory, in the Institut national de Sante publique du Quebec, accredited by the Standards Council of Canada. The chemicals selected for analysis in the MIREC study were identified as priority chemicals in Health Canada's Chemical Management Plan (161). Priority substances are those that are determined to be 'persistent, bioaccumulative in the food chain, inherently toxic to the environment, and are known to be in commerce in Canada; and/or a high hazard to humans with a high likelihood of exposure to individuals in Canada' (162).

In addition to providing consent for participation in the MIREC study and collection of laboratory data, study participants provided separate consent to have additional biospecimens collected and stored for use in future research in the MIREC Data and Biological Specimens Bank (161). This research accessed umbilical cord blood samples from the MIREC Biobank for the analysis of the immune system biomarkers. As noted in Chapter Two this analysis was undertaken by J. Ashley-Martin with assistance from the technical staff of Dr. Marshall's lab at the Department of Microbiology and Immunology at Dalhousie University. The protocol that was developed for the analysis of the cord blood samples is provided in Appendix A.

This thesis received ethical approval from Health Canada, St. Justine's Hospital (Montreal, QC), and the IWK Health Centre (Halifax, NS). In addition, approval was granted from the MIREC Biobank Management Committee to access the cord blood biobank samples.

Chapter Five. Umbilical cord blood levels and predictors of Interleukin-33, Thymic Stromal Lymphopoietin and Immunoglobulin E

As of the writing of this thesis, this manuscript was submitted to *Pediatric Allergy and Immunology*.

J. Ashley-Martin performed the ELISA analysis of umbilical cord blood samples, conducted the statistical analysis, and wrote the manuscript. L. Dodds provided supervisory input regarding the cohort, statistical analysis and critical feedback on the manuscript. T. Arbuckle, co-principal investigator of the MIREC study, provided guidance on the study cohort as well as reviewed and edited the manuscript. A. Levy assisted in structuring and writing the manuscript. R. Platt reviewed the statistical methods. J. Marshall provided lab space, materials, and technical support to facilitate the completion of the ELISA analysis and critically reviewed early versions of the manuscript. All authors were involved with the study design and methodology.

Umbilical cord blood levels and predictors of Interleukin-33, Thymic Stromal Lymphopoietin and Immunoglobulin E

Jillian Ashley-Martin[1], Linda Dodds*[2], Tye E. Arbuckle[3], Adrian R. Levy[4], Robert W.Platt[5], Jean S. Marshall[6]

Author Affiliations:

1. Interdisciplinary PhD Program, Dalhousie University
2. Department of Obstetrics & Gynaecology and Paediatrics, Dalhousie University
3. Healthy Environments and Consumer Safety Branch, Health Canada
4. Department of Community Health & Epidemiology, Dalhousie University
5. Department of Epidemiology and Biostatistics, McGill University
6. Department of Microbiology & Immunology, Dalhousie University

*Corresponding author l.dodds@dal.ca Perinatal Epidemiology Research Unit, 7th Floor Women's Site, IWK Health Centre, 5980 University Ave. PO Box 9700, Halifax, NS, Canada B3H 6R8

Running Title: Cord Blood TSLP, IL-33, and IgE

Word count: 2500

Number of tables & figures: 3 tables, 3 figures

Umbilical cord blood levels and predictors of Interleukin-33, Thymic Stromal Lymphopoietin and Immunoglobulin E

Ashley-Martin J, Dodds L, Arbuckle TE, Levy AR, Platt RW, Marshall JS.
Pediatr Allergy Immunol

Abstract:
Background: The fetal immune system is a critical window of development. The epithelial cell derived cytokines thymic stromal lymphopoietin (TSLP) and interleukin-33 (IL-33) have received attention for their role in allergic responses but not been studied during this critical window. The objectives were to assess correlations among IL-33, TSLP and IgE in umbilical cord blood samples and identify prenatal predictors of these biomarkers.

Methods: This study utilized data and banked cord blood collected in the Maternal-Infant Research on Environmental Chemicals (MIREC) Study, a trans-Canada cohort study of 2001 pregnant women. Our analytic sample comprised the 1254 women with a singleton, term birth with a cord blood sample. Spearman correlation coefficients (SCC) and logistic regression models were used to examine associations between biomarkers and identify potential predictors of elevated biomarker levels.

Results: TSLP and IL-33 were more strongly correlated with each other (SCC=0.75, $P<0.0001$) than with IgE (IL-33 SCC=0.14, TSLP SCC= 0.21). Maternal allergy, self-reported exposure to heavy street traffic and elevated birth weight were significantly associated with jointly elevated TSLP and IL-33 levels, whereas maternal age and female infant sex were inversely associated with elevated IgE.

Conclusions: In this population of Canadian women and infants, TSLP and IL-33 were detectable in cord blood, more strongly correlated with each other than with IgE, and associated with maternal characteristics indicative of inflammatory responses. This study motivates investigation into the value of cord blood IL-33 and TSLP levels as childhood allergy predictors and raises interesting questions regarding *in utero* coordinated regulation of these cytokines.

Key words: allergy, immunoglobulin E, interleukin-33, thymic stromal lymphopoietin, umbilical cord blood

Introduction

The fetal time period is a critical window of immune system development (45,50,163). *In utero* perturbations to the regulation of immune responses and inflammatory mediators may underlie susceptibility to childhood allergic diseases. This susceptibility may manifest as alterations in fetal antibody and cytokine levels (98). Previous research into biomarkers of childhood allergic disease has primarily focused on elevated levels of cord blood immunoglobulin E (IgE) and T cell derived cytokines (90,102,103,164). Recent research has demonstrated that the epithelial cell produced cytokines, thymic stromal lymphopoietin (TSLP) and interleukin-33 (IL-33) contribute to inflammatory related conditions (77). TSLP has been implicated in atopic dermatitis (165), eosinophilic esophagitis (89), and allergic airway disease (68). A recent clinical trial of children treated with anti-TSLP antibodies demonstrated that, compared to controls, treated children had less bronchoconstriction and airway inflammation following an allergen challenge test (68). IL-33 has been implicated in atopic dermatitis (166), asthma (83), and inflammatory bowel disease (167). Neither TSLP nor IL-33, however, has been studied in neonates.

Though most research has examined the independent roles of these cytokines, the interaction between TSLP and IL-33 can exacerbate inflammatory responses. Specifically, IL-33 can induce TSLP and sensitize mast cells to TSLP. TSLP can, in conjunction with IL-33, induce mast cell production of Th2 cytokines (77). Despite this experimental evidence, there is a lack of knowledge regarding the correlation of these cytokines in the developing immune system. Investigation into this correlation may provide insight into early life origins of IL-33 and TSLP cross-regulation. Mechanistic

interaction between these cytokines *in utero* could potentially promote an allergic phenotype in childhood.

In recognition of evidence regarding 1) the inflammatory roles of TSLP and IL-33 and 2) the fetal time period as a critical window of immune system development, we sought to explore the nature of these cytokines at birth. The specific objectives of this exploratory analysis were to: 1) determine umbilical cord blood levels of and correlations among IL-33, TSLP and IgE and 2) identify statistical associations between maternal and infant characteristics and elevated cord blood levels of IL-33, TSLP, and IgE. Given the lack of evidence explaining the higher prevalence of allergic diseases, including asthma, among boys (168) a secondary objective was to determine whether these statistical associations differed by infant sex.

Method

Study Design

Biospecimens and data for this study were from the Maternal-Infant Research on Environmental Chemicals (MIREC) Study Biobank, a trans-Canada cohort study of 2001 pregnant women from ten Canadian cities during 2008 to 2011, as previously described (169). Briefly, women were eligible for inclusion if they were < 14 weeks gestation at time of recruitment, ≥ 18 years of age, able to communicate in French or in English, and planning on delivering at a local hospital (169). Participants included in the present investigation were mothers who had a singleton, live, term birth (≥37 weeks) and a cord blood sample suitable for analysis. Cord blood samples (n=5) with immunoglobulin A (IgA) levels ≥ 10 µg/mL were excluded to minimize the possibility of contamination from maternal blood (106). Ethical approval was obtained from

Health Canada, Sainte Justine's Hospital (Montreal, QC), and the IWK Health Centre (Halifax, NS).

DATA COLLECTION

Trained research staff interviewed participants during the first and third trimester to obtain data on maternal lifestyle, medical history, demographics, and ambient environment characteristics. Clinical data on the mother and infant were obtained from chart review.

Immune system biomarkers were measured in the plasma of umbilical cord blood samples in the Department of Microbiology & Immunology, Dalhousie University, Halifax, NS using specific ELISAs. TSLP concentrations were assessed using a commercial kit (Biolegend, San Diego, CA, USA). IL-33 concentrations were assessed an R & D systems duoset (Minneapolis, MN, USA). ELISA kits (EBioscience, San Diego, CA, USA) were also used to assess both total IgE and IgA concentrations. ELISA assays were performed according to manufacturer's instructions with the exception that plates were coated with sodium bicarbonate buffer (pH 8.3-8.5) and blocked with 2% BSA in PBS instead of manufacturer's coating and blocking buffers. Extended standard curves were used to improve sensitivity. The inter-assay and intra-assay coefficients of variation (CVs) for IL-33 were 5.9% and 11.3% respectively; for TSLP inter-assay and intra-assay CVs were 6.0% and 8.1% respectively. The inter-assay and intra-assay CVs were 3.2% and 6.5% respectively for IgE and 5.2% and 10.4% respectively for IgA.

STATISTICAL ANALYSIS

Ambient maternal exposures, reproductive and medical history characteristics as well as newborn characteristics determined *a priori* to be potential determinants of childhood allergic diseases were included in statistical models (20, 25). Maternal age, household income, self-reported exposure to street traffic, parental smoking, ownership of at least one pet, pre-pregnancy body mass index (BMI) (170), maternal allergy, parity, mode of delivery, infant sex, and fetal growth were included in the analysis. The association between maternal fever and infection and elevated biomarker concentrations was examined to assess the potential influence of underlying infection-related processes.

Descriptive statistics for the immune system biomarkers were calculated using medians and interquartile ranges (IQR) due to the skewed distribution of the data. Spearman correlation coefficients (SCC) were calculated to determine correlation among continuous measures of TSLP, IL-33, and IgE. Due to the high percentage of samples below the limit of detection (LOD), each biomarker was categorized into a binary variable. The 80^{th} percentile was used to identify the subset of samples with elevated concentrations of TSLP (572 pg/mL) and IL-33 (942 pg/mL) due to the lack of established cut-off points. This cut-off was used instead of the detection limit because the objective was to assess prenatal predictors of elevated cytokine levels. A composite variable was developed to identify samples with elevated concentrations of both TSLP and IL-33. A sensitivity analysis was conducted to examine TSLP and IL-33 as binary variables defined at their respective LODs (TSLP=63 pg/mL; IL-33=45 pg/mL). The cut-off percentile for IgE was defined at 1.2 ng/mL (0.5 kU/L) (171,172), a cut-off point previously used in studies of cord blood IgE (90,102). A bivariate analysis of all prenatal

predictors and elevated IL-33, TSLP and IgE was conducted using logistic regression. All variables with a P-value of <0.1 in this analysis were assessed for inclusion in a multivariate logistic regression model using stepwise logistic regression with a P-value criteria of 0.1 for variable inclusion and retention. To assess whether the relationship between prenatal predictors and immune system biomarkers differed by infant sex, the P-value of the product term was assessed and sex-stratified analysis were conducted. All analyses were done using SAS software v. 9.2 (SAS Institute, Cary, NC, USA).

Results

Of the 2001 women recruited into MIREC, 18 withdrew and asked that their data and biospecimens be destroyed. Of the remaining 1983 subjects, 1363 women had provided a cord blood sample. Of these 1363 samples, 104 were excluded for a high IgA concentration, pre-term birth (< 37 weeks), multiple birth, or samples that were inadequate for analysis, leaving 1259 subjects for the present study.

The percentages of TSLP, IL-33 and IgE samples above the limit of detection were 42%, 52%, and 18%, respectively. The median and inter-quartile ranges of the IL-33 (pg/mL), TSLP (pg/mL), and IgE (ng/mL) were 50.7 (425.3), 63.0 (236.0), and 0.30 (2.4), respectively. The percentage of samples with IgE concentrations that exceeded 1.2 ng/mL was 16%. As shown in table 5.1, the correlation between TSLP and IL-33 (SCC=0.75, p<0.0001) was stronger than the correlations between IgE and either TSLP (SCC=0.21) or IL-33 (SCC=0.14). Eighty percent of samples that were categorized as having a high TSLP value had a high IL-33 value.

Maternal allergy and birth weight were associated with elevated TSLP concentrations in a bivariate analysis (P-value of <0.1). Household income, maternal

allergy, and birth weight were associated with elevated IL-33. The following variables were associated with jointly elevated TSLP and IL-33: household income, self-reported exposure to street traffic, maternal allergy, parity, and birth weight (Table 5.2). Maternal age, household income, traffic exposure, and infant sex were associated with elevated IgE. There were no significant associations between either maternal fever or maternal infection and any of the tested biomarkers (data not shown).

Birth weight was the only variable that remained in the multivariate analysis of elevated IL-33 while maternal allergy and birth weight remained in the analysis of elevated TSLP (Table 5.3). In the multivariate analysis of jointly elevated TSLP and IL-33, significant predictors were maternal allergy (OR=2.1; 95% CI: 1.1-3.9 vs no allergy), self-reported exposure to traffic (heavy traffic OR=1.8; 95% CI: 1.2-2.8 vs no traffic), and birth weight (≥4000g OR=2.2; 95% CI: 1.2-4.0 vs < 3000). A significant dose-response relationship between birth weight (test for trend p=0.001) and jointly elevated TSLP and IL-33 was observed (Table 5.3). The relationships between birth weight, traffic and maternal allergy and jointly elevated TSLP and IL-33 did not differ by sex as evidenced by a non-statistically significant product term and comparable magnitudes of effects (Figures 5.1-5.3). In the sensitivity analysis using LOD rather than the 80th, birth weight was the only variable significantly associated with detectable concentrations of either TSLP or IL-33. In the multivariate IgE model, maternal age (test for trend P-value= 0.02) (maternal age ≥35 OR=0.5; 95% CI: 0.3-1.0 vs ≤24) and female sex (OR=0.8; 95% CI:0.6-1.0 vs male sex) were inversely related to elevated IgE levels. The association between maternal age and IgE did not differ by infant sex.

Discussion

In this prospective cohort study of Canadian women, we observed several novel findings. First, IL-33 and TSLP levels were detectable at birth and highly correlated with each other. Second, elevated levels of the composite TSLP and IL-33 variable were significantly and positively associated with maternal allergy, birth weight, and self-reported exposure to traffic. The magnitudes of these associations were greater in the joint IL-33 TSLP model than in the independent cytokine models. Further, cord blood IgE levels were associated with maternal age and female sex, but not the presence of maternal allergy, in a multivariate model.

The observed strong correlation between TSLP and IL-33 provides preliminary evidence that TSLP and IL-33 may be operating in a dependent manner in the developing immune system. These speculations require further investigation to clarify whether the observed levels of TSLP and IL-33 persist into childhood and promote inflammatory responses that manifest as allergic disease. The link with higher birth weight might also suggest an early inflammatory component involving TSLP and IL-33 that is linked to adiposity. The observed dose-response relationship between birth weight and elevated cytokine levels in the primary and sensitivity analysis is consistent with evidence of common phenotypes among both childhood asthma and obesity (173,174). The observed association raises the question of whether a childhood asthma - obesity association is rooted in fetal development

Though, to our knowledge, there are no published investigations of TSLP or IL-33 levels in cord blood, the present findings are consistent with evidence regarding allergic disease determinants. The observed statistically significant association between maternal allergy and joint elevation of levels of TSLP and IL-33 suggests that maternal

allergy has an influence on these biomarkers that is detectable at birth. This finding builds upon evidence of a genetic component to childhood allergic disease (175). The lack of significant association in the sensitivity analysis may indicate that the subset of infants with elevated cytokine levels have a different risk profile that than those with detectable concentrations.

The finding between self-reported street traffic exposure and jointly elevated TSLP and IL-33 levels, though lacking a strong dose-response relationship, is supported by toxicological evidence that air pollution, specifically diesel exhaust and particulate matter, can induce inflammatory processes including epithelial cell production of TSLP (74). The lack of dose-response relationship may be explained by the potential challenge in differentiating between 'low' and 'medium' levels of traffic exposure and resulting exposure misclassification (176). Given the coherence between the present results and the literature on allergic disease determinants, further investigation is warranted to elucidate: 1) the mechanisms underlying the observed statistical associations; 2) the long term implications of elevated cord blood levels of TSLP and IL-33 s and 3) whether immune system sequelae are enhanced when TSLP and IL-33 are elevated.

Consistent with the present study, a US-based study reported that both maternal age and female sex were inversely related to elevated cord blood IgE (177). The same study reported that residence in low income areas, Hispanic ethnicity, and maternal total IgE were associated with elevated cord blood IgE levels (177). The MIREC study did not measure maternal total IgE as it was not part of the original cohort analyses.

Moreover, the MIREC study population is primarily urban, Caucasian and of higher household income levels than the general population (23).

The present study benefited from the relatively large sample size and comparatively rich covariate data. The sample size exceeds any previous analysis of either TSLP or IL-33 in humans. The MIREC study population represents multiple geographic regions within the country. We were able to restrict the analysis to cord blood samples not contaminated by maternal blood by excluding samples with elevated IgA concentrations. We were also able to rule out the role of underlying inflammatory responses in promoting high biomarker concentrations. This study was, however, subject to at least four limitations common to observational studies. First, as data on childhood allergic diagnoses were not available in the MIREC study, we were not able to examine these outcomes. Second, both maternal allergy and traffic were self-reported. Given that the estimated allergy prevalence in the MIREC study (4.3%) is lower than other national estimates of allergy medication use (4-9%) (178), it is unlikely that any resulting bias from the self-reported nature of the variable overestimated allergy prevalence. Furthermore, the available data on traffic exposure in the MIREC study does not capture time or duration of exposure. Third, the study sample included higher income, more educated women, who were less likely to smoke than the general population. Though this potential selection bias is unlikely to have influenced the observed associations, which operate independently of socioeconomic status, it does restrict examination of certain prenatal characteristics. Fourth, the influence of residual confounding on the observed associations from unmeasured variables such as maternal stress or vitamin D cannot be ruled out. There are also unmeasured factors, such as endogenous

corticosteroids and other inflammatory cytokines, which could modify IL-33 and TSLP simultaneously. For example, the epithelial derived cytokine IL-25 (IL-17E) and IL-17 family member has received attention for interacting with IL-33 and TSLP to enhance inflammatory responses (77).

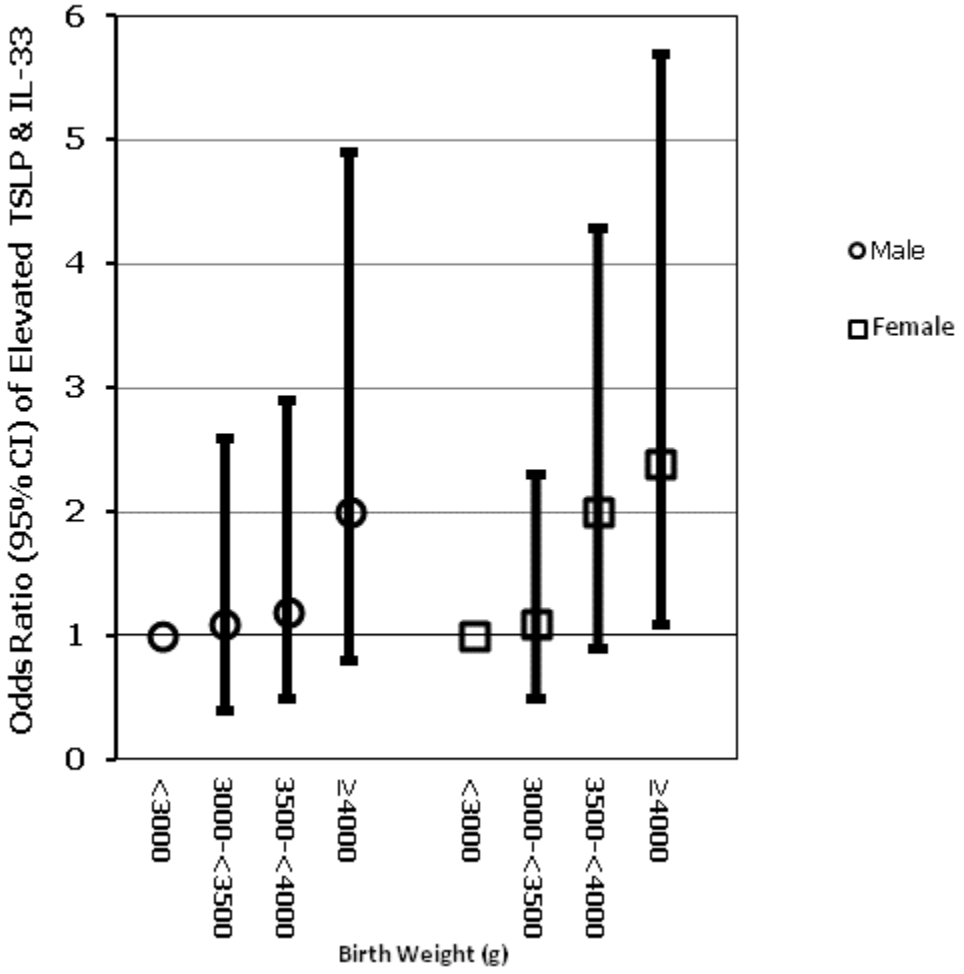
In conclusion, the present findings suggest that neonates with elevated cord blood levels of TSLP and IL-33 may represent an immunologically distinct subset. These cytokines were associated with maternal and infant characteristics that reflect underlying inflammation and/or increased risk of allergic disease development. Thus, cord blood levels of TSLP and IL-33 warrant further investigation as potential predictive factors for inflammatory and allergic disease. Given what is known regarding the integral role of TSLP and IL-33 in allergic disease and inflammatory processes, the present findings also raise interesting questions regarding *in utero* coordinated regulation of these cytokines and motivate further research on this topic.

Acknowledgements

We would like to recognize the valuable contributions of Yisong Wei, Nong Xu (and other members of the Marshall lab) for their assistance in the analysis of the cord blood samples. We would also like to acknowledge the MIREC Study Group and the MIREC Biobank for access to the cord blood samples as well as the MIREC study participants for their dedication.

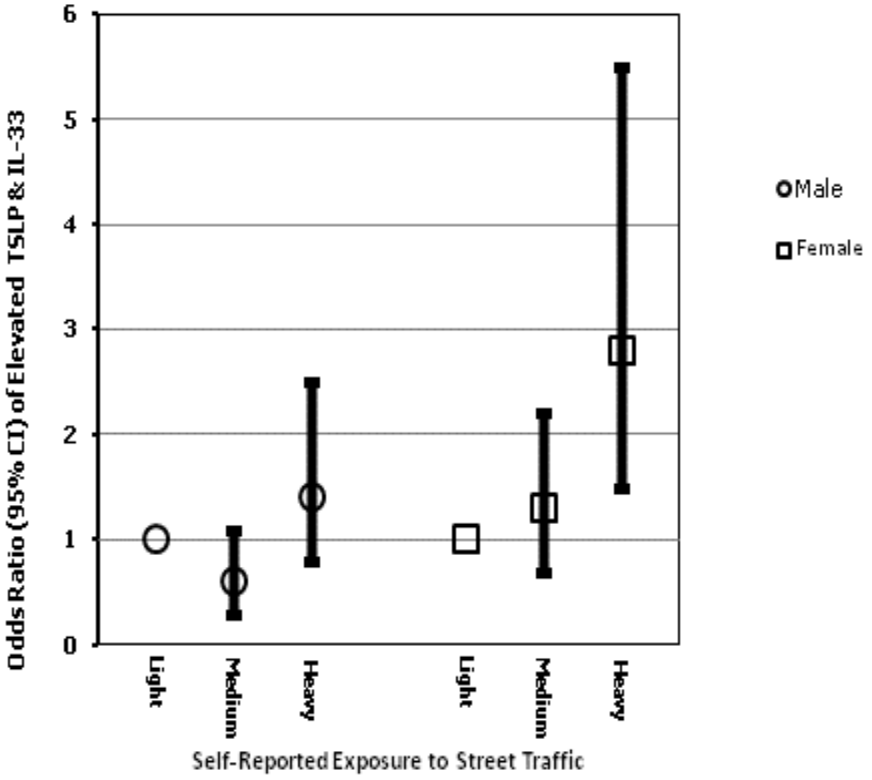
List of Tables and Figures:

Figure 5.1 Adjusted odds ratios (95% CI) of birth weight and elevated TSLP and IL-33 stratified by infant sex



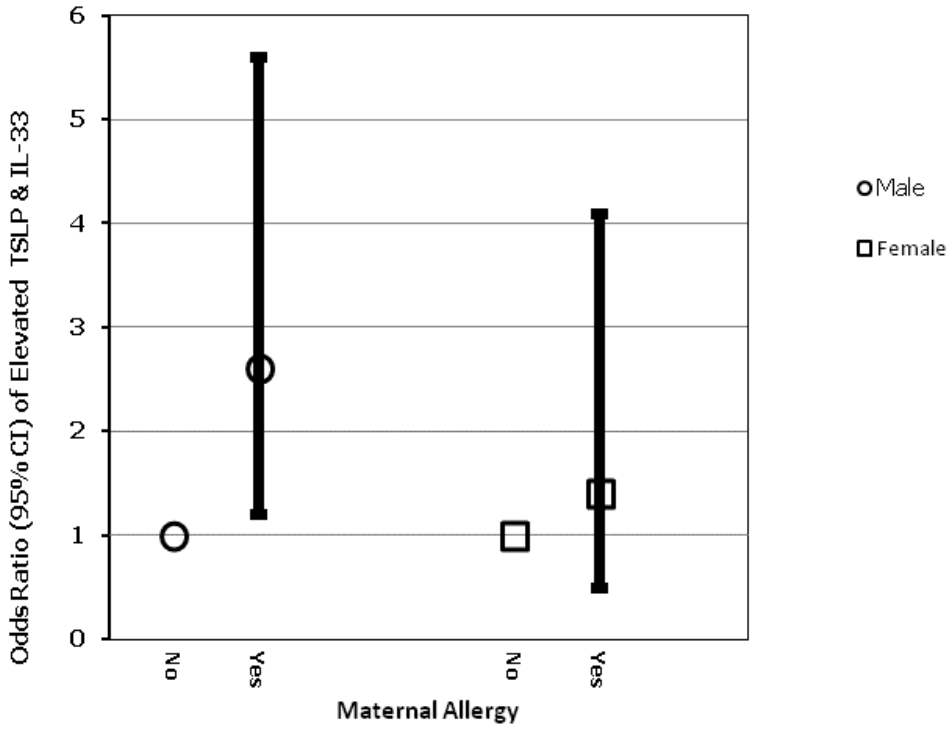
Adjusted for traffic exposure, maternal allergy, and birth weight

Figure 5.2 Adjusted odds ratios (95% CI) of traffic exposure and elevated TSLP and IL-33 stratified by infant sex



Adjusted for traffic exposure, maternal allergy, and birth weight

Figure 5.3 Adjusted odds ratios (95% CI) of maternal allergy and elevated TSLP and IL-33 stratified by infant sex



Adjusted for traffic exposure, maternal allergy, and birth weight

Table 5.1 Spearman rank correlation (p-value) among umbilical cord blood levels of IL-33, TSLP and IgE, MIREC Study, Canada, 2008-2011 (n=1259)

Biomarker	TSLP (pg/mL)	IL-33 (pg/mL)	IgE (ku/L)
TSLP	1.0	0.75 <.0001	0.21 <.0001
IL-33	0.75 <.0001	1.0	0.14 <.0001
IgE	0.21 <.0001	0.14 <.0001	1.0

Table 5.2 Unadjusted odds ratios (95% CI) of prenatal predictors and umbilical cord blood levels of high TSLP (≥80%) (pg/mL), IL-33 (≥80%) (pg/mL), and IgE (≥0.5 ku/L), MIREC Study, Canada, 2008-2011

Characteristic	N	(%)	High TSLP OR (95% CI)	High IL33 OR (95% CI)	High IL33 / TSLP OR (95% CI)	High IgE OR (95% CI)
Maternal Demographic, Reproductive & Medical History						
Maternal Age						
≤24	60	(4.8)	1.0	1.0	1.0	1.0
25-29	270	(21.5)	1.0 (0.5-1.9)	0.7 (0.4-1.3)	0.8 (0.4-1.5)	0.8 (0.4-1.6)
30-34	453	(36.0)	0.7 (0.4-1.9)	0.7 (0.4-1.2)	0.6 (0.3-1.2)	0.7 (0.4-1.4)
≥35	476	(37.8)	0.8 (0.4-1.5)	0.7 (0.4-1.2)	0.7 (0.3-1.3)	0.6 (0.3-1.1)*
Pre-Pregnancy BMI						
Underweight (<18.5)	27	(2.3)	1.0	1.0	1.0	1.0
Normal (18.5 to 24.9)	719	(60.5)	1.1 (0.4-3.0)	1.1 (0.4-2.9)	1.1 (0.4-3.3)	0.5 (0.2-1.2)
Overweight (25 to 29.9)	272	(22.9)	1.1 (0.4-3.1)	1.1 (0.4-3.0)	1.1 (0.4-3.3)	0.6 (0.2-1.5)
Obese (≤ 30)	171	(14.4)	0.9 (0.3-2.5)	1.0 (0.3-2.8)	0.9 (0.3-2.8)	0.4 (0.2-1.1)
Maternal Allergy[1]						
No	1205	(95.7)	1.0	1.0	1.0	1.0
Yes	54	(4.3)	1.9 (1.1-3.4)*	1.8 (1.0-3.2)*	2.1 (1.1-3.9) *	1.5 (0.8-3.0)
Parity						
Nulliparous	527	(41.9)	1.0	1.0	1.0	1.0
Primiparous	513	(40.8)	1.1 (0.8-1.5)	1.1 (0.8-1.5)	1.3 (0.9-1.8)	0.9 (0.7-1.3)
Multiparous	217	(17.3)	0.7 (0.4-1.0)	0.7 (0.5-1.1)	0.7 (0.4-1.1) *	1.0 (0.7-1.6)
Mode of Delivery						
Spontaneous	900	(71.5)	1.0	1.0	1.0	1.0
Caesarean	359	(25.5)	1.0 (0.7-1.3)	1.0 (0.7-1.4)	0.9 (0.7-1.3)	1.0 (0.7-1.4)
Household Income						
≤30,000	90	(7.4)	1.0	1.0	1.0	1.0
30,001-50,000	117	(9.7)	1.7 (0.8-3.3)	2.8 (1.3-6.0)*	2.6 (1.1-5.8)*	1.2 (0.6-2.4)*
50,001-100,000	515	(42.6)	1.0 (0.5-1.9)	1.5 (0.8-3.0)	1.5 (0.7-3.1)	0.7 (0.4-1.2)
>100,000	488	(40.3)	1.2 (0.7-2.0)	1.9 (0.6-3.6)	1.8 (0.9-3.7)	0.7 (0.4-1.2)
Ambient Maternal Exposures						
Traffic[2]						
Light	860	(68.4)	1.0	1.0	1.0	1.0
Medium	250	(19.9)	0.8 (0.6-1.2)	0.9 (0.7-1.4)	0.9 (0.6-1.3)	1.0 (0.7-1.5)
Heavy	147	(11.7)	1.3 (0.9-2.0)	1.5 (1.0-2.2)	1.7 (1.1-2.6)*	1.7 (1.1-2.6) *
Parental Smoking [3]						
No	1009	(80.1)	1.0	1.0	1.0	1.0
Yes	250	(19.9)	1.1 (0.88-1.5)	1.2 (0.9-1.7)	1.2 (0.8-1.7)	1.2 (0.9-1.8)
Pet Ownership						
No	560	(44.5)	1.0	1.0	1.0	1.0
Yes	699	(55.5)	1.1 (0.8-1.5)	1.1 (0.8-1.4)	1.0 (0.7-1.3)	0.8 (0.6-1.1)

[1] Defined by the self-reported use an anti-allergen medication
[2] Defined as self-reported residential street traffic intensity
[3] Defined as yes if either the mother or father smoked during the pregnancy,
* P-value <0.1

Table 2 (cont'd)

Characteristic	N (%)		High TSLP OR (95% CI)	High IL33 OR (95% CI)	High IL33 / TSLP OR (95% CI)	High IgE OR (95% CI)
Infant Characteristics						
Infant Sex						
Male	673	(53.5)	1.0	1.0	1.0	1.0
Female	585	(46.5)	1.2 (0.9-1.6)	1.0 (0.8-1.3)	1.1 (0.8-1.5)	0.8 (0.6-1.0)*
Birth Weight (g)						
<3000	138	(11.0)	1.0	1.0	1.0	1.0
3000-<3500	479	(38.1)	0.9 (0.6-1.3)	1.0 (0.6-1.8)	1.1 (0.7-1.9)	1.0 (0.6-1.7)
3500-<4000	450	(35.7)	1.3 (0.8-2.1)	1.2 (0.8-2.0)	1.4 (0.8-2.4)	1.3 (0.8-2.2)
≥ 4000	192	(15.3)	1.9 (1.1-3.2)*	1.7 (1.0-3.0)*	2.1 (1.2-3.9) *	1.2 (0.6-2.2)

Table 5.3 Adjusted odds ratios of prenatal predictors and umbilical cord blood levels of high IL-33 (≥80%) (pg/mL), TSLP (≥80%) (pg/mL), and IgE (≥0.5 ku/L), MIREC Study, Canada, 2008-2011*

Characteristics	High IL-33	High TSLP	High IL-33 &TSLP OR (95% CI)	IgE High OR (95% CI)
Maternal Reproductive & Medical History				
Maternal Age				
≤24	--	--	--	1.0
25-29				0.8 (0.4-1.6)
30-34				0.7 (0.4-1.4)
≥35				0.5 (0.3-1.0)
Test for trend				**0.02**
Maternal Allergy				
No	--	1.0	1.0	
Yes		**1.8 (1.0-3.3)**	**2.1 (1.1-3.9)**	--
Parity				
Nulliparous	--	--	--	--
Primiparous				
Multiparous				
Household Income				
≤30,000	--	--	--	--
30,001-50,000				
50,001-100,000				
>100,000				
Test for trend				
Ambient Maternal Exposures				
Traffic				
Light	--	--	1.0	--
Medium			0.9 (0.6-1.3)	
Heavy			**1.8 (1.2-2.8)**	
Test for trend			**0.04**	
Infant Characteristics				
Sex				
Male	**--**	--	--	1.0
Female				0.8 (0.6-1.0)
Birth Weight (g)				
<3000	1.0	1.0	1.0	--
3000-<3500	1.0 (0.6-1.5)	0.9 (0.6-1.3)	1.1 (0.6-1.9)	
3500-<4000	1.2 (0.8-2.0)	1.3 (0.8-2.1)	1.5 (0.8-2.6)	
≥ 4000g	**1.7 (1.0-3.0)**	**1.9 (1.1-3.2)**	**2.2 (1.2-4.0)**	
Test for trend	**0.006**	**0.001**	**0.001**	

*Models adjusted for those variables identified as having p<0.1 based on findings in Table 2. IL-33 model adjusted for birth weight, TSLP model adjusted for allergy and birth weight, IL-33/TSLP model adjusted for allergy, traffic, and birth weight, IgE model adjusted for maternal age and sex.

Chapter Six. Prenatal Exposure to Phthalates, Bisphenol A and Perfluorinated Compounds and Cord Blood Levels of IgE, TSLP and IL-33

This manuscript has been submitted to Health Canada for scientific peer and management review. The authors' contributions are as follows:

J. Ashley-Martin performed the ELISA analysis of umbilical cord blood samples, conducted the statistical analysis, and wrote the manuscript. L. Dodds provided supervisory input regarding the cohort, statistical analysis and critical feedback on the manuscript. A. Levy assisted in structuring and writing the manuscript. R. Platt provided guidance on the Bayesian hierarchical modeling and reviewed the statistical methods. J. Marshall provided lab space, materials, and technical support to facilitate the completion of the ELISA analysis and critically reviewed early versions of the manuscript. T. Arbuckle, co-principal investigator of the MIREC study, provided guidance on the study cohort as well as reviewed and edited the manuscript. All authors were involved with the study design and methodology.

Authors: Jillian Ashley-Martin[1], Linda Dodds*[2], Adrian R. Levy[3], Robert W. Platt[4], Jean S. Marshall[5], Tye E. Arbuckle[6]

Author Affiliations:

7. Interdisciplinary PhD Program, Dalhousie University
8. Department of Obstetrics & Gynaecology and Paediatrics, Dalhousie University
9. Department of Community Health & Epidemiology, Dalhousie University
10. Department of Epidemiology and Biostatistics, McGill University
11. Department of Microbiology & Immunology, Dalhousie University
12. Healthy Environments and Consumer Safety Branch, Health Canada

Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

1. IDPhD c/o Faculty of Graduate Studies Room 314 Henry Hicks Building 6299 South St Halifax, NS B3H 4H6
2. Perinatal Epidemiology Research Unit, 7th Floor Women's Site, IWK Health Centre, 5980 University Ave. PO Box 9700, Halifax, NS, Canada B3H 6R8 (l.dodds@dal.ca)
3. Department of Community Health and Epidemiology Centre for Clinical Research 5790 University Avenue
   Halifax, NS B3H 1V7 (Adrian.levy@dal.ca)
4. Department of Epidemiology and Biostatistics, McGill University Purvis Hall 1020 Pine Ave. West
   Montreal, QC H3A 1A2 (robert.platt@mcgill.ca)
5. Department of Microbiology & Immunology Dalhousie University Sir Charles Tupper Medical Building, Room 7-C 5850 College Street Halifax, Nova Scotia Canada (jean.marshall@dal.ca)
6. Population Studies Division, Healthy Environments and Consumer Safety Branch, Health Canada, 50 Colombine Dr., AL 0801A, Ottawa ON K1A 0K9 Canada (Tye.Arbuckle@hc-sc.gc.ca)

Abstract:
The fetal time period is a critical window of immune system development and resulting heightened susceptibility to the adverse effects of environmental exposures. Epidemiologists and toxicologists have hypothesized that phthalates, bisphenol A (BPA) and perfluorinated compounds have immunotoxic properties. Immunotoxic effects of chemicals may manifest in an altered immune system profile at birth. Immunoglobulin E, thymic stromal lymphopoietin (TSLP), and interleukin-33 (IL-33) are integral in the etiology of childhood allergy and detectable at birth. The objective of this study was to determine the relationship between maternal levels of phthalates, bisphenol A (BPA), and perfluorinated compounds and elevated umbilical cord blood levels of IgE, TSLP, and IL-33. This study utilized data collected in the Maternal-Infant Research on Environmental Chemicals (MIREC) Study, a trans-Canada cohort study of 2,001 pregnant women. Of these women, 1258 had a singleton, term birth and cord blood sample. A Bayesian hierarchical model was employed to determine associations between log-transformed continuous variables and immune system biomarkers while adjusting for potential confounding from correlated environmental contaminants. Inverse, nonlinear associations were observed between maternal urinary MCPP levels and elevated levels of both IL-33/TSLP and IgE and between maternal urinary BPA levels and elevated levels of IL-33/TSLP. In this primarily urban Canadian population of pregnant women and their newborns, maternal urinary and plasma concentrations of phthalate metabolites, BPA, and perfluorinated compounds were not associated with immunotoxic effects that manifest as increased odds of elevated levels of IgE, TSLP or IL-33. Determining whether this absence of effect persists into childhood requires follow-up in a cohort of children.

5 Key words: phthalates, bisphenol A, perfluorinated compounds, immunoglobulin E, cytokines

Word count ~ 3800

Sources of Funding: This work was funded by the Nova Scotia Health Research Foundation, the Izaak Walton Killam (IWK) Health Centre, and the Nova Scotia Atlee Foundation. The MIREC study was funded by Health Canada, the Canadian Institutes for Health Research (CIHR) and the Ontario Ministry of the Environment. The Dalhousie University Department of Microbiology and Immunology receives support from the Canadian Institutes of Health Research. Robert Platt is a Chercheur-national of the Fonds de Récherche du Quebec – Santé (FRQ-S), and is a member of the Research Institute of the McGill University Health Centre, which receives core support from the FRQ-S.
This work received ethical approval from Health Canada, St. Justine's Hospital (Montreal, QC), and the IWK Health Centre (Halifax, NS).

Introduction[4]

The fetal time period is a critical window of immune system development and heightened susceptibility to the adverse effects of environmental exposures (45,50,163). This susceptibility stems from the immaturity of fetal organ systems and undeveloped detoxification systems (109). Exposure to environmental contaminants during this time can promote permanent, irreversible changes to immune system development and increase risk of an allergic phenotype (8,53,179). Exploration of *in utero* exposures that may precipitate these changes is, therefore, critical to understanding the etiology of childhood allergic disease (180).

Epidemiologists and toxicologists have hypothesized that phthalates (124,181), bisphenol A (BPA) (133) and perfluorinated compounds (10,182) may have immunotoxic properties. These contaminants are ubiquitous in the environment; the majority of adults have detectable concentrations in their urine or plasma (183–185). Moreover, maternal urinary or plasma concentrations of phthalates, BPA, and perfluorinated compounds results in fetal exposure as these contaminants are known to cross the placenta (186–188).

Despite the ubiquity of these contaminants and potential for immunotoxic effects, the epidemiologic literature regarding the association between prenatal exposure to these contaminants and childhood allergic diagnoses is limited (128,134,135,140,189). Moreover, the validity of studies that examine childhood allergic diagnosis is challenged by: 1) confounding from childhood exposures to allergy risk factors and 2) outcome misclassification inherent in the diagnosis of childhood allergic disease (190–192).

[4] TSLP=thymic stromal lymphopoietin, IL-33=interleukin-33, IgE-=immunoglobulin E

The immunotoxic effects of *in utero* environmental contaminant exposure may manifest in altered levels of immune system biomarkers at birth. An altered immune system profile at birth is a risk factor for childhood allergy (46,53,98,180). The immune system biomarkers, immunoglobulin E (IgE), interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP), are detectable in cord blood (193). Elevated levels are associated with inflammatory processes and allergic disease in later life (16,69,194). Cord blood IgE levels have been previously used as a means of assessing the immunotoxic effects of *in utero* environmental contaminant exposure (140,141,195). TSLP and IL-33 have recently been recognized for their etiologic role in atopic dermatitis, the earliest manifestation of childhood allergy (70,87). Unlike other cytokines that are produced from hematopoietic cells (196), epithelial cell production of TSLP and IL-33 is not dependent on the presence of a functioning, developed immune system function (67). Analysis of TSLP and IL-33 levels in cord blood provides a novel means for examining the susceptibility of the newborn immune system to the potential adverse effects of *in utero* exposure to environmental contaminants.

The objective of this study was to determine the relationship between maternal levels of phthalates, bisphenol A (BPA), and perfluorinated compounds and elevated umbilical cord blood levels of IgE, TSLP, and IL-33 in Canadian birth cohort. Given that childhood allergy prevanelce differs according to infant sex (168), a secondary objective was to determine how relationships may differ by infant sex. Due to the co-occurrence of phthalate metabolites within common household products (e.g. cosmetics, plastics), individuals are routinely exposed to multiple phthalate metabolites simultaneously (107). The concentrations of multiple perfluorinated compounds in household dust have been

shown to be correlated (138). We employed an analytical approach to account for the potential effects of correlated exposures.

Methods

STUDY POPULATION AND DATA SOURCES

This study used data and biospecimens from the Maternal-Infant Research on Environmental Chemicals (MIREC) Study Biobank, a trans-Canada cohort study of 2,001 pregnant women from ten Canadian cities recruited during 2008-2011 (169). The target population was pregnant women and their newborns who reside in these ten urban regions and surrounding catchment areas. Approximately 6% of study participants lived in a rural area according to postal forward sortation area. Study participants were contacted throughout pregnancy at pre-specified time points to obtain data and biospecimens. Briefly, women were eligible for inclusion if they were < 14 weeks gestation at time of recruitment, ≥ 18 years of age, able to communicate in French or English, and planning on delivering at a local hospital (169). The population in the present investigation was mothers who had a singleton, live, term birth (≥37 weeks) and a cord blood sample suitable for analysis. Cord blood samples (n=5) that were determined to be contaminated with maternal blood based on an elevated immunoglobulin A (IgA) level (≥ 10 μg/mL) were excluded from the analysis (Ownby, et al, 19961996). This study received ethical approval from Health Canada, St. Justine's Hospital (Montreal, QC), and the IWK Health Centre (Halifax, NS) and all participants signed informed consent forms.

ENVIRONMENTAL CONTAMINANT EXPOSURE

BPA and eleven phthalate metabolites were measured in maternal urine collected during the 1st trimester as shown in Table 6.1 and as previously described (197). Briefly, chemical analysis of urine samples was carried out at the Laboratoire de Toxicologie, Institut National de Santé Publique du Québec (Québec, QC, Canada), accredited by the Standards Council of Canada. Phthalates in urine were analyzed by LC-MS/MS with an Ultra Performance Liquid Chromatography (UPLC) coupled with a tandem mass spectrometer and Quattro Premier XE following enzymatic deconjugation. Total BPA in urine were measured with a GC-MS-MS instrument with a GC Agilent 6890 N (Agilent Technologies; Mississauga, ON, Canada) coupled with a tandem mass spectrometer Quattro Micro GC (Waters; Milford, Massachusetts, USA). An enzymatic hydrolysis freed the conjugated compounds in the urine, the samples were then derivatized and the derivatives extracted and analyzed. Perfluorinated compounds were measured in study participant 1st trimester plasma using a Water Acquity UPLC-MS-MS operated in the MRM mode with an electrospray ion source in negative mode.

FETAL MARKERS OF IMMUNE SYSTEM FUNCTION

Immune system biomarkers were measured in the plasma of umbilical cord blood samples in the Department of Microbiology & Immunology, Dalhousie University, Halifax, NS using ELISA. TSLP concentrations were determined using a commercial antibody kit (Biolegend; San Diego, CA, USA). IL-33 concentrations were assessed using antibodies from an R & D systems duoset (Minneapolis, MN, USA). ELISA kits (EBioscience; San Diego, CA, USA) were also used to assess both total IgE and IgA concentrations. ELISA assays were performed according to manufacturer's instructions with the exception that plates were coated with sodium bicarbonate buffer (pH 8.3-8.5)

and blocked with 2% BSA in PBS instead of using the manufactures coating and blocking buffers. The inter-assay and intra-assay CVs for IL-33 were 5.9% and 11.3% respectively; for TSLP inter and intra-assay CVs were 6.0% and 8.1% respectively. The inter-assay and intra-assay CVs were 3.2% and 6.5% respectively for IgE and 5.2% and 10.4% respectively for IgA.

STATISTICAL ANALYSIS

Due to the high percentage of samples below the limit of detection (LOD), each immune system biomarker was categorized into a binary variable. A composite variable was developed to identify samples with elevated concentrations of both TSLP and IL-33 (IL-33/TSLP) as these cytokines are highly correlated (spearman correlation coefficient=0.8). Elevated levels of TSLP and IL-33 were defined as samples that exceeded the 80th percentile for both cytokines (TSLP=554 pg/mL; IL-33=879 pg/mL) because there are no pre-existing thresholds. Elevated IgE samples were defined as those that exceeded 1.2 ng/mL (0.5 kU/L) (171,172), a cut-off point previously used in studies of cord blood IgE (90,104).

Maternal urinary measures of eleven phthalate metabolites and BPA collected from a spot urine sample in the 1st trimester were available in the MIREC study (Table 6.1). Four phthalate metabolites (MCHP, MOP, MNP, MMP) were not examined in multivariate analysis due to the low proportion of values above the limit of detection (% >LOD MCHP=7.4, MOP=3.0, MNP=1.1, MMP=14.0). Three of the phthalate metabolites are primary (MEHP) and secondary (MEHHP, MEOHP) metabolites of the parent compound Di(2-ethylhexyl)phthalate (DEHP) (198). Considering the high correlation (Pearson correlation coefficient=0.9) between these metabolites, they were

not analyzed as individual metabolites. Rather, metabolite concentrations were summed to create an index of DEHP metabolite exposure as previously reported (199). Maternal measures of three perfluorinated compounds were collected from 1st trimester plasma (Table 6.2). All values less than the LOD were imputed as LOD/2.

As the distribution of the environmental contaminants was non-normal, chemical concentrations were log-transformed to calculate descriptive statistics and perform multivariate analysis. Geometric means and standard deviations were calculated for the all environmental contaminants according to high and low levels of the immune system biomarkers. Concentrations of phthalate metabolites and bisphenol A were adjusted for urinary specific gravity according to the following formula $P_c = P_i\ [(SG_m - 1)/(SG_i - 1)]$ where: P_c = SG adjusted metabolite concentration (μg/ml), P_i = observed metabolite concentration, SG_i = specific gravity of the urine sample, and SG_m = median SG for the cohort (200).

The Pearson correlation coefficients of log-transformed phthalate metabolites and bisphenol A ranged from 0.3 (between MEP and BPA) to 0.6 (between MBP and DEHP metabolites). The correlation coefficients of the log-transformed perfluorinated compounds ranged from 0.5 (between PFOA and PFHxS) to 0.6 (between PFOA and PFOS). Bayesian hierarchical logistic regression models were employed to estimate odds ratios (OR) and 95% credible intervals (CI) for the association between the environmental contaminants and immune system biomarkers. The outcome variables were elevated IL-33/TSLP and elevated IgE (as defined above). This approach facilitates inclusion of correlated exposures and is not subject to the challenges of convergence and unstable estimates faced by maximum likelihood regression models (201). A separate

hierarchical model was developed for each chemical class (e.g. phthalates, perfluorinated compounds) and outcome (IL-33/TSLP and IgE). As the correlation between BPA and phthalate metabolites was comparable to the within-phthalate correlation (e.g. MBP-BPA correlation coefficient = 0.5), BPA was included in the phthalate model.

The Bayesian model was run with three chains and 5,000 iterations per chain with the first 500 iterations discarded as a burn-in period. We used a normal distribution (0, Φ) as prior for the parameters, with Φ assumed to follow a half-normal distribution (with the underlying normal having mean=0, variance=100) (202). This uninformative prior distribution was chosen to reflect lack of prior knowledge regarding the association between these chemicals and newborn immune system development. The distribution is uninformative due to the large variance. In comparison to models that apply a fixed variance and resulting fixed degree of estimate shrinkage toward the prior mean, this approach allows the degree of shrinkage to vary depending on how closely the data fit the prior distribution (201). Model convergence was assessed by visual assessment of trace plots and by convergence diagnostic tests (Gelman-Rubin convergence test <1.05; R-hat=1)(203). Details on the model are provided in Appendix 1.

Prior to inclusion in the hierarchical models, the linearity of each contaminant-immune system biomarker relationship was assessed using restricted cubic spline models (204) with knots set at the 5^{th}, 50^{th}, and 95^{th} percentiles. Environmental contaminants that met the criteria for linearity were log-transformed and those that did not were modeled with a quadratic term.

Potential confounders were identified using a hypothesized causal model (205). The causal models were constructed using evidence regarding predictors of the exposures

(197,206) and predictors of the immune system biomarkers (193,207). The minimal adjustment sets for confounding were identified using DAGitty (208). Specific gravity was forced into the adjusted phthalate/BPA model to account for heterogeneity in urinary dilution (197). As childhood allergy prevalence differs by sex (168), analyses were also stratified by sex.

Descriptive statistics were performed in SAS V.9.2 (Cary, NC). Bayesian modeling was performed using R v.3.0.3 (The R Foundation for Statistical Computing) and Openbugs (v 3.2.1 Members of OpenBUGS Project Management Group).

Results

Of the 2,001 women recruited, 18 withdrew and asked that all their data and biospecimens be destroyed. Of the remaining 1983 subjects, 1363 women had a cord blood sample. Of these 1363 samples, a total of 104 samples were excluded for a high IgA concentration, pre-term birth (< 37 weeks), multiple birth, or samples with insufficient sample for analysis, and no chemical data for either phthalates, BPA and the perfluorinated compounds leaving 1258 subjects for inclusion in the statistical analysis. An additional 121 women did not have maternal urinary measurements of phthalates and an additional 16 women did not have maternal plasma measurements of perfluorinated compounds.

Table 6.1 depicts geometric mean concentrations of phthalates and perfluorinated compounds according to categories of the immune system biomarkers. Maternal demographic, reproductive, and infant characteristics are depicted in Table 6.2. The majority of study participants were greater than 30 years of age, had a household income greater than $50,000, were non-smokers, and of normal BMI.

Significant non-linear associations were observed between the phthalate metabolite MCPP (P-value=0.008 test for linearity; P-value =0.01 test for association) and BPA (P-value test for linearity=0.02, P-value test for association 0.05) and odds of elevated IL-33/TSLP (Figures 6.1-6.2). MCPP was also observed to have a significant, non-linear relationship with odds of elevated IgE (P-value linearity = 0.01, P-value association=0.02) (Figure 6.3). Thus, quadratic terms for both MCPP and BPA were included in the IL-33/TSLP model and a quadratic term for MCPP was included in the IgE model. There were no significant non-linear associations between the perfluorinated compounds and either IL-33/TSLP or IgE.

Tables 6.3 and 6.4 depict results of the Bayesian hierarchical models. The quadratic term for maternal urinary BPA concentrations was inversely associated with odds of elevated IL-33/TSLP (aOR=0.8; 95% CI: 0.5-1.0) (Table 6.3). The quadratic term for MCPP was inversely associated with odds of elevated IgE (aOR=0.7; 95% CI: 0.5-1.0) (Table 6.5). Credible intervals for all other phthalates and perfluorinated compounds included the null value (Tables 6.3-6.4). Results were similar among male and female infants in all statistical models (Supplementary tables 6.1.1-6.1.4).

Discussion

In this study, we sought to determine the relationship between maternal levels of phthalates, BPA, and perfluorinated compounds and elevated umbilical cord blood levels of IgE, TSLP and IL-33. This question was motivated by the fact that *in utero* environmental contaminant exposure may alter newborn immune system development and promote risk of an allergic phenotype (8,53). Consistent with the inverted U-shaped curve observed in the restricted cubic spline analyses, we found an inverse, non-linear

association between maternal urinary MCPP levels and elevated levels of both IL-33/TSLP and IgE in the Bayesian hierarchical model. An inverse, non-linear association was also observed between maternal urinary BPA levels and elevated levels of IL-33/TSLP in the Bayesian hierarchical model, similarly consistent with the association observed in the restricted cubic spline analysis. We did not observe any associations between the contaminants of interest and elevated levels of IgE, or IL-33/TSLP where the credible interval did not include the null.

PRENATAL EXPOSURE TO PHTHALATE METABOLITES AND MEASURES OF CHILDHOOD ALLERGY

The potential role of phthalate exposure in childhood allergy etiology is supported by experimental evidence demonstrating that these chemicals exhibit adjuvant-like properties (181). Epidemiological evidence regarding prenatal phthalate exposure is, however, equivocal. To our knowledge, authors of two birth cohort studies in New York (128,129) and Taiwan (189) have examined the association between prenatal phthalate exposure and measures of childhood allergy. The Taiwanese birth cohort reported no statistically significant associations between phthalate metabolites and cord blood IgE (189). Investigators of the New York city cohort reported a significant association between maternal urinary phthalate metabolite (MBzP) concentrations and eczema at two but not five years of age (128). In a later follow-up of this cohort, authors report a significant association between prenatal MBzP exposure and childhood asthma diagnosed between the ages of 5 and 11 (129). Considering that the present study also identified a slight positive association between cord blood IgE levels and MBzP exposure (OR=1.2 95% CI: 0.9-1.7), further investigation into the role of this metabolite in childhood allergy etiology is warranted. Divergence in other results among the New York (128),

Taiwanese (189) and present study may be explained by the heterogeneity in target populations, exposure levels, or outcome measures (Table 6.5).

PRENATAL EXPOSURE TO BPA AND MEASURES OF CHILDHOOD ALLERGY

Experimental evidence has demonstrated that BPA may induce an asthmatic phenotype by elevating IgE levels and promoting eosinophilic inflammation (132,133). However, epidemiological literature regarding prenatal BPA exposure and childhood allergy is limited and inconsistent. A birth cohort study from the mid-west US (134) reported a positive association between maternal urinary BPA levels and childhood wheeze whereas the NY birth cohort investigators (135) reported an inverse association. In contrast, in the present study we identified a non-linear relationship between BPA and elevated IgE. In addition to differences outlined in Table 6.5, a possible explanation for the heterogeneity in results is exposure misclassification. The short half-life (130), complexity of metabolism (209), and potential contamination of urinary samples with external sources of BPA (e.g. laboratory equipment) (210) create material challenges to accurately estimating fetal BPA levels.

PRENATAL EXPOSURE TO PERFLUORINATED COMPOUNDS AND MEASURES OF CHILDHOOD ALLERGY

In experimental models, perfluorinated compounds have been shown to induce immune system profile changes indicative of an allergic phenotype (10,139). Two previous birth cohort studies reported that the relationship between gestational perfluorinated compound exposure and cord blood IgE levels differed by infant sex (Table 6.5) (140,141). These findings may be explained by the fact that PFOA has a longer half-live and slower renal clearance in males (206). The lack of notable

differences by sex in the PFOA and IgE model in the present study may be due to the relatively low percentage of cord blood samples with levels of IgE above 0.5 ku/L.

STRENGTHS AND LIMITATIONS

This study benefited from the relatively large sample size in the MIREC study, the comparatively rich covariate data, and the use of novel immune system biomarkers. The study population was predominantly from urban regions in Canada. We employed analytical methods to control for correlated exposures (201) and assess non-linear relationships. Furthermore, by shrinking effect estimates towards each other, Bayesian hierarchical models lower the likelihood of identifying a significant association by chance (211).

This study was subject to at least three limitations common to observational studies. First, the short half life and rapid elimination of phthalates and BPA creates a source of potential misclassification bias as the body burden of these chemicals may vary on a daily basis (130,212–214). In pregnant women, the reported intraclass correlation (ICCs) for BPA has been very low, ranging from 0.11 to 0.24 (212,215–217). The phthalate metabolite MCPP was reported to have considerable within person variability based on an ICC of 0.20 in a study of serial measurements in pregnant women (218). Considering, however, that 1) that exposure misclassification is non-differential and 2) this study employed continuous rather than polytomous exposure variables, any potential misclassification bias likely attenuated effect estimates towards the null (58,219). Reproduction of the present findings in cohort studies with serial phthalate and BPA measurements would be valuable to further investigate the influence of this potential information bias.

Second, the MIREC study population was of higher income and more educated than the target population. This potential selection bias is unlikely to have biased the observed associations because the associations did not seem to be confounded by income or education. The socioeconomic characteristics of the MIREC population did, however, preclude our ability to assess associations among certain population subgroups including low income women with minimal secondary education. Third, the influence of residual confounding cannot be ruled out. We did not have information on certain risk factors for childhood allergy such as vitamin D (220) and maternal stress (221). Considering that a relationship between these variables and exposure to the contaminants of interest is not well established, we have no reason to believe our observed associations are invalidated by lack of control for these variables.

PUBLIC HEALTH INTERPRETATIONS

Due to the lack of Canadian health-based biomonitoring guidelines for the contaminants of interest, interpretation of the present findings within a public health context is a challenge. On average, the phthalate and BPA exposure levels observed within the MIREC study are lower than other birth cohorts discussed (Table 6.5) and consistent with the temporal trend of declining body burden levels of many phthalates and BPA (183,222). Health Canada recently took regulatory action to minimize exposure levels of BPA and certain phthalates to children (223,224). Though these actions are unlikely to directly affect prenatal exposure levels in the current study, the regulations may promote heightened consumer awareness and subsequent attempts to minimize use of BPA and phthalate-laden products.

Plasma concentrations of PFOA and PFOS have also been decreasing over time, but concentrations of other perfluorinated compounds (e.g. perfluorononanoic acid (PFNA)) have been on the rise (183,225,226). The decrease in PFOA and PFOS concentrations is explained by the cessation of PFOA and PFOS production within the last ten years (227,228). The persistent nature and long half-life of these compounds (206) explains the continued presence of detectable concentrations of these chemicals in maternal plasma. The second cycle of the Canadian Health Measures Study reported that the majority of Canadians have detectable concentrations to some of these less common compounds such as PFNA (185). As such, continuing biomonitoring efforts are warranted to determine trends in levels and sources of exposure as well as investigate potential health effects.

The novel finding of a non-linear relationship between MCPP, BPA and the immune system biomarkers of interest in this study is consistent with evidence that endocrine disrupting chemicals may operate in a non-monotonic manner (229). Further elucidation of these dose-response relationships and underlying biological mechanisms will be a valuable contribution to the development of health based biomonitoring guidelines for these contaminants.

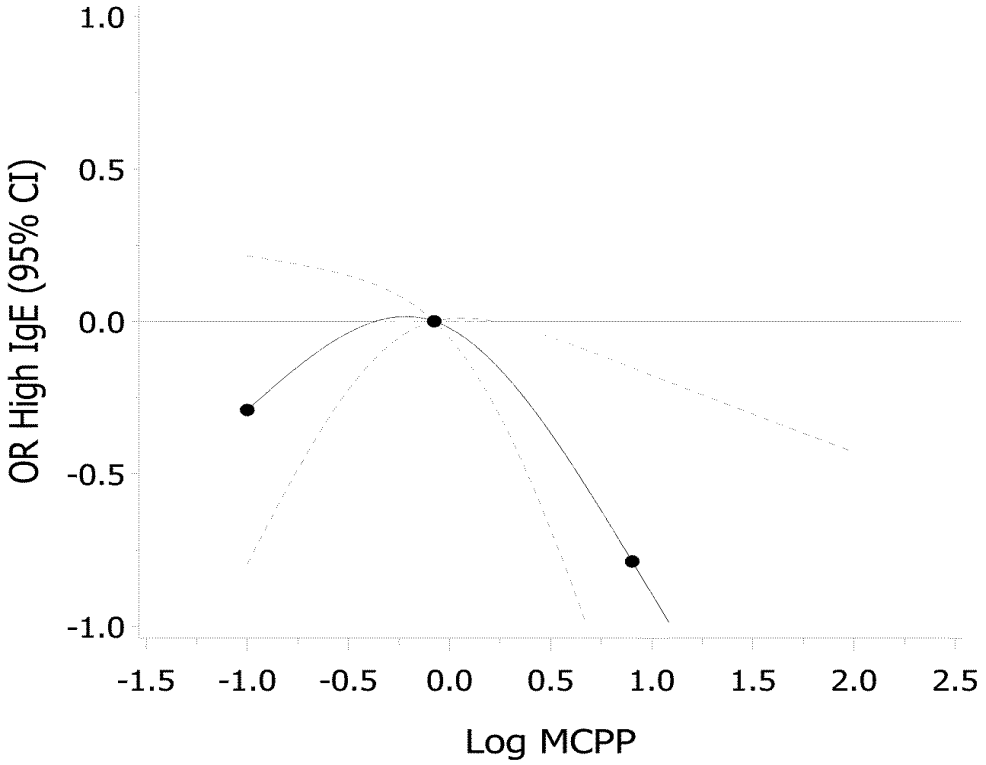
Acknowledgements

We would like to acknowledge the valuable contributions of Yisong Wei, Nong Xu (and other members of the Marshall lab) for their assistance in the analysis of the cord blood samples. We would also like to acknowledge the MIREC biobank committee for granting access to the cord blood samples as well as the MIREC study participants for their dedication.

List of Tables and Figures

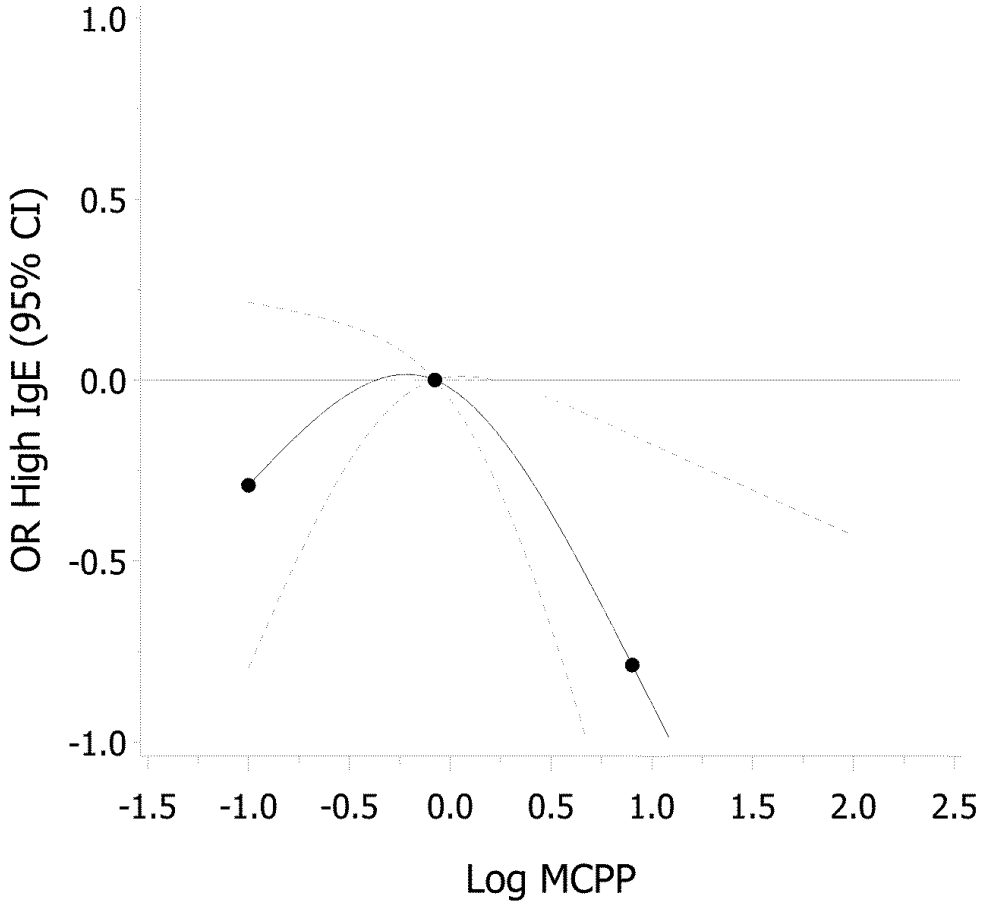
SUPPLEMENTARY TABLES

Figure 6.1 Restricted cubic spline curve of association between maternal urinary log_{10} MCPP concentrations and high IL-33/TSLP cord blood concentrations (MIREC study, 2008-2011)



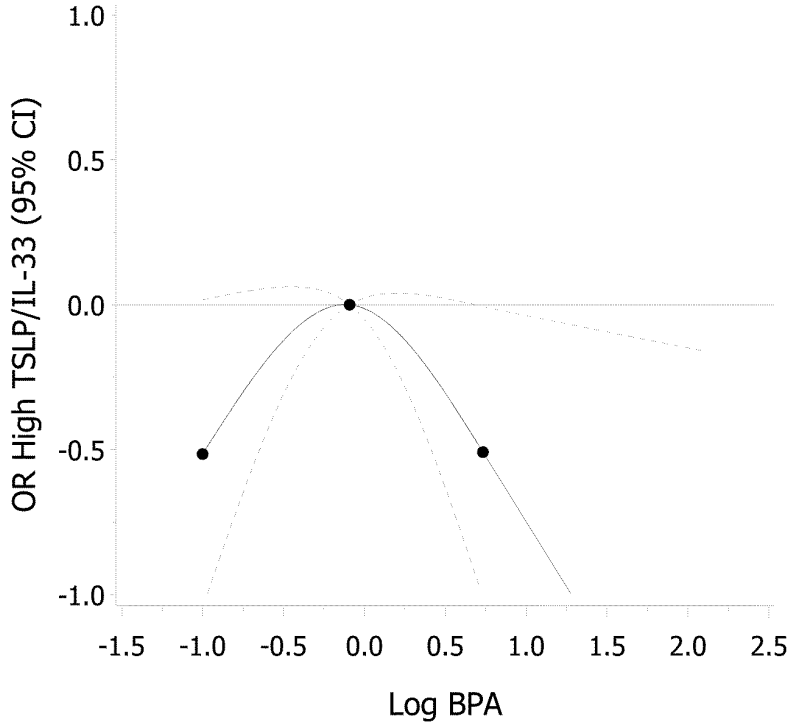
*adjusted for maternal age, specific gravity. Dotted lines represent 95% confidence interval, dots represent knots defined at the 5th, 50th, and 95th percentiles.

Figure 6.2 Restricted cubic spline curve of association between maternal urinary log_{10} MCPP urinary concentrations and high IgE cord blood levels (MIREC study, 2008-2011)



*adjusted for maternal age, specific gravity. Dotted lines represent 95% confidence interval, dots represent knots defined at the 5th, 50th, and 95th percentiles.

Figure 6.3 Restricted cubic spline curve of association between maternal urinary log_{10} BPA concentrations and high IL-33/TSLP cord blood concentrations (MIREC study, 2008-2011)



*adjusted for maternal age, specific gravity. Dotted lines represent 95% confidence interval, dots represent knots defined at the 5th, 50th, and 95th percentiles.

Table 6.1 Geometric mean of environmental contaminants (µg/L) by categories of TSLP, IL-33, and IgE (MIREC study, 2008-2011)

			IL33-TSLP (pg/mL)		IgE (ku/L)	
Chemical (µg/L)	**LOD[a]**	**%> LOD**	**≥ 80%**	**< 80%**	**≥ 0.5 ku/L**	**<0.5ku/L**
			Geometric Mean (SD)	Geometric Mean (SD)	Geometric Mean (SD)	Geometric Mean (SD)
Phthalate Metabolites & BPA (n=1137)[b]						
Mono-ethyl phthalate (MEP)	0.5	99.8	33.9 (3.9)	39.0 (4.1)	32.4 (3.8)	39.3 (4.1)
Mono-n-butyl phthalate (MBP)	0.2	99.7	12.6 (2.5)	13.3 (2.3)	12.9 (2.4)	13.2 (2.3)
Mono-benzyl phthalate (MBzP)	0.2	99.5	5.5 (2.8)	6.0 (2.7)	6.4 (3.0)	5.9 (2.6)
Mono- 3-carboxypropyl (MCPP)	0.2	83.9	0.9 (2.6)	1.0 (3.0)	0.9 (2.7)	1.0 (3.0)
Mono-2-ethylhexyl phthalate (MEHP)	0.2	98.3	2.5 (2.6)	2.7 (2.5)	2.6 (2.7)	2.6 (2.5)
Mono-(2-ethyl-5-hydroxyhexyl) (MEHHP)	0.4	99.3	9.4 (2.6)	10.7 (2.5)	10.4 (2.7)	10.6 (2.4)
Mono-(2-ethyl-5-oxohexyl) (MEOHP)	0.2	99.7	6.8 (2.5)	7.5 (2.3)	7.4 (2.5)	7.4 (2.3)
Bisphenol A (BPA)	0.2	86.6	0.9 (2.6)	0.9 (2.8)	0.9 (2.6)	0.9 (2.8)
Mono cyclohexyl phthalate (MCHP)	0.2	7.4				
Mono-n-octyl phthalate (MOP)	0.6	3.0				
Mono-isononyl phthalate (MNP)	0.4	1.1				
Mono-methyl phthalate (MMP)	5.0	14.0				
Perfluorinated Compounds (n=1242)						
Perfluorooctanoic acid (PFOA)	0.3	99.8	1.7 (1.9)	1.7 (1.8)	1.7 (1.9)	1.6(1.8)
Perfluorooctane sulfonate (PFOS)	0.1	99.8	4.7 (1.9)	4.5 (1.8)	4.6 (1.9)	4.6 (1.8)
Perfluorohexane sulfanoate (PFHxS)	0.3	96.0	1.0 (2.2)	1.0 (2.3)	1.0 (2.4)	1.0 (2.3)

[a]LOD=limit of detection

[b]Geometric means and standard deviations are adjusted for specific gravity

Table 6.2 Maternal demographic characteristics, MIREC Study, Canada, 2008-2011 (n=1258) [a]

Characteristic	N	(%)
Maternal Demographic		
Maternal Age (yrs.)		
<=24	60	(4.8)
25-29	270	(21.5)
30-34	452	(35.9)
≥35	476	(37.8)
Household Income ($CAD)		
≤30,000	90	(7.4)
30,001-50,000	117	(9.7)
50,001-100,000	515	(42.6)
>100,000	487	(40.3)
Parental Smoking[b]		
No	1008	(80.1)
Yes	249	(19.9)
Pet Ownership		
No	560	(44.5)
Yes	698	(55.5)
Maternal Reproductive & Medical History		
Pre-Pregnancy BMI[c]		
Underweight (<18.5)	27	(2.3)
Normal (18.5 to 24.9)	718	(60.4)
Overweight (25 to 29.9)	272	(22.9)
Obese (≥ 30)	171	(14.4)
Maternal Allergy[d]		
No	1204	(95.7)
Yes	54	(4.3)
Parity		
Nulliparous	526	(41.9)
Primiparous	512	(40.8)
Multiparous	217	(17.3)
Infant Characteristics		
Infant Sex		
Male	672	(53.5)
Female	585	(46.5)
Birth Weight (g)		
≤2500	138	(11.0)
2500-3000	479	(38.1)
3500-4000	449	(35.7)
≥ 4000	192	(15.3)

[a] Subgroup total may not equal 1258 due to missing data[b] Defined as either the mother or father smoking during pregnancy [c] World Health Organization Classification (WHO, 2006)
[d] Defined as use of maternal allergy medication

Table 6.3 Bayesian odds ratios of elevated (≥80%) cord blood IL-33/TSLP (pg/mL) and maternal concentrations of log_{10} environmental contaminants (µg/L) (MIREC study, 2008-2011)

Contaminant	Unadjusted OR (95% CI)	Adjusted OR [a] (95% CI)
Phthalates & BPA (n=1137)*		
MEP	0.9 (0.7-1.1)	0.9 (0.7-1.1)
MBP	1.0 (0.6-1.4)	1.0 (0.7-1.4)
MBzP	1.0 (0.7-1.3)	0.9 (0.7-1.3)
MCPP	0.9 (0.7-1.2)	0.9 (0.7-1.2)
MCPP*MCPP	0.8 (0.5-1.0)	0.8 (0.5-1.1)
∑ DEHP	0.9 (0.6-1.2)	0.9 (0.7-1.3)
BPA	1.0 (0.7-1.3)	1.0 (0.7-1.3)
BPA*BPA	0.8 (0.5-1.0)	0.8 (0.5-1.0)
*Perfluorinated compounds** (n=1242)*		
PFOA	1.1 (0.6-1.9)	1.1 (0.6-1.8)
PFOS	1.1 (0.6-1.9)	1.1 (0.6-1.9)
PFHxS	1.0 (0.7-1.4)	1.0 (0.7-1.4)

[a]adjusted for maternal age, specific gravity
[b] adjusted for maternal age, sex,

Table 6.4. Bayesian odds ratio of elevated (≥0.5 ku/L) cord blood IgE (ku/L) and maternal concentrations of log_{10} environmental contaminants (µg/L) (MIREC study, 2008-2011)

Contaminant	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Phthalates & BPA[a] (n=1137)		
MEP	0.8 (0.6-1.0)	0.8 (0.6-1.0)
MBP	1.0 (0.6-1.4)	0.9 (0.6-1.3)
MBzP	1.3 (0.9-1.8)	1.2 (0.9-1.7)
MCPP	0.8 (0.6-1.1)	0.8 (0.6-1.1)
MCPP*MCPP	0.7 (0.5-1.0)	0.7 (0.5-1.0)
∑ DEHP	1.0 (0.7-1.4)	1.0 (0.7-1.5)
BPA	1.0 (0.8-1.4)	1.0 (0.7-1.3)
Perfluorinated compounds[b] (n=1242)		
PFOA	1.1 (0.6-2.0)	1.1 (0.6-1.9)
PFOS	1.1 (0.6-2.0)	1.1 (0.6-1.9)
PFHxS	1.0 (0.7-1.4)	1.0 (0.7-1.4)

[a]adjusted for maternal age, specific gravity
[b]adjusted for maternal age, sex

Table 6.5 Birth cohort studies examining the association between prenatal exposure to phthalates, BPA or perfluorinated compounds and measures of childhood allergy

Study	Target Population	Maternal Exposure Concentrations (Median)	Outcome Measures	Major Finding
MIREC	Primarily Caucasian, urban women residing in Canada	BPA = 0.8 ug/L MBzP =5.0 ug/L MCPP = 0.9 ug/L PFOA=1.7 ug/L, PFOS=4.6 ug/L, PFHxS=1.0 ug/L	Cord blood IgE, TSLP, IL-33	
Whyatt 2014	Dominican African American women residing in New York city	MBzP =13.6 ug/L MnBP = 37.5 ug/L	Diagnosed asthma	MBzP asthma RR =1.17 95% CI:1.01-1.35 MnBP asthma RR=1.25 95% CI:1.04-1.51
Just et al., 2012 Donohue et al., 2013	Dominican African American women residing in New York city	MBzP =13.6 ug/L BPA =1.8 ng/mL	Atopic eczema ages 2,5 Wheeze in childhood	MBzP and eczema age 2 RR= 95% CI:1.2-1.9 BPA and wheeze age 5 OR= 0.7 95% CI:0.5-0.9
Spanier et al., 2012	Mid-West US	BPA =2.2 ug/g creatinine	Wheeze	BPA and wheeze birth to age 3 OR= 1.2 95% CI:1.0-1.5
Okada et al., 2012	Japanese	PFOS= 5.2 ng/mL PFOA= 1.3 ng/mL	Cord Blood IgE	PFOA and IgE Males β (95% CI) = 1.3 (-2.2,4.7) Females β (95% CI)=-3.1(-5.4- -0.7)
Wang et al., 2011	Taiwanese	PFOA = 1.7 ng/mL, PFOS = 5.5 ng/mL PFHxS = 0.055 ng/mL	Cord Blood IgE	PFOA and IgE Males β (p-value)=0.2(0.02) Females β (p-value)=0.06(0.9)

SUPPLEMENTARY TABLES

Table 6.1.1 Bayesian odds ratios of elevated IL-33/TSLP and maternal urinary concentrations of log_{10} phthalate metabolites and Bisphenol A by sex (MIREC study, 2008-2011)

	Males (n=604)		Females (n=532)	
Contaminant	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Unadjusted OR(95% CI)	Adjusted OR (95% CI)
MEP	1.0 (0.8-1.4)	1.0 (0.7-1.4)	0.8 (0.6-1.1)	0.8 (0.6-1.1)
MBP	1.1 (0.7-1.6)	1.1 (0.7-1.6)	0.9 (0.5-1.4)	0.9 (0.5-1.4)
MBzP	1.1 (0.7-1.6)	1.1 (0.7-1.6)	0.8 (0.5-1.2)	0.8 (0.5-1.2)
MCPP	0.8 (0.5-1.1)	0.8 (0.5-1.1)	1.1 (0.7-1.6)	1.1 (0.7-1.7)
MCPP*MCPP	0.8 (0.5-1.1)	0.8 (0.5-1.1)	0.9 (0.5-1.3)	0.9 (0.5-1.3)
∑ DEHP	0.9 (0.6-1.3)	0.9 (0.6-1.3)	1.0 (0.6-1.4)	0.9 (0.6-1.4)
BPA	1.0 (0.6-1.4)	1.0 (0.6-1.4)	1.0 (0.7-1.4)	1.0 (0.7-1.4)
BPA*BPA	0.7 (0.4-1.1)	0.7 (0.4-1.1)	0.9 (0.5-1.3)	0.9 (0.6-1.3)

*adjusted for maternal age, specific gravity,

Table 6.1.2 Bayesian odds ratio of elevated IgE and maternal urinary concentrations of log_{10} phthalate metabolites and Bisphenol A by sex (MIREC study, 2008-2011)

	Males (n=604)		Females (n=532)	
Contaminant	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
MEP	0.8 (0.6-1.1)	0.8 (0.6-1.1)	0.9 (0.6-1.2)	0.9 (0.6-1.2)
MBP	1.2 (0.7-1.7)	1.1 (0.7-1.7)	0.8 (0.4-1.2)	0.8 (0.4-1.2)
MBzP	1.2 (0.8-1.8)	1.2 (0.8-1.7)	1.2 (0.8-1.9)	1.2 (0.8-2.0)
MCPP	0.8 (0.5-1.1)	0.8 (0.5-1.2)	1.0 (0.6-1.4)	0.9 (0.6-1.4)
MCPP*MCPP	0.7 (0.5-1.0)	0.7 (0.5-1.0)	0.8 (0.5-1.2)	0.8 (0.5-1.2)
∑ DEHP	1.0 (0.6-1.5)	1.0 (0.7-1.5)	1.0 (0.6-1.6)	1.0 (0.7-1.6)
BPA	1.0 (0.7-1.5)	1.0 (0.7-1.5)	1.0 (0.6-1.4)	1.0 (0.6-1.4)

*adjusted for maternal age, specific gravity

Table 6.1.3 Bayesian odds ratio of elevated IL-33/TSLP and maternal plasma concentrations of log_{10} perfluorinated compounds by sex (MIREC study, 2008-2011)

	Males (n=664)		Females (n= 578)	
Contaminant	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
PFOA	1.1 (0.5-2.0)	1.1 (0.5-2.0)	1.1 (0.5-2.1)	1.1 (0.5-2.1)
PFOS	1.1 (0.5-2.1)	1.1 (0.4-2.0)	1.1 (0.5-2.1)	1.1 (0.7-2.1)
PFHxS	1.3 (0.8-2.1)	1.2 (0.8-2.0)	0.8 (0.4-1.2)	0.8 (0.2-1.2)

*adjusted for maternal age, sex

Table 6.1.4 Bayesian odds ratio of elevated IgE and maternal plasma concentrations of log_{10} perfluorinated compounds by sex (MIREC study, 2008-2011)

	Males (n=664)		Females (n=578)	
Contaminant	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
PFOA	1.2 (0.6-2.1)	1.2 (0.6-2.3)	1.0 (0.5-1.9)	1.0 (0.5-1.9)
PFOS	1.2 (0.6-2.3)	1.2 (0.7-2.3)	1.0 (0.5-1.9)	1.0 (0.4-1.9)
PFHxS	1.2 (0.7-1.8)	1.1 (0.7-1.7)	0.9 (0.5-1.4)	0.9 (0.5-1.4)

*adjusted for maternal age, sex

Appendix 1. Bayesian methods

Hierarchical Model

$$Yi|\beta j \sim N(\sum_{J=1}^{k} \beta x_{ij}, \sigma^2)$$

$\beta \sim N(0, \Phi^2)$
$\Phi^2 \sim N(0,0.01)$

The model was a logistic regression model of the form as described in Maclehose (2009):

Logit Pr(Yi = 1|xi1 + xi2 + xi3...) = βo + β1xi1 + β2xi2..)

The Bayesian hierarchical model was run using OpenBUGS version 322 and R v. 3.0.3. The R2OpenBUGS package was used in R to run OpenBUGS from R. The hierarchical model was run with the following OpenBUGS code.

```
model
{
for (i in 1:n) {
# Linear regression on logit
logit(p[i]) <- alpha + b[1]*x1[i]  + b[2]*x2[i] + b[3]*x3[i] + b[4]*x4[i] + b[5]*x5[i] + b[6]*x6[i]

# Likelihood function for each data point
y[i] ~ dbern(p[i])
}
for (j in 1:J) {
b[j] ~ dnorm (0,phi) }  # Prior for betas
alpha ~ dnorm(0.0,0.01) # Prior for intercept
phi ~ dnorm(0,0.01)I(0,)

#Calculate odds ratios for three betas
ORx1<-exp(b[1])
ORx2<-exp(b[2])
ORx3<-exp(b[3])
ORx4<-exp(b[4])
ORx5<-exp(b[5])
ORx6<-exp(b[6])}
```

Chapter Seven. Maternal Exposure to Metals and Persistent Pollutants and Cord Blood Levels of IgE, TSLP and IL-33: The MIREC Study

This manuscript not yet been submitted to Health Canada or a journal for review.

J. Ashley-Martin performed the ELISA analysis of umbilical cord blood samples, conducted the statistical analysis, and wrote the manuscript. L. Dodds provided supervisory input regarding the cohort, statistical analysis and critical feedback on the manuscript. A. Levy assisted in structuring and writing the manuscript. R. Platt provided guidance on the maximum likelihood modeling and reviewed the statistical methods. J. Marshall provided lab space, materials, and technical support to facilitate the completion of the ELISA analysis and critically reviewed early versions of the manuscript. T. Arbuckle, co-principal investigator of the MIREC study, provided guidance on the study cohort as well as reviewed and edited the manuscript. All authors were involved with the study design and methodology.

Authors: Jillian Ashley-Martin[1], Adrian R. Levy[2], Tye E. Arbuckle[3], Robert W. Platt[4], Jean S. Marshall[5], Linda Dodds*[6]

Author Affiliations:

[1]Interdisciplinary PhD Program, Dalhousie University

IDPhD c/o Faculty of Graduate Studies Room 314 Henry Hicks Building 6299 South St Halifax, NS B3H 4H6

[2]Department of Community Health & Epidemiology, Dalhousie University

Department of Community Health and Epidemiology Centre for Clinical Research 5790 University Avenue Halifax, NS B3H 1V7 (Adrian.levy@dal.ca)

[3]Healthy Environments and Consumer Safety Branch, Health Canada

Population Studies Division, Healthy Environments and Consumer Safety Branch, Health Canada, 50 Colombine Dr., AL 0801A, Ottawa ON K1A 0K9 Canada (Tye.Arbuckle@hc-sc.gc.ca)

[4]Department of Epidemiology and Biostatistics, McGill University

Department of Epidemiology and Biostatistics, McGill University Purvis Hall 1020 Pine Ave. West Montreal, QC H3A 1A2 (robert.platt@mcgill.ca)

[5]Department of Microbiology & Immunology, Dalhousie University

Department of Microbiology & Immunology Dalhousie University Sir Charles Tupper Medical Building, Room 7-C 5850 College Street Halifax, Nova Scotia Canada (jean.marshall@dal.ca)

[6]Department of Obstetrics & Gynaecology and Paediatrics, Dalhousie UniversityPerinatal Epidemiology Research Unit, 7th Floor Women's Site, IWK Health Centre, 5980 University Ave. PO Box 9700, Halifax, NS, Canada B3H 6R8 (l.dodds@dal.ca)

Abstract

The fetal time period is a critical window of immune system development and resulting heightened susceptibility to the adverse effects of environmental exposures. Epidemiologists and toxicologists have hypothesized that persistent organic pollutants, pesticides and metals have immunotoxic properties. Immunotoxic effects may manifest as an altered immune system profile at birth. Immunoglobulin E, thymic stromal lymphopoietin (TSLP), and interleukin-33 (IL-33) may be implicated in the etiology of childhood allergy and detectable at birth. The objective of this study was to examine the potential relationship between maternal levels of metals, persistent organic pollutants, and pesticides and elevated umbilical cord blood levels of IgE, TSLP, and IL-33 in a Canadian birth cohort. This study utilized data collected in the Maternal-Infant Research on Environmental Chemicals (MIREC) Study, a trans-Canada cohort study of 2,001 pregnant women. Of these women, 1258 had a singleton, term birth and cord blood sample. Logistic regression was used to determine associations between log-transformed continuous variables and immune system biomarkers. A trend towards an inverse association between lead and elevated levels of IL-33/TSLP was observed. No significant associations were observed between any of the other environmental contaminants and the immune system biomarkers of interest. In this primarily urban Canadian population of pregnant women and their newborns, maternal blood or urine concentrations of persistent organic pollutants, pesticides, and metals were not associated with immunotoxic effects that manifest as increased odds of elevated levels of IgE, TSLP or IL-33.

Key words: persistent organic pollutants, metals, pesticides, immunoglobulin E, thymic stromal lymphopoietin, interleukin-33, pregnancy, cohort

Word count: 3200

Introduction

The fetal immune system is susceptible to the adverse effects of environmental contaminants due to the immaturity of detoxification enzymes and fetal organ systems (109). Immunotoxic effects of environmental contaminants occur at lower doses in fetuses than adults (230). *In utero* exposures to certain contaminants can trigger permanent, irreversible changes to the developing immune system. These changes may promote the persistence of skewed immune system responses and subsequent increased risk of an allergic phenotype (5,8,179).

Toxicologists and epidemiologists have hypothesized that metals (8,48,111), pesticides (150,231), and polychlorinated biphenyls (PCBs) (37,143) have immunotoxic properties. Anthropogenic and biogenic sources of metals result in detectable blood concentrations in the majority of North American individuals (183,232). Organophosphate pesticide exposure is common, even in urban populations, due to ingestion of pesticide residues on treated food (154) and presence in household dust (155). Due to regulatory changes enacted in the 1970s, use of persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs) (233) and certain organochlorine pesticides (149) has curtailed in many developed countries. Many adults, however, still have detectable concentrations of these chemicals due to their lipophilic and persistent nature (183).

The epidemiologic literature regarding associations between *in utero* exposure to the aforementioned contaminants and measures of childhood allergy is limited and inconsistent. Investigations that have explored the role of environmental contaminants in childhood allergic disease etiology are challenged by the transient nature of certain

diagnoses, such as wheeze, and resulting misclassification (234,235). Disentangling the confounding effects of childhood from *in utero* environmental exposures poses yet another challenge.

Determining the nature of the relationship between prenatal environmental contaminant exposures and fetal immune system biomarkers may facilitate insight into the susceptibility of the fetal immune system to potential immunotoxicants. The effects of *in utero* environmental contaminant exposure may manifest in altered levels of immune system biomarkers at birth. An altered immune system profile at birth is a risk factor for childhood allergy (20–22,23). Specifically, elevated cord blood levels of certain inflammatory T helper 2 (Th2) cytokines have been found to be predictors of infantile atopic dermatitis (236). The immune system biomarkers, immunoglobulin E (IgE), interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP), have been detected at birth (193) and are integral in the mechanisms underlying allergic responses (16,69,194). Cord blood IgE levels have been previously used as a means of assessing the immunotoxic effects of *in utero* environmental contaminant exposure (55,140,141,195). TSLP and IL-33 have recently been recognized for their etiologic role in atopic dermatitis, the earliest manifestation of childhood allergy (70,87). Moreover, a recent clinical trial demonstrated that children treated with anti-TSLP antibodies had less bronchoconstriction and airway inflammation following an allergen challenge test than children who did not receive the treatment (68).

In response to the lack of epidemiologic evidence regarding 1) intrauterine influences on childhood asthma risk (54) and 2) determinants of cord blood levels of TSLP and IL-33, we sought to examine fetal immune system susceptibility to *in utero*

environmental contaminant exposure. Specifically, the objective of the present study was to examine the association between maternal levels of pesticides, PCBs, and metals and elevated cord blood levels of IgE, TSLP, and IL-33 using data from a Canadian birth cohort. As childhood allergy prevalence differs by sex (57,28,30), a secondary objective was to assess whether these associations differed by infant sex.

Materials and Methods

Study Population

Data and biospecimens were obtained from the Maternal-Infant Research on Environmental Chemicals (MIREC) Study, a trans-Canada cohort study of 2,001 pregnant women from ten Canadian cities recruited between 2008 and 2011 (169). The target population and exclusion criteria for the MIREC study and present analysis have been described previously (169,193). Cord blood samples that were determined to be contaminated with maternal blood based on an elevated immunoglobulin A (IgA) level (≥ 10 μg/mL) were excluded from the analysis (106). As pre-term and multiple birth infants had lower cord blood samples of the immune system biomarkers and may have a differing allergic disease risk profile (237,238), these samples were excluded. This study received ethical approval from Health Canada, St. Justine's Hospital (Montreal, QC), and the IWK Health Centre (Halifax, NS) and all participants provided consent.

Environmental Contaminant Exposure

In the present investigation, four organochlorine pesticides, four PCB congeners, five organophosphate pesticides metabolites, and four metals were analyzed. These organochlorine pesticides and PCB congeners were measured in first trimester plasma

(Table 7.1). The non-dioxin like PCB congeners 138, 153, and 180 were summed to create an index of PCB exposure. Plasma samples were enriched with internal standards and halogenated organic compounds were retrieved by liquid-liquid extraction with a mixture of ammonium sulfate: ethanol:hexane (1: 1: 3). The extracts were concentrated, automatically purified on florisil column, and then analyzed by gas chromatography coupled to a mass spectrometer (Agilent 6890N/5973 GC-MS). Measurements of ions generated after negative chemical ionization were performed in selective ion mode (SIM).

The organophosphate pesticide metabolites were measured in first trimester urine (Table 7.1). Index measures of the dimethyl alkylphosphate (DMAP) and diethyl alkylphosphate (DEAP) metabolites were calculated by summing the respective metabolites on a molar basis (239). As only 2% of samples had detectable concentrations of the organophosphate metabolite DEDTP, this metabolite was not analysed. Chemical analysis of organophosphate metabolites in urine samples was carried out at the Laboratoire de Toxicologie, Institut National de Santé Publique du Québec (Québec, QC, Canada), accredited by the Standards Council of Canada. Organophosphate metabolites in urine were measured with a GC-MS-MS instrument with a GC Agilent 6890 N (Agilent Technologies; Mississauga, ON, Canada) coupled with a tandem mass spectrometer Quattro Micro GC (Waters; Milford, Massachusetts, USA).

Metals (lead, mercury, arsenic, and cadmium) were measured in whole blood collected from the first and third trimesters. These two measurements were averaged to create an index of exposure throughout pregnancy. Metal concentrations were analyzed

using inductively coupled plasma mass spectrometry (PerkinElmer ELAN ICP-MS DRC II).

Fetal Markers of Immune System

Immune system biomarkers were measured in the plasma of umbilical cord blood samples in the Department of Microbiology & Immunology, Dalhousie University, Halifax, NS using ELISA. TSLP concentrations were determined using a commercial antibody kit (Biolegend, San Diego, CA, USA). IL-33 concentrations were analyzed using antibodies from an R & D systems duoset (Minneapolis, MN, USA). ELISA kits (EBioscience, San Diego, CA, USA) were also used to assess both total IgE and IgA concentrations. ELISA assays were performed according to manufacturer's instructions with the exception that plates were coated with sodium bicarbonate buffer (pH 8.3-8.5) and blocked with 2% BSA in PBS instead of using the manufacture's coating and blocking buffers. The inter-assay and intra-assay CVs for IL-33 were 5.9% and 11.3%, respectively; for TSLP inter and intra-assay CVs were 6.0% and 8.1%, respectively. The inter-assay and intra-assay CVs were 3.2% and 6.5%, respectively, for IgE and 5.2% and 10.4%, respectively, for IgA.

Statistical Analysis

For the descriptive statistics, urinary concentrations of the organophosphate metabolites were adjusted for specific gravity to account for heterogeneity in urinary dilution according to the following formula $P_c = P_i\,[(SG_m - 1)/(SG_i - 1)]$, where: P_c = SG adjusted metabolite concentration (µg/ml), P_i = observed metabolite concentration,, SG_i = specific gravity of the urine sample, and SG_m = median SG for the cohort (200).

Due to the high percentage of cord blood samples below the limit of detection (LOD), each immune system biomarker was categorized into a binary variable. A composite variable was developed to identify samples with elevated concentrations of both TSLP and IL-33 (IL-33/TSLP) as these cytokines are highly correlated (Spearman correlation coefficient=0.8) and known to interact in experimental models (77). Elevated levels of TSLP and IL-33 were defined as samples that exceeded the 80th percentile for both cytokines (TSLP=554 pg/mL; IL-33=879 pg/mL) because there are no pre-existing thresholds. Elevated IgE samples were defined as those that exceeded 1.2 ng/mL (0.5 kU/L) (171,172), a cut-off point previously used in studies of cord blood IgE (90,104).

Logistic regression was employed to estimate the association between log-transformed continuous variables and elevated levels of IgE and IL-33/TSLP with the exception of hexachlorobenzene. Due to the high percentage of samples below the LOD for this chemical, a binary variable categorized at the LOD was created. For all other chemicals, maximum likelihood estimation was used, assuming a log-normal distribution for sample concentrations. This method of accounting for samples with values below the LOD is less prone to bias than substitution methods that use LOD/2 or LOD/$\sqrt{2}$ (240). For environmental contaminants with 2 LODs (e.g. mercury, DETP), all values less than the higher LOD were treated as below the limit of detection. For the summary measures of PCBs congeners and organophosphate metabolites, a value was treated as less than the LOD if each of the individual metabolite values were undetectable.

Potential covariates were identified using hypothesized causal models (205). The causal models were constructed using evidence regarding predictors of the environmental contaminant exposures (organochlorine pesticides (241), organophosphorous pesticides

(242), PCBs (243), metals (244–246)) and predictors of the immune system biomarkers (193,207). The minimal adjustment sets for confounding were identified using DAGitty (208). Maternal blood lipid levels were included in the organochlorine and PCB models to account for the lipophilic nature of these chemicals and intra-individual heterogeneity in lipids (247). Maternal urinary specific gravity was included into the organophophate pesticide metabolite models to account for heterogeneity in urinary dilution (197). The linearity of the relationship between each environmental contaminant and immune system biomarker was assessed using restricted cubic splines (204). Exposures that did not meet the criteria for linearity ($P < 0.05$) were modeled with a quadratic term. To assess whether relationships differed by infant sex, the P-value of the sex*exposure interaction term was assessed and sex-stratified analyses were conducted. If the upper or lower confidence limit was equal to one, an additional analyses using tertiles of exposure was conducted. This was done for ease of interpretation and visualization of results.

Results

Of the 2,001 women recruited, 18 withdrew and asked that all their data and biospecimens be destroyed. Of the remaining 1,983 subjects, 1,363 women had a cord blood sample. Of these 1,363 samples, 107 were excluded for missing chemical exposure data, a high IgA concentration, pre-term birth (< 37 weeks), multiple birth, or samples with insufficient sample for analysis.

Table 7.1 shows descriptive statistics of the environmental contaminants. The percentage of samples above the limit of detection ranged from 29.7% for hexachlorobenzene to 100% for lead. Maternal demographic, reproductive, and infant characteristics are depicted in Table 7.2. The majority of study participants were 30

years or older, had a household income greater than $50,000, were non-smokers, and of normal BMI.

As no chemical met the criteria for non-linearity, all exposures were entered into the model as continuous variables without a quadratic term. Results of the IL-33/TSLP and IgE models are presented in Tables 7.3 and 7.4 respectively. All models were adjusted for age and, depending on the specific chemical, specific gravity or lipids. A significant inverse association was observed between prenatal blood lead levels and elevated concentrations of IL-33/TSLP in the unadjusted model (OR=0.7, 95% CI: 0.5-0.9) but not the adjusted model (OR=0.7, 95% CI: 0.5-1.0). No significant associations were observed between any of the other environmental contaminants and elevated cord blood levels of either IL-33/TSLP or IgE in the adjusted models. There was no observed effect modification by sex or material differences in the sex-stratified analysis (data not shown).

Tables 7.5 and 7.6 depict the results of the tertile analysis for those chemicals with borderline significant associations. Results are consistent with the analysis of continuous exposures. Though a suggestion of an inverse finding was observed, none of the effect measures were statistically significant.

Discussion

In this study, we sought to characterize the association between prenatal exposure to PCBs, pesticides and metals and elevated levels of IgE, IL-33 and TSLP. We observed that there was no significant association between any of the environmental contaminants and the immune system biomarkers of interest in the multivariate models. We also observed that these associations did not differ by sex. These findings suggest that

contemporary maternal levels of these environmental contaminants do not induce developmental immunotoxic effects as detected by elevated levels of the immune system biomarkers of interest. Due to the lack of literature specific to the immune system biomarkers, comparison with previous results is not possible. As detailed below, the present findings are globally consistent with studies that have demonstrated an absence of an effect between prenatal environmental contaminant exposures and measures of childhood allergy. Divergence across this broad body of literature is likely explained by heterogeneity in timing and type of exposure and outcome measurements.

Studies of Prenatal Metal Exposure and Childhood Allergy

Experimental evidence has demonstrated that gestational lead exposure may elevate IgE levels (111,248) and enhance Th2 cell responses (249). A significantly elevated risk of childhood atopy was observed among children with elevated maternal and cord blood lead levels in a Polish birth cohort (250). In addition, significant correlations between childhood lead levels and both elevated IgE (114,115,251) and atopic dermatitis (252) have been identified in cross-sectional analyses. In light of these previous findings, the observed lack of association between lead and IgE and trend towards an inverse association between lead and IL-33/TSLP in the present study was unexpected. The present findings may be explained by the relatively low level of maternal lead exposure among MIREC study participants. Average lead levels in the MIREC study (geometric mean = 0.88 µg/dL) were lower than observed in the Polish birth cohort (geometric mean = 1.60 µg/dL).

Epidemiologic evidence on the other metals of interest is limited. The relatively few studies of the association between childhood allergy and mercury (31 ,68 ,73),

arsenic (119,254), and cadmium (255) are not suggestive of an association between metal exposure and immune system changes consistent with allergic disease.

Studies of Prenatal PCB Exposure and Childhood Allergy

There is experimental evidence that PCBs may enhance production of Th2 cell cytokines involved in allergic responses (143). Similar associations have not, however, been consistently observed in epidemiologic studies. For example, consistent with the present findings, a meta-analysis of ten European cohort studies concluded that prenatal PCB-153 exposure was not associated with infant wheeze or bronchitis (153). Conversely, authors of a recent Dutch cohort study reported a positive association between PCB-153 and allergy after twenty years of follow-up. A limitation of this study is that allergy diagnosis was based on self-reported medication use between the ages of 6 and 20. Thus, there is no ability to pinpoint the timing of outcome or examine early life outcomes (144). Maternal concentrations of PCB-153 were notably higher in the Dutch cohort study (median=1.37 μg/L) (144) than in the MIREC study (median=0.04 μg/L). The difference in exposure levels and resulting effect estimates is likely explained by the differences in timing of recruitment. Study participants in the Dutch cohort study were recruited during the early 1980s, prior to the complete cessation of PCB manufacture in Europe (256).

Studies of Prenatal Organochlorine Pesticide Exposure and Childhood Allergy

Experimental evidence has demonstrated that organochlorine pesticide exposure, specifically DDE, may also induce allergic type responses via enhanced production of Th2 cytokines (150). Evidence from epidemiologic studies is not strongly suggestive of a

positive association. Though positive associations between prenatal DDE exposure and measures of childhood allergic disease have been reported in two birth cohort studies (61 ,62), a meta-analysis of 10 cohort studies reported that this association was limited to children less than 18 months old (153). In addition, authors of the Dutch cohort study reported no significant association between prenatal DDE exposure (median = 2.47 ng/mL) and asthma medication use (144). The present finding of a trend towards an inverse relationship between DDE and elevated IL-33/TSLP levels may be explained by the low exposure levels (median DDE = 0.3 μg/L) in MIREC study participants.

Studies of Prenatal Organophosphate Pesticide Exposure and Childhood Allergy

While no study has examined the association between organophosphate metabolites and childhood allergy, authors of the US Agricultural health study report that these pesticides are associated with an increased risk of adult-onset asthma and wheeze (Hoppin et al., 2009; Hoppin et al., 2006). Moreover, exposure to common organophosphate pesticides has been shown to exacerbate atopic dermatitis and increase IgE levels in experimental models (158).

Strengths & Limitations

This study benefited from the relatively large sample size and the comparatively rich covariate data. Considering the relatively long-half for the persistent organic pollutants, there is unlikely to be considerable within-person variability in exposure throughout pregnancy. We employed an analytical method to model samples with concentrations below the LOD. This approach is prone to less bias than substituting a constant (e.g. LOD/2) for undetectable samples (240).

It is worth exploring whether the lack of significant associations in the present study may be due to an actual absence of effect or are a result of the following study limitations. First, the MIREC study population was of higher income and more educated than the target population of primarily urban Canadian women. This potential selection bias is unlikely to have biased the observed associations because the associations did not seem to be confounded by income or education. Second, we did not have information on certain risk factors for childhood allergy such as maternal vitamin D (220) and stress (221). Considering that a relationship between these variables and exposure to the contaminants of interest is not well established, it is unlikely that our observed associations are invalidated by lack of control for these variables. The trend towards an inverse relationship between some of the environmental contaminants (e.g. lead, DDE) and cord blood immune system biomarkers raises questions regarding the potential role of confounding due to other unmeasured protective factors for allergic disease. Third, though the persistent organic pollutants and metals have long half-lives and, therefore, little expected variation throughout pregnancy, the organophosphorous metabolites have relatively short half-lives (156) and may vary during pregnancy (257). Furthermore, first trimester urinary measures may not be representative of exposure levels during critical windows of immune system development. It is likely that any resulting bias is non-differential and may attenuate effect estimates towards from the null (219).

The observed absence of effect is not necessarily reflective of an absence of developmental immune system toxicity. It is possible that the immunotoxicity of the environmental chemicals of interest do not manifest as elevated levels of cord blood levels of IgE, TSLP or IL-33. Future studies that assess serial measurements of a wider

panel of immune system biomarkers and include clinical allergic diagnosis data would have the capacity to assess whether immunotoxic effects appear in different outcomes and at different time points.

PUBLIC HEALTH CONTEXT

Manufacture and use of DDT, PCBs, and hexachlorobenzene was curtailed three decades ago (149,233,258). Thus, the residual exposure levels observed in the MIREC population largely represent historical use. Given the lack of Canadian biomonitoring guidelines for these contaminants, interpretation of the health implications of the observed values is challenging. The lower levels of maternal exposure in the MIREC study compared to more historical cohorts (116,144) suggests that exposure levels have been declining since the cessation of production.

In contrast to these POPs, contemporary sources of exposure to metals exist. All MIREC study participants had detectable concentrations of lead but these levels were notably lower than the Canadian blood lead intervention level of 10 ug/dL (259). Moreover, the median value in this study (0.62 μg/dL) is lower than 1 to 2 ug/dL, values which have recently been reported to be associated with adverse health effects (259). Similarly, the median level of blood mercury concentration in the MIREC study population (0.70 ug/L) is also well below 20 ug/L, the current Canadian guideline for blood mercury concentrations in adults (260). Though there are no Canadian biomonitoring guidelines for blood levels of cadmium, median values in the MIREC population are lower than reported in the Canadian Health Measures Survey (232) and comparable to that reported in the US NHANES (183). Median blood arsenic levels in

the MIREC study are slightly higher than those measured in the Canadian Health Measures Survey (12).

Conclusions

In this primarily urban Canadian population of pregnant women and their newborns, maternal concentrations of persistent organic pollutants, pesticides, and metals were not associated with potential immunotoxic effects manifested as increased odds of elevated IgE, TSLP or IL-33. Future studies that examine the trajectory of these biomarkers from birth to childhood will complement the present findings. It is possible that any immunotoxic effect of prenatal exposure to these chemicals manifests in a different manner or is delayed until later in life. Determination of whether the observed absence of effect at birth persists into childhood requires follow-up in either a nested case-control study or a cohort of children with clinical assessment of allergic disease.

Table 7.1 Descriptive statistics of environmental contaminants (MIREC study, 2008-2011)

Chemical (µg/L)	N	LOD	% >LOD	Min	Median	Max	IQR
Pesticides							
Organochlorine Pesticides (plasma)							
Trans-nonachlor	1232	0.01	84.58	LOD	0.02	0.23	0.01
DDE	1233	0.09	99.19	LOD	0.30	26.00	0.28
Hexachlorobenzene	1232	0.04	29.71	LOD	0.02	0.61	0.02
Oxychlordane	1231	0.005	92.04	LOD	0.01	0.13	0.01
Organophosphate Pesticide Metabolites (urine)[a]							
Dimethylphosphate (DMP)	1229	1.00	79.55	LOD	3.25	43.33	4.44
Dimethylthiphosphate (DMTP)	1227	0.60	80.49	LOD	3.44	141.82	7.22
Diethyldithiophosphate (DEDTP)	1230	0.30	2.27	LOD	0.15	4.02	0.18
Diethylthiophosphate (DETP)	1229	0.6	52.92	LOD	0.65	31.89	0.85
Diethylphosphate (DEP)	1230	1.00	76.64	LOD	2.38	2104.76	2.90
Polychlorinated biphenyls (plasma)							
PCB 118	1233	0.01	74.45	LOD	0.01	0.22	0.02
PCB 138	1233	0.01	93.19	LOD	0.03	0.28	0.02
PCB 153	1233	0.01	99.19	LOD	0.04	0.53	0.04
PCB 180	1233	0.01	92.70	LOD	0.03	0.77	0.03
Metals (whole blood)							
Lead (µg/dL)	1241	0.10	100.00	0.17	0.62	4.14	0.41
Cadmium	1241	0.04	97.42	LOD	0.20	5.06	0.17
Arsenic	1241	0.22	93.63	LOD	0.82	34.46	0.66
Mercury	1241	0.12	90.57	LOD	0.70	10.03	1.02

[a]adjusted for specific gravity (200)

Table 7.2 Maternal demographic characteristics, MIREC Study, Canada, 2008-2011 (n=1256)[a]

Characteristic	N	%
Maternal Demographic		
Maternal Age (yrs.)		
<=24	60	4.8
25-29	270	21.5
30-34	450	35.8
≥35	476	37.9
Household Income ($CAD)		
≤30,000	90	7.5
30,001-50,000	116	9.6
50,001-100,000	514	42.6
>100,000	487	40.4
Parental Smoking [b]		
No	1006	80.1
Yes	250	19.9
Pet Ownership		
No	559	44.5
Yes	697	55.5
Maternal Reproductive & Medical History		
Pre-Pregnancy BMI [c]		
Underweight (<18.5)	27	2.3
Normal (18.5 to 24.9)	717	60.5
Overweight (25 to 29.9)	272	22.9
Obese (≥ 30)	170	14.3
Maternal Allergy [d]		
No	1202	95.7
Yes	54	4.3
Parity		
Nulliparous	526	41.9
Primiparous	512	40.8
Multiparous	216	17.2
	2	
Infant Characteristics		
Infant Sex		
Male	671	53.5
Female	584	46.5
Birth Weight (g)		
≤2500	138	11.0
2500-3000	478	38.1
3500-4000	448	35.7
≥ 4000	192	15.3

[a] Subtotals may not sum to total due to missing data, [b] defined as either the mother or father smoking during pregnancy, [c] World Health Organization Classification (WHO, 2006), [d] Defined as maternal use of allergy medication

Table 7.3 Odds ratio of log_{10} maternal contaminant concentrations and elevated (≥80%) cord blood levels of IL-33/TSLP

Chemical (µg/L)	N	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Pesticides			
Organochlorine Pesticides [a]			
Trans-nonachlor	1232	0.8 (0.6-1.1)	0.8 (0.5-1.1)
DDE	1233	0.8 (0.6-1.0)	0.8 (0.6-1.0)
Hexachlorobenzene (binary)	1232	0.8 (0.6-1.1)	0.8 (0.6-1.1)
Oxychlordane	1231	0.8 (0.6-1.1)	0.8 (0.5-1.1)
Organophosphate Pesticide Metabolites[b]			
Dimethylphosphate (DMP)	1229	0.9 (0.7-1.0)	0.9 (0.7-1.0)
Dimethylthiphosphate (DMTP)	1227	1.0 (0.9-1.1)	1.0 (0.8-1.1)
Diethylthiophosphate (DETP)	1229	0.9 (0.8-1.1)	1.0 (0.7-1.2)
Diethylphosphate (DEP)	1230	0.8 (0.7-1.0)	0.7 (0.7-1.0)
ΣDMP&DMTP	1226	0.9 (0.8-1.0)	0.9 (0.8-1.1)
ΣDEP&DETP	1229	0.8 (0.7-1.0)	0.9 (0.7-1.0)
Polychlorinated biphenyls[a]			
PCB 118	1233	0.8 (0.6-1.0)	0.7 (0.4-1.0)
PCB 138	1233	0.8 (0.7-1.0)	0.9 (0.6-1.1)
PCB 153	1233	0.9 (0.7-1.0)	0.9 (0.7-1.1)
PCB 180	1233	0.9 (0.7-1.0)	0.9 (0.7-1.0)
ΣPCB (non-dioxin – 138,153,180)	1233	0.9 (0.7-1.1)	0.9 (0.7-1.1)
Metals [a]			
Lead	1241	0.7 (0.5-0.9)	0.7 (0.5-1.0)
Cadmium	1241	0.9 (0.7-1.1)	0.9 (0.7-1.1)
Arsenic	1241	0.9 (0.7-1.1)	0.9 (0.7-1.2)
Mercury	1241	0.9 (0.8-1.1)	1.0 (0.8-1.1)

[a]adjusted for age and lipids, [b]adjusted for age and specific gravity

Table 7.4 Odds ratio of log_{10} maternal contaminant concentrations and elevated (≥0.5 ku/L) cord blood levels of IgE

Chemical (µg/L)	N	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Pesticides			
Organochlorine Pesticides [a]			
Trans-nonachlor	1232	0.8 (0.6-1.0)	0.9 (0.5-1.2)
DDE	1233	0.9 (0.8-1.1)	1.0 (0.8-1.2)
Hexachlorobenzene (binary)	1232	0.9 (0.6-1.2)	0.9 (0.6-1.3)
Oxychlordane	1231	0.8 (0.6-1.1)	1.0 (0.6-1.4)
Organophosphate Pesticide Metabolites[b]			
Dimethylphosphate (DMP)	1229	1.0 (0.8-1.1)	1.0 (0.8-1.2)
Diethylphosphate (DEP)	1230	0.9 (0.8-1.0)	0.9 (0.7-1.1)
Dimethylthiphosphate (DMTP)	1227	1.0 (0.9-1.1)	0.9 (0.8-1.1)
Diethylthiophosphate (DETP)	1229	1.0 (0.8-1.1)	1.0 (0.7-1.2)
ΣDMP&DMTP	1226	1.0 (0.8-1.1)	1.0 (0.8-1.1)
ΣDEP&DETP	1229	1.0 (0.8-1.1)	0.9 (0.7-1.0)
Polychlorinated biphenyls[a]			
PCB 118	1233	1.0 (0.7-1.2)	1.1 (0.7-1.5)
PCB 138	1233	0.9 (0.7-1.1)	1.1 (0.8-1.4)
PCB 153	1233	0.9 (0.7-1.1)	1.0 (0.8-1.3)
PCB 180	1233	0.9 (0.7-1.1)	1.1 (0.8-1.3)
ΣPCB (non-dioxin – 138,153,180)	1233	1.0 (0.7-1.2)	1.1 (0.8-1.3)
Metals [a]			
Lead	1241	0.9 (0.6-1.2)	1.0 (0.7-1.3)
Cadmium	1241	0.9 (0.7-1.1)	0.9 (0.7-1.1)
Arsenic	1241	1.2 (0.9-1.4)	1.1 (0.9-1.4)
Mercury	1241	1.0 (0.8-1.1)	1.0 (0.8-1.1)

[a]adjusted for age and lipids, [b] adjusted for age and specific gravity

Table 7.5 Odds ratios of tertiles of exposure and elevated (≥80%) cord blood levels of IL-33/TSLP for contaminants with borderline significant associations using continuous exposure variables

Contaminant	Tertiles	Adjusted OR (95% CI)
DDE[b] (ug/L)	≤0.23	1.0
(n=1233)	0.24-0.39	0.8 (0.5-1.1)
	>0.39	0.7 (0.5-1.0)
DMP (ug/L)	≤0.1.60	1.0
(n=1229)	1.61-4.70	0.8 (0.6-1.2)
	>4.70	0.7 (0.5-1.1)
ΣDEP&DETP[c] (ug/L)	≤12.59	1.0
(n=1229)	12.7-26.89	0.8 (0.5-1.2)
	>26.89	0.9 (0.6-1.4)
PCB 180[b] (ug/L)	≤ 0.02	1.0
(n=1233)	0.03-0.04	0.9 (0.6-1.4)
	>0.04	0.8 (0.5-1.3)
PCB 118 (ug/L)	≤ 0.01	1.0
(n=1233)	0.01-0.02	0.8 (0.6-1.2)
	>0.02	0.7 (0.5-1.1)
Lead[a] (ug/dL)	≤0.68	1.0
(n=1241)	0.69-1.08	0.8 (0.6-1.2)
	>1.08	0.8 (0.5-1.1)

[a] adjusted for age, [b] adjusted for age and lipids, [c] adjusted for age and specific gravity

Table 7.6 Odds ratios of tertiles of exposure and elevated cord blood levels of elevated (≥0.5 ku/L) IgE for contaminants with borderline significant associations using continuous exposure variables

Contaminant	Tertiles	Adjusted OR (95% CI)
ΣDEP&DETP[c] (ug/L)	≤12.59	1.0
(n=1229)	12.7-26.89	0.9 (0.6-1.3)
	>26.89	0.9 (0.5-1.4)

[c] adjusted for age and specific gravity

Chapter Eight. Public Health Context

The ultimate goal of such work shall be to inform regulatory decisions and reduce risks associated with chemical exposures. To position this investigation within the entirety of the research cycle (Figure 1.1), this chapter will provide an overview of the process involved in translating epidemiologic research into public health guidelines and some of the inherent challenges.

Public health guidelines are developed and implemented through the processes of risk assessment and risk management. The US National Research Council formalized these processes in 1983 in an effort to bridge the gap between scientific knowledge and government regulation. Risk assessment is defined as the synthesis of scientific evidence to understand potential human health effects resulting from exposure to hazardous materials and situations (261). The fundamental question of interest to risk assessors is 'What is the risk of disease Y in the presence of agent X relative to the risk of disease Y in absence of X?' (262). The ability to answer this question requires an understanding of the toxicity (hazard) of the agent as well as the likelihood of population exposure to the agent. The aforementioned National Research Council Report outlined four steps to risk assessment; hazard identification, dose-response assessment, exposure assessment, and risk characterization (261).

Risk management combines the results of risk assessment with economic, social and technological considerations to develop policy recommendations (263). The goal of risk management is not to completely eliminate risks but to establish levels of risk that are considered acceptable based on a balance of economic, social, political and technological considerations. This is well exemplified by the ALARA principle

commonly used in assessing risk to ionizing radiation which states that the exposure must be 'As Low As Reasonably Achievable' while accounting for social and economic benefits and availability of existing technology. Risk management approaches include source control, point-of-use control, educational strategies, legally enforceable limits, regulations, standards, operating targets, guidelines and a request for further research (264).

Hazard Identification

Hazard identification, the first stage of risk assessment, attempts to answer the question of 'What adverse health effects are associated with exposure (developmental toxicity, neurotoxicity, cancer)?' by incorporating available toxicological and epidemiological data to create an assessment of hazards resulting from exposure. Epidemiological studies offer valuable contributions to this step through the capacity to 1) assess the range of exposures of interest in real-life settings 2) incorporate genetic diversity and 3) assess effect modification and resulting health effects in susceptible populations (265). Though regulatory agencies such as Health Canada, the US Environmental Protection Agency (EPA), International Agency for Research on Cancer (IARC) and Institute of Medicine (IOM) may each undertake a slightly different approach to hazard identification, the typical result is a qualitative weight of evidence narrative and corresponding descriptor. For example, the IARC *Monographs on the Evaluation of Carcinogenic Risks to Humans* (2006) conclude with a narrative discussing the methodological strengths and weaknesses of the scientific evidence, the length of time studies were conducted, routes of exposures, biological modes of action and severity of effects. Based on an evaluation of the strength

of evidence, the substance is then categorized into one of the following groups (266):Group 1: The agent is carcinogenic to humans

Group 2A: The agent is probably carcinogenic to humans

Group 2B: The agent is possibly carcinogenic to humans

Group 3: The agent is not classifiable as to its carcinogenicity to humans.

Group 4: The agent is probably not carcinogenic to humans

The process undertaken to synthesize this diverse body of literature and choose an appropriate descriptor is largely qualitative. While the IARC notes that the descriptor should be reviewed in conjunction with the corresponding narrative, it is quite likely that the final concluding descriptor receives the bulk of attention. A recent report examining the environmental risks of breast cancer similarly conducted a qualitative review of literature and noted the limitations inherent in this process (267). Determination of causality in this manner is, to some degree, a judgment call (263). A similarly challenging process applies to determining immunotoxicity. Determination of developmental immunotoxicity, in particular, is not routine for pharmaceuticals or environmental contaminants despite consistent evidence of the sensitivity of the developing immune system to xenobiotics (46). One of the barriers to establishing immunotoxicity may be the diversity of immune system mediated diseases and underlying physiological processes.

In addition to the challenges of synthesizing epidemiological and toxicological literature, risk assessment relies on appropriate interpretation of epidemiological results. Due to the potential role of chance, bias, confounding, exposure misclassification and lack of power, null results from epidemiological analyses can be difficult to interpret and

cannot be used to prove the absence of toxic effects (265). The null results from the present thesis provide an example. The observed null findings do not necessarily reflect an absence of effect. It is possible that immunotoxic effects may have been observed with other biomarkers, observed effects may not be detectable until childhood, or the study was underpowered to detect an association.

Reliance on Animal Models

Due to ethical considerations as well as the duration of time and resources required to assess human health effects of substances, contributions from epidemiological studies to hazard identification are often limited. The resulting reliance on animal models results in either an inability to classify substances or a classification that is characterized by considerable uncertainty. Of the 942 agents that have been classified according to carcinogenicity by the IARC, the majority (508) are classified as Group 3 'not classifiable' (266) .

In the face of insufficient human data, the process of risk assessment relies on default options, invoked by regulatory agencies when human data is lacking. An US EPA example of a default states that positive effects in animal cancer studies indicate that the chemical of interest is carcinogenic to humans. Conversely, if cancer effects are not identified in animal studies and epidemiological data is lacking, the default option is that the agent of interest does not exhibit carcinogenic potential (268). The IARC follows a similar approach in determining carcinogenicity to humans in the absence of adequate human data (262). While these options do provide guidance in situations of insufficient data, they are clearly limited by the reliance on extrapolating results from animal models to humans (268).

Challenges related to use of animal data manifest in the creation of regulatory reference doses as well. Reference doses are calculated for substances determined to have a threshold level for adverse health effects. The reference dose represents: 'an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious effects during a lifetime'(269).

Reference doses, such as a tolerable daily intake (TDI) are calculated by applying uncertainty factors, usually ranging from 1 to 10, to account for interspecies variations, inadequacies in the database, or potential interaction with other chemical substances, to the no-observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) (270). The goal of this approach is to determine whether application of the uncertainty factor provides sufficient protection to vulnerable populations from the potential adverse effects of environmental exposures. Limited evidence suggests an uncertainty factor of 10-fold would be insufficient to protect the developing fetus from adverse immune system related effects of dioxin (46).

Dose-Response Assessment

The goal of the second step of risk assessment, dose-response, is to determine the health effects of different doses and to determine whether there is a threshold below which no adverse effects occur. The two steps employed in assessing the dose-response relationships are 1) to model the dose-response relationship and 2) to extrapolate from observed data to exposures within the range of human experiences. Dose-response models have traditionally assumed the presence of a threshold point, below which no adverse effects occur, for non-cancer outcomes. Development and interpretation of dose-

response relationships have also traditionally hinged on the assumption that the nature, number, severity and incidence of effects are positively associated with increases in the dose, duration and frequency of exposure (271). Both of these assumptions have recently come under scrutiny.

The assumption that carcinogenic and mutagenic endpoints have no threshold was based on the theory that carcinogenesis occurs when a small number of molecules can lead to changes in cells and resulting, uncontrolled cellular proliferation. In contrast, it is assumed that individual homeostatic, compensatory and adaptive mechanisms must be overcome in order for systemic endpoints (e.g. neurodevelopmental toxicity, immunotoxicity) to occur. Authors of the National Research Council Report *'Science and Decisions, Advancing Risk Assessment'* have recently advocated an approach to dose response assessment that does not distinguish between carcinogenic and non-carcinogenic outcomes (272). The rationale for this unified approach is backed by recent research examining the non-cancer health outcomes associated with exposure to agents such as particulate matter, ozone and lead. The Canadian blood lead intervention level (10 μg/dL) for blood lead levels was established in 1994 by Health Canada based on evidence of adverse health effects occurring between 10-15 μg/dL (273). Recent research suggests that adverse health effects (neurotoxic, cardiovascular, renal, and reproductive) occur at levels as low as 1 μg/dL. In fact, the slope of the dose-response curve may be steeper at levels below the accepted guidelines of 10 μg/dL (274,275). The recent 'Final Human Health State of the Science Report on Lead' authored by Health Canada concludes that there is no evidence of a threshold below which no adverse effects from lead exposure occur (273).

Traditional approaches to risk assessment that rely on monotonically increasing adverse outcomes with increasing exposure are similarly being revisited in the face of evidence regarding non-linear dose-response relationships. Endocrine disruptors have been shown to be associated with adverse health effects in a non-linear fashion and to have effects at low doses (276). Moreover, inverted U-shaped dose response curves have been observed between exposure to certain environmental contaminants (e.g. lead) and changes to the immune system (277). Results from the present thesis similarly suggest that certain phthalates and BPA may not operate in a linear fashion. There is also evidence to suggest that the toxic effect of contaminants may plateau at higher concentrations when the physiological compensatory mechanisms become saturated. This phenomenon is depicted in Figure 8.1. A doubling in hormone concentration results in a 50% increase in hormone receptor occupancy from 0-50% whereas an increase from 4- to 5-fold results in only a four percent increase (78-82%) (229). This evidence provides further impetus to lessen the emphasis on the traditional adage 'the dose makes the poison.' Specifically, careful investigation of dose-response relationships that are not reliant on the assumptions of linearity and the presence of a threshold is necessary.

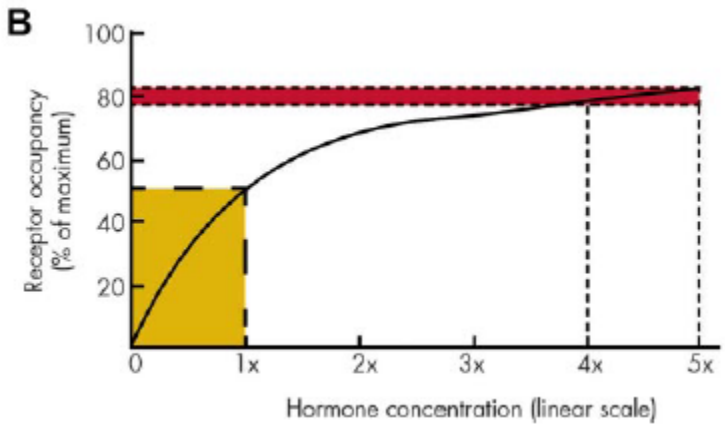


Figure 8.1. Characteristics of Hormones Source: (229)**, p.385**

Chemical mixtures

Understanding the health effects of chemical mixtures is of critical importance due to the potential additive and/or synergistic relationships between chemicals. Furthermore, exposure to multiple chemicals is the exposure scenario of reality. From a risk assessment perspective, the fundamental goal for exploring the human health effects of chemical mixtures is to understand whether the uncertainty factors provide sufficient public health protection from cumulative effects. Figure 8.2 illustrates the potential effect that co-exposures and biological susceptibility can have on the percent of the population with adverse outcomes. By examining the health effects of one chemical without consideration for underlying biological susceptibilities, traditional risk assessment approaches may be underestimating the percentage of the population that incur health effects from environmental exposures (278). The effect of phthalate exposure on the development of the male reproductive system is an example where cumulative risk assessment has been determined to be necessary to accurately understand the magnitude of the relationship between phthalate exposure and male reproductive toxicity (279). Current approaches to risk assessment, however, have little ability to

accurately account for co-exposures. The challenges of understanding the health effects of multiple exposures are substantial. Specifically, methodology and application is challenged by lack of knowledge regarding 1) the magnitude, duration, frequency and timing of cumulative exposure, 2) whether chemical mixtures are characterized by antagonist, synergistic, or additive relationships, and 3) mechanisms of interaction (280) .

One example of regulatory efforts to address this challenge are the 'Interaction Profiles' produced by the US Agency for Toxic Substances and Disease Response (ATSDR). Examples of final interaction profiles include 'Persistent chemicals found in breast milk,' 'Persistent chemicals found in fish,' and 'Arsenic, chromium, cadmium, and lead'. Given the paucity of epidemiological data and methodology targeted at chemical mixtures, the predominant type of evidence and methodology employed in these interaction profiles are toxicology and in vitro studies (281). In order to provide substantial contributions to public health policy, epidemiological analysis of interactions between chemicals is necessary. Toxicological evidence, as outlined in cumulative risk assessment or interaction profiles, provides a reasonable foundation for epidemiologic investigation into synergy between chemicals.

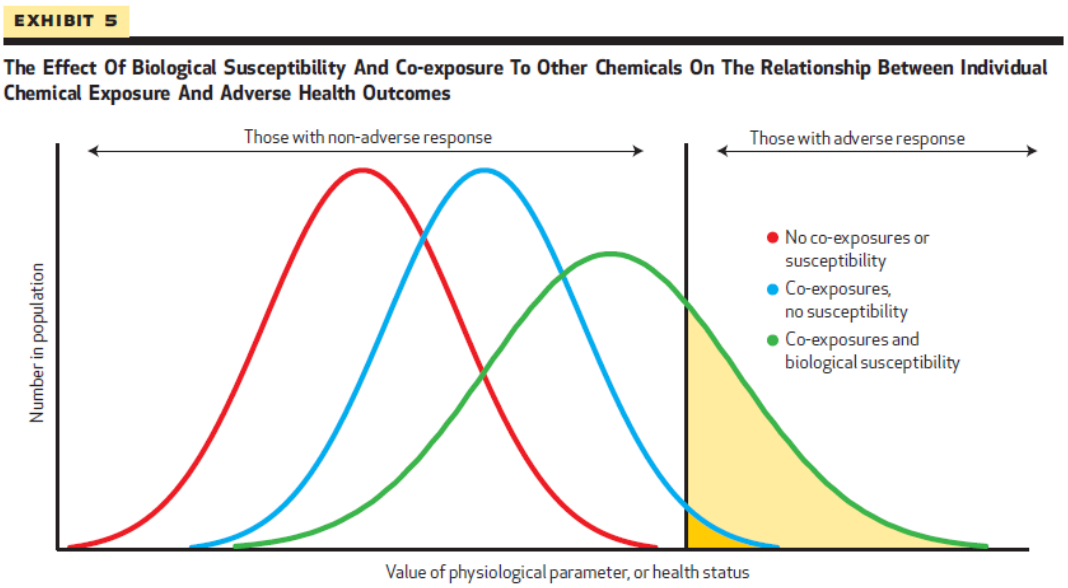


Figure 8.2. Biologic Susceptibility and co-exposure to other chemicals on the relationship between individual chemical exposure and adverse health outcomes Source (278)**, p. 961.**

The topic of chemical mixtures is of particular interest to this thesis because one of the original objectives was to examine potential synergy between environmental chemicals. Specifically, the thesis proposal objective was to determine whether those individuals jointly exposed to two environmental chemicals were at a greater risk of health outcome than an individual exposed to only one environmental chemical. This objective was going to be achieved by calculating the relative excess risk due to interaction between chemicals with a priori evidence of potential interaction based on toxicological studies. However, this objective was intentionally not carried out for the reasons noted below.

First, as there were no statistically significant effects observed between the chemicals of interest and the immune system biomarkers, detecting a statistically significant joint

effect is unlikely. When two chemicals have a weak effect, the interaction effect will be similarly weak on both an additive and a multiplicative scale (282). Second, considering the paucity of toxicological data and the vast number of exposures, evaluating interaction between two specific chemicals was determined to be reductionist and overly simplistic. The Bayesian hierarchical model was an alternative approach to account for co-exposure to highly correlated chemicals. Though a hierarchical model and an assessment of synergism address different research questions, the former approach was deemed to be a methodologically sound and appropriate method for dealing with multiple exposures.

Interaction analyses are also subject to challenges beyond those relevant to the present research. First, the power required to detect an association in interaction analysis is estimated to be an order of magnitude below the power required to detect main effects (283). It is not uncommon for true interactions to not be identified. Greenland (283) suggests addressing this issue by establishing subgroups a priori and attempting to develop sample sizes large enough to assess interactions (283). Funding and resource limitations, however, frequently trump the goal of achieving an adequate sample size. The MIREC study is one of the larger birth cohorts with prenatal exposure data. When missing data are accounted for, subgroup sample size is notably underpowered to detect an interaction. For example, in the present analysis there were 604 male infants with available prenatal MBzP exposure. Based on this sample size, this study would have 5% power to detect a main effect risk ratio of 1.3 based on 25% of the population being 'exposed.' This calculation is a notable underestimate due to reliance on an arbitrary definition of 'exposure' and a categorical rather than continuous exposure variable. Moreover, the notion of statistical power, as defined in a frequentist setting, cannot be

applied to Bayesian analysis. Despite these differences, this example does illustrate that the challenges of small sample size are exacerbated when attempting to detect interaction. Second, both differential and non-differential measurement error can further exacerbate the probability of identifying spurious interactions and not identifying true interactions (283). Third, epidemiology, by design, examines rates of diseases rather than individual-level susceptibilities (58). As such, epidemiologic assessment of interaction can only be done at the population level. Understanding the physiological mechanisms underlying interaction requires inclusion of experimental evidence.

Given the vast challenges in identifying interaction between chemicals in interaction analysis, alternative methodological approaches for dealing with chemical mixtures are warranted. Some of these measures have been applied in the present research including using approaches to account for the correlated nature of co-exposures and summing metabolites where there is supporting biologic rationale. Interaction analyses are likely not warranted unless there is adequate evidence of a strong main effect. Last, continued efforts among and collaboration between toxicologists, epidemiologists, statisticians and risk assessors is necessary to continue to develop approaches for examining chemical synergy and to translate these findings into risk assessment processes.

Concluding Thoughts & Recommendations

Despite the aforementioned challenges, there have been notable successes in the regulation of environmental contaminants. Emissions of key air pollutants, including cadmium, lead, mercury, hexachlorobenzene, and dioxins, have declined between 1985 and 2012 in Canada. These trends are primarily due to reduced emissions from industrial sources (284). Under the goals set out under the Chemicals Management Plan

established in 2006, Canada aims to continue to identify priority chemicals and implement necessary measures to reduce health risks (285). Recommendations that may further enhance risk assessment have been outlined in recent commentaries (272,278). Briefly, these recommendations include a unified approach to dose-response, transparent characterization of uncertainty, use of default options to avoid inaction in the face of uncertainty, and a focus on vulnerable populations (272,278). In addition, a growing body of evidence has highlighted the need for and potential benefits of developmental immunotoxicity testing (DIT) of chemicals (46,61,163,286). Current risk assessments of the immunotoxic properties of chemicals are largely derived from adult models. Characterizing risks in adults does not have the capacity to sufficiently identify risks in early life given the immaturity and resulting vulnerability of the developing immune system (287). Moreover, the traditional focus on immunotoxicity testing has been on immunosuppression not hypersensitivity or inflammatory responses (288). Details of the methods involved in DIT testing, which is beyond the scope of this chapter, are reviewed elsewhere (289). The present research has attempted to identify immunotoxic effects in the developing immune system subsequent to exposure. Testing for developmental immunotoxicity may provide an additional tool for identifying chemicals, particularly new substances, with severe immune system related toxicity prior to exposure. This approach may ultimately reduce exposure to immune system toxicants and minimize the public health burden of chronic diseases such as childhood allergic disease.

Chapter Nine. Conclusions

This thesis made contributions to original knowledge in the fields of immunology and perinatal/environmental epidemiology. The present research offers the first evidence of TSLP and IL-33 in cord blood samples. By demonstrating that these cytokines are detectable and correlated at birth, the present findings provide motivation for further research into the value of these cytokines in predicting childhood allergic disease. These findings also raise interesting questions regarding the mechanistic interaction between these cytokines in early life. The observed dose-response relationship with birth weight suggests that these cytokines may have a shared inflammatory component with adiposity in early life. This thesis was the first assessment of the relationship between prenatal exposure to multiple environmental contaminants and elevated levels of IL-33, TSLP, and IgE. Determining whether the observed absence of effect is the result of confounding, bias, or random error requires confirmation in future studies.

These findings are presented in light of the limitations that have been raised in the discussion section of the manuscript chapters. I will reiterate the primary limitations here that pertain to the thesis as a whole. First, as this thesis presents the first measurement of TSLP and IL-33 at birth, it is not known whether these biomarkers are accurate predictors of childhood allergic disease. Given that this thesis focused on the snapshot of time from prenatal exposures to birth, the lack of prospective data in childhood does not invalidate this thesis research. Second, this thesis analyzed multiple chemical exposures. The challenge associated with multiple comparisons was addressed by using a Bayesian hierarchical model in the phthalate and perfluorinated compound analysis. The analysis of metals, pesticides and PCBs was subject to an increased probability of finding a

significant association by chance due to the number of statistical tests conducted. Given that no significant associations were observed, it is unlikely that our results are invalidated by type 1 error. In addition, statistical associations of individual chemicals rather than chemical mixtures were examined. Though this approach may be limited in its ability to examine potential confounding effects of multiple exposures, it was deemed appropriate due to the exploratory nature of this research.

This thesis provides motivation for further investigation into 1) the trajectory of TSLP and IL-33 from birth to childhood 2) the role of TSLP and IL-33 in childhood allergic diseases and 3) the potential role of prenatal exposure to these contaminants in childhood allergic disease etiology. When combined with these complementary investigations, this research will offer notable contributions to etiological knowledge of childhood allergic disease.

References

1. Garner R, Kohen D. Changes in the prevalence of asthma among Canadian children Changes in the prevalence of asthma among. Health Reports. 2008; Catalogue no. 820003-X.

2. Cookson W, Moffatt M, Strachan DP. Genetic risks and childhood-onset asthma. J Allergy Clin Immunol.2011;128:266–70.

3. Backman H, Hedman L, Jansson S, Lindberg A, Lundbäck B, Rönmark E. Prevalence trends in respiratory symptoms and asthma in relation to smoking - two cross-sectional studies ten years apart among adults in northern Sweden. World Allergy Organ J. 2014;7:1–10.

4. Subbarao P, Mandhane PJ, Sears MR. Asthma: epidemiology, etiology and risk factors. CMAJ. 2009;181:E181–90.

5. Ho S-M. Environmental epigenetics of asthma: an update. J Allergy Clin Immunol. 2010 ;126:453–65.

6. Bisgaard H, Bønnelykke K. Long-term studies of the natural history of asthma in childhood. J Allergy Clin Immunol. 2010;126:187–97.

7. Yeatts K, Sly P, Shore S, Weiss S, Martinez F, Geller A, et al. A brief targeted review of susceptibility factors, environmental exposures, asthma incidence, and recommendations for future asthma incidence research. Environ Health Perspect. 2006;114:634–40.

8. Luebke RW, Chen DH, Dietert R, Yang Y, King M, Luster MI. The comparative immunotoxicity of five selected compounds following developmental or adult exposure. J Toxicol Environ Health B Crit Rev. 2006; 9:1–26.

9. Golub MS, Wu KL, Kaufman FL, Li L-H, Moran-Messen F, Zeise L, et al. Bisphenol A: developmental toxicity from early prenatal exposure. Birth Defects Res B Dev Reprod Toxicol. 2010;89:441–66.

10. Dewitt JC, Peden-Adams MM, Keller JM, Germolec DR. Immunotoxicity of perfluorinated compounds: recent developments. Toxicol Pathol. 2012;40:300–11.

11. Woodruff TJ, Zota AR, Schwartz JM. Environmental chemicals in pregnant women in the United States: NHANES 2003-2004. Environ Health Perspect. 2011;119:878–85.

12. Health Canada. Report on Human Biomonitoring of Environmental Chemicals in Canada [Internet]. 2010 [cited 2014 Jul 24]. Available from: http://www.hc-sc.gc.ca/ewh-semt/pubs/contaminants/chms-ecms/section8-eng.php#n8_1_2

13. Tugwell P, Bennet K, Sackett D, Haynes R. The measurement iterative loop: a framework for the critical appraisal of need, benefits and costs of health interventions. J Chron Dis. 1985;38:339–51.

14. Health Canada. Overview of the Second Report on Human Biomonitoring of Environmental Chemicals in Canada [Internet]. 2013 [cited 2014 Oct 9]. Available from: http://www.hc-sc.gc.ca/ewh-semt/pubs/contaminants/chms-ecms-cycle2/overview-vue-eng.php

15. Ker J, Hartert T V. The atopic march: what's the evidence? Ann Allergy Asthma Immunol 2009;103:282–9.

16. Murphy K. Janeway's Immunobiology. 8th ed. Garland Science Taylor & Science Group; 2012.

17. Zheng T, Yu J, Oh MH, Zhu Z. The atopic march: progression from atopic dermatitis to allergic rhinitis and asthma. Allergy Asthma Immunol Res. 2011;3:67–73.

18. Immune Tolerance Network . Learning Early About Peanut Allergy [Internet]. 2014 [cited 2014 Oct 1]. Available from: http://www.leapstudy.co.uk/amarch.html

19. Ebert CS, Pillsbury HC. Epidemiology of allergy. Otolaryngol Clin North Am. 2011 ;44:537–48.

20. Wallace DV, Dykewicz MS, Bernstein DI, Blessing-Moore J, Cox L, Khan D a, et al. The diagnosis and management of rhinitis: an updated practice parameter. J Allergy Clin Immunol 2008;122(2 Suppl):S1–84.

21. Wang H-Y, Pizzichini M, Becker A, Duncan J, Ferguson A, Greene J, et al. Disparate geographic prevalences of asthma , allergic rhinoconjunctivitis and atopic eczema among adolescents in five Canadian cities. Pediatr Allergy Immunol. 2010;867–77.

22. Soller L, Ben-Shoshan M, Harrington DW, Fragapane J, Joseph L, St Pierre Y, et al. Overall prevalence of self-reported food allergy in Canada. J Allergy Clin Immunol. 2012;130:986–8.

23. Soller L, Ben-Shoshan M, Knoll M, Harrington D, Fragapane J, Joseph L, et al. PD16 - Prevalence of childhood food allergy in Canada: a focus on under-represented populations. Clin Transl Allergy. 2014;4(Suppl):P16.

24. Seung S, Mittman N. Urgent care costs of uncontrolled asthma in Canada, 2004. Can Respir J. 2005;12:2004–5.

25. Mancini AJ, Kaulback K, Chamlin SL. The socioeconomic impact of atopic dermatitis in the United States: a systematic review. Pediatr Dermatol. 2008;25:1–6.

26. Palmer C, Irvine A, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee S, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet. 2006;38:441–6.

27. Arshad S, Kurukulaaratchy R, Fenn M, Matthews S. Early life risk factors for current wheeze, asthma, and bronchial hyperresponsiveness at 10 years of age. Chest. 02005;127:502–8.

28 Burke H, Leonardi-Bee J, Hashim a., Pine-Abata H, Chen Y, Cook DG, et al. Prenatal and passive smoke exposure and incidence of asthma and wheeze: systematic review and meta-analysis. Pediatrics . 2012;129:735–44.

29 Brunst KJ, Ryan PH, Lockey JE, Bernstein DI, McKay RT, Khurana Hershey GK, et al. Unraveling the relationship between aeroallergen sensitization, gender, second-hand smoke exposure, and impaired lung function. Pediatr Allergy Immunol. 2012; 00; 1-9.

30. Dell SD, Jerrett M, Beckerman B, Brook JR, Foty RG, Gilbert NL, et al. Presence of other allergic disease modifies the effect of early childhood traffic-related air pollution exposure on asthma prevalence. Env Int. 2014;65:83–92.

31. Chung EK, Miller RL, Wilson MT, McGeady SJ, Culhane JF. Antenatal risk factors, cytokines and the development of atopic disease in early childhood. Arch Dis Child Fetal Neonatal Ed. 2007;92:F68–73.

32. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet. 1999;354:541–5.

33. Jackson D, Gangnon R, Evans M, Roberg K, Anderson E, Pappas T. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. Am R Respir Crit Care Med. 2008;178:667–72.

34 Karmaus W, Arshad H, Mattes J. Does the Sibling Effect Have Its Origin In Utero ? Investigating Birth Order , Cord Blood Immunoglobulin E Concentration , and Allergic Sensitization at Age 4 Years. Am J Epidemiol. 2001;154:909–15.

35. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. New Engl J Med. 2002;347: 869-77.

36. Illi S, Weber J, Zutavern A, Genuneit J, Schierl R, Strunz-Lehner C, et al. Perinatal influences on the development of asthma and atopy in childhood. Ann Allergy, Asthma Immunol. 2014;112:132–139.e1.

37. Hertz-Picciotto I, Park H-Y, Dostal M, Kocan A, Trnovec T, Sram R. Prenatal exposures to persistent and non-persistent organic compounds and effects on immune system development. Basic Clin Pharmacol Toxicol. 2008;102:146–54.

38. Peden D. Development of atopy and asthma: candidate environmental influences and important periods of exposure. Environ Health Perspect. 2000;108 (suppl 3):475–82.

39. Wigle DT, Arbuckle TE, Turner MC, Bérubé A, Yang Q, Liu S, et al. Epidemiologic evidence of relationships between reproductive and child health outcomes and environmental chemical contaminants. J Toxicol Environ Health B Crit Rev. 2008; 11: 373–517.

40. Barker D. The fetal origins of coronary heart disease. Acta Paediatr. 1997;422:78–82.

41. Turner S. Perinatal programming of childhood asthma: early fetal size, growth trajectory during infancy, and childhood asthma outcomes. Clin Dev Immunol. 2012;2013:1–9.

42. Barker DJ. A new model for the origins of chronic disease. Med Health Care Philos 2001;4:31–5.

43. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA., et al. Developmental plasticity and human health. Nature. 2004 22;430:419–21.

44. Ben-Shlomo Y. Rising to the challenges and opportunities of life course epidemiology. Int J Epidemiol. 2007;36:481–3.

45. Selgrade MK. Immunotoxicity: the risk is real. Toxicol Sci. 2007;100:328–32.

46. Dietert R. Developmental immunotoxicology: focus on health risks. Chem Res Toxicol. 2009;22:17–23.

47. Stick S. The contribution of airway development to paediatric and adult lung disease. Thorax. 2000;55:587–94.

48. Klaasen C. Casarett and Doull's Essentials of Toxicology. 2nd ed. New York: McGraw-Hill Medical; 2010.

49. Chung EK, Miller RL, Wilson MT, McGeady SJ, Culhane JF. Antenatal risk factors, cytokines and the development of atopic disease in early childhood. Arch Dis Child Fetal Neonatal Ed. 2007;92(1):F68–73.

50. Dietert RR, Etzel R, Chen D, Halonen M, Holladay S, Jarabek A. Workshop to identify critical windows of exposure for children's health: Immune and respiratory system work group summary. Environ Health Perspect. 2000;108(suppl 3):483–90.

51. Barouki R, Gluckman P, Grandjean P, Hanson M, Heindel J. Developmental origins of non-communicable disease:implications for research and public health. Env Heal. 2012;11:42.

52. Grandjean P, Bellinger D, Bergman A, Cordier S, Davey-Smith G, Eskenazi B, et al. The faroes statement: human health effects of developmental exposure to chemicals in our environment. Basic Clin Pharmacol Toxicol. 2008;102:73–5.

53. Martino D, Prescott S. Epigenetics and prenatal influences on asthma and allergic airways disease. Chest. 2011;139:640–7.

54. Bracken MB. Genetic and Perinatal Risk Factors for Asthma Onset and Severity: A review and theoretical analysis. Epidemiol Rev. 2002;24:176–89.

55. Grandjean P, Poulsen LK, Heilmann C, Steuerwald U, Weihe P. Allergy and sensitization during childhood associated with prenatal and lactational exposure to marine pollutants. Environ Health Perspect. 2010;118:1429–33.

56. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69:89–95.

57. Last J. A Dictionary of Epidemiology. Oxford: Oxford University Press; 2001.

58. Rothman KJ, Greenland S, Lash T. Modern Epidemiology. 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2008.

59. National Research Council. Human biomonitoring for environmental chemicals [Internet]. 2006 [cited 2011 Nov 22]. Available from: http://books.nap.edu/catalog/11700.html

60. National Research Council. Biological Markers in Environmental Health Research. Environ Health Perspect. 1987;74:3–9.

61. Dietert R. Developmental immunotoxicity, perinatal programming, and noncommunicable diseases: focus on human studies. Adv Med. 2014;867805:1–18.

62. Metcalf SW, Orloff KG. Biomarkers of exposure in community settings. J Toxicol Environ Health A. 2010;67:715–26.

63. Duramad P, Holland N. Biomarkers of immunotoxicity for environmental and public health research. Int J Env Res Public Health. 2011;8:1288–401.

64. Chu DK, Llop-Guevara A, Walker TD, Flader K, Goncharova S, Boudreau JE, et al. IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization. J Allergy Clin Immunol. 2013;131:187–200.e1–8.

65. Muto T, Fukuoka A, Kabashima K, Ziegler SF, Nakanishi K, Matsushita K, et al. The role of basophils and proallergic cytokines, TSLP and IL-33, in cutaneously sensitized food allergy. Int Immunol. 2014;26:539–49.

66. Huston DP, Liu YJ. Thymic stromal lymphopoietin:a potential therapeutic target for allergy and asthma. Curr Allergy Asthma Rep. 2006;6:372–6.

67. Oliphant CJ, Barlow JL, McKenzie ANJ. Insights into the initiation of type 2 immune responses. Immunology. 2011;134:378–85.

68. Gauvreau GM, O'Byrne PM, Boulet L-P, Wang Y, Cockcroft D, Bigler J, et al. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. N Engl J Med. 2014;370:2102–10.

69. Lambrecht BN, Hammad H. Asthma: The importance of dysregulated barrier immunity. Eur J Immunol. 2013;43:3125–37.

70. Brandt EB, Sivaprasad U. Th2 Cytokines and Atopic Dermatitis. J Clin Cell Immunol 2011;2:110.

71. Salimi M, Barlow J, Saunders S, Xue L, Gutowska-Owsiak D, Wang X, et al. A role for IL-25 and IL-33 driven type-2 innate lymphoid cells in atopic dermatitis. J Exp Med. 2013;210:2939–50.

72. Comeau MR, Ziegler SF. The influence of TSLP on the allergic response. Mucosal Immunol 2010;3:138–47.

73. Iijama K, Kobayashi T, Hara K, Kephart G, Ziegler S, McKenzie A, et al. IL-33 and thymic stromal lymphopoietin mediate immune pathology in response to crhonic airborne allergen exposure. J Immunol. 2014;193:1549–59.

74. Bleck B, Grunig G, Chiu A, Liu M, Gordon T, Kazeros A, et al. MicroRNA-375 regulation of thymic stromal lymphopoietin by diesel exhaust particles and ambient particulate matter in human bronchial epithelial cells. J Immunol. 2013 Apr;190(7):3757–63.

75. Licona-Limón P, Kim LK, Palm NW, Flavell R a. TH2, allergy and group 2 innate lymphoid cells. Nat Immunol. 2013;14:536–42.

76. Cayrol C, Girard J-P. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. Curr Opin Immunol. 2014;31:31–7.

77. Saenz SA, Taylor BC, Artis D. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. Immunol Rev. 2009;226:172–90.

78. Thomas C, editor. Taber's cyclopedic medical dictionary. Philadelphia, PA: FA Davis; 1997.

79. Nakae S, Morita H, Ohno T, Arae K, Matsumoto K, Saito H. Role of interleukin-33 in innate-type immune cells in allergy. Allergol Int. 2013;62:13–20.

80. Lloyd C. IL-33 family members and asthma - bridging innate and adaptive immune responses. Curr Opin Immunol. 2010;22:800–6.

81. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. Nat Rev Immunol. 2010;10:103–10.

82. Tamagawa-Mineoka R, Okuzawa Y, Masuda K, Katoh N. Increased serum levels of interleukin 33 in patients with atopic dermatitis. J Am Acad Dermatol. 2014;70:882–8.

83. Kurowska-Stolarska M, Stolarski B, Kewin P, Murphy G, Corrigan CJ, Ying S, et al. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. J Immunol. 2009;183:6469–77.

84. Leonard W. TSLP: finally in the limelight. Nat Immunol. 2002;3:605–7.

85. Wang W, Li H, Zhang M, Gao P, He S, Zhang T, et al. Thymic stromal lymphopoietin: A promising therapeutic target for allergic diseases. Int Arch Allergy Immunol. 2013;160:18–26.

86. Soumelis A, Reche P, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell-mediated allergic inflammation by producing TSLP. Nat Immunol. 2002;3:673–80.

87. Lee EB, Kim KW, Hong JY, Jee HM, Sohn MH, Kim K-E. Increased serum thymic stromal lymphopoietin in children with atopic dermatitis. Pediatr Allergy Immunol. 2010;21:e457–60.

88. Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, et al. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. J Immunol. 2005;174:8183–90.

89. Noti M, Wojno EDT, Kim BS, Siracusa MC, Giacomin PR, Nair MG, et al. Thymic stromal lymphopoietin-elicited basophil responses promote eosinophilic esophagitis. Nat Med. 2013;19:1005–13.

90. Pesonen M, Kallio MJT, Siimes MA., Elg P, Björksten F, Ranki A. Cord serum immunoglobulin E as a risk factor for allergic symptoms and sensitization in children and young adults. Pediatr Allergy Immunol. 2009;20:12–8.

91. Odelram H, Björkstén B, Leander E, Kjellman NI. Predictors of atopy in newborn babies. Allergy. 1995;50:585–92.

92. Hansen L, Halken S, Host A, Moller K, Osterballe O. Prediction of allergy from family history and cord blood IgE levels. Pediatr Allergy Immunol. 1993;4:34–40.

93. Bergmann R, Schulz J, Giinther S, JD, Ke B. Determinants of cord-blood IgE concentrations in 6401 German neonates. Allergy. 1995;50:65–71.

94. Edenharter G, Bergmann R, Bergmann K, Wahn V, Forster J, Zepp F, et al. Cord blood IgE as risk factor and predictor for atopic diseases. Clin Exp Allergy. 1998;28:671–8.

95. Tariq S, Arshad S. Elevated cord serum IgE increases the risk of aeroallergen sensitization without increasing respiratory allergic symptoms in early childhood. Exp Allergy. 1999;29:1042–8.

96. Kaan A., Dimich-Ward H, Manfreda J, Becker A., Watson W, Ferguson A., et al. Cord blood IgE: its determinants and prediction of development of asthma and other allergic disorders at 12 months. Ann Allergy, Asthma Immunol. 2000;84:37–42.

97. Holt PG. Prenatal versus postnatal priming of allergen specific immunologic memory: the debate continues. J Allergy Clin Immunol. 2008;122:717–8.

98. Warner JO. The early life origins of asthma and related allergic disorders. Archives of disease in childhood. 2004;89:97–102.

99. Delespesse G, Sarfati M, Lang G, Sehon A. Prenatal and neonatal synthesis of IgE. Monogr Allergy. 1983;17:83–95.

100. Miller D, Hirvonen T, Gitlin D. Synthesis of IgE by the human conceptus. J Allergy Clin Immunol. 973;52:182–8.

101. Ngoc L, Gold D, Tzianabos A, Weiss S, Celedon J. Cytokines, allergy, and asthma. Curr Opin Allergy Clin Immunol. 2005;5:161–6.

102. Edenharter G, Bergmann R, Bergmann K, Wahn V, Forster J, Zepp F, et al. Cord blood-IgE as risk factor and predictor for atopic diseases. Clin Exp Allergy. 1998;28:671–8.

103. Ferguson A, Dimich-Ward H, Becker A, Watson W, DyBuncio A, Carlsten C, et al. Elevated cord blood IgE is associated with recurrent wheeze and atopy at 7 yrs in a high risk cohort. Pediatr Allergy Immunol. 2009;20:710–3.

104. Sadeghnejad A, Karmaus W, Davis S, Kurukulaaratchy R, Matthews S, Arshad S. Raised cord serum immunoglobulin E increases the risk of allergic sensitisation at ages 4 and 10 and asthma at age 10. Thorax. 2004;59:936–42.

105. Avrech O, Samra Z, Lazarovich Z, Caspi E, Jacobovich A, Sompolinsky D. Efficacy of the placental barrier for immunoglobulins: Correlations between maternal, paternal, and fetal immunoglobulin levels. Int Arch Allergy Immunol. 1994;103:160–5.

106. Ownby DR, McCullough J, Johnson CC, Peterson EL. Evaluation of IgA measurements as a method for detecting maternal blood contamination of cord blood samples. Pediatr Allergy Immunol. 1996;7:125–9.

107. Dodson RE, Nishioka M, Standley LJ, Perovich LJ, Brody JG, Rudel RA. Endocrine disruptors and asthma-associated chemicals in consumer products. Environ Health Perspect. 2012;120:935–43.

108. CDC. Fourth National Report on Human Exposure to Environmental Chemicals [Internet]. 2009. Available from: http://www.cdc.gov/exposurereport/

109. Barr DB, Bishop A, Needham LL. Concentrations of xenobiotic chemicals in the maternal-fetal unit. Reprod Toxicol. 2007;23:260–6.

110. Needham L, Grandjean P, Heinzow B, Jørgensen P, Flemming N, Patterson D, et al. Partition of environmental chemicals between maternal and fetal blood and tissues. Environ Sci Technol. 2011;45:1121–6.

111. Dietert RR, Lee J-E, Hussain I, Piepenbrink M. Developmental immunotoxicology of lead. Toxicol Appl Pharmacol. 2004;198:86–94.

112. Rabinowitz MB, Allred EN, Bellinger DC, Leviton a, Needleman HL. Lead and childhood propensity to infectious and allergic disorders: is there an association? Bull Environ Contam Toxicol. 1990;44:657–60.

113. Beeh KM, Ksoll M, Buhl R. Elevation of total serum immunoglobulin E is associated with asthma in nonallergic individuals. Eur Respir J. 2000;16:609.

114. Lutz PM, Wilson TJ, Ireland J, Jones a L, Gorman JS, Gale NL, et al. Elevated immunoglobulin E (IgE) levels in children with exposure to environmental lead. Toxicology. 1999;134:63–78.

115. Sun L. Influence of exposure to environmental lead on serum immunoglobulin in preschool children. Environ Res. 2003;92:124–8.

116. Jedrychowski W, Perera F, Maugeri U, Miller RL, Rembiasz M, Flak E, et al. Intrauterine exposure to lead may enhance sensitization to common inhalant allergens in early childhood: a prospective prebirth cohort study. Environ Res. 2011;11:119–24.

117. Jomova K, Jenisova Z, Feszterova M, Baros S, Liska J, Hudecova D, et al. Arsenic: toxicity, oxidative stress and human disease. J Appl Toxicol. 2011;31:95–107.

118. Islam LN, Nabi a HMN, Rahman MM, Zahid MSH. Association of respiratory complications and elevated serum immunoglobulins with drinking water arsenic toxicity in human. J Environ Sci Health A Tox Hazard Subst Environ Eng . 2007;42:1807–14.

119. Soto-Peña G a, Luna AL, Acosta-Saavedra L, Conde P, López-Carrillo L, Cebrián ME, et al. Assessment of lymphocyte subpopulations and cytokine secretion in children exposed to arsenic. FASEB J. 2006;20:779–81.

120. Belles-isles M, Québec-chul D, Ayotte P, Dewailly E, Weber J. Cord blood lymphocyte functions in a newborns from a remote maritime population exposed to organochlorines and methylmercury. J Toxicol Environ Heal Part A. 2002;54:165–82.

121. Hauser R, Calafat AM. Phthalates and human health. 2005; 62:806–18.

122. Heudorf U, Mersch-Sundermann V, Angerer J. Phthalates: Toxicology and exposure. Int J Hyg Environ Health. 2007;210:623–34.

123. Chalubinski M, Kowalski ML. Endocrine disrupters--potential modulators of the immune system and allergic response. Allergy. 2006;61:1326–35.

124. Bornehag CG, Nanberg E. Phthalate exposure and asthma in children. Int J Androl 2010;33:333–45.

125. Jaakkola JJK, Verkasalo PK, Jaakkola N. Plastic Wall Materials in the Home and Respiratory Healtli in Young Children. Am J Public Heal. 2000;90:797–800.

126. Bertelsen RJ, Calafat AM, Hoppin JA. Urinary biomarkers for phthalates associated with asthma in Norwegian children. Env Heal Perspect. 2013;121:251–6.

127. Jaakkola JJK, Ieromnimon A, Jaakkola MS. Interior surface materials and asthma in adults: a population-based incident case-control study. Am J Epidemiol. 2006;164:742–9.

128. Just AC, Whyatt RM, Perzanowski MS, Calafat AM, Perera FP, Goldstein IF, et al. Prenatal exposure to butylbenzyl phthalate and early eczema in an urban cohort. Environ Health Perspect. 2012; 120:1475-80.

129. Whyatt RM, Perzanowski MS, Just AC, Rundle AG, Donohue KM, Calafat AM, et al. Asthma in inner-city children at 5-11 years of age and prenatal exposure to phthalates: The Columbia Centre for Children's Environmental Health Cohort. Environ Health Perspect. 2014; 122:1141-6.

130. Stahlhut RW, Welshons W V, Swan SH. Bisphenol A data in NHANES suggest longer than expected half-life, substantial nonfood exposure, or both. Environ Health Perspect. 2009;117:784–9.

131. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons W V. Human exposure to bisphenol A (BPA). Reprod Toxicol. 2007;24:139–77.

132. Nakajima Y, Goldblum RM, Midoro-Horiuti T. Fetal exposure to bisphenol A as a risk factor for the development of childhood asthma: an animal model study. Environ Health; 2012;11:8.

133. Midoro-Horiuti T, Tiwari R, Watson CS, Goldblum RM. Maternal Bisphenol A exposure promotes the development of experimental asthma in mouse pups. Environ Health Perspect. 2009;118:273–7.

134. Spanier AJ, Kahn RS, Kunselman AR, Hornung R, Xu Y. Prenatal exposure to bisphenol A and child wheeze from birth to three years. Environ Health Perspect. 2012; 120:916-920

135. Donohue KM, Miller RL, Perzanowski MS, Just AC, Hoepner LA, Arunajadai S, et al. Prenatal and postnatal bisphenol A exposure and asthma development among inner-city children. J Allergy Clin Immunol. 2013;131:736–42.

136. US ATSDR. Draft toxicological profile for perfluoroalkyls [Internet]. 2009 [cited 2014 Sep 22]. Available from: www.atsdr.cdc.gov/toxprofiles/tp200.pdf

137. Fromme H, Tittlemier S, Völkel W, Wilhelm M, Twardella D. Perfluorinated compounds--exposure assessment for the general population in Western countries. Int J Hyg Environ Health 2009;212:239–70.

138. D'Hollander W, Voogt P De, Coen W De, Bervoets L. Perflourinated substances in human food and other sources of exposures. In: De Voogt P, editor. Review of environmental contamination and toxicology volume 208. New York, NY: Springer New York; 2010 p. 179–215. Available from: http://link.springer.com/10.1007/978-1-4419-6880-7

139. Fairley KJ, Purdy R, Kearns S, Anderson SE, Meade B. Exposure to the immunsuppresant, perfluorooctanoic acid, enhances the murine IgE and airway hyperreactivity response to ovalbumin. Toxicol Sci. 2007;97:375–83.

140. Okada E, Sasaki S, Saijo Y, Washino N, Miyashita C, Kobayashi S, et al. Prenatal exposure to perfluorinated chemicals and relationship with allergies and infectious diseases in infants. Environ Res. 2012;112:118–25.

141. Wang I-J, Hsieh W-S, Chen C-Y, Fletcher T, Lien G-W, Chiang H-L, et al. The effect of prenatal perfluorinated chemicals exposures on pediatric atopy. Environ Res. 2011;111:785–91.

142. US ATSDR. Public Health Statement Polychlorinated Biphenls (PCBs) [Internet]. 2000 [cited 2014 Sep 22]. Available from: www.atsdr.cdc.gov/ToxProfiles/tp17-c1-b.pdf

143. Gaspar-Ramírez O, Pérez-Vázquez FJ, Pruneda-Álvarez LG, Orta-García ST, González-Amaro R, Pérez-Maldonado IN. Effect of polychlorinated biphenyls 118 and 153 on Th1/Th2 cells differentiation. Immunopharmacol Immunotoxicol. 2012;34:627–32.

144. Hansen S, Strøm M, Olsen SF, Maslova E, Rantakokko P, Kiviranta H. Maternal concentrations of persistent organochlorine pollutants and the risk of asthma in offspring: Results from a prospective cohort with 20 years. Environ Health Perspect. 2014;122:93–9.

145. Stølevik SB, Nygaard UC, Namork E, Haugen M, Kvalem HE, Meltzer HM, et al. Prenatal exposure to polychlorinated biphenyls and dioxins is associated with increased risk of wheeze and infections in infants. Food Chem Toxicol. 2011;49:1843–8.

146. Weisglas-Kuperus N, Vreugdenhil HJI, Mulder PGH. Immunological effects of environmental exposure to polychlorinated biphenyls and dioxins in Dutch school children. Toxicol Lett. 2004;149:281–5.

147. Environment Canada. Plans and Policies. 2014. Available from: http://www.ec.gc.ca/lcpe-cepa/default.asp?lang=En&n=3E4FEE25-1

148. US ATSDR. Public health statement for DDT,DDE, and DDD. 2011. Available from: http://www.atsdr.cdc.gov/phs/phs.asp?id=79&tid=20

149. US ATSDR. Toxicological profile for DDT, DDE, and DDD. 2002 [cited 2014 Jul 9]. Available from: http://www.atsdr.cdc.gov/toxprofiles/tp35.pdf

150. Corsini E, Sokooti M, Galli CL, Moretto a, Colosio C. Pesticide induced immunotoxicity in humans: a comprehensive review of the existing evidence. Toxicology. 2013;307:123–35.

151. Noakes PS, Taylor P, Wilkinson S, Prescott SL. The relationship between persistent organic pollutants in maternal and neonatal tissues and immune responses to allergens: A novel exploratory study. Chemosphere. 2006;63:1304–11.

152. Sunyer J, Garcia-Esteban R, Alvarez M, Guxens M, Goñi F, Basterrechea M, et al. DDE in mothers' blood during pregnancy and lower respiratory tract infections in their infants. Epidemiology. 2010;21:729–35.

153. Gascon M, Sunyer J, Casas M, Martínez D, Ballester F, Basterrechea M, et al. Prenatal exposure to DDE and PCB 153 and respiratory health in early childhood: A meta-Analysis. Epidemiology. 2014;25:544–53.

154. Lu C, Barr DB, Pearson MA, Waller LA. Dietary intake and its contribution to longitudinal organophosphorus pesticide exposure in urban/suburban children. Environ Health Perspect. 2008;116:537–42.

155. Stout DM, Bradham KD, Egeghy PP, Jones P a, Croghan CW, Ashley PA, et al. American healthy homes survey: a national study of residential pesticides measured from floor wipes. Environ Sci Technol. 2009;43:4294–300.

156. Lambert WE, Lasarev M, Muniz J, Scherer J, Rothlein J, Santana J, et al. Variation in organophosphate pesticide metabolites in urine of children living in agricultural communities. Environ Health Perspect. 2005;113:504–8.

157. US ATSDR. Cholinesterase Inhibitors [Internet]. 2012 [cited 2014 Sep 22]. Available from: www.atsdr.cdc.gov/csem/csem.asp?csem=11&po=5.

158. Fukuyama T, Tajima Y, Hayashi K, Ueda H, Kosaka T. Prior or coinstantaneous oral exposure to environmental immunosuppressive agents aggravates mite allergen-induced atopic dermatitis-like immunoreaction in NC/Nga mice. Toxicology. 2011;289(2-3):132–40.

159. Hoppin JA, Umbach DM, London SJ, Lynch CF, Alavanja MCR, Sandler DP. Pesticides associated with wheeze among commercial pesticide applicators in the Agricultural Health Study. Am J Epidemiol. 2006;163:1129–37.

160. Hoppin JA, Umbach DM, London SJ, Henneberger PK, Kullman GJ, Coble J, et al. Pesticide use and adult-onset asthma among male farmers in the Agricultural Health Study. Eur Respir J. 2009;34:1296–303.

161. Arbuckle T, Fraser W. Cohort Profile: The Maternal-Infant Research on Environmental Chemicals Research Platform. Paediatr Perinat Epidemiol. 2013;27:415–25.

162. Government of Canada. Chemical Substances. 2012 [cited 2014 Sep 22]. Available from: http://www.chemicalsubstanceschimiques.gc.ca/plan/plan-eng.php

163. Holsapple MP, Paustenbach DJ, Charnley G, West LJ, Luster MI, Dietert RR, et al. Symposium summary: children's health risk--what's so special about the developing immune system? Toxicol Appl Pharmacol. 2004;199:61–70.

164. Fu Y, Lou H, Wang C, Lou W, Wang Y, Zheng T, et al. T cell subsets in cord blood are influenced by maternal allergy and associated with atopic dermatitis. Pediatr Allergy Immunol. 2013;24:178–86.

165. Yoo J, Omori M, Gyarmati D, Zhou B, Aye T, Brewer A, et al. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. J Exp Med. 2005;202:541–9.

166. Cevikbas F, Steinhoff M. IL-33: a novel danger signal system in atopic dermatitis. J Invest Dermatol. 2012;132:1326–9.

167. Pastorelli L, De Salvo C, Cominelli M a, Vecchi M, Pizarro TT. Novel cytokine signaling pathways in inflammatory bowel disease: insight into the dichotomous functions of IL-33 during chronic intestinal inflammation. Therap Adv Gastroenterol. 2011;4:311–23.

168. Kynyk JA, Mastronarde JG, McCallister JW. Asthma, the sex difference. Curr Opin Pulm Med. 2011;17:6–11.

169. Arbuckle TE, Fraser WD, Fisher M, Davis K, Liang CL, Lupien N, et al. Cohort Profile : The Maternal-Infant Research on Environmental Chemicals Research Platform. Paediatr Perinat Epidemiol 2013; 27:415–25.

170. World Health Organization. WHO BMI Classification [Internet]. 2006 [cited 2014 Sep 8]. Available from: http://apps.who.int/bmi/index.jsp?introPage=intro_3.html

171. Seagroatt V, Anderson S. The second international reference preparation for human serum immunoglobulin E and the first British standard for human serum immunoglobulin E. J Biol Stand. 1981;4:431–7.

172. Amarasekera M. Immunoglobulin E in health and disease. Asia Pac Allergy. 2011;1:12–5.

173. Rasmussen F, Hancox RJ. Mechanisms of obesity in asthma. Curr Opin Allergy Clin Immunol. 2014;14:35–43.

174. Reilly JJ, Armstrong J, Dorosty AR, Emmett PM, Ness A, Rogers I, et al. Early life risk factors for obesity in childhood : cohort study. BMJ. 2005;330:1357.

175. Westman M, Kull I, Lind T, Melén E, Stjärne P, Toskala E, et al. The link between parental allergy and offspring allergic and nonallergic rhinitis. Allergy. 2013 68:1571–8.

176. Cesaroni G, Badaloni C, Porta D, Forastiere F, Perucci C a. Comparison between various indices of exposure to traffic-related air pollution and their impact on respiratory health in adults. Occup Environ Med. 2008;65:683–90.

177. Scirica CV, Gold DR, Ryan L, Abulkerim H, Celedón JC, Platts-Mills TE, et al. Predictors of cord blood IgE levels in children at risk for asthma and atopy. J Allergy Clin Immunol. 2007;119:81–8.

178. Manfreda J, Becklake MR, Sears MR, Chan-Yeung M, Dimich-Ward H, Siersted HC, et al. Prevalence of asthma symptoms among adults aged 20-44 years in Canada. CMAJ. 2001;164:995–1001.

179. Holladay S, Smialowicz R. Development of the murine and human immune system: differential effects of immunotoxicants depend on time of exposure. Env Heal Perspect2. 2000;108:463–73.

180. Prescott SL, Clifton V. Asthma and pregnancy: emerging evidence of epigenetic interactions in utero. Curr Opin Allergy Clin Immunol. 2009;9:417–26.

181. Kimber I, Dearman RJ. An assessment of the ability of phthalates to influence immune and allergic responses. Toxicology. 2010;271:73–82.

182. Fletcher D, Steenland K, Savitz D. Status Report: PFOA and immune biomarkers in adults exposed to PFOA in drinking water in the mid Ohio valley. 2009. Available from: http://www.c8sciencepanel.org/pdfs/Status_Report_C8_and_Immune_markers_March2009.pdf

183. NHANES. Fourth national report on human exposure to environmental chemicals [Internet]. 2013 [cited 2014 May 20]. Available from: http://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Sep2013.pdf,

184. Saravanabhavan G, Guay M, Langlois É, Giroux S, Murray J, Haines D. Biomonitoring of phthalate metabolites in the Canadian population through the Canadian Health Measures Survey (2007 – 2009). Int J Hyg Environ Health. 2013;216:652–61.

185. Health Canada. Second Report on Human Biomonitoring of Environmental Chemicals in Canada. 2013 [cited 2014 Sep 4]. Available from: http://www.hc-sc.gc.ca/ewh-semt/pubs/contaminants/chms-ecms-cycle2/index-eng.php

186. Mørck TJ, Sorda G, Bechi N, Rasmussen BS, Nielsen JB, Ietta F, et al. Placental transport and in vitro effects of Bisphenol A. Reprod Toxicol. 2010;30:131–7.

187. US ATSDR. Toxicological profile for diethyl phthalate . 1995 [cited 2014 May 30]. Available from: http://www.atsdr.cdc.gov/toxprofiles/tp73.pdf

188. Li L-X, Chen L, Meng X-Z, Chen B-H, Chen S-Q, Zhao Y, et al. Exposure levels of environmental endocrine disruptors in mother-newborn pairs in China and their placental transfer characteristics. PLoS One. 2013;8:e62526.

189. Wang I-J, Lin C-C, Lin Y-J, Hsieh W-S, Chen P-C. Early life phthalate exposure and atopic disorders in children: a prospective birth cohort study. Environ Int. 2014;62:48–54.

190. Reed CE. The natural history of asthma. J Allergy Clin Immunol. 2006;118:543–8;

191. Ballmer-Weber BK. Value of allergy tests for the diagnosis of food allergy. Dig Dis. 2014;32:84–8.

192. Lipozencić J, Wolf R. The diagnostic value of atopy patch testing and prick testing in atopic dermatitis: facts and controversies. Clin Dermatol. 2010;28:38–44.

193. Dietert RR. Developmental immunotoxicology: focus on health risks. Chemical research in toxicology. 2009;22:17–23.

194. Ashley-Martin J, Dodds L, Arbuckle T, Levy A, Platt R, Marshall J. Umbilical cord blood levels and predictors of Interleukin-33, Thymic Stromal Lymphopoietin and Immunoglobulin E. Pediatr Allergy Immunol. 2014;submitted.

195. Bartemes KR, Kita H. Dynamic role of epithelium-derived cytokines in asthma. Clin Immunol. 2012;143:222–35.

196. Herr CEW, Ghosh R, Dostal M, Skokanova V, Ashwood P, Lipsett M, et al. Exposure to air pollution in critical prenatal time windows and IgE levels in newborns. Pediatr Allergy Immunol. 2011;22:75–84.

197. Arbuckle TE, Davis K, Marro L, Fisher M, Legrand M, Leblanc A, et al. Phthalate and bisphenol A exposure among pregnant women in Canada - Results from the MIREC study. Environ Int. 2014;68C:55–65.

198. Hauser R, Calafat A. Phthalates and human health. Occup Environ Med. 2005;62:806–18.

199. Hoppin JA, Jaramillo R, London SJ, Bertelsen RJ, Salo PM, Sandler DP, et al. Phthalate exposure and allergy in the U.S. population: results from NHANES 2005-2006. Environ Health Perspect. 2013;121:1129–34.

200. Just AC, Adibi JJ, Rundle AG, Calafat AM, Camann DE, Hauser R, et al. Urinary and air phthalate concentrations and self-reported use of personal care products among minority pregnant women in New York city. J Expo Sci Environ Epidemiol. 2010;20:625–33.

201. Maclehose RF, Dunson DB, Herring AH, Hoppin JA. Bayesian methods for highly correlated exposure data. Epidemiology. 2007;18:199–207.

202. Spiegelhalter D, Abrams K, Myles J. Bayesian approaches to clinical trials and health-care evalutations. West Sussex, England: John Wiley & Sons Ltd; 2004.

203. Yau C. R Tutorial. 2014 [cited 2014 Jun 5]. Available from: http://www.r-tutor.com/bayesian-statistics/openbugs

204. Desquilbet L, Mariotti F. Dose-response analyses using restricted cubic spline functions in public health research. Stat Med. 2010;29:1037–57.

205. Greenland S, Pearl J, Robins J. Causal diagrams for epidemiologic research. Epidemiology. 1999;10:37–48.

206. Kudo N, Kawashima Y. Toxicity and toxicokinetics of perfluorooctanoic acid in humans and animals. J Toxicol Sci. 2003;28:49–57.

207. Scirica CV, Gold DR, Ryan L, Abulkerim H, Celedón JC, Platts-Mills T a E, et al. Predictors of cord blood IgE levels in children at risk for asthma and atopy. J Allergy Clin Immunol. 2007;119:81–8.

208. Textor J, Hardt J. DAGitty: A graphical tool for analyzing causal diagrams. Epidemiology. 2011;22:745–51.

209. Zalko D, Soto AM, Dolo L, Dorio C, Rathahao E, Debrauwer L, et al. Biotransformations of Bisphenol A in a mammalian model: Answers and new questions raised by low-dose metabolic fate studies in pregnant CD1 mice. Environ Health Perspect. 2003;111:309–19.

210. Ye X, Zhou X, Hennings R, Kramer J, Calafat AM. External contamination with bisphenol A and other ubiquitous organic environmental chemicals during biomonitoring analysis: An elusive laboratory challenge. Environ Health Perspect. 2013;121:283–7.

211. Gelman A, Carlin J, Stern H, Rubin D. Bayesian Data Analysis. 2nd ed. London: Chapman & Hall; 2004.

212. Braun JM, Kalkbrenner AE, Calafat AM, Bernert JT, Ye X, Silva MJ, et al. Variability and predictors of urinary bisphenol A concentrations during pregnancy. Environ Health Perspect. 2011;119:131–7.

213. Ye X, Wong L, Bishop AM, Calafat AM. Variability of urinary concentrations of bisphenol A in spot samples, first morning voids, and 24-hour collections. Environ Health Perspect. 2011;119:983–9.

214. Volkel W, Colnot T, Csana A, Filser JG, Dekant W. Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. Chem Res Toxicol. 2002;15:1281–7.

215. Meeker JD, Cantonwine DE, Rivera-gonzález LO, Ferguson KK, Mukherjee B, Calafat AM, et al. Distribution, variability and predictors of urinary concentrations of phenols and parabens among pregnant women in Puerto Rico. Env Sci Technol. 2014;47:3439–47.

216. Quirós-Alcalá L, Eskenazi B, Bradman A, Ye X, Calafat AM, Harley K. Determinants of urinary bisphenol A concentrations in Mexican/Mexican--American pregnant women. Environ Int. 2013;59:152–60.

217. Salehi F, Turner M, Phillips K, Wigle D, Krewski D, Aronson K. Review of the etiology of breast cancer with special attention to organochlorines as potential endocrine disruptors. J Toxicol Env Heal Part B. 2008;11:276–300.

218. Cantonwine DE, Cordero JF, Rivera-González LO, Anzalota Del Toro L V, Ferguson KK, Mukherjee B, et al. Urinary phthalate metabolite concentrations among pregnant women in Northern Puerto Rico: distribution, temporal variability, and predictors. Environ Int. 2014;62:1–11.

219. Wacholder S. When measurement errors correlate with truth:suprising effects of nondifferential misclassification. Epidemiology. 1995;6:157–61.

220. Muehleisen B, Gallo RL. Vitamin D in allergic disease: shedding light on a complex problem. J Allergy Clin Immunol. 2013;131:324–9.

221. Von Hertzen LC. Maternal stress and T-cell differentiation of the developing immune system: Possible implications for the development of asthma and atopy. J Allergy Clin Immunol. 2002;109:923–8.

222. Zota AR, Calafat AM, Woodruff TJ. Temporal trends in phthalate exposures: findings from the National Health and Nutrition Examination Survey, 2001-2010. Environ Health Perspect. 2014 122:235–41.

223. Health Canada. Phthalate regulations [Internet]. 2011 [cited 2014 Jun 2]. Available from: http://www.hc-sc.gc.ca/ahc-asc/media/nr-cp/_2011/2011_07fs-eng.php

224. Health Canada. Bisphenol A [Internet]. 2010. Available from: http://www.hc-sc.gc.ca/ahc-asc/media/nr-cp/_2011/2011_07fs-eng.php

225. Glynn A, Berger U, Bignert A, Ullah S, Aune M, Lignell S, et al. Perfluorinated alkyl acids in blood serum from primiparous women in Sweden: serial sampling during pregnancy and nursing, and temporal trends 1996-2010. Environ Sci Technol. 2012;46:9071–9.

226. Okada E1, Kashino I, Matsuura H, Sasaki S, Miyashita C, Yamamoto J, Ikeno T, Ito YM, Matsumura T, Tamakoshi A KR. Temporal trends of perfluoroalkyl acids in plasma samples of pregnant women in Hokkaido, Japan, 2003-2011. Env Int. 2013;60:89–96.

227. 3M. What is 3M Doing? [Internet]. 2014 [cited 2014 Jun 2]. Available from: http://solutions.3m.com/wps/portal/3M/en_US/PFOS/PFOA/Information/Action

228. Health Canada. Perfluorooctane Sulfonate (PFOS) and Health [Internet]. 2007 [cited 2014 Jun 2]. Available from: http://www.hc-sc.gc.ca/ewh-semt/pubs/contaminants/perflurooctane_sulfonate-eng.php

229. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee D-H, et al. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr Rev. 2012;33:378–455.

230. WHO IPCS Harmonization Project. Guidance for immunotoxicity risk assessment for chemicals [Internet]. 2012 [cited 2014 Jul 10]. Available from: http://www.who.int/ipcs/methods/harmonization/areas/guidance_immunotoxicity.pdf?ua=1

231. Vine MF, Stein L, Weigle K, Schroeder J, Degnan D, Tse CJ, et al. Plasma 1,1-Dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE) levels and immune response. Am J Epidemiol. 2001;153:53–63.

232. Statistics Canada. Canadian Health Measures Survey: Cycle 2 Data Tables 2009 to 2011 [Internet]. 2013 [cited 2014 Jul 9]. Available from: http://www.statcan.gc.ca/pub/82-626-x/2013001/t060-eng.pdf

233. Dyke PH, Foan C, Fiedler H. PCB and PAH releases from power stations and waste incineration processes in the UK. Chemosphere. 2003;50:469–80.

234. Henderson J, Granell R, Heron J, Sherriff a, Simpson a, Woodcock a, et al. Associations of wheezing phenotypes in the first 6 years of life with atopy, lung function and airway responsiveness in mid-childhood. Thorax. 2008;63:974–80.

235. Taussig LM, Wright AL, Holberg CJ, Halonen M, Morgan WJ, Martinez FD. Tucson children's respiratory study: 1980 to present. J Allergy Clin Immunol. 2003;111:661–75.

236. Sugiyama M, Arakawa H, Ozawa K, Mizuno T, Mochizuki H, Tokuyama K, et al. Early-life risk factors for occurrence of atopic dermatitis during the first year. Pediatrics. 2007;119:e716–23.

237. Been JV, Lugtenberg MJ, Smets E, van Schayck CP, Kramer BW, Mommers M, et al. Preterm birth and childhood wheezing disorders: a systematic review and meta-analysis. PLoS Med. 2014 Jan;11:e1001596.

238. Algert CS, Bowen JR, Lain SL, Allen HD, Vivian-Taylor JM, Roberts CL. Pregnancy exposures and risk of childhood asthma admission in a population birth cohort. Pediatr Allergy Immunol. 2011;22:836–42.

239 . Arcury TA, Grzywacz ÃJG, Davis SW, Barr DB, Quandt SA. Organophosphorus pesticide urinary metabolite levels of children in farmworker households in eastern North Carolina. Am J Ind Med. 2006;49:751–60.

240. Cole SR, Chu H, Nie L, Schisterman EF. Estimating the odds ratio when exposure has a limit of detection. Int J Epidemiol. 2009;38:1674–80.

241. Wolff MS, Deych E, Ojo F, Berkowitz GS. Predictors of organochlorines in New York City pregnant women, 1998-2001. Environ Res. 2005;97:170–7.

242. Bradman A, Castorina R, Barr DB, Chevrier J, Harnly ME, Eisen E, et al. Determinants of organophosphorus pesticide urinary metabolite levels in young children living in an agricultural community. Int J Environ Res Public Health. 2011;8:1061–83.

243. Rylander C, Lund E, Frøyland L, Sandanger TM. Predictors of PCP, OH-PCBs, PCBs and chlorinated pesticides in a general female Norwegian population. Environ Int. 2012;43:13–20.

244. Callan AC, Hinwood a L, Ramalingam M, Boyce M, Heyworth J, McCafferty P, et al. Maternal exposure to metals--concentrations and predictors of exposure. Environ Res. 2013;126:111–7.

245. Taylor CM, Golding J, Hibbeln J, Emond AM. Environmental factors predicting blood lead levels in pregnant women in the UK: the ALSPAC study. PLoS One. 2013;8:e72371.

246. Razzaghi H, Tinker SC, Crider K. Blood mercury concentrations in pregnant and nonpregnant women in the United States: National Health and Nutrition Examination Survey 1999-2006. Am J Obstet Gynecol. 2014;210:357.e1–9.

247. Schisterman EF, Whitcomb BW, Buck Louis GM, Louis T. Lipid adjustment in the analysis of environmental contaminants and human health risks. Environ Health Perspect 2005;113:853–7.

248. Miller TE, Golemboski K a, Ha RS, Bunn T, Sanders FS, Dietert RR. Developmental exposure to lead causes persistent immunotoxicity in Fischer 344 rats. Toxicol Sci. 1998;42:129–35.

249. Gao D, Mondal TK, Lawrence D. Lead effects on development and function of bone marrow-derived dendritic cells promote Th2 immune responses. Toxicol Appl Pharmacol. 2007;222:69–79.

250. Jedrychowski W, Perera F, Maugeri U, Miller RL, Rembiasz M, Flak E, et al. Intrauterine exposure to lead may enhance sensitization to common inhalant allergens in early childhood: a prospective prebirth cohort study. Environ Res.2011;111:119–24.

251. Karmaus W, Brooks KR, Nebe T, Witten J, Obi-Osius N, Kruse H. Immune function biomarkers in children exposed to lead and organochlorine compounds: a cross-sectional study. Environ Health. 2005;4:5.

252. Hon K-LE, Wang SS, Hung ECW, Lam HS, Lui HHK, Chow C-M, et al. Serum levels of heavy metals in childhood eczema and skin diseases: friends or foes. Pediatr Allergy Immuno]. 2010;21:831–6.

253. Weidinger S, Kramer U, Dunnemann L, Mohrenschlager M, Ring J, Behrendt H. Body burden of mercury is associated with acute atopic eczema and total IgE in children from southern Germany. J Allergy Clin Immunol. 2004;114:457–457.

254. Farzan SF, Korrick S, Li Z, Enelow R, Gandolfi J, Madan J, et al. In utero arsenic exposure and infant infection in a United States cohort: a prospective study. Environ Res . 2013;126:24–30.

255. Leung TF, Chan IHS, Liu TC, Lam CWK, Wong GWK. Relationship between passive smoking exposure and urinary heavy metals and lung functions in preschool children. Pediatr Pulmonol. 2013;48:1089–97.

256. Environment EC on Polychlorinated biphenyls and polychlorinated terphenyls (PCBs / PCTs). 2014 [cited 2014 Jul 14]. Available from: http://ec.europa.eu/environment/waste/pcbs/index.htm

257. Rauch SA, Braun JM, Barr DB, Calafat AM, Khoury J, Montesano MA. Associations of prenatal exposure to organophosphate pesticide metabolites with gestational age and birth weight. Env Heal Perspect. 2012;120(7):1055–61.

258. US ATSDR. Draft toxicological profile for hexachlorobenzene [Internet]. 2013 [cited 2014 Jul 11]. Available from: http://www.atsdr.cdc.gov/toxprofiles/tp90.pdf

259. Health Canada. What is lead [Internet]. 2013 [cited 2014 Jul 9]. Available from: http://www.hc-sc.gc.ca/ewh-semt/contaminants/lead-plomb/index-eng.php

260. Environment Canada. Risk management strategy for mercury [Internet]. 2010 [cited 2014 Jul 14]. Available from: http://www.ec.gc.ca/doc/mercure-mercury/1241/index_e.htm#goto313

261. National Research Council. Risk assessment in the federal government: Manageing the Process [Internet]. 1983 [cited 2011 Nov 10]. Available from: http://www.nap.edu/openbook.php?record_id=366&page=1

262. Hertz-Piccioto I. Epidemiology and quantitative risk assessment: A bridge from science to policy. Am J Public Heal. 1995;85:484–91.

263. Thomas D. Statistical Methods in Environmental Epidemiology. New York: Oxford University Press; 2009.

264. Health Canada. Assessment and management of risks from radiological and chemical hazards [Internet]. 1998 [cited 2011 Nov 14]. Available from: http://www.hc-sc.gc.ca/ewh-semt/pubs/radiation/98ehd-dhm216/index-eng.php

265. Calderon RL. Measuring risks in humans: the promise and practice of epidemiology. Food Chem Toxicol. 2000; 38:S59–63.

266. International Agency Research Cancer. IARC Monographs on the evaluation of carcinogenic risks to humans [Internet]. 2006 [cited 2011 Nov 23]. Available from: http://monographs.iarc.fr/ENG/Preamble/CurrentPreamble.pdf

267. Institute of Medicine. Breast cancer and the environment. The scientific evidence, research methodology, and future directions. [Internet]. 2011 [cited 2011 Dec 8]. Available from: http://www.nap.edu/catalog.php?record_id=13263

268. US EPA. Guidelines for carcinogen risk assessment. 2005; Available from: http://www.epa.gov/raf/publications/pdfs/CANCER_GUIDELINES_FINAL_3-25-05.PDF [November23, 2011

269. US EPA. Framework for cumulative risk assessment [Internet]. 2003 [cited 2011 Nov 23]. Available from: http://www.epa.gov/raf/publications/framework-cra.htm

270. US EPA. Glossary [Internet]. 2014 [cited 2014 Sep 22]. Available from: www.epa.gov/risk_assessment/glossary.htm#u

271. Health Canada. Determination of "Toxic" for the purposes of new substances provisions (Chemicals and polymers) under then Canadian Environmental Protection Act [Internet]. 2007 [cited 2011 Nov 14]. Available from: http://www.hc-sc.gc.ca/ewh-semt/pubs/contaminants/toxic-toxique/index-eng.php

272. Abt E, Rodricks J V, Levy JI, Zeise L, Burke T . Science and decisions: advancing risk assessment. Risk Anal. 2010;30:1028–36.

273. Health Canada. Final human health state of the science report on lead [Internet]. 2013 [cited 2014 Sep 22]. Available from: www.hc-sc.gc.ca/ewh-semt/pubs/contaminants/dhhssrl-rpecsecsh/index-eng.php#a12

274. Rice D, Schoeny R, Mahaffey K. Methods and rationale for derivation of a reference dose for methylmercury by the US EPA. Risk Anal. 2003;23:107–15.

275. Canfield R, Henderson C, Cory-slechta D, Cox C, Jusko T, Lanphear BP. Intellectual impairment in children with blood lead concentrations below 10ug per deciliter. N Engl J Med. 2003;348:1517–26.

276. Phillips KP, Foster WG, Leiss W, Sahni V, Karyakina N, Turner MC, et al. Assessing and managing risks arising from exposure to endocrine-active chemicals. J Toxicol Env Heal Part B. 2008;11:351–72.

277. Calabrese EJ. Hormetic dose-response relationships in immunology: occurrence, quantitative features of the dose response, mechanistic foundations, and clinical implications. Crit RevToxicol. 2005;35:89–295.

278. Woodruff TJ, Burke T a, Zeise L. The need for better public health decisions on chemicals released into our environment. Health Aff. 2011;30:957–67.

279. National Research Council. Phthalates and cumulative risk assessment [Internet]. 2008 [cited 2014 Sep 26]. Available from: www.nap.edu/download.php?record_id=12528#

280. Sexton K, Hattis D. Assessing cumulative health risks from exposure to environmental mixtures - three fundamental questions. Environ Health Perspect. 2007;115:825–32.

281. US ATSDR. Interaction profiles for toxic substances [Internet]. 2011 [cited 2012 May 7]. Available from: http://www.atsdr.cdc.gov/interactionprofiles/index.asp

282. Weinberg CR. Interaction and Exposure Modification: Are We Asking the Right Questions? Am J Epidemiol. 2012; 175:602-5

283. Greenland S. Basic problems in interaction assessment. Environ Health Perspect. 1993;101 Suppl 59–66.

284. Environment Canada. 2012 Air pollutant emission summaries and historical emission trends [Internet]. 2014 [cited 2014 Oct 2]. Available from: http://www.ec.gc.ca/inrp-npri/default.asp?lang=En&n=F98AFAE7-1

285. UN. Canada National reporting to CSD-18/19 thematic profile on chemicals [Internet]. 2011 [cited 2014 Oct 2]. Available from: http://www.un.org/esa/dsd/dsd_aofw_ni/ni_pdfs/NationalReports/canada/Chemicals.pdf

286. Dietert R, DeWitt J. Developmental immunotoxicity (DIT): The why, when, and how of DIT testing. Methods Mol Biol. 2010;598:17–25.

287. Dietert RR, Zelikoff JT. Early-life environment, developmental immunotoxicology, and the risk of pediatric allergic disease including asthma. Birth Defects Res B Dev Reprod Toxicol. 2008;83:547–60.

288. Dietert RR, DeWitt JC, Germolec DR, Zelikoff JT. Breaking patterns of environmentally influenced disease for health risk reduction: immune perspectives. Environ Health Perspect. 2010;118:1091–9.

289. Dietert RR, Holsapple MP. Methodologies for developmental immunotoxicity (DIT) testing. Methods. 2007;41:123–31.

Appendix A. ELISA Protocol

Generic ELISA protocol (modified for Jillian's analysis):

Reagents:

A) 0.1M Sodium Bicarbonate Coating Buffer:

50 mL ddH_20
0.42g $NaHCO_3$
1.46g NaCl
- pH to 8.3- 8.5.
- Make once every week.

B) Washing buffers

i. 0.05% Tween 20 in PBS

C) Blocking Buffer:

2% BSA in PBS

D) Assay Buffer:

0.1% Tween-20, 0.2% BSA, in PBS. (do a 1/10 dilution of Blocking buffer with PBS before adding Tween-20).

TOTAL Assay buffer required for 8 plates= samples (40 ml) + standards (20 ml) + 2nd Ab (48 ml) + HRP Avidin (only used for 4 plates) (24 ml) = 132 ml

H) Stop solution

- 2 N H_2SO_4
  - add acid slowly to water as it is a strong acid and generates a lot of heat; **DO NOT ADD WATER TO ACID.**

ELISA PROTOCOL:

Day one

1. Make coating buffer. For 3 runs of 8 plates/run, at 6 ml/plate, 24*6=144 ml. Make 200 ml total – quadruple above recipe to be:

   200 mL ddH_20
   1.68 g $NaHCO_3$
   5.84g NaCl make in 2 100 ml bottles

2. Coat ELISA plate with 50ul 1st AB (coating buffer is the diluent) over night at +4°C. Gently tap sides of plate to ensure even coating of wells. Seal plate with para-film.

Antibody	Stock Conc.	Des. Conc.	Calc. Volume/1 plate	Volume/2 plates
IL-33	144 ug/mL	0.4 ug/mL	16.67 ul	33.3 ul
TSLP		1:200	30 ul	60 ul
IgE		1:250	24 ul	48 ul
IgA		1:250	24 ul	48ul

*(vol. (6 mL) *Des Conc.))/stock conc.) add to 6 mL coating buffer, mix, and add 50 ul/well (assuming 1 plate)
* For running 8 plates, add 12 ml of coating buffer to each 15 ml tubes, then add corresponding amount of 1st antibody. Vortex briefly.

*Determine what samples will be analyzed during run. Create plate layout sheets of samples. Print out elisa layout sheets with all sample ids to mark on during elisa runs. Keep all layout sheets in binder on Yisong's desk

Day two

1. Make 2% BSA/PBS. Yisong will make 500 ml 2% BSA/PBS, filter it, then aliquot them to 50 mL tubes to keep at 4° C.

2. Wash plate with 0.05% Tween 20 /PBS 3 times. (wash plates in batches of 4 at a time, fill all then dump)

3. Block plate with 100ul 2% BSA/PBS blocking buffer for 1-2 hr at R/T.

4. Dilute remaining 2% BSA/PBS to 0.2% and add 0.1% Tween 20. Give Yisong at least 70 ml 0.2% diluents for standard and sample dilution. Keep remainder for 2nd Ab dilution on day 3.

5. See Yisong's protocol for CBMC sample dilution. Wash plate with 0.05% Tween 20 /PBS 3 times

Antibody	Stock Conc. STANDARD	Dilution	0.2% diluent to add to Tube A	0.2% diluent to add to Tube B-H	Highest Conc.	Calc. Volume std. add to Tube A	Serial transfer volume (LV. TUBE h BLK)	Total diluents vol. needed
IL-33	1 ug/mL	1:2	2000 ul	500 ul	1000 pg/mL	2 ul	500 ul	4.5 ml
TSLP	75 ng/Ml (new lot # B60293)	1:2	1000 ul	500 ul	250 pg/mL	3.3 ul	500 ul	4.5 ml
	30 ng/mL (lot# B149882)					8.3 ul		
IgE	1000 ng/mL	1:2	875 ul	500 ul	125 ng/mL	125 ul	500 ul	4.375 ml
IgA	200 ng/mL	1:2	350	700 ul	100 ng/mL	500 ul	332.5 ul	3.2 mL

Add 50 ul to columns 1-2

Day three

1. Wash plates with 0.05% Tween 20 /PBS 3 times. Dump contents of plate into 10% bleach solution in basin in sink.

2. Add 50ul 2nd Ab (biotinylated) (0.2% BSA/PBS assay buffer with 0.1% Tween 20 is the diluent) to plate. Incubate plate at R/T for 1 hr.

Antibody	Stock Conc.	Des. Conc.	Calc. Volume/1 plate	Volume/2 plates
IL-33	36 ug/mL	50 ng/mL	8.3 ul	16.6
TSLP		1:200	30 ul	60 ul
IgE		1:125	48 ul	96 ul
IgA		1:250	24 ul	48 ul

3. Wash plate with 0.05% Tween 20 /PBS 3 times.

4. Amplification system differs for IgE/IgA and TSLP/IL-33.
Start development for TSLP/IL-33 plates first since they take longer.

Development Plan

	TSLP / IL33			IgE/IgA	
Starting Time	Job	Duration	Starting Time	Job	Duration
9:00	**Prepare 2nd Ab, wash plates, load 2nd Ab**	30 Min.			
9:30	Incubation	1 hr	9:30	**Prepare 2nd Ab, wash plates, load 2nd Ab**	30 min.
10:00	**Wash plates, prepare and load Avidin-HRP (4 plates – 24 ml)**	30 min.	10:00	Incubate	1 hr
10:30	Incubate	30 min.	10:30	**Wash plates, load substrate**	30 min.
11:00	**Wash plates, load substrate**	30 min.	11:00	Incubate	15 minutes IgA room T 30 minutes IgE 37 degrees
11:30	Incubate in dark	15 minutes – Watch until blue turns dark	11:30	**Stop**	
12:00	**Stop**		12:00		
		READ on Gene V Wavelength 450			

TSLP/IL-33

5. Dilute Avidin-HRP in diluent (0.2% PBS/BSA w/ Tween 20) (1:1000).
   For two plates, dilute 12 ul HRP in 12 ml diluent.
   For four plates, dilute 24 ul HRP in 24 diluent.

6. .Add 50 ul of Avidin-HRP to TSLP and IL-33 plates. Incubate for 30 minutes.

7. Wash with wash buffer.

8. Add 50 ul TMB substrate solution and incubate in the dark for 15 minutes.
   For 2 plates, 12 ml required, For 4 plates, 24 ml required.

9.Add 50 ul stop solution. Read at 450 nm.

IgE/IgA

4.Add 50 ul of kit Substrate solution. Incubate 15 minutes.
For 2 plates, 12 ml required, for 4 plates, 24 ml required.

5. Add 50 ul of stop solution. Read at 450 nm.

Analysis

Use GeneV softwar

Determination of Limits of Detection for ELISA curves:
The lower limit of detection of IL-33 and TSLP was set to the lowest point on the standard curve. IL-33 = 63 pg/ml TSLP = 45 pg/mL

To extend the sensitivity of the IgE curve, detectable samples were defined as all samples that were statistically different (95%) from zero. This was determined by the following formula.

[(sample mean) - 2*(sample SD)] - standard curve mean concentration at 0.0001

I used the standard concentration that corresponded to each sample. Many times even though the standard is defined as 0.001, the software gives a very low concentration e.g. 0.4 - so I standardized each sample to be 2 SDs above whatever concentration was given for 0.0001. All samples with one duplicate <0.0001 were excluded because an SD can't be calculated with one value.

Quality Assurance Practices

- Storage/Freeze-thaw
  - All samples stored at -80° C
  - All biomarkers analyzed simultaneously to minimize freeze-thaw cycles
  - Analysis done over condensed time period

- Inter-assay Variation
  - 1 technician diluted and loaded all samples
  - Analysis done over condensed time period

- Intra-assay Variation
  - All samples run in duplicate
  - Standard curve and sample control run in duplicate on every plate
  - All plates analyzed using reference wave length

- CV=Coefficient of Variation= (Mean SD/Mean Conc)*100
- Inter (between plates): CV values from standard curve
- Intra (within plates): CV values from sample control duplicates