Immunotoxic effects of dietary toxicants:

focus on prenatal exposure to

acrylamide, polychlorinated biphenyls and dioxins

by

Solvor Berntsen Stølevik

Dissertation submitted for the degree of Philosophiae Doctor

Faculty of Medicine

University of Oslo

Department of Environmental Immunology

Division of Environmental Medicine

Norwegian Institute of Public Health

2012

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1325

ISBN 978-82-8264-298-9

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Unipub.
The thesis is produced by Unipub merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

‘It would be nice if the Food and Drug Administration
stopped issuing warnings about toxic substances
and just gave me the names of one or two things
still safe to eat.’

Robert Fuoss (1912-1980)

Acknowledgements

The present work was funded by the EU integrated project NewGeneris and the Norwegian Institute of Public Health (NIPH). I want to specially thank the former Department of Environmental Immunology (MIMI) at the NIPH for excellent working facilities.

To my splendid supervisors at the NIPH; thank you so much for your great guidance and support through all these years in this 'not so straightforward' project. I have learned a lot from you. You really complement each other.

- Professor Martinus Løvik, thank you for all useful discussions and for your valuable contribution with up to date knowledge, information about the history of immunotoxicology and your special capability to improve manuscripts and the thesis. I want to thank you for always listening to my opinions, thoughts and questions, and for all your optimism and labour to give me finance through these years.
- Dr. Unni Cecilie Nygaard, Dr. Berit Granum and Dr. Ellen Namork, thank you for always having time for me, for all the hours with valuable discussions, frustrations, critical reading of manuscripts and the thesis, and social events such as lunch, conferences and meetings. Special thanks to Unni for all your support and work within the *in vitro* study, for always being positive and for keeping me company on several flights. Berit, I want to give you special thanks for all your work with the establishment of the BraMat cohort and during the follow-up study. Ellen, you have given me excellent guidance on English writing, and thank you for your valuable assistance with laboratory work and telephone interviews. Finally, I will thank all three of you for being my 'guarding angels' and your never ending patience with me.

I have been very lucky to get to know several fantastic people both at the NIPH and from other places in Europe during these years. You have all contributed to make these years valuable and unforgettable. At the NIPH, I will specially express my gratitude to my colleagues at MIMI. You have all contributed to excellent working environment. Anneli Pellerud and Bodil Hasseltvedt, you have performed excellent work regarding the *in vitro* study and immunophenotyping of a lot of blood samples. I will also give my greatest thanks to Åse Eikeset, Berit Arvesen Stensby, Astri Grestad and Else-Carin Groeng for your assistance with laboratory work. Nina E. Vinje, thank you for all your encouraging words and all cosy dinners at the NIPH. Monica Andreassen, Dr. Randi J. Bertelsen and Dr. Johanna

Bodin, thank you for all good talks and discussions. Dr. Jitka Stilund Hansen, Dr. Gro Tunheim and Lisa Nome, you are the absolutely best ‘roommates’!

I will express my great gratitude to all co-authors. It has been very interesting and informative to work together with all of you. Professor Henk van Loveren, thank you for contributing with your knowledge in discussions, interpretation of the results and writing of the manuscripts. Dr. Margaretha Haugen, thank you for your contribution with the exposure calculations and knowledge.

I want to express my gratitude to the children and their parents participating in the BraMat birth cohort. Furthermore, I want to thank everyone who has contributed to the establishment of the BraMat cohort, blood sampling and laboratory work. I will also thank Hans Christian Dalsbotten Aass and Petter Mowinckel at Oslo University Hospital, Ullevål, for performing the cytokine release analyses and statistical analysis (mixed model), respectively. Professor Per Nafstad, thank you for being my contact supervisor at the Faculty of Medicine, University of Oslo.

Finally, I want to express my greatest gratitude to my family and friends. Special thanks to Dr. Sveinung B. Stølevik and Dr. Hilde Løvdal, for reading and commenting on the thesis, and my parents who took care of our two children when we needed it the most. Thank you Harald, you are the most understanding man in the world, allowed me to work all day and night when necessary, and gave me what I needed at all times. Last but not least, thank you Kristine and Elias for giving me your love and understanding every day, either face to face or by phone. My family is the best!

Oslo, May 2012
Solvor B. Stølevik

Contents

SUMMARY **1**

ABBREVIATIONS **3**

LIST OF PAPERS **4**

1 INTRODUCTION **5**

1.1 THE IMMUNE SYSTEM 5
1.1.1 Immune cells and mediators *5*
1.1.2 Immune response *6*
1.2 IMMUNOTOXICOLOGY 7
1.2.1 Study approaches in immunotoxicology *9*
1.3 THE DEVELOPING IMMUNE SYSTEM 10
1.4 TOXICANTS IN FOOD 11
1.4.1 PCBs and dioxins *12*
1.4.2 Acrylamide *15*

2 AIMS **17**

3 SUBJECTS AND METHODS **18**

3.1 THE NEWGENERIS PROJECT 18
3.2 SUBJECTS 18
3.3 IN VITRO STUDY 19
3.4 BRAMAT STUDY DESIGN 21
3.5 QUESTIONNAIRES AND HEALTH OUTCOMES 21
3.6 DIETARY EXPOSURE ASSESSMENT 22
3.7 BLOOD ANALYSES 23
3.8 STATISTICAL ANALYSES 23
3.9 ETHICAL ISSUES 25

4 RESULTS **27**

4.1 IN VITRO EXPOSURE AND CYTOKINE RELEASE 27
4.2 PRENATAL EXPOSURE AND IMMUNOTOXICITY IN CHILDREN 27
4.2.1 PCBs and dioxins – health outcomes *29*
4.2.2 PCBs and dioxins – blood parameters at three years of age *30*
4.2.5 Acrylamide *31*

5 METHODOLOGICAL CONSIDERATIONS **36**

5.1 IN VITRO ASSAY 36
5.2 QUESTIONNAIRES 37
5.3 BLOOD PARAMETERS 38
5.4 STATISTICAL ANALYSES 39

6 DISCUSSION **41**

6.1 IMMUNOTOXICITY OF DIETARY TOXICANTS IN VITRO 41
6.2 PRENATAL EXPOSURE TO ACRYLAMIDE AND IMMUNOTOXICITY 42
6.3 PRENATAL EXPOSURE TO PCBS AND DIOXINS AND IMMUNOTOXICITY 44
6.3.1 Susceptibility to infections *44*
6.3.2 Wheeze *44*
6.3.3 Blood parameters *45*

6.3.4 Foetal stage, a critical time window? *46*
6.3.5 Mechanisms *47*
6.3.6 Confounding exposure factors *49*
6.3.7 Implications of the findings *50*
6.4 FUTURE PERSPECTIVES 50
7 CONCLUSIONS **52**
8 REFERENCES **53**

Summary

Food contains toxicants which may exert adverse effects on the immune system. The result can be immune-related diseases such as allergy, asthma and autoimmune conditions, or increased susceptibility to infectious diseases and cancer. The overall aim of the present work was to investigate the immunotoxic potential of dietary toxicants, with focus on prenatal exposure to toxicants from the maternal diet. Prenatal exposure to immunotoxicants is of particular concern since the foetus may be especially vulnerable due to an extensively developing immune system.

To examine whether release of cytokines in an *in vitro* system can be used as a marker of immunotoxic properties of dietary toxicants, human peripheral blood mononuclear cells were exposed *in vitro* to 12 dietary toxicants. Both immunotoxic and non-immunotoxic substances, classified according to published *in vivo* studies, were included. All 12 dietary toxicants affected the release of one or more of the nine cytokines included, and the exposure to each of the toxicants resulted in different cytokine release patterns. Although the effects on the release of each cytokine were examined separately and in combinations of cytokines, the *in vitro* cytokine release could not be used to differentiate between the immunotoxic and the non-immunotoxic substances.

Of the 12 dietary toxicants, the environmental pollutants polychlorinated biphenyls (PCBs) and dioxins, as well as acrylamide formed during food preparation, were chosen for further examination in a birth cohort. The birth cohort BraMat (n=205), a sub-cohort of the Norwegian Mother and Child Cohort Study (MoBa), was established to investigate whether prenatal exposure to these toxicants from the maternal diet increases the risk of immune-related diseases in the child. The children were followed using annual questionnaires covering health outcomes during their three first years of life. Immune-related blood parameters were examined at three years of age. The maternal intake of the toxicants during pregnancy was calculated using a validated food frequency questionnaire from MoBa, and the levels were assumed to be representative for the general population. Prenatal dietary exposure to PCBs and dioxins was found to be associated with an increased risk of wheeze (periods of more than 10 days with dry cough, chest tightness or wheeze, or shortness of breath), the childhood disease exanthema subitum and more frequent upper respiratory tract infections during the three first years of life. Further, at three years of age, exposure to PCBs and dioxins was found to be associated with a reduced antibody response to measles vaccine. No associations were found between prenatal exposure to PCBs and dioxins and immunophenotype data

including levels of regulatory T cells, allergic sensitization or antibody responses to other vaccines than measles. Prenatal acrylamide exposure was not found to be associated with any of the children's health outcomes or blood parameters.

In conclusion, the *in vitro* study, within the limitations of the study design, does not support the replacement of *in vivo* studies with *in vitro* cytokine release studies for identification of immunotoxic substances. In the BraMat cohort, prenatal exposure to acrylamide from the maternal diet was not found to be associated with immune-related health outcomes or blood parameters, but the statistical power may be too low to conclude on negative findings. However, prenatal exposure to PCBs and dioxins from the maternal diet may increase the risk of wheeze and the susceptibility to infectious diseases during early childhood. Overall, continued efforts to reduce the exposure to PCBs and dioxins from food for women of fertile age may be beneficial for their children's health.

Abbreviations

AhR	aryl hydrocarbon receptor
BaP	benzo[a]pyrene
BMI	body mass index
bw	body weight
CBMC	cord blood mononuclear cells
CD	cluster of differentiation
CI	confidence interval
dl-PCBs	dioxin-like PCBs
ELISA	enzyme-linked immunosorbent assay
FFQ	food frequency questionnaire
FICZ	6-formylindolo[3,2-b]carbazole
Hib	Haemophilus influenzae type B
HSC	hematopoietic stem cell
IL	interleukin
MBRN	the Medical Birth Registry of Norway
MoBa	the Norwegian Mother and Child Cohort Study
ndl-PCBs	non-dioxin-like PCBs
NK cells	natural killer cells
OR	odds ratio
PBMC	peripheral blood mononuclear cells
PCA	principal component analysis
PCBs	polychlorinated biphenyls
PCDDs/PCDFs	polychlorinated dibenzo-*p*-dioxins/dibenzofurans
PHA	phytohemagglutinin
TEQ	toxic equivalents
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
URTI	upper respiratory tract infections

List of papers

The thesis is based on the following original publications (Papers I-III):

Paper I

Stolevik, S.B., Nygaard, U.C., Namork, E., Granum, B., Pellerud, A., van Leeuwen, D.M., Gmuender, H., van Delft, J.H., van Loveren, H., Lovik, M. (2011). *In vitro* cytokine release from human peripheral blood mononuclear cells in the assessment of the immunotoxic potential of chemicals. *Toxicol. In Vitro* ***25****, 555-562.*

Paper II

Stolevik, S.B., Nygaard, U.C., Namork,E., Haugen, M., Kvalem, H.E., Meltzer, H.M., Alexander, J., van Delft, J.H., van Loveren, H., Lovik, M., Granum, B. (2011). Prenatal exposure to polychlorinated biphenyls and dioxins is associated with increased risk of wheeze and infections in infants. *Food Chem. Toxicol.* ***49****, 1843-1848.*

Paper III

Stolevik, S.B., Nygaard, U.C., Namork, E., Haugen, M., Meltzer, H.M., Alexander, J., Knutsen, H.K., Aaberge, I., Vainio, K., van Loveren, H., Lovik, M., Granum, B. Immunosuppressive effects of prenatal exposure to polychlorinated biphenyls and dioxins from the maternal diet persist into early childhood. *Submitted.*

1 Introduction

1.1 The immune system

The immune system is protecting the body against infectious agents and the development of tumors (Bogen and Munthe, 2007; Murphy *et al.*, 2008). The immune system consists of a number of lymphoid organs, a variety of cells and soluble mediators. The lymphoid organs can be divided into central and peripheral lymphoid organs. The central organs consist of the bone marrow and the thymus, and the peripheral organs of the lymph nodes, the spleen and the mucosal lymphoid tissues.

The immune system can be divided into the adaptive and the innate immune system. The adaptive immune system develops an immune response with a high degree of specificity against an infectious agent, which may result in specific, long-lasting protection (immunological memory). In contrast, the innate immune system develops a faster, non-specific response to a wide range of agents which does not result in immunological memory.

1.1.1 Immune cells and mediators

The cells of the immune system are called white blood cells or leukocytes, which can be divided into several types and subtypes with different effector functions (Bogen and Munthe, 2007; Murphy *et al.*, 2008). The two main categories of leukocytes are the myeloid and the lymphoid lineages. The myeloid lineage comprises the majority of the cells of the innate immune system and consists of monocytes, macrophages (mature form of monocytes), granulocytes (neutrophils, basophils and eosinophils), mast cells and dendritic cells. The lymphoid lineage consists of the lymphocytes of the adaptive immune system and the natural killer (NK) cells of the innate immune system. The two main types of lymphocytes are B lymphocytes (B cells) and T lymphocytes (T cells). The immune cells express clusters of differentiation (CD) markers on their surface (membrane molecules), and the various expressions of CD markers on the immune cells can be used to distinguish between the different subtypes and maturation states.

The leukocytes communicate via direct cell-cell contact and via secretion and binding of cytokines and chemokines (Bogen and Munthe, 2007; Murphy *et al.*, 2008). Cytokines are small proteins produced by a cell that affect the function of cells with the appropriate receptor, while chemokines are small proteins that attract cells bearing their receptor. The different types of leukocytes release different sets of cytokines which are important for their

respective functions. In Table 2 (section 3.3), examples of cytokines, their producer cells and their effects are shown.

1.1.2 Immune response

The innate and the adaptive immune system is cooperating in protecting the human body against infectious agents (Bogen and Munthe, 2007; Murphy *et al.*, 2008). The innate immune system acts immediately upon exposure to invading infectious agents. The main functions of the cells of the innate immune system are to destroy pathogens by phagocytosis, induce inflammation, activate and recruit other leukocytes, and to present antigens to T cells and activate them. Antigens are usually molecules from the pathogens which the immune system may respond to. The antigen presenting cells, such as dendritic cells, form a crucial link between the innate and the adaptive immune response.

In general, the adaptive immune response reinforces the innate immune response. The adaptive immune system is capable of eliminating infections more efficiently than the innate immune system, but an adaptive immune response may take days to develop (Bogen and Munthe, 2007; Murphy *et al.*, 2008). The adaptive immune response is divided into cell-mediated and humoral (antibody-mediated) responses. The T cells are crucial for developing the cell-mediated responses. T-helper (h) 1 cells can fight intracellular bacterial infections in macrophages by e.g. activating infected macrophages to destroy the pathogens. Cytotoxic T cells may directly kill cells infected with viruses if they display viral antigens (Horst *et al.*, 2011). Th17 cells seem to be important in recruiting neutrophils to control the early stages of an infection (Miossec, 2009). Unlike Th1 cells, cytotoxic T cells and Th17 cells, regulatory T cells restrain inflammation and maintain tolerance to harmless antigens, including self antigens (Curotto de Lafaille and Lafaille, 2009; Sanchez and Yang, 2011). In the humoral immune response, antibodies are the important factors for fighting extracellular invading infectious agents and harmful molecules. Th2 cells, but also Th1 cells, (Mosmann *et al.*, 1986) stimulate B cells to produce such antibodies (Bogen and Munthe, 2007; Murphy *et al.*, 2008). The antibodies may bind to pathogens or their products and neutralize them by blocking their access to cells. Furthermore, different parts of the antibodies may bind to antigens and elements of the innate immune system simultaneously, which results in an effective elimination of the pathogens by e.g. ingestion by phagocytes (opsonization) or activation of the complement system. The complement system is part of the innate immune system and consists of a cascade system of plasma proteins that interact to opsonize pathogens, to kill directly by lysis and to induce a series of inflammatory responses to fight

infections. Antibodies are divided into the different classes called IgD, IgM, IgG, IgA and IgE, and all classes have different effector functions. IgM is the first class of antibody secreted during an immune response and is important in activating the complement system. Later, IgG and IgA are the predominant antibody classes. IgG is mainly involved in fighting pathogens in the blood and the extracellular fluid, whereas IgA is mainly involved in neutralizing pathogens in the airways and the gastro-intestinal tract (mucosal antibody). IgE antibodies are usually present at low levels in the blood and the extracellular fluid, but may bind to mast cells, basophils and activated eosinophils. Antigens when cross-binding cell-bound IgE, can (further) activate the cells. IgE-mediated responses are characteristic for allergic diseases (Akdis, 2006), however, allergic reactions can also be independent of IgE (see section 1.2) (Bogen and Munthe, 2007; Murphy *et al.*, 2008).

1.2 Immunotoxicology

Immunotoxicology is a relatively new discipline in toxicology (Burleson and Dean, 1995; Descotes, 1999). Adverse effects of chemicals on the immune system were reported in several studies, and in 1977 Vos *et al.* concluded that standard toxicity testing in animals underestimated chemical effects on the immune system (Vos, 1977). The first scientific symposia on this topic were at the Annals of the New York Academy of Science meeting in 1979, followed by a Gordon Research Conference, and reported by Dean *et al.* (1979) in a special issue of the journal of Drug and Chemical Toxicology. This special issue may be regarded as one of the foundation texts of the discipline of immunotoxicology. The 'birth' of this discipline was formally announced by Davies in 1983 (Davies, 1983). In 1984, a seminar was held in Luxembourg, which is widely regarded as a hallmark in the history of immunotoxicology (Descotes, 1999).

Several definitions of immunotoxicology exist. This discipline of toxicology can informally be defined as the science that deals with changes of the immune system induced by substances (Flaherty, 2005). A change refers to any adverse effect on the structure or function of the immune system, or on other systems as a result of immune system dysfunction. The adverse effects include immunosuppression, hypersensitivity and autoimmunity (Figure 1). Immunosuppression may result in increased susceptibility to infections and cancer. Accordingly, an increased risk of cancer and severe infections in transplant recipients receiving immunosuppressive therapy to avoid transplant rejections have been reported (Buell *et al.*, 2005; Engels *et al.*, 2011; Horl *et al.*, 2002; Vial and Descotes, 2003). Hypersensitivity

is defined as an inappropriate or excessive response of the adaptive immune system to various foreign substances (Vohr, 2005). Hypersensitivity reactions mediated by IgE-antibodies can result in allergic diseases such as rhinoconjunctivitis, asthma, food allergy, urticaria and systemic anaphylaxis (Murphy *et al.*, 2008), whereas T-cell mediated hypersensitivity reactions may result in diseases such as allergic contact dermatitis. Autoimmunity is an immune response to antigens expressed by cells and tissues of the body (Rose, 2005). A critical function of the immune system is to discriminate normal self from altered self and non-self, but if the immune system fails, tissue-specific or systemic autoimmune diseases such as type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus may develop.

Sometimes also immunostimulation is included as an immunotoxic endpoint (De Jong and van Loveren, 2007; Descotes, 1999; Descotes, 2005), but adverse immunostimulatory effects are assumed to mainly result in hypersensitivity and autoimmunity. Other immunostimulating effects such as flu-like reactions or the more severe cytokine storm, exacerbation of underlying disease and inhibition of hepatic drug metabolism, have been reported for some drugs and vaccines (Descotes, 1999; Descotes, 2005).

Immunotoxicity may be divided into direct and indirect immunotoxicity (Figure 1). A substance is considered to exert direct immunotoxicity when effects on organs or cells of the immune system or immune function are observed at doses not causing overt general toxicity (De Jong and van Loveren, 2007; Karras and Holsapple, 1996). Indirect immunotoxicity is when the immune system is influenced by other organs affected by the substance. The differentiation between direct and indirect immunotoxicity is to a certain extent artificial since chemicals may exert both types of immunotoxicity (De Jong and van Loveren, 2007). From an epidemiological point of view, it may be more important to investigate whether a chemical is immunotoxic or not, rather than whether it exerts direct or indirect immunotoxicity. With regard to mechanistic studies, however, direct versus indirect immunotoxicity is an issue of importance. *In vitro* studies may have an advantage compared to *in vivo* studies with regard to examination of direct immunotoxic effects, since the organs or cells of interest are isolated and will not be influenced by effects on other organs (Gennari *et al.*, 2005; Lankveld *et al.*, 2010).

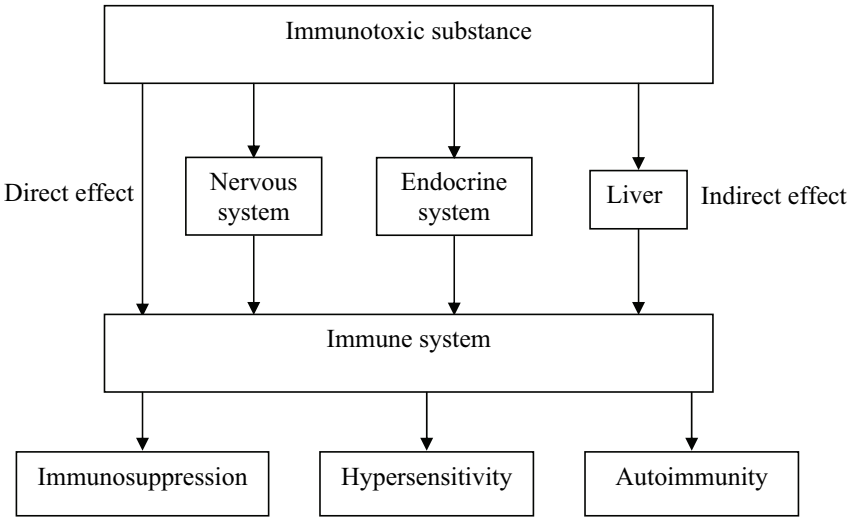

Figure 1. *Direct and indirect effects of immunotoxic chemicals on the immune system, and possible outcomes (modified from Karras and Holsapple (1996)).*

1.2.1 Study approaches in immunotoxicology

The field of immunotoxicology is explored using *in vitro* and *in vivo* studies in animals and humans (Gennari *et al.*, 2005; Luster *et al.*, 1992; Luster *et al.*, 2005). An important topic in research is the three R's; reduce, refine or replace the use of animals in experimental studies (Balls *et al.*, 1995). This is due to animal welfare considerations, the question of relevance to risk assessment for humans, as well as economical reasons. The European Community regulation REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances), which will require reassessment of thousands of existing chemicals and the use of millions of animals (European Commission, 2009), illustrates the need for *in vitro* methods as an alternative to animal models. *In vitro* methods using human cells may give more relevant data for humans compared to animal models regarding direct immunotoxicity. It may be a challenge, however, to assess whether the immunotoxic effects observed *in vitro* are biologically relevant (Gennari *et al.*, 2005). Furthermore, the complex interaction between immune cells and tissues are difficult to recreate *in vitro*. Human studies are important for risk assessment, but may be time-consuming and expensive, have insufficient exposure data, many influencing factors have to be considered, and can not be used for novel substances without a

history of human exposure (Descotes, 2006). The four approaches (*in vitro* and *in vivo* studies in animals and humans) often provide different and supplementary information to a given hypothesis (Figure 2) (Adami *et al.*, 2011).

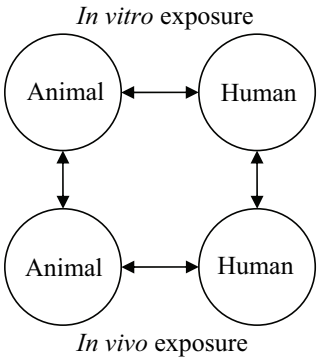

Figure 2. *Schematic illustration of the relationship between the different study approaches (modified from Selgrade et al. (1995)).*

1.3 The developing immune system

Herbst *et al.* reported in 1971 that the treatment of women before and during pregnancy with stilbestrol (drug to prevent pregnancy complications) was associated with an increased risk of vaginal carcinoma of their daughters (Herbst *et al.*, 1971). This was one of the first observations in humans of possible effects of prenatal exposure to a chemical (Tomatis, 1979). In studying the maternal diet during pregnancy, David Barker in 1989 was the first to report that the lower the weight of a baby at birth and during infancy, the higher the risk for coronary heart disease in later life (Barker *et al.*, 1989). These results led to the 'Foetal origins hypothesis' or the 'Barker hypothesis' which states that coronary heart disease is associated with a specific pattern of disproportionate foetal growth that results from foetal undernutrition in middle to late gestation (Barker, 1995).

Many toxicants cross the placenta and reach the developing foetus (Annola *et al.*, 2008; Annola *et al.*, 2009; Mathiesen *et al.*, 2009; Park *et al.*, 2008), and may be associated with increased risk of diseases in later life. Foetal exposure to immunotoxicants is of concern since the immune system develops extensively during the foetal stage (Dietert, 2008;

Holsapple *et al.*, 2004; van Loveren and Piersma, 2004; West, 2002). Prenatal immunotoxic exposure may result in effects that differ with respect to both the duration of the effect and the spectrum of effects compared to exposure in later life (Dietert and Piepenbrink, 2006). Furthermore, the foetus may be sensitive to lower doses of immunotoxicants than adults. Hence, the prenatal immune system should be viewed as different from that of adults regarding risk assessment.

Since different stages of immune system development may differ in their vulnerability, several critical time windows in the foetal stage have been proposed for immunotoxic exposure. During the foetal stage, development of the human immune system begins with hematopoietic stem cell (HSC) formation, which gives rise to the leukocytes (Holt and Jones, 2000; Leibnitz, 2005). The HSCs expand and differentiate, and colonize lymphoid organs such as the bone marrow and the thymus. Within the thymus, a selection process of T cells takes place to eliminate potentially self-reactive cells (Metzger and Anderson, 2011; Murphy *et al.*, 2008). This process is very important to avoid the development of autoimmune conditions. The foetus exerts Th2 skewed immune responses for protection against toxicity to placenta caused by Th1 immune responses (Leibnitz, 2005). At birth, the immune system has achieved a considerable level of maturity, but is still not fully developed (Holt and Jones, 2000; Leibnitz, 2005). The Th1 immune responses will be increasingly expressed, but the Th1 cytokine IFN-γ may not reach adult levels until three years of age or later (Leibnitz, 2005; Miyawaki *et al.*, 1985). The newborn child has low levels of all self-produced antibodies, such as IgG, which does not reach adult levels before 4-6 years of age (Holladay and Smialowicz, 2000; Leibnitz, 2005).

1.4 Toxicants in food

Food items may contain small amounts of toxicants. Dietary toxicants can originate from environmental pollution or be formed during food preparation such as baking and broiling, or may be life style factors such as alcohol. Table 1 shows examples of 12 dietary toxicants as selected within the EU-funded project NewGeneris (Merlo *et al.*, 2009), of which the present work was a part (described in section 3.1). In the present work, we have investigated all 12 substances in an *in vitro* system, as well as dietary exposure to polychlorinated biphenyls (PCBs), dioxins (polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)) and acrylamide in a human study.

Table 1. *Examples of dietary toxicants as selected within the NewGeneris project (described in section 3.1), their chemical classes and dietary sources.*

Chemical class	Dietary toxicant	Dietary sources
Acrylamides	Monoacrylamide	Generated in plant-derived food rich in carbohydrates during heat treatment (Mottram *et al.*, 2002; Stadler *et al.*, 2002)
Alcohols	Ethanol	Alcoholic beverages
DNA reactive aldehydes	4-hydroxynonenal (4-HNE) Malondialdehyde (MDA)	Generated in food containing polyunsaturated fatty acids during storage and preparation (Govaris *et al.*, 2004; Guillen and Goicoechea, 2008)
Heterocyclic amines	2-amino-3-methylimidazo-[4,5-f]quinoline (IQ) 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP)	Generated in food rich in proteins (meat and fish) during heat treatment (Kim and Lee, 2010; Ni *et al.*, 2008)
Mycotoxins	Aflatoxins Deoxynivalenol (DON)	Fungal toxins that may be found in fungus-infected food (Schollenberger *et al.*, 2005; Thuvander *et al.*, 2001)
Nitrosamines	Dimethylnitrosamine (DMNA)	Generated in nitrite-containing food during e.g. digestion and heat treatment (Lijinsky, 1999)
Organochlorines	Dioxins Polychlorinated biphenyls (PCBs)	Environmental pollutants contaminating the food chain (Charnley and Doull, 2005; Domingo and Bocio, 2007)
Polycyclic aromatic hydrocarbons	Benzo[a]pyrene (BaP)	Environmental pollutant contaminating the food chain, generated during food preparation (Kazerouni *et al.*, 2001)

1.4.1 PCBs and dioxins

The main environmental reservoirs of PCBs and dioxins are soils and sediments (Ross, 2004). PCBs and dioxins are highly lipophilic and have long half-lives (years) due to resistance to degradation processes (Milbrath *et al.*, 2009; Ritter *et al.*, 2011). PCBs and dioxins accumulate in adipose tissue as they move up in the food chain, therefore food of animal origin is usually the predominant source of exposure in humans. In general, the diet is assumed to provide more than 90% of the total human exposure to PCBs and dioxins (Domingo and Bocio, 2007; Liem *et al.*, 2000).

Dioxins have never been produced intentionally, but are unwanted by-products of industrial and combustion processes like burning of waste, chlorine bleaching of paper pulp and the manufacturing of organochlorines such as PCBs and some herbicides and pesticides (Hites, 2011; WHO, 2010). The first evidence of man-made dioxins is from as early as 1827 and comes from a German chemical production plant that manufactured washing soda (White and Birnbaum, 2009). Dioxins are also produced during natural processes, such as volcanic eruptions and forest fires, but human activities have been primarily responsible for the environmental contamination by this class of chemicals over the past two centuries (van den Berg *et al.*, 1994; White and Birnbaum, 2009).

Depending on which carbon atoms in the aromatic rings the chlorine atoms are bound to (Figure 3 A and B), 210 chemically different congeners of dioxins can be formed, of which 17 congeners (7 PCDDs and 10 PCDFs) are considered highly toxic (Hites, 2011; van den Berg *et al.*, 1994; van den Berg *et al.*, 2006). Most, if not all, immunotoxic and other toxic effects of these congeners have been reported to be mediated through the aryl hydrocarbon receptor (AhR), a transcription factor which regulates the transcription of several genes (Denison *et al.*, 2011; Marshall and Kerkvliet, 2010; Stockinger *et al.*, 2011). Its ligands may be both xenobiotics and natural molecules such as the natural photoproduct FICZ (6-formylindolo[3,2-b]carbazole).

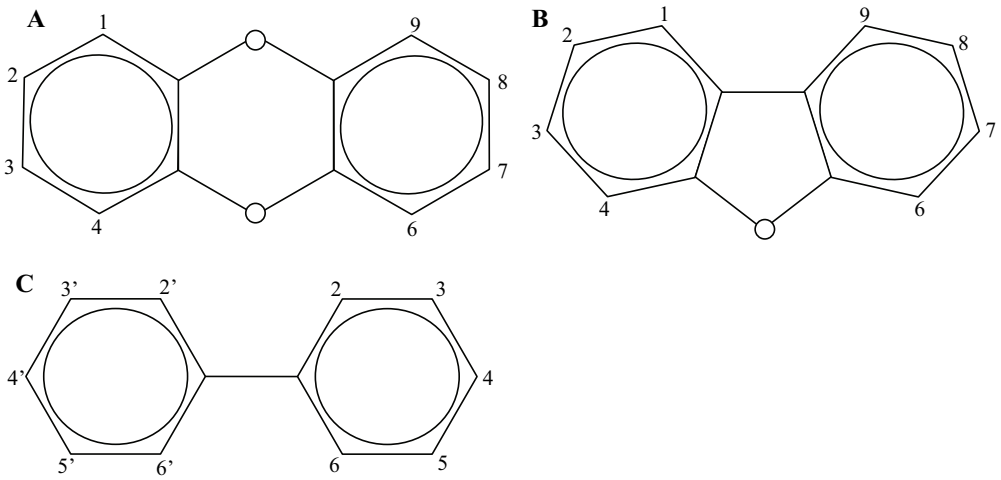

Figure 3. *General molecular structure of PCDDs (A), PCDFs (B) and PCBs (C). The numbers indicate the possible positions for the chlorine atoms.*

In contrast to dioxins, PCBs were produced intentionally and were widely used in many industrial products since the commercial usage started in 1929 (Ross, 2004). PCBs were used particularly as electrical insulation fluids and as heat-exchange fluids due to their remarkable electrical insulating properties and their flame resistance. The US Environmental Protection Agency (EPA) ultimately banned the manufacture of PCBs in 1979 (US EPA, 2011), and new usage of PCBs was forbidden in Norway in 1980 (Ministry of the environment, 2011). Even today, materials containing PCBs are in use, but there are strict regulations on handling and disposal of PCB-containing waste. Thus, the levels of PCBs are decreasing worldwide, but still these toxicants are found in humans (Dallaire *et al.*, 2003; Llobet *et al.*, 2008; Polder *et al.*, 2008; Ulaszewska *et al.*, 2011).

There are 209 chemically different PCB congeners depending on which carbon atom in the two connected aromatic rings the chlorine atoms are bound to (Figure 3C). Twelve of these congeners are considered highly toxic and called dioxin-like PCBs (dl-PCBs) due to their similar toxicological properties to the dioxins (van den Berg *et al.*, 2006). The comparable toxicological properties of dioxins and dl-PCBs allow the combined exposure to be expressed as toxic equivalents (TEQ) (van den Berg *et al.*, 1998; van den Berg *et al.*, 2006).

PCB congeners that do not have similar properties to dioxins also exist. These are called non-dioxin-like PCBs (ndl-PCBs) (EFSA, 2005). Ndl-PCBs are suggested to be less immunotoxic than dioxins and dl-PCBs due to their low binding affinity to the AhR. Most epidemiological studies do not differentiate between the exposure to dioxins and dl-PCBs, and ndl-PCBs since they may be highly correlated, which complicate the investigation of causal relationships. However, immunotoxic effects of ndl-PCBs in animal studies and in *in vitro* studies are reported, which suggest an immunotoxic mechanism independent of AhR (Ferrante *et al.*, 2011; Fischer *et al.*, 1998; Levin *et al.*, 2005; Lyche *et al.*, 2004; Lyche *et al.*, 2006).

There are well known episodes where animals and humans have accidently been exposed to high levels of dioxins and PCBs (Hites, 2011; White and Birnbaum, 2009). The herbicide Agent Orange was used by the American military as a defoliant in Vietnam to reduce enemy ground cover. Agent Orange was contaminated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the dioxin congener regarded as most potent, and human exposure was reported to be associated with diseases such as cancer and diabetes (Michalek and Pavuk, 2008). In 1968 in Japan, a rice bran oil company's supplies became contaminated with PCBs and dioxins, and in Taiwan in 1979, a similar contamination of rice

oil occurred (White and Birnbaum, 2009). The two latter exposures were reported to be associated with symptoms such as skin lesions, fatigue, retarded development in children, altered reproductive and immunologic function, and cancer (Aoki, 2001; Guo *et al.*, 2004; Onozuka *et al.*, 2009). The condition of the exposed humans was called the 'Yusho' disease in Japan and 'Yucheng' disease in Taiwan.

Since the developing immune system of the foetus appears to be especially vulnerable to toxicant exposure (Holsapple *et al.*, 2004; van Loveren and Piersma, 2004; West, 2002), it is of particular concern that the immunotoxicants PCBs and dioxins can cross the placenta and reach the foetus (Covaci *et al.*, 2002; Park *et al.*, 2008; Suzuki *et al.*, 2005). Immunotoxic effects of prenatal exposure to accidental and high non-accidental levels of PCBs and dioxins in humans have been reported (Aoki, 2001; Dallaire *et al.*, 2004; Dallaire *et al.*, 2006; Grandjean *et al.*, 2010; Guo *et al.*, 2004; Heilmann *et al.*, 2006). Even though the levels of exposure to PCBs and dioxins are decreasing worldwide (Dallaire *et al.*, 2003; Llobet *et al.*, 2008; Polder *et al.*, 2008; Ulaszewska *et al.*, 2011), effects of prenatal exposure to PCBs and dioxins on immunological parameters in humans have been reported also for the general population (Glynn *et al.*, 2008; Miyashita *et al.*, 2011; ten Tusscher *et al.*, 2003; Weisglas-Kuperus *et al.*, 2000; Weisglas-Kuperus *et al.*, 2004). Previous studies on immunotoxic effects of pre- and postnatal exposure to PCBs and dioxins have used measurements in biological samples such as blood and breast milk, which indicate the body burden of these toxicants (Ayotte *et al.*, 2003; Patterson, Jr. *et al.*, 1988; van den Berg *et al.*, 1994). To our knowledge, however, immunotoxic effects of PCBs and dioxins from the diet quantified by means of a food frequency questionnaire (FFQ) have not been examined. A possible advantage of using FFQ data is that the results may indicate more directly than body burden (as measured in blood/breast milk) how exposure to toxicants from the maternal diet affects the health of the children. This is valuable information to be used in counselling women of fertile age regarding their diet.

1.4.2 Acrylamide

In 1997, there was a leakage of acrylamide in the Hallandsås tunnel in Sweden (Reynolds, 2002). Measurements of acrylamide levels in the exposed tunnel workers were compared with non-accidently exposed humans, which resulted in the findings that humans in general have unexpectedly high levels of acrylamide. Furthermore, it was found that acrylamide was a component in tobacco smoke. These findings led to the now confirmed hypothesis that acrylamide is generated in food during heat treatment (Hogervorst *et al.*, 2010). Acrylamide is

formed in plant-derived food rich in carbohydrates during heat treatment at temperatures above 120 °C (Mottram *et al.*, 2002; Stadler *et al.*, 2002; Tareke *et al.*, 2002). Typical food items are coffee, crispbread, crisps and deep-fried potatoes. Acrylamide can be generated as a result of the Maillard reaction between reducing sugars and amino acids, especially asparagine, a major amino acid in potatoes and cereals. Acrylamide is also manufactured by the industry, and has since the 1950 been used in the production of polyacrylamides. Polyacrylamides are used as flocculants for purifying drinking water and in industrial applications such as in the production of textiles and plastics, and grouting (Carere, 2006; Hogervorst *et al.*, 2010; Parzefall, 2008). Consequently, non-food exposure to acrylamide may exist (Carere, 2006), although the diet is assumed to be the major source of exposure to acrylamide for the general non-smoking population.

Acrylamide is metabolized to a chemically reactive epoxide, glycidamide (Figure 4), in a reaction catalyzed by cytochrome P450 2E1 (Hogervorst *et al.*, 2010; Sumner *et al.*, 1999). Acrylamide binds to proteins, while glycidamide binds to both DNA and proteins (Doerge *et al.*, 2005; Tornqvist *et al.*, 2002). Free acrylamide and glycidamide have half-lives in the scale of hours (Calleman, 1996; Fennell *et al.*, 2006).

Figure 4. *Structures of acrylamide (left) and its major metabolite glycidamide (right).*

Carcinogenic, genotoxic, neurotoxic and reproductive toxic properties of acrylamide have been reported (Carere, 2006; Hogervorst *et al.*, 2010; Parzefall, 2008). Glycidamide is assumed to be responsible for the genotoxic properties of acrylamide and for its carcinogenic properties (Paulsson *et al.*, 2001). Information on immunotoxicity of acrylamide is scarce. To our knowledge, only Zaidi *et al.* (1994) have reported on immunotoxic effects of acrylamide using a rat model. Acrylamide has been shown to cross the placenta and reach the foetus (Annola *et al.*, 2008; Schettgen *et al.*, 2004; Sorgel *et al.*, 2002), but studies on possible immunotoxic effects of prenatal exposure to acrylamide have not been published.

2 Aims

The overall aim of the work was to investigate the immunotoxic potential of dietary toxicants, with a special focus on prenatal exposure to toxicants from the maternal diet.

The specific aims were:

- To explore whether cytokine release from human peripheral blood mononuclear cells in an *in vitro* system can be used as a marker of the immunotoxic properties of 12 selected dietary toxicants (Paper I)

- To establish a mother-child birth cohort and employ this cohort to investigate whether exposure to acrylamide, PCBs and dioxins from the maternal diet during pregnancy increases the risk of immune-related diseases during the three first years of life, and to explore whether these toxicants affect immune-related blood parameters in the three-year-old children (Papers II and III)

3 Subjects and methods

3.1 The NewGeneris project

The present work is part of the EU-funded project NewGeneris, which is the acronym for 'Newborns and Genotoxic Exposure Risks' (Merlo *et al.*, 2009). The main aim of NewGeneris was to investigate whether maternal exposure to dietary toxicants results in *in utero* exposure and in molecular events in the unborn child, leading to increased risks of cancer and immune disorders in childhood. The aims of our workpackage were to develop biomarkers of immunotoxic risk based on both *in vitro* and *in vivo* studies and to investigate possible immunotoxic effects of prenatal exposure to dietary toxicants (Papers II and III). Initially, to develop biomarkers, gene expression (transcriptomics) (Hochstenbach *et al.*, 2010), protein expression (proteomics) and cytokine release (Paper I) were examined in peripheral blood mononuclear cells (PBMCs) exposed *in vitro* to 12 dietary toxicants to find candidate biomarker genes and proteins. The candidate biomarkers were then investigated in a birth cohort called BraMat. The biomarkers will finally be applied on several European birth cohorts to achieve the main aim of the NewGeneris project.

3.2 Subjects

In the *in vitro* study (Paper I), healthy non-smoking Caucasian males (n=25) and females (n=35) (20-35 years of age) were used as blood donors. 'Healthy' was defined as absence of self-reported infections, chronic diseases like autoimmune disorders or use of medication at the time of blood sampling.

For the birth cohort BraMat (Papers II and III), invitations to participate were sent by regular mail to all pregnant women already enrolled in the Norwegian Mother and Child Cohort Study (MoBa) (April 2007 - March 2008), and who were scheduled to give birth at Oslo University Hospital Ullevål or Akershus University Hospital (25% participation rate). MoBa is a prospective population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health, including 108 000 children born between 1999 - 2008 from all over the country (38.5% participation rate) (Magnus *et al.*, 2006). Invitations to the BraMat birth cohort were sent in week 37 of gestation, thus only infants that had experienced a full term pregnancy (37^{th} - 42^{nd} week of gestation) were included. There were no plurality births.

Exclusion criteria for the BraMat cohort were autoimmune diseases of the mother and use of steroids, anti-inflammatory drugs or epileptic drugs during pregnancy.

3.3 In vitro study

The design of the *in vitro* study is shown in Figure 5. The *in vitro* methods are described in more detail in Paper I. In short, PBMCs were isolated from venous blood of adults. The 12 selected substances (Table 1; PCBs: PCB-153; dioxins: TCDD) were classified into immunotoxic and non-immunotoxic substances based on published *in vivo* data. The concentrations of these substances and the exposure duration used in the experiments were determined based on preliminary experiments (described in Paper I). To select the concentration ranges, we first wanted to determine the concentrations causing 10% cytotoxicity. Since all concentrations tested caused <10% cytotoxicity based on the trypan blue exclusion technique, other criteria were used to select the concentration ranges (described in Paper I). The cells were exposed to three concentrations (tenfold dilution series) of each of the 12 substances. Each cell culture contained 10% freshly made S9-mix (human liver S9-fraction and co-factors) for biotransformation of the substances to mimic the process after peroral exposure in the *in vivo* situation. The cell cultures were incubated for 20 hours at 37˚C in a humidified atmosphere with 5% CO_2. The tubes were kept at a 20° angle to avoid formation of a dense cell pellet. For each substance, cells from four donors were individually exposed to all three concentrations of the substance and its respective solvent.

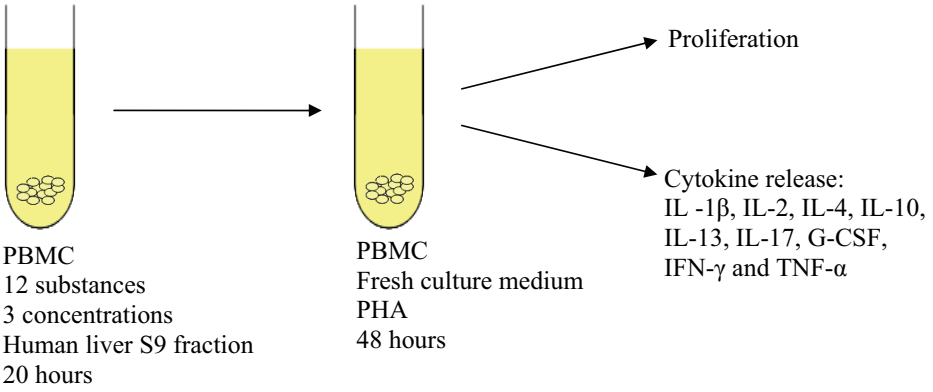

Figure 5. *The design of the in vitro study.*

After exposure, the substances and S9-mix were removed from the cell cultures. The cells were resuspended in culture medium containing 10 μg/ml of the mitogen phytohemagglutinin (PHA; Sigma-Aldrich, St.Louis, MO, USA) and incubated for 48 hours at 37˚C in a humidified atmosphere with 5% CO_2.

Mitogen-induced proliferation was determined using a colorimetric immunoassay based on BrdU incorporation during DNA synthesis (Roche Diagnostics GmbH, Penzberg, Germany). In the supernatants, nine cytokines IL-1β, IL-2, IL-4, IL-10, IL-13, IL-17, G-CSF, IFN-γ and TNF-α (Table 2) were quantified using a Bio-Plex Human Cytokine Assay (Bio Rad, Hercules, CA, USA). These cytokines were selected to reflect different types of immune responses such as Th1, Th2, regulatory and proinflammatory responses and to enable the use of a single dilution (1:2) to determine cytokine release (described in Paper I). Due to donor variation of mitogen-induced cytokine release and proliferation in the solvent controls, the parameters were expressed relative to the corresponding solvent control.

Table 2. *The nine selected cytokines, their producer cells and their effects (Murphy et al., 2008; Oppenheim et al., 2001).*

Cytokine	Producer cells	Effects
IL -1β	Macrophages, epithelial cells	Fever, T cell activation, macrophage activation
IL-2	T cells	Proliferation, differentiation and survival of T cells and NK cells
IL-4	T cells, mast cells, basophils	Regulation of IgE and IgG1 (mice) production, induces differentiation into Th2 cells
IL-10	Monocytes, T cells	Suppression of immune responses, but possesses also stimulatory activities
IL-13	Th2 cells, mast cells, NK cells	Regulation of IgE secretion by B cells, modulation of Th2 cell development, suppression of inflammatory responses due to regulation of macrophage function, plays a central role in Th2 responses
IL-17	T cells	Induces production of several proinflammatory and hematopoietic bioactive molecules by stromal cells
IFN-γ	T cells, NK cells	Macrophage activation, increased expression of MHC molecules and antigen presenting components, Ig class switching, suppresses Th2 responses
G-CSF	Fibroblasts, monocytes, endothelial cells	Stimulates neutrophil development and differentiation
TNF-α	Macrophages, NK cells, T cells	Promotes inflammation and endothelial activation

3.4 BraMat study design

We used a prospective cohort study design (Figure 6). Maternal intake of the toxicants during the first four months of pregnancy was calculated using a food frequency questionnaire (FFQ). The children were followed annually during the three first years of life using questionnaires covering immune-related health outcomes. Furthermore, at three years of age, immune-related blood parameters were examined: concentrations of allergen specific IgE antibodies, vaccine antibody levels and immunophenotype data.

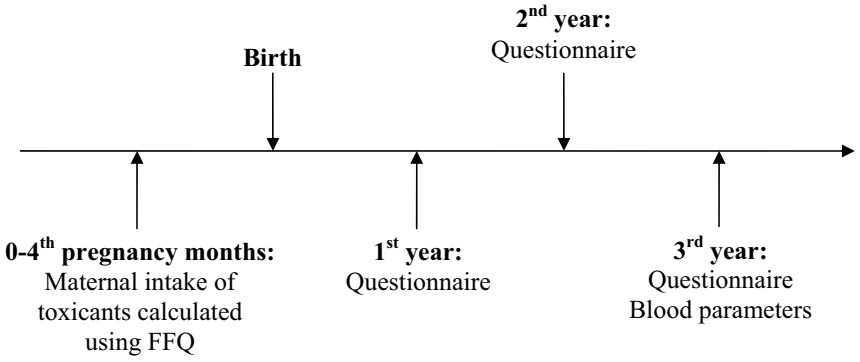

Figure 6. The study design for the BraMat cohort.

3.5 Questionnaires and health outcomes

When the children in the BraMat cohort were one, two and three years of age, a questionnaire was sent to the mothers (Appendix A-D). The mothers could either fill in the questionnaire and return it by regular mail, or give the answers by a telephone interview. The questionnaire covered topics for the last year related to the child's infectious diseases, allergy, asthma and other chronic diseases and the use of medications. Concerning infectious diseases, the mothers were asked if the child had experienced the following diseases/complaints and the number of episodes: colds and other upper respiratory tract infections, otitis media, pneumonia, gastroenteritis with vomiting or diarrhoea and urinary tract infection. The mothers were also asked if the child had experienced any childhood diseases, such as chicken pox and exanthema subitum (roseola infantum). Concerning allergy, asthma and other chronic diseases, the mothers were asked: 'Has the child been diagnosed with asthma, asthma-bronchitis or bronchial hyperreactivity by a doctor?' (Bronchial hyperreactivity was not

included in the questionnaire for the first year.) 'Has the child had periods of more than 10 days with dry cough, chest tightness or wheeze, or shortness of breath? Has the child had eczema or itching in the face or at joints (e.g. the groin, hollow of the knee, ankle, elbow and wrist)? Has the child been diagnosed with atopic dermatitis by a doctor? Has the child been diagnosed with allergy by a doctor? Has the child any other chronic disease?' Data for the first year (Paper II), cumulative data (0-3 years of age, Paper III) and data for the last year only (2-3 years of age, Paper III) were used to examine possible associations between prenatal toxicant exposure and health outcomes during early childhood.

In addition to BraMat questionnaires, potential confounding variables were extracted from MoBa questionnaires filled in by the mothers during pregnancy (~15th and 30th week of gestation) and about six months after birth. Birth-related information was extracted from the Medical Birth Registry of Norway (MBRN).

3.6 Dietary exposure assessment

Of the 12 substances selected within the project NewGeneris, alcohol, acrylamide, PCBs and dioxins were examined further in the BraMat cohort due to availability of exposure data and a wish to include both persistent and non-persistent toxicants. Concerning alcohol, only 25 (12.2%) of the mothers reported consuming alcoholic beverages during pregnancy, regarded to be too low a number to perform statistical analyses.

Maternal intake of the dietary toxicants polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDDs/PCDFs or dioxins) and acrylamide was calculated from a validated FFQ used in MoBa (The Norwegian Mother and Child Cohort Study, 2011a; The Norwegian Mother and Child Cohort Study, 2011b). Description and validation of the FFQ is reported elsewhere (Brantsaeter *et al.*, 2008a; Meltzer *et al.*, 2008). The method for calculating dietary exposure to acrylamide, PCBs and dioxins has been described in Brantsaeter *et al.* (2008b) and Kvalem *et al.* (2009), respectively. As described in section 1.4.1, PCBs and dioxins can be divided into two groups according to their toxicological properties: 1) dioxins and dioxin-like PCBs (dl-PCBs) and 2) non-dioxin-like PCBs (ndl-PCBs). The most toxic congeners were included in the TEQ calculations for dioxins and dl-PCBs: all 17 of the 2,3,7,8-substituted PCDD/PCDFs; non-*ortho*-substituted PCBs: PCB-77, -81, -126 and -169; mono-*ortho*-substituted PCBs: PCB-105, -114, -118, -123, -156, -157, -167 and -189. For ndl-PCBs, the sum of exposure to PCB-28, -52, -101, -138, -153 and -180 was used. The exposure was expressed relative to the mother's self-

reported body weight (bw) before pregnancy, thus the exposure to dioxins and dl-PCBs was expressed as pg TEQ/kg bw/day, exposure to ndl-PCBs as ng/kg bw/day and acrylamide as µg/kg bw/day.

3.7 Blood analyses

Blood sampling and analyses are described in more detail in Paper III. In short, venous blood was collected from the three-year-old children either at their doctor's office, at home by a clinical laboratory technician or at a commercial laboratory (Fürst Medical Laboratory, Oslo).

Levels of specific IgG antibodies to measles, rubella, tetanus toxoid, pneumococcal polysaccharides and *Haemophilus influenzae* type b (Hib) were determined in the sera of the children using ELISA techniques.

Allergen-specific IgE antibodies were analysed in the sera of the children using ImmunoCAP Phadiatop® Infant (Phadia AB, Uppsala, Sweden) comprising 11 allergens selected to be relevant for young children (house dust mite, cat, dog, hen's egg, cow's milk, peanut, shrimp, timothy, birch, ragweed and wall pellitory (*Parietaria judaica*)). Sera from the children found to be sensitized using Phadiatop Infant were analysed for specific IgE antibodies to the 'individual' allergen preparations from house dust mite, cat, dog, hen's egg, cow's milk, peanut, timothy and birch. Determination of the serum concentration of specific IgE to allergens is used to diagnose allergic sensitization. A positive test does therefore not necessarily mean that the tested individual has an allergic disease (Sicherer and Sampson, 2010).

Immunophenotyping using flow cytometry was performed on whole blood of the children within 24 hours after blood collection according to the protocol of the manufacturer (BD, Franklin Lakes, NJ, USA). The percentage and absolute number of monocytes ($CD14^+$) and the following lymphocyte subsets were determined: T cells ($CD3^+$), T-helper cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$), B cells ($CD19^+$), natural killer (NK) cells ($CD16^+CD56^+$) and natural killer T cells ($CD3^+CD16^+CD56^+$). Furthermore, the percentage of regulatory T cells of $CD4^+$cells were determined ($CD4^+CD25^{high}CD127^{low}$).

3.8 Statistical analyses

Principal component analysis (PCA) with the log-transformed values of the *in vitro* cytokine release data relative to solvent control was performed to investigate clustering of the cell

cultures based on effects on cytokine release (Genedata Expressionist® software). To enable exclusion of cytotoxic concentrations, proliferation data were visualised (colour coded) in a PCA plot to examine if proliferation was in concordance with the clustering (Paper I). Furthermore, *in vivo*-based (literature) classification of the immunotoxic potential of the substances was visualised (colour coded) in a PCA plot to investigate if all cytokines combined could be used to distinguish the immunotoxic from the non-immunotoxic substances.

A multivariate random effects model (mixed model) was applied to assess the influence of *in vitro* exposure to the 12 substances on the subsequent mitogen-stimulated release of each of the nine cytokines and the proliferation. One analysis was performed per substance. A nested model was used since one donor was exposed to all three concentrations of one substance resulting in dependent variables. To examine the associations, the Cook's D and the multivariate DfFits statistics (Belsley *et al.*, 1980) as well as the Covtrace and the Covratio statistics were used. The post hoc test Simes' procedure was used to adjust for multiple testing. The analyses were performed in Statistical Analysis System (SAS, Cary, NC, USA) version 9.1.3. A change in cytokine release relative to the corresponding solvent control was considered to be statistically significant if the value 1 was not included in the 95% confidence interval.

In Papers II and III, logistic regression analyses were applied to assess the influence of exposure on the different binary health outcomes for the children. The exposure to the dietary toxicants were categorised using the 80^{th} percentile to compare the highest exposed children, who constituted the upper tail of the exposure distribution, with the remaining children ($\geq 80^{th}$ percentile and $<80^{th}$ percentile, respectively). If no statistically significant associations were found, also the continuous exposure variables were examined since the use of continuous variables usually increase the statistical power compared to categorised variables. For eczema in the first year of life, the exposure to the dietary toxicants was categorised using the tertiles due to apparently nonlinear associations (the reference category was the lowest exposure category). Highly correlated variables (correlation coefficients ≥ 0.7) were not included in the same multivariate analysis. Separate multivariate analyses were therefore performed for the dietary toxicants dioxins and dl-PCBs, and ndl-PCBs ($\tau_b = 0.81$, $p<0.001$). Since parity, previous breast-feeding and number of older siblings/children living together with the child were also highly correlated ($\tau_b \sim 0.9$, $p<0.001$), only parity was included in the multivariate analyses. The criterion for inclusion of potential confounding variables in the multivariate

regression analyses was $p<0.250$ in bivariate analysis. The manual backward deletion method was used starting with all included variables in the model. At each deletion step, the least significant variable in the multivariate model was manually removed until only statistically significant ($p<0.05$) variables remained in the model. Hosmer-Lemeshow test, Cook's D and residuals were used to investigate the robustness of the multivariate logistic regression models.

For the outcome variables 'numbers of upper respiratory tract infections' (URTI) and 'numbers of episodes of gastroenteritis', linear regression analyses were applied. The variable 'numbers of upper respiratory tract infections' was ln-transformed (ln (URTI+1)) except for the data for the first year of life. For the transformed variables, the reported results are back-transformed values (ratio of effect). The exposure variables and the inclusion criterion for potential confounding variables in the multivariate analyses were the same as for logistic regression analyses (described above). Cook's D, Leverage values and residuals were used to investigate the robustness of the multivariate linear regression models.

The immune-related blood parameters were investigated by linear and logistic regression analyses. Due to small sample size, ln-transformed exposure variables were used in the statistical analyses to avoid results being strongly influenced by only a few observations. The outcome variable was ln-transformed if necessary to fulfil the criteria of normally distributed residuals. For the transformed outcome variables, back-transformed values are reported. Multivariate regression analyses were performed as described above.

Results of the logistic and linear regression analyses were considered statistically significant at $p<0.05$. The statistical analyses were performed using the statistical software PASW Statistics 17 (SPSS Inc., Chicago, IL, USA).

3.9 Ethical issues

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics and the Data Inspectorate. All blood donors and mothers enrolled in the BraMat cohort gave their written informed consent.

To recruit pregnant mothers to the BraMat cohort, the mothers received a brochure presenting the study, including the information that potential harmful substances in the maternal diet may exert adverse effects resulting in increased risk of immune-related diseases in the child. This information can make the parents worried, and in the brochure, we therefore focused on that the BraMat cohort was established to assure that dietary exposure to the

substances of investigation is associated with no or low risk of immune-related diseases. The parents also received contact information to persons who could answer possible questions. Mothers with family relations or co-workers to personnel involved in the project were excluded. It was ensured that the mothers who chose to give the answer on the questionnaires by a telephone interview, were interviewed by a person unknown to them.

Regarding blood sampling, qualified health personnel were used to reduce unnecessary discomfort for the three-year-old children. Individual results of blood analyses were given to parents on request. Only results with diagnostic values (IgE-test) were given to the parents, followed by a detailed explanation of the test result.

The blood samples and the questionnaires were labelled with an ID-code, and the ID-code was also used in the data files. Only a few persons have access to the file (key-file) containing the link between ID-codes and personal data. The key-file is stored in a secure zone at the institute's server.

4 Results

4.1 In vitro exposure and cytokine release

For some concentrations of the 12 substances, the proliferation and the release of most, if not all, nine cytokines were strongly reduced relative to solvent control, which suggests cytotoxic effects. These concentrations were excluded in further analyses based on clustering of the cell cultures in a PCA plot (described in section 3.8). After exclusion, all substances significantly affected the cytokine release relative to solvent control for one or more cytokines, and the cytokine release patterns differed depending on substance and concentration (Paper I). To investigate if it was possible to distinguish immunotoxic from non-immunotoxic substances using *in vitro* cytokine release, the significant effects on release of each cytokine were examined separately and in combinations of cytokines. However, we did not find any common effect pattern on cytokine release for either the immunotoxic or the non-immunotoxic substances. Neither when using PCA, the release of the nine selected cytokines could distinguish the immunotoxic from the non-immunotoxic substances.

4.2 Prenatal exposure and immunotoxicity in children

BraMat questionnaires were received for 195 (95%), 184 (90%) and 180 (88%) of the 205 children for the first, second and third year, respectively. All three questionnaires were received for 162 (79%) children. Since few children had experienced pneumonia, urinary tract infection and 'other chronic diseases', statistical analyses were not performed on these health outcomes. For the same reason, analyses for the health outcomes asthma and allergy diagnosed by a doctor and numbers of episodes of gastroenteritis were performed on cumulative data (0-3 years of age) only. Furthermore, analyses on frequency of childhood diseases were performed on first year data and on cumulative data. All other health outcomes were investigated for first year, third year and cumulative data.

Data on health outcomes and potential confounders for some of the children were missing since some of the questionnaires were incomplete. Exposure data (intake/bw) could not be calculated for five of the mothers since the body weight was missing, and they were therefore excluded from the statistical analyses. The calculated maternal dietary intake of

dioxins and dl-PCBs, ndl-PCBs and acrylamide is presented in Table 3, and the dietary sources of exposure to the toxicants in the BraMat cohort are shown in Figure 7.

Table 3. *The calculated dietary intake of ndl-PCBs, dioxins and dl-PCBs, and acrylamide for the mothers in the BraMat cohort (n=200).*

Dietary toxicant	Median[a]	Min	Max	IQR	80th P
Ndl-PCBs (ng/kg bw/day) [b]	2.59	0.53	30.12	1.80-4.09	4.37
Dioxins and dl-PCBs (pg TEQ/kg bw/day) [c]	0.58	0.15	3.07	0.45-0.81	0.90
Acrylamide (µg/kg bw/day)	0.56	0.07	2.05	0.41-0.72	0.79

IQR: interquartile range; P: percentile
[a] The median values are presented since the data were not normally distributed.
[b] PCB-28, -52, -101, -138, -153 and -180.
[c] All 17 of the 2,3,7,8-substituted PCDD/PCDFs; non-ortho-substituted PCBs: PCB-77, -81, -126 and -169; mono-ortho-substituted PCBs: PCB-105, -114, -118, -123, -156, -157, -167 and -189.

Seafood was a major source of exposure to dioxins and dl-PCBs, and ndl-PCBs, of which fat fish such as salmon, trout, mackerel and herring was the most important. Other dietary sources such as cereals, eggs (including seagull eggs), milk and dietary products also contributed to the exposure to these toxicants. For acrylamide, important sources were crispbread, crisps and other snacks. Coffee is usually an important source of acrylamide exposure (JECFA, 2005), but not for the pregnant women in the BraMat cohort. One reason may be the recommendations of low coffee consumption during pregnancy by the Norwegian authorities (Norwegian food safety authority, 2011a).

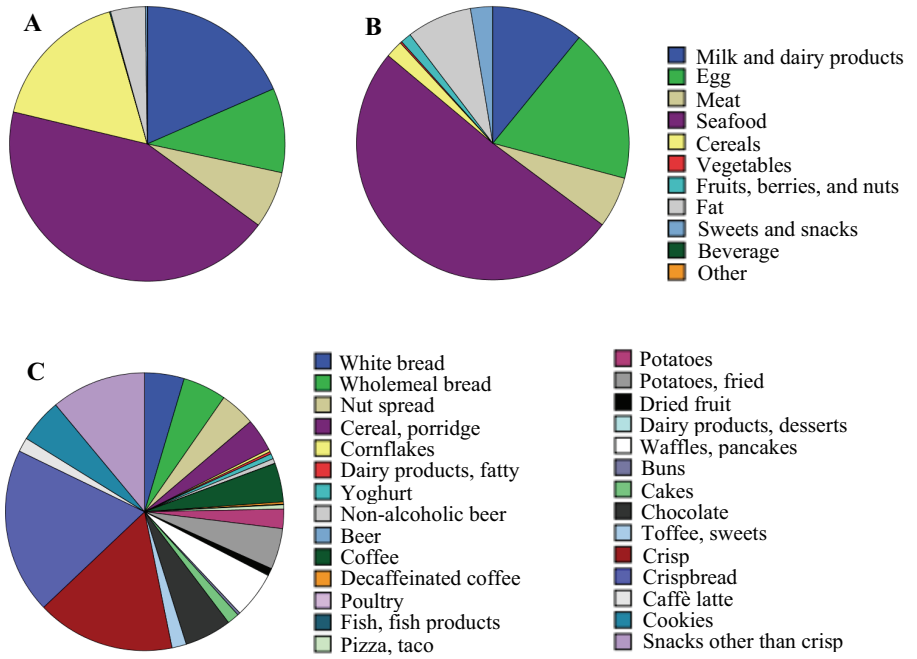

Figure 7. *The dietary sources of exposure to dioxins and dl-PCBs (A), ndl-PCBs (B) and acrylamide (C) for the mothers in the BraMat cohort (n=205). Other: dietary supplements, spices and baking powder etc.*

4.2.1 PCBs and dioxins – health outcomes

Results of bivariate analyses and statistically significant multivariate analyses are shown in Table 4-6. Prenatal dietary exposure to dioxins and dl-PCBs, and ndl-PCBs was significantly associated with an increased risk of wheeze (periods of more than 10 days with dry cough, chest tightness or wheeze, or shortness of breath) during the first year of life (Paper II) and the three first years of life (Paper III), whereas no associations were found for the third year only (Paper III).

Regarding infections, prenatal dietary exposure to dioxins and dl-PCBs, and ndl-PCBs was significantly associated with increased numbers of upper respiratory tract infections up to three years of age (Papers II and III). Prenatal exposure to dioxins and dl-PCBs, and ndl-PCBs was also associated with an increased risk of the childhood disease exanthema subitum during the first year of life (Paper II). Furthermore, prenatal exposure to dioxins and dl-PCBs, and ndl-PCBs was associated with increased numbers of episodes

of gastroenteritis in the three first years of life (Paper III). However, the associations with numbers of episodes of gastroenteritis were not robust as indicated by the residuals and Cook's D.

Increased prenatal exposure to dioxins and dl-PCBs, and ndl-PCBs was also associated with a decreased risk of eczema or itching for the first year of life in bivariate logistic regression analyses when the levels of exposure to dietary toxicants were categorised using the tertiles (Table 5). However, when adjusted for maternal BMI in the final models, the associations were not statistically significant (ndl-PCBs: OR (95% CI) *p*-value; 1st vs 2nd tertile: 0.45 (0.18, 1.14) 0.091; 1st vs 3rd tertile: 0.83 (0.36, 1.91) 0.668; dioxins and dl-PCBs: 1st vs 2nd tertile: 0.49 (0.20, 1.22) 0.127; 1st vs 3rd tertile: 0.76 (0.32, 1.78) 0.521). Finally, prenatal exposure to ndl-PCBs was associated with an increased risk of doctor diagnosed allergy up to three years of age (Table 6), but the association was not robust as indicated by residuals and Cook's D (Paper III).

4.2.2 PCBs and dioxins – blood parameters at three years of age

Blood was collected from 112 (56%) of the 205 children. Due to logistic challenges, immunophenotyping and regulatory T cell assessment could only be performed for 81 and 78 samples, respectively. Vaccine-induced antibody levels and concentrations of allergen specific IgE antibodies were measured in 111 samples (110 samples for rubella and measles antibodies). There were no statistically significant differences with regard to frequency of health outcomes in children giving and not giving a blood sample at three years of age (results not shown).

No significant associations were found between prenatal exposure to PCBs and dioxins and the levels of different subpopulations of leukocytes in peripheral blood at three years of age (Paper III). Regarding vaccine-induced antibody levels, prenatal exposure to ndl-PCBs, and dioxins and dl-PCBs was associated with reduced levels of anti-measles antibodies in multivariate analyses (ndl-PCBs: β (95% CI) *p*-value; -0.12 (-0.23, -0.01) 0.032; dioxin and dl-PCBs: -0.15 (-0.29, -0.01) 0.036). None of the other vaccine-induced antibody levels were significantly associated with prenatal exposure to dioxins and dl-PCBs, and ndl-PCBs (Paper III).

Twenty-three of 111 (20.7 %) children were found to be sensitized using Phadiatop® Infant. In bivariate logistic regression analyses, no significant associations were found between prenatal exposure to dioxins and dl-PCBs, and ndl-PCBs and sensitization (Paper III). Furthermore, when the positive sera were analysed for specific IgE antibodies to the

‘individual’ allergen preparations, 15 out of the 23 children were found to be sensitized to at least one of the food allergens tested, nine to at least one of the respiratory allergens tested, whereas five children were not sensitized to any of the ‘individual’ allergen preparations tested.

4.2.5 Acrylamide

No significant associations were found between prenatal dietary exposure to acrylamide and the investigated immune-related health outcomes or blood parameters at any age (Papers II and III).

Table 4. *Results of bivariate analyses of prenatal dietary exposure to ndl-PCBs, dioxins and dl-PCBs, and acrylamide and health outcomes. The dietary toxicants were categorised using the 80th percentile.*

Health outcome	Dietary toxicant	0-1		0-3		2-3	
		OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**
Eczema	Ndl-PCBs	1.17 (0.50-2.72)	0.721	0.75 (0.34-1.68)	0.488	1.00 (0.45-2.23)	0.996
	Dioxins and dl-PCBs	0.96 (0.40-2.31)	0.932	0.57 (0.25-1.31)	0.188	1.00 (0.45-2.23)	0.996
	Acrylamide	1.34 (0.59-3.04)	0.492	1.36 (0.60-3.06)	0.461	1.06 (0.47-2.36)	0.893
Atopic eczema	Ndl-PCBs			0.62 (0.22-1.74)	0.362 [a]	0.85 (0.30-2.42)	0.757
	Dioxins and dl-PCBs			0.62 (0.22-1.74)	0.362	1.11 (0.41-2.99)	0.837
	Acrylamide			1.21 (0.49-2.98)	0.680	1.42 (0.55-3.66)	0.472
Allergy	Ndl-PCBs			1.52 (0.45-5.11)	0.498		
	Dioxins and dl-PCBs			1.00 (0.27-3.78)	0.997		
	Acrylamide			0.57 (0.12-2.64)	0.469		
Wheeze	Ndl-PCBs	**2.79 (1.20-6.49)**	**0.018**	**3.20 (1.42-7.22)**	**0.005**	1.95 (0.83-4.58)	0.126
	Dioxins and dl-PCBs	**3.35 (1.45-7.74)**	**0.005**	**2.71 (1.21-6.04)**	**0.015**	1.95 (0.83-4.58)	0.126
	Acrylamide	1.82 (0.76-4.35)	0.180	1.01 (0.45-2.25)	0.990	1.06 (0.42-2.67)	0.909
Asthma	Ndl-PCBs			1.44 (0.48-4.31)	0.519		
	Dioxins and dl-PCBs			1.94 (0.68-5.54)	0.217		
	Acrylamide			1.03 (0.32-3.32)	0.964		
Asthma medicine	Ndl-PCBs			0.94 (0.37-2.41)	0.902	1.44 (0.59-3.56)	0.425
	Dioxins and dl-PCBs			1.45 (0.60-3.49)	0.412	2.16 (0.91-5.13)	0.080
	Acrylamide			1.18 (0.48-2.91)	0.726	1.16 (0.46-2.95)	0.756

Otitis media	Ndl-PCBs	1.85 (0.67-5.17)	0.238	1.56 (0.67-3.64)	0.301	1.05 (0.39-2.80)	0.931
	Dioxins and dl-PCBs	2.41 (0.90-6.51)	0.082	1.56 (0.67-3.64)	0.301	0.80 (0.28-2.27)	0.675
	Acrylamide	0.97 (0.31-3.07)	0.958	1.35 (0.59-3.08)	0.483	0.80 (0.28-2.27)	0.675
Chicken pox	Ndl-PCBs	1.34 (0.41-4.37)	0.631	0.57 (0.21-1.50)	0.255		
	Dioxins and dl-PCBs	0.90 (0.24-3.30)	0.869	0.72 (0.28-1.82)	0.487		
	Acrylamide	0.23 (0.03-1.83)	0.166	0.42 (0.15-1.16)	0.095		
Exanthema subitum	Ndl-PCBs	1.43 (0.56-3.66)	0.460	1.01 (0.42-2.42)	0.979		
	Dioxins and dl-PCBs	1.12 (0.42-3.00)	0.819	1.01 (0.42-2.42)	0.979		
	Acrylamide	0.61 (0.20-1.88)	0.390	1.39 (0.60-3.21)	0.439		
Gastroenteritis	Ndl-PCBs	0.85 (0.40-1.78)	0.664			1.10 (0.48-2.50)	0.824
	Dioxins and dl-PCBs	0.98 (0.47-2.04)	0.953			1.10 (0.48-2.50)	0.824
	Acrylamide	0.80 (0.38-1.68)	0.558			1.38 (0.60-3.20)	0.450
		0-1		**0-3**		**2-3**	
		β (95% CI)	***p*-value**	**β (95% CI)**	***p*-value**	**β (95% CI)**	***p*-value**
URTI	Ndl-PCBs	**1.10 (0.12-2.08)**	**0.028**	1.19 (0.92-1.54)	0.191 [b]	**1.27 (1.00-1.61)**	**0.050** [b]
	Dioxins and dl-PCBs	0.80 (-0.18-1.78)	0.107	1.20 (0.92-1.56)	0.175 [b]	**1.28 (1.01-1.62)**	**0.042** [b]
	Acrylamide	0.17 (-0.77-1.11)	0.721	1.09 (0.85-1.40)	0.481 [b]	1.12 (0.89-1.42)	0.337 [b]
Gastroenteritis	Ndl-PCBs			0.92 (-0.02-1.85)	0.054		
	Dioxins and dl-PCBs			**1.09 (0.14-2.03)**	**0.024**		
	Acrylamide			-0.02 (-1.00-0.97)	0.977		

Bold font, $p<0.05$; URTI, upper respiratory tract infections.

[a] 1-3 years of age.

[b] The outcome variable was ln-transformed, back-transformed values are reported (ratio of effect).

Table 5. *Results of bivariate analyses of prenatal dietary exposure to ndl-PCBs, dioxins and dl-PCBs, and health outcomes when using categorisations of dietary toxicant variables other than the 80th percentile.*

Health outcome	Dietary toxicant	0-1		0-3		2-3	
		OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**
Eczema	Ndl-PCBs (33-66th P)	**0.34 (0.14-0.83)**	**0.018**				
Eczema	Ndl-PCBs (>66th P)	0.67 (0.31-1.47)	0.320				
Eczema	Dioxins and dl-PCBs (33-66th P)	**0.40 (0.17-0.94)**	**0.035**				
Eczema	Dioxins and dl-PCBs (>66th P)	0.60 (0.27-1.33)	0.209				
Allergy	Ndl-PCBs (C)			**1.12 (1.02-1.23)**	**0.021**		
	Dioxins and dl-PCBs (C)			2.12 (0.85-5.26)	0.106		
Asthma medicine	Ndl-PCBs (C)					1.09 (1.00-1.19)	0.059
	Dioxins and dl-PCBs (C)					2.05 (0.93-4.50)	0.075
Exanthema subitum	Ndl-PCBs (C)	**1.10 (1.02-1.20)**	**0.018**				
	Dioxins and dl-PCBs (C)	**2.38 (1.11-5.13)**	**0.026**				
		0-1		**0-3**		**2-3**	
		β (95% CI)	***p*-value**	**β (95% CI)**	***p*-value**	**β (95% CI)**	***p*-value**
URTI	Ndl-PCBs (C)			1.03 (1.00-1.06)	0.061 [a]	**1.03 (1.00-1.06)**	**0.025** [a]
	Dioxins and dl-PCBs (C)	**1.23 (0.33-2.13)**	**0.008**	**1.31 (1.03-1.68)**	**0.031** [a]		

Bold font, $p<0.05$; P, percentile; C, continuous dietary toxicant variable was used; URTI, upper respiratory tract infections.

[a] The outcome variable was ln-transformed, back-transformed values are reported (ratio of effect).

Table 6. *Results of significant multivariate analyses [a] ($p<0.05$) of prenatal dietary exposure to ndl-PCBs, dioxins and dl-PCBs, and acrylamide and health outcomes. The dietary toxicants were categorised using the 80th percentile if not stated otherwise.*

Health outcome	Dietary toxicant	0-1			0-3			2-3		
		n	**aOR (95% CI)**	***p*-value**	**n**	**aOR (95% CI)**	***p*-value**	**n**	**aOR (95% CI)**	***p*-value**
Wheeze	Ndl-PCBs	190	2.79 (1.20-6.49)	0.018	149	4.03 (1.59-10.23)	0.003			
	Dioxins and dl-PCBs	190	3.35 (1.45-7.74)	0.005	148	2.69 (1.03-7.07)	0.044			
Exanthema subitum	Ndl-PCBs	188	1.10 (1.02-1.20)	0.018 [b]						
	Dioxins and dl-PCBs	188	2.38 (1.11-5.13)	0.026 [b]						
Allergy	Ndl-PCBs				153	1.12 (1.00-1.26)	0.043 [b, c]			
		0-1			**0-3**			**2-3**		
		n	**aβ (95% CI)**	***p*-value**	**n**	**aβ (95% CI)**	***p*-value**	**n**	**aβ (95% CI)**	***p*-value**
URTI	Ndl-PCBs	177	1.16 (0.16-2.16)	0.023	126	1.04 (1.01-1.08)	0.007 [b, d]	160	1.03 (1.00-1.06)	0.025 [b, d]
	Dioxins and dl-PCBs	177	1.31 (0.40-2.22)	0.005 [b]	126	1.42 (1.11-1.83)	0.006 [b, d]	160	1.28 (1.01-1.62)	0.042 [d]
Gastroenteritis	Ndl-PCBs				135	0.94 (0.02-1.85)	0.046 [c]			
	Dioxins and dl-PCBs				135	1.01 (0.08-1.95)	0.034 [c]			

n, number of children included in the final model; aOR: adjusted odds ratio (logistic regression); aβ: adjusted linear regression coefficient; URTI, upper respiratory tract infections.

[a] Variables initially included in the multivariate analyses were: parity, maternal asthma and allergy, maternal age, maternal smoking, maternal passive smoking, maternal education, maternal BMI, child's gender, birth season, type of delivery, apgar score, breast-feeding of the child and day-care attendance. For the third year and all three years merged, also paternal asthma and allergy, mean parental gross income for the three years of the study, gestational age and birth weight were included.

[b] Continuous dietary toxicant variable was used.

[c] Significant association, but the multivariate model was not robust.

[d] The outcome variable was ln-transformed, back-transformed values are reported (ratio of effect).

5 Methodological considerations

5.1 In vitro assay

The overall aim of the work was to investigate the immunotoxic potential of dietary toxicants, with special focus on prenatal exposure to toxicants from the maternal diet. Therefore, in principle, cord blood would have been the best source of mononuclear cells (CBMC). Due to low volumes of cord blood available in the present work, PBMCs from adults were used to obtain sufficient amounts of mononuclear cells. Since CBMCs are immature and may therefore respond differently to exposure than PBMCs from adults (Bessler *et al.*, 2002; Bessler *et al.*, 2007; Goldberg *et al.*, 2008), the possibility of different effects of exposure to the 12 investigated substances for the CBMCs and PBMCs cannot be excluded.

We did not include more than four donors per substance since such an *in vitro* screening assay should be practically feasible with regard to donor recruitment and amount of labour. Cells from both genders were used for all substances due to possible gender differences in the response to toxicant exposure (Aldridge *et al.*, 2003). Due to individual variation, including possible gender differences, and low statistical power, some immunotoxic effects may not have been detected in our *in vitro* assay.

Human liver S9-fraction was used in all cell cultures to allow biotransformation of the 12 substances since it may be the metabolites of a substance that cause the adverse effects after peroral exposure *in vivo* (Coecke *et al.*, 2006). Effects of BaP on gene expression (mRNA) were found to be weaker in cultures without S9-fraction in our *in vitro* system, which suggests that BaP was successfully biotransformed by the enzymes in the S9-fraction (Hochstenbach *et al.*, 2010). This may suggest that the enzymes were able to exert biotransformation under our cell culture conditions. In preliminary experiments, however, the S9-fraction appeared to influence cytokine release and proliferation also in the solvent controls (results not shown). Independent of whether biotransformation was necessary for the substance to exert their effects or not, S9-fraction was added to all cell cultures to minimize cell culture variations.

All 12 substances selected within the NewGeneris project were included in the *in vitro* study. Of these, four were classified as non-immunotoxic substances and were used to investigate whether it was possible to distinguish immunotoxic from non-immunotoxic substances. Since all the substances were classified into carcinogenic and/or immunotoxic substances, and the solvent controls were used in the calculation of the results of the cytokine

release and the proliferation, an improvement might have been to include a substance with no known effects as a negative control.

Viability was measured initially using the trypan blue exclusion technique, which is based on the principle that live cells possess intact cell membranes that exclude the dye trypan blue, whereas dead cells do not (Stober, 1997). This technique has limitations due to the indirect measure of viability based on cell membrane integrity, and the subjective assessment of viability (dye exclusion). Still, this method was selected since the use of objective and less time-consuming assays (Lactate dehydrogenase (LDH) and Alamar blue) were found not to be suitable due to interference by the S9-mix in the cell cultures. Trypan blue uptake, however, was for four of the substances compared with the objective method propidium iodide uptake determined by flow cytometry. The results from the two methods were similar, indicating that subjective interpretation was not a problem in the present work. The viability determined by trypan blue was >90% after exposure to all concentrations of the substances. Still, for some of the concentrations used, proliferation and release of most, if not all, nine cytokines were strongly inhibited. The viability of the cells may be affected, such as cell functions, even if its membrane integrity is maintained as measured by trypan blue exclusion technique (Stober, 1997). The observed inhibition may therefore suggest cytotoxic effects, and these concentrations were excluded (described in section 3.8).

5.2 Questionnaires

The potential selection bias (i.e. a systematic error due to the procedures used to select subjects and from factors that influence study participation which result in a mistaken observed association (Gordis, 2000)) in MoBa due to self-selection of participation has been evaluated by Nilsen *et al.* (2009). Eight well-known exposure-outcome associations were estimated separately for MoBa participants and the total population, such as prenatal smoking and low birth weight (<2500g), chronic hypertension and gestational diabetes and parity and pre-eclampsia. No statistically significant relative differences in association measures were found between MoBa participants and the total population, and the authors suggested that the results of MoBa are not influenced by selection bias (Nilsen *et al.*, 2009). Selection bias due to the low recruitment rate from MoBa into the BraMat cohort, however, cannot be excluded.

Calculation of the dietary exposure using FFQ may give inaccurate exposure estimates due to possible misreporting of the dietary intake, the use of standard portion sizes and the use of expected representative concentrations of toxicants for specific food items. However, the

FFQ used in the present work has been well validated (Brantsaeter *et al.*, 2008a), and calculated intake of PCBs and dioxins from the diet has been found to be correlated with blood concentrations (e.g. dioxins and dl-PCBs: ρ=0.34, p=0.017), and calculated intake of acrylamide correlated with concentrations of acrylamide metabolites in urine (acrylamide: ρ=0.26, p=0.005) (Brantsaeter *et al.*, 2008b; Kvalem *et al.*, 2009). Even though, in general, dietary intake over a certain period and concentrations in biological samples are different measures of exposure and should be compared with caution, the correlations previously reported suggest that the calculated intake of the toxicants reflects the exposure from the diet.

The mothers had the choice of returning the BraMat questionnaire by regular mail or to answer the questionnaire by telephone interview. To avoid differences in the answers for the two answering opportunities, the interviewers were instructed to ask the questions as written in the questionnaires and not to discuss the answers. No statistically significant differences were found with regard to frequency of health outcomes when comparing the written questionnaires and the telephone interviews in the one-year follow-up in the BraMat cohort (Paper II).

5.3 Blood parameters

Antibody responses to vaccines were included as a measure of immune function. Vaccine responses are a promising tool in immunotoxicity testing, including developmental immunotoxicology studies (Luster *et al.*, 2005; van Loveren *et al.*, 2001). It is unlikely that toxicants have the intrinsic capability to disrupt completely the responses to vaccines, but reduced levels of vaccine-induced antibodies may indicate a capacity of immunosuppression. In the present work, it was important to measure the antibody levels at corresponding times with regard to vaccinations for all children to reduce variability in the phase of the immune responses to vaccines. The dates of vaccinations were not available, however, and the time intervals between vaccination and blood sampling could therefore not be adjusted for in the multivariate analyses. The five selected vaccines in the Norwegian Childhood Vaccination Program are generally administered previous to 16 months of age, whereas the blood samples in the present work were collected at about three years of age (33-43 months). Thus, variations in vaccination responses due to differences in time intervals are assumed to be of less importance. Two viral (attenuated measles and rubella), one bacterial toxin (tetanus toxoid) and two bacterial polysaccharide-protein conjugate vaccines (pneumococcus and *Haemophilus influenzae* type b (Hib)) were selected. Different vaccines, including adjuvants,

may stimulate distinct components of the immune system resulting in vaccine-dependent differences in the effects of a toxicant. In addition, exposure to different toxicants may exert different effects on the various vaccine responses.

Immunophenotyping using flow cytometry may be useful in diagnosing individuals with severe immunodeficiency disorders such as HIV, but the method has not always been successful in identifying minor immunodeficiencies in humans, including those associated with toxicant exposures (Luster *et al.*, 2005). However, immunophenotyping was performed in peripheral blood sampled at three years of age, since at the population level immunophenotyping may be valuable to detect immunotoxic effects (Luster *et al.*, 2005). If statistically significant associations between exposure and absolute or relative numbers of different leukocyte populations are found, the associations may be of importance even though the absolute or relative numbers are within normal reported ranges and thus difficult to interpret with regard to biological significance. The numbers of cells in tissues, however, may not be reflected in blood. Furthermore, the absolute or relative numbers of leukocyte populations are only indirect measures of immune function which is more decisive.

5.4 Statistical analyses

Based on other studies reporting associations between prenatal exposure to PCBs and dioxins and immune-related endpoints (Dallaire *et al.*, 2004; ten Tusscher *et al.*, 2003; Weisglas-Kuperus *et al.*, 1995; Weisglas-Kuperus *et al.*, 2000; Weisglas-Kuperus *et al.*, 2004), a follow-up study including 200 participants were considered to have sufficient statistical power to reveal the strongest associations. A study with 200 participants may, however, have too low statistical power to be able to conclude on negative findings, and it may be difficult to properly assess and adjust for all possible confounding factors. To achieve a precise effect estimate, it is possible that all known potential confounding factors have to be included and properly adjusted for in the final models (Budtz-Jorgensen *et al.*, 2007). In the present work, however, potential confounding variables not significant in the multivariate analyses were excluded using backward elimination method to gain statistical power.

On count data, Poisson or negative binomial regression analyses are often used due to the distribution of the data, while linear regression analyses are most appropriate if the data/residuals are normally distributed. The latter was not the case for numbers of upper respiratory tract infections (0-3 and 2-3 years of age). Neither did these data fulfil the assumption of the Poisson regression analysis (equal mean and variance of the Poisson

distribution). The negative binomial regression allows data with a greater variance, as in the present work. However, the results of linear regression analyses with ln-transformed data using the statistical software SPSS were similar to negative binominal regression analyses using the statistical software Stata. Linear regression analyses were therefore chosen for practical reasons.

6 Discussion

6.1 Immunotoxicity of dietary toxicants in vitro

As a result of today's search for alternative methods to the use of animals in toxicity testing of chemicals (Balls *et al.*, 1995), many studies have been performed to establish *in vitro* assays to predict *in vivo* effects of substances on the immune system (Carfi' *et al.*, 2007; Fischer *et al.*, 2011; Galbiati *et al.*, 2010; Hymery *et al.*, 2006; Koeper and Vohr, 2009; Langezaal *et al.*, 2001; Lankveld *et al.*, 2010; Lebrec *et al.*, 1995; Ringerike *et al.*, 2005; Wagner *et al.*, 2006). Assessment of cytokine release from leukocytes *in vitro* has been proposed to be a useful method to examine immunotoxic effects of substances (Corsini and House, 2010; Gennari *et al.*, 2005; House, 1999). Since different substances act via different mechanisms, several cytokines may have to be analyzed to detect immunotoxicity (Ringerike *et al.*, 2005). In the present work, we explored whether effects on the release of nine selected cytokines from human PBMCs in an *in vitro* system could be used as a marker of the immunotoxic properties of dietary toxicants. However, the effects of the 12 selected substances on *in vitro* cytokine release did not reflect the *in vivo*-based classification of their immunotoxic potential. None of the cytokines, alone or in any combination, were identified as a candidate biomarker of immunotoxic exposure. Within the limitations of the study design, our findings do therefore not support the replacement of *in vivo* studies with *in vitro* cytokine release for identification of immunotoxic substances. In contrast, a transcriptomic profile of 48 genes indicative of immunotoxic exposure was found in PBMCs from the same cell cultures (Hochstenbach *et al.*, 2010).

One major challenge with *in vitro* assays is to distinguish immunotoxic from general toxic effects. Initially, viability was determined using the trypan blue exclusion technique to select suitable concentrations of the selected substances to be used in the experiments without causing general toxic effects. Still, proliferation and the release of most, if not all, nine cytokines were strongly inhibited relative to solvent control for some of the concentrations, also by the non-immunotoxic substances. PCA based on cytokine release and proliferation was therefore used in a further attempt to identify and exclude these possible general toxic effects. After exclusion, proliferation was not inhibited by the non-immunotoxic substances, indicating that the remaining concentrations did not cause general toxic effects. On the other hand, immunotoxic effects may also have been excluded. When effects on cytokine release were examined, however, also the non-immunotoxic substances significantly affected

cytokine release relative to solvent control for one or more cytokines, possibly reflecting general toxic effects. Therefore, in our *in vitro* system, general toxic effects may still be a possible explanation for not being able to distinguish the immunotoxic from the non-immunotoxic substances. A recently published study reported that inhibition of mitogen-induced cytokine release from human PBMCs could be used to detect immunotoxic substances by comparing the 50% inhibitory concentration (IC_{50}) with the estimated human 50% lethal concentration (LC_{50}) (Kooijman *et al.*, 2010). If IC_{50} was lower than LC_{50}, the substance was considered to be immunotoxic. With this approach, it may be possible to differentiate between general toxic and immunotoxic effects, however, since the approach is dependent on availability of human data regarding LC_{50}, it is not suitable for novel substances.

Another limitation of *in vitro* systems is the difficulty to extrapolate the relevance of observed *in vitro* effects on cells to organs and cells of the immune system and immune function *in vivo.* In the present work, the observed effects on cytokine release after *in vitro* exposure to the non-immunotoxic substances may therefore not be relevant *in vivo*, which may explain that also the non-immunotoxic substances affected the *in vitro* cytokine release.

Although it was not possible to distinguish the immunotoxic from non-immunotoxic substances in the present work, observed effects of immunotoxic substances on *in vitro* cytokine release were in agreement with published *in vivo* and *ex vivo* animal studies (discussed in Paper I). Exposure to TCDD, as an example, was found to suppress the release of the cytokines IL-4, IFN-γ and IL-17 in our *in vitro* system which is in accordance with the *ex vivo* studies by Ito *et al.* (2002), Nohara *et al.* (2002) and Quintana *et al.* (2008). The agreement between studies indicates that *in vitro* cytokine release may be a useful parameter in mechanistic studies of substances causing direct effects on the immune system. This notion is in accordance with published literature (Gennari *et al.*, 2005; Lankveld *et al.*, 2010; Levin *et al.*, 2008).

6.2 Prenatal exposure to acrylamide and immunotoxicity

Exposure to acrylamide from the maternal diet during pregnancy was not found to be associated with immune-related health outcomes during the three first years of life. Furthermore, no associations were found between prenatal exposure to acrylamide and the immune-related blood parameters investigated at three years of age. To our knowledge, only Zaidi *et al.* (1994) have reported immunosuppressive properties of acrylamide, but this study

was performed in rats. Furthermore, 50 mg/kg bw was administered intraperitoneally (i.p.) daily for 10 days (this dose was found to be an optimum dose to induce hind limb paralysis within 10 days), resulting in a high dose compared to estimated dietary intake for the general human population (1 μg/kg bw/day (JECFA, 2005)) and for the present BraMat cohort (0.56 μg/kg bw/day).

Since the literature about immunotoxic properties of acrylamide *in vivo* is scarce in contrast to literature about carcinogenic, genotoxic, neurotoxic and reproductive toxic properties (Carere, 2006; Hogervorst *et al.*, 2010; Parzefall, 2008), acrylamide was defined in the *in vitro* study as a non-immunotoxic substance (Paper I). Shortage of publications, however, is not sufficient to conclude that acrylamide does not exert immunotoxic effects, and could be a result of a lack of studies and a lack of publication of negative results (publication bias). The proposed carcinogenic and neurotoxic properties of acrylamide may suggest that acrylamide also has immunotoxic properties since the immune system is both involved in killing tumor cells and interacts closely with the nervous system (Murphy *et al.*, 2008; Rosas-Ballina *et al.*, 2011; Wong *et al.*, 2011). Carcinogenic effects of acrylamide, or its metabolite glycidamide, in animal studies have been reported. Furthermore, acrylamide exposure has also been reported to be associated with increased risk of cancer in humans (Hogervorst *et al.*, 2007; Hogervorst *et al.*, 2008; Hogervorst *et al.*, 2010; Wilson *et al.*, 2010). Regarding neurotoxicity, it has been reported neurotoxic effects in humans occupationally exposed to high levels of acrylamide (Hagmar *et al.*, 2001; Kjuus *et al.*, 2004). To our knowledge, neurotoxic effects have not been observed in the general non-smoking population where food is assumed to be the major source of acrylamide exposure. Indirect immunotoxic effects due to the proposed neurotoxic properties may therefore not be very likely in our study population. Although the statistical power may be too low to conclude on negative findings, our study is in line with the notion that dietary acrylamide does not exert immunotoxic effects in the general non-smoking population. Irrespective of whether acrylamide is an immunotoxicant or not, the present nutritional guidelines for pregnant women may ensure low exposure since important sources of acrylamide are food items considered to be unhealthy, such as potato crisps and deep-fried potatoes (Norwegian food safety authority, 2011a).

6.3 Prenatal exposure to PCBs and dioxins and immunotoxicity

6.3.1 Susceptibility to infections

Prenatal dietary exposure to PCBs and dioxins was found to be associated with increased numbers of upper respiratory tract infections up to three years of age. Furthermore, exposure to PCBs and dioxins was associated with an increased risk of the common childhood disease exanthema subitum (roseola infantum) during the first year of life, and a tendency of increased numbers of gastroenteritis episodes up to three years of age. The results suggest that prenatal exposure to PCBs and dioxins is associated with an increased risk of infections, indicating immunosuppressive effects (discussed in section 6.3.5), persisting up to the third year of life. In agreement with our findings, other studies have reported associations between the levels of PCBs and dioxins in blood or breast milk and increased frequency of infections, especially lower respiratory tract infections and otitis media (Dallaire *et al.*, 2004; Dallaire *et al.*, 2006; Miyashita *et al.*, 2011; Weisglas-Kuperus *et al.*, 2000; Weisglas-Kuperus *et al.*, 2004). Glynn *et al.* (2008), however, reported divergent results regarding respiratory infections when prenatal exposure to individual congeners of PCBs in Swedish children was investigated.

6.3.2 Wheeze

Prenatal exposure to PCBs and dioxins from the maternal diet was found to be associated with an increased risk of wheeze during the first year and cumulatively during the three first years of life. No associations were found, however, between prenatal exposure to PCBs and dioxins and wheeze in the third year only. Wheeze may be divided into different phenotypes, and the most common are transient infantile wheeze, viral-associated wheeze and atopic wheeze (Sly *et al.*, 2008; Stein and Martinez, 2004). However, the division into three phenotypes does not mean that the groups are exclusive. Viral-associated wheeze is thought to occur due to changes in the airways associated with viral infections. Atopic wheeze is associated with sensitisation to aeroallergens and with other atopic diseases, whereas transient infantile wheeze is associated with reduced lung function before any event of lower respiratory illness has occurred. Any insult that decreases airway diameter or alters airway wall compliance could lead to wheeze. In the present work, we were not able to distinguish between the wheeze phenotypes (discussed in Papers II and III). When wheeze was combined with the number of upper respiratory tract infections (< 7 or ≥ 7 episodes of infections) during the first year of life, however, we found a significantly stronger association between prenatal exposure

to PCBs and dioxins and wheeze along with seven or more episodes of infection compared to wheeze along with less than seven episodes (results not shown). Although the statistical power of the analysis is low, our results may suggest that the observed association between prenatal dietary exposure to PCBs and dioxins and wheeze may be mainly due to respiratory infections. The influence of respiratory infections in inducing wheeze may be of less importance in later childhood since the diameter of the airways increases as the child grows older. The observed decreasing tendency with time of wheeze associated with exposure to PCBs and dioxins may therefore further support the notion that the exposure is associated with wheeze mainly induced by respiratory infections.

Weisglas-Kuperus *et al.* (2000) reported dioxin exposure, as measured in breast milk, to be associated with a higher occurrence of coughing, chest congestion and phlegm lasting for at least 10 days in 3-4-year-old children. At both 3-4 years of age and at school age, however, they reported that prenatal PCB exposure was associated with less shortness of breath with wheeze (Weisglas-Kuperus *et al.*, 2000; Weisglas-Kuperus *et al.*, 2004). It is difficult to compare our findings and the findings of the two studies of Weisglas-Kuperus *et al.* (2000 and 2004) due to differently formulated questions in the questionnaires. The association between exposure and a higher occurrence of coughing, chest congestion and phlegm lasting for at least 10 days, however, may reflect respiratory infections, which is in concordance with the present work. The associations between exposure and less shortness of breath with wheeze at 3-4 years of age and at school age may indicate that the effects of prenatal PCB exposure on wheeze may change as the child grows older and the influence of respiratory infections in inducing wheeze may become less important.

6.3.3 Blood parameters

Antibody responses to five different vaccines included in the Norwegian Childhood Vaccination Program were analysed in three-year-old children. Prenatal exposure to PCBs and dioxins was found to be associated with a reduced antibody response to the measles vaccine. Reduced vaccination responses may be a result of immunosuppression (discussed in section 6.3.5) and may indicate a decreased host capacity for immune responses against pathogens, which is in accordance with the observed increased number of infections. Associations between PCB exposure and reduced antibody responses to vaccines have also been reported in other human studies (Heilmann *et al.*, 2006; Heilmann *et al.*, 2010; Weisglas-Kuperus *et al.*, 2000). No significant associations were found for antibody

responses to vaccines tested other than measles. Effects of exposure to PCBs and dioxins may differ for different vaccines as discussed in section 5.3.

No associations were found between prenatal exposure to PCBs and dioxins and immunophenotype data, including regulatory T cell data. Similarly, no associations were found between prenatal exposure and allergic sensitization in the three-year-old children. In contrast, associations between prenatal exposure to PCBs and dioxins and the levels of different leukocyte populations in blood have been reported in other human studies (Glynn *et al.*, 2008; Weisglas-Kuperus *et al.*, 1995; Weisglas-Kuperus *et al.*, 2000). One might expect to find effects on immunophenotype data since effects on other immune-related endpoints were observed. Since immunophenotyping was only performed on about 80 samples in the present work, low statistical power may be a reason for the lack of associations. Immunophenotyping was also performed on cord blood in the BraMat cohort and another Norwegian NewGeneris cohort (n=295). Despite a larger sample size of cord blood (compared to blood samples at three years), no associations were found between exposure to PCBs and dioxins and the levels of different leukocyte populations in blood (results not shown). Our results indicate that functional endpoints such as vaccine antibody responses may be more sensitive in identifying associations with toxicant exposures than non-functional endpoints as immunophenotype distribution (see discussion in section 5.3).

6.3.4 Foetal stage, a critical time window?

As discussed in Paper III, only 5 of the participating mothers had a slightly higher intake than the tolerable weekly intake (TWI) of 14 pg TEQ/kg bw/week (set by the EU Scientific Committee on Food (Scientific Committee on Food, 2001)). Considering the relative low exposure levels, assumed to be representative for the general population (Kvalem, 2010), it gives reason for concern that prenatal exposure to PCBs and dioxins was found to be associated with changes in immune-related endpoints, even as late as at three years of age. The result may be in line with the notion that the foetal stage is a critical time window of immune system vulnerability to immunotoxicant exposures (Dietert, 2008; Holsapple *et al.*, 2004; van Loveren and Piersma, 2004; West, 2002). It is challenging in a cohort study like ours, however, to differentiate between prenatal and postnatal dietary exposure of the child. Breast-fed children may be exposed to PCBs and dioxins postnatally since these toxicants are transferred to breast milk (Ayotte *et al.*, 2003; Polder *et al.*, 2008; Tsukimori *et al.*, 2011), and the mother's diet is probably similar to the diet during pregnancy. Breast-feeding of the children was therefore adjusted for in the multivariate analyses. In addition, the children in the

present work have also been exposed through their own diet, other than breast milk, to these toxicants. Since postnatal exposure data on PCBs and dioxins were not available for the BraMat cohort, it was not possible to identify the role of postnatal exposure. However, in a subgroup of the BraMat children (n=111), changes in expression of immune-related genes in cord blood were found to correlate with both prenatal exposure to PCBs and TCDD and the antibody response to the measles vaccine at three years of age (Hochstenbach *et al*., in preparation). This may suggests that the immunosuppression observed in the present work, at least in part, was caused by prenatal exposure to PCBs and dioxins.

Prenatal exposure may result in different types of effects on the immune system compared to exposure later in life. An illustrating example is exposure to TCDD and the onset of autoimmune diseases in mice. Perinatal exposure in mice is reported to possibly increase the risk of developing autoimmune diseases, in contrast to exposure in later life which may lower the risk of developing autoimmune diseases (Gogal, Jr. and Holladay, 2008; Holladay *et al.*, 2011; Kerkvliet *et al.*, 2009; Li and McMurray, 2009; Quintana *et al.*, 2008; Zhang *et al.*, 2010). Humans are exposed to PCBs and dioxins throughout life, and it is therefore challenging to distinguish the effects of early life exposure from the effects of exposure in later life.

6.3.5 Mechanisms

As discussed in Papers II and III, our findings that prenatal PCB and dioxin exposure is associated with an increased risk of wheeze and infections, and with reduced levels of vaccine-induced antibodies are in accordance with the proposed immunosuppressive properties of dioxins and dl-PCBs. Immunosuppression in general is expected to result in more infections and less inflammatory diseases such as allergy and autoimmunity. Based on animal studies, a proposed mechanistic explanation is that dioxins and dl-PCBs bind to the aryl hydrocarbon receptor (AhR) which results in expansion of the population of regulatory T cells and subsequently suppressed immune responses (Funatake *et al.*, 2005; Ho and Steinman, 2008; Kerkvliet *et al.*, 2009; Marshall *et al.*, 2008; Marshall and Kerkvliet, 2010; Quintana *et al.*, 2008). No associations were found in our study between prenatal exposure to PCBs and dioxins and relative number of regulatory T cells in blood. The function and number of regulatory T cells in tissues with an ongoing inflammatory response, however, may not be reflected in blood (Seddiki and Kelleher, 2008), which may explain the lack of association between exposure and relative number of regulatory T cells in the present work. Furthermore, low statistical power can also be a reason.

It is interesting that different high affinity ligands of AhR, such as TCDD (a dioxin) and FICZ (a natural photoproduct), may exert divergent effects on the immune system (Quintana *et al.*, 2008). In contrast to TCDD, FICZ have been reported to expand the population of Th17 cells resulting in increased inflammatory responses. It has earlier been proposed that the different effects of TCDD compared to FICZ are due to high toxicity of TCDD (Veldhoen *et al.*, 2008). A proportional shift in regulatory T cells may be due to the death of other cells rather than an actual expansion in numbers. However, given a major difference in half-lives of these ligands *in vivo*, it is likely that the effects of the prolonged exposure to TCDD compared to FICZ can explain the apparent functional differences (Stockinger *et al.*, 2011).

In the present work, a tendency of lowered risk of eczema or itching with increasing prenatal exposure to PCBs and dioxins was found during the first year of life, but no significant associations were found for either eczema/itching or doctor diagnosed atopic eczema in later childhood. On the other hand, a tendency of increased risk of doctor diagnosed allergy was found during the three first years of life, but no significant association was found between prenatal exposure to PCBs and dioxins and allergic sensitization at three years of age. With regard to allergic diseases in humans, exposure to PCBs and dioxins in general has been found to be associated with a reduced risk of allergy and atopic eczema, which is confirmed in animal studies (Grandjean *et al.*, 2010; Luebke *et al.*, 2001; Schulz *et al.*, 2011; Tarkowski *et al.*, 2010; ten Tusscher *et al.*, 2003; Weisglas-Kuperus *et al.*, 2000). However, exposure to PCBs and dioxins has also been found to be associated with increased levels of total IgE and a possible increased risk of food allergy (Grandjean *et al.*, 2010; Miyashita *et al.*, 2011). Furthermore, it has been reported that TCDD exposure in mice may impair the oral tolerance induction, which results in increased risk of sensitization to food allergens (Chmill *et al.*, 2010; Kinoshita *et al.*, 2006). Increased levels of total IgE antibodies and increased risk of food allergy may suggest that perinatal exposure to PCBs and dioxins also affects the Th1/Th2-balance upon birth which may result in Th2 skewness in the foetus and early childhood, persisting into later child- and adulthood. Th2 skewness may also be an explanation for immunosuppressive effects such as increased susceptibility to infections (Luster *et al.*, 2005). Conflicting results regarding allergy in humans may also be due to exposure to ndl-PCBs, which may be highly correlated to dioxins and dl-PCBs, but may exert effects independent of AhR (Ferrante *et al.*, 2011; Fischer *et al.*, 1998; Levin *et al.*, 2005; Lyche *et al.*, 2004; Lyche *et al.*, 2006).

6.3.6 Confounding exposure factors

In the present work, dioxins and dl-PCBs, and ndl-PCBs were found to be highly correlated (τ_b= 0.81, p<0.001). Separate statistical analyses were therefore performed and conclusions drawn without differentiating between the two groups of PCBs and dioxins. As discussed above, our findings on immune-related parameters and prenatal PCB and dioxin exposure are in accordance with the proposed immunosuppressive properties of dioxins and dl-PCBs. We cannot exclude, however, the possibility that ndl-PCBs may exert similar immunotoxic effects independent of AhR. In risk assessments for humans, it may be more important to examine the effects of the mixture of PCBs and dioxins than investigating effects of different PCB and dioxin congeners individually. The majority of individual PCBs and dioxins possess their own intrinsic toxicities, and they can interact additively, synergistically and/or antagonistically, resulting in high variation in the effects of mixtures (White and Birnbaum, 2009).

An important limitation, which is generally valid for all epidemiological studies, is the uncertainty about whether other substances or factors contribute to the associations found. n-3 fatty acids are suggested to have anti-inflammatory properties (Chapkin *et al.*, 2009; Klemens *et al.*, 2011; Wall *et al.*, 2010). The calculated maternal intake from food items of the n-3 long-chain fatty acids eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (described in Haugen *et al*. (2008)) was found to be highly correlated with the maternal intake of dioxins and dl-PCBs, and ndl-PCBs (ρ = 0.78, p<0.001, and ρ =0.80, p<0.001, respectively) as expected due to their common dietary source (seafood) and the lipophilic properties of the compounds. Therefore, n-3 fatty acids may possibly influence the associations found between exposure to PCBs and dioxins and immune-related endpoints in the present work. Due to the high correlation, we could not adjust for these n-3 fatty acids in the statistical analyses. However, when investigating prenatal exposure to the long chain n-3 fatty acids from food items and health outcomes (0-3 and 2-3 years of age), a significant association was only found for wheeze during the three first years of life (bivariate analysis; OR (95% CI) p-value; 2.37 (1.03-5.46) 0.043). Interestingly, no associations were found between long chain n-3 fatty acids from dietary supplements and health outcomes (results not shown). Since dietary supplements containing n-3 fatty acids are purified to remove toxicants such as PCBs and dioxins, these results may suggest that exposure to PCBs and dioxins, rather than n-3 fatty acids, is most important for the immunosuppression found in the present work.

6.3.7 Implications of the findings

The findings in the present work may suggest that prenatal exposure to levels of PCBs and dioxins from the maternal diet representative for the general population increases the risk of wheeze and infections during the three first years of life. Reducing the level of dietary exposure to these toxicants for women of fertile age may therefore be beneficial for their children's health. For the pregnant women in the BraMat cohort, fat fish was an important source of exposure to PCBs and dioxins. Since fish also provides a healthful source of dietary proteins and is high in nutrients that may have beneficial effects on human health, such as n-3 fatty acids (Costa, 2007), reducing or avoiding fish in the diet, in general, is not advised by the Norwegian authorities (Norwegian food safety authority, 2011b). However, for women of fertile age it is recommended to reduce or avoid consumption of food items with possible high levels of PCBs and dioxins such as the most polluted fish, brown meat of crabs and food items containing fish liver (Norwegian food safety authority, 2011a; Norwegian food safety authority, 2011c). Furthermore, it is recommended to avoid consumption of seagull eggs, which may be highly contaminated with PCBs and dioxins. Consumption of seagull eggs was reported by 5 of the mothers in the BraMat cohort. The results of the present work are in line with the dietary advice from the authorities to women of fertile age.

6.4 Future perspectives

In the *in vitro* study, it was not possible to distinguish immunotoxic from non-immunotoxic substances by investigating effects on *in vitro* cytokine release. However, a transcriptomic profile indicative of immunotoxic exposure was identified in PBMCs from the same cell cultures (Hochstenbach *et al.*, 2010). Considering today's search for alternative methods to the use of animals in toxicity testing of chemicals (Balls *et al.*, 1995), it is important to continue working on developing reliable *in vitro* methods able to predict immunotoxicity, such as methods based on the promising tool transcriptomics.

The number of participants in the BraMat birth cohort was found to be sufficient to find associations between toxicant exposure and health outcomes and blood parameters. Still, studies should preferentially be performed in a cohort with more participants to increase the statistical power, especially regarding acrylamide, to be able to conclude on the negative findings. More statistical power may also be needed to further investigate the associations and the tendencies of associations found for PCBs and dioxins to sufficiently adjust for all

possible confounders in the final multivariate model to obtain more precise effect estimates and narrower CI. Furthermore, additional associations may be found in a larger study population, and it may be possible to identify vulnerable subgroups.

Dietary exposure to PCBs and dioxins during pregnancy was investigated in the present work, whereas exposure data later in childhood were not available. If possible, also postnatal dietary exposure to the toxicants should be determined to investigate whether the prenatal, postnatal or both periods are critical time windows of exposure. In addition, it would be of interest to compare the relative importance of maternal blood levels (body burden) and the dietary intake during pregnancy of these toxicants and their immune-related effects in the child.

Regarding acrylamide, it is of interest to study whether immunotoxic effects in occupationally exposed humans occur, since neurotoxic effects in these highly exposed humans have been reported. Occupational exposure may not be comparable to prenatal dietary exposure since the exposure routes are different, the foetus may be more sensitive to toxicant exposure than adults, and different effects may appear. However, if no immunotoxicity is observed in occupationally exposed humans, it may suggest that prenatal exposure to acrylamide from food does not exert immunotoxic effects in the general population. Furthermore, more animal studies may be valuable to gain knowledge about whether prenatal dietary exposure to acrylamide exerts immunotoxic effects.

7 Conclusions

Based upon the results from the present work and the literature, as discussed in the present thesis, the following conclusions may be drawn:

- The effect of the 12 selected substances on *in vitro* cytokine release from PBMCs did not reflect the *in vivo*-based classification of their immunotoxic potential according to published data. Within the limitations of the study design, our results therefore do not support the replacement of *in vivo* studies with *in vitro* cytokine release for identification of immunotoxic substances.

- Prenatal exposure to acrylamide from the maternal diet was not found to be associated with immune-related health outcomes or blood parameters during the three first years of life, but the statistical power may be too low to conclude on negative findings. Prenatal exposure to PCBs and dioxins from the maternal diet was found to be associated with an increased risk of wheeze up to three years of age (0-1 and 0-3 years), but not during the third year of life (2-3 years). The data suggest that the wheeze mainly was infection-induced. Furthermore, prenatal exposure to PCBs and dioxins was found to be associated with increased frequency of infections during the three first years of life. Prenatal exposure to PCBs and dioxins was also associated with reduced levels of vaccine-induced antibodies to measles at three years of age. Overall, the results suggest that prenatal exposure to PCBs and dioxins may result in immunosuppression and thereby increased susceptibility to infections in early childhood. A continued effort to reduce the exposure to PCBs and dioxins from food for women of fertile age appears therefore to be beneficial for their children's health.

8 References

Adami, H. O., Berry, S. C., Breckenridge, C. B., Smith, L. L., Swenberg, J. A., Trichopoulos, D., Weiss, N. S., and Pastoor, T. P. (2011). Toxicology and epidemiology: improving the science with a framework for combining toxicological and epidemiological evidence to establish causal inference. *Toxicol Sci* **122,** 223-234.

Akdis, C. A. (2006). Allergy and hypersensitivity: mechanisms of allergic disease. *Curr Opin. Immunol* **18,** 718-726.

Aldridge, J. E., Gibbons, J. A., Flaherty, M. M., Kreider, M. L., Romano, J. A., and Levin, E. D. (2003). Heterogeneity of toxicant response: sources of human variability. *Toxicol Sci* **76,** 3-20.

Annola, K., Heikkinen, A. T., Partanen, H., Woodhouse, H., Segerback, D., and Vahakangas, K. (2009). Transplacental transfer of nitrosodimethylamine in perfused human placenta. *Placenta* **30,** 277-283.

Annola, K., Karttunen, V., Keski-Rahkonen, P., Myllynen, P., Segerback, D., Heinonen, S., and Vahakangas, K. (2008). Transplacental transfer of acrylamide and glycidamide are comparable to that of antipyrine in perfused human placenta. *Toxicol Lett.* **182,** 50-56.

Aoki, Y. (2001). Polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans as endocrine disrupters--what we have learned from Yusho disease. *Environ Res* **86,** 2-11.

Ayotte, P., Muckle, G., Jacobson, J. L., Jacobson, S. W., and Dewailly, E. (2003). Assessment of pre- and postnatal exposure to polychlorinated biphenyls: lessons from the Inuit Cohort Study. *Environ Health Perspect* **111,** 1253-1258.

Balls, M., Goldberg, A. M., Fentem, J. H., Broadhead, C. L., Burch, R. L., Festing, M. F., Frazier, J. M., Hendriksen, C. F., Jennings, M., van der Kamp, M. D., Morton, D. B., Rowan, A. N., Russell, C., Russell, W. M., Spielmann, H., Stephens, M. L., Stokes, W. S., Straughan, D. W., Yager, J. D., Zurlo, J., and van Zutphen, B. F. (1995). The three Rs: the way forward: the report and recommendations of ECVAM Workshop 11. *Altern. Lab. Anim.* **23,** 838-866.

Barker, D. J. (1995). Fetal origins of coronary heart disease. *BMJ* **311,** 171-174.

Barker, D. J., Winter, P. D., Osmond, C., Margetts, B., and Simmonds, S. J. (1989). Weight in infancy and death from ischaemic heart disease. *Lancet* **2,** 577-580.

Belsley, D. A., Kuh, E., and Welsh, R. E. (1980). Regression Diagnostics: Identifying Influential Data and Sources of Collinearity, p. 32. John Wiley & Sons, New York.

Bessler, H., Wyshelesky, G., Osovsky, M., Prober, V., and Sirota, L. (2007). A comparison of the effect of vitamin A on cytokine secretion by mononuclear cells of preterm newborns and adults. *Neonatology.* **91,** 196-202.

Bessler, H., Ziyada, S., Bergman, M., Punsky, I., and Sirota, L. (2002). Indomethacin and ibuprofen effect on IL-1ra production by mononuclear cells of preterm newborns and adults. *Biol Neonate* **82,** 73-77.

Bogen, B., and Munthe, L. A. (2007). Immunologi, pp. 1-334. Universitetsforlaget AS, Oslo.

Brantsaeter, A. L., Haugen, M., Alexander, J., and Meltzer, H. M. (2008a). Validity of a new food frequency questionnaire for pregnant women in the Norwegian Mother and Child Cohort Study (MoBa). *Matern. Child Nutr* **4,** 28-43.

Brantsaeter, A. L., Haugen, M., Mul, A., Bjellaas, T., Becher, G., Klaveren, J. V., Alexander, J., and Meltzer, H. M. (2008b). Exploration of different methods to assess dietary acrylamide exposure in pregnant women participating in the Norwegian Mother and Child Cohort Study (MoBa). *Food Chem. Toxicol.* **46,** 2808-2814.

Budtz-Jorgensen, E., Keiding, N., Grandjean, P., and Weihe, P. (2007). Confounder selection in environmental epidemiology: assessment of health effects of prenatal mercury exposure. *Ann Epidemiol* **17,** 27-35.

Buell, J. F., Gross, T. G., and Woodle, E. S. (2005). Malignancy after transplantation. *Transplantation* **80,** S254-S264.

Burleson, G. R., and Dean, J. H. (1995). Immunotoxicology: Past, Present, and Future. In *Methods in Immunotoxicology* (G.R.Burleson, J.H.Dean, and A.E.Munson, Eds.), pp. 3-10. Wiley-Liss, New York.

Calleman, C. J. (1996). The metabolism and pharmacokinetics of acrylamide: implications for mechanisms of toxicity and human risk estimation. *Drug Metab Rev* **28,** 527-590.

Carere, A. (2006). Genotoxicity and carcinogenicity of acrylamide: a critical review. *Ann Ist. Super. Sanita* **42,** 144-155.

Carfi', M., Gennari, A., Malerba, I., Corsini, E., Pallardy, M., Pieters, R., van Loveren, H., Vohr, H. W., Hartung, T., and Gribaldo, L. (2007). In vitro tests to evaluate immunotoxicity: a preliminary study. *Toxicology* **229,** 11-22.

Chapkin, R. S., Kim, W., Lupton, J. R., and McMurray, D. N. (2009). Dietary docosahexaenoic and eicosapentaenoic acid: emerging mediators of inflammation. *Prostaglandins Leukot. Essent. Fatty Acids* **81,** 187-191.

Charnley, G., and Doull, J. (2005). Human exposure to dioxins from food, 1999-2002. *Food Chem. Toxicol.* **43,** 671-679.

Chmill, S., Kadow, S., Winter, M., Weighardt, H., and Esser, C. (2010). 2,3,7,8-Tetrachlorodibenzo-p-dioxin impairs stable establishment of oral tolerance in mice. *Toxicol Sci* **118,** 98-107.

Coecke, S., Ahr, H., Blaauboer, B. J., Bremer, S., Casati, S., Castell, J., Combes, R., Corvi, R., Crespi, C. L., Cunningham, M. L., Elaut, G., Eletti, B., Freidig, A., Gennari, A., Ghersi-Egea, J. F., Guillouzo, A., Hartung, T., Hoet, P., Ingelman-Sundberg, M., Munn, S., Janssens, W., Ladstetter, B., Leahy, D., Long, A., Meneguz, A., Monshouwer, M., Morath, S., Nagelkerke, F., Pelkonen, O., Ponti, J., Prieto, P., Richert, L., Sabbioni, E., Schaack, B.,

Steiling, W., Testai, E., Vericat, J. A., and Worth, A. (2006). Metabolism: a bottleneck in in vitro toxicological test development. The report and recommendations of ECVAM workshop 54. *Altern. Lab. Anim.* **34,** 49-84.

Corsini, E., and House, R. V. (2010). Evaluating cytokines in immunotoxicity testing. *Methods Mol Biol* **598,** 283-302.

Costa, L. G. (2007). Contaminants in fish: risk-benefit considerations. *Arh. Hig. Rada Toksikol.* **58,** 367-374.

Covaci, A., Jorens, P., Jacquemyn, Y., and Schepens, P. (2002). Distribution of PCBs and organochlorine pesticides in umbilical cord and maternal serum. *Sci Total Environ* **298,** 45-53.

Curotto de Lafaille, M. A., and Lafaille, J. J. (2009). Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* **30,** 626-635.

Dallaire, F., Dewailly, E., Muckle, G., and Ayotte, P. (2003). Time trends of persistent organic pollutants and heavy metals in umbilical cord blood of Inuit infants born in Nunavik (Quebec, Canada) between 1994 and 2001. *Environ Health Perspect* **111,** 1660-1664.

Dallaire, F., Dewailly, E., Muckle, G., Vezina, C., Jacobson, S. W., Jacobson, J. L., and Ayotte, P. (2004). Acute infections and environmental exposure to organochlorines in Inuit infants from Nunavik. *Environ. Health Perspect.* **112,** 1359-1365.

Dallaire, F., Dewailly, E., Vezina, C., Muckle, G., Weber, J. P., Bruneau, S., and Ayotte, P. (2006). Effect of prenatal exposure to polychlorinated biphenyls on incidence of acute respiratory infections in preschool Inuit children. *Environ. Health Perspect.* **114,** 1301-1305.

Davies, G. E. (1983). mmunotoxicity: Undesirable effects of inappropriate responsesI. *Immunol Today* **4,** 1-2.

De Jong, W. H., and van Loveren, H. (2007). Screening of xenobiotics for direct immunotoxicity in an animal study. *Methods* **41,** 3-8.

Dean, J. H., Padarathsingh, M. L., and Jerrells, T. R. (1979). Assessment of immunobiological effects induced by chemicals, drugs or food additives. I. Tier testing and screening approach. *Drug Chem Toxicol* **2,** 5-17.

Denison, M. S., Soshilov, A. A., He, G., Degroot, D. E., and Zhao, B. (2011). Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol Sci* **124,** 1-22.

Descotes, J. (1999). An introduction to immunotoxicology, Taylor & Francis, London.

Descotes, J. (2005). Immunotoxicology: role in the safety assessment of drugs. *Drug Saf* **28,** 127-136.

Descotes, J. (2006). Methods of evaluating immunotoxicity. *Expert Opin. Drug Metab Toxicol* **2,** 249-259.

Dietert, R. R. (2008). Developmental immunotoxicology (DIT): windows of vulnerability, immune dysfunction and safety assessment. *J Immunotoxicol.* **5,** 401-412.

Dietert, R. R., and Piepenbrink, M. S. (2006). Perinatal immunotoxicity: why adult exposure assessment fails to predict risk. *Environ Health Perspect* **114,** 477-483.

Doerge, D. R., da Costa, G. G., McDaniel, L. P., Churchwell, M. I., Twaddle, N. C., and Beland, F. A. (2005). DNA adducts derived from administration of acrylamide and glycidamide to mice and rats. *Mutat. Res* **580,** 131-141.

Domingo, J. L., and Bocio, A. (2007). Levels of PCDD/PCDFs and PCBs in edible marine species and human intake: a literature review. *Environ Int* **33,** 397-405.

EFSA (2005). Opinion of the scientific panel on contaminants in the food chain on a request from the Commission related to the presence of non dioxin-like polychlorinated biphenyls (PCB) in feed and food. *EFSA J.* **284,** 1-137.

Engels, E. A., Pfeiffer, R. M., Fraumeni, J. F., Jr., Kasiske, B. L., Israni, A. K., Snyder, J. J., Wolfe, R. A., Goodrich, N. P., Bayakly, A. R., Clarke, C. A., Copeland, G., Finch, J. L., Fleissner, M. L., Goodman, M. T., Kahn, A., Koch, L., Lynch, C. F., Madeleine, M. M., Pawlish, K., Rao, C., Williams, M. A., Castenson, D., Curry, M., Parsons, R., Fant, G., and Lin, M. (2011). Spectrum of cancer risk among US solid organ transplant recipients. *JAMA* **306,** 1891-1901.

European Commission (2010).
http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm.

Fennell, T. R., Sumner, S. C., Snyder, R. W., Burgess, J., and Friedman, M. A. (2006). Kinetics of elimination of urinary metabolites of acrylamide in humans. *Toxicol Sci* **93,** 256-267.

Ferrante, M. C., Mattace, R. G., Esposito, E., Bianco, G., Iacono, A., Clausi, M. T., Amero, P., Santoro, A., Simeoli, R., Autore, G., and Meli, R. (2011). Effects of non-dioxin-like polychlorinated biphenyl congeners (PCB 101, PCB 153 and PCB 180) alone or mixed on J774A.1 macrophage cell line: modification of apoptotic pathway. *Toxicol Lett.* **202,** 61-68.

Fischer, A., Koeper, L. M., and Vohr, H. W. (2011). Specific antibody responses of primary cells from different cell sources are able to predict immunotoxicity in vitro. *Toxicol In Vitro* **25,** 1966-1973.

Fischer, L. J., Seegal, R. F., Ganey, P. E., Pessah, I. N., and Kodavanti, P. R. (1998). Symposium overview: toxicity of non-coplanar PCBs. *Toxicol. Sci.* **41,** 49-61.

Flaherty, D. K. (2005). Immunotoxicology. In *Encyclopedic reference of immunotoxicology* (H.W.Vohr, Ed.), pp. 340-342. Springer, Heidelberg.

Funatake, C. J., Marshall, N. B., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2005). Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin generates a population of CD4+ CD25+ cells with characteristics of regulatory T cells. *J Immunol* **175,** 4184-4188.

Galbiati, V., Mitjans, M., and Corsini, E. (2010). Present and future of in vitro immunotoxicology in drug development. *J Immunotoxicol.* **7,** 255-267.

Gennari, A., Ban, M., Braun, A., Casati, S., Corsini, E., Dastych, J., Descotes, J., Hartung, T., Hooghe-Peters, R., House, R., Pallardy, M., Pieters, R., Reid, L., Tryphonas, H., Tschirhart, E., Tuschl, H., Vandebriel, R., and Gribaldo, L. (2005). The Use of In Vitro Systems for Evaluating Immunotoxicity: The Report and Recommendations of an ECVAM Workshop. *J. Immunotoxicol.* **2,** 61-83.

Glynn, A., Thuvander, A., Aune, M., Johannisson, A., Darnerud, P. O., Ronquist, G., and Cnattingius, S. (2008). Immune cell counts and risks of respiratory infections among infants exposed pre- and postnatally to organochlorine compounds: a prospective study. *Environ Health* **7,** 62.

Gogal, R. M., Jr., and Holladay, S. D. (2008). Perinatal TCDD exposure and the adult onset of autoimmune disease. *J Immunotoxicol.* **5,** 413-418.

Goldberg, M. R., Nadiv, O., Luknar-Gabor, N., Zadik-Mnuhin, G., Tovbin, J., and Katz, Y. (2008). Correlation of Th1-type cytokine expression and induced proliferation to lipopolysaccharide. *Am. J. Respir. Cell Mol. Biol.* **38,** 733-737.

Gordis, L. (2000). Epidemiology, W.B. Saunders Company, Philadelphia.

Govaris, A., Botsoglou, N., Papageorgiou, G., Botsoglou, E., and Ambrosiadis, I. (2004). Dietary versus post-mortem use of oregano oil and/or alpha-tocopherol in turkeys to inhibit development of lipid oxidation in meat during refrigerated storage. *Int J Food Sci Nutr* **55,** 115-123.

Grandjean, P., Poulsen, L. K., Heilmann, C., Steuerwald, U., and Weihe, P. (2010). Allergy and sensitization during childhood associated with prenatal and lactational exposure to marine pollutants. *Environ Health Perspect* **118,** 1429-1433.

Guillen, M. D., and Goicoechea, E. (2008). Toxic oxygenated alpha,beta-unsaturated aldehydes and their study in foods: a review. *Crit. Rev. Food Sci. Nutr.* **48,** 119-136.

Guo, Y. L., Lambert, G. H., Hsu, C. C., and Hsu, M. M. (2004). Yucheng: health effects of prenatal exposure to polychlorinated biphenyls and dibenzofurans. *Int Arch Occup Environ Health* **77,** 153-158.

Hagmar, L., Tornqvist, M., Nordander, C., Rosen, I., Bruze, M., Kautiainen, A., Magnusson, A. L., Malmberg, B., Aprea, P., Granath, F., and Axmon, A. (2001). Health effects of occupational exposure to acrylamide using hemoglobin adducts as biomarkers of internal dose. *Scand J Work Environ Health* **27,** 219-226.

Haugen, M., Brantsaeter, A. L., Alexander, J., and Meltzer, H. M. (2008). Dietary supplements contribute substantially to the total nutrient intake in pregnant Norwegian women. *Ann Nutr Metab* **52,** 272-280.

Heilmann, C., Budtz-Jorgensen, E., Nielsen, F., Heinzow, B., Weihe, P., and Grandjean, P. (2010). Serum concentrations of antibodies against vaccine toxoids in children exposed perinatally to immunotoxicants. *Environ Health Perspect* **118,** 1434-1438.

Heilmann, C., Grandjean, P., Weihe, P., Nielsen, F., and Budtz-Jorgensen, E. (2006). Reduced antibody responses to vaccinations in children exposed to polychlorinated biphenyls. *PLoS. Med.* **3,** e311.

Herbst, A. L., Ulfelder, H., and Poskanzer, D. C. (1971). Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *N Engl J Med* **284,** 878-881.

Hites, R. A. (2011). Dioxins: an overview and history. *Environ Sci Technol* **45,** 16-20.

Ho, P. P., and Steinman, L. (2008). The aryl hydrocarbon receptor: a regulator of Th17 and Treg cell development in disease. *Cell Res* **18,** 605-608.

Hochstenbach, K., van Leeuwen, D. M., Gmuender, H., Stolevik, S. B., Nygaard, U. C., Lovik, M., Granum, B., Namork, E., van Delft, J. H., and van, L. H. (2010). Transcriptomic profile indicative of immunotoxic exposure: in vitro studies in peripheral blood mononuclear cells. *Toxicol Sci* **118,** 19-30.

Hogervorst, J. G., Baars, B. J., Schouten, L. J., Konings, E. J., Goldbohm, R. A., and van den Brandt, P. A. (2010). The carcinogenicity of dietary acrylamide intake: a comparative discussion of epidemiological and experimental animal research. *Crit Rev Toxicol* **40,** 485-512.

Hogervorst, J. G., Schouten, L. J., Konings, E. J., Goldbohm, R. A., and van den Brandt, P. A. (2007). A prospective study of dietary acrylamide intake and the risk of endometrial, ovarian, and breast cancer. *Cancer Epidemiol Biomarkers Prev* **16,** 2304-2313.

Hogervorst, J. G., Schouten, L. J., Konings, E. J., Goldbohm, R. A., and van den Brandt, P. A. (2008). Dietary acrylamide intake and the risk of renal cell, bladder, and prostate cancer. *Am J Clin Nutr* **87,** 1428-1438.

Holladay, S. D., Mustafa, A., and Gogal, R. M., Jr. (2011). Prenatal TCDD in mice increases adult autoimmunity. *Reprod. Toxicol* **31,** 312-318.

Holladay, S. D., and Smialowicz, R. J. (2000). Development of the murine and human immune system: differential effects of immunotoxicants depend on time of exposure. *Environ Health Perspect* **108 Suppl 3,** 463-473.

Holsapple, M. P., Paustenbach, D. J., Charnley, G., West, L. J., Luster, M. I., Dietert, R. R., and Burns-Naas, L. A. (2004). Symposium summary: children's health risk--what's so special about the developing immune system? *Toxicol Appl. Pharmacol* **199,** 61-70.

Holt, P. G., and Jones, C. A. (2000). The development of the immune system during pregnancy and early life. *Allergy* **55,** 688-697.

Horl, M. P., Schmitz, M., Ivens, K., and Grabensee, B. (2002). Opportunistic infections after renal transplantation. *Curr Opin. Urol.* **12,** 115-123.

Horst, D., Verweij, M. C., Davison, A. J., Ressing, M. E., and Wiertz, E. J. (2011). Viral evasion of T cell immunity: ancient mechanisms offering new applications. *Curr Opin. Immunol* **23,** 96-103.

House, R. V. (1999). Theory and practice of cytokine assessment in immunotoxicology. *Methods* **19,** 17-27.

Hymery, N., Sibiril, Y., and Parent-Massin, D. (2006). Improvement of human dendritic cell culture for immunotoxicological investigations. *Cell Biol. Toxicol.* **22,** 243-255.

Ito, T., Inouye, K., Fujimaki, H., Tohyama, C., and Nohara, K. (2002). Mechanism of TCDD-induced suppression of antibody production: effect on T cell-derived cytokine production in the primary immune reaction of mice. *Toxicol. Sci.* **70,** 46-54.

JECFA (2005). Summary and conclusions of the sixty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). http://www.who.int/foodsafety/chem/jecfa/summaries/summary_report_64_final.pdf.

Karras, J. G., and Holsapple, M. P. (1996). Structure and function of the immune system. In *Experimental immunotoxicology* pp. 3-12. CRC Press, Boca Raton.

Kazerouni, N., Sinha, R., Hsu, C. H., Greenberg, A., and Rothman, N. (2001). Analysis of 200 food items for benzo[a]pyrene and estimation of its intake in an epidemiologic study. *Food Chem. Toxicol.* **39,** 423-436.

Kerkvliet, N. I., Steppan, L. B., Vorachek, W., Oda, S., Farrer, D., Wong, C. P., Pham, D., and Mourich, D. V. (2009). Activation of aryl hydrocarbon receptor by TCDD prevents diabetes in NOD mice and increases Foxp3+ T cells in pancreatic lymph nodes. *Immunotherapy.* **1,** 539-547.

Kim, S., and Lee, K. G. (2010). Effects of cooking variables on formation of heterocyclic amines (HCA) in roasted pork and mackerel. *J Toxicol Environ Health A* **73,** 1599-1609.

Kinoshita, H., Abe, J., Akadegawa, K., Yurino, H., Uchida, T., Ikeda, S., Matsushima, K., and Ishikawa, S. (2006). Breakdown of mucosal immunity in gut by 2,3,7,8-tetraclorodibenzo-p-dioxin (TCDD). *Environ Health Prev Med* **11,** 256-263.

Kjuus, H., Goffeng, L. O., Heier, M. S., Sjoholm, H., Ovrebo, S., Skaug, V., Paulsson, B., Tornqvist, M., and Brudal, S. (2004). Effects on the peripheral nervous system of tunnel workers exposed to acrylamide and N-methylolacrylamide. *Scand J Work Environ Health* **30,** 21-29.

Klemens, C. M., Berman, D. R., and Mozurkewich, E. L. (2011). The effect of perinatal omega-3 fatty acid supplementation on inflammatory markers and allergic diseases: a systematic review. *BJOG* **118,** 916-925.

Koeper, L. M., and Vohr, H. W. (2009). Functional assays are mandatory for a correct prediction of immunotoxic properties of compounds in vitro. *Food Chem. Toxicol.* **47,** 110-118.

Kooijman, R., Devos, S., and Hooghe-Peters, E. (2010). Inhibition of in vitro cytokine production by human peripheral blood mononuclear cells treated with xenobiotics: Implications for the prediction of general toxicity and immunotoxicity. *Toxicol In Vitro* **24,** 1782-1789.

Kvalem, H. E. (2010). From food to blood - diet as a predictor of blood concentrations of dioxins and PCBs. Dissertation

Kvalem, H. E., Knutsen, H. K., Thomsen, C., Haugen, M., Stigum, H., Brantsaeter, A. L., Froshaug, M., Lohmann, N., Papke, O., Becher, G., Alexander, J., and Meltzer, H. M. (2009). Role of dietary patterns for dioxin and PCB exposure. *Mol Nutr Food Res* **53,** 1438-1451.

Langezaal, I., Coecke, S., and Hartung, T. (2001). Whole blood cytokine response as a measure of immunotoxicity. *Toxicol. In Vitro* **15,** 313-318.

Lankveld, D. P., van Loveren, H., Baken, K. A., and Vandebriel, R. J. (2010). In vitro testing for direct immunotoxicity: state of the art. *Methods Mol. Biol.* **598,** 401-423.

Lebrec, H., Roger, R., Blot, C., Burleson, G. R., Bohuon, C., and Pallardy, M. (1995). Immunotoxicological investigation using pharmaceutical drugs. In vitro evaluation of immune effects using rodent or human immune cells. *Toxicology* **96,** 147-156.

Leibnitz, R. (2005). Development of the human immune system. In *Developmental immunotoxicology* (S.D.Holladay, Ed.), pp. 21-42. CRC Press, Boca Raton.

Levin, M., Leibrecht, H., Ryan, J., van Dolah, F., and De, G. S. (2008). Immunomodulatory effects of domoic acid differ between in vivo and in vitro exposure in mice. *Mar. Drugs* **6,** 636-659.

Levin, M., Morsey, B., Mori, C., Nambiar, P. R., and De, G. S. (2005). Non-coplanar PCB-mediated modulation of human leukocyte phagocytosis: a new mechanism for immunotoxicity. *J Toxicol Environ Health A* **68,** 1977-1993.

Li, J., and McMurray, R. W. (2009). Effects of chronic exposure to DDT and TCDD on disease activity in murine systemic lupus erythematosus. *Lupus* **18,** 941-949.

Liem, A. K., Furst, P., and Rappe, C. (2000). Exposure of populations to dioxins and related compounds. *Food Addit Contam* **17,** 241-259.

Lijinsky, W. (1999). N-Nitroso compounds in the diet. *Mutat. Res* **443,** 129-138.

Llobet, J. M., Marti-Cid, R., Castell, V., and Domingo, J. L. (2008). Significant decreasing trend in human dietary exposure to PCDD/PCDFs and PCBs in Catalonia, Spain. *Toxicol Lett.* **178,** 117-126.

Luebke, R. W., Copeland, C. B., Daniels, M., Lambert, A. L., and Gilmour, M. I. (2001). Suppression of allergic immune responses to house dust mite (HDM) in rats exposed to 2,3,7,8-TCDD. *Toxicol Sci* **62,** 71-79.

Luster, M. I., Johnson, V. J., Yucesoy, B., and Simeonova, P. P. (2005). Biomarkers to assess potential developmental immunotoxicity in children. *Toxicol. Appl. Pharmacol.* **206,** 229-236.

Luster, M. I., Portier, C., Pait, D. G., White, K. L., Jr., Gennings, C., Munson, A. E., and Rosenthal, G. J. (1992). Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. *Fundam. Appl. Toxicol.* **18,** 200-210.

Lyche, J., Larsen, H., Skaare, J. U., Tverdal, A., Dahl, E., Johansen, G., and Ropstad, E. (2004). Effects of perinatal exposure to low doses of PCB 153 and PCB 126 on lymphocyte proliferation and hematology in goat kids. *J. Toxicol. Environ. Health A* **67,** 889-904.

Lyche, J. L., Larsen, H. J., Skaare, J. U., Tverdal, A., Johansen, G. M., and Ropstad, E. (2006). Perinatal exposure to low doses of PCB 153 and PCB 126 affects maternal and neonatal immunity in goat kids. *J. Toxicol. Environ. Health A* **69,** 139-158.

Magnus, P., Irgens, L. M., Haug, K., Nystad, W., Skjaerven, R., and Stoltenberg, C. (2006). Cohort profile: the Norwegian Mother and Child Cohort Study (MoBa). *Int. J. Epidemiol.* **35,** 1146-1150.

Marshall, N. B., and Kerkvliet, N. I. (2010). Dioxin and immune regulation: emerging role of aryl hydrocarbon receptor in the generation of regulatory T cells. *Ann N Y. Acad. Sci* **1183,** 25-37.

Marshall, N. B., Vorachek, W. R., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2008). Functional characterization and gene expression analysis of CD4+ CD25+ regulatory T cells generated in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Immunol* **181,** 2382-2391.

Mathiesen, L., Rytting, E., Mose, T., and Knudsen, L. E. (2009). Transport of benzo[alpha]pyrene in the dually perfused human placenta perfusion model: effect of albumin in the perfusion medium. *Basic Clin. Pharmacol. Toxicol.* **105,** 181-187.

Meltzer, H. M., Brantsaeter, A. L., Ydersbond, T. A., Alexander, J., and Haugen, M. (2008). Methodological challenges when monitoring the diet of pregnant women in a large study: experiences from the Norwegian Mother and Child Cohort Study (MoBa). *Matern. Child Nutr* **4,** 14-27.

Merlo, D. F., Wild, C. P., Kogevinas, M., Kyrtopoulos, S., and Kleinjans, J. (2009). NewGeneris: a European study on maternal diet during pregnancy and child health. *Cancer Epidemiol Biomarkers Prev* **18,** 5-10.

Metzger, T. C., and Anderson, M. S. (2011). Control of central and peripheral tolerance by Aire. *Immunol Rev* **241,** 89-103.

Michalek, J. E., and Pavuk, M. (2008). Diabetes and cancer in veterans of Operation Ranch Hand after adjustment for calendar period, days of spraying, and time spent in Southeast Asia. *J Occup Environ Med* **50,** 330-340.

Milbrath, M. O., Wenger, Y., Chang, C. W., Emond, C., Garabrant, D., Gillespie, B. W., and Jolliet, O. (2009). Apparent half-lives of dioxins, furans, and polychlorinated biphenyls as a function of age, body fat, smoking status, and breast-feeding. *Environ Health Perspect* **117,** 417-425.

Ministry of the environment (2011). http://www.miljostatus.no/.

Miossec, P. (2009). IL-17 and Th17 cells in human inflammatory diseases. *Microbes. Infect* **11,** 625-630.

Miyashita, C., Sasaki, S., Saijo, Y., Washino, N., Okada, E., Kobayashi, S., Konishi, K., Kajiwara, J., Todaka, T., and Kishi, R. (2011). Effects of prenatal exposure to dioxin-like compounds on allergies and infections during infancy. *Environ Res* **111,** 551-558.

Miyawaki, T., Seki, H., Taga, K., Sato, H., and Taniguchi, N. (1985). Dissociated production of interleukin-2 and immune (gamma) interferon by phytohaemagglutinin stimulated lymphocytes in healthy infants. *Clin Exp Immunol* **59,** 505-511.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136,** 2348-2357.

Mottram, D. S., Wedzicha, B. L., and Dodson, A. T. (2002). Acrylamide is formed in the Maillard reaction. *Nature* **419,** 448-449.

Murphy, K., Travers, P., and Walport, M. (2008). Janeway's Immunobiology, Garland Science, Taylor & Francis Group, LLC, New York.

Ni, W., McNaughton, L., LeMaster, D. M., Sinha, R., and Turesky, R. J. (2008). Quantitation of 13 heterocyclic aromatic amines in cooked beef, pork, and chicken by liquid chromatography-electrospray ionization/tandem mass spectrometry. *J Agric Food Chem* **56,** 68-78.

Nilsen, R. M., Vollset, S. E., Gjessing, H. K., Skjaerven, R., Melve, K. K., Schreuder, P., Alsaker, E. R., Haug, K., Daltveit, A. K., and Magnus, P. (2009). Self-selection and bias in a large prospective pregnancy cohort in Norway. *Paediatr. Perinat. Epidemiol* **23,** 597-608.

Nohara, K., Fujimaki, H., Tsukumo, S., Inouye, K., Sone, H., and Tohyama, C. (2002). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on T cell-derived cytokine production in ovalbumin (OVA)-immunized C57Bl/6 mice. *Toxicology* **172,** 49-58.

Norwegian food safety authority (2011a). http://matportalen.no/rad_til_spesielle_grupper/tema/gravide/.

Norwegian food safety authority (2011b). http://www.matportalen.no/matvaregrupper/tema/fisk_og_skalldyr/helhetsvurdering_av_helseeffektene_ved_aa_spise_fisk_2006_-_spis_mer_fisk.

Norwegian food safety authority (2011c). http://www.matportalen.no/uonskedestoffer_i_mat/tema/miljogifter/dioksin_og_pcb.

Onozuka, D., Yoshimura, T., Kaneko, S., and Furue, M. (2009). Mortality after exposure to polychlorinated biphenyls and polychlorinated dibenzofurans: a 40-year follow-up study of Yusho patients. *Am J Epidemiol* **169,** 86-95.

Oppenheim, J. J., Feldmann, M., Durum, S. K., Hirano, T., Vilcek, J., and Nicola, N. A. (2001). Cytokine Reference, pp. 1-1436. Academic Press, London, San Diego.

Park, J. S., Bergman, A., Linderholm, L., Athanasiadou, M., Kocan, A., Petrik, J., Drobna, B., Trnovec, T., Charles, M. J., and Hertz-Picciotto, I. (2008). Placental transfer of polychlorinated biphenyls, their hydroxylated metabolites and pentachlorophenol in pregnant women from eastern Slovakia. *Chemosphere* **70,** 1676-1684.

Parzefall, W. (2008). Minireview on the toxicity of dietary acrylamide. *Food Chem. Toxicol.* **46,** 1360-1364.

Patterson, D. G., Jr., Needham, L. L., Pirkle, J. L., Roberts, D. W., Bagby, J., Garrett, W. A., Andrews, J. S., Jr., Falk, H., Bernert, J. T., Sampson, E. J., and . (1988). Correlation between serum and adipose tissue levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin in 50 persons from Missouri. *Arch Environ Contam Toxicol* **17,** 139-143.

Paulsson, B., Granath, F., Grawe, J., Ehrenberg, L., and Tornqvist, M. (2001). The multiplicative model for cancer risk assessment: applicability to acrylamide. *Carcinogenesis* **22,** 817-819.

Polder, A., Thomsen, C., Lindstrom, G., Loken, K. B., and Skaare, J. U. (2008). Levels and temporal trends of chlorinated pesticides, polychlorinated biphenyls and brominated flame retardants in individual human breast milk samples from Northern and Southern Norway. *Chemosphere* **73,** 14-23.

Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., Caccamo, M., Oukka, M., and Weiner, H. L. (2008). Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* **453,** 65-71.

Reynolds, T. (2002). Acrylamide and cancer: tunnel leak in Sweden prompted studies. *J Natl. Cancer Inst.* **94,** 876-878.

Ringerike, T., Ulleras, E., Volker, R., Verlaan, B., Eikeset, A., Trzaska, D., Adamczewska, V., Olszewski, M., Walczak-Drzewiecka, A., Arkusz, J., van Loveren, H., Nilsson, G., Lovik, M., Dastych, J., and Vandebriel, R. J. (2005). Detection of immunotoxicity using T-cell based cytokine reporter cell lines ("Cell Chip"). *Toxicology* **206,** 257-272.

Ritter, R., Scheringer, M., MacLeod, M., Moeckel, C., Jones, K. C., and Hungerbuhler, K. (2011). Intrinsic human elimination half-lives of polychlorinated biphenyls derived from the temporal evolution of cross-sectional biomonitoring data from the United Kingdom. *Environ Health Perspect* **119,** 225-231.

Rosas-Ballina, M., Olofsson, P. S., Ochani, M., Valdes-Ferrer, S. I., Levine, Y. A., Reardon, C., Tusche, M. W., Pavlov, V. A., Andersson, U., Chavan, S., Mak, T. W., and Tracey, K. J. (2011). Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science* **334,** 98-101.

Rose, N. R. (2005). Autoimmunity, autoimmune diseases. In *Encyclopedic reference of immunotoxicology* (H.W.Vohr, Ed.), pp. 79-82. Springer, Heidelberg.

Ross, G. (2004). The public health implications of polychlorinated biphenyls (PCBs) in the environment. *Ecotoxicol. Environ Saf* **59,** 275-291.

Sanchez, A. M., and Yang, Y. (2011). The role of natural regulatory T cells in infection. *Immunol Res* **49,** 124-134.

Schettgen, T., Kutting, B., Hornig, M., Beckmann, M. W., Weiss, T., Drexler, H., and Angerer, J. (2004). Trans-placental exposure of neonates to acrylamide--a pilot study. *Int Arch Occup Environ Health* **77,** 213-216.

Schollenberger, M., Muller, H. M., Rufle, M., Suchy, S., Planck, S., and Drochner, W. (2005). Survey of Fusarium toxins in foodstuffs of plant origin marketed in Germany. *Int J Food Microbiol* **97,** 317-326.

Schulz, V. J., Smit, J. J., Willemsen, K. J., Fiechter, D., Hassing, I., Bleumink, R., Boon, L., van den Berg, M., van Duursen, M. B., and Pieters, R. H. (2011). Activation of the aryl hydrocarbon receptor suppresses sensitization in a mouse peanut allergy model. *Toxicol Sci* **123,** 491-500.

Scientific Committee on Food (2001). Opinion for the SCF on the risk assessment of dioxins and dioxin-like PCBs in food, update based on new scientific information available since the SCF opinion of 22nd November 2000.

Seddiki, N., and Kelleher, A. D. (2008). Regulatory T cells in HIV infection: who's suppressing what? *Curr Infect Dis Rep* **10,** 252-258.

Selgrade, M. K., Cooper, K. D., Devlin, R. B., van, L. H., Biagini, R. E., and Luster, M. I. (1995). Immunotoxicity--bridging the gap between animal research and human health effects. *Fundam. Appl Toxicol* **24,** 13-21.

Sicherer, S. H., and Sampson, H. A. (2010). Food allergy. *J Allergy Clin Immunol* **125,** S116-S125.

Sly, P. D., Boner, A. L., Bjorksten, B., Bush, A., Custovic, A., Eigenmann, P. A., Gern, J. E., Gerritsen, J., Hamelmann, E., Helms, P. J., Lemanske, R. F., Martinez, F., Pedersen, S., Renz, H., Sampson, H., von, M. E., Wahn, U., and Holt, P. G. (2008). Early identification of atopy in the prediction of persistent asthma in children. *Lancet* **372,** 1100-1106.

Sorgel, F., Weissenbacher, R., Kinzig-Schippers, M., Hofmann, A., Illauer, M., Skott, A., and Landersdorfer, C. (2002). Acrylamide: increased concentrations in homemade food and first evidence of its variable absorption from food, variable metabolism and placental and breast milk transfer in humans. *Chemotherapy* **48,** 267-274.

Stadler, R. H., Blank, I., Varga, N., Robert, F., Hau, J., Guy, P. A., Robert, M. C., and Riediker, S. (2002). Acrylamide from Maillard reaction products. *Nature* **419,** 449-450.

Stein, R. T., and Martinez, F. D. (2004). Asthma phenotypes in childhood: lessons from an epidemiological approach. *Paediatr. Respir. Rev* **5,** 155-161.

Stober, W. (1997). Current Protocols in Immunology, John Wiley & Sons, Inc., New York.

Stockinger, B., Hirota, K., Duarte, J., and Veldhoen, M. (2011). External influences on the immune system via activation of the aryl hydrocarbon receptor. *Semin. Immunol* **23,** 99-105.

Sumner, S. C., Fennell, T. R., Moore, T. A., Chanas, B., Gonzalez, F., and Ghanayem, B. I. (1999). Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice. *Chem Res Toxicol* **12,** 1110-1116.

Suzuki, G., Nakano, M., and Nakano, S. (2005). Distribution of PCDDs/PCDFs and Co-PCBs in human maternal blood, cord blood, placenta, milk, and adipose tissue: dioxins showing high toxic equivalency factor accumulate in the placenta. *Biosci. Biotechnol. Biochem* **69,** 1836-1847.

Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., and Tornqvist, M. (2002). Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J Agric Food Chem* **50,** 4998-5006.

Tarkowski, M., Kur, B., Nocun, M., and Sitarek, K. (2010). Perinatal exposure of mice to TCDD decreases allergic sensitisation through inhibition of IL-4 production rather than T regulatory cell-mediated suppression. *Int J Occup Med Environ Health* **23,** 75-83.

ten Tusscher, G. W., Steerenberg, P. A., van, L. H., Vos, J. G., von dem Borne, A. E., Westra, M., van der Slikke, J. W., Olie, K., Pluim, H. J., and Koppe, J. G. (2003). Persistent hematologic and immunologic disturbances in 8-year-old Dutch children associated with perinatal dioxin exposure. *Environ Health Perspect* **111,** 1519-1523.

The Norwegian Mother and Child Cohort Study (2011a). Questionnaire 2. http://www.fhi.no/dokumenter/253304bd64.pdf.

The Norwegian Mother and Child Cohort Study (2011b). Spørreskjema 2. http://www.fhi.no/dokumenter/a4a9fd0336.pdf.

Thuvander, A., Moller, T., Barbieri, H. E., Jansson, A., Salomonsson, A. C., and Olsen, M. (2001). Dietary intake of some important mycotoxins by the Swedish population. *Food Addit Contam* **18,** 696-706.

Tomatis, L. (1979). Prenatal exposure to chemical carcinogens and its effect on subsequent generations. *Natl. Cancer Inst. Monogr* 159-184.

Tornqvist, M., Fred, C., Haglund, J., Helleberg, H., Paulsson, B., and Rydberg, P. (2002). Protein adducts: quantitative and qualitative aspects of their formation, analysis and applications. *J Chromatogr. B Analyt. Technol Biomed. Life Sci* **778,** 279-308.

Tsukimori, K., Uchi, H., Mitoma, C., Yasukawa, F., Fukushima, K., Todaka, T., Kajiwara, J., Yoshimura, T., Hirata, T., Wake, N., and Furue, M. (2011). Comparison of the concentrations of polychlorinated biphenyls and dioxins in mothers affected by the Yusho incident and their children. *Chemosphere* **84,** 928-935.

Ulaszewska, M. M., Zuccato, E., and Davoli, E. (2011). PCDD/Fs and dioxin-like PCBs in human milk and estimation of infants' daily intake: A review. *Chemosphere* **83,** 774-782.

US EPA (2011). http://www.epa.gov/history/topics/pcbs/01.html.

van den Berg, M., Birnbaum, L., Bosveld, A. T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X., Liem, A. K., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski, T. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Perspect.* **106,** 775-792.

van den Berg, M., Birnbaum, L. S., Denison, M., De, V. M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N., and Peterson, R. E. (2006). The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol Sci* **93,** 223-241.

van den Berg, M., De Jongh, J., Poiger, H., and Olson, J. R. (1994). The toxicokinetics and metabolism of polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) and their relevance for toxicity. *Crit Rev Toxicol* **24,** 1-74.

van Loveren, H., and Piersma, A. (2004). Immunotoxicological consequences of perinatal chemical exposures. *Toxicol. Lett.* **149,** 141-145.

van Loveren, H., van Amsterdam, J. G., Vandebriel, R. J., Kimman, T. G., Rumke, H. C., Steerenberg, P. S., and Vos, J. G. (2001). Vaccine-induced antibody responses as parameters of the influence of endogenous and environmental factors. *Environ. Health Perspect.* **109,** 757-764.

Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J. C., and Stockinger, B. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* **453,** 106-109.

Vial, T., and Descotes, J. (2003). Immunosuppressive drugs and cancer. *Toxicology* **185,** 229-240.

Vohr, H. W. (2005). Hypersensitivity reactions. In *Encyclopedic reference of immunotoxicology* (H.W.Vohr, Ed.), pp. 302-305. Springer, Heidelberg.

Vos, J. G. (1977). Immune suppression as related to toxicology. *CRC Crit Rev Toxicol* **5,** 67-101.

Wagner, W., Walczak-Drzewiecka, A., Slusarczyk, A., Biecek, P., Rychlewski, L., and Dastych, J. (2006). Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. *Toxicol. Lett.* **162,** 55-70.

Wall, R., Ross, R. P., Fitzgerald, G. F., and Stanton, C. (2010). Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutr Rev* **68,** 280-289.

Weisglas-Kuperus, N., Patandin, S., Berbers, G. A., Sas, T. C., Mulder, P. G., Sauer, P. J., and Hooijkaas, H. (2000). Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children. *Environ. Health Perspect.* **108,** 1203-1207.

Weisglas-Kuperus, N., Sas, T. C., Koopman-Esseboom, C., van der Zwan, C. W., De Ridder, M. A., Beishuizen, A., Hooijkaas, H., and Sauer, P. J. (1995). Immunologic effects of background prenatal and postnatal exposure to dioxins and polychlorinated biphenyls in Dutch infants. *Pediatr. Res.* **38,** 404-410.

Weisglas-Kuperus, N., Vreugdenhil, H. J., and Mulder, P. G. (2004). Immunological effects of environmental exposure to polychlorinated biphenyls and dioxins in Dutch school children. *Toxicol. Lett.* **149,** 281-285.

West, L. J. (2002). Defining critical windows in the development of the human immune system. *Hum Exp Toxicol* **21,** 499-505.

White, S. S., and Birnbaum, L. S. (2009). An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. *J Environ Sci Health C Environ Carcinog. Ecotoxicol. Rev* **27,** 197-211.

WHO (2010). http://www.who.int/mediacentre/factsheets/fs225/en/.

Wilson, K. M., Mucci, L. A., Rosner, B. A., and Willett, W. C. (2010). A prospective study on dietary acrylamide intake and the risk for breast, endometrial, and ovarian cancers. *Cancer Epidemiol Biomarkers Prev* **19,** 2503-2515.

Wong, C. H., Jenne, C. N., Lee, W. Y., Leger, C., and Kubes, P. (2011). Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. *Science* **334,** 101-105.

Zaidi, S. I., Raisuddin, S., Singh, K. P., Jafri, A., Husain, R., Husain, M. M., Mall, S. A., Seth, P. K., and Ray, P. K. (1994). Acrylamide induced immunosuppression in rats and its modulation by 6-MFA, an interferon inducer. *Immunopharmacol. Immunotoxicol.* **16,** 247-260.

Zhang, L., Ma, J., Takeuchi, M., Usui, Y., Hattori, T., Okunuki, Y., Yamakawa, N., Kezuka, T., Kuroda, M., and Goto, H. (2010). Suppression of experimental autoimmune uveoretinitis by inducing differentiation of regulatory T cells via activation of aryl hydrocarbon receptor. *Invest Ophthalmol Vis. Sci* **51,** 2109-2117.

I

II

III

Article title

Immunosuppressive effects of prenatal exposure to polychlorinated biphenyls and dioxins from the maternal diet persist into early childhood

Names

Solvor Berntsen Stølevik,* Unni Cecilie Nygaard,* Ellen Namork,* Margaretha Haugen,* Helle Margrete Meltzer,* Jan Alexander,* Helle Katrine Knutsen,* Ingeborg Aaberge,* Kirsti Vainio,* Henk van Loveren,[†,‡] Martinus Løvik,*[,§] and Berit Granum *

Affiliations

* Norwegian Institute of Public Health, NO-0403 Oslo, Norway

[†] Maastricht University, 6200 MD Maastricht, The Netherlands

[‡] National Institute of Public Health and the Environment, 3720 BA Bilthoven, The Netherlands

[§] Norwegian University of Science and Technology, NO-7491 Trondheim, Norway

E-mail addresses

S.B. Stølevik; solvor.berntsen@fhi.no

U.C. Nygaard; unni.cecilie.nygaard@fhi.no

E. Namork; ellen.namork@fhi.no

M. Haugen; margaretha.haugen@fhi.no

H.M. Meltzer; helle.margrete.meltzer@fhi.no

J. Alexander; jan.alexander@fhi.no

H.K. Knutsen; helle.knutsen@fhi.no

I. Aaberge; ingeborg.aaberge@fhi.no

K. Vainio; kirsti.vainio@fhi.no

H. van Loveren; henk.van.loveren@rivm.nl

M. Løvik; martinus.lovik@fhi.no

B. Granum; berit.granum@fhi.no

Corresponding author

S.B. Stølevik; Department of Environmental Immunology, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, NO-0403 Oslo, Norway; E-mail: solvor.berntsen@fhi.no; Telephone: +47 21076257; Fax: +47 21076686.

Short title

Prenatal toxicant exposure and immunotoxicity

Abstract

We investigated whether prenatal exposure from the maternal diet to the toxicants acrylamide, polychlorinated biphenyls (PCBs) and dioxins affected the development of immune-related diseases in childhood. Children participating in BraMat, a sub-cohort of the Norwegian Mother and Child Cohort Study (MoBa), were followed in the three first years of life using annual questionnaires (0-3 years; n = 162, 2-3 years; n = 180), and blood parameters were examined at three years of age (n = 114). The maternal intake of the toxicants was calculated using a validated food frequency questionnaire from MoBa. Prenatal dietary exposure to PCBs and dioxins was found to be associated with an increased risk of wheeze (defined as periods of more than 10 days of dry cough, chest tightness, or wheeze) and more frequent upper respiratory tract infections during the three first years of life. Furthermore, at three years of age, prenatal exposure to PCBs and dioxins was associated with reduced antibody response to the measles vaccine. No associations were found between prenatal exposure and immunophenotype data including regulatory T cells, allergic sensitization, and vaccine antibody responses other than measles. Prenatal acrylamide exposure was neither found to be associated with health outcomes nor blood parameters. Our results suggest that prenatal dietary exposure to PCBs and dioxins may increase the risk of wheeze and the susceptibility to infectious diseases during early childhood.

Keywords

Polychlorinated biphenyls; dioxins; acrylamide; prenatal; diet; immunotoxicity;

Abbreviations

BMI: body mass index

bw: body weight

dioxins: PCDDs/PCDFs: polychlorinated dibenzo-p-dioxins/dibenzofurans

dl-PCBs: dioxin-like PCBs

ELISA: enzyme-linked immunosorbent assay

FFQ: food frequency questionnaire

FSC: forward scatter

Hib: Haemophilus influenzae type B

MBRN: the medical birth registry of Norway

MoBa: the Norwegian mother and child cohort study

ndl-PCBs: non-dioxin-like PCBs

PCBs: polychlorinated biphenyls

SSC: side scatter

TEQ: toxic equivalents

URTI: upper respiratory tract infections

1. Introduction

Food items may contain environmental toxicants like the organochlorines polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins/dibenzofurans (PCDDs/PCDFs, commonly called dioxins), as well as toxicants generated during food preparation like acrylamide. PCBs and dioxins are highly lipophilic and accumulate in the food chain. The diet is assumed to be the main source of human exposure to these substances, providing more than 90% of the total exposure (Domingo and Bocio, 2007). The diet is also assumed to be the main source of exposure to acrylamide for the general non-smoking population. Acrylamide is formed during heat treatment at temperatures above 120 °C, particularly in plant-derived foods rich in carbohydrates (Mottram *et al.*, 2002; Stadler *et al.*, 2002).

Studies have shown that acrylamide, PCBs and dioxins cross the placenta and reach the foetus (Annola *et al.*, 2008; Covaci *et al.*, 2002; Suzuki *et al.*, 2005). Prenatal exposure to immunotoxicants is of concern since the foetus may be especially vulnerable due to an extensively developing immune system (Holsapple *et al.*, 2004; van Loveren and Piersma, 2004). Adverse effects on the immune system may result in immune-related diseases like allergy, asthma, and autoimmune conditions, or increased susceptibility to infectious diseases.

We have previously examined in the birth cohort BraMat whether exposure to acrylamide, PCBs and dioxins from the maternal diet during pregnancy exert immunotoxic effects in the children's first year of life. No associations between prenatal dietary exposure to acrylamide and immune-related health outcomes were found. In other studies, it has been reported on carcinogenic, genotoxic, neurotoxic, and reproductive toxic properties of acrylamide (reviewed by Hogervorst *et al*. (2010) and Parzefall (2008)). Information on immunotoxicity of acrylamide is scarce. The carcinogenic and neurotoxic properties of acrylamide may indicate that acrylamide also has immunotoxic properties since the immune

system is involved in killing harmful tumor cells, and the nervous system may influence the immune system (Murphy *et al.*, 2008).

The results in our previous study suggested that prenatal dietary exposure is associated with an increased risk of wheeze and infections during the first year of life (Stolevik *et al.*, 2011). In agreement with these findings, health effects of prenatal exposure to PCBs and dioxins in humans have been reported by others such as increased frequency of infections, less atopic dermatitis, and reduced antibody responses to vaccines (Dallaire *et al.*, 2004; Dallaire *et al.*, 2006; Glynn *et al.*, 2008; Grandjean *et al.*, 2010; Heilmann *et al.*, 2006; ten Tusscher *et al.*, 2003; Weisglas-Kuperus *et al.*, 2000; Weisglas-Kuperus *et al.*, 2004). These studies have measured the concentrations of the toxicants in biological media such as cord blood and breast milk, indicating the body burden of the toxicants. In contrast, we investigated the effect of exposure through the diet using an extensive and well validated food frequency questionnaire (FFQ) (Brantsaeter *et al.*, 2008a; Meltzer *et al.*, 2008). An advantage of using FFQ data are that the results may indicate how exposure to toxicants from the diet affects the health of the children, which is valuable information to be used in counselling women in fertile age regarding their diet.

The present study is part of the EU-funded project NewGeneris with the main aim to investigate whether maternal exposure to dietary toxicants results in *in utero* exposure and molecular events in the unborn child, leading to an increased risk of cancer and immune disorders in childhood (Merlo *et al.*, 2009). The present paper reports the results from the three-year follow-up of the children participating in the birth cohort BraMat. To investigate immunotoxic effects of PCBs and dioxins, as well as acrylamide, questionnaire data, concentrations of allergen specific IgE antibodies, vaccine antibody titers, and immunophenotype data including regulatory T cells, were examined.

2. Materials and methods

2.1. Study population

The BraMat cohort (n=205) was established between April 2007 and March 2008. Invitations to participate in the birth cohort BraMat were sent by regular mail to all pregnant women already enrolled in the Norwegian Mother and Child Cohort Study (MoBa) and who were scheduled to give birth at Oslo University Hospital Ullevål or Akershus University Hospital (recruitment rate ~25%). MoBa is a prospective population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health, including 108.000 children from all over the country born between 1999-2008 (Magnus *et al.*, 2006). Invitations to the BraMat birth cohort was sent in week 37 of gestation, thus only infants who had experienced a full term pregnancy (37th - 42nd week of gestation) were included. There were no plurality births. Exclusion criteria for the BraMat cohort were autoimmune diseases of the mother and use of steroids, anti-inflammatory, or epileptic drugs during pregnancy. The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics and the Data Inspectorate. All mothers gave their written informed consent both to MoBa and BraMat.

2.2. Blood sampling and handling

Venous blood was collected from the three year old children (age mean (range), 36 (33-43) months) either at their doctor's office, at home by a technician, or at a commercial laboratory (Fürst medical laboratory). Blood was collected into BD Vacutainer®SST™II serum gel separation tubes with butterfly blood collection sets (BD, Franklin Lakes, NJ, USA). The blood was allowed to clot for at least 30 min before centrifugation at 1000-1300*g* for 10 min at room temperature. Aliquots of the serum samples were stored at -20 °C until further

analyses. Blood was also collected into EDTA BD Vacutainers® (BD) and kept at room temperature until analyses were performed.

2.3. Determination of vaccine responses

As a measure of the immune function of the children, antibody responses to five of the vaccines in the Norwegian Childhood Vaccination Program were examined.

2.3.1 Viral vaccine antibody titers

The serum samples were analyzed for anti-measles IgG antibodies using Enzygnost Anti-Measles Virus IgG ELISA (Siemens, Marburg, Germany) and for anti-rubella IgG antibodies using Serion Rubella Virus IgG ELISA (Virion/Serion, Würzburg, Germany). Both assays were performed as recommended by the manufacturers. With the measles assay, the cut-off for qualitative evaluation of positivity was an optical density (OD) of 0.2 at 450 nm, corresponding to approximately 400 U/ml, as recommended by the manufacturer. With the rubella assay, the cut-off values to identify rubella positivity were calculated separately for each run, according to manufacturer's instructions.

2.3.2 Bacterial vaccine antibody titers

Concentrations of specific IgG antibodies to tetanus toxoid (Simonsen *et al.*, 1986), pneumococcal polysaccharides (Aaberge *et al.*, 1996), and *Haemophilus influenzae* type b (Hib) (modified from (Phipps *et al.*, 1990)) were determined using ELISA techniques. The antibody concentration to tetanus toxoid was given as IU/ml. IgG antibodies to a mix of 23 serotypes of pneumococcal polysaccharides (PPV23, Pneumovax®, Sanofi Pasteur MSD) were measured after C polysaccharide (CPS) adsorption of the sera (Statens Serum Institut, Copenhagen, Denmark). The IgG antibody concentration to PPV23 was given as arbitrary

units (AU/ml). Regarding the anti-Hib assay, microtiter plates were coated with Hib oligosaccharide conjugated to human albumin (1 mg/ml) (NIBSC, Potters Bar, UK). The serum samples were diluted two-fold starting at dilution 1:10. Goat anti-human IgG-alkaline phosphatase (ALP) (Sigma-Aldrich, St. Louis, MO, USA) was used as conjugate. The concentration of specific antibodies was calculated using human anti-Hib capsular polysaccharide serum as standard (LOT no. 1983; FDA, Silver Spring, MD, USA).

2.4. Determination of allergen specific IgE

The serum samples were analysed for allergen-specific IgE antibodies using ImmunoCAP Phadiatop® Infant (Phadia AB, Uppsala, Sweden) containing 11 allergens selected to be relevant for young children (house dust mite, cat, dog, hen's egg, cow's milk, peanut, shrimp, timothy, birch, ragweed, and *Parietaria judaica*). The concentrations of antibodies were expressed as Phadia Arbitrary Units/l (PAU/l) indicating the degree of sensitisation. Children with antibody concentrations ≥ 0.35 PAU/l were considered to be sensitized.

Sera from the children found to be sensitized using Phadiatop Infant, were analysed for specific IgE antibodies to the single allergens from house dust mite, cat, dog, hen's egg, cow's milk, peanut, timothy, and birch. The concentrations of antibodies were expressed as kU/l, and children with antibody concentrations ≥ 0.35 kU/l were considered to be sensitized. The analyses were performed as recommended by the manufacturer.

2.5. Immunophenotyping by flow cytometry

Within 24h, immunophenotyping was performed on whole blood samples according to the protocol of the manufacturer (BD). In short, the blood samples were stained with the antibodies in the Multitest 6-color TBNK reagent (FITC-labelled anti-CD3, PE-labelled anti-CD16 and anti-CD56, PerCP-Cy5.5-labelled anti-CD45, PE-Cy7-labelled anti-CD4, APC-

labelled anti-CD19, APC-Cy7-labelled anti-CD8; BD) and Pacific Blue-labelled anti-CD14 (BD) for 15 min in the dark at room temperature. Red blood cells were lysed in FACS lysing solution (BD) by incubation for 15 min in the dark at room temperature. The cells were counted using a BD LSRII flow cytometer with BD FACSDiva Software version 6.1.2. The lymphocytes were gated based on side light scatter (SSC) and CD45 expression, and monocytes were gated based on CD14 and CD45 expression. The percent and absolute numbers of the following lymphocyte subsets were determined: T-lymphocytes ($CD3^+$), T-helper cells ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), B-lymphocytes ($CD19^+$), natural killer (NK) cells ($CD16^+CD56^+$), and natural killer T-lymphocytes ($CD3^+CD16^+CD56^+$). Absolute numbers of the leukocytes (cells/μl) were determined by the use of Truecount tubes (BD).

For assessment of regulatory T cells, the blood samples were stained with the antibodies in the Human Regulatory T cell Coctail (FITC-labelled anti-CD4, PE-Cy7-labelled anti-CD25, Alexa647-labelled anti-CD127; BD) for 30 min in the dark at room temperature. After lysing of red blood cells, the cells were washed twice and centrifuged at 200*g* for 5 min at 18 °C, and resuspended in 400 μl washing buffer. The percent of regulatory T cells was determined using a four steps gating strategy. The lymphocytes were gated based on their light scatter properties (side scatter (SSC)/forward scatter (FSC)), and duplicates of lymphocytes were excluded by gating based on FSC-width and FSC-area. $CD4^+$ cells were then further gated based on SSC and CD4 expression. Finally, regulatory T cells were determined as percent CD127 low CD25 high cells of $CD4^+$ cells.

2.7. Assessment of exposure to the dietary toxicants

Maternal intake of the dietary toxicants PCBs, dioxins, and acrylamide, was calculated from a validated food frequency questionnaire (FFQ) used in MoBa. Description and validation of

the FFQ is given elsewhere (Brantsaeter *et al.*, 2008a; Meltzer *et al.*, 2008). The FFQ covers the dietary intake of the mothers during the first four months of pregnancy. The method for estimation of exposure to PCBs, dioxins, and acrylamide has been described in Kvalem *et al*. (2009) and Brantsaeter *et al*. (2008), respectively. PCBs and dioxins can be divided into two groups according to their toxicological properties: 1) dioxins and dioxin-like PCBs (dl-PCBs) and 2) non-dioxin-like PCBs (ndl-PCBs). Similar toxicological properties of dioxins and dl-PCBs allow the combined exposure to be expressed as toxic equivalents (TEQ) (van den Berg *et al.*, 2006). The exposure was expressed relative to the mother's self-reported body weight (bw) before pregnancy, thus the exposure to dioxins and dl-PCBs was expressed as pg TEQ/kg bw/day, exposure to ndl-PCBs as ng/kg bw/day, and acrylamide as μg/kg bw/day.

2.8. Health outcomes from questionnaires

When the children in the BraMat cohort were one, two, and three years of age, a questionnaire was sent to the mothers. The mothers had the choice of filling in the questionnaire and returning it by regular mail, or to give the answers by a telephone interview. The questionnaire covered topics related to the child's infectious diseases, allergy, asthma, and other chronic diseases, and the use of medications. Concerning infectious diseases, the mothers were asked if the child had experienced the following diseases/complaints, and how many episodes: colds and other upper respiratory tract infections, otitis media, pneumonia, gastroenteritis with vomiting or diarrhoea, and urinary tract infection. The mothers were also asked if the child had experienced any children's diseases, such as chicken pox and exanthema subitum (roseola infantum). Concerning allergy, asthma, and other chronic diseases, the mothers were asked: "Has the child been diagnosed with asthma, asthma bronchitis, or bronchial hyperreactivity by a doctor? Has the child had periods of more than 10 days of dry cough, chest tightness, or wheeze (hereafter called wheeze)? Has the child had

eczema or itchiness (in the face or at joints such as the groin, popliteal fossa, ankle, elbow, and wrist)? Has the child been diagnosed with atopic dermatitis by a doctor? Has the child been diagnosed with allergy by a doctor? Has the child any other chronic disease?" Cumulative data (0-3 years of age) and data for the last year only (2-3 years of age) were investigated.

2.9. Potential confounding variables

Potential confounding variables were extracted from the BraMat questionnaires and MoBa questionnaires filled in by the mothers during pregnancy (~15th and 30th week of gestation) and ~ 6 months after birth (version 5 of the quality-controlled MoBa data files). Birth-related information was extracted from the Medical Birth Registry of Norway (MBRN). Potential confounding variables included in the analyses were mother's previous breast-feeding, parity, maternal and paternal asthma and allergy, maternal age, maternal smoking, maternal passive smoking, maternal education, maternal BMI before pregnancy, mean parental gross income for the three years of the study, child's gender, season of birth, type of delivery, apgar score, gestational age, birth weight, number of older siblings, breast-feeding of the child, and day-care attendance. The categories of the variables used in the analyses are shown in Table 1.

2.10. Statistical analyses

If $p<0.1$ in bivariate analyses, multivariate logistic regression analyses were applied to assess the influence of exposure on the different binary health outcomes for the children. The exposure levels of the dietary toxicants were categorised using the 80^{th} percentile to compare the highest exposed participants constituting the upper tail of the exposure distribution, with the remaining participants ($\geq 80^{th}$ percentile and $<80^{th}$ percentile respectively). If no

statistically significant associations were found, also the continuous exposure variables were examined. The criterion for inclusion of potential confounding variables in the multivariate regression analyses was $p<0.25$ in bivariate analyses. Highly correlated variables (correlation coefficients ≥ 0.7) were not included in the same multivariate analysis. Since the exposure levels of dioxins and dl-PCBs, and ndl-PCBs were highly correlated, separate multivariate analyses were performed for the two groups of dietary toxicants. Parity, previous breast-feeding, and number of older siblings were also highly correlated, therefore only parity was included in the multivariate analyses. The manual backward deletion method was used starting with all included variables in the model. At each deletion step, the least significant potential confounding variable in the multivariate model was manually removed until only statistically significant ($p<0.05$) variables remained in the model. Hosmer-Lemeshow test, Cooks'D, and residuals were used to investigate the robustness of the results.

The outcome variables "numbers of upper respiratory tract infections (URTI)" and "numbers of gastroenteritis" consist of count data, but linear regression analyses were applied since the residuals were approximately normally distributed. Since the numbers of URTI were ln-transformed, the reported results for the numbers of URTI are back-transformed values (ratios). The multivariate analyses were performed as described above. Cooks'D, Leverage values and residuals were used to investigate the robustness of the results.

Linear and logistic regression analyses were performed on immunophenotype data, vaccine antibody titers, and concentrations of allergen specific IgE antibodies. Due to small sample size, ln-transformed exposure variables were used to avoid results to be strongly influenced by only a few observations. The outcome variable was ln-transformed when necessary to fulfil the criteria of normally distributed residuals. For the transformed outcome variables, back-transformed values are reported (ratios). Multivariate regression analyses were performed as described above. Regarding vaccine responses, children reported not to follow

the Norwegian Childhood Vaccination Program (n=2) were excluded from the statistical analyses.

Results were considered statistically significant at $p<0.05$. The statistical analyses were performed using the statistical software PASW Statistics 17 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Questionnaires and blood samples

BraMat questionnaires were received from 195 (95%), 184 (90%), and 180 (88%) of the 205 participants for the first, second, and third year, respectively. All three questionnaires were received from 162 (79%) participants. Some of the questionnaires were incomplete. Exposure data were given as intake per kg bodyweight. Since the body weight was unknown for five of the mothers, exposure data could not be calculated and they were excluded from the statistical analyses. Data on health outcomes and potential confounders for some of the children were also missing. The total numbers of children included in the different multivariate analyses are given in Table 4.

Blood was collected from 112 (56%) children with exposure data. Due to logistic challenges, immunophenotyping and regulatory T cell assessment was only performed for 81 and 78 samples, respectively. Vaccine antibody titers and concentrations of allergen specific IgE antibodies were measured in 110-111 samples. There were no statistically significant differences with regard to frequency of health outcomes in children giving and not giving a blood sample at three years of age.

3.2. Demographics, frequencies of health outcomes, and exposure levels

Demographics and the frequencies of the health outcomes for the children included in the statistical analyses are shown in Table 1. Few children had experienced pneumonia, urinary tract infection, or “other chronic diseases”, therefore statistical analyses were not performed for these health outcomes. For the same reason, analyses for the health outcomes “doctor diagnosed asthma and allergy, frequency of children’s diseases, and numbers of

gastroenteritis" were performed on cumulative data (0-3 years) only. The calculated maternal dietary intake of dioxins and dl-PCBs, ndl-PCBs, and acrylamide is presented in Table 2.

3.3. Health outcomes

The results of bivariate logistic or linear regression analyses are presented in Table 3. No significant associations between prenatal dietary exposure to acrylamide and the investigated health outcomes were found.

3.3.1. Cumulative data (0-3 years of age)

In multivariate analyses, prenatal exposure to dioxins and dl-PCBs, and ndl-PCBs was significantly associated with an increased risk of wheeze (Table 4). In the final multivariate model, the OR was adjusted for maternal asthma and allergy and maternal education, and also gross income for dioxins and dl-PCBs. Furthermore, prenatal exposure to dioxins and dl-PCBs, as well as ndl-PCBs was associated with increased numbers of URTI when the continuous variables of the toxicants were used. In the final models, the β was adjusted for maternal BMI, maternal education, and gestational age, and also birth season for ndl-PCBs. Also, prenatal exposure to dioxins and dl-PCBs, and ndl-PCBs was associated with increased numbers of gastroenteritis, however, the associations were not robust as indicated by the residuals and Cooks'D. The β was adjusted for apgar score in the final models. Finally, prenatal exposure to ndl-PCBs was associated with an increased risk of doctor diagnosed allergy, but the association was not robust as indicated by residuals and Cooks'D. The OR was adjusted for maternal smoking (ever smoked), type of delivery, and day-care attendance in the final model.

3.3.2. Last year (2-3 years of age)

In multivariate analyses, prenatal exposure to dioxins and dl-PCBs, and ndl-PCBs was associated with increased numbers of URTI (Table 4). However, for ndl-PCBs, a significant association was found only when the continuous exposure variable of the dietary toxicants was used. The dietary toxicant variables were the only variables remaining in the final multivariate models. No significant associations were found for wheeze during the third year (Table 3).

3.4. Blood parameters

No significant associations were found between prenatal exposure to dioxins and dl-PCBs, ndl-PCBs, or acrylamide and the levels of different subpopulations of leukocytes in peripheral blood (results not shown). Regarding vaccine antibody titers, prenatal exposure to dioxins and dl-PCBs, and ndl-PCBs were associated with reduced levels of anti-measles antibodies in multivariate analyses (Table 5). The dietary toxicant variables were the only variables remaining in the final multivariate models. None of the other vaccine antibody titers were significantly associated with exposure to dioxins and dl-PCBs, ndl-PCBs, or acrylamide. Twenty three of 111 (20.7 %) children were found to be sensitized using Phadiatop Infant. Of these 23 children, 15 were found to be sensitized to at least one food allergen, 9 to at least one inhalant allergen, whereas 5 children were not found to be sensitized to any individual allergens tested. In bivariate logistic regression analyses, no significant associations were found between exposure to the dietary toxicants and sensitization (Table 5).

4. Discussion

In this study, we found that prenatal dietary exposure to PCBs and dioxins was associated with an increased risk of wheeze (periods of more than 10 days of dry cough, chest tightness, or wheeze) and numbers of upper respiratory tract infections (URTI) during the three first years of life, and reduced levels of vaccine-induced anti-measles antibodies at three years of age. No associations were found with immunophenotype data or allergic sensitization. Furthermore, no associations were observed between prenatal dietary exposure to acrylamide and the health outcomes or the blood parameters investigated.

The association found between exposure to PCBs and dioxins and an increased risk of wheeze during the three first years of life is in accordance with previous reported results from this birth cohort (BraMat), where prenatal dietary exposure to PCBs and dioxins was associated with an increased risk of wheeze during the first year of life (Stolevik *et al.*, 2011). This association, however, was not found when investigating only the third year. Wheeze may be divided into distinct phenotypes (reviewed by Sly *et al*. (2008)). It was not possible to differentiate between wheeze phenotypes in the present study, however, the decreasing tendency with time of wheeze associated with exposure to PCBs and dioxins may suggest that the exposure was mainly associated with infection-induced wheeze. Since the diameter of the airways increases as the child grows older, the influence of infections in inducing wheeze may be of less importance in later childhood. Weisglas-Kuperus *et al*. (2000 and 2004) have also reported on wheeze and wheeze-related symptoms, however, it is difficult to compare the results in the present study and the two studies of Weisglas-Kuperus *et al*. (2000 and 2004) due to differences in the formulations of questions in the questionnaires. To examine whether prenatal dietary exposure to PCBs and dioxins is associated with persistent wheeze or asthma, a follow-up into later childhood is needed.

In the present study, prenatal dietary exposure to PCBs and dioxins was found to be associated with increased numbers of URTI. During the three first years of life, also a tendency of increased numbers of gastroenteritis was found. This is in accordance with previous findings in this cohort at 1 year of age (Stolevik *et al.*, 2011) and findings in other studies, especially for lower respiratory tract infections and otitis media (Dallaire *et al.*, 2004; Dallaire *et al.*, 2006; Miyashita *et al.*, 2011; Weisglas-Kuperus *et al.*, 2000; Weisglas-Kuperus *et al.*, 2004). We also found that prenatal exposure to PCBs and dioxins was associated with reduced levels of vaccine-induced anti-measles antibodies at three years of age which is in agreement with results reported in other studies (Heilmann *et al.*, 2006; Heilmann *et al.*, 2010; Weisglas-Kuperus *et al.*, 2000). The present findings on reduced antibody response to the measles vaccine as well as increased numbers of URTI may indicate that prenatal exposure to PCBs and dioxins is associated with immunosuppression that may result in increased susceptibility to infectious diseases.

In contrast to other studies, prenatal dietary exposure to PCBs and dioxins was not found to be associated with the levels of different subpopulations of leukocytes in peripheral blood in the present study (Glynn *et al.*, 2008; Weisglas-Kuperus *et al.*, 2000). Since immunophenotyping in the present study was only performed on 78-80 samples, low power may be a reason for the different results. In general, changes in the levels of different leukocytes may be expected when effects on frequency and prevalence of diseases are observed, but the method may have insufficient sensitivity (reviewed by Luster *et al*. (2005)). Overall, our results indicate that in humans functional immunological endpoints like vaccine responses are more sensitive in identifying associations with chemical exposures than non-functional endpoints like immunophenotype data.

The associations found between prenatal dietary exposure to PCBs and dioxins and increased risk of wheeze, increased numbers of URTI, and reduced antibody response to a

vaccine are all in accordance with the proposed immunosuppressive properties of dioxins and dl-PCBs. A suggested mechanistic explanation is that dioxins and dl-PCBs bind to the aryl hydrocarbon receptor (AhR) with a resulting expansion of the population of regulatory T cells with subsequent down-regulation of immune responses (Kerkvliet *et al.*, 2009; Quintana *et al.*, 2008).

No significant associations were found between prenatal dietary exposure to acrylamide and immunological health outcomes or blood parameters. The study population may be too small, however, to conclude on negative findings. New studies should be performed in a cohort with more participants to improve the statistical power.

It gives rise to concern that prenatal exposure to PCBs and dioxins was found to be associated with immune-related endpoints, even for the third year of life, especially since only 5 of 205 participating mothers had slightly higher intake than the tolerable weekly intake (TWI) of 14 pg TEQ/kg bw/week (set by the EU Scientific Committee on Food (Scientific Committee on Food, 2001)). This supports the notion that the foetal stage is a critical time window for exposure (Dietert, 2008; Holsapple *et al.*, 2004; van Loveren and Piersma, 2004). It is difficult, however, to differentiate between prenatal and postnatal exposure from the child's diet. In the present study, the children have been exposed to PCBs and dioxins also postnatally from breast milk (Ayotte *et al.*, 2003) and food items. Thus, based on the exposure data available for the birth cohort used, it was not possible to identify the existence of a critical time window during foetal stage and early childhood for exposure to PCBs and dioxins.

The strengths of our study are that the food frequency questionnaire used is well validated (Brantsaeter *et al.*, 2008a), and that extensive information about the children and their mothers is available from MoBa and MBRN. Furthermore, the potential selection bias in MoBa has been evaluated, and no differences regarding 8 well-known associations between

MoBa participants and all women giving birth in Norway was found, suggesting no influential selection bias in MoBa (Nilsen *et al.*, 2009). However, selection bias due to the low recruitment rate to the BraMat cohort (25% of the invited MoBa participants) cannot be excluded. Regarding information bias, we have previously reported that no significant differences were found for frequency of health outcomes when comparing written questionnaires and telephone interviews for the birth cohort BraMat at 1 year of age (Stolevik *et al.*, 2011).

Long-chain n-3 fatty acids from fish may influence the associations found in the present study due to their suggested anti-inflammatory effects (reviewed by Chapkin *et al.* (2009)). As expected due to their common source sea food and the lipohilic properties of the toxicants, the maternal intake during pregnancy from food items of the n-3 long-chain fatty acids eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) (calculated as described in (Haugen *et al.*, 2008)) were highly correlated with the intake of dioxins and dl-PCBs and ndl-PCBs ($\rho = 0.8$). Therefore, we could not adjust for these n-3 fatty acids in the statistical analyses. However, when it comes to health outcomes, a significant association was only found between the prenatal exposure to n-3 fatty acids from food items and wheeze during the three first years of life (bivariate analysis; OR (95% CI) p-value; 2.37 (1.03-5.46) 0.043) and no other health outcomes found to be associated with PCB and dioxin exposure (results not shown). No associations were found for n-3 fatty acids from dietary supplements (purified to remove PCBs and dioxins), which may suggest that exposure to PCBs and dioxins, rather than n-3 fatty acids, is most important for the immunosuppression found in the present study.

In conclusion, exposure to acrylamide from the maternal diet during pregnancy was not found to be associated with either immune-related health outcomes or with blood parameters in children during the three first years of life. Our study suggests that prenatal

dietary exposure to PCBs and dioxins is associated with immunosuppression that persists into early childhood. Overall, a continued effort to reduce the exposure to PCBs and dioxins from the diet for women in fertile age may be beneficial for their child's health.

Funding

This work was supported by the EU Integrated Project NewGeneris, 6th Framework Programme, Priority 5: Food Quality and Safety (FOOD-CT-2005-016320). NewGeneris is the acronym for "Newborns and Genotoxic exposure risks", http://www.newgeneris.org. The Norwegian Institute of Public Health also contributed to the funding of the study. The Norwegian Mother and Child Cohort Study is supported by the Norwegian Ministry of Health and the Ministry of Education and Research, NIH/NIEHS (contract no. NO-ES-75558), NIH/NINDS [grant no. 1 UO1 NS 047537-01], and the Norwegian Research Council/FUGE [grant no. 151918/S10].

Acknowledgements

We thank children and parents for participating in the BraMat birth cohort. We thank Bodil Hasseltvedt, Else-Carin Groeng, Astri Grestad, Berit Arvesen Stensby, and Åse Eikeset at the Norwegian Institute of Public Health, Ulla-Maj Sundstrøm at the Oslo University Hospital, and Nærmil Ghadani and Elin Hareton at Akershus University Hospital for their assistance in blood sampling and processing. We thank Anne-Cathrine Kristoffersen for analysis of bacterial vaccine antibody titers. We thank Jorid Eide and Ragnhild Hovengen at Norwegian Institute of Public Health, and the staff at the maternity ward and the laboratory at Oslo and Akershus University Hospital for their contribution to recruitment of participants.

References

Aaberge, I. S., Steinsvik, T. E., Groeng, E. C., Leikvold, R. B., and Lovik, M. (1996). Human antibody response to a pneumococcal vaccine in SCID-PBL-hu mice and simultaneously vaccinated human cell donors. *Clin Exp Immunol* **105,** 12-17.

Annola, K., Karttunen, V., Keski-Rahkonen, P., Myllynen, P., Segerback, D., Heinonen, S., and Vahakangas, K. (2008). Transplacental transfer of acrylamide and glycidamide are comparable to that of antipyrine in perfused human placenta. *Toxicol Lett.* **182,** 50-56.

Ayotte, P., Muckle, G., Jacobson, J. L., Jacobson, S. W., and Dewailly, E. (2003). Assessment of pre- and postnatal exposure to polychlorinated biphenyls: lessons from the Inuit Cohort Study. *Environ Health Perspect* **111,** 1253-1258.

Brantsaeter, A. L., Haugen, M., Alexander, J., and Meltzer, H. M. (2008a). Validity of a new food frequency questionnaire for pregnant women in the Norwegian Mother and Child Cohort Study (MoBa). *Matern. Child Nutr* **4,** 28-43.

Brantsaeter, A. L., Haugen, M., Mul, A., Bjellaas, T., Becher, G., Klaveren, J. V., Alexander, J., and Meltzer, H. M. (2008b). Exploration of different methods to assess dietary acrylamide exposure in pregnant women participating in the Norwegian Mother and Child Cohort Study (MoBa). *Food Chem. Toxicol.* **46,** 2808-2814.

Chapkin, R. S., Kim, W., Lupton, J. R., and McMurray, D. N. (2009). Dietary docosahexaenoic and eicosapentaenoic acid: emerging mediators of inflammation. *Prostaglandins Leukot. Essent. Fatty Acids* **81,** 187-191.

Covaci, A., Jorens, P., Jacquemyn, Y., and Schepens, P. (2002). Distribution of PCBs and organochlorine pesticides in umbilical cord and maternal serum. *Sci Total Environ* **298,** 45-53.

Dallaire, F., Dewailly, E., Muckle, G., Vezina, C., Jacobson, S. W., Jacobson, J. L., and Ayotte, P. (2004). Acute infections and environmental exposure to organochlorines in Inuit infants from Nunavik. *Environ. Health Perspect.* **112,** 1359-1365.

Dallaire, F., Dewailly, E., Vezina, C., Muckle, G., Weber, J. P., Bruneau, S., and Ayotte, P. (2006). Effect of prenatal exposure to polychlorinated biphenyls on incidence of acute respiratory infections in preschool Inuit children. *Environ. Health Perspect.* **114,** 1301-1305.

Dietert, R. R. (2008). Developmental immunotoxicology (DIT): windows of vulnerability, immune dysfunction and safety assessment. *J Immunotoxicol.* **5,** 401-412.

Domingo, J. L., and Bocio, A. (2007). Levels of PCDD/PCDFs and PCBs in edible marine species and human intake: a literature review. *Environ Int* **33,** 397-405.

Glynn, A., Thuvander, A., Aune, M., Johannisson, A., Darnerud, P. O., Ronquist, G., and Cnattingius, S. (2008). Immune cell counts and risks of respiratory infections among infants exposed pre- and postnatally to organochlorine compounds: a prospective study. *Environ Health* **7,** 62.

Grandjean, P., Poulsen, L. K., Heilmann, C., Steuerwald, U., and Weihe, P. (2010). Allergy and sensitization during childhood associated with prenatal and lactational exposure to marine pollutants. *Environ Health Perspect* **118,** 1429-1433.

Haugen, M., Brantsaeter, A. L., Alexander, J., and Meltzer, H. M. (2008). Dietary supplements contribute substantially to the total nutrient intake in pregnant Norwegian women. *Ann Nutr Metab* **52,** 272-280.

Heilmann, C., Budtz-Jorgensen, E., Nielsen, F., Heinzow, B., Weihe, P., and Grandjean, P. (2010). Serum concentrations of antibodies against vaccine toxoids in children exposed perinatally to immunotoxicants. *Environ Health Perspect* **118,** 1434-1438.

Heilmann, C., Grandjean, P., Weihe, P., Nielsen, F., and Budtz-Jorgensen, E. (2006). Reduced antibody responses to vaccinations in children exposed to polychlorinated biphenyls. *PLoS. Med.* **3,** e311.

Hogervorst, J. G., Baars, B. J., Schouten, L. J., Konings, E. J., Goldbohm, R. A., and van den Brandt, P. A. (2010). The carcinogenicity of dietary acrylamide intake: a comparative discussion of epidemiological and experimental animal research. *Crit Rev Toxicol* **40,** 485-512.

Holsapple, M. P., Paustenbach, D. J., Charnley, G., West, L. J., Luster, M. I., Dietert, R. R., and Burns-Naas, L. A. (2004). Symposium summary: children's health risk--what's so special about the developing immune system? *Toxicol Appl. Pharmacol* **199,** 61-70.

Kerkvliet, N. I., Steppan, L. B., Vorachek, W., Oda, S., Farrer, D., Wong, C. P., Pham, D., and Mourich, D. V. (2009). Activation of aryl hydrocarbon receptor by TCDD prevents diabetes in NOD mice and increases Foxp3+ T cells in pancreatic lymph nodes. *Immunotherapy.* **1,** 539-547.

Kvalem, H. E., Knutsen, H. K., Thomsen, C., Haugen, M., Stigum, H., Brantsaeter, A. L., Froshaug, M., Lohmann, N., Papke, O., Becher, G., Alexander, J., and Meltzer, H. M. (2009). Role of dietary patterns for dioxin and PCB exposure. *Mol Nutr Food Res* **53,** 1438-1451.

Luster, M. I., Johnson, V. J., Yucesoy, B., and Simeonova, P. P. (2005). Biomarkers to assess potential developmental immunotoxicity in children. *Toxicol. Appl. Pharmacol.* **206,** 229-236.

Magnus, P., Irgens, L. M., Haug, K., Nystad, W., Skjaerven, R., and Stoltenberg, C. (2006). Cohort profile: the Norwegian Mother and Child Cohort Study (MoBa). *Int. J. Epidemiol.* **35,** 1146-1150.

Meltzer, H. M., Brantsaeter, A. L., Ydersbond, T. A., Alexander, J., and Haugen, M. (2008). Methodological challenges when monitoring the diet of pregnant women in a large study: experiences from the Norwegian Mother and Child Cohort Study (MoBa). *Matern. Child Nutr* **4,** 14-27.

Merlo, D. F., Wild, C. P., Kogevinas, M., Kyrtopoulos, S., and Kleinjans, J. (2009). NewGeneris: a European study on maternal diet during pregnancy and child health. *Cancer Epidemiol Biomarkers Prev* **18,** 5-10.

Miyashita, C., Sasaki, S., Saijo, Y., Washino, N., Okada, E., Kobayashi, S., Konishi, K., Kajiwara, J., Todaka, T., and Kishi, R. (2011). Effects of prenatal exposure to dioxin-like compounds on allergies and infections during infancy. *Environ Res* **111,** 551-558.

Mottram, D. S., Wedzicha, B. L., and Dodson, A. T. (2002). Acrylamide is formed in the Maillard reaction. *Nature* **419,** 448-449.

Murphy, K., Travers, P., and Walport, M. (2008). Janeway's Immunobiology, 7th ed., Garland Science, Taylor & Francis Group, LLC, New York.

Nilsen, R. M., Vollset, S. E., Gjessing, H. K., Skjaerven, R., Melve, K. K., Schreuder, P., Alsaker, E. R., Haug, K., Daltveit, A. K., and Magnus, P. (2009). Self-selection and bias in a large prospective pregnancy cohort in Norway. *Paediatr. Perinat. Epidemiol* **23,** 597-608.

Parzefall, W. (2008). Minireview on the toxicity of dietary acrylamide. *Food Chem. Toxicol.* **46,** 1360-1364.

Phipps, D. C., West, J., Eby, R., Koster, M., Madore, D. V., and Quataert, S. A. (1990). An ELISA employing a Haemophilus influenzae type b oligosaccharide-human serum albumin conjugate correlates with the radioantigen binding assay. *J Immunol Methods* **135,** 121-128.

Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., Caccamo, M., Oukka, M., and Weiner, H. L. (2008). Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* **453,** 65-71.

Scientific Committee on Food (2001). Opinion for the SCF on the risk assessment of dioxins and dioxin-like PCBs in food, update based on new scientific information available since the SCF opinion of 22nd November 2000.

Simonsen, O., Bentzon, M. W., and Heron, I. (1986). ELISA for the routine determination of antitoxic immunity to tetanus. *J Biol Stand.* **14,** 231-239.

Sly, P. D., Boner, A. L., Bjorksten, B., Bush, A., Custovic, A., Eigenmann, P. A., Gern, J. E., Gerritsen, J., Hamelmann, E., Helms, P. J., Lemanske, R. F., Martinez, F., Pedersen, S., Renz, H., Sampson, H., von, M. E., Wahn, U., and Holt, P. G. (2008). Early identification of atopy in the prediction of persistent asthma in children. *Lancet* **372,** 1100-1106.

Stadler, R. H., Blank, I., Varga, N., Robert, F., Hau, J., Guy, P. A., Robert, M. C., and Riediker, S. (2002). Acrylamide from Maillard reaction products. *Nature* **419,** 449-450.

Stolevik, S. B., Nygaard, U. C., Namork, E., Haugen, M., Kvalem, H. E., Meltzer, H. M., Alexander, J., van Delft, J. H., Loveren, H., Lovik, M., and Granum, B. (2011). Prenatal exposure to polychlorinated biphenyls and dioxins is associated with increased risk of wheeze and infections in infants. *Food Chem. Toxicol.* **49,** 1843-1848.

Suzuki, G., Nakano, M., and Nakano, S. (2005). Distribution of PCDDs/PCDFs and Co-PCBs in human maternal blood, cord blood, placenta, milk, and adipose tissue: dioxins showing high toxic equivalency factor accumulate in the placenta. *Biosci. Biotechnol. Biochem* **69,** 1836-1847.

ten Tusscher, G. W., Steerenberg, P. A., van, L. H., Vos, J. G., von dem Borne, A. E., Westra, M., van der Slikke, J. W., Olie, K., Pluim, H. J., and Koppe, J. G. (2003). Persistent hematologic and immunologic disturbances in 8-year-old Dutch children associated with perinatal dioxin exposure. *Environ Health Perspect* **111,** 1519-1523.

van den Berg, M., Birnbaum, L. S., Denison, M., De, V. M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N., and Peterson, R. E. (2006). The 2005

World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol Sci* **93,** 223-241.

van Loveren, H., and Piersma, A. (2004). Immunotoxicological consequences of perinatal chemical exposures. *Toxicol. Lett.* **149,** 141-145.

Weisglas-Kuperus, N., Patandin, S., Berbers, G. A., Sas, T. C., Mulder, P. G., Sauer, P. J., and Hooijkaas, H. (2000). Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children. *Environ. Health Perspect.* **108,** 1203-1207.

Weisglas-Kuperus, N., Vreugdenhil, H. J., and Mulder, P. G. (2004). Immunological effects of environmental exposure to polychlorinated biphenyls and dioxins in Dutch school children. *Toxicol. Lett.* **149,** 281-285.

Table 1. Demographics of the BraMat study population included in the statistical analyses on cumulative (n = 158) and last year (n = 176) questionnaire data, and blood parameters (n = 112).

Demographics	Cumulative n (%)	Last year n (%)	Blood parameters n (%)
Eczema/Itchiness (yes)	74 (49.0)	54 (31.4)	36 (33.3)
Doctor diagnosed atopic eczema (yes)	33 (21.2)	28 (16.0)	18 (16.4)
Doctor diagnosed allergy (yes)	16 (10.3)	13 (7.4)	8 (7.2)
Wheeze (yes)	61 (38.6)	34 (19.3)	22 (19.8)
Use of asthma medicine (yes)	37 (23.4)	32 (18.2)	16 (14.4)
Doctor diagnosed asthma (yes)	20 (12.7)	16 (9.1)	9 (8.1)
Upper respiratory tract infections (mean (min-max))	10 (0-35)	3 (0-20)	3 (0-20)
Otitis media (yes)	54 (35.8)	30 (17.1)	20 (18.2)
Gastroenteritis (yes)	138 (92.0)	118 (69.0)	78 (72.2)
Gastroenteritis (mean (min-max))	3 (0-11)	1 (0-5)	2 (0-5)
Pneumonia (yes)	11 (7.3)	4 (2.3)	4 (3.6)
Urinary tract infection (yes)	10 (6.7)	6 (3.5)	6 (5.5)
Other chronic diseases (yes)	8 (5.1)	9 (5.1)	3 (2.7)
Children's diseases (yes)	76 (50.3)	36 (20.6)	25 (22.7)
Chicken pox	43 (28.5)	26 (14.9)	19 (17.3)
Exanthema subitum	45 (29.8)	8 (4.6)	5 (4.5)
Gender (boy)	68 (43.0)	75 (42.6)	41 (36.6)
Type of delivery (caesarean section)	18 (11.4)	21 (11.9)	14 (12.5)
Birth season			
March-August	27 (17.1)	34 (19.3)	21 (18.8)
September-February	131 (82.9)	142 (80.7)	91 (81.3)
Apgar score 1 min (<7)	7 (4.4)	8 (4.5)	6 (5.4)
Gestational age (mean (min-max))	40 (37-42) wks	40 (37-42) wks	40 (37-42) wks
Birth weight (mean (min-max))	3554 (2292-4870) g	3555 (2292-4870) g	3525 (2292-4870) g
Breast-feeding (no or <6 months)	19 (13.1)	21 (13.0)	15 (13.9)
Start day-care centre (<12 months)	30 (19.4)	33 (19.1)	25 (22.5)
Number older siblings (≥1)	72 (52.9)	83 (54.2)	49 (51.0)
Parity (≥1)	82 (51.9)	93 (52.8)	56 (50.0)
Previous breast-feeding			
No	82 (51.9)	91 (51.7)	61 (54.5)
≤12 months	39 (24.7)	45 (25.6)	27 (24.1)
>12 months	37 (23.4)	40 (22.7)	24 (21.4)
Maternal asthma and allergy (yes)	66 (41.8)	68 (38.6)	45 (40.2)
Paternal asthma and allergy (yes)	41 (26.8)	47 (27.5)	27 (24.8)
Maternal age			
<30	44 (27.8)	47 (26.7)	31 (27.7)
30-35	81 (51.3)	94 (53.4)	55 (49.1)
>35	33 (20.9)	35 (19.9)	26 (23.2)
Maternal BMI			
<20.0	27 (17.2)	30 (17.1)	17 (15.2)
20.0-24.9	95 (60.5)	101 (57.7)	69 (61.6)
>24.9	25 (22.3)	44 (25.1)	26 (23.2)
Maternal education (years)			
<13	31 (20.4)	38 (22.4)	14 (13.2)
13-16	65 (42.8)	68 (40.0)	49 (46.2)
>16	56 (36.8)	64 (37.6)	43 (40.6)
Ever smoked (yes)	65 (41.1)	75 (42.6)	42 (37.5)
Smoking during pregnancy (yes)	2 (1.3)	4 (2.3)	1 (0.9)
Smoking last three months before pregnancy (yes)	24 (15.5)	27 (15.7)	12 (10.9)
Passive smoking (yes)	10 (6.4)	11 (6.3)	9 (8.0)
Gross income of the household (>700 000 NOK)	105 (68.2)	116 (67.4)	76 (69.7)

Table 2. The calculated dietary intake of ndl-PCBs, dioxins and dl-PCBs, and acrylamide of the mothers in the BraMat cohort (n=200).

Dietary toxicant	Median[a]	Min-max	IQR	80th percentile
Ndl-PCBs (ng/kg bw/day) [b]	2.59	0.53-30.12	1.80-4.09	4.37
Dioxins and dl-PCBs (pg TEQ/kg bw/day) [c]	0.58	0.15-3.07	0.45-0.81	0.90
Acrylamide (μg/kg bw/day)	0.56	0.07-2.05	0.41-0.72	0.79

Note. IQR, interquartile range

[a] The median values are presented since the data were not normally distributed.

[b] PCB-28, 52, 101, 138, 153, and 180.

[c] All 17 of the 2,3,7,8-substituted PCDD/PCDFs, non-ortho-substituted PCBs (PCB-77, 81, 126, and 169), and mono-ortho-substituted PCBs (PCB-105, 114, 118, 123, 156, 157, 167, and 189).

Table 3. Results of bivariate analyses of prenatal dietary exposure to ndl-PCBs, dioxins and dl-PCBs, and acrylamide and health outcomes.

		Acrylamide		Ndl-PCBs		Dioxins and dl-PCBs	
Years of age	Health outcome	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
0-3 years	Eczema	1.36 (0.60-3.06)	0.461	0.75 (0.34-1.68)	0.752	0.57 (0.25-1.31)	0.188
	Atopic eczema [b]	1.21 (0.49-2.98)	0.680	0.62 (0.22-1.74)	0.362	0.62 (0.22-1.74)	0.362
	Allergy	0.57 (0.12-2.64)	0.469	1.12 (1.02-1.23)	**0.021** [a]	2.12 (0.85-5.26)	0.106 [a]
	Wheeze	1.01 (0.45-2.25)	0.990	3.20 (1.42-7.22)	**0.005**	2.71 (1.21-6.04)	**0.015**
	Asthma	1.03 (0.32-3.32)	0.964	1.44 (0.48-4.31)	0.519	1.94 (0.68-5.54)	0.217
	Asthma medicine	1.18 (0.48-2.91)	0.726	0.94 (0.37-2.41)	0.902	1.45 (0.60-3.49)	0.412
	Otitis media	1.35 (0.59-3.08)	0.483	1.56 (0.67-3.64)	0.301	1.56 (0.67-3.64)	0.301
	Chicken pox	0.42 (0.15-1.16)	0.095	0.57 (0.21-1.50)	0.255	0.72 (0.28-1.82)	0.487
	Exanthema subitum	1.39 (0.60-3.21)	0.439	1.01 (0.42-2.42)	0.979	1.01 (0.42-2.42)	0.979
2-3 years	Eczema	1.06 (0.47-2.36)	0.893	1.00 (0.45-2.23)	0.996	1.00 (0.45-2.23)	0.996
	Atopic eczema	1.42 (0.55-3.66)	0.472	0.85 (0.30-2.42)	0.757	1.11 (0.41-2.99)	0.837
	Wheeze	1.06 (0.42-2.67)	0.909	1.95 (0.83-4.58)	0.126	1.95 (0.83-4.58)	0.126
	Asthma medicine	1.16 (0.46-2.95)	0.756	1.09 (1.00-1.19)	0.059 [a]	2.05 (0.93-4.50)	0.075 [a]
	Otitis media	0.80 (0.28-2.27)	0.675	1.05 (0.39-2.80)	0.931	0.80 (0.28-2.27)	0.675
	Gastroenteritis	1.38 (0.60-3.20)	0.450	1.10 (0.48-2.50)	0.824	1.10 (0.48-2.50)	0.824
		β (95% CI)	*p*-value	β (95% CI)	*p*-value	β (95% CI)	*p*-value
0-3 years	URTI	1.09 0.85-1.40)	0.481	1.03 (1.00-1.06)	0.061 [a]	1.31 (1.03-1.68)	**0.031** [a]
	Gastroenteritis	-0.02 (-1.00-0.97)	0.977	0.92 (-0.02-1.85)	0.054	1.09 (0.14-2.03)	**0.024**
2-3 years	URTI	1.12 (0.89-1.42)	0.337	1.03 (1.00-1.06)	**0.025** [a]	1.28 (1.01-1.62)	**0.042**

Note. Bold font, $p<0.05$; URTI, upper respiratory tract infections, back-transformed values are reported (ratios).

[a] Continuous exposure variables were used in the statistical analyses.

[b] 1-3 years of age.

Table 4. Results of multivariate analyses[a] of prenatal dietary exposure to ndl-PCBs, dioxins and dl-PCBs, and acrylamide and health outcomes.

Years of age	Health outcome	n	Dietary toxicant	Dietary toxicant categories [b]	aOR (95% CI)	*p*-value
0-3	Wheeze	149	Ndl-PCBs	P80	4.03 (1.59-10.23)	**0.003**
		148	Dioxins and dl-PCBs	P80	2.69 (1.03-7.07)	**0.044**
	Allergy	153	Ndl-PCBs	Continuous	1.12 (1.00-1.26)	**0.043**
	Chicken pox	151	Acrylamide	P80	0.40 (0.14-1.15)	0.088
2-3	Asthma medicine	170	Ndl-PCBs	Continuous	1.10 (0.99-1.24)	0.090
		170	Dioxins and dl-PCBs	Continuous	1.98 (0.85-4.60)	0.112
					aβ (95% CI)	*p*-value
0-3	URTI	126	Ndl-PCBs	Continuous	1.04 (1.01-1.08)	**0.007**
		126	Dioxins and dl-PCBs	Continuous	1.42 (1.11-1.83)	**0.006**
	Gastroenteritis	135	Ndl-PCBs	P80	0.94 (0.02-1.85)	**0.046**
		135	Dioxins and dl-PCBs	P80	1.01 (0.08-1.95)	**0.034**
2-3	URTI	160	Ndl-PCBs	Continuous	1.03 (1.00-1.06)	**0.025**
		160	Dioxins and dl-PCBs	P80	1.28 (1.01-1.62)	**0.042**

Note. Bold font, $p<0.05$; n, number of participants included in the final model; P80, 80th percentile; URTI, upper respiratory tract infections, back-transformed values are reported (ratios); aOR, adjusted OR; aβ, adjusted β.

[a] Variables initially included in the multivariate analyses were: parity, maternal and paternal asthma and allergy, maternal age, maternal smoking, maternal passive smoking, maternal education, maternal BMI, mean parental gross income for the three years of the study, child's gender, birth season, type of delivery, apgar score, gestational age, birth weight, breast-feeding of the child, and day-care attendance.

[b] The reference category is the lowest exposure category.

Table 5. Results of bivariate and multivariate[a] analyses of prenatal dietary exposure to ndl-PCBs, dioxins and dl-PCBs, and acrylamide and sensitization (Phadiatop Infant) and vaccine titres.

Serum analyses	Dietary toxicant	OR (95% CI)	p-value	aOR (95% CI)	p-value
Sensitization	Ndl-PCBs	1.72 (0.90–3.26)	0.099		
	Dioxins and dl-PCBs	1.52 (0.65-3.55)	0.329		
	Acrylamide	0.71 (0.29-1.74)	0.453		
		β (95% CI)	p-value	aβ (95% CI)	p-value
Measles	Ndl-PCBs	-0.12 (-0.23- -0.01)	**0.032**	-0.12 (-0.23- -0.01)	**0.032**
	Dioxins and dl-PCBs	-0.15 (-0.29- -0.01)	**0.036**	-0.15 (-0.29- -0.01)	**0.036**
	Acrylamide	-0.13 (-0.29-0.02)	0.095	-0.13 (-0.29-0.02)	0.095
Rubella	Ndl-PCBs	-0.05 (-0.16-0.06)	0.373		
	Dioxins and dl-PCBs	-0.07 (-0.21-0.07)	0.335		
	Acrylamide	-0.04 (-0.19-0.11)	0.620		
Tetanus [b]	Ndl-PCBs	1.11 (0.92-1.32)	0.268		
	Dioxins and dl-PCBs	1.22 (0.97-1.54)	0.083	1.22 (0.97-1.54)	0.083
	Acrylamide	1.25 (0.98-1.59)	0.076	1.25 (0.98-1.59)	0.076
HiB [b]	Ndl-PCBs	0.76 (0.56-1.02)	0.070	0.78 (0.58-1.04)	0.085 [c]
	Dioxins and dl-PCBs	0.75 (0.51-1.11)	0.150		
	Acrylamide	1.22 (0.80-1.85)	0.349		
Pneumococcus [b]	Ndl-PCBs	0.96 (0.81-1.15)	0.687		
	Dioxins and dl-PCBs	0.94 (0.75-1.18)	0.590		
	Acrylamide	0.94 (0.74-1.20)	0.627		

Note. Bold font, $p<0.05$; aOR, adjusted OR; aβ, adjusted β.

[a] Variables initially included in the multivariate analyses were: parity, maternal and paternal asthma and allergy, maternal age, maternal smoking, maternal passive smoking, maternal education, maternal BMI, mean parental gross income for the three years of the study, child's gender, birth season, type of delivery, apgar score, gestational age, birth weight, breast-feeding of the child, and day-care attendance.

[b] The outcome variable was ln-transformed, back-transformed values are reported (ratios).

[c] Adjusted for gestational age and day-care attendance.

Appendix A: 1-year follow-up questionnaire (Norwegian)

BraMat

Et delprosjekt i Den norske mor og barn-undersøkelsen

Oppfølging av barnet ved fylte 1 år

Vi gratulerer dere med 1 årsdagen!

Vi har fra april 2007 til mars 2008 rekruttert 200 mødre og deres barn. Blodprøvene vi fikk av deg og barnet i forbindelse med fødselen blir nå analysert. Inntaket av fremmedstoffer (som steke-mutagener og andre miljøgifter) under svangerskapet beregnes ut fra spørreskjemaene som du har sendt inn til oss.

Vi ønsker å følge barnet ditt til det er 3 år gammelt. I denne perioden vil vi følge utviklingen av barnets immunforsvar mot sykdom, spesielt allergi og infeksjoner, for å undersøke om fremmedstoffene kan ha påvirket barnets immunsystem.

I denne studien opprettes også tilsvarende mor-barn prosjekter i Spania, Storbritannia, Hellas og Danmark. Data fra alle prosjektene vil bli sammenstilt. På den måten kan vi oppdage forskjeller og likheter mellom de ulike landene.

Om kort tid vil vi kontakte deg per telefon for å spørre om hvilke sykdommer eller helseplager barnet ditt har hatt i løpet av sitt første leveår. På de neste sidene kan du lese de spørsmålene vi kommer til å stille deg i intervjuet. Bruk notatboken og helsestasjonskortet når du krysser av for spørsmålene.

Dersom du ikke ønsker et telefonintervju, ber vi om at du fyller ut skjemaet og sender det til oss i vedlagte returkonvolutt innen en uke.

ID-nummer

BRAMAT

den norske Mor & barn undersøkelsen

NewGeneris
Newborns and Genotoxic exposure risks

folkehelseinstituttet

Har barnet hatt noen av de følgende sykdommer/helseplager i alderen 0-12 mnd?	**Ja**	**Nei**	**Antall ganger**
Forkjølelse eller annen luftveissykdom *f.eks. falsk krupp, halsbetennelse, influensa, kikhoste*	☐	☐	
Ørebetennelse	☐	☐	
Lungebetennelse	☐	☐	
Omgangssyke med oppkast eller diaré	☐	☐	
Urinveisinfeksjon	☐	☐	

Astma, allergi og andre kroniske sykdommer i alderen 0-12 mnd	**Ja**	**Nei**
Har barnet fått diagnosen astma eller astmabronkitt av lege?	☐	☐
Hvis ja, hvor mange måneder var barnet når det fikk diagnosen?		
Har barnet hatt perioder, som har vart i mer enn 10 dager, med tørrhoste, tetthet eller piping i brystet, eller kortpustethet?	☐	☐
Har barnet eksem og kløe i ansiktet eller i bøyefurene på armer og ben? (f.eks. lyske, knehase, ankel, albubøy, håndledd)	☐	☐
Har barnet fått diagnosen allergi av lege?	☐	☐
Hvis ja, hvor mange måneder var barnet når det fikk diagnosen?		
Har barnet noen annen kronisk sykdom? *f.eks. diabetes (sukkersyke) eller leddgikt*	☐	☐

Har barnet hatt noen barnesykdommer i alderen 0-12 mnd?	Ja ☐	Nei ☐
Hvis ja, hvilke?		

Medisinbruk	**Ja**	**Nei**
Bruker eller har barnet brukt astmamedisiner?	☐	☐
Bruker eller har barnet brukt allergimedisiner?	☐	☐
Har barnet fått antibiotika (penicillin eller lignende) mot infeksjoner? *f.eks. Apocillin, Calcipen, Weifapenin, Aboticin, Ery-Max, Imacillin*	☐	☐
Hvis ja, ved hvor mange infeksjoner har barnet fått behandling med antibiotika?		

Vaksinasjon	
Kryss av for hvilke vaksiner barnet har fått i alderen 0-12 mnd	☐
Ikke fått noen vaksine	☐
DTP (Difteri, stivkrampe, kikhoste)	☐
DT (Difteri, stivkrampe)	☐
Polio – Hib (Hemophilus influenzae)	☐
PKV (Pneumokokk)	☐
MMR (Meslinger, kusma og røde hunder)	☐
Hepatitt B (Engerix-B)	☐
Annen vaksine	☐
Hvis annen vaksine, beskriv:	

Fikk barnet bivirkninger av vaksineringen?	Ja ☐	Nei ☐
Hvis ja, beskriv:		

Kommentarer

Hva er barnets alder ved utfylling av dette skjemaet?

Antall måneder	

Hvis dere lurer på noe i forbindelse med 1 års oppfølgingen, kan dere ringe til prosjektkoordinator Berit Granum, tlf. **21 07 66 96**, stipendiat Solvor Berntsen Stølevik, tlf **21 07 62 57**, eller sende e-post til **bramat@fhi.no**.

Tusen takk for samarbeidet så langt!

Appendix B: 1-year follow-up questionnaire (English)

Has the child had any of the following diseases/complaints at 0-12 months of age?	**Yes**	**No**	**No. of times**
Cold or other respiratory diseases *e.g. false croup, throat infection, influenza ,whooping cough*	☐	☐	
Otitis media	☐	☐	
Pneumonia	☐	☐	
Gastroenteritis with vomiting or diarrhoea	☐	☐	
Urinary tract infection	☐	☐	

Asthma, allergy and other chronic diseases at 0-12 months of age	**Yes**	**No**
Has the child been diagnosed with asthma or asthma bronchitis by a doctor?	☐	☐
If yes, how old was the child when diagnosed?		
Has the child had periods of more than 10 days with dry cough, chest tightness or wheeze, or shortness of breath?	☐	☐
Has the child had eczema or itching in the face or at joints? (e.g. groin, hollow of the knee, ankle, elbow, wrist)	☐	☐
Has the child been diagnosed with allergy by a doctor?	☐	☐
If yes, how old was the child when diagnosed?		
Does the child have any other chronic diseases? *e.g. diabetes or arthritis*	☐	☐

Has the child had any childhood diseases at 0-12 months of age?	**Yes** ☐	**No** ☐
If yes, which ones?		

Medication	**Yes**	**No**
Does the child use or has used asthma medicine?	☐	☐
Does the child use or has used allergy medicine?	☐	☐
In case of infection, has the child used antibiotics (penicillin or similar)? *e.g. Apocillin, Calcipen, Weifapenin, Aboticin, Ery-Max, Imacillin*	☐	☐
If yes, how many infections have been treated?		

Vaccinations	
Tick off for the vaccines the child has been given at 0-12 months of age	☐
No vaccines	☐
DTP (Diphtheria, tetanus, whooping cough)	☐
DT (Diphtheria, tetanus)	☐
Polio – Hib (Hemophilus influenzae)	☐
Pneumococcal vaccine	☐
MMR (Measles, mumps, rubella)	☐
Hepatitis B (Engerix-B)	☐
Other vaccines	☐
If other vaccines, describe:	

Did the child experience any side-effects upon vaccination?	**Yes** ☐	**No** ☐
If yes, describe:		

Comments

Fill in the child's age

No. of months	

Appendix C: 3-year follow-up questionnaire (Norwegian)

BraMat

Et delprosjekt i Den norske mor og barn-undersøkelsen

Oppfølging av barnet etter fylte to år

Om kort tid vil vi kontakte deg på telefon for å spørre om hvilke sykdommer eller helseplager barnet ditt har hatt etter fylte to år. Dersom du ikke ønsker et telefonintervju, ber vi om at du fyller ut skjemaet og sender det til oss i vedlagt returkonvolutt innen en uke. Bruk notatboken og helsestasjonskortet når du krysser av for spørsmålene.

ID-nummer

Opplysninger om barnet:

Har barnet hatt noen av de følgende sykdommer/helseplager etter at det fylte to år?	**Ja**	**Nei**	**Antall ganger**
Forkjølelse eller annen luftveissykdom *f.eks. falsk krupp, halsbetennelse, influensa, kikhoste, bihulebetennelse*	☐	☐	
Ørebetennelse	☐	☐	
Lungebetennelse	☐	☐	
Omgangssyke med oppkast eller diaré	☐	☐	
Urinveisinfeksjon	☐	☐	
Feber uten andre symptomer enn slapphet	☐	☐	

Astma, allergi og andre kroniske sykdommer etter fylte to år	**Ja**	**Nei**
Har barnet fått diagnosen astma, astmabronkitt eller bronkial hyperreaktivitet av lege?	☐	☐
Hvis ja, hvor mange episoder har barnet hatt det siste året?		
Hvis ja, hvor gammelt var barnet da det fikk diagnosen?		
Har barnet hatt perioder, som har vart i mer enn 10 dager, med tørrhoste, tetthet eller piping i brystet, eller kortpustethet?	☐	☐
Hvis ja, hvor mange episoder har barnet hatt det siste året?		
Har barnet hatt eksem og kløe i ansiktet eller i bøyefurene på armer og ben? (f.eks. lyske, knehase, ankel, albubøy, håndledd)	☐	☐
Har barnet fått diagnosen atopisk eksem av lege?	☐	☐
Hvis ja, hvor gammelt var barnet da det fikk diagnosen?		
Har barnet fått diagnosen allergi av lege?	☐	☐
Hvis ja, hvor gammelt var barnet da det fikk diagnosen?		
Hvis ja, hva er barnet allergisk mot?		
Har barnet noen annen kronisk sykdom? *f.eks. diabetes (sukkersyke) eller leddgikt*	☐	☐
Hvis ja, hvilke?		

Har barnet hatt noen barnesykdommer etter fylte to år?	**Ja** ☐	**Nei** ☐
Hvis ja, hvilke?		

Medisinbruk etter fylte to år	**Ja**	**Nei**
Bruker eller har barnet brukt astmamedisiner?	☐	☐
Bruker eller har barnet brukt allergimedisiner (ikke salver)?	☐	☐
Har barnet fått antibiotika (penicillin eller lignende) mot infeksjoner? *f.eks. Apocillin, Calcipen, Weifapenin, Aboticin, Ery-Max, Imacillin*	☐	☐
Hvis ja, ved hvor mange infeksjoner har barnet fått behandling med antibiotika?		

Hvis ja, ved hvilke infeksjoner har barnet fått behandling med antibiotika?

Forkjølelse/annen luftveissykdom	Ørebetennelse	Lungebetennelse/bronkitt	Urinveisinfeksjon
☐	☐	☐	☐

Hvis andre, hvilke?

Har barnet fått andre medisiner? *f.eks. febernedsettende og smertelindrende*	**Ja** ☐	**Nei** ☐

Hvis ja, oppgi navn på medisin

Vaksiner etter fylte to år		
Har barnet fått noen vaksiner?	**Ja** ☐	**Nei** ☐
Hvis ja, hvilke?		
Fikk barnet bivirkninger av vaksineringen?	**Ja** ☐	**Nei** ☐
Hvis ja, beskriv:		

Informasjon om barnets hverdag fra fødsel fram til i dag	
I hvor mange måneder ammet du barnet?	

Hvor har barnet vært passet på dagtid (fra fødsel)?

Barnets alder (mnd) Fra - til	Hjemme	Dagmamma	Familiebarnehage	Barnehage
	☐	☐	☐	☐
	☐	☐	☐	☐
	☐	☐	☐	☐
	☐	☐	☐	☐

Hvor mange eldre søsken/barn bor sammen med barnet?	

Oppholder barnet seg i rom hvor noen røyker?

Nei	Ja, av og til	Ja, daglig
☐	☐	☐

Opplysninger om barnets mor:

Har du noen av disse sykdommene/helseplagene?	**Ja**	**Nei**
Har du fått diagnosen astma av lege?	☐	☐
Har du fått diagnosen allergi av lege?	☐	☐

Hvis ja, hvilken type allergi har du?

Luftveisallergi *(f.eks. pollen, dyr, midd)*	Matallergi	Annen *(f.eks. medisiner, kosmetikk, insekter)*
☐	☐	☐

Hvis annen, hva?

Har du noen annen kronisk sykdom? *f.eks. diabetes (sukkersyke) eller leddgikt*	☐	☐

Hvis ja, hvilke?

Opplysninger om barnets far:

Har barnets far noen av disse sykdommene/helseplagene?	**Ja**	**Nei**
Har barnets far fått diagnosen astma av lege?	☐	☐
Har barnets far fått diagnosen allergi av lege?	☐	☐

Hvis ja, hvilken type allergi har barnets far?

Luftveisallergi *(f.eks. pollen, dyr, midd)*	Matallergi	Annen *(f.eks. medisiner, kosmetikk, insekter)*
☐	☐	☐

Hvis annen, hva?

Har barnets far noen annen kronisk sykdom? *f.eks. diabetes (sukkersyke) eller leddgikt*	☐	☐

Hvis ja, hvilke?

Husholdningens brutto inntekt

Hva er husholdningens totale brutto inntekt per år (gjennomsnitt for de siste tre årene)?

< 300 000	300 000-700 000	700 001-1 100 000	>1 100 000
☐	☐	☐	☐

Kommentarer

Hva er barnets alder ved utfylling av dette skjemaet?

Antall måneder	

Hvis dere lurer på noe i forbindelse med oppfølgingen, kan dere ringe til prosjektkoordinator Berit Granum, tlf. **21 07 66 96**, stipendiat Solvor Berntsen Stølevik, tlf **21 07 62 57**, eller sende e-post til **bramat@fhi.no**.

Appendix D: 3-year follow-up questionnaire (English)

Information about the child:

Has the child had any of the following diseases/complaints after turning 2 years of age?	Yes	No	No. of times
Cold or other respiratory diseases *e.g. false croup, throat infection, influenza ,whooping cough, sinusitis*	☐	☐	
Otitis media	☐	☐	
Pneumonia	☐	☐	
Gastroenteritis with vomiting or diarrhoea	☐	☐	
Urinary tract infection	☐	☐	
Fever without other symptoms than feeling weak	☐	☐	

Asthma, allergy and other chronic diseases after turning 2 years of age	Yes	No
Has the child been diagnosed with asthma, asthma bronchitis, or bronchial hyperreactivity by a doctor?	☐	☐
If yes, how many episodes during the last year?		
If yes, how old was the child when diagnosed?		
Has the child had periods of more than 10 days with dry cough, chest tightness or wheezing, or shortness of breath?	☐	☐
If yes, how many episodes during the last year?		
Has the child had eczema or itching in the face or at joints? (e.g. groin, hollow of the knee, ankle, elbow, wrist)	☐	☐
Has the child been diagnosed with atopic eczema by a doctor?	☐	☐
If yes, how old was the child when diagnosed?		
Has the child been diagnosed with allergy by a doctor?	☐	☐
If yes, how old was the child when diagnosed?		
If yes, what is the child allergic to?		
Does the child have any other chronic diseases? *e.g. diabetes or arthritis*	☐	☐
If yes, which ones?		

Has the child had any childhood diseases after turning 2 years of age	Yes ☐	No ☐
If yes, which ones?		

Use of medication after turning 2 years of age	**Yes**	**No**
Does the child use or has used asthma medication?	☐	☐
Does the child use or has used allergy medication (not salves)?	☐	☐
In case of infection, has the child used antibiotics (penicillin or similar)? *e.g. Apocillin, Calcipen, Weifapenin, Aboticin, Ery-Max, Imacillin*	☐	☐
If yes, how many infections have been treated?		

If yes, what kind of infections has been treated with antibiotics?

Cold/other airway diseases	Otitis media	Pneumonia/bronchitis	Urinary tract infection
☐	☐	☐	☐

Others?

Has the child been given other medicines? *e.g. reducing fever or soothing pain*	**Yes** ☐	**No** ☐

If yes, give the name on the medicine

Vaccines after turning 2 years of age		
Has the child been given any vaccines?	**Yes** ☐	**No** ☐
If yes, what kind of vaccines?		
Did the child experience any side-effects upon vaccination?	**Yes** ☐	**No** ☐
If yes, describe:		

Information about the child's everyday life from birth till today	
How many months did you breast-feed your child?	

Where has the child stayed in the daytime (from birth)?

Child's age (months) From-till	At home	Childminder	Kindergarten (small group)	Kindergarten
	☐	☐	☐	☐
	☐	☐	☐	☐
	☐	☐	☐	☐
	☐	☐	☐	☐

How many older siblings/children live together with the child?	

Does the child stay in rooms together with somebody smoking?

No	Yes, sometimes	Yes, daily
☐	☐	☐

Information about the mother:

Do you have any of these diseases/complaints	**Yes**	**No**
Have you been diagnosed with asthma by a doctor?	☐	☐
Have you been diagnosed with allergy by a doctor?	☐	☐
If yes, what kind of allergy do you have?		
Respiratory allergy *(e.g. pollen, animals, mite)* ☐	Food allergy ☐	Other *(e.g. medicines, cosmetics, insects)* ☐
If others, describe:		
Do you have any other chronic diseases? *e.g. diabetes or arthritis*	☐	☐
If yes, describe:		

Information about the father:

Has the father any of these diseases/complaints	**Yes**	**No**
Has the father been diagnosed with asthma by a doctor?	☐	☐
Has the father been diagnosed with allergy by a doctor?	☐	☐
If yes, what kind of allergy do you have?		
Respiratory allergy *(e.g. pollen, animals, mite)* ☐	Food allergy ☐	Other *(e.g. medicines, cosmetics, insects)* ☐
If others, describe:		
Has the father any other chronic diseases? *e.g. diabetes or arthritis*	☐	☐
If yes, describe:		

Gross income of the household
What is the total gross income of the household per year (mean of three last years)?
< 300 000 ☐ 300 000-700 000 ☐ 700 001-1 100 000 ☐ >1 100 000 ☐

Comments

Fill in the child's age

No. of months	