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LIFESTYLE, DIETARY AND ENVIRONMENTAL EXPOSURES IN INFANCY AND THE DEVELOPMENT OF ALLERGIC SENSITIZATION

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Lifestyle, dietary and environmental exposures in infancy and the development of allergic sensitization

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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“Our bodies are as unknown to us as the ocean,
both familiar and strange; the sea inside ourselves.”

Philip Hoare

SUMMARY

Allergy related diseases have increased in the Western world, affecting nearly half of the children. Lifestyle, dietary and environmental changes are thought to be important for disease risk and disease development. The aim of the prospective ALADDIN (Assessment of Lifestyle and Allergic Disease During INfancy) cohort is to study how lifestyle and environmental factors during pregnancy and early childhood affect the development of allergic disease in children.

The aim of this thesis was to study lifestyle, environmental and dietary exposures during pregnancy and infancy in relation to the development of allergic sensitization in the ALADDIN birth cohort.

In **study I** we investigated if there are differences in concentrations of toxic and essential metals in maternal blood, placenta and cord blood between 40 mother-child pairs with and 40 without an anthroposophic lifestyle. Metal concentrations were analyzed using inductively coupled plasma mass spectrometry. We found higher concentrations of Cd, Pb and Co in samples from mother-child pairs with an anthroposophic lifestyle. None of the studied lifestyle factors explained the higher concentrations observed in this study.
In **study II** we investigated if the long chain fatty acid composition in breast milk was associated with allergic sensitization in the child at two years of age. 225 mother-child pairs were included in this study. We found an inverse association between the concentration of omega-3 fatty acids and child sensitization at two years of age. However, this association could not explain the lower prevalence of sensitization among children of anthroposophic families.
In **study III** we investigated if the incidence and prevalence of food, animal and pollen sensitization differed with lifestyle and age of the children. 100 children from anthroposophic, 209 from partly anthroposophic and 165 children from non-anthroposophic families were included. We found a lower incidence of food allergen sensitization among children of anthroposophic families. The lower prevalence of sensitization in children from anthroposophic families was largely explained by the lower incidence of food sensitization before one year of age.
In **study IV** we studied the development specific IgE to egg, milk and peanut from six months to five years of age in 372 children, with a particular interest in low levels of IgE. IgE concentrations were divided into non-sensitized (≤0.09 kU/L), low levels (0.1-0.34 kU/L) and sensitized (≥0.35 kU/L). At six months, 5% of the children had low IgE levels to egg, 14% to milk and 4% to peanut. Low levels to egg seemed to be more transient than low levels to milk. Early low levels to egg and milk seemed to decrease over time, but might increase the probability of sensitization to inhalant allergens.

In conclusion, this thesis together with previous publications from the ALADDIN cohort lead to better understanding of risk and protective factors during pregnancy and infancy for the development of allergic disease in children.

LIST OF PUBLICATIONS

I. **Fagerstedt S**, Kippler M, Scheynius A, Gutzeit C, Mie A, Alm J, Vahter M. Anthroposophic lifestyle influences the concentration of metals in placenta and cord blood. *Environmental Research*. 2014 Jan; 136:88-96.

II. Rosenlund H, **Fagerstedt S**, Alm J, Mie A. Breast milk fatty acids in relation to sensitization – the ALADDIN birth cohort. *Allergy*. 2016 Oct;71(10):1444-52.

III. **Fagerstedt S**, Hesla HM, Ekhager E, Rosenlund H, Mie A, Benson L, Scheynius A, Alm J. Anthroposophic lifestyle is associated with a lower incidence of food allergen sensitization in early childhood. *Journal of Allergy and Clinical Immunology*. 2016 Apr;137(4):1253-6 e1-3.

IV. **Fagerstedt Nilsson S,** Lilja G, Järnbert-Pettersson H, Alm J. Relevance of low specific IgE levels to egg, milk and peanut in infancy. *Manuscript submitted*.

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ALA	Alpha-linolenic acid
ALADDIN	Assessment of Lifestyle and Allergic Disease During INfancy
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Antigen presenting cell
As	Arsenic
Cd	Cadmium
CI	Confidence interval
Co	Cobalt
CLA	Conjugated linoleic acid
DGLA	Dihomo-gammalinolenic acid
DHA	Docosahexaenoic acid
DIT	Developmental immunotoxicity
DMT1	Divalent metal transporter 1
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
Fe	Iron
GEE	Generalized estimating equations
HPA-axis	Hypothalamus-pituitary-adrenal axis
Ig	Immunoglobulin
ICPMS	Inductively coupled plasma mass spectrometry
IL	Interleukin
LA	Linoleic acid
MCHCC	Maternal child health care centre
MHC	Major histocompatibility complex
OR	Odds ratio
Pb	Lead
PUFA	Polyunsaturated fatty acid
RA	Rumenic acid
RR	Risk ratio
VA	Vaccenic acid

1 INTRODUCTION

The focus, as well as the title of this thesis is lifestyle and environmental exposures in infancy in relation to allergic sensitization. The prevalence of allergic diseases has increased during the last decades, affecting nearly half of the children in Western countries[1]. The reason for disease development and the rapid increase in prevalence is still not entirely known. Genetic predisposition is an important risk factor but does not explain the rapid increase. Therefore, changes in lifestyle and exposure to environmental factors are thought to influence the risk for disease development[2].

The overall aim of the ALADDIN study is to increase our understanding of how the lifestyle of the parents and the child's environment are associated with the development of allergic disease.

I will limit the background to IgE sensitization and IgE mediated allergy. Regarding the exposures, I will only briefly discuss genetic and epigenetic factors behind allergy as none of my studies are in those fields of research. I go through how the known environmental risk and protective factors are believed to contribute to the development of the immune system in health and disease and, in some cases, preventive strategies to reduce the risk of allergic disease in childhood.

2 BACKGROUND

2.1 ALLERGY

Allergy is an unwanted immunological reaction directed towards a non-harmful environmental substance called an allergen[3,4]. Allergens are usually small, stable proteins present in low amounts, but some carbohydrates have been identified to cause allergic symptoms as well[3,5]. Allergy can be atopic, which involves the production of allergen specific immunoglobulin E (IgE) antibodies, or it can be non-atopic where no antibodies are produced but where there is a cellular or a tissue/epithelial reaction[3,4].

Genetic factors are of great importance in the development of allergic disease[6], where maternal atopy is believed to be a stronger risk factor for female offspring and vice versa[7]. Parental atopy has a dose response relationship to the risk of atopy in the offspring. Several studies also report gender related differences in allergy prevalence and that the female/male ratios may be age dependent[8,9]. Additionally, environmental exposures affect the transcription and translation of genes important for immune function[10].

2.1.1 Prevalence and clinical aspects

During the past half century, the prevalence of allergic diseases have increased and, for asthma and rhinitis, reached a plateau in some countries[1,11]. Food allergy though still seems to be increasing in prevalence[11-14]. There are great variations in prevalence of allergy related diseases, with higher prevalence in Western countries[1]. In Sweden, the population based birth cohort BAMSE reported that 15% of the children were sensitized to an inhalant allergen at four years of age and that proportion increased to 25% at eight years of age[15]. Food allergies affect nearly 8% of the children in Western countries[11] and 6% in Europe[13]. At four years of age, 17% of the children in the BAMSE cohort were sensitized to a food allergen[16] and food allergy in their early childhood (0-4 years) was reported for 6.8% of the children[17].

The clinical symptoms/atopic manifestations include food allergy, eczema, asthma and allergic rhinitis[4]. Food allergy is most prevalent in early childhood and commonly affects the oral mucosa, skin and the gastrointestinal tract[18]. Rapidly occurring symptoms include itching of mouth, gastrointestinal symptoms, urticaria, angioedema, airway symptoms and depending on the severity of the allergy, anaphylaxis. As many plant proteins causing allergies have structural similarities, pollen allergic individuals may experience cross reactivity to other similar allergens in food[19].

To understand who is at risk of developing allergic disease there is a need for diagnostic tools and parameters that can be followed over time. Skin prick-test, serum IgE and assessing asthma by spirometry are widely used in the clinic to diagnose and evaluate disease development[18,20]. These methods, accompanied by more experimental techniques, are also used in research settings.

2.1.2 Sensitization

General references concerning sensitization development are Abbas *et al*. 2014[21] and Holgate *et al*. 2011[22].

The immune system is divided into innate and adaptive immunity, and both play important roles in health maintenance and disease development. The innate immunity acts as a chemical and physical barrier to infectious agents. Important functions of the innate immune cells are antigen presentation and activation of the adaptive immune system. The adaptive immunity directs a specific response to the identified threat.

All cellular responses involving the production of antibodies, including allergic sensitization, start similarly (Figure 1). Both endogenous and exogenous substances are constantly taken up by antigen presenting cells (APC) and presented to immune cells to identify potential threats. Allergens can enter through the skin, airway epithelia, digestive tract/intestinal epithelia and other mucous tissues. In the tissue the allergen is taken up by APCs, mainly by macrophages and dendritic cells (Figure 1, A). The APCs serve as a bridge between the innate and adaptive immune response. The APCs migrate to lymphoid tissue where they present the processed allergen to naïve T-cells on their major histocompatibility complex (MHC) II (Figure 1, B). The naïve T-cells, that through the T-cell selection have become specific for that particular antigen, recognize the antigen on their T-cell receptor (Fc-receptor) and can be activated. The activation of T-cells also requires cytokine signalling. Interleukin 4 (IL-4) is produced by the APC.

The activated T-cell, now a Th-2 cell, can in turn activate antigen specific B-cells to mount a humoral response, i.e. the production of allergen specific IgE antibodies (Figure 1, C). Several different antibodies can be produced to an allergen. Although an allergen is a small protein or peptide, it provides several possible epitopes for antibody binding. It is not known what regulates the "choice" of epitope. Some individuals produce only one type of antibody to an allergen while others produce several different antibodies. In allergic individuals, knowledge of the specific antibodies produced by a patient is of importance in guiding the clinician to the correct therapy for the patient.

In the first phase of sensitization, antibodies released to the surroundings are bound by Fc-receptors on mast cells and basophiles (Figure 1, D). Antibodies also cover the cell membranes of the B-cells. The activated B- and T-cells circulate in the body and eventually encounter the same allergen.
This initiates the second step in the sensitization process. The allergen will bind to the IgE antibodies on the mast cells and cross-link the Fc-receptors (Figure 1, E). This will activate the degranulation of the mast cells releasing histamine and pro-inflammatory mediators in the surrounding tissue (Figure 1, F).

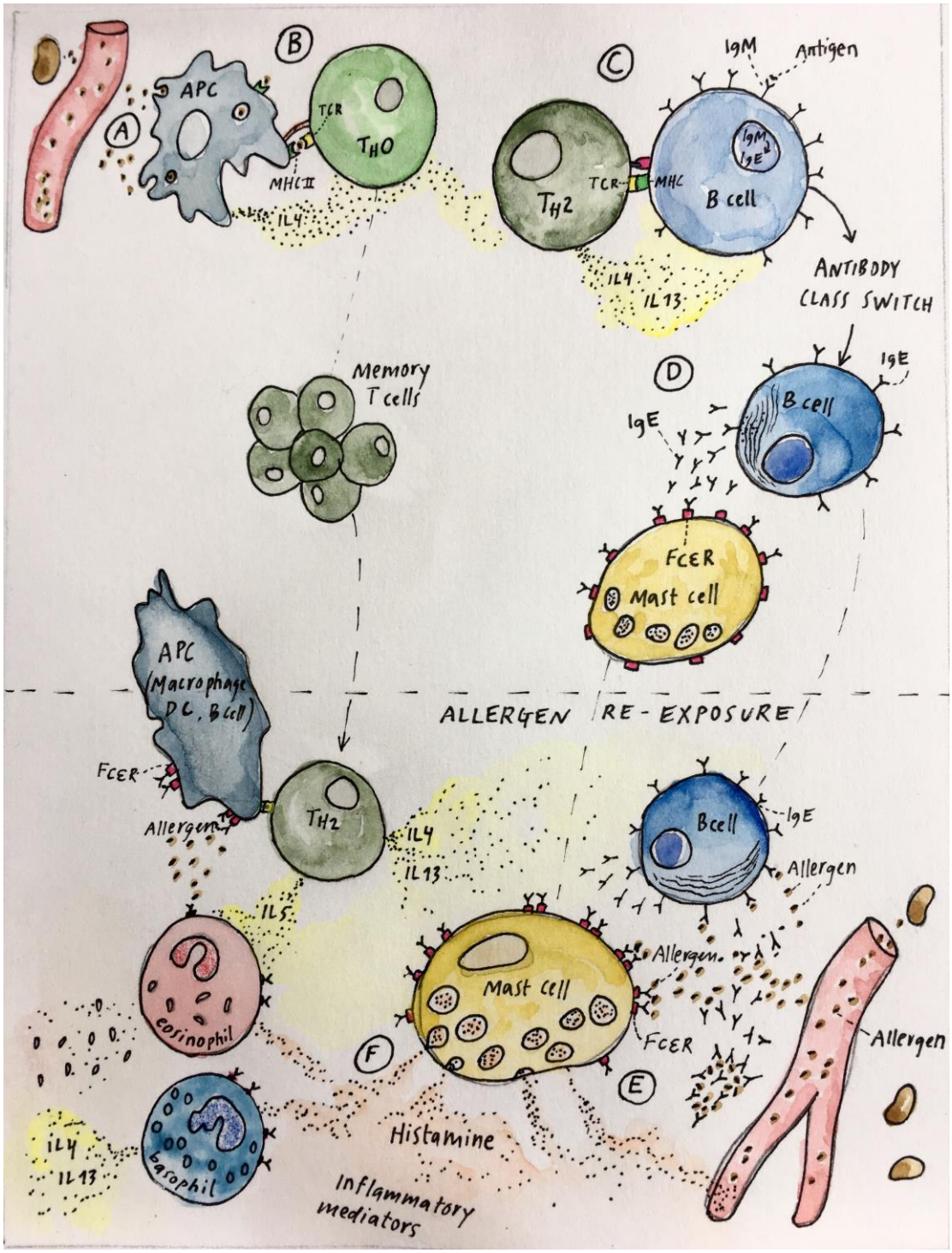

Figure 1 Schematic illustration of the cellular type I hypersensitivity reaction. In the upper half, the allergen is encountered for the first time and re-encountered in the lower half of the figure. Figure by Sara Fagerstedt Nilsson.

Histamine causes vasodilation, itching and mucous secretion. In cases of severe allergic reactions, mast cell and basophil degranulation causes anaphylaxis, where airways can become obstructed by tissue swelling and smooth muscle contraction, and blood pressure can drop resulting in unconsciousness[23]. Untreated anaphylaxis is a life-threatening condition.

Activated B-cells can become antibody producing plasma cells. They can also form memory B-cells that last in the body for years.

2.1.2.1 Natural course of sensitization and the "atopic march"

Production of IgE antibodies precedes allergic symptoms. The likelihood of allergic symptoms increases with increasing allergen specific IgE levels although no clear dose response relationship exists[24,25]. Several large cohort studies have gathered knowledge about the natural course of sensitization. On a population level, sensitization often begins with IgE to milk and/or egg in early infancy and later followed or replaced by IgE to indoor and outdoor inhalant allergens[26,27]. A subject of discussion throughout the years has been the "atopic march". Accordingly, allergic disease commonly starts with IgE sensitization to common food allergens followed by atopic manifestations like eczema and later rhinitis and asthma[27,28]. This event chain is seen on a population level but it is unclear if the atopic march exists on an individual level. Should these manifestations be regarded as separate events or a causal chain of events? What is clear though is that having a sensitization or eczema are risk factors for food allergy, and that food allergy is associated with the development of rhinoconjunctivitis and asthma, so to some extent, an association exists[29-32]. Especially sensitization to hen's egg in early infancy seems to predict later sensitization to aeroallergens[33].

Monosensitization to food allergens is rare. Analogous to the atopic march, the "food allergic march" describes the sensitization process to food allergens[29]. Commonly the age of onset of food allergy to egg, milk and peanut is during the first two years of life, followed by tree nuts, seeds, fish, seafood and fruits later in childhood and adolecense[29,34].

Some children outgrow their sensitization and allergy. For egg and milk, tolerance is usually achieved around school start[35-37]. Sensitization to peanut is of a more persistent character, although it has been demonstrated that also peanut allergy can be outgrown in around 20% of peanut allergic children[38].

2.1.3 Pre- and postnatal immune development

During the prenatal period and for some time thereafter, the immune system is plastic and highly adaptive. Environmental exposure during this time-window may affect organ development[10,30]. Maternal and foetal immune systems are in close contact and maternal tolerance to paternal alloantigens is mediated through regulatory T-cells[39].

Development of the immune system starts early in foetal life. Early blood cell progenitors can be found in the circulation in gestational week 4 and the adaptive immune system occurs already between gestational week 15 and 20[40]. Membrane bound IgM is expressed on liver B-cells at week 10 and at week 12, B-cells can be found in the circulation[40]. Maternal antibodies may cross the placenta and can be found in the foetal circulation. At birth, the IgM, IgA and IgE levels are low and most IgG is of maternal origin. Nevertheless, foetal IgG can be found in the spleen at gestational week 10 and IgE can be detected in foetal liver and lungs at week 11[40]. The neonatal immune cells are, however, rather immature and the immune response is limited[40]. Interestingly, the skin and gastrointestinal tract are more immunologically mature than the airway epithelia, perhaps explaining the earlier occurrence of atopic manifestations in these organs[40].

After birth and during the first years, the immune system develops towards an adult immune system with establishment of immune competence and formation of the immunological memory. As the immune system is highly dynamic, it continues to develop and adapt throughout life.

2.2 ENVIRONMENT – RISK AND PROTECTION

2.2.1 The hygiene hypothesis

During the last century, the lifestyle in Westernized countries have gone through major changes including urbanization, decreased family size and decreased animal contact resulting in a change in microbial exposure compared to a rural environment. During this same period, the prevalence of asthma and allergic rhinitis increased too rapidly to be of an evolutionary origin. In year 1989, epidemiologist David Strachan stated that the risk of hay fever was related to family size where the risk of hay fever was reduced in children having older siblings as they would increase the exposure of the youngest child to both infectious and non-infectious microorganisms[41]. This "hygiene hypothesis" has since been revised to "the microbial hypothesis" as more knowledge through additional cohorts and epidemiological studies has been gathered[42,43].

2.2.2 Developmental origin of health and disease

The development and function of a healthy immune system depends largely on interactions with the surrounding environment. Industrialization and urbanization have caused major changes in our environmental exposures and thus, a child born today is exposed to a vast array of chemical compounds that did not exist a hundred years ago. Developmental immunotoxicity (DIT) is a term used to describe the effect of various environmental toxicants on the vulnerable pre- and postnatal immune system development[44]. DIT is not only thought to influence development of allergic diseases. A diverse range of other disorders with low-grade chronic inflammation are also thought to have a developmental origin[45].

Harmful environmental exposures are thought to facilitate postnatal maintenance of the Th2 polarization seen during pregnancy, altering the pre- and postnatal maturation of innate immune cells and maintaining low grade inflammatory processes[46]. DIT is induced by exposure to biological materials, drugs, chemicals, medical devices, physical factors (like radiation) etc., extensively reviewed by Dietert and Zelikoff[47]. Toxicity testing for developmental effects have shown that extrapolation from adult data on dose sensitivity contains a large uncertainty[44]. In addition, toxic exposure in early life might cause more persistent adverse effects compared to adult exposure[44].

2.2.3 Exposure factors

A complex interplay between the innate and adaptive immune system and environmental exposure shapes the maturation of the immune system. Several exposure factors have been, and are studied, in relation to allergic disease, often

closely related to the *hygiene/microbial hypothesis* and developmental immunotoxicity but also to genetic/epigenetic modifications (Figure 2).

Figure 2 Exposure factors in the early life with possible associations to allergic disease development. Figure by Sara Fagerstedt Nilsson.

2.2.3.1 Toxic compounds

During the same time frame that the allergic diseases have increased in prevalence, the number of chemical compounds in our environment have dramatically increased (Information from Swedish Chemicals Agency, European Chemicals Agency and REACH). Persistent organic pollutants have repeatedly been detected in maternal blood and cord blood from newborns[48]. In addition to toxic organic compounds, toxic metals are also found in the placenta and cord blood. Some metals, like cadmium, accumulates in the placenta which might affect the transport of essential micronutrients to the foetus [49]. Cadmium might also alter hormonal regulation and cortisol by suppressing the transcription of the cortisol converting enzyme 11β-hydroxysteroid dehydrogenase type 2 in placenta syncytiotrophoblast cells[50]. Other metals, like lead and arsenic, pass through the placenta[51]. Lead exposure has been shown to shift the immune balance to Th2 dependent responses[52]. In experimental animal models, lead has been shown to induce adverse immunotoxic effects in the offspring depending on the gestational age at exposure[53].

Prenatal arsenic exposure may induce oxidative stress and inflammatory processes in the placenta[54]. Arsenic also seems to reduce the number of placental T-cells and alter cord blood cytokines[54]. Pre- and early postnatal arsenic exposure has been shown to induce oxidative stress and inflammation, causing damage in the developing lung tissue[55]. Arsenic exposure has in cohort settings been associated with a reduced lung function with gender related differences[55].

Outdoor air pollution, as exposure to traffic related air pollution, particulate matter, ozone, nitrogen dioxide and sulphur dioxide have been associated with asthma in children and adults[56]. The effects are thought to be mediated through increased oxidative stress in the airway epithelium.

Smoking during pregnancy has in several studies showed a clear association to wheeze and asthma[57,58]. Adding important information to this risk factor is a study where the methylation effects of *in utero* exposure to smoking was dependent on smoking at a certain stage of pregnancy[59]. This knowledge reinforces/emphasise the importance of toxicity testing during different stages of gestation.

2.2.3.2 Microbial exposure

The maturing immune system learns to distinguish between self and non-self, as well as harmful and innocuous molecules and compounds. As all surfaces of the human body that are in contact with the external world are inhabited by microbes, the "diplomatic" relations to these inhabitants are extremely important for the wellbeing of the host[60]. The microbiome shows geographical differences and comparing the maps of allergy prevalence worldwide and microbiome in different countries, one can easily understand why microbiota is of such interest to allergy researchers[1,61]. It has become increasingly clear during the last years that the microbial exposures ought to take place during a certain developmental phase or time window, although the timing is currently unknown[60]. It is thought that that the microbial diversity is more important than exposure to some certain bacterial strains or viruses[62,63].

It was previously believed that the intrauterine milieu was sterile, but recent research has showed that the microbial exposure may begin already *in utero*[64]. More established knowledge though, is the importance of a vaginal delivery and the transfer of maternal microbial flora to the newborn[65,66].
Studies have demonstrated differences in gut microbiota comparing healthy and allergic children[67,68]. In a study by Kalliomäki *et al*, the differences were observed prior to the occurrence of symptoms[67]. The results of prospective studies indicate that the time window for healthy gut colonization is in the early life[69-71]. This early programming of the immune system by exposure to microbes, perhaps occurring already *in utero*, may be important for tolerance development in infancy[62].

The rural farm environment has been shown to hold allergy protective features, and has thus been studied extensively[72,73]. A comprehensive review by von Mutius and Vercelli contain a table on studies investigating the effect of childhood farm exposure on allergic disease[73]. These studies have shown that contact with livestock, animal feed and consumption of unprocessed farm milk reduce the risk of allergic disease in childhood[73,74]. Interestingly, the farm effect was independent of several other

traditional risk factors as day care, family size, breastfeeding and heredity of allergies. Both pre- and postnatal farm environment exposure seems to have allergy protective effects. Maternal exposure to barn/stable environment and consumption of unprocessed cow's milk during pregnancy has been shown to induce immunomodulatory, allergy protective effects[75-77]. Child consumption of unprocessed cow's milk has also been shown to be allergy protective[78,79]. It was previously demonstrated in the ALADDIN study that living on a farm and parental sensitization modulate the gene expression in the placenta[80].

Figure by Sara Fagerstedt Nilsson.

Mechanistic evidence for farm related allergy protection are scarce, but in a recent article Schuijs *et al*. demonstrated in mice how continuous low dose bacterial endotoxin exposure inhibited dendritic cell activation and therefore supressed Th2 immunity[81]. The group found that a variant of a gene encoding an enzyme in the lung epithelia mediated this effect. They investigated blood samples from the GABRIELA study and found that the farm children also had the genetic variant that mediated the protective effect[81].

Effects mediated by exposure to bacterial endotoxin was also thought to explain the differences in atopy seen between Amish and Hutterite children[82]. Both Amish and Hutterite people originate from European farmers that during the Protestant Reformation migrated to the United States, where they have lived isolated and strongly preserved their traditions. Both groups have similar genetic background and lifestyle but the prevalence of sensitization and asthma was significantly lower in the Amish children. A difference was found in the dust samples where those from the Amish homes contained almost seven times the amount of endotoxin found in the Hutterite homes[82].

Antibiotics negatively effects gut microbiota and has thus been suggested to have a role in allergy development. Prenatal maternal antibiotics may be associated with an increased risk of asthma and wheeze in the child[83]. Several prospective studies have found associations between antibiotics and antipyretics and asthma/wheezing, but it cannot be excluded that it is due to reverse causation[84-86]. Children with respiratory symptoms have an increased risk of having prescription antibiotics/antipyretics. Antibiotic use in infancy shows no association with eczema or sensitization[84].

2.2.3.3 Pathogenic viral exposure

The immune system continues to learn, develop and adapt to the environment. Early contact with pathogens strongly shapes the developing immune system. Viral respiratory infections have been extensively studied as risk factors for asthma[87]. Bronchiolitis in the first year of life has been associated with increased asthma risk[88]. Respiratory syncytial virus and human rhinovirus are the most common viruses to cause bronchiolitis in infants[89]. Additionally, rhinovirus infection was demonstrated to be associated with an increased sensitization to aeroallergens[90].

Although several studies have shown positive or negative associations between certain microbial and viral exposure, it is currently not known what type of infections are important in programming health and disease and if there are specific time windows for that exposure[87].

2.2.3.4 Exposure to allergens

Not only microbes influence the developing immune system. Potential allergenic compounds are inhaled, ingested and taken up through skin or mucosal tissue. Exposure through the skin rather than oral exposure has been shown to increase the risk of food allergy and sensitization[91].

Maternal avoidance diets have failed to limit the increase of food allergy and a repeated Cochrane review concluded that avoidance diet in high-risk women during pregnancy was unlikely to reduce the risk of atopic disease in the child[92]. Recent randomized trials are indeed suggesting the opposite[93]. Increased food diversity in the first year of life have been inversely associated with asthma, food allergy and food sensitization[94]. Delayed introduction of cow's milk and other foods was shown to be associated with an increased risk of atopic manifestations in the first two years of life in the KOALA study[95]. Additionally, a Finnish study showed that reduced food diversity during the first year of life was associated with a higher risk of allergy and asthma in childhood[96]. Early introduction of allergenic foods in the diet has been a success in the case of peanut and the LEAP study on high risk children[97] but also raising doubts as results were less convincing for other foods than peanut in a non-selected population[98]. However, the study reported a lower prevalence of egg and peanut allergy in the per-protocol group[98]. Notable is that the National Institute of Allergy and Infectious Diseases (NIAID) in the US have changed the guidelines regarding peanut based on a single trial (LEAP study).

Although not the focus of this thesis, it should be added that indoor and outdoor allergen exposure is also of importance. Modern homes are far different from the dwellings of our ancestors. Additionally, a significant proportion of the time is spent indoors and therefore the immune system is exposed to indoor inhalant allergens from house dust mites, cockroaches and pets[99]. The timing, duration and amount of exposure seems to be of importance[99,100]. Interestingly there seem to be a dose dependent association in the exposure to allergens demonstrated in Figure 3[101-104].

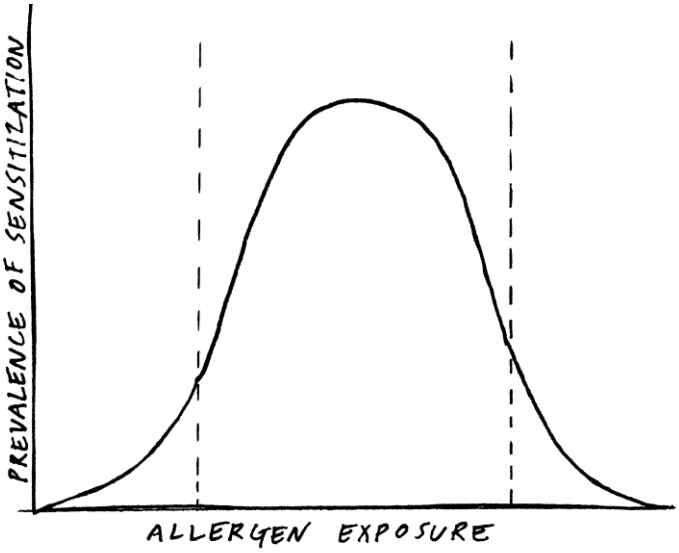

Figure 3 A non-linear dose dependent relationship between prevalence of sensitization and exposure level to allergens.

2.2.3.5 Breast feeding and breast milk

Exclusive breastfeeding for the first six months of life and continued complementary breastfeeding thereafter has been recommended by the World Health Organization, since breastfeeding reduces morbidity and mortality in infancy especially where clean drinking water is a scarce commodity. Breastfeeding for at least six months has been a standard guideline in many countries including Sweden.

Human milk contains important nutritional and bioactive compounds[105]. The milk that is produced during the first few days *post partum* is the colostrum. It contains secretory IgA, leukocytes, lactoferrin, developmental factors like growth factors and higher levels of sodium, chloride and magnesium than mature milk[105]. After a few weeks *post partum*, the milk is considered fully mature[105]. The mature milk contains approximately 0.9-1.2 g protein, 3.2-3.6 g fat and 6.7-7.8 g lactose per 100 ml[105]. The mature milk also contains a variety of immunomodulatory factors thought to influence the development of oral tolerance to food[106].

It was previously demonstrated in the ALADDIN cohort that the phenotype of breast milk exosomes varied with lifestyle and maternal sensitization[107]. Exosomes are small

membrane vesicles budding from the endosomal compartment of various cells and used in cell-to-cell communication[108].

2.2.3.6 Fatty acids in breast milk

The fatty acid (FA) profile in breast milk varies in relation to maternal diet. In many parts of the world, the ratio of omega-3 to omega-6 long chain polyunsaturated fatty acids (LC-PUFA) has shifted towards omega-6 FAs due to the decreasing levels of omega-3 FAs in the diet[109]. This is the basis of the lipid hypothesis by Hodge *et al*.[110] and further developed by Black and Sharpe[109] stating that the low intake of omega-3 PUFA could shift the inflammatory balance towards a pro-inflammatory state, leading to allergic disease. Both omega-3 and omega-6 (Figure 4) are precursor molecules for inflammatory mediators and therefore their availability could regulate the inflammatory status.

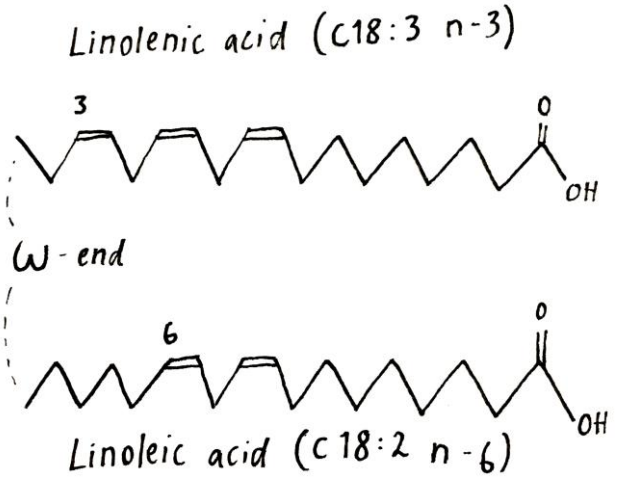

Figure 4 Example of an omega-3 FA (linolenic acid) and an omega-6 FA (linoleic acid).

The association between breastfeeding and allergic disease has been evaluated in several studies with somewhat contradictory conclusions. Prolonged breastfeeding has been associated with an increased risk of atopic eczema[111,112]. On the other hand, it has also been demonstrated that exclusive breastfeeding for at least three months reduced the risk of atopic dermatitis in high-risk infants[113]. The same study saw no such association in children without atopic parents. The association between breastfeeding and asthma has been evaluated in several meta-analyses concluding that breastfeeding protects from childhood asthma[114]. The study that reported an increased risk of eczema also reported that breastfeeding reduced the risk of wheezy disorders in high-risk infants[112]. This Danish study saw no associations between FA composition and their outcomes.

The FA composition in breast milk in relation to allergic disease has been evaluated in several large studies[115-119]. Two recent Cochrane reviews concluded that neither pre- and postnatal supplementation[120] nor supplementation in infancy[121] of omega-3 PUFA reduced allergy, asthma, eczema or food allergy in children and that the quality of

evidence was low[121]. Studies on high-risk infants support their findings and report that higher omega-3 in colostrum was associated with increased risk of allergic disease[122,123]. On the contrary, a report recently published by Bisgaard *et al*, (the COPSAC cohort) from a randomized trial on omega-3 supplementation found that omega-3 supplementation in the third trimester reduced the risk of persistent wheeze or asthma and lower respiratory tract infections[124]. The allergy protective effect of omega-3 and/or fatty fish consumption is supported by several Nordic studies[125-128]. Interestingly, a recent publication from the PASTURE study reported that the higher omega-3 content might contribute to the allergy protective effect of unprocessed cow's milk (farm milk)[129].

In addition to omega-3 PUFAs, the breast milk also contains other molecules with suggested immunomodulatory effects. A study on mice found that breast milk soluble IgA had long lasting positive effects on the gut microbiome[130]. Also in humans, where the breast milk previously was considered sterile, research has now revealed that both colostrum and mature milk contain numerous bacterial species with possible probiotic features (for a list, see Table 1 in Fernández *et al*)[131]. Apart from the breast milk bacterial contribution to a healthy microbiota in the infant gut, the bacteria have been shown to reduce infections and influence the maturation of the immune system[131]. Although not entirely clear, the origins of the breast milk bacteria are thought to be the infant oral microflora, the breast skin microbiota and the maternal gut microflora[131]. In addition to IgE and bacteria, breast milk also contains a large variety of non-digestible prebiotic oligosaccharides. The oligosaccharides promote a healthy microbial environment by "cleaning" the gut from pathogenic bacteria and also promote colonization of bifidobacteria and lactobacilli[60].

2.2.3.7 Dietary exposure

Diet is one of the most important environmental exposure factors. As mentioned earlier, maternal food allergen avoidance diets have proven inefficient in preventing atopy and instead, earlier introduction of foods is suggested to induce tolerance[92,132,133]. Maternal diet before and during pregnancy is thought to influence the health and disease risk in the offspring through epigenetic mechanisms[134]. Probiotic bacteria, prebiotic oligosaccharides, PUFAs, antioxidants and vitamin D have all been suggested to influence immune development and function[132]. Dietary changes in the Western world have led to a decreased intake of fatty fish, seeds, nuts, fibres, fruits and vegetables containing much of the aforementioned components[132].

Fruits and vegetables are important sources of antioxidants like vitamin E and C, selenium, zinc and β-carotene[132]. There is some evidence of associations between lower intake of antioxidant rich foods and allergic disease but at the moment, recommendations of a balanced diet has better evidence than supplementation with specific vitamins or minerals[132].

The National Food Agency in Sweden recommends eating fish 2-3 times per week although it is important, especially for pregnant women and children, to avoid fish and seafood from certain "polluted" waters. Fish is also a good source of vitamin D. In addition to the role of vitamin D in calcium absorption and maintaining bone health, vitamin D has important functions in the immune system[135]. Several studies

have investigated the association between serum vitamin D levels and allergic disease[135]. Low serum levels have repeatedly been measured in atopic individuals[135]. Many studies have reported inadequate levels in relation to national recommendations, especially in wintertime in certain geographical regions when sunlight is scarce. Interestingly, a Finnish study reported a non-linear relationship between IgE sensitization and vitamin D[136]. Studies on asthma and vitamin D show inconclusive results[137,138]. More studies are needed to strengthen the evidence if vitamin D has a role in allergic disease[135]. Perhaps prospective studies starting with maternal vitamin D status during pregnancy could shed more light on this issue.

Food can also be an exposure source for environmental toxicants such as pesticides and persistent organic pollutants[139]. In our cohort study, many of the anthroposophic and partly anthroposophic families choose organically or biodynamically produced food. Organically produced food contains significantly lower levels of pesticides compared to conventionally produced food[140,141]. Consumers of organic food also have less pesticide metabolites in their urine[142]. The association between consumption of organic food and atopic disease has been investigated in the KOALA birth cohort[143]. Kummeling *et al.* reported that consumption of organic dairy products was associated with a lower eczema risk in children up to 2 years. No associations were found for total organic diet or other specific organic foods.

The role of microbiota for maintaining a healthy immune system has been investigated in several studies, both in observational studies and in trials[132]. Probiotics have been suggested as primary prevention strategy for allergic disease but currently there is not enough evidence proving such therapy to be effective and have lasting effects[132]. Fermented vegetables contain live bacteria and/or yeast[144]. The process of fermentation occurs naturally by microorganisms in the food or by adding bacteria/yeast culture[144]. The microorganisms use sugars in the food for energy, producing other compounds like lactic acid instead[144]. Possible health benefits from consumption of fermented foods have been studied with the most positive outcomes from the consumption of fermented dairy products[144].

2.3 ANTHROPOSOPHY

Anthroposophy is a holistic view of life that was founded in the early 20th century by Rudolf Steiner. Most aspects of life are encompassed by anthroposophy including medicine, healthcare, childbirth, parenting, education, art, architecture, agricultural methods and food. The general idea behind anthroposophic medicine can be described by the Latin proverb *"medicus curat, natura sanat"*, translated as "the physician cares for the patient but nature heals". The anthroposophic medicine therefore has a restrictive use of "modern" medicine such as antibiotics, antipyretics and vaccinations, instead using homeopathic/nature medicine[145]. The aim is to treat the cause and the patient holistically, not only the symptom (e.g. fever). Integrated in the anthroposophic medicine is also the therapy through art including painting, music and eurythmy.

Vaccinations are sometimes refused by parents who believe that infectious diseases are beneficial for the development of the immune system [146]. Especially the measles, mumps and rubella (MMR) vaccine is avoided with the explanation that these diseases

are less serious than those in the diphtheria-tetanus-pertussis (DTP) vaccination[146]. However, studies demonstrate that there is no evidence of protective effects from MMR diseases on atopy, or that vaccinations would cause atopic diseases[146,147]. There are even studies showing that MMR vaccination may have a protective effect against allergy and asthma in childhood[148].

Biodynamic or organic farming/cultivation are the preferred agricultural methods. A peculiar characteristic of dietary preferences is the use of fermentation to preserve vegetables. Fermented vegetables are also consumed by the children.

The Swedish anthroposophic community has its centre in Järna, south of Stockholm. Sweden's only anthroposophic hospital, Vidarkliniken, is also located in Järna.

Figure by Sara Fagerstedt Nilsson

2.3.1 The ALADDIN cohort

The hygiene hypothesis inspired the search for allergy protective factors within the anthroposophic population. It started with a cross-sectional study comparing children from Steiner schools with children from public schools in the same area regarding allergy outcomes (asthma, allergic rhinoconjunctivitis, atopic dermatitis, food allergy, allergic urticaria, skin prick-test and IgE sensitization)[149]. The study revealed that the anthroposophic lifestyle was associated with a lower prevalence of both clinical symptoms of allergy, allergic sensitization and positive skin prick-test[149]. However, the study design could not point out any specific lifestyle exposure factors responsible for the observed differences.

A larger cross-sectional European study, PARSIFAL, was conducted to find what aspects of the anthroposophic lifestyle contributed to the observed allergy protection. In this study of 4606 Steiner school children and 2024 reference children, the authors could confirm the results of the previous study by Alm *et al*. 1999 adding that the restrictive use of antibiotics and antipyretics was associated with the reduced risk of allergic disease in the children[150].

Later, in 2006, results from the PARSIFAL study concluded that the most pronounced allergy protection was associated with growing up on a farm. The lower prevalence of allergic disease and sensitization in the anthroposophic population was less clear in this study and there were geographical variations[151].

With the results from the cross-sectional studies in mind, the prospective birth cohort **A**ssessment of **L**ifestyle and **A**llergic **D**isease **D**uring **IN**fancy (ALADDIN) study was founded in 2004 recruiting 330 pregnant women in their third trimester and later additionally 222 families who were included at two months *post partum*. The cohort is described in detail in the materials and methods section.

2.3.1.1 Previous results of the ALADDIN study

The anthroposophic lifestyle aims to reduce negative stress for the infant. This could be confirmed in the ALADDIN cohort where the infants from anthroposophic families had significantly lower levels of salivary cortisol[152]. The parents had similar levels across lifestyle groups, indicating that the lifestyle related stress reduction is more relevant for the children. Additionally, Stenius *et al*, showed that salivary cortisol was positively associated with allergic sensitization and allergic symptoms at two years of life, although there was no lifestyle related effect on this association[153]. Altered regulation of the hypothalamus-pituitary-adrenal (HPA) axis could influence the development of allergic disease[154-156]. Altered levels of stress hormone is expected in many diseases and it is possible that the association between stress and allergic disease is bi-directional[157].

The findings of the two cross-sectional studies[149,150] were confirmed also in prospective cohort settings, establishing that the anthroposophic lifestyle is associated with a reduced risk of allergic sensitization in infancy[158]. Additionally, Marell Hesla *et al*. showed that the anthroposophic and partly anthroposophic lifestyles were associated with a reduced risk of parent reported food hypersensitivity and recurrent wheeze up to two years of age[159]. Interestingly, this study found that delayed body wash of the newborn was associated with a reduced risk of sensitization[159], possibly associated with the differences seen in vernix proteins in children with atopic eczema and differences in lipid composition related to lifestyle differences[160,161].

Several characteristics of the anthroposophic lifestyle, like homebirth, stress reducing environment, restrictive use of antibiotics, prolonged breast feeding and consumption of fermented vegetables may influence the gut microbiota. Gut microbiota was analysed in stool samples from 128 mother-child pairs (child samples at 6 days, 3 weeks, 2 and 6 months *post partum*). Marell Hesla *et al*. found that caesarean section and breastfeeding, but not lifestyle, had a significant impact on gut microbiota[162].

3 PROBLEM FORMULATION

During the last decades, there has been a notable increase in prevalence of immune related diseases. That increase cannot only be due to genetic adaptation/evolution. Therefore, answers are sought in changes in our way of life during the same time-period. Urbanization, intensive agriculture and the vast number of chemical compounds in our daily life and environment have caused major changes in what we are exposed to. Of particular concern is harmful exposure during the sensitive prenatal period that may have unfavourable developmental effects. Priming of the immune system towards an inflammatory phenotype starts early in life. Therefore, we are investigating if and how exposures during pregnancy and infancy can affect development of allergic sensitization. Regarding this thesis, the following research questions have been raised.

I. Immunomodulatory effects of several toxic metals have been studied both *in vivo* and *in vitro*. Metals are taken up by plants and animals and thus food is a major source of exposure. Children from families with an anthroposophic lifestyle have a lower risk for sensitization. Our hypothesis was that the anthroposophic population have a lower level of exposure to immunomodulatory environmental pollutants, e.g. toxic metals, and that this could partly explain the sensitization differences.

II. Changes in both eating habits and dietary preferences over the last decades have resulted in a shift in omega-3 to omega-6 ratio. According to the lipid hypothesis this might be one possible contribution to the increase in immune related diseases as allergy and asthma. Therefore, we wanted to investigate if the lower prevalence of allergic sensitization seen in children in families with an anthroposophic lifestyle could be explained by fatty acid composition in breast milk.

III. Sensitization to food allergens is still increasing and is the most prevalent form of allergy in young children. In addition, according to the atopic march and seen on a population level, sensitization to food allergens may be followed by sensitization to other allergens and/or developed into allergic disease later in life. The cause for this developmental pattern is not known and few studies have prospective sampling from infancy. Therefore, we wanted to study if the association between lifestyle and sensitization to food, animal and pollen allergens is dependent on the age of the child.

IV. In Study III we found that an anthroposophic lifestyle is associated with a lower incidence of food sensitization in infancy and we wanted to investigate early food sensitization further to increase our knowledge in the area. In study IV we followed the development of specific IgE to egg, milk and peanut from six months until five years. Our focus was on low levels (0.1-0.34 kU/L) of specific IgE as concentrations below 0.35 kU/L are less investigated and not well understood.

4 AIMS

The aim of this thesis was to study the associations between lifestyle, environmental and dietary exposures during pregnancy and infancy and development of allergic sensitization in the ALADDIN study.

The specific aims of this thesis were:

I. To elucidate if mothers with an anthroposophic lifestyle have a lower exposure to potentially immunomodulatory toxic metals (i.e. foetal exposure to toxic metals and essential metals), **Article I**.

II. To measure the long chain fatty acid composition in breast milk samples and to assess relationship between fatty acid concentrations (particularly omega-3, omega-6 and ruminant fatty acids) and child sensitization up to 24 months of age, **Article II**.

III. To investigate if sensitization to food, animal and/or pollen allergens differed with the child's age and parental lifestyle, **Article III**.

IV. To follow different levels of specific IgE concentrations to egg, milk and peanut from six months to five years, **Article IV** (in manuscript).

5 MATERIAL AND METHODS

5.1 THE ALADDIN COHORT

All four studies are based on the prospective birth cohort study ALADDIN.

5.2 STUDY DESIGN

Between September 2004 and March 2011, a total of 552 families were recruited from anthroposophic Maternal-Child Health Care Centres (MCHCC's) (n=312) in Järna (Vidarkliniken and Kirstens Familjehälsa) and Stockholm (Vidarkliniken at Hälsans Hus and Rosenlund hospital) and from conventional MCHCC's (n=240) in Järna (Järna Vårdcentral) and Södertälje (Oxbackskliniken). In the second trimester, their midwife asked the parents if they wanted information about the ALADDIN study.

The initial 330 families (ALADDIN Original) were enrolled in the study at gestational week 28-32. Inclusion criteria were no severe illness before or during pregnancy and child born ≥ gestational week 35. Additionally 222 families (ALADDIN Plus) were included at two months *post partum*. An overview of the data and sample collection from the participants is provided in Figure 5.

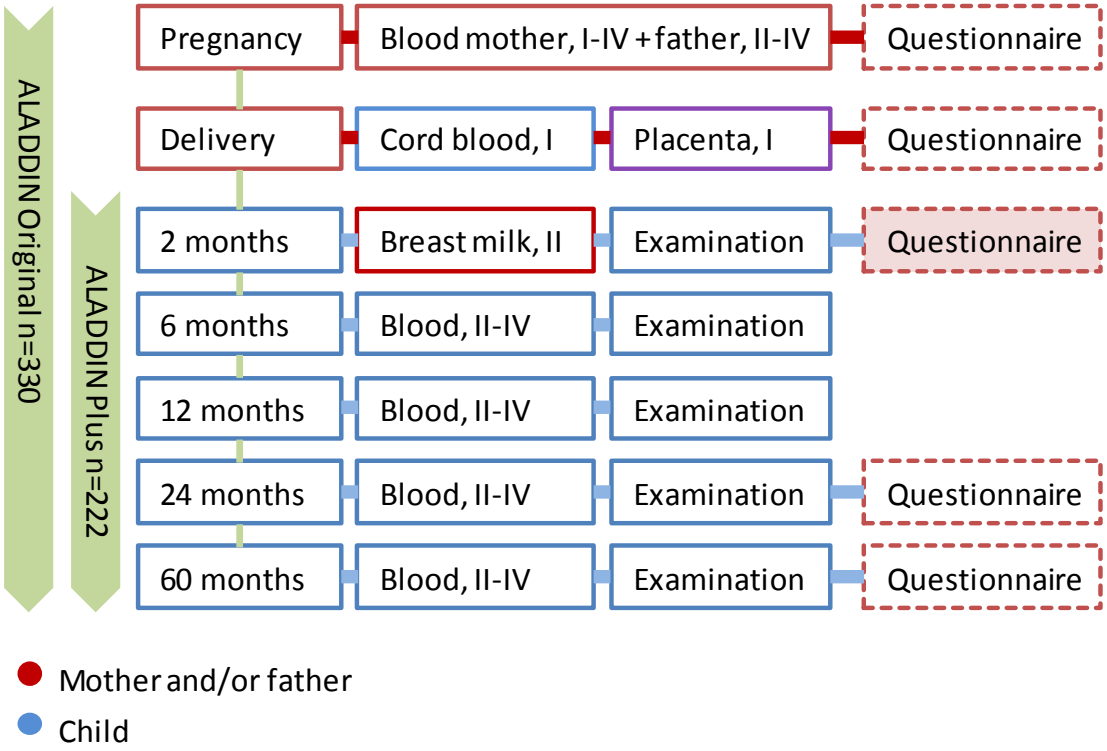

Figure 5 Flow chart of the samples and questionnaire data used for studies included in this thesis. Roman numbers denote article numbers.

5.2.1 Lifestyle

To evaluate the degree of anthroposophy of the participants in the ALADDIN cohort, the families were divided into three lifestyle groups based on following questions in the questionnaire.

1.” What kind of preschool/school will your child probably go to?”

a) Public preschool/school.
b) Waldorf/Steiner preschool/school.
c) Other private school.

2. “Do any of the parents, no matter what preschool/school you have planned for your child, have an anthroposophic view of life? (Yes/No)

3. “Is the family’s daily life influenced by an anthroposophic view of life?” (Yes/No)

If the parents answered they will choose an anthroposophic (Steiner or Waldorf school), and “yes” on the following two questions and attended an anthroposophic M-CHC, the family was regarded as having an anthroposophic lifestyle. If they answered that they will choose a public school or private other than anthroposophic and “no” on the following questions and went to a conventional M-CHC, the family was regarded as not having an anthroposophic view of life. All other combinations were regarded as partly anthroposophic.

5.2.2 Study populations

All studies in my thesis are based on the ALADDIN study. Depending on specific aims, inclusion criteria and sample availability, different populations have been chosen for the individual studies.

5.2.2.1 Article I

Mothers eligible for randomization for study I were mothers with and without an anthroposophic lifestyle with available placenta samples. Placentas were collected at 270 of the 328 deliveries (Figure 6). Exclusion criteria for study I were maternal smoking before or during pregnancy and/or paternal or other person smoking indoors during pregnancy. Available number of placentas for study I were 52 placentas from mothers with and 66 placentas from mothers without an anthroposophic lifestyle. We randomly selected 40 placentas from each lifestyle group.

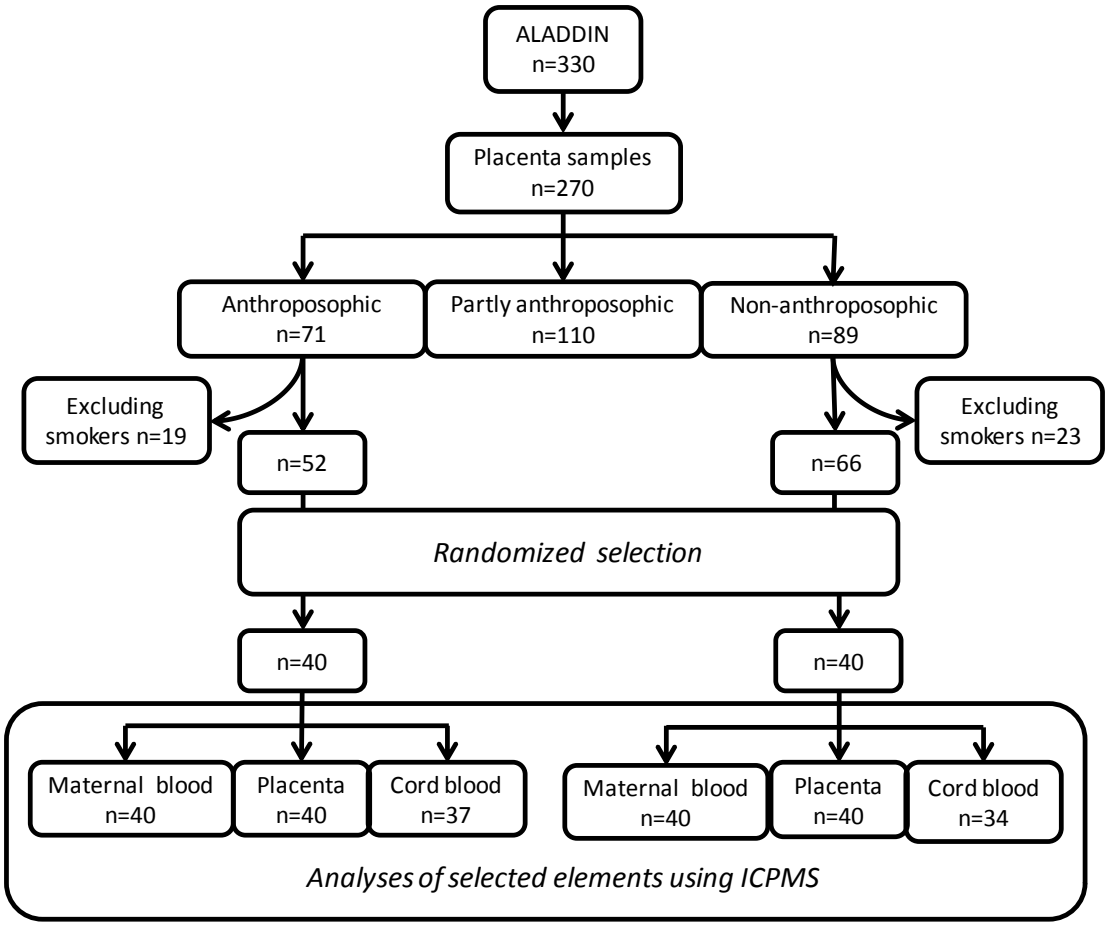

Figure 6 The selection process of the 40 anthroposophic and 40 non-anthroposophic mother-child pairs for study I. (ICPMS = inductively coupled plasma mass spectrometry).

5.2.2.2 Article II

Inclusion criteria for study II were having a lifestyle categorization, providing a breast milk sample at two months post-partum and at least one blood sample from the child at any of the time-points (6, 12 or 24 months). In total, 225 mother-child pairs provided both breast milk and blood samples.

5.2.2.3 Article III

Inclusion criteria for study III were having a lifestyle categorization and at least one blood sample taken at any of the time-points; 6, 12, 24 or 60 months. At time of study III, a number of children had not yet completed the five-year follow-up. Of the eligible 552 children, 100 from anthroposophic, 209 from partly anthroposophic and 165 from non-anthroposophic families were included in study III. The inclusion process and number of available blood samples for study III are presented in the flowchart in Figure 7.

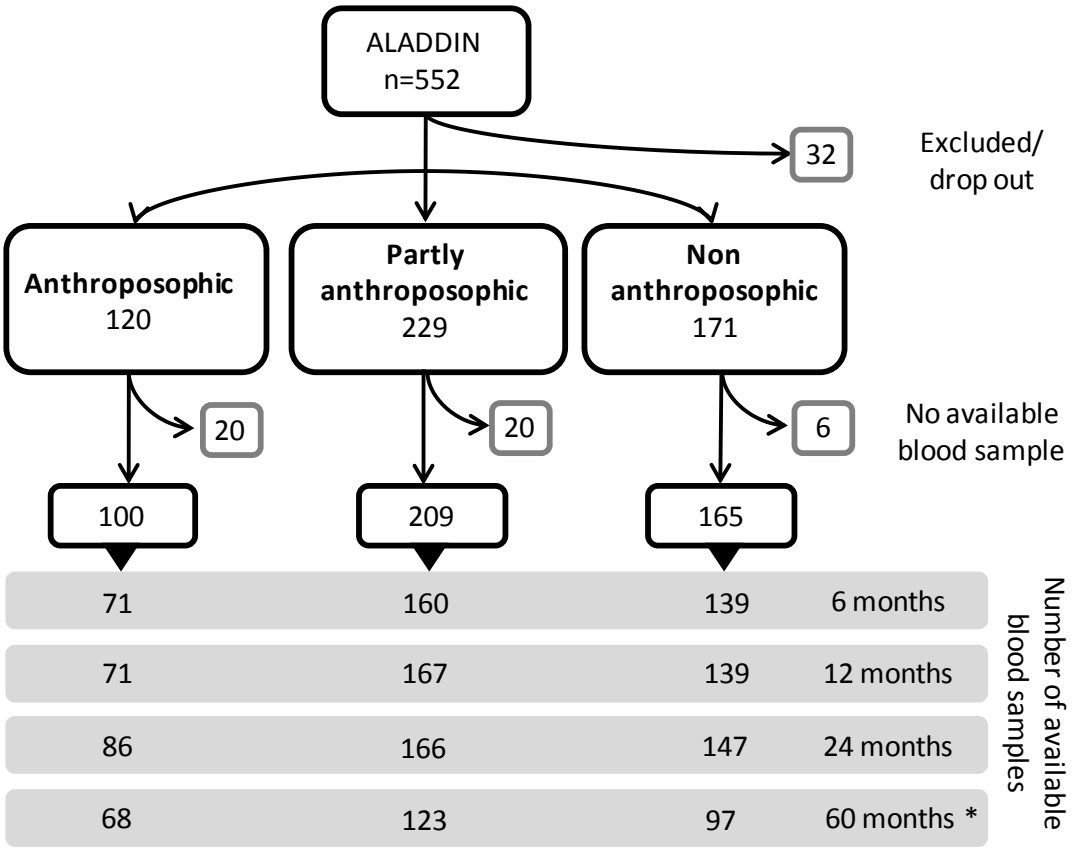

Figure 7 Flowchart of the inclusion process and available blood samples at each time-point for the three lifestyle groups.
* Not all children had completed the five year follow-up at the time of this study.

5.2.2.4 Article IV

Inclusion criteria for study IV were having an analysed blood sample at six months and providing at least one additional blood sample at a later time-point to enable longitudinal analysis.

5.3 SAMPLES USED IN THIS THESIS

Article I evaluates trace elements in maternal blood, placenta and cord blood. I did all sample preparations from the samples stored in our biobank. The metal analysis was carried out at Marie Vahter's laboratory; metals and health, Institute of Environmental Medicine, Karolinska Institutet.

Article II relates fatty acids in breast milk to sensitization up to two years of age. Lonneke Janssen Duijghuijsen and Axel Mie did the lipid extraction from breast milk and the fatty acid analysis was done at Swedish University of Agricultural Sciences (SLU).

Blood IgE analyses used in **Articles II, III and IV** were continuously done by laboratory technicians at the clinical immunology laboratory at Karolinska University Hospital and at Forskningscentrum Södersjukhuset.

5.3.1 Placenta samples

Placenta sample collection is described in detail in article I. Briefly, placenta samples were collected by midwives after delivery. A cross-sectional sample was cut out, washed, frozen in liquid nitrogen and stored at -80°C [163]. In contrast to the procedures for sample collecting for trace element analysis, where the risk of external metal contamination is minimized (e.g. using acid washed titanium knives), the ALADDIN samples were cut with a scalpel. To compare sample collecting methods we conducted a pilot study with three samples using each method. No significant differences in metal concentrations between the two methods were found.

5.3.2 Blood samples

Blood samples from the parents were collected at inclusion during the third trimester or during the neonatal period. At delivery, midwives collected umbilical cord blood by aspiration, and blood from the children were collected at 6, 12, 24 and 60 months of age. All blood samples were non-fasting. Blood samples were collected in sodium heparin tubes. The blood samples were separated using Ficoll-Paque (General Electric Healthcare Bio-Sciences, Uppsala, Sweden) into plasma and blood cells and stored at -20°C. Ficoll-Paque was compared with direct centrifugation to investigate if Ficoll-Paque separation could influence trace element concentrations. Ficoll-Paque did not influence elements analysed in article I [164].

5.3.3 Breast milk sample collection and lipid analysis

Breast milk samples were collected at two months *post partum* by the participants and kept at +4°C. The milk samples were frozen within 24 h from collection and stored at -80°C. The lipids have to be extracted from the milk sample for analysis of the FAs. Lipids were extracted in hexane using a procedure described by Hara and Radin[165]. Lipids are as such not suitable for gas chromatography and therefore they have to be transformed to fatty acid methyl esters (FAMEs). The lipids were saponificated using sodium hydroxide and derivatised to FAMEs using methanol and the esterification reaction was catalysed using boron trifluoride. The esterification procedure was described by Appelqvist[166]. The FAMEs were analysed with gas chromatography with a flame ionisation detector (GC-FID). The FAMEs are vaporised at injection in the gas chromatograph column where the FAMEs are separated. The time it takes for the FAMEs to pass through the column depends on their boiling point and their interactions with the stationary phase in the column. For detection, the FAMEs are burned in a hydrogen flame and thereafter the electric current generated by the resulting ions is measured. The method is described in detail by Fredriksson Eriksson and Pickova[167].

5.3.4 Trace element analysis

The procedure for trace element analysis is described in detail in article I. Briefly, maternal and cord blood was alkali diluted and placenta tissue samples were acid digested before trace element analysis. Toxic elements cadmium (Cd), arsenic (As) and lead (Pb) and essential elements calcium (Ca), cobalt (Co), copper (Cu), iron (Fe),

magnesium (Mg), manganese (Mn), molybdenum (Mo), selenium (Se) and zinc (Zn) were quantified in placenta and blood samples using inductively coupled plasma mass spectrometry (Agilent Technologies, Tokyo, Japan).

5.3.5 Sensitization

IgE concentrations were analysed in blood plasma from the parents, cord blood and blood from the children. Parental sensitization was determined using Phadiatop (Thermo Fisher Scientific, Uppsala, Sweden) for 11 common inhalant allergens. If the Phadiatop was ≥0.35 kU/L, s-IgE to 9 allergens were analysed in the sample (birch, timothy, mugwort, mould, two species of house dust mite, cat, dog and horse). Sensitization at 6, 12 and 24 months was determined using ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) for analysis of specific IgE concentrations to egg, milk, peanut, cat, dog, birch and timothy. At five years of age, blood samples from the children were analysed using Phadiatop for inhalant allergens and fx5, a mix for egg, milk, peanut, soy, wheat and cod. If Phadiatop and/or fx5 were positive, specific IgE for Phadiatop/fx5 allergens in that sample were analysed. Subjects with allergen specific IgE levels ≥0.35 kU/L were regarded as being sensitized.

5.4 STATISTICAL ANALYSES

5.4.1 Study I

The trace element concentrations were not normally distributed and therefore non-parametric statistical analyses were applied. To compare metal concentrations between two independent groups we used Mann-Whitney U-test. To evaluate the correlations between trace element concentrations we used Spearman's rank correlation test. To study the influence of lifestyle, gestational age in placenta and cord blood, gestational age at blood sampling, maternal age at delivery, parity, herbal medicine intake and occupation on the trace element concentrations we used analysis of covariance (ANCOVA). To achieve normal distribution of the residuals of the ANCOVAs the metal concentrations were log transformed in all cases except for placenta Pb and cord blood Co where we used 1/ √metal concentration and for Co in maternal blood where we used √Co. For Cd in placenta we used non-transformed concentrations. The transformations did not substantially change the results. Statistical analyses were performed using SPSS Statistics (IBM SPSS Statistics, version 21, Illinois, USA). Significance level was set at 0.05.

5.4.2 Study II

Fatty acids (FAs) were grouped as omega-3 PUFAs, omega-6 PUFAs and ruminant FAs and the concentrations were divided in quartiles. The **omega-3 PUFAs** were following; C18:3 n-3 (alpha linolenic acid (ALA)), C18:4 n-3, C20:3 n-3, C20:4 n-3, C20:5 n-3 (eicosapentaenoic acid (EPA)), C22:5 n-3 (docosapentaenoic acid (DPA)) and C22:6 (docosahexaenoic acid (DHA)). The **omega-6 PUFAs** were C18:2 n-6 (linoleic acid (LA)), C18:3 n-6, C20:2 n-6, C20:3 n-6 (dihomo-gammalinolenic acid

(DGLA)), C20:4 n-6 (arachidonic acid (AA) and C22:4 n-6. **Ruminant FAs** were C18:1 n-7trans (vaccenic acid (VA)) and C18:2 cis9 trans11 (conjugated linoleic acid (CLA)). The exact handling of the FAs in the analyses is described in detail in article II. Parental and child sensitization was defined as having specific IgE concentration ≥0.35 kU/L for at least one allergen. To be defined as non-sensitized, IgE for all seven allergens had to be <0.35 kU/L.

Variables considered to be traditional risk factors or influencing sensitization outcome were included in the statistical model. The final model was adjusted for the following variables; lifestyle, maternal smoking during pregnancy, living on a farm with animals during pregnancy, sex, presence of siblings or other children in the household at birth, exclusive breastfeeding at two months, parental education and parental sensitization. Maternal dietary factors were not included in the model as they might directly influence the FA composition. Distribution of demographic variables and potential confounders across the lifestyle groups were evaluated using chi-2 test.

The associations between FA concentration in breast milk (exposure) and sensitization up to two years of age (outcome) were assessed longitudinally using generalized estimating equations (GEE) with a log-link and a poisson distribution[168]. All analyses were performed using R v 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria). Significance level was set at 0.05.

5.4.3 Study III

Children with allergen specific IgE concentration ≥ 0.35 kU/L were regarded as sensitized. The allergens were grouped into three categories; food (hen's egg, cow's milk and/or peanut), animal (cat and/or dog) and pollen (birch and/or timothy). Seven allergens were analysed at six months, one and two years of age and for longitudinal comparison we decided to restrict allergens to those seven also at five years.

Incidences and prevalences for the three allergens were calculated for all four time-points. Prevalence was calculated as the number of sensitized children by the number of non-missing children at each time-point. Incidence proportions were calculated as the number of first time sensitized children (cases) during a time-period divided by the number of children at risk. Children with previous missing samples were considered at risk for the time-period up to the following non-missing sample given that they had not been sensitized earlier.
Chi-square test and analysis of variance (ANOVA) were used for comparison between demographic and lifestyle related factors.

We used GEE to investigate if the association between lifestyle and prevalence of sensitization for food, animal and pollen sensitization was affected by age of the child. Statistical analyses were performed using R v 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria) and SAS v9.4 (SAS Institute, Cary, NC). The significance level was set at 0.05.

5.4.4 Study IV

The allergen specific IgE concentrations were divided into three categories. Children with concentrations below the limit of quantification, 0-0.09 kU/L, were regarded as non-sensitized. Levels between 0.1 and 0.34 kU/L were regarded as low levels and those ≥ 0.35 kU/L were regarded as sensitized. As described in section "5.3.5 Sensitization", the allergens included in the fx5/Phadiatop were analysed only if the fx5/Phadiatop was positive. In study IV we replaced the missing negative values at five years with the fx5/Phadiatop result divided by the number of allergens included in the test (six in fx5/nine in Phadiatop). To evaluate the change in mean egg or milk concentrations from six months to five years we used non-parametric Wilcoxon signed rank test since the concentrations were not normally distributed. Statistical analyses were performed using SPSS Statistics (IBM SPSS Statistics version 23, Illinois, USA). The significance level was set at 0.05.

5.5 ETHICAL CONSIDERATIONS

All studies require ethical considerations irrespective of the health status of the subjects. The benefits from a study must be greater than its possible adverse effects and downsides. Although some participants have found filling out questionnaires to be time consuming and some sample collection to be somewhat awkward, most examinations and sample collections were non-invasive. The blood sample collection and skin prick-tests were performed by an experienced paediatric nurse. Emla-adhesive plaster with local anaesthetic was used prior to blood sampling to minimize discomfort.

Extensive amounts of questionnaire data and biological samples have been collected. The data is stored in encrypted databases separated from personal identity information. Social security numbers can only be accessed by the cohort steering group.
In the ALADDIN cohort, the parents were given oral and written information about the study and written consent from both parents was required for participation.
The study was approved by the local research ethics committee at the Huddinge University hospital and the Regional Ethical Review Board, Karolinska Institutet, both in Stockholm, Sweden.

6 RESULTS AND DISCUSSION

Demographic characteristics of the subpopulations in study I, II and IV can be found in the articles. In general terms, reporting a vegetarian diet, preference of organic cultivation, home delivery and delayed washing of infants after birth was more common in the anthroposophic group. The distribution of families living on a farm with animals and maternal age at delivery was similar in the studied lifestyle groups.

6.1 ASSOCIATIONS BETWEEN TRACE ELEMENT CONCENTRATIONS AND LIFESTYLE, ARTICLE I

The concentrations of trace elements in maternal blood, placenta and cord blood are presented in Tables 2a, b and c in article I. In contrast to our hypothesis, we found that the concentrations of the toxic elements Cd and Pb and the essential element Co were higher in the anthroposophic group.

Cadmium

The Cd concentrations in maternal blood and placenta were higher in the anthroposophic group compared to the non-anthroposophic group ($p<0.001$). The median Cd concentrations in cord blood were low in both lifestyle groups.

When we studied how the elements correlated within or between tissues we could see a linear association between Cd in maternal blood and in placenta ($r_s=0.36$, $p=0.001$). Cd accumulates in the placenta and in our study we found that the Cd concentrations in the placentas were on average 12 times higher.

Factors influencing metal concentrations were evaluated using ANCOVA. Results are presented in Table 1.

We could see that only lifestyle was significantly associated with maternal blood Cd concentrations ($p=0.010$). In placenta both lifestyle ($p=0.011$) and working with art ($p=0.029$) was significantly associated with the Cd concentrations.

Parity, maternal age at delivery, diet or consumption of herbal medicine could not explain the measured Cd concentrations in this study. It is known that a preference of vegetarian diet as well as a high intake of fibres are associated with higher Cd concentrations[169,170]. Vegetarian diet was not significantly associated with Cd concentrations in our study. 45% of the anthroposophic mothers were vegetarians and in this sub-population, the vegetarians (0.48µg/kg) and omnivores (0.40 µg/kg) had similar Cd concentrations. We speculated that the anthroposophic families might have a higher intake of plant-derived food regardless of other dietary preferences.

Unfortunately, we did not have information on maternal blood iron status so it remains unclear whether the vegetarian mothers had lower iron status or if iron status alone could explain part of the difference in Cd concentrations.

Divalent metals are taken up from the intestine by the divalent metal transporter 1 (DMT1)[171]. DMT1 is the main iron transporter but it is not specific for iron and therefore also transports toxic metals like Cd (and Pb)[171]. DMT1expression is up regulated during pregnancy and in iron deficiency/low iron stores. The transport of metals (toxic and essential) is mainly dependent on their availability in diet[172,173].

Lead

The concentrations of Pb were higher in the maternal blood (p=0.005) and cord blood (p=0.004) in the anthroposophic group.

Pb passes through the placenta and therefore we found no differences in placental Pb concentrations between the lifestyle groups. As expected, maternal and cord blood Pb concentrations were highly correlated r_s=0.55, p<0.001).

We did not find any explanation for the higher levels of Pb in the anthroposophic group. A low calcium diet might influence the gastrointestinal absorption of Pb[174,175] but as the diet in the Nordic countries is rich in calcium sources and since the calcium levels were similar in the two lifestyle groups, we do not believe that the calcium metabolism could explain the differences seen in Pb.

Working with art and craft is a common and important feature of the anthroposophic lifestyle. Mothers could have been exposed to Pb in old paint, working with metals or welding. However, working with art could not explain the higher Pb concentrations.

Cobalt

The concentrations of Co were significantly higher in all studied tissues in the anthroposophic group compared to the non-anthroposophic group.

Co followed a slightly different pattern with high correlation between maternal blood and placenta (r_s=0.47, p<0.001) where concentrations in placenta were on average 40 times those in maternal blood. Also, maternal blood concentrations correlated well with those in cord blood (r_s=0.35, p=0.003) where the concentrations were quite similar.

Our results showing positive correlations between Cd and Co both in maternal blood and placenta indicate that these metals share similar uptake and transport routes as shown in previous reports [176-178].

A higher intake of vegetables might also explain the higher Co concentrations seen in the anthroposophic mothers as green leafy vegetables, legumes, seeds, nuts and cereals are some of the main sources of Co[179,180].

Despite the higher levels of toxic Pb and Cd seen in the anthroposophic group, all of the measured trace element concentrations in our study are similar to concentrations reported by other Nordic studies[181,182].

In this study, we did not relate the result to allergic sensitization. It is possible that exposure to toxic metals could interfere with prenatal immune development. Cd in

placental syncytiotrophoblast cells has been found to suppress transcription of the cortisol converting enzyme 11β-hydroxysteroid dehydrogenase type 2[50,183]. We have previously shown that children with low salivary cortisol have a lower prevalence of allergic sensitization in the first two years of life[153]. Evaluating this theory in our material was however not possible due to time differences in collection and sample preparation of the placentas.

Table 1 Analysis of covariance for factors influencing cadmium, cobalt and lead concentrations in maternal blood erythrocytes, placenta and cord blood erythrocytes.

			Maternal blood			Placenta			Cord blood		
	Variable	**Type**	**Effect size**	**95% Conf. Int.**	**p-value**	**Effect size**	**95% Conf. Int.**	**p-value**	**Effect size**	**95% Conf. Int.**	**p-value**
Cd	**Lifestyle (anthroposophic)**	Cat.	0.148	(0.036-0.259)	**0.010**	1.385	(0.323-2.447)	**0.011**	0.042	(-0.06-0.145)	0.412
	Diet (omnivore)	Cat.	-0.047	(-0.165-0.071)	0.426	-0.662	(-1.737-0.414)	0.224	0.035	(-0.070-0.140)	0.508
	Herbal medicine (not taken)	Cat.	0.056	(-0.053-0.166)	0.308	0.231	(-0.755-1.217)	0.642	-0.005	(-0.099-0.089)	0.917
	Parity (one child)	Cat.	-0.031	(-0.151-0.089)	0.607	-0.201	(-1.287-0.885)	0.713	-0.024	(-0.127-0.080)	0.648
	(two children)	"	-0.075	(-0.174-0.024)	0.137	-0.221	(-1.115-0.674)	0.624	-0.056	(-0.142-0.030)	0.197
	Occupation (art)	Cat.	0.112	(-0.008-0.0233)	0.067	1.25	(0.132-2.367)	**0.029**	0.104	(-0.007-0.214)	0.066
	Gestational age*	Cont.	0.002	(0.000-0.004)	0.121	0.007	(-0.078-0.093)	0.868	0.000	(-0.007-0.008)	0.918
	Maternal age	Cont.	-0.001	(-0.010-0.008)	0.851	-0.022	(-0.069-0.024)	0.339	0.000	(-0.005-0.004)	0.958
	Intercept		*-0.849*	*(-1.489-(-0.209))*	*0.010*	*10.114*	*(-2.598-22.825)*	*0.117*	*-1.586*	*(-2.846-(-0.326))*	*0.015*
Co	**Lifestyle (anthroposophic)**	Cat.	0.036	(-0.02-0.092)	0.205	0.156	(0.048-0.264)	**0.005**	-0.301	(-0.734-0.0123)	0.169
	Diet (omnivore)	Cat.	-0.059	(-0.118-0.000)	0.051	-0.016	(-0.126-0.094)	0.774	-0.133	(-0.577-0.311)	0.551
	Herbal medicine (not taken)	Cat.	-0.001	(-0.056-0.054)	0.982	-0.002	(-0.103-0.098)	0.962	0.255	(-0.142-0.652)	0.204
	Parity (one child)	Cat.	-0.032	(-0.093-0.028)	0.286	-0.019	(-0.130-0.092)	0.734	-0.063	(-0.502-0.375)	0.774
	(two children)	"	0.022	(-0.027-0.072)	0.373	-0.011	(-0.102-0.080)	0.814	0.054	(-0.310-0.418)	0.768
	Occupation (art)	Cat.	0.007	(-0.054-0.068)	0.82	-0.025	(-0.139-0.089)	0.665	0.297	(-0.170-0.765)	0.208
	Gestational age*	Cont.	0.001	(0.000-0.003)	**0.018**	-0.01	(-0.018-(-0.001))	**0.033**	0.047	(0.014-0.080)	**0.007**
	Maternal age	Cont.	-0.003	(-0.007-0.002)	0.272	0.004	(0.000-0.009)	0.070	-0.012	(-0.031-0.008)	0.242
	Intercept		*0.078*	*(-0.243-0.399)*	*0.630*	*-0.514*	*(-1.811-0.784)*	*0.432*	*5.478*	*(0.152-10.803)*	*0.044*
Pb	**Lifestyle (anthroposophic)**	Cat.	0.105	(-0.005-0.215)	0.060	0.142	(-1.185-1.468)	0.832	0.165	(0.023-0.306)	**0.023**
	Diet (omnivore)	Cat.	0.085	(-0.032-0.201)	0.151	-0.087	(-0.198-0.024)	0.122	0.09	(-0.055-0.235)	0.219
	Herbal medicine (not taken)	Cat.	-0.013	(-0.1210.095)	0.809	-0.075	(-0.188-0.037)	0.185	-0.003	(-0.132-0.127)	0.968
	Parity (one child)	Cat.	0.103	(-0.015-0.221)	0.086	0.01	(-0.093-0.113)	0.851	-0.028	(-0.171-0.115)	0.697
	(two children)	"	-0.073	(-0.171-0.024)	0.138	-0.089	(-0.202-0.025)	0.123	-0.063	(-0.182-0.056)	0.295
	Occupation (art)	Cat.	0.116	(-0.003-0.235)	0.056	0.018	(-0.075-0.111)	0.702	0.033	(-0.12-0.186)	0.670
	Gestational age*	Cont.	0.001	(-0.001-0.004)	0.249	-0.057	(-0.173-0.060)	0.335	-0.003	(-0.013-0.008)	0.642
	Maternal age	Cont.	0.004	(-0.005-0.013)	0.344	0.003	(-0.006-0.012)	0.448	0.001	(-0.006-0.007)	0.821
	Intercept		*0.668*	*(0.038-1.298)*	*0.038*	*0.002*	*(-0.003-0.006)*	*0.524*	*0.666*	*(-1.075-2.406)*	*0.447*

For detailed information on statistics, see statistics section.

*Gestational age at blood sampling for maternal blood and gestational age at birth (days) for placenta and cord blood.

The significance level was set at 0.05

6.2 BREAST MILK FATTY ACID COMPOSITION IN RELATION TO SENSITIZATION, ARTICLE II

The 225 mother-child pairs included in this study did not differ from those not included regarding lifestyle group and sensitization outcome up to two years of age. Since a breast milk sample was required for inclusion, breastfeeding at two months was more common among those included. On the other hand, smoking during pregnancy and organic diet during breastfeeding was more common among those not included. Background and sensitization data on the included mother-child pairs is presented in Table 1 in the article.

We found higher concentrations of omega-6 PUFAs compared to omega-3 and ruminant FAs in our study. The concentrations of omega-3 and omega-6 PUFAs were positively correlated and both were negatively correlated with concentrations of ruminant FAs. The total concentrations of FAs are presented in Table 2 and detailed information can be found in the article in Figure 1 and in supplementary Table S1. The highest concentrations of omega-3 were found in the partly anthroposophic group.

Table 2 Concentrations of grouped omega-6, omega-3 and ruminant FAs in breast milk samples in the three lifestyle groups.

Lifestyle	**ω-6**	**ω-3**	**ruminant**	**p-value***
	wt%, stdev	wt%, stdev	wt%, stdev	
Non-anthroposophic	11.38 ± 2.32	2.28 ± 0.63	0.76 ± 0.23	*0.796*
Partly anthroposophic	11.55 ± 2.58	2.40 ± 1.04	0.86 ± 0.31	***0.037***
Anthroposophic	11.28 ± 2.63	2.06 ± 0.83	0.94 ± 0.28	***0.002***

*ANOVA was used to test differences in mean values between the three lifestyle groups. The concentrations were log transformed.

The result from the longitudinal analysis of overall effect of breast milk FAs on sensitization up to two years is presented in Table 3. We found an inverse association between omega-3 PUFAs in breast milk and sensitization (adjusted RR, highest vs. lowest quartile 0.49, 95% CI 0.023-1.05, p=0.024). Interestingly, a higher AA/EPA ratio was associated with a higher risk of sensitization (adjusted RR upper median vs. lowest quartile 2.64, 95% CI 1.15-6.07, p=0.029). There were no other significant associations between omega-6 PUFAs, ruminant FAs, single FAs or other ratios to sensitization in the child.

Table 3 Longitudinal analyses of the relative risks between breast milk FA concentrations and sensitisation in the child up to 24 months of age in the ALADDIN birth cohort (N=225).

	Quartiles of fatty acid concentration				
	1	2	3	4	
	Ref	RR (95% CI) [1]	RR (95% CI) [1]	RR (95% CI) [1]	p-value
Fatty acids					
Omega-3 PUFA[2]	1.0	0.89 (0.44-1.80)	0.49 (0.23-1.04)	0.49 (0.23-1.05)	*0.024*
Omega-6 PUFA[2]	1.0	1.39 (0.72-2.67)	0.62 (0.27-1.42)	0.89 (0.41-1.92)	*0.315*
Ruminant FA[2]	1.0	0.89 (0.41-1.95)	1.26 (0.60-2.66)	1.07 (0.50-2.31)	*0.676*
Omega-3					
C18:3 n-3 (ALA)	1.0	1.14 (0.56-2.30)	0.90 (0.44-1.85)	0.67 (0.29-1.58)	*0.254*
C22:5 n-3 (DPA)	1.0	1.10 (0.51-2.37)	1.25 (0.59-2.63)	1.21 (0.56-2.59)	*0.574*
C22:6 n-3 (DHA)	1.0	1.00 (0.49-2.05)	0.91 (0.44-1.86)	0.62 (0.26-1.48)	*0.249*
LC omega-3[3]	1.0	0.82 (0.41-1.65)	0.72 (0.38-1.36)	0.58 (0.29-1.15)	*0.111*
Omega-6					
C18:2 n-6 (LA)	1.0	1.44 (0.72-2.87)	0.99 (0.45-2.18)	0.84 (0.38-1.89)	*0.428*
C20:3 n-6 (DGLA)	1.0	0.60 (0.29-1.25)	0.87 (0.42-1.82)	0.86 (0.43-1.75)	*0.911*
C20:4 n-6 (AA)	1.0	0.89 (0.39-2.05)	0.85 (0.38-1.86)	1.36 (0.65-2.87)	*0.397*
LC omega-6[3]	1.0	1.02 (0.43-2.43)	1.40 (0.64-3.06)	1.14 (0.49-2.65)	*0.598*
Omega 6/3 ratio	1.0	0.64 (0.27-1.52)	1.51 (0.78-2.93)	1.42 (0.69-2.91)	*0.070*
AA/EPA ratio[4]	1.0	2.92 (1.24-6.87)	2.64 (1.15-6.07)	*0.029*	
Ruminant					
C18:1 n-7 trans (VA)	1.0	0.82 (0.43-1.59)	0.91 (0.45-1.83)	0.74 (0.33-1.65)	*0.523*
C18:2 cis 9 trans 11 (CLA)	1.0	0.68 (0.33-1.40)	0.69 (0.32-1.48)	1.10 (0.54-2.26)	*0.848*

GEE was used to get RRs and CIs and test for linear trend was used to get P values.
1=lowest quartile, 4=highest quartile.
[1]Adjusted for lifestyle, sex, parental sensitisation, maternal smoking during pregnancy, living on a farm with animals during pregnancy, older children in the household, exclusive breastfeeding at 2 months and parental education.
[2] The group of omega-3 PUFAs includes C18:3 n-3, C18:4 n-3, C20:3 n-3, C20:4 n-3, C20:5 n-3, C22:5 n-3 and C22:6 n-3. The group of omega-6 PUFAs includes C18:2 n-6, C18:3 n-6, C20:2 n-6, C20:3 n-6 and C20:4 n-6 and C22:4 n-6. Ruminant FAs include C18:1n-7tr and C18:2 cis9 trans11.
[3] Long-chain omega-3 FA includes c20:3 n-3, c20:4 n-3, c20:5 n-3, C22:5 n-3 and C22:6 n-3. Long-chain omega-6 FA includes C20:2 n-6, C20:3 n-6 and C20:4 n-6 and C22:4 n-6.
[4] EPA concentrations were below the LOQ=0.05 in 47% of samples. These were set to LOQ/√2=0.0354 for the purpose of this analysis. All of these samples were in quartiles 3 and 4 of AA/EPA ratios. These quartiles were therefore combined.

It would have been desirable to stratify on lifestyle in the longitudinal analysis but as the number of sensitized children in the anthroposophic and partly anthroposophic group was low, such analysis was not possible. Instead, the effect of FAs on lifestyle was assessed in a separate GEE analysis (results presented in Table 3 in the article). In this model we adjusted for traditional risk factors (see statistics section).

As in previous studies[158], we found that an anthroposophic lifestyle was associated with a lower risk of sensitization up to two years of age also in this sup-population (adjusted RR anthro. vs. non-anthro. 0.44, 95% CI 0.21-0.90). When separately including omega-3, omega-6 and ruminant FAs in the model, the RRs remained almost unchanged which shows that the lower prevalence of sensitization in the anthroposophic group was not a result of FA differences in breast milk. Results from the longitudinal analyses are presented in detail in Table 3 in the article.

The observed effect of omega-3 FAs in breast milk on sensitization in our study is in line with previous reports and supports the lipid hypothesis[109,115,116]. It is nevertheless difficult to draw conclusions on the effect of FAs in breast milk as several studies show

conflicting results. Opposing results have been observed in studies on high-risk populations. The Dutch PIAMA cohort study on allergic and non-allergic mothers and their infants up to four years reported that omega-3 PUFAs in breast milk was inversely associated with asthma/atopy up to four years in children to allergic mothers[184]. They also reported that alpha linolenic acid (ALA) was positively associated with sensitization in children to non-allergic mothers. Although in a later report, with results up to 14 years of age, they found no association between ALA and sensitization[185]. Nevertheless, they confirm their previous findings in the children to allergic mothers and indeed see that asthma is less prevalent if the breast milk contained higher concentrations of omega-3 PUFAs and likewise there was a positive association between omega-6 PUFAs and asthma in the children to non-allergic mothers.

It has been shown that living on a farm with animals, especially with exposure to farm milk, has an allergy protective effect[72,74]. A report from the KOALA cohort showed that ruminant FAs in breast milk, in addition to omega-3 FAs, were associated with a lower risk of atopic manifestations in infancy[115].
Interestingly, a report by Brick *et al* from the PASTURE study show that the protective effect of farm milk could be explained by its higher content of omega-3 PUFAs[129].

The association between ruminant FAs and allergy has not yet been extensively studied although vaccenic acid (VA) and rumenic acid (RA) have been studied in other diseases such as cardiovascular disease[186]. One study reported that VA in maternal plasma during pregnancy was protecting against atopic eczema in infancy[187]. However, in our study we could not see any association between ruminant FAs (or VA or CLA) and sensitization.

Consuming fatty fish or fish oil supplements during lactation (≥1-3 times/month) was less common in the anthroposophic group compared to the non- and partly anthroposophic. This could explain the slightly lower concentrations of omega-3 PUFAs in the anthroposophic group (Table 2).
A Cochrane review by Gunaratne *et al* from 2015 that studied maternal pre and/or postnatal omega-3 supplementation for preventing allergies in childhood could not see any clear protective effect[120]. They found that omega-3 supplementation reduced their primary outcome of *any allergy* in children up to 36 months of age and that there was a reduction in sensitization to egg and *any sensitization* during this same time-period. The modest observed effect of omega-3 supplementation suggest that there are other factors and sources of omega-3 that might be important for the development of sensitization.

It was significantly more common among mothers in the partly anthroposophic and in the anthroposophic group to eat organically produced food during lactation. We could not attribute the sensitization protective effect of lifestyle to the differences in FA content of breast milk and we could not include organic diet in the analysis. It would have been interesting to study the FA content in breast milk of mothers consuming organic dairy products as a recent meta-analysis report higher concentrations of PUFA, omega-3 PUFA and CLA in organic milk[188].

6.3 ASSOCIATION BETWEEN INCIDENCE OF SENSITIZATION AND LIFESTYLE, ARTICLE III

Demographic data for the 474 included children and their parents are presented in Table 4. The table was not included in the original article. Of these 474 children, 100 came from families with an anthroposophic, 209 from families with a partly anthroposophic and 165 from families with a non-anthroposophic lifestyle. Having older siblings and being exclusively breastfed at six months were significantly more common in the anthroposophic group. In this group, it was also significantly less common to receive milk formula during the first week of life. No significant differences between the lifestyle groups were found for parental sensitization, maternal education, smoking and living on a farm during pregnancy.

Table 4 Demographic data on the 474 children and their parents included in study III.

Demographic data	Antroposophic	Partly antroposophic	Non-antroposophic	P-value*
Female sex	49/100 (49%)	107/209 (51%)	87/165 (53%)	0.84
Parental sensitization†	65/100 (65%)	121/198 (61%)	97/162 (60%)	0.70
Mothers education				0.10
Elementary school	4/99 (4%)	6/207 (3%)	8/163 (5%)	
Gymnasium	29/99 (29%)	75/207 (36%)	72/163 (44%)	
University	66/99 (67%)	126/207 (61%)	83/163 (51%)	0.10
Mother smoking during pregnancy	5/99 (5%)	11/206 (5%)	10/163 (6%)	0.92
Mother living on a farm during pregnancy	7/97 (7%)	14/204 (7%)	11/160 (7%)	0.99
Mother's age at delivery (years)	33±5.8	32±5.5	31±4.3	0.06
Having older siblings	77/99 (78%)	120/204 (59%)	102/161 (63%)	<0.01
Exclusive breastfeeding at 6 mo	40/100 (40%)	69/206 (33%)	20/165 (12%)	<0.01
Milk formula during 1st week	14/97 (14%)	41/207 (20%)	52/163 (32%)	<0.01

*Categorical variables presented with n/N (%) and tested with Chi-2 test; continuous variables presented with mean ± standard deviation and tested with ANOVA.

†Parental sensitization was defined as mother and/or father with an IgE level ≥0.35 kU/L against a mix of 11 inhalant allergens (ImmunoCAP Phadiatop®, Thermo Fisher Scientific)

Incidence proportions and prevalences of sensitization are presented in Figure 8. The incidence proportions of food sensitization in the anthroposophic group were low during the first year of life (1% at 0-6 months and 1% at 6-12 months). During the same time-period, the incidence proportions in the non-anthroposophic group were 15% and 16%. In the anthroposophic group, the incidence proportion increased from one to two years of age to 8% and remained stable up to five years (9%). In contrast, incidence proportions in the non-anthroposophic children decreased from one to two years of age (5%) but increased thereafter to 16% from two to five years. In the partly anthroposophic group, the incidence proportions were stable and at around 7% in all time-periods.

Incidence proportions to animal and pollen allergens were similar for the lifestyle groups and sensitization seemed to occur later than food sensitization, as expected.

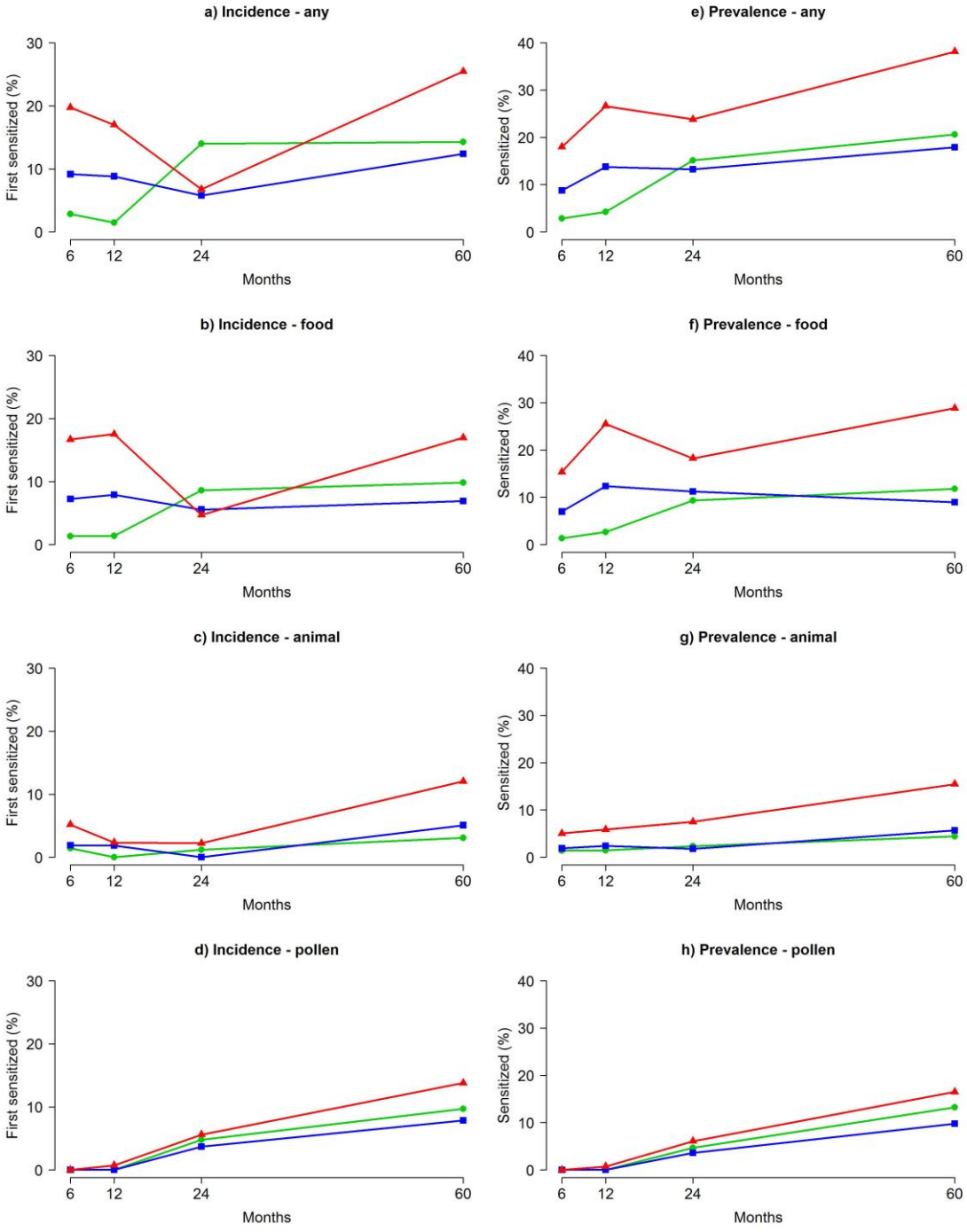

Figure 8 Incidence proportions (a-d) and prevalences (e-h) of sensitization to food, animal and pollen allergens in children of families with an anthroposophic (green), partly anthroposophic (blue) and non-anthroposophic (red) lifestyle.

We used a GEE model to evaluate if the association between lifestyle and sensitization was modified by time (the age of the child). We found that age significantly modified the association between lifestyle and food allergen sensitization (p=0.020). The anthroposophic and partly anthroposophic groups were compared to the non-anthroposophic group in the model.

The results are presented in Figure 9. The association between anthroposophic lifestyle and food allergen sensitization was stronger in the first year of life (odds ratios (ORs); 0.16 at 6 months, 0.18 at one year, 0.25 at two years and 0.58 at five years of age). The associations seen for the partly anthroposophic group were similar for all time-points. We have previously shown that an anthroposophic lifestyle is associated with a lower prevalence of allergic sensitization[149,158]. Results from this study suggests that this may, to a great extent, be related to food allergen sensitization in infancy which indicates that the protective effect of an anthroposophic lifestyle may be greater in early life.

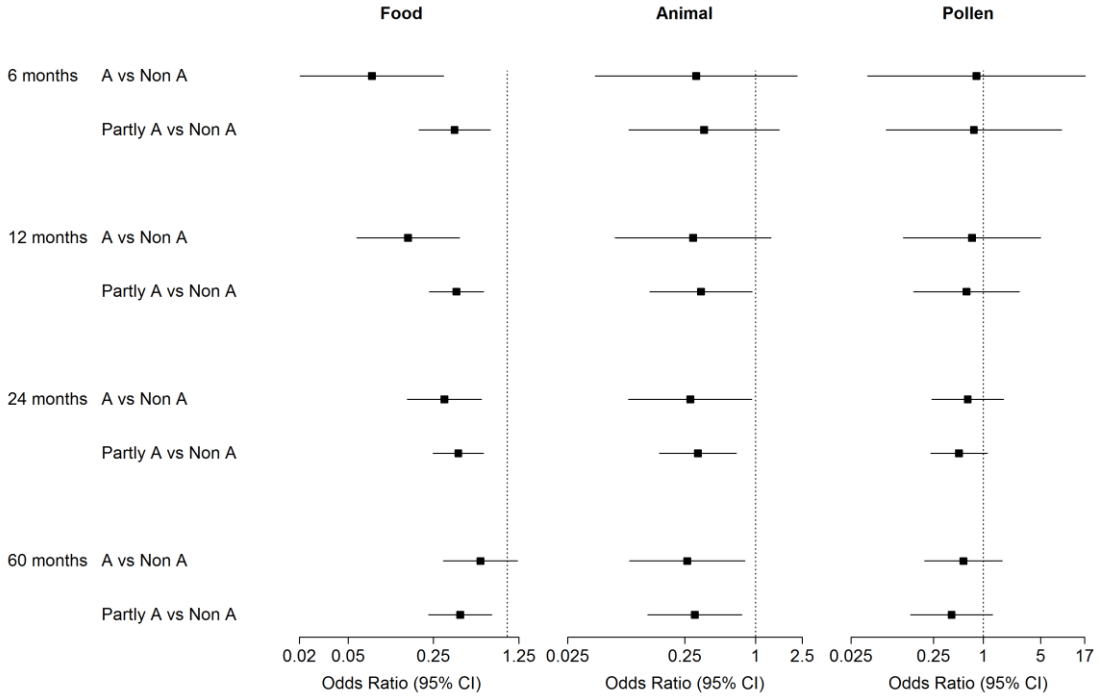

Figure 9 Associations between lifestyle and prevalences of food, animal and pollen sensitization at 6, 12, 24 and 60 months of age. A = anthroposophic, Partly A = partly anthroposophic and Non A = non-anthroposophic. ORs and 95% CIs from a generalized estimating equation model.

The pattern of allergic sensitization, starting with sensitization against food allergens followed by animal and pollen allergen sensitization, has been described earlier[14,26,27]. Few studies have reported the prevalence of allergen sensitization in the first year of life[13]. A study on Estonian and Swedish infants reported the prevalence of milk and egg sensitization at six months of age to be as high as 41% (21/51) and that of milk, egg, cat and birch allergen sensitization at one year to be 18% (21/114) and at two years 15% (18/117)[189]. This is in accordance with the prevalences in the non-anthroposophic group in our study.

In the Dutch KOALA cohort, 27% (215/786) of the children were sensitized to one or more common allergens at the age of two years[190], which is similar to the corresponding prevalence, 24%, in the non-anthroposophic group.

In the MAS study[26], including 216 children born in 1990, the prevalence of any sensitization at age one year was 11%. In our study, the prevalence in the non-anthroposophic group was higher, 27%, which may partly be explained by the use of a

higher cut-off level for sensitization (0.70 kUA/L) in the German study. However, a general rise in the prevalence of sensitization during the last 15 years cannot be ruled out.

Tolerance to cow´s milk and hen´s egg usually develops at four to six years of age[35,37]. This tendency could not be seen in this study. Interestingly, both the prevalence and incidence of food allergen sensitization in the non-anthroposophic group increased up to five years of age.

Another thing worth noting is that both prevalence and incidence of food sensitization at five years was slightly higher in the anthroposophic group compared to the partly anthroposophic group. The partly anthroposophic group is indeed larger which gives better statistical power. One explanation to the age-related differences could be that sensitization develops later in the children of anthroposophic families.
More questions than answers came out of this study and therefore we decided to further study the early development of food sensitization in the fourth study.

6.4 THE RELEVANCE OF LOW SPECIFIC IGE LEVELS TO EGG, MILK AND PEANUT IN INFANCY, ARTICLE IV

372 children were included in study IV. Demographic data is presented in Table 1 in the manuscript. Children were divided into three categories of IgE concentrations at six months and followed prospectively until five years. Sensitization development is presented in Figures 1-3 in the article. As expected, the prevalence of sensitization at six months was low for all three followed allergens.

6.4.1 Development of s-IgE to egg

At six months, 5% (18/369) of the children had low level s-IgE (0.1-0.34 kU/L) (Figure 10) to egg and 5% (20/369) were sensitized (≥0.35 kU/L). Diagrams for non-sensitized and sensitized children are presented in the manuscript in Figure 1.

Following the children with low level s-IgE to egg at six months (Figure 10), we can see that at one year the IgE to egg increases in four children but also decreases in the same number of children. Thereafter the number of sensitized children and children with low levels in this group decreases. At five years, the IgE to egg have decreased to below 0.1 in 11/14 of the children. On the other hand, it seems like early low sensitization is associated with an increased risk for sensitization to another allergen at five years. Five children had a sensitization to another food allergen and ten to an inhalant allergen.

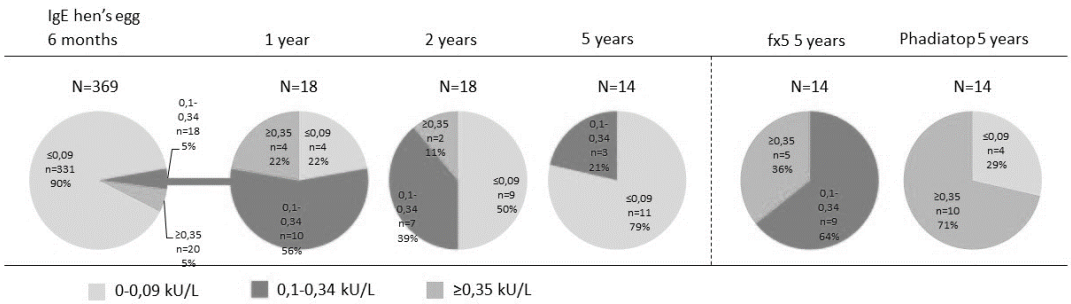

Figure 10 Development of low levels of s-IgE during the first five years to egg.

Following the children sensitized at six months, the prognosis is less positive compared to those with low levels. Egg sensitization is considered to have a worse prognosis, increasing the risk for sensitization to inhalant allergens[30,31,36]. Although limited to 20 children, similar patterns can be seen in this population. The children sensitized at six months were also to a high extent sensitized later even though the ratio of sensitized children decreased. Indeed, a majority of these children had a sensitization to food or inhalant allergens at five years of age.

For children with samples at both time-points, we compared the median s-IgE concentrations at six months to those at five years and found that there was a significant decrease in median s-IgE to egg in the children with low levels at six months (p=0.011). In the sensitized group the median s-IgE to egg decreased from 1.2 kU/L to 0.34 kU/L, however the change was not statistically significant. (Figure 4a and b in the manuscript).

6.4.2 Development of s-IgE to milk

At six months, 14% (51/372) of the children had low level s-IgE (0.1-0.34 kU/L) (Figure 11) to milk and 5% (20/372) were sensitized. Diagrams for non-sensitized and sensitized children are presented in the manuscript in Figure 2.

Similar to the low levels to egg, the number of children with low levels to milk decreased over time (Figure 11). Interestingly, the proportion developing sensitization in this low level group remained at around 20% from 1-5 years. Furthermore, in contrast to egg, 12/41 were sensitized to a food allergen at five years where indeed milk was the most common allergen. At five years, 15/41 children had developed sensitization to an inhalant allergen.

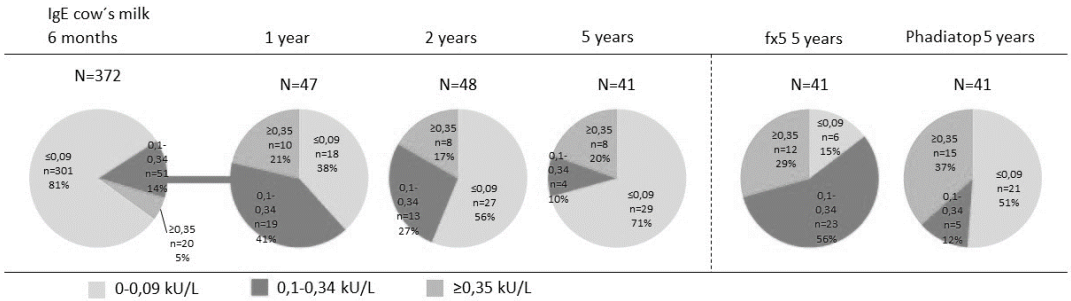

Figure 11 Development of low levels of s-IgE during the first five years to milk.

Twenty children were sensitized to milk at six months of age and also here the number of sensitized children decreased over time. A recent article by Caubet *et al.* investigating tolerance development to milk pointed to an importance of the diversity of IgE and IgG4 antibodies to milk protein binding sites[191].

In the children sensitized at six months, 9/15 had a sensitization to food (mainly milk) and 4/15 were sensitized to an inhalant allergen at five years.
The decrease in median s-IgE to milk from six months to five years in the children sensitized at six months was significant (p=0.004). (Figure 5a and b in the manuscript).

6.4.3 Development of s-IgE to peanut

At six months, 4% (15/367) of the children had low level s-IgE (0.1-0.34 kU/L) (Figure 12) to peanut and 1% (4/367) were sensitized. Diagrams for non-sensitized and sensitized children are presented in the manuscript in Figure 3.
In the non-sensitized group of children at six months, the number of sensitized children increased over time as expected. The majority of those children were multisensitized.

As for egg and milk, there was a trend towards a decrease in s-IgE in the low level group. Although, comparing median concentrations between six months and five years showed no statistically significant difference (p=0.062). (Figure 6a the manuscript).
A majority of the children in the low level category were sensitized to a food and/or an inhalant allergen at five years (Figure 12).

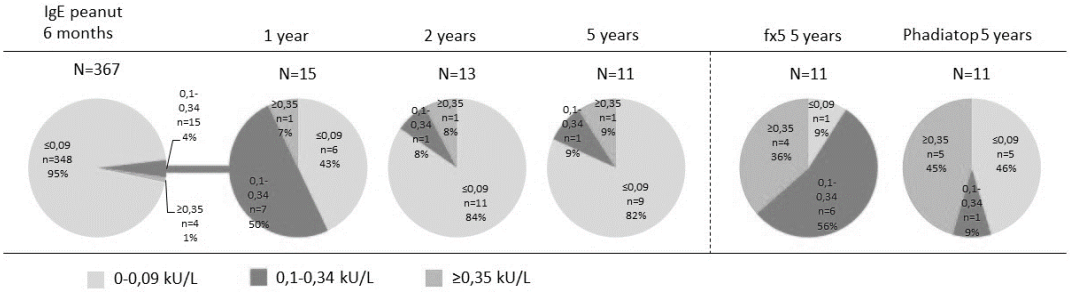

Figure 12 Development of low levels of s-IgE during the first five years to peanut.

In this study, only four children were sensitized to peanut at six months of age. In these children, the s-IgE levels increased during the five-year period. Despite the low number of peanut sensitized children at six months, the six months and five years median values were compared, showing no significant differences (p=0.465). (Figure 6b the manuscript).

Low levels of s-IgE has previously been investigated in another Swedish study[192]. Authors for that study categorized low levels as 0.1-0.7 kU/L, thus not entirely comparable with our study. Nevertheless, their results were similar showing an increased risk of sensitization at five years in the children with levels between 0.1 and 0.7 kU/L at six months of age.

Reactions to food is the most common form of allergy in infancy where egg and milk are the most common allergens. Egg sensitization is considered more persistent and associated with a worse prognosis compared to milk sensitization[26,193].
This study shows that following s-IgE concentrations, although low, in selected patient groups over time can be helpful guiding the physician. It can be discussed what the most appropriate cut-off level should be as 0.35 kU/L has little clinical relevance[194].
Stratification on lifestyle was not possible due to the limited number of sensitized children.

6.5 METHODOLOGICAL CONSIDERATIONS

The ALADDIN study has a prospective cohort design.
Before studies are initiated, a "statistical power" calculation is made to estimate the number of participants needed in the study which depends on the occurrence of the outcome in the population. The confidence intervals reflect precision of the studied association. In all studies, we have set the significance level at 0.05 which is standard. This means if we would do 100 analyses, 5 of the found associations would be due to chance.

There are, however, certain types of errors that may occur in epidemiological studies. Selection bias, misclassification bias and confounding in relation to my thesis will be addressed in the following section.

Selection bias
Selection bias means there is an unequal distribution of the exposure or outcome variables in the participants compared to the non-participants. An example would be that parental allergy would affect the decision to participate in a cohort study in only one of the studied (exposure) groups. In cohort studies, the risk of selection bias based on the outcome is small since the outcome has not occurred at inclusion.

Selection bias can occur at each follow-up as all longitudinal studies have some degree of drop-out. The participation rate based on blood sampling and distribution of background variables among the participants and non-participants are presented in Table 5.

In some cases, parents did not consent to blood sampling in the early age. Adding to that, some of the (blood sample) volumes were insufficient to analyse all allergens. In those cases, the most prevalent allergens, milk and egg, were prioritized.
There is a significantly lower number of blood samples at the first three sampling occasions from children of anthroposophic parents, a tendency also seen in the PARSIFAL study[150]. However, comparing those parents who did and did not consent to blood sampling showed no difference in parental sensitization, family history of atopic disease or reported allergies in the parents. Therefore, we do not believe participation in blood sampling would introduce selection bias. In addition, consent to blood sampling was made before the parents received any results regarding their own or their child's blood sample analyses.

Table 5 Distribution of baseline characteristics among the children and their parents at 6 months, 1, 2 and 5 years.

	6 months		1 year		2 years		5 years	
	n/N	%	n/N	%	n/N	%	n/N	%
Blood sample analyzed	370/552	*67*	377/552	*68*	399/552	*72*	336/552	*61*
Lifestyle								
Anthroposophic	71/120*	*60*	71/120*	*60*	86/120*	*72*	74/120	*62*
Partly anthro.	160/229	*70*	167/229	*73*	166/229	*73*	150/229	*66*
Non-anthro.	139/170	*81*	139/170	*81*	147/170	*86*	112/170	*66*
No lifestyle data	0/32	*0*	0/32	*0*	0/32	*0*	0/32	*0*
Mother sensitized	108/370	*30*	116/377	*31*	113/399	*28*	104/336	*31*
Father sensitized	163/359	*45*	170/368	*46*	176/387	*46*	149/325	*46*
Mother university educated	185/367	*50*	188/372	*51*	195/395	*49*	166/332	*50*
Mother smoking during pregnancy	19/366	*5*	19/371	*5*	22/395	*6*	15/332	*5*
Having older siblings	237/364	*65*	230/367	*63*	254/390	*65*	215/329	*65*
Gender (female)	186/370	*50*	192/377	*51*	202/399	*51*	178/336	*49*
Child received antibiotics <2mo	15/362	*4*	16/368	*4*	14/387	*4*	11/326	*3*
Child received antipyretics <2mo	18/361	*5*	22/366	*6*	18/386	*5*	16/325	*5*
Breastfeeding 6m								
Fully	96/369	*26*	96/376	*26*	104/396	*26*	102/333	*31*
Partly	211/369	*57*	217/376	*58*	229/396	*58*	183/333	*55*

*Significant at the 0.05 level.

Chi-2 test was used to compare distribution.

Selection bias could also be introduced by inclusion criteria for the studies. In all studies, we have compared the non-participants to the participants regarding background and exposure variables, and found no significant differences concerning parental sensitization or later sensitization in the children between those two groups. In study I, we randomized the selection of mother-child pairs to minimize selection bias. In study II we repeated the longitudinal analysis of the association between FAs in breast milk and sensitization up to two years in children with blood samples at all time-points and found that it did not substantially change our results. Similarly, in study III we repeated the analyses with a very strict inclusion criteria that required analysed blood samples at all four time-points which gave similar results, although broader CIs, as the original analyses.

Misclassification/information bias

Information bias means that the information collected about/from the participants is incorrect. This can lead to under/overestimating the studied association. In a cohort study, the exposure data is collected before the outcome occurs. The primary exposure in the ALADDIN study is lifestyle. In the cross-sectional, pre-ALADDIN study on atopy in anthroposophic school children, Alm *et al*. defined the degree of anthroposophy based on 15 exposure factors characteristic of an anthroposophic

lifestyle. At the planning of the ALADDIN cohort, the categorization of lifestyle was changed to minimize misclassification bias. Instead of researchers choosing lifestyle characteristics, the parents were asked about their lifestyle. Then, in the questionnaire answers, researchers could search for important exposure variables.

Most of our variables are based on questionnaire answers. It is always a balance between asking too detailed questions/taking too much time to answer, and still getting information accurate enough to be able to create reasonably good variables, a best estimate of reality.

Sensitization was the main outcome variable in articles II-IV. IgE in a serum sample is an objective measure with little or no degree of misclassification. IgE levels could serve as a marker for allergy that is not dependent on parents' or doctor's views on allergic disease, especially at young ages when clinical symptoms are sometimes difficult to interpret.
What is relevant though is the interpretation of the IgE value. A good question is what is the best, and most clinically relevant cut-off for sensitization?

In study I, the trace element concentrations were measured with ICP-MS in red blood cells and placenta. Like the IgE measurement, also mass spectrometry is an objective quantification method. The metal concentrations were used as quantitative variables and thus, no classification was made. The samples were analysed blinded and in a randomized order. It is standard to use quality control samples and blanks in the analysis.

It is also relevant to consider if the analysed sample was representative of the true metal concentration in the tissue. Although the placenta samples were washed several times to drain blood residue, it is possible that the sample contained clotted blood which could influence the results. The number of placenta samples available was limited in our study, which could make it more difficult to find true associations.

In study II the exposure variable was fatty acids in breast milk samples measured with gas chromatography with a flame ionisation detector. The samples were analysed in a randomized order.
We do not have information on the exact time of breast milk sample collection, if it was taken before or after meal intake, what the mothers ate that day etc. Detailed information would have given the opportunity to exclude that diurnal variation would have affected the composition. There are several studies on variation in milk FAs. The fatty acid composition does not show diurnal variation although the amount of milk fat varies over the course of the day[195]. The proportions of omega-3 and omega-6 fatty acids remain constant over time in mature milk[196]. It has also been shown that the milk FA composition does not change during a feeding[197]. In our study, we conducted a small pilot to see if foremilk or hindmilk would differ in FA concentration. We found that it did not affect the FA composition. A variation in FA composition could introduce bias in the association between FA composition and sensitization if the variation would be systematic. However, we do not believe such systematic variation exists.

Confounding

A confounder is a risk factor for the outcome that is also associated with the exposure but not an effect of the exposure. Confounding is always a possible problem in epidemiological studies. Known confounders can be included in the analyses or can be used for stratification. The problem occurs when the confounder(s) is/are unknown. The result of an analysis where confounders have been taken into account/adjusted for, is the true effect of the exposure and/or the effect of the exposure plus the effect of an unknown confounder. In the ALADDIN study, the main exposure variable is lifestyle. If separate lifestyle factors are included in a model and they are true confounders, it means they explain the "anthroposophic effect". The "anthroposophic effect" might be a combination of lifestyle factor effects, present in the anthroposophic lifestyle, that cannot be separated. A specific problem can arise if a variable is strongly correlated with lifestyle. In study I, we decided not to include organic/biodynamic diet in the analysis since it was too correlated with anthroposophic lifestyle.

Figure 13 shows the exposure and outcome variables for each study and the variables/confounders included in the analyses. However, unknown confounders can still be present.

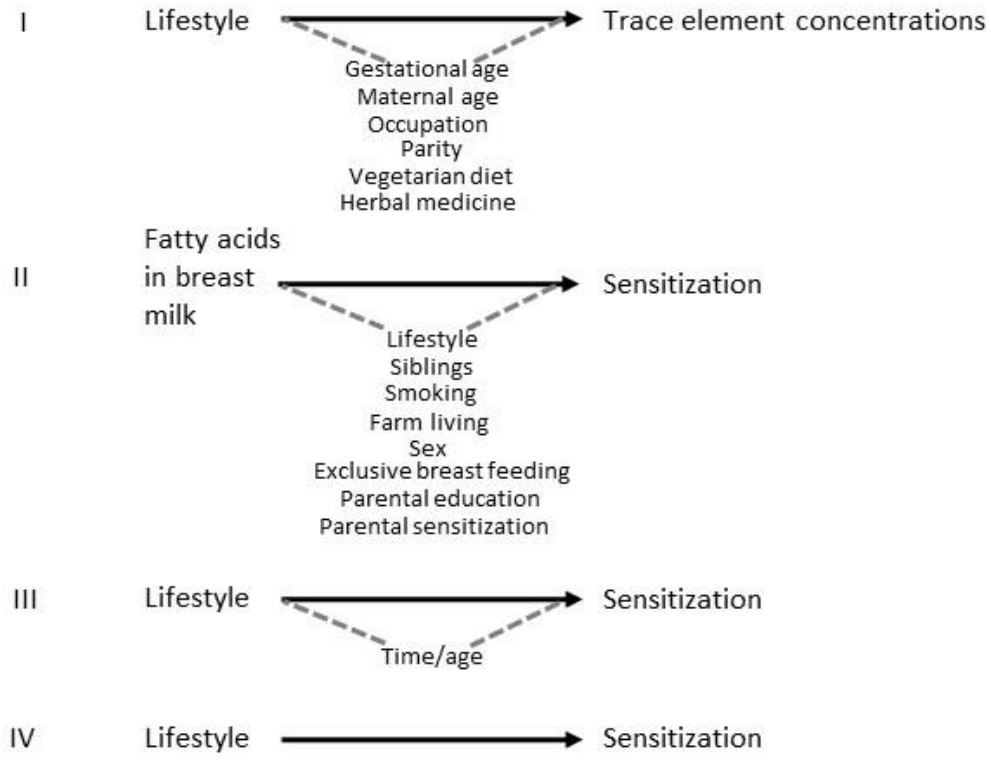

Figure 13 Exposure and outcome variables for studies I-IV and the variables/confounders included in the analyses.

6.5.1 Generalizability

Importantly, the populations included in the studies in this thesis did not differ from the total ALADDIN cohort regarding the main exposure variable, lifestyle, and the main outcome variable, sensitization, and thus the results of the studies are generalizable for the ALADDIN cohort. Additionally, the populations did not differ regarding parental sensitization.

Caution should always be taken when generalizing to a larger population. Compared to other epidemiological studies, the ALADDIN study is on the smaller side. The non-anthroposophic population (n=171) should be representative of the general population. Results can be strengthened by comparing to other studies showing similar results or by pooling results from similar studies.

Concerning the anthroposophic population studied and its' representativeness of other anthroposophic populations, we can compare our study to the previous cross-sectional studies[149,150]. There seem to be some differences between the Swedish anthroposophic population compared to other European anthroposophic populations which shows as differences in the protective effect associated with the anthroposophic lifestyle.

7 CONCLUSIONS

Based on the research and the articles included in this thesis, the following conclusions can be drawn.

- We found higher concentrations of cadmium, cobalt and lead in maternal blood and placenta samples from women with an anthroposophic lifestyle and in cord blood samples from their infants. None of the factors studied explained the lifestyle related differences in metal concentrations but one possible explanation could be a diet rich in plant-derived foods.

- We found an inverse association between omega-3 fatty acids in breast milk and sensitization in the child up to two years of age. This is in line with previous published results of many studies suggesting omega-3 PUFAs might protect the child from allergic disease in childhood. However, in our study this association did not explain the low prevalence of sensitization among children of families with an anthroposophic lifestyle.

- The lower prevalence of allergic sensitization seen among the anthroposophic children could be explained by the lower incidence of food allergen sensitization in the infancy in this group. It is possible that the allergy protective effect of the anthroposophic lifestyle is strongest in the early life.

- Early low levels of s-IgE to milk and egg seem to decrease over time although possibly increasing the probability of sensitization to inhalant allergens. Low levels to egg seem to be more transient than low levels to milk.

8 FUTURE PERSPECTIVES

The results from my thesis adds to previous knowledge about the development of allergic sensitization as well as about the characteristics of an anthroposophic population. No strong conclusions can be drawn from just single studies and therefore, the added knowledge is of great importance for public health work.

A general conclusion from my thesis, and likewise a conclusion in several other allergy cohorts, is that the prenatal time and infancy are important time windows for immune system development and maturation. The changes and differences seen early often persist as seen in prospective settings. Therefore, I believe detailed research in this time-frame is of importance for future allergy research.

An area receiving much attention today is food and diet. A majority of the risk factors for allergic disease are related to food and diet. I believe research has a great responsibility to provide correct and objective information about what is known in the field. Information is easily available over the Internet and therefore, it is important that authorities are consistent in their recommendations.

Methods today offer possibilities we could not dream of when ALADDIN started in 2004. Now it is possible to study single cells in detail, conduct metabolomic, epigenomic and genome wide analyses. Some of these methods can be applied to the existing material, broadening our knowledge about sensitization development in our cohort even more.

I would like to end with a section from Susan Prescott's article on developmental origin of allergic disease[45].

"..holistic approaches are more likely to simultaneously modify a variety of innate immune responses – analogous to the multiple environmental changes currently acting simultaneously on many organ systems to exert chronic inflammatory changes".

The anthroposophic lifestyle might be just that.

9 SVENSK SAMMANFATTNING

Allergisjukdomar har stadigt ökat under det senaste seklet för att nu utgöra en del av vår tids folksjukdomar. Allergier drabbar nu nästan vartannat barn i västvärlden och debuterar ofta som födoämnesallergi, speciellt mot mjölk och ägg.

Grunden i sjukdomen är ett avvikande beteende hos immunsystemet där kroppens immunceller börjat försvara sig mot ofarliga ämnen (allergener) i vår omgivning. Om så kallade IgE antikroppar produceras mot allergenet, benämns det allergisk sensibilisering.

Orsaken till allergiökningen är fortfarande till stor del okänd. Ärftlighet är en viktig faktor men kan inte förklara den snabba ökningen. Orsaken tros vara förändringar i livsstil, som i kombination med omgivningsfaktorer påverkar vårt immunsystem. Till sådana faktorer kan räknas mikrober, infektioner, förlossningssätt, amning, stress, rökning, miljöföroreningar och kemikalier i vår hemmiljö. Studier har visat att barn uppväxta på bondgårdar med nära kontakt till djur och som dricker opastöriserad komjölk har en lägre förekomst av allergier.

Vi har tidigare visat att barn i familjer med en antroposofisk livsföring har en låg förekomst av allergier. Den antroposofiska livsstilen innefattar flera hypotetiskt intressanta aspekter, bl.a. väljer de flesta att äta ekologiskt eller biodynamiskt producerad mat och mjölksyrade grönsaker innehållande levande bakteriekultur. Barnen föds ofta hemma och den antroposofiska sjukvården har en restriktiv syn på användningen av vaccinationer, antibiotika och febernedsättande läkemedel. I stället används ofta homeopatiska naturläkemedel.

Mitt avhandlingsarbete utgår från den prospektiva födelsekohorten ALADDIN (Assessment of Lifestyle and Allergic Disease During INfancy) där vi undersöker och försöker identifiera livsstils- och omgivningsfaktorer som påverkar allergiutvecklingen.

I **arbete I** undersökte vi om mammor med en antroposofisk livsstil har en lägre exponering för potentiellt immunstörande toxiska metaller. Vi mätte koncentrationerna av tre toxiska och nio essentiella metaller i blod från mamman, i placenta och i navelsträngsblod hos 40 mamma-barnpar med och 40 utan en antroposofisk livsstil. Vi fann högre nivåer av kadmium, bly och kobolt i mamma-barnpar med en antroposofisk livsstil. Orsaken till denna skillnad kunde inte förklaras med någon av de specifika livsstilsfaktorer vi registrerat. Trots de högre nivåerna var ändå alla metallkoncentrationer inom nivåer som anses normala.

I **arbete II** undersökte vi om den lägre förekomsten av allergisk sensibilisering hos barn i antroposofiska familjer kunde bero på en skillnad i bröstmjölkens fettsyresammansättning. Vi mätte koncentrationerna av långkedjade fleromättade fettsyror i bröstmjölksprover från 225 mammor och undersökte förekomsten av allergisk sensibilisering fram till två års ålder hos barnet. Vi såg att högre halter av

omega-3-fettsyror i bröstmjölken var kopplat till en lägre risk för allergisk sensibilisering. Detta förhållande kunde dock inte förklara den lägre förekomsten av sensibilisering hos barn i antroposofiska familjer.

I **arbete III** undersökte vi om incidensen (insjuknandet) och prevalensen (förekomsten) av födoämnes-, djur- och pollensensibilisering fram till fem års ålder påverkades av barnets ålder. Vi undersökte 100 antroposofiska, 209 delvis antroposofiska och 165 icke-antroposofiska barn. Vi fann en lägre incidens av födoämnessensibilisering hos barn tillhörande antroposofiska familjer. Vi kunde därmed förklara att den lägre förekomsten av allergisk sensibilisering hos barn från antroposofiska familjer nästan uteslutande beror på en lägre risk för födoämnessensibilisering före ett års ålder.

I **arbete IV** har vi beskrivit förloppet av IgE-sensibilisering mot ägg, mjölk och jordnöt fram till fem års ålder hos 372 barn. Vi delade in IgE-koncentrationerna i icke-sensibiliserade ($\leq$ 0,09 kU/L), låga nivåer (0,1-0,34 kU/L) och sensibiliserade ($\geq$ 0,35 kU/L). Vi kunde se att de barn som hade låga IgE-nivåer av ägg och mjölk vid sex månaders ålder blev färre till antalet över tid, men att det samtidigt kunde öka risken att senare sensibiliseras mot ett luftburet allergen. Låga nivåer mot ägg visade sig vara mer övergående än låga nivåer mot mjölk.

Sammanfattningsvis beskriver denna avhandling, tillsammans med tidigare arbeten inom ALADDIN-kohorten, att tidig exponering för livsstil och omgivningsfaktorer har stor betydelse för immunsystemets mognad och utveckling av allergisk sjukdom.

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