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LIFESTYLE AND ALLERGY- IN RELATION TO VIRAL INFECTIONS AND GUT MICROBIOTA

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LIFESTYLE AND ALLERGY- IN RELATION TO VIRAL INFECTIONS AND GUT MICROBIOTA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Allergy-related diseases such as food allergy, eczema, asthma and rhinoconjunctivitis affect nearly half of Swedish children before twelve years of age and are more prevalent in populations with westernized lifestyle. Reduced microbial exposure very early in life is believed to play a crucial role for this increased risk. Children of families with an anthroposophic lifestyle are less commonly affected. The aim of this thesis was to study associations between this lifestyle and development of allergy-related disease and the possible role of microbial exposure in the form of herpesviruses and gut microbiota. All four papers in this thesis are based on the prospective birth cohort study ALADDIN (Assessment of Lifestyle and Allergic Disease during INfancy) in which children with anthroposophic and non-anthroposophic lifestyles have been followed up with questionnaires, examinations, parental interviews and blood- and fecal samples.

In **paper I** we determined IgG-levels towards Epstein-Barr virus, HHV6, HHV7 and cytomegalovirus in blood samples from 62 anthroposophic and 95 non-anthroposophic children at one and two years of age and from their parents. Seroprevalence of these herpesviruses was similar in the lifestyle groups among both children and parents and is therefore unlikely to explain the reduced risk of sensitization among anthroposophic children. In **paper II** we analyzed the bacterial composition in fecal samples from 55 anthroposophic and 73 non-anthroposophic infants at six days, three weeks, two months and six months of age and from their mothers, using pyrosequencing of 16S rRNA genes. Mode of delivery and breastfeeding were stronger determinants of the infant gut microbiota than anthroposophic lifestyle. At six months anthroposophic lifestyle was associated with higher abundance of *Bifidobacterium* and lower abundance of *Bacteroides.* No associations with anthroposophic lifestyle were seen up to two months of age or in the mothers. Global microbiota diversity was not influenced by anthroposophic lifestyle and is therefore unlikely to mediate the risk-reducing effect on sensitization. Further studies, with higher taxonomic resolution and deeper coverage, could better clarify a potential role of gut microbiota. In **paper III** we investigated the effect of lifestyle on the risk of clinical allergy-related manifestations up to two years of age in 116 anthroposophic, 212 partly anthroposophic and 162 non-anthroposophic children. Risk of food hypersensitivity and recurrent wheeze, but not eczema, was reduced among children with anthropsophic and partly anthroposophic lifestyle. Delayed wash of the newborn's whole body was associated with reduced risk of food hypersensitivity, eczema and sensitization whereas recurrent wheeze was associated with maternal level of education and child having had milk formula during the first week of life. The 'anthroposophic effect' however remains largely unexplained. In **paper IV** we described incidence and prevalence of sensitization to food, animal and pollen allergens up to five years of age for 100 anthroposophic, 209 partly anthroposophic and 165 non-anthroposophic children. The effect of lifestyle on food sensitization differed significantly with age of the child. The reduced prevalence of sensitization among children from families with an anthroposophic lifestyle was mainly explained by a low risk of food allergen sensitization before one year of age.

In conclusion, this thesis illustrates the strong influence of very early lifestyle exposures on allergy-related outcomes, but also the complexity of studying lifestyle in relation to disease. The convincing findings of association between anthroposophic lifestyle and allergy-related outcomes make the ALADDIN cohort a 'model' for studying how lifestyle affects the development of allergy, regardless of what the 'anthroposophic factor' might be.

LIST OF SCIENTIFIC PAPERS

I. **Hesla HM**, Gutzeit C, Stenius F, Scheynius A, Dahl H, Linde A, Alm J. Herpesvirus infections and allergic sensitization in children of families with anthroposophic and non-anthroposophic lifestyle - the ALADDIN birth cohort. *Pediatric allergy and immunology*. 2013;24(1):61-65.

II. **Hesla HM**, Stenius F, Jäderlund L, Nelson R, Engstrand L, Alm J, Dicksved J. Impact of lifestyle on the gut microbiota of healthy infants and their mothers – the ALADDIN birth cohort. *FEMS Microbiol Ecol*. 2014;90(3):791-801

III. **Hesla HM**, Stenius F, Järnbert-Pettersson H, Alm J. Allergy-related disease in relation to early life exposures – the ALADDIN birth cohort. (Submitted manuscript)

IV. Fagerstedt S, **Hesla HM**, Ekhager E, Rosenlund H, Mie A, Benson L, Scheynius A, Alm J. Lifestyle Reduces Sensitization to food allergens in infancy – the ALADDIN cohort. (Submitted manuscript)

CONTENTS

LIST OF ABBREVIATIONS

ALADDIN	Assessment of Lifestyle and Allergic Disease During INfancy
CI	Confidence Interval
CMV	Cytomegalovirus
EBV	Epstein-Barr Virus
GEE	General Estimating Equations
HHV	Human Herpesvirus
IgE	Immunoglobulin E
IgG	Immunoglobulin G
kU_A/L	Kilo-units of antibodies per liter
MCHC	Mother-Child Health Center
OR	Odds ratio
PCoA	Principal coordinates analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational multivariate analysis of variance
SCORAD	SCORing Atopic Dermatitis

1 BACKGROUND

1.1 ALLERGY-RELATED DISEASES

The allergy-related diseases constitute a heterogeneous entity of symptoms, signs and reactions which include asthma, food allergy, dermatitis (eczema) and rhinoconjunctivitis. They are symptoms of hypersensitivity, meaning that they are initiated by exposure to a defined stimulus at a dose tolerated by normal persons, and usually mediated by inflammation[1]. Asthma, rhinoconjunctivitis and dermatitis are classified as *allergic* if the hypersensitivity reactions are initiated by specific immunologic mechanisms and can in turn be *atopic* (IgE-mediated) or *non-atopic* (non-IgE-mediated). Atopy is a personal inclination to respond with IgE antibody production to usually tolerated proteins (allergens)[1].

Asthma is a heterogeneous disease, especially in children. Recurrent wheeze during the first years of life is commonly non-atopic and related to airway infections and commonly outgrown before school-age. Onset of wheeze after two years of age or in combination with other allergy-related disease is more strongly associated with persistent disease[2].

Food allergy is commonly diagnosed by history of reactions to exposure to a food allergen in combination with detection of IgE-antibodies, either by measurement in serum or by skin prick test, or in uncertain cases, by oral food challenge, which is used both for confirming and for excluding food allergy. Of the most common childhood food allergies, cow's milk and hen's egg allergies are usually outgrown, whereas peanut allergy is more persistent. Food allergy can also be non-IgE-mediated.[3].

Eczema is a term that is used in parallel with atopic dermatitis and is defined as a chronic, itching inflammatory skin condition, localized to typical skin areas often in combination with personal or family history of other allergy-related disease[4]. The SCORing of Atopic Dermatitis (SCORAD) index is a validated instrument for measuring severity of eczema[5,6].

Allergic rhinoconjunctivitis is an IgE-mediated inflammatory reaction in nasal membranes and the conjunctiva. Intermittent allergic rhinoconjunctivitis is commonly triggered by pollen allergens, such as classic hay fever, and persistent allergic rhinoconjunctivitis is often triggered by indoor allergens such as pets, dust mite or mold[7].

Sensitization is occurrence of allergen specific IgE-antibodies which can be detected with skin prick test or in serum/plasma. In a skin prick test a small amount of allergen-extract is introduced into the skin. If the person has allergen specific IgE-antibodies bound to mast cells in the skin, histamine will be released and cause a local reaction in form of a wheal and redness, which is a positive reaction. Skin prick testing has the advantage of an immediate result, but is limited to a number of available standardized allergen extracts. Blood sample based IgE-analysis, which is available for several hundred different allergens, uses enzyme-linked anti-IgE antibodies and allows for quantitative measurement of allergen specific IgE from a concentration of less than 0.1 kU_A/L. Traditionally a cut-off level of 0.35 kU_A/L has been used for determination of sensitization. Detection of allergen specific IgE in

serum/plasma or by skin prick test is not equal to allergy but the levels of IgE are related to likelihood of clinical symptoms. In addition, screening panels for common allergens have a high negative predictive value for clinical IgE-mediated allergy[8].

An age associated variation in allergy-related symptoms in childhood is typically seen in a population and referred to as the 'atopic march'. The first allergy-related symptoms are commonly eczema and food allergy, later followed by asthma and rhinoconjunctivitis[9,10]. The idea of 'atopic march' is however not necessarily applicable at an individual level[11].

There is a wide range in severity of the allergy-related diseases. Symptoms range from very mild, such as an itching nose a few days during birch pollen season or a few spots of dry, itchy skin during the winter season, to disabling disease such as severe treatment resistant asthma or eczema and life-threatening anaphylactic reactions.

Large geographical variations in prevalence of the allergy-related diseases have been demonstrated, with higher occurrence in westernized countries[12]. In a Swedish population-based cohort as many as 58 % of 12-year-olds had ever had one of the allergy-related manifestations eczema, asthma or rhinitis. Eczema was the most common manifestation and was most prevalent up to four years of age (15-18 %) and then decreasing. Asthma prevalence was rather stable from two to twelve years of age, around 6 %, whereas prevalence of rhinitis increased with age and was reported for 20 % of the twelve-year-olds[13]. In the same cohort, prevalence of doctors-diagnosed food allergy was 3.1 % at one year and 7.6 % at eight years of age[14].

1.2 LIFESTYLE AND ALLERGY

1.2.1 The 'hygiene hypothesis'

It is a general understanding that allergy-related diseases were rare a hundred years ago and that there has been a substantial increase in prevalence the last half century[15]. Prevalence of asthma and airway allergies appears to have plateaued in high risk areas but food allergies still seem to be increasing worldwide[16-18]. Even if heredity is a significant risk factor for allergy-related disease, the large increase during the last half century cannot be explained by genetics alone, since such large changes to our genome can unlikely have occurred in such short time. Lifestyle-related and environmental exposures are therefore believed to interact with our genes in the development of allergy-related disease [19], and westernized lifestyle has been attributed a substantial part of the increase. Studies of populations of same ethnicity but differential exposure to modern, urban, westernized lifestyle have revealed a lower prevalence of allergy-related disease in East *vs* West Germany[20], Russian *vs* Finnish Karelia[21], mainland China *vs* Hong Kong[22] and among children growing up in farming *vs* non-farming environment[23,24].

The prevailing hypothesis is that a decreased exposure to infectious and non-infectious micro-organisms is a possible explanation for the 'allergy-promoting' effect of modern lifestyle[25]. This is commonly called the 'hygiene hypothesis' even if other, perhaps more

suitable, names have been suggested, such as ‘old friends hypothesis’ and ‘biodiversity hypothesis’. The original ‘hygiene hypothesis’ was stipulated in 1989 by the British epidemiologist David Strachan based on his findings in an epidemiological study that the risk of hay fever in children was inversely related to number of older siblings[26]. He hypothesized that unhygienic contact with older siblings leads to transmission of common viral infections which in turn leads to reduced risk of hay fever. The association between older siblings and allergy-related disease has been verified in several studies, but mechanistic explanations for this association are lacking[27-29] and smaller family size is not considered a major factor for the increased allergy burden[30] .

1.2.2 Lifestyle-related exposures

Most lifestyle-related exposures that have been studied in relation to development of allergy-related disease are directly or indirectly related to infections or non-infectious exposure to microbes, but also other factors associated with modern lifestyle such as dietary factors, tobacco smoke, air pollution, timing for - and extent of - allergen exposure and exposure to stress have been studied.

The underlying immunologic mechanisms for the lifestyle-induced increase and for the gene-environment interactions in the development of allergy-related disease are not fully understood. Allergic inflammation is a Th2 weighted immune response, and it is believed that microbial exposure regulates the balance between Th1 and Th2 immune response by suppression via regulatory T cells and cells of the innate immune system[31]. Epigenetic mechanisms could, at least to some extent, mediate the interaction between environmental factors and genetics in the pathogenesis of allergy-related diseases[19].

1.2.2.1 Infections

In line with the original ‘hygiene hypothesis’ some studies indicated an inverse relationship between number of reported early childhood infections and risk of atopy[32,33] but others did not[34,35] and the mere quantity of early unspecified respiratory viral infections is no longer believed to explain neither the sibling-effect nor the ‘westernization-effect’[36]. Presence of IgG against hepatitis A, *Toxoplasma gondii* and *Helicobacter pylori*[37,38] has been negatively associated with atopy. However, since these infections are more common under less hygienic conditions, seropositivity to these antigens may rather be markers of a more diverse microbial exposure[31]. Some helminth (parasitic worms) infections have been negatively associated with allergy-related disease with strong evidence of a protective effect for early and chronic infection[25,39].

Herpesviruses

There are eight known human herpesviruses (HHV)[40]. They all establish lifelong infection in the host and could be regarded as part of the human ‘microbiome’. The ability of latency requires evasion or neutralization of the host’s immune system through immuno-modulatory mechanisms[40]. Epstein-Barr virus (EBV) is a herpesvirus that has been related to the risk of

sensitization to allergens. Early infection, before two years of age, has been associated with reduced risk of sensitization whereas later EBV infection rather seems to promote allergy development[34,41-44]. One possible immune-modulatory mechanism could be that EBV DNA encodes a protein that resembles human interleukin-10, a cytokine that is important in regulating immune responses[40]. Studies of other herpesviruses and association with risk of sensitization have been inconclusive, but potential association has been seen for cytomegalovirus (CMV) and HHV6[34,41,45]. HHV7 is a more recently discovered herpesvirus which is widespread among healthy children and whose relevance in allergy development has not been studied[46]. Nearly 100 % of adults worldwide have been infected with EBV but in children seroprevalence (the proportion of seropositive individuals in a population) is dependent on lifestyle[47]. In affluent countries, where allergic diseases are more prevalent, children seroconvert later to EBV and CMV than in non-affluent countries[47-49].

1.2.2.2 Non-infectious microbial exposure

Most microbes in our environment are not pathogenic but nevertheless interact with our immune system. High diversity of microbial exposure has been shown to mediate the protective effect of farming on asthma development[50]. A recent study demonstrated an inverse relationship between amount of green environment (forest and agricultural land) around the family home and the risk of sensitization in children, possibly mediated by environmental diversity[51]. One important route of exposure to microbes and other antigens is via the gastrointestinal canal, where indeed almost 70 % of the human lymphoid tissue is found[52].

Gut microbiota

The adult human intestinal canal is inhabited by an estimated 10^{14} microbes, mainly bacteria and mainly in the colon[53]. The magnitude of the number of microbes is ten times the estimated number of human cells in our body. The gut microbiota has several beneficial functions for the host, including fermentation of indigestible carbohydrates into short chain fatty acids, immune system maturation and protection from invasion of exogenous microbes[54]. The gut of the newborn is considered sterile, although there is some evidence of bacterial exposure *in utero*[55,56]. Colonization of the infant gut starts at delivery and the gut microbiota gradually develops up to two to three years of age when it resembles the adult one[55]. The gut microbiota composition is influenced by heredity[57,58] but also by environmental exposures such as delivery mode, diet and antibiotics[59-62]. The establishment of the gut microbiota is considered important for the development of the immune system and induction of oral tolerance[63,64].

The ‘hygiene hypothesis’ led to large interest in the role of the gut microbiota in the development of allergy-related disease. Both earlier, culture-based, and more recent, culture independent molecular-based studies have indicated that reduced diversity of early infant gut microbiota is associated with increased risk of later allergic disease, especially eczema[65-70]. Species of *Bifidobacterium*, *Lactobacillus* and *Bacteroides* have been regarded as beneficial in the aspect of allergy-related disease, whereas *Clostridium difficile* and *Staphylococcus*

aureus have been associated with allergy development[65,66,71] but findings have been inconsistent and comparisons between studies are difficult due to large methodological differences.

Traditionally gut microbiota studies have been culture-based. However such studies do not give a representative picture, since only around 20 % of gut microbes can be cultivated[72]. In recent decades, there has been a tremendous development in the biotechnological field which has led to development of molecular techniques to explore the composition and function of the gut microbiota. Most commonly they are based upon the analysis of the prokaryotic 16S ribosomal RNA (16SrRNA) gene that is present in all prokaryotes. This gene has evolutionarily conserved regions that enable accurate alignment and at the same time sufficient variable regions for species detection. The most common techniques rely on sequencing of PCR-amplicons of this gene, which can be matched with an extensive public DNA database to determine phylogenetic origin of the sequences. These high throughput sequencing techniques, one of which is 454-pyrosequencing[73], allow for larger study populations, but also generate large datasets that require advanced biometrical tools for interpretation[74].

1.2.2.3 Allergen exposure

The importance of timing and type of exposure to different allergens has been studied in relation to development of allergy-related outcomes. Keeping pets during pregnancy and infancy has been associated with reduced risk of later sensitization to aero-allergens, but results have been conflicting concerning protection against allergy-related disease[75,76]. A possible association between pet ownership and atopy could however also be mediated through microbial exposure, since pet exposure has been demonstrated to have an impact on infant gut microbiota[77]. Introduction of food and the relation to breastfeeding has also been studied in relation to atopy, and evidence for a protective effect of delaying introduction of solids beyond four to six months of age is lacking and may rather increase the risk.[3,78] The 'dual allergen exposure hypothesis' proposes that allergic sensitization to food occurs through cutaneous exposure, whereas oral exposure induces tolerance[79].

1.2.2.4 Diet/breastfeeding

Dietary factors are highly influenced by lifestyle and a possible means for allergy-prevention. Recent research indicates that maternal intake of fish and fish oil[80] and antioxidants[81,82] during pregnancy could be associated with risk of allergy in the child, however data is insufficient for conclusions. Vitamin D deficiency, attributable to use of sunscreen and more time spent indoors, has been associated with increased risk of sensitization[83]. Such an effect is however less likely in populations with widespread vitamin D supplementation to infants such as the Swedish one. Probiotics seem to have a beneficial effect on childhood sensitization, if administered prenatally, but not on asthma and not if only administered postnatally to infant[84]. Diversity of food in infancy was recently shown to be inversely related to later food allergy and asthma[85]. Duration and extent of breastfeeding has been

extensively studied in relation to development of allergy-related outcomes, but results are conflicting and evidence of a protective effect is weak other than for early childhood upper respiratory infection-associated wheeze[78].

1.2.2.5 Smoking and air pollution

Prenatal and early life exposure to second hand tobacco smoking increases the risk of asthma, rhinitis and eczema, especially in early childhood, but also persisting into adolescence[86]. Studies have been less consistent about association between exposure to tobacco smoke and sensitization in some studies[87-89]. Similarly, exposure to air pollutants is associated with both exacerbation and incidence of asthma, but evidence is less convincing for allergic asthma and sensitization[90].

1.2.2.6 Mode of delivery

Rates of caesarean section have increased from 5% in 1973 to 17% in 2013 in Sweden[91] and delivery by caesarean section has been shown to strongly influence the infant gut microbiota[59,60]. A possible effect of mode of delivery on allergy-related disease has been attributed to effects on microbial exposure, including gut microbiota establishment[92]. Results from cohort studies on the association between caesarean mode of delivery and risk of atopy have been inconsistent, where some show no association[93,94] and others indicate a higher risk[95,96] possibly modified by hereditary status of the child. For asthma, studies have also found adverse association with caesarean delivery, however the effect of delivery mode has been smaller than for atopy and likely confounded by the indication for the caesarean section[96,97].

1.2.2.7 Antibiotics/paracetamol

Early exposure to antibiotics and paracetamol have been associated with increased prevalence of subsequent wheeze and asthma[98-100], however the effect of confounding by early respiratory viral infections is difficult to address. In a recent publication the association between early antibiotics and later asthma seemed completely based upon reversed causation[101]. Antibiotics exposure does not seem to influence the risk of sensitization or eczema[98].

1.2.2.8 Stress

Stress is an environmental factor which is associated with morbidity in already existing disease like asthma and eczema but also thought to play a role in the development of allergy-related disease[102]. Differential immunologic responses to stress have been demonstrated between atopic and non-atopic individuals, possibly mediated by differential cortisol excretion[103]. In the ALADDIN birth cohort, higher salivary cortisol levels at six months of age was associated with increased risk of later sensitization[104]. Cortisol-levels were however not associated with parental sense of coherence, which could indicate that other mechanisms than stress might mediate the increased salivary cortisol levels[105].

1.2.3 Anthroposophic lifestyle and allergy

Several of the lifestyle-related exposures mentioned above are associated with anthroposophic lifestyle, which in turn has been associated with reduced risk of allergy-related disease in children[106-109]. Anthroposophy is a holistic philosophy founded in the beginning of the 20th century by the Austrian philosopher Rudolf Steiner. The philosophy applies to many aspects of life such as medicine, education, art, architecture, agriculture. Anthroposophic medicine is a form of alternative medicine that often includes highly diluted substances, similar to homeopathic, often produced by anthroposophic pharmaceutical companies. It includes restrictive usage of antibiotics, antipyretics and vaccines and often home births. Anthroposophic schools are called Steiner or Waldorf schools. Food is typically produced by biodynamic farming which is organic but also has a spiritual perspective. In Järna, south of Stockholm in Sweden, an anthroposophic center is situated with a large community of anthroposophic followers, an anthroposophic cultural center and an anthroposophic hospital – Vidarkliniken[106,109,110].

1.2.3.1 Previous studies

The idea that children in anthroposophic schools seemed to have lower prevalence of allergic diseases came from personal observations by members of our study group and after discussions with a teacher at an anthroposophic school in Järna in the mid-90's[109]. The hypothesis that anthroposophic lifestyle, which includes many aspects interesting from the point of view of the 'hygiene hypothesis', is associated with reduced risk of allergic disease was tested, and confirmed, in a cross-sectional study of 295 Steiner school children and 380 children from conventional schools in 1997[107]. Steiner school children, who often come from families with an anthroposophic lifestyle, had lower prevalence of reported asthma, atopic dermatitis and allergic rhinoconjunctivitis and also of allergic sensitization. The findings were confirmed in a large European cross-sectional multicenter study, PARSIFAL[108]. However, despite the obvious association between anthroposophic lifestyle and allergic disease, specific exposure factors responsible for this association could not be identified.

Three limitations were identified with the cross-sectional studies, when it came to identifying specific allergy-related lifestyle factors: 1) Exposure data was collected retrospectively, which introduces the risk of recall bias, for example allergic status of the child and/or the parent's choice of lifestyle could influence how he or she reports duration of breastfeeding, smoking or use of antibiotics for the child's first years. 2) Anthroposophic lifestyle is characterized by a wide variety of typical exposures, so commonly in families who have chosen an anthroposophic lifestyle; the children are often exposed to the same typical factors. For example, in an anthroposophic family the child is commonly born at home and served fermented vegetables but also vaccinated to a lesser extent. This would make it difficult to point out one (or a few) factors that could explain the reduced allergy-risk, even if there was one, unless the study-population is very large. 3) The studies were conducted among school-aged children. The prevailing theory is that very early in life, possibly even during pregnancy, is an important time for the development of the immune system in relation to environmental

exposures. Thus thorough characterization of anthroposophic lifestyle at this time of life could serve as a means of identifying specific lifestyle exposures that are associated with development of allergy-related disease.

Since anthroposophic lifestyle appeared to be representative of non-westernized lifestyle, or at least of non-allergy-promoting lifestyle, and there was need for a better understanding of the origin of allergy-related disease and its relation to lifestyle, the birth cohort study Assessment of Lifestyle and Allergic Disease During INfancy (ALADDIN) was initiated in 2004. The birth cohort design addressed limitation 1 above by prospective collection of exposure data, before allergy-related disease develops and limitation 3 by collecting exposure data already during pregnancy. Limitation 2 was addressed through the population based design, with inclusion of not only the most purely anthroposophic families, but all families who attend anthroposophic or conventional maternal-child health centers in Järna. In this way part of the cohort was expected to be variably exposed to typical anthroposophic lifestyle factors, in other words 'partly anthroposophic'.

Results from this cohort study have confirmed a lower prevalence of allergic sensitization among children of families with an anthroposophic lifestyle, and this was seen already at six months of age[111]. Again, no specific lifestyle factors could be identified that explained the reduced risk of sensitization, but the anthroposophic children had significantly lower levels of salivary cortisol at six months of age[112] and salivary cortisol could in turn be associated with sensitization, eczema and food hypersensitivity[104].

After initiation of the study decision was taken to extend the inclusion number from 330 to 550 families, allowing for better studies of the clinical allergy-related outcomes which occur less frequently than sensitization, and also for better chances of identifying specific allergy-associated lifestyle factors.

1.3 PROBLEM FORMULATION

One could consider the high prevalence of allergy-related disease a 'price worth paying' for the beneficial health effects, including low child mortality rates and long life expectancy, of westernized lifestyle. However farming, which has consistently been associated with reduced risk of allergy-related outcomes, is not associated with lower life expectancy[113]. It is therefore unlikely that the 'allergy-protective' factors of farming and anthroposophic lifestyle have serious adverse health effects and thorough characterization of these lifestyles could give rise to strategies for allergy-prevention. The finding of large differences in sensitization already at six months of age between children of families with anthroposophic and non-anthroposophic lifestyle, who had equal heredity[111], supports the idea that protective or promoting factors should be sought among pre- or perinatal and very early life exposures.

- Timing for herpesvirus infections, such as EBV-infection, and the establishment of infant gut microbiota have been associated with both lifestyle and development of

atopy. We therefore hypothesized that herpesvirus infections and/or gut microbiota could mediate the 'allergy-protective' effect of anthroposophic lifestyle. This was investigated in **papers I and II** respectively. Due to the rapid development of methods for analysis and interpretation of gut microbiota there is need for studies of the establishment of infant gut microbiota and its determinants, using high throughput sequencing technology. This was also approached in **paper II**.

- A risk-reducing effect on sensitization was seen already at six months of age, but the effect of anthroposophic lifestyle on allergy-related symptoms such as food allergy, wheeze and eczema before school-age has not yet been studied. In addition, specific lifestyle-related exposures that could explain the 'allergy-protective' effect of anthroposophic lifestyle have not yet been identified. Both these problems were approached in **paper III**.

- The findings on sensitization in ALADDIN indicated that the effect of anthroposophic lifestyle was more pronounced at six months of age than later, and for food-allergens more than for animal- or pollen-allergens. This was further explored in paper IV.

2 AIMS

The overall aim of the ALADDIN-study, and of this thesis, is to increase the understanding of how lifestyle exposures during pregnancy and early childhood can influence the development of allergy-related disease. Specific aims in this thesis were:

- To investigate if anthroposophic lifestyle is associated with seroprevalence of the four herpesviruses EBV, CMV, HHV6 and HHV7 among children at one and two years of age (I).

- To study associations between certain lifestyle-related exposures, including anthroposophic lifestyle, and the gut microbiota composition in infants up to six months of age and in their mothers (II).

- To analyze associations between anthroposophic lifestyle, sensitization and the allergy-related manifestations food hypersensitivity, recurrent wheeze and eczema up to two years of age (III).

- To investigate if the effect of anthroposophic lifestyle on sensitization to food, animal and/or pollen allergens in children up to five years of age differs with age of the child (IV).

3 MATERIALS AND METHODS

3.1 THE ALADDIN BIRTH COHORT

The four papers in this thesis are based on the prospective birth cohort study ALADDIN (Assessment of Lifestyle and Allergic Disease During INfancy), which was started in 2004.

3.1.1 Study design

Between November 2004 and March 2011, 552 children were consecutively, and in parallel, enrolled from anthroposophic Maternal-Child Health Centers (MCHCs) (N=312) in Järna and Stockholm and from conventional MCHCs (N=240) in Järna and Södertälje. Of these, 444 were recruited during pregnancy, and 108 after birth. The families were informed by the midwife or nurse about the study and then, if they agreed, given oral and written information from one of the study doctors before enrollment. Exclusion criteria were delivery before gestational week 36 or miscarriage.

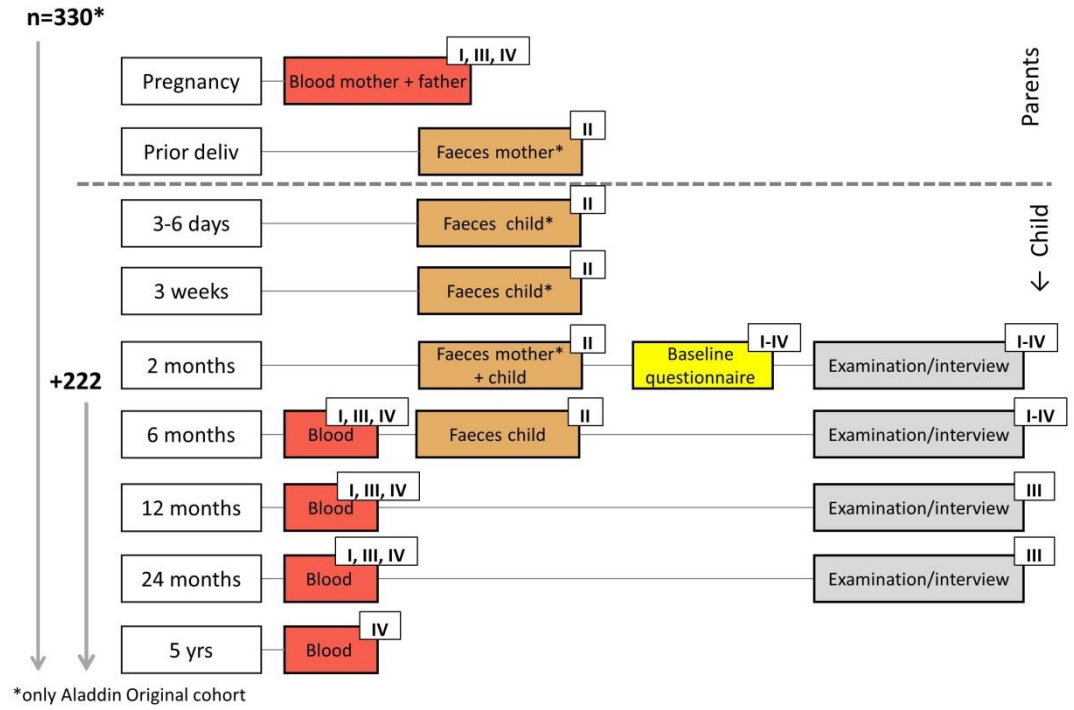

Figure 1. Flow-chart of data collection in ALADDIN for the studies in this thesis (depicted by roman numbers I-IV).

3.1.2 Classification of anthroposophic lifestyle exposure

The classification of the exposure anthroposophic lifestyle was based upon, in addition to choice of antenatal clinic, the parents' answers to three baseline questionnaire questions that were filled in before two months of age of the child. These three questions were: 1) 'What

kind of preschool/school will your newborn child probably go to?', 2) 'Has any of the parents, no matter which type of school you have planned for your child, an anthroposophic view of life?', and 3) 'Is the family's everyday life influenced by an anthroposophic view of life?'. Families answering 'anthroposophic school' to question 1 and 'yes' to questions 2 and 3, and also attending anthroposophic MCHCs were defined as 'anthroposophic'. Families answering conventional or any other non-anthroposophic type of school to question 1, 'no' to questions 2 and 3, and attending conventional MCHCs were defined as 'non-anthroposophic'. Families with any other combination of answers were defined as partly anthroposophic.

3.2 STUDY POPULATIONS PAPERS I-IV

Paper I
Eligible: anthroposophic and non-anthroposophic groups of original cohort. Partly anthroposophic group was not eligible. Exclusion criteria: no available child plasma from 12 or 24 months. This resulted in 157 families, 62 anthroposophic and 95 non-anthroposophic.
Paper II
Eligible: same as paper I. Exclusion criteria: fecal samples missing from more than one of the following six sampling occasions: mother before birth, mother when infant was two months, infant at six days, three weeks, two months and six months of age. This resulted in 128 families, 55 anthroposophic and 73 non-anthroposophic.
Paper III
Eligible: entire cohort. Exclusion criteria: baseline questionnaire not completed and having attended neither one- nor two-year-follow-up. This resulted in 490 families; 116 anthroposophic, 212 partly anthroposophic and 162 non-anthroposophic included in the study.
Paper IV
Eligible: entire cohort. Exclusion criteria: data for lifestyle-classification not available and no blood-sample available from child. This resulted in 474 families in the study; 100 with anthroposophic, 209 with partly anthroposophic and 165 with non-anthroposophic lifestyle.

3.3 HERPESVIRUS SEROLOGY (PAPER I)

Serological analyses of EBV, HHV6, HHV7 and CMV were performed in plasma samples from children at 12 and 24 months of age, and from parents in families where child samples from both ages were available. Titers of IgG against the EBV, HHV6 and HHV7 were determined by immunofluorescence. A specific fluorescence in dilution of 1/20 was regarded as a sign of seropositivity. For CMV IgG detection the method was based on enzyme-linked immunosorbent assay and a sample was considered positive if the absorbance was >0.2 at a dilution of 1/100 (see paper I for details).

3.4 GUT MICROBIOTA (PAPER II)

Fecal samples were collected from the infants at ages six days, three weeks, two months and six months and from the mothers one week before delivery and when their child was two

months of age. Parents were instructed to freeze these samples within 20 minutes of collection and store at home at -20°C. They were later transported in a frozen state for storage at -70°C.

The method for analyzing bacterial components of the fecal samples is described in paper II. In brief, DNA was isolated from 250 mg of stool from each sample and the V3 and V4 regions of the 16S rRNA genes were amplified. The amplified DNA-sequences (amplicons) were sequenced using the Roche/454 GS Titanium technology platform (Branford, CT, USA). The obtained sequences from the 454-pyrosequencing analysis were processed and taxonomically classified. Statistical evaluation of the data was performed on data taxonomically classified to genus level. Samples with less than 500 sequences were excluded from analysis.

Gut microbiota outcomes

Relative abundance

Using this method, the abundance of a taxon (see table for definition) is not measured as an absolute number, but as the proportion of the total number of sequences analysed in the sample. The relative abundances can be measured for the different levels of taxonomy; in our case we used phylum and genus level.

Table 1. Hierarchic classification system for bacteria

Category (Taxon)	***Example***
Domain	*Bacteria*
Phylum	*Firmicutes*
Class	*Bacilli*
Subclass[a]	
Order/Subsection	*Lactobacillales*
Suborder[a]	
Family	*Lactobacillaceae*
Genus	*Lactobacillus*
Species	*Lactobacillus casei*
Subspecies	*Lactobacillus casei strain 12 A*

[a]Subclass and Suborder are only used for phylum Actinobacteria

Diversity and similarity

The microbial diversity of a sample can be described by a diversity index, a mathematical measure that takes into account not only the number of taxa in the sample (richness), but also the relative abundances of the taxa (evenness). There are several diversity indices in use in microbiology, of which one of the most commonly used in gut microbiota analysis is the one we used, the Shannon Weiner diversity index. It was calculated using the formula $- \Sigma \ln (p_i) p_i$ where p is the relative abundance of taxon i in a sample.

The similarity between two samples was calculated as Bray-Curtis index of similarity using the formula $1 - \frac{\sum_i |p_{ji} - p_{ki}|}{2}$ where p_i is the relative abundance of taxon i in samples j and k respectively. It takes a value between 0 and 1 where 1 means the two samples have the same composition and 0 means the two samples don't share any taxa.

The diversity and similarity calculations were done at genus level. Before calculating diversity index in a sample, adjustment has to be made for the number of sequences in the sample. In a sample with a high number of sequences, taxa with low abundance can be detected that would not be detected in a sample with few sequences. To avoid the influence of the number of sequences in the samples on the diversity-parameters, the relative abundances in each sample were recalculated to correspond to a sample with 500 sequences, so the lowest detectable relative abundance was 0.2 %.

3.5 ALLERGY-RELATED OUTCOMES (PAPERS I, III AND IV)

Sensitization
Levels of IgE in plasma towards seven common allergens (hen's egg, cow's milk, peanut, cat, dog, birch and timothy) were determined in blood samples from the children collected at six months **(papers III and IV)**, one year **(papers III and IV)**, two years **(papers I,III,IV)** and five years **(paper IV)**, using ImmunoCAP®(Phadia AB, Uppsala, Sweden). The definition of a positive sample (sensitized) was IgE levels ≥0.35 kU_A/L towards at least one of the seven allergens. For **paper IV**, the allergens were categorized into food-allergens (cow's milk, hen's egg and peanut), animal-allergens (cat and dog) and pollen-allergens (birch and timothy). Parents' blood samples, collected at the time for inclusion, were analyzed by ImmunoCAP Phadiatop® (Phadia AB) containing a mix of eleven inhalant allergens and determined positive or negative **(papers III and IV)**.

Food hypersensitivity
Food hypersensitivity was defined as reported acute onset of symptoms such as skin reactions, wheezing, vomiting or diarrhea on more than one occasion after ingestion or contact with a particular type of food and based on parental report and doctor's evaluation at one and two years of age **(paper III)**.

Recurrent wheeze
Recurrent wheeze was defined as three or more episodes of wheeze since the last examination at one and two years of age respectively and based on parental report and doctor's evaluation **(paper III)**.

Eczema
Eczema was defined as a SCORAD-score[5] of > 0 at the time for doctor's examination and determined at two months, six months, one year and two years of age **(paper III)**.

3.6 STATISTICAL ANALYSES

3.6.1 Paper I

Exposure: lifestyle as a dichotomous variable (anthroposophic or non-anthroposophic). *Outcomes:* serostatus (positive or negative) for four herpesviruses (EBV, CMV, HHV6 and HHV7) at two time points (one and two years of age). Logistic regression was used for calculating odds ratios (ORs) and 95% confidence intervals (CIs). Fischer's exact test was used for calculating p-values for differences in the background variables and parental virus serology between the two exposure groups.

3.6.2 Paper II

Exposures: age (six days, three weeks, two months, six months or adult), lifestyle (anthroposophic or non-anthroposophic) and, in addition, nine exposure factors chosen for their potential association with microbiota and/or allergy: Living on a farm, Mother vegetarian, Antibiotics during pregnancy, Birthplace (home/hospital), Birth mode (vaginal/caesarean), Sex, Milk formula 1st week, Breastfeeding at two and six months of age (fully/partly/not). *Outcomes:* relative abundance for all detected taxa down to genus level at the six different sampling occasions and Shannon Weiner diversity index at the six different sampling occasions.

Principal coordinate analysis (PCoA) based on abundance data from sequences classified to genus level, was performed to find clustering patterns among the subjects. To evaluate which factors that were associated with the composition of the microbiota a permutational multivariate analysis of variance (PERMANOVA) based on Bray Curtis distances and 1000 permutations was performed. For differences in relative abundance of specific bacterial taxa, we used Wilcoxon tests and linear regressions. To correct for multiple testing we calculated false discovery rates, i e the fraction of positive tests expected to be false positive, and q-values[114].

We also calculated Bray-Curtis index of similarity within the family for the following eight comparisons: mother before and after birth, mother and the different samples from her infant, as well as between the consecutive samples of the infant.

The multivariate statistical software Past version 2.17 (University of Oslo, Norway) was used for calculation of similarity indices, diversity, ordination and PERMANOVA. The Wilcoxon tests and linear regression were conducted using the R statistical framework (R-project 2013). Associations between the different exposures and Shannon Weiner diversity index were tested with Mann Whitney's test or Kruskal Wallis' test, using IBM SPSS Statistics 21 software (Chicago, IL, USA).

3.6.3 Paper III

Exposures: main exposure variable was lifestyle with three values (anthroposophic, partly anthroposophic or non-anthroposophic), but in addition all exposures registered in the baseline questionnaire and at the two-months-follow-up were studied (the 64 variables in tables 1a and 1b of paper III). *Outcomes:* food hypersensitivity up to two years of age (two measurements), recurrent wheeze up to two years of age (two measurements), eczema up to two years of age (four measurements) and sensitization up to two years of age (three measurements). In the sub analyses of associations between sensitization and the three clinical manifestations sensitization was defined as having had any positive sample during the follow-up and used as exposure variable.

Fisher's exact test (for categorical variables) and Kruskal Wallis test (for continuous variables) were used for comparisons of distributions of the investigated exposure factors between the three lifestyle groups. Generalized estimating equations (GEE), with unstructured matrix correlation, were used to study the associations between each exposure and the respective outcomes together with a time variable. The unadjusted models for lifestyle represented our main findings. Each of the 64 exposure variables from tables 1a and 1b in the manuscript was studied separately in GEE-models for each outcome (food hypersensitivity, recurrent wheeze, eczema and IgE-sensitization). Exposure factors that were significantly associated ($p<0.05$) with the outcome in the crude models were then adjusted for lifestyle in bivariate models and those that were significantly associated with lifestyle in the bivariate models were kept in addition to lifestyle in final, adjusted GEE models for each outcome. No correction was done for multiple testing.

Calculations were done using IBM SPSS Statistics for Windows (version 21.0, Armonk, NY: IBM Corp).

3.6.4 Paper IV

Exposure: lifestyle variable with three values (anthroposophic, partly anthroposophic or non-anthroposophic). *Outcomes:* 1. Incidence rates of sensitization against food (cow's milk, hen's egg or peanut), animal (cat or dog) and pollen (birch or timothy) allergens for four age periods (before six months, six months to one year, one to two years and two to five years) measured in cases per 100 person years. Incidence rates were only described, no statistical inference was calculated. 2. Prevalence of sensitization to the three allergen categories.

Prevalence was calculated as the number of sensitized children at each age through the number of non-missing observations at the respective age. Incidence rates were calculated as the number of first time sensitized children (cases) during the time period through person time at risk. The children with missing previous samples were considered at risk for that time period, as long as they had not previously been sensitized and had a non-missing value for the age at the end of the time period. We assumed that cases were sensitized half-way between the observed ages and therefore contributed with only half the person time compared to non-

cases. In order to evaluate if the association between lifestyle and prevalence of sensitization of the respective categories of allergens varied with age we used generalized estimating equations (GEE) models, using unstructured correlation matrix, and included an interaction term between lifestyle and age.

Statistical analyses were performed in R v 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria) and SAS v 9.4 (SAS Institute Inc., Cary, NC, USA).

The level of significance (alpha-level) was set to 0.05 for all statistical calculations in this thesis.

3.7 ETHICAL CONSIDERATIONS

Performing examination of healthy children for research purpose requires ethical considerations. Oral and written information about the study and its procedures was given to the parents and written consent from both parents was required for participation. The benefits must overweigh the adverse effects. Most of the examination of the children was non-invasive and did not cause discomfort. Considering the heterogeneity of allergy-related outcomes and risk of misclassifications based on parental reports, blood-samples are highly valuable for objective classification of outcomes. The blood-sampling was performed by an experienced nurse after application of topical anesthetic and few of the children showed signs of experienced pain. Extensive collection of sensitive personal data was performed through questionnaires and interviews. This data is stored in encrypted data-bases, and personal identity information is stored in a separate database which is only accessed by the project steering group, for use in future follow-ups. The study was approved by the Research Ethical Committee at Huddinge University Hospital, Stockholm, Sweden.

4 RESULTS AND DISCUSSION

Data on demographics, parental atopy and early life exposures are presented for the respective lifestyle groups in table 1 in each paper. The most thorough description of the cohort is found in Tables 1a and 1b in paper III.

Parental atopy, as a measure of heredity, was equally distributed in the lifestyle groups. The prevalence of sensitization among the mothers was 32.8 %, 26.4 % and 30.2 % in the anthroposophic, partly anthroposophic and non-anthroposophic groups respectively. Prevalence was higher among fathers; 50.9 %, 46.2 % and 41.1 % respectively. Reported allergy-related manifestations (eczema, asthma, rhinoconjunctivitis and food reactions) were also similarly distributed in the lifestyle groups.

4.1 ASSOCIATION BETWEEN ANTHROPOSOPHIC LIFESTYLE AND HERPES VIRUS INFECTIONS (PAPER I)

The seroprevalence at one and two years of age for the four herpesviruses is presented for the two lifestyle groups in Figure 2.

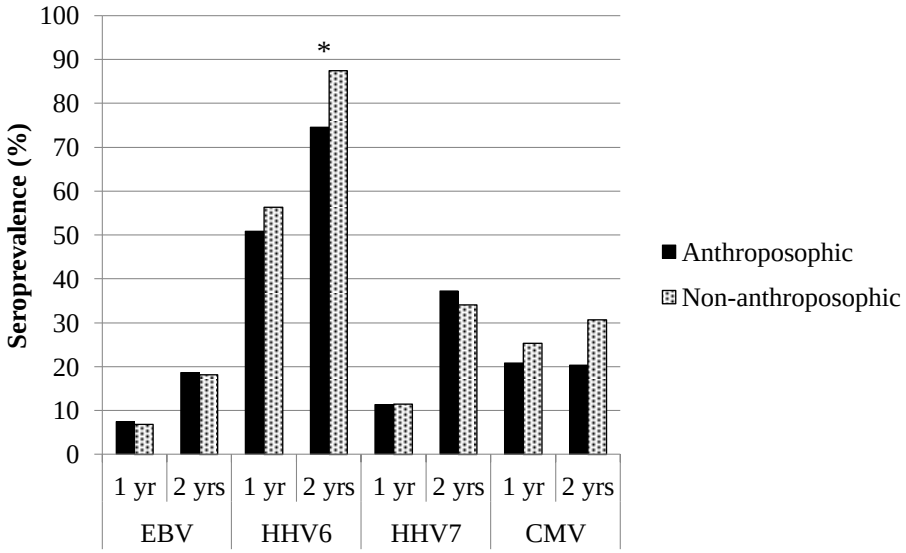

Figure 2. Seroprevalence (%) of the four respective herpesviruses at one and two years of age among children of families with anthroposophic and non-anthroposophic lifestyle. *p-value 0.048

We found no significant differences in seroprevalence for EBV, HHV7 or CMV at any age between the two lifestyle groups. The seroprevalence for HHV6 at 24 months of age was 74.6 % in the anthroposophic group, which was significantly lower than in the non-anthroposophic group (87.5 %) ($p = 0.048$). We also looked at the associations between seroprevalence for the four viruses and IgE-sensitization at 24 months of age, although this was not our primary aim and the power of the study was not enough to draw conclusions. For HHV6-, HHV7- and CMV- seropositivity, odds ratios for being IgE-sensitized at 24 months were close to one, indicating no association (range from 0.83 to 1.27) with p-values ranging from 0.65 to 0.93.

For EBV, however, the odds ratio was 0.55 with a lower p-value (0.30), indicating that there might be an association.

Even if the seroprevalence of HHV 6 and CMV was higher in the non-anthroposophic group, significant only for HHV 6 at 24 months (p 0.048), neither of these viruses were associated with sensitization so therefore we concluded that seroprevalence for these viruses do not seem to be a mediator for the association between anthroposophic lifestyle and sensitization.

When we analyzed the association between EBV seroprevalence and IgE-sensitization at 24 months of age separately in the two lifestyle groups the results indicated that EBV could have an effect in the non-anthropsophic but not in the anthroposophic group (Figure 3).

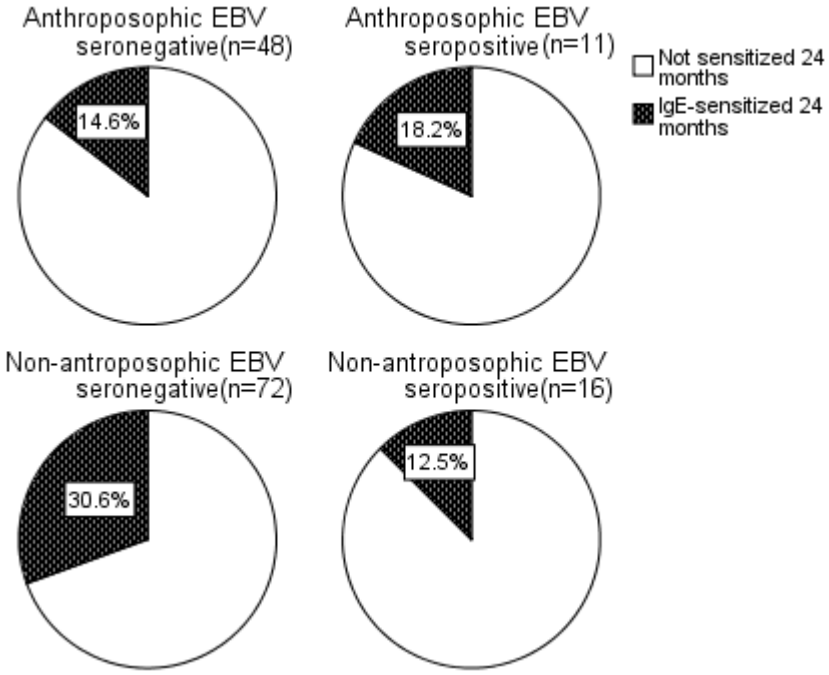

Figure 3. Proportions sensitized among EBV seropositive and EBV seronegative children at 24 months of age stratified by lifestyle group.

Even if our study population was too small to draw conclusions on these associations, the findings would be in line with earlier studies where support for a protective effect of early EBV-infection is more convincing than for the other viruses[34,41,42]. Extended studies of the entire cohort would give better information about potential associations between these virus infections and allergy-related outcomes and also about the possible interaction effect between anthroposophic lifestyle and EBV on the association with sensitization. Such an interaction effect would indicate either that an early EBV-infection can be replaced by some other immune-modulatory factor(s) in mediating the allergy-protective effect or that the EBV infection itself is not causative for the allergy-protective effect, but rather a proxy for some other protective factor.

4.2 ASSOCIATION BETWEEN LIFESTYLE AND GUT MICROBIOTA (PAPER II)

Of the 128 mother-infant pairs (55 anthroposophic and 73 non-anthroposophic) the numbers of samples that were included were 116 from mothers before delivery; 116 from mothers after delivery; and 110, 101, 113 and 109 from the infants at ages six days, three weeks, two months and six months, respectively. The mean number of sequences per sample was 2670 (505-14 300). First, we described the gut microbiota at the investigated time points, showing the influence of age on gut microbiota from different aspects. Large differences were seen between adult and infant microbiota (Figure 4a,b,c) with Firmicutes as the dominating phylum in the adult gut microbiota whereas Actinobacteria was the dominating phylum in infants (Figure 4a). The mean Shannon diversity index in the mothers' samples was significantly higher than in infants'. A significantly increased diversity was seen from two to six months of age (Figure 4b). The Bray Curtis index of similarity was calculated for comparisons within the family; between the two mother samples, between the mother and the samples from her infant, as well as between the consecutive samples of the infant. The mean similarity was highest between the two mother samples. The infant's microbiota was more similar to itself over time than to its mother's, but became more similar to its mother's with increasing age (Figure 4d).

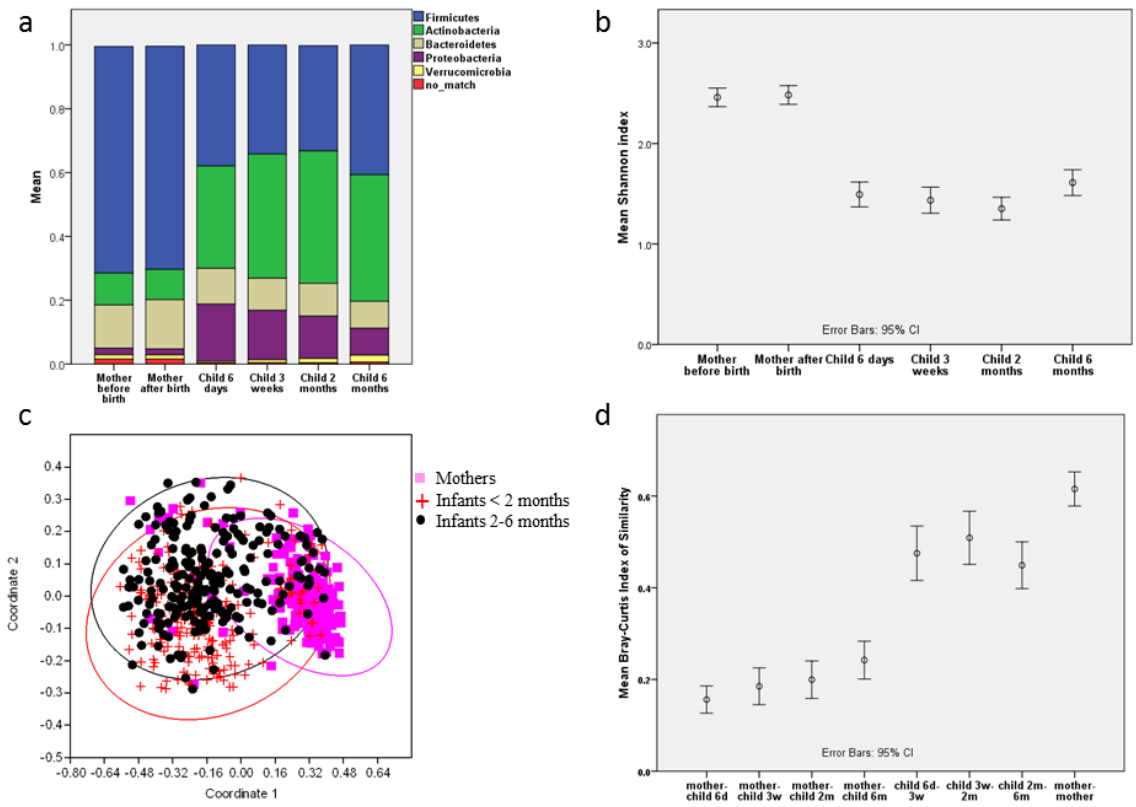

Figure 4. (a) Mean relative abundances of phyla. (b) Mean Shannon diversity index for the different ages. (c) Principal coordinate analysis plot (Bray Curtis distances). Ellipses represent 95% confidence interval. (d) Comparison of similarity between infants at the different ages and their respective mothers (1st to 4th circles from left), between two consecutive samples from the infants (5th to 7th circles) and between mother before and two months after delivery (8th circle). Circles represent mean Bray Curtis index of similarity and error bars represent 95% confidence interval.

Then we studied associations between different lifestyle-factors, including our main exposure, namely anthroposophic lifestyle, and gut microbiota. In the first step, we looked at the global microbiota, using a multivariate approach with principal coordinate analysis and PERMANOVA to sort out with which, if any, of the lifestyle-factors it was significantly associated. No apparent clustering was observed for anthroposophic lifestyle. In the PERMANOVA strong associations were seen for mode of delivery (caesarean vs vaginal) at six days ($p<0.001$), three weeks ($p<0.001$) and two months of age ($p=0.02$) and for breastfeeding at six months of age ($p<0.01$) whereas none of the p-values for association between anthroposophic lifestyle and global gut microbiota were significant after correction for multiple testing.

The more traditional description of the global microbiota, Shannon diversity index, was similar in anthroposophic and non-anthroposophic samples at all investigated ages (Figure 5a). None of the investigated exposure factors were significantly associated with Shannon diversity index after correction for multiple testing, but there was a clear trend for an inverse dose-response relationship between breastfeeding and diversity (Figure 5b).

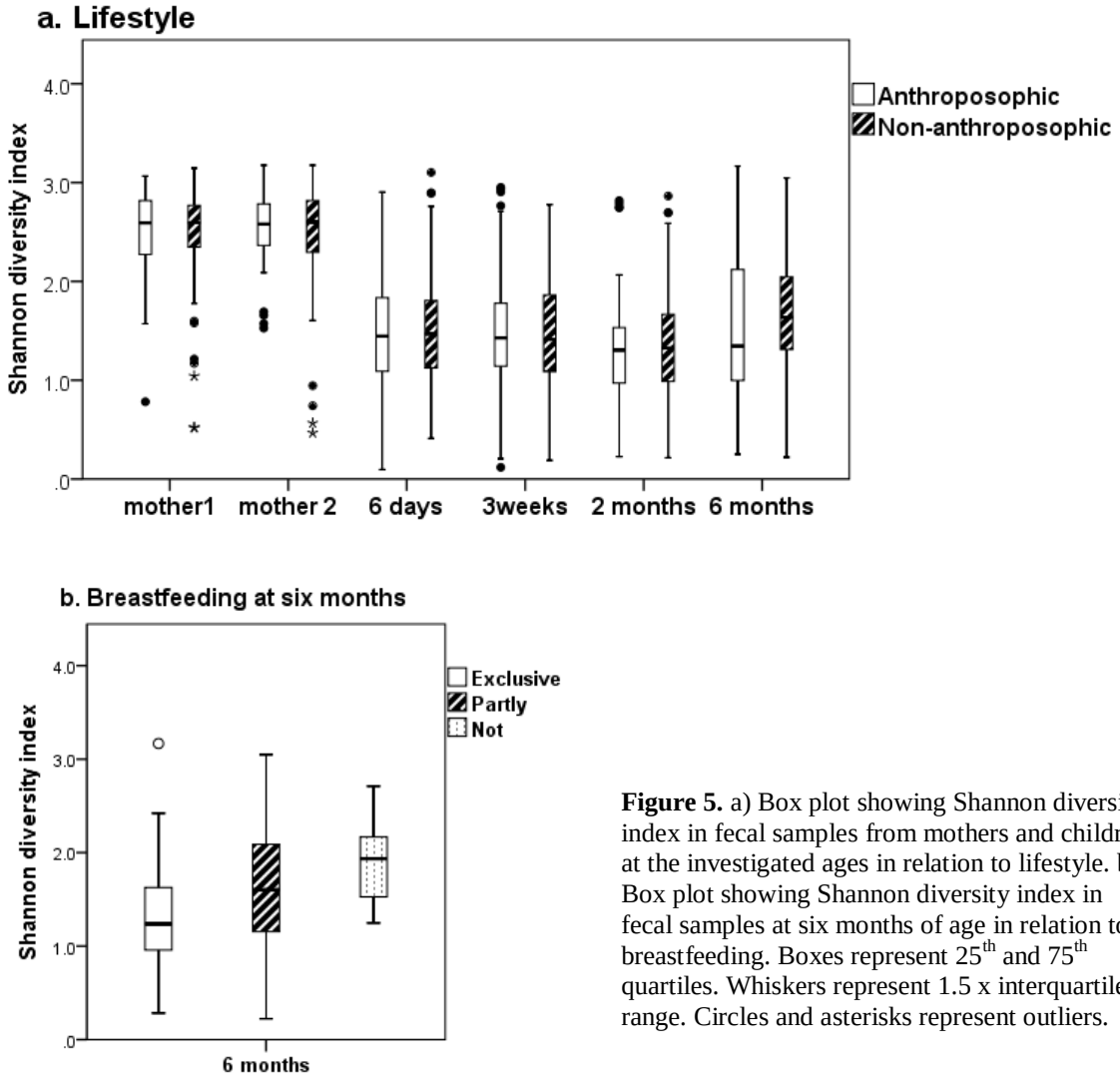

Figure 5. a) Box plot showing Shannon diversity index in fecal samples from mothers and children at the investigated ages in relation to lifestyle. b) Box plot showing Shannon diversity index in fecal samples at six months of age in relation to breastfeeding. Boxes represent 25th and 75th quartiles. Whiskers represent 1.5 x interquartile range. Circles and asterisks represent outliers.

We then wanted to test which bacterial taxa were responsible for the associations with overall microbiota pattern that were identified for mode of delivery and breastfeeding in the PERMANOVA. This was done by relating the relative abundances of the most abundant taxa to these exposures and in addition to anthroposophic lifestyle, since this was our main exposure factor. At all investigated ages, the infants delivered by caesarean section had lower relative abundance of *Bacteroides* (although not significantly at six months) and higher relative abundance of unclassified *Enterobacteriaceae* (significantly at all ages) and *Clostridium* (significantly at three weeks and two months) than the vaginally delivered infants. In addition, at six days and three weeks of age, these infants had significantly lower abundance of *Bifidobacterium* and significantly higher abundance of *Haemophilus* and *Veillonella*, than the vaginally delivered. Breastfed children had higher relative abundances of *Bifidobacterium* and *Streptococcus* but instead lower relative abundances of *Clostridiales*, Clostridiaceae1 and unclassified *Lachnospiraceae* at six months age. Six months old children in families with anthroposophic lifestyle had a significantly higher relative abundance of *Bifidobacterium* and lower relative abundance of *Bacteroides* and *Veillonella*. At the earlier ages, and among mothers, no significant association was seen between anthroposophic lifestyle and any of the most abundant taxa.

Based on previous studies, where species of both *Bifidobacterium* and *Bacteroides* have been reported as beneficial in relation to atopy development[69,115], our findings are somewhat difficult to interpret. It was not known at the time when this study was conducted that eczema was not significantly associated with anthroposophic lifestyle up to two years in this cohort, as was seen in paper III. The most convincing evidence for an association between gut microbiota and allergy-related outcomes has been demonstrated for eczema whereas studies have been more conflicting for sensitization[70]. However one recent study demonstrated an association between gut microbiota composition at three months of age and positive skin prick test for food allergens at one year[116]. In our study only differences down to genus-level could be detected. Even if differences have been demonstrated at the same taxonomic depth between infants who did or did not develop eczema[69] most studies have been at species, or even sub-species level. Prospective studies have shown that composition of the gut microbiota is different *before* development of eczema and recently also food allergen sensitization. Without interventional studies, however, a causal relationship cannot be established. In one study the gut microbiota of infants with allergic heredity differed from that of infants with no allergic heredity[58]. It is therefore possible that to some extent the differences in gut microbiota composition that have been observed between atopic and non-atopic individuals could be due to the genetic predisposition for atopy.

4.3 LIFESTYLE AND ALLERGY-RELATED SYMPTOMS (PAPER III)

The main finding in this paper was that anthroposophic lifestyle was associated with a reduced risk of food hypersensitivity and recurrent wheeze, but not eczema, in children up to two years of age (Figure 6). The overall risk of food hypersensitivity during the first two years of life was reduced for children from families with an anthroposophic lifestyle

compared with the non-anthroposophic children with an OR of 0.38 (95 % CI 0.12-1.2), p-value 0.11. The corresponding OR for the partly anthroposophic group was 0.35 (0.13-0.92), p-value 0.03. For recurrent wheeze, the risk was significantly reduced in both the anthroposophic, OR 0.38 (0.16-0.91) p-value 0.03, and partly anthroposophic, OR 0.51 (0.26-0.99), p-value 0.048 groups compared with the non-anthroposophic group whereas eczema up to two years of age was not significantly associated with lifestyle (p-values 0.50 and 0.24 respectively). In accordance with the results that have already been published for the first 302 children in the cohort[111], the risk of IgE-sensitization was significantly reduced in the anthroposophic, OR 0.37 (0.19-0.70), p-value 0.002 and partly anthroposophic group, OR 0.46 (0.27-0.74), p-value 0.002.

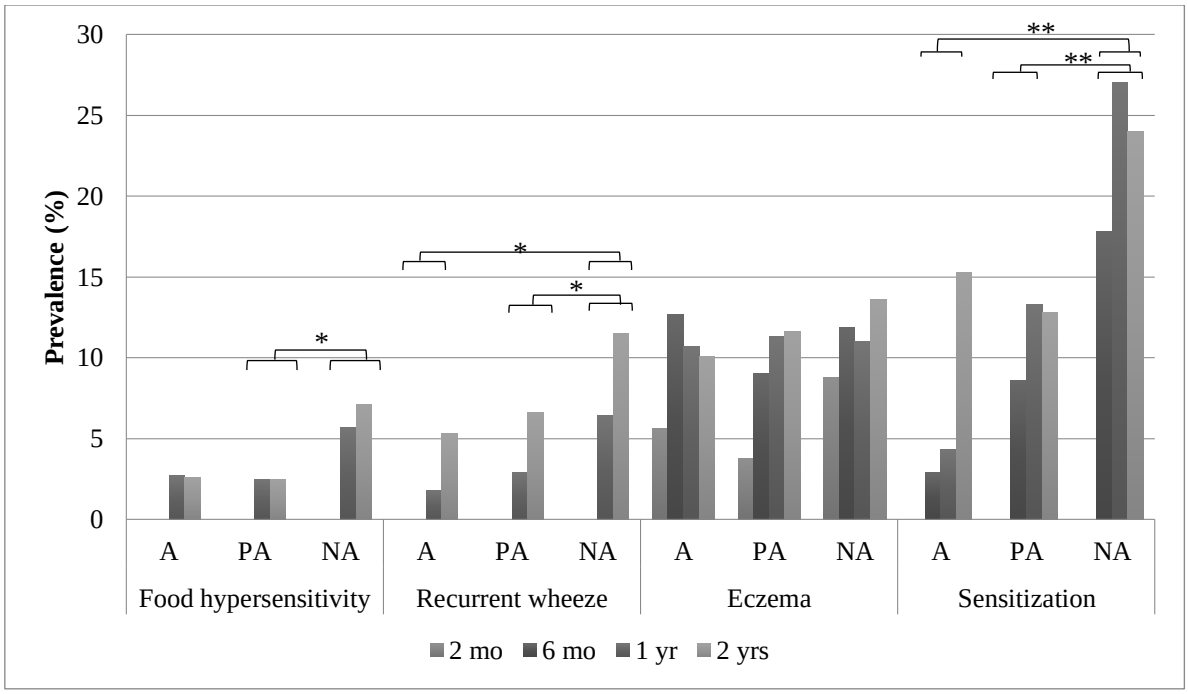

Figure 6. Prevalence of food hypersensitivity, recurrent wheeze, eczema and IgE-sensitization at the different ages for the children in the three lifestyle groups. A =Anthroposophic, PA = Partly Anthroposophic and NA = Non-Anthroposophic lifestyle group. *p-value < 0.05, **p-value<0.01 from GEE for association with outcome up to two years of age.

Furthermore we demonstrated that sensitization to any of the seven investigated allergens was associated with food hypersensitivity, OR 6.8 (95 % CI 2.7-17.0) and eczema, OR 2.7 (1.7-4.3) up to two years of age but not recurrent wheeze, OR1.1 (0.55-2.3) (Figure 7).

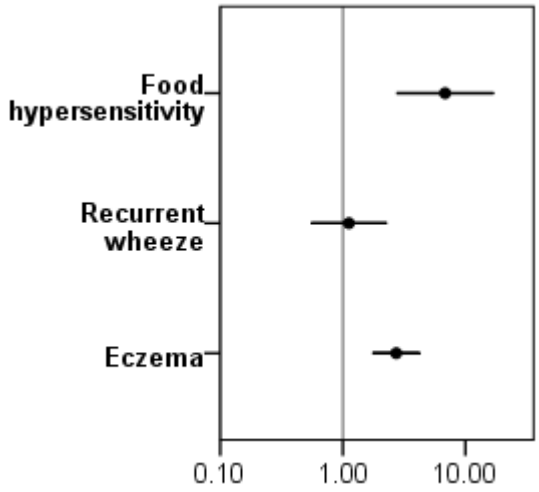

Figure 7**.** Relative risk (odds ratio and 95 % CI) of allergy-related symptoms up to two years among children who were ever sensitized to cow's milk, hen's egg, peanut, cat, dog, birch and/or timothy compared with those who were never sensitized (reference line).

Of the 64 investigated lifestyle exposures, depicted in Tables 1a and 1b in paper III**,** those that were significantly associated with the respective allergy-related outcome, after adjusting for lifestyle, were included in final GEE models for each outcome. The risk estimates (odds ratios) and 95 % confidence intervals are graphically illustrated in Figure 8.

Having older siblings was associated with a significantly reduced risk of food hypersensitivity, adjusted OR 0.40 (95 % CI 0.17-0.96). However, the risk estimates for food hypersensitivity for anthroposophic and partly anthroposophic lifestyle compared to non-anthroposophic remained largely unchanged in the adjusted model, so having older siblings does not seem to be an explanatory factor for the association between anthroposophic lifestyle and food hypersensitivity (Figure 8a).

A significantly reduced risk of recurrent wheeze was seen for children that had a mother who is university-educated, adjusted OR 0.29 (0.14-0.58) and for having a mother who used olive oil as main cooking fat during pregnancy, adjusted OR 0.45 (0.20-0.99). An increased risk of recurrent wheeze was seen for the children who had been admitted to neonatal care, adjusted OR 3.0 (1.2-7.3) and those who had received milk formula during the first week of life, adjusted OR 2.3 (1.2-4.6). In contrast to what was seen for food hypersensitivity, the association between recurrent wheeze and anthroposophic and partly anthroposophic lifestyle was attenuated in the adjusted model, adjusted odds ratios 0.59 (0.18-1.9) for the anthroposophic and 0.77 (0.33-1.8) for the partly anthroposophic group. This indicates that these exposure factors could be partly explanatory for the association (Figure 8b).

The risk of eczema was significantly increased among children who lived in an apartment in comparison with those who lived in a house, OR 1.7 (1.08-2.6). An increased risk was also seen for children of mothers who had received antibiotics during pregnancy OR 2.0 (1.2-3.3) and for children who had a wash of the whole body before one week of age, OR 1.6 (1.1-2.4). For children of families who kept pets in their household a reduced risk of eczema was observed, OR 0.57 (0.37-0.89) (Figure 8c).

The risk of sensitization was significantly increased among children whose mother worked (was gainfully employed) during pregnancy, OR 3.0 (1.1-8.1) and among those who were wash of the whole body before one week of age, OR 2.1 (1.2-3.5). The associations between sensitization and anthroposophic, OR 0.56 (0.28-1.1) and partly anthroposophic lifestyle, OR 0.60 (0.35-1.02) were slightly attenuated in the final model. Again this indicates that much of the 'anthroposophic effect' on sensitization remains unexplained (Figure 8d).

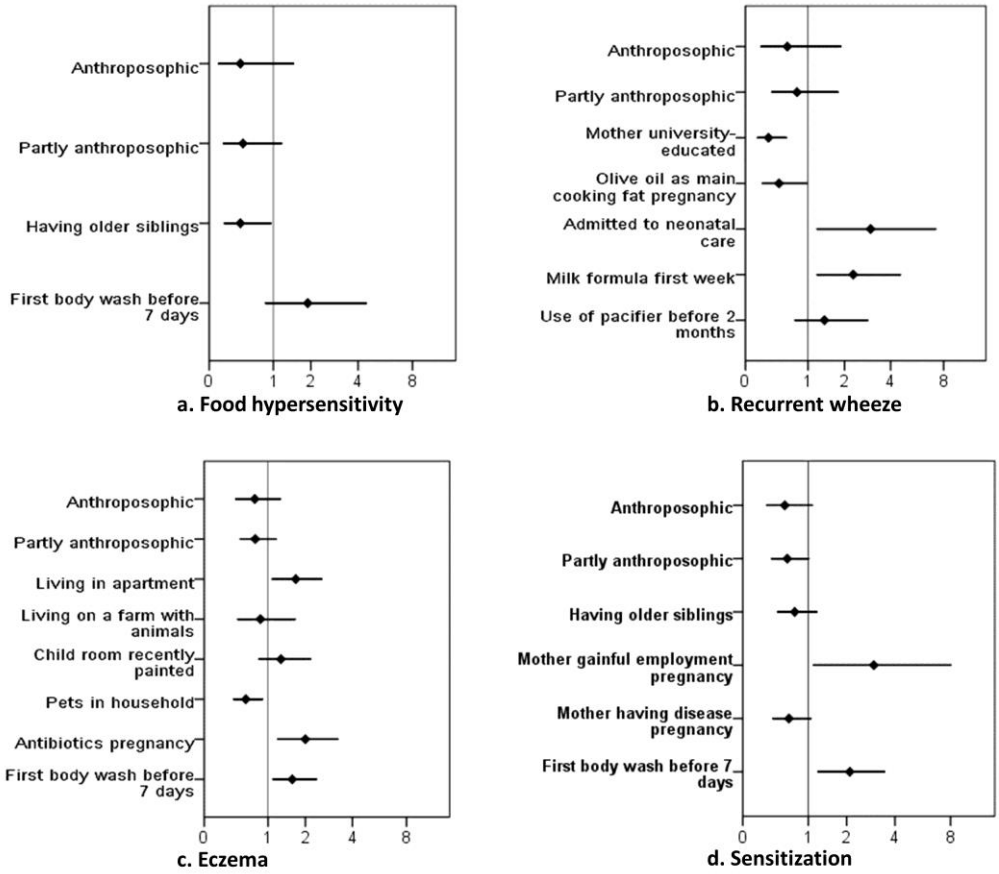

Figure 8. Effect of different lifestyle exposures for developing food hypersensitivity (a), recurrent wheeze (b), eczema (c) and sensitization (d) up to two years of age presented as odds ratios (diamonds) and 95 % confidence intervals from adjusted GEE models.

The discrepant findings on recurrent wheeze and eczema seem somewhat puzzling since eczema but not recurrent wheeze was associated with sensitization for which indeed the most convincing association with anthroposophic lifestyle so far has been demonstrated. The inverse association with recurrent wheeze is however in line with findings that farm exposure is inversely associated with not only atopy but also transient wheeze and non-atopic asthma in children[117]. However the exposures that seemed to largely explain the protective effect of anthroposophic lifestyle (maternal level of education, maternal consumption of olive oil during pregnancy, receiving milk formula during the first week of life and having been admitted to neonatal care) are unlikely to be mediated by microbial exposure which was the case for farming exposure and asthma[50]. One must also consider possible misclassification of the outcomes. In the case of recurrent wheeze parents with anthroposophic lifestyle could be

less likely to report wheezing symptoms. The fact that classification was done by one (of the four) examining study doctors would decrease, but not eliminate that risk of misclassification. Concerning eczema, classification was entirely based on symptoms at the time for examination. Misclassification could be caused by differential usage of topical corticosteroids in the lifestyle groups, and unfortunately no such information was collected. However, another possible explanation could be that the contribution of genetics is differential for the outcomes at this age, and genetic predisposition is presumed to be equally distributed between the lifestyle groups, since sensitization was equally distributed between parents.

Since no correction was done for multiple testing, the interpretation of the results from the adjusted models should be done with caution. However, one interesting finding was that age at first wash of whole body was associated with food hypersensitivity, eczema and sensitization. These associations could be in accordance with recent findings in this cohort that development of eczema was associated with the protein composition of the vernix[118], and the theory that sensitization to food allergens occurs through cutaneous exposure[79].

4.4 IMPACT OF AGE ON ASSOCIATION BETWEEN LIFESTYLE AND SENSITIZATION (PAPER IV)

118 of the 474 children developed any allergic sensitization up to 5 years of age (18 anthroposophic, 44 partly and 56 non-anthroposophic). High incidence of food sensitization was seen up to one year of age among the partly and non-anthroposophic children but not in the anthroposophic group (Figure 9).

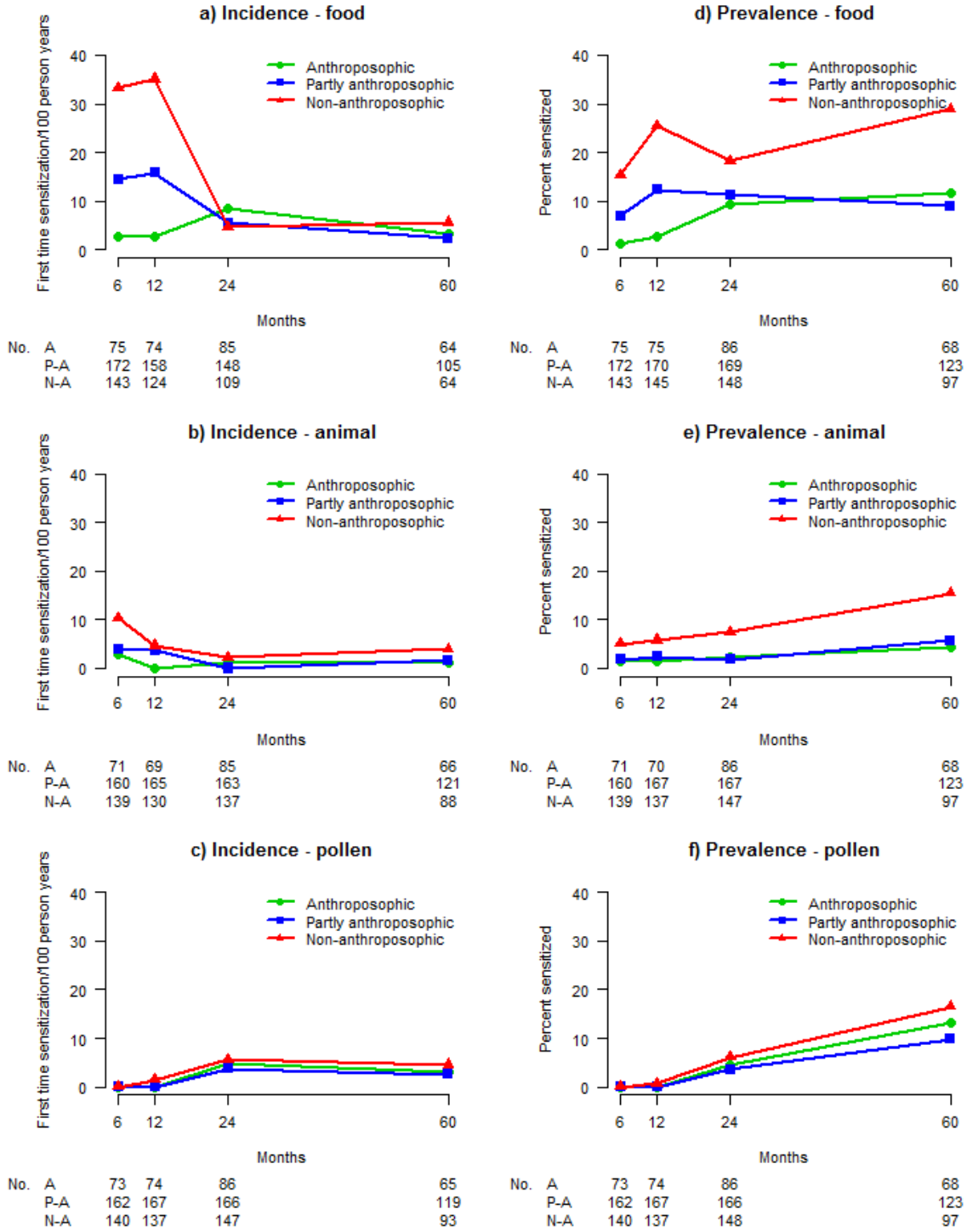

Figure 9. Incidence rates and point prevalence of food, animal and pollen allergen sensitization at 6, 12, 24 and 60 months of age among children with anthroposophic (A), partly anthroposophic (P-A) and non-anthroposophic (N-A) lifestyle.

To test if the effect of lifestyle on allergen sensitization differed with age, we used GEE-models with prevalence of sensitization as outcome and added an interaction term for lifestyle and age. There was significant interaction between lifestyle and age for the association with food (p = 0.02), but not animal (p = 0.89) or pollen (p = 0.91) allergen sensitization.

In an additional analysis, which is not included in the current version of the manuscript, the children were classified as early food sensitized (n= 68) if the 6- and/or the 12-months samples were positive for a food allergen. Thereby it was possible to adjust the effect of lifestyle on overall risk of any sensitization for the effect of early (before one year of age) food allergen sensitization. The relative risk of being sensitized to any of the seven allergens up to five years of age was significantly reduced in the anthroposophic, OR 0.42 (0.24-0.73) and partly anthroposophic groups, OR 0.43 (0.28-0.67) compared with the non-anthroposophic group (Figure 10a). However no risk-reducing effect was observed after adjusting for early food allergen sensitization (Figure 10b). This further supports that the effect of anthroposophic lifestyle on sensitization up to five years of age is almost exclusively on food allergen sensitization before one year of age.

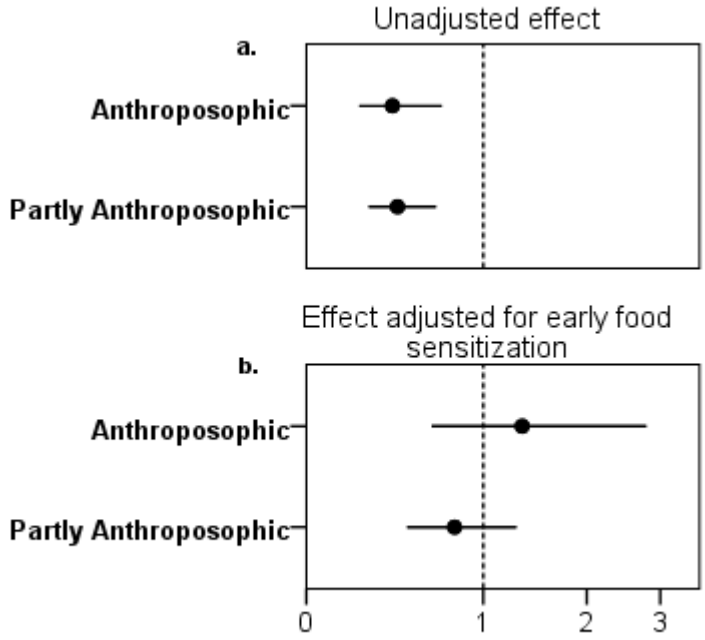

Figure 10. This figure shows the relative risk of any sensitization up to five years of age among anthroposophic and partly anthroposophic children compared to non-anthroposophic (reference-line) as odds ratios with 95 % confidence intervals without (a) and with (b) adjusting for the effect of early (up to 12 months) food allergen sensitization.

It is possible that a substantial part of the 'anthroposophic effect' seen in school children is associated with the very early sensitization to food allergens. However, incidence of animal and pollen allergen sensitization was very low, as expected at this age[10], and therefore no

conclusion can be drawn about the importance of early food sensitization for animal and pollen sensitization. The clinical relevance of very early food sensitization is unclear. Allergies to cow's milk and hen's egg are usually outgrown before school age, but there is some evidence that they are becoming more persistent[3]. Even if studies of sensitization in infancy are scarce, high incidence of food sensitization early in life has been reported previously for high-risk populations[119,120], and is therefore likely to be relevant for the development of allergy-related disease. In the ALADDIN cohort only a fraction of the food sensitized children had symptoms of allergy at one or two years of age, as reported in paper III. Future follow-ups will likely increase understanding about the relevance of early food allergen sensitization on other allergen sensitization and clinical allergy.

4.5 METHODOLOGICAL CONSIDERATIONS

The cohort study is the design of choice for studying an uncommon exposure, such as anthroposophic lifestyle and a common outcome, such as sensitization and allergy-related disease. The classification of lifestyle was based exclusively upon the parents' answers about the family's view/way of life. The investigators' opinions about what is characterizing for anthroposophic lifestyle was therefore not considered for this classification. The design of studies I and II were based on the findings of a markedly reduced prevalence of sensitization among the anthroposophic children, a risk reduction that was most pronounced already at six months of age. A potentially explanatory, or mediating, exposure-factor, such as herpesvirus infection was therefore expected to be unequally distributed between the lifestyle groups.

A confounding variable (confounder) is a risk-factor for the outcome that is also associated with the exposure but not an effect of the exposure[121]. For confounding to occur, the confounder must be unequally distributed between the exposure groups. The problem with confounding must always be approached in non-randomized studies. If a potential confounder is measured it can be controlled for, either by stratification or in regression models. However unmeasured confounders cannot be controlled for (other than by randomization) so an adjusted effect of an exposure is either the 'real' effect of that exposure or the effect of some unmeasured confounder(s) or a mix of these effects. In our studies the exposure was anthroposophic lifestyle. The effect of anthroposophic lifestyle on the allergy-related outcomes is surely confounded, because parents' choice of maternal-child health center and their opinions about their lifestyle cannot be biologically causative for the development of allergy. However, adjusting for the measured potential confounders would not necessarily result in a more accurate effect estimate for the association between anthroposophic lifestyle and for example sensitization or herpesvirus seroprevalence. On the contrary, the crude (unadjusted) effect is probably the most accurate one for the compound exposure factor 'anthroposophic lifestyle'. In paper III the regression analyses were used for identifying risk-factors for the allergy-related outcomes by adjusting for anthroposophic lifestyle and other potential risk-factors. Only a few of the identified risk-factors were also confounders, meaning that they to some extent explained the 'anthroposophic effect'. This could of course

be completely due to unmeasured confounding, but there are also some important sources of error for these analyses. The level of significance was set to 0.05 which is custom. We tested 64 exposures for each of the four outcomes. This means that we would expect to find three risk-factors for each outcome just by chance (alpha-error). On the other hand, the subgroups for these analyses were relatively small, and the statistical power is dependent on the size of the study population and using a p-value of 0.05 as cut-off, as we did, could have excluded 'true' risk factors from further analysis (beta-error). Statistical correction for multiple testing would have increased the risk of beta-errors and was therefore not done. It is possible that more advanced multivariate statistical analysis could add information.

4.5.1 Future perspectives

Our results indicate that the impact of lifestyle is largest very early in life, before six months of age. Analyzing markers of allergic inflammation in cord blood, which is available for the original cohort, could give an estimate of the extent of the lifestyle-effect that is achieved already in utero. Such studies are ongoing in broad translational research collaborations.

Clinical allergy-related outcomes in five-year-olds, including lung function, will be analyzed when all the children have reached five years of age in March 2016. At this age some children will be diagnosed with asthma and allergic rhinoconjunctivitis, outcomes that have not yet been investigated. Furthermore, ten-year-follow-ups of the cohort will start this year, which will provide information about the persistence of our findings.

Analyzing EBV-serology in the whole cohort would help clarifying its potential role in allergy-development.

Similarly, analyzing fecal microbiota for the whole cohort will give the opportunity to study associations with allergy-related outcomes. But also further analyses of the already purified DNA from the 128 mother-child pairs, with methods that give deeper coverage and/or higher taxonomical resolution, could better clarify if gut microbiota is a mediator for the effect of anthroposophic lifestyle on allergy-related outcomes.

5 CONCLUSIONS

Based on the papers included in this thesis, the following conclusions can be drawn:

- Since seroprevalence of EBV, HHV6, HHV7 and CMV up to two years of age was similar among anthroposophic and non-anthroposophic children, timing for these infections is unlikely to contribute to the reduced risk of sensitization that is associated with anthroposophic lifestyle (I).
- Mode of delivery and infant feeding seem to be stronger determinants of infant gut microbiota composition than anthroposophic lifestyle. Global gut microbiota diversity was not influenced by anthroposophic lifestyle and is unlikely to explain the reduced risk of sensitization. However other methods for gut microbiota analysis might better clarify the effect of anthroposophic lifestyle on gut microbiota (II).
- Anthroposophic lifestyle is associated with reduced risk of sensitization, food hypersensitivity and recurrent wheeze up to two years of age. The 'anthroposophic effect' remains unexplained, but delayed first wash of the newborn's whole body could play a role (III).
- The reduced prevalence of sensitization among children from families with an anthroposophic lifestyle was explained by a low risk of food allergen sensitization before one year of age. This low risk of food allergen sensitization during the first year of life is likely to be relevant for the reduced risk of allergy-related disease that has been observed among school aged children with an anthroposophic lifestyle (IV).

6 SVENSK SAMMANFATTNING

Allergi-relaterade sjukdomar såsom födoämnesallergi, eksem, astma och allergisk rinokonjunktivit har ökat i befolkningar med ”västerländsk” livsstil och drabbar nära hälften av svenska barn upp till tolv års ålder. Minskad mikrobiell exponering tidigt i livet tros spela en viktig roll för denna ökade förekomst. Barn som växer upp i familjer med antroposofisk livsstil har visat sig ha minskad risk för allergi-relaterad sjukdom. Syftet med denna avhandling var att studera samband mellan denna livsstil och tidig allergiutveckling samt en möjlig roll av mikrobiell exponering, i form av herpesvirusinfektioner och tarmflora. De fyra delarbetena i denna avhandling baseras på födelsekohorten ALADDIN (Assessment of Lifestyle and Allergic Disease during INfancy) där barn från familjer med olika livsstil följts upp med bl. a. frågeformulär, kliniska undersökningar, föräldraintervjuer och blod- och avföringsprover.

Livsstilsfaktorer påverkar hur tidigt man infekteras med herpesvirus. Vissa herpesvirus, särskilt Epstein-Barr virus (EBV), har associerats med allergi-risk hos barn. I **arbete I** mätte vi nivåer av IgG mot EBV, HHV6, HHV7 och cytomegalovirus i blodprov vid ett och två års ålder från 62 antroposofiska och 95 icke-antroposofiska barn och från deras föräldrar. Vi fann liknande förekomst av IgG mot dessa virus i de båda livsstilsgrupperna bland både föräldrar och barn. Det är därför osannolikt att exponering för dessa virus förklarar skillnaden i förekomst av allergisk sensibilisering mellan antroposofiska och icke-antroposofiska barn.

Den tidiga etableringen av tarmfloran har betydelse för immunsystemets utveckling och påverkas av livsstilsfaktorer. I **arbete II** analyserade vi bakteriell sammansättning i avföringsprov tagna från 55 antroposofiska och 73 icke-antroposofiska spädbarn vid sex dagars, tre veckors, två månaders och sex månaders ålder samt från deras mammor med pyrosekvensering av 16SrRNA-genen. Kejsarsnittsförlossning och amning hade större påverkan än antroposofisk livsstil på barnens tarmflora. Vid sex månaders ålder var de relativa nivåerna högre för *Bifidobacterium* och lägre för *Bacteroides* hos de antroposofiska barnen. Inga samband mellan antroposofisk livsstil och tarmflora sågs hos barnen till och med två månaders ålder eller hos mammorna. Tarmflorans diversitet (mångfald) var inte påverkad av antroposofisk livsstil och tycks därför inte förklara varför antroposofiska barn har lägre risk för allergisk sensibilisering. Fler studier, med annan metodologi, skulle behövas för att klargöra huruvida tarmfloran medierar den minskade sensibiliseringsrisken.

I **arbete III** studerade vi samband mellan livsstil och kliniska allergi-relaterade manifestationer upp till två års ålder hos 116 antroposofiska, 212 delvis antroposofiska och 162 icke-antroposofiska barn. Barn med antroposofisk eller delvis antroposofisk livsstil hade lägre risk för födoämnesöverkänslighet och obstruktiva luftrörsbesvär än icke-antroposofiska barn, men risken för eksem var liknande för alla tre grupperna. Att vänta minst en vecka med första helkroppstvätt av det nyfödda barnet var förenat med en minskad risk för födoämnesöverkänslighet och eksem och dessa manifestationer var associerade med allergisk sensibilisering. Risken för obstruktiva luftrörsbesvär var associerad med mammans utbildningsnivå och huruvida barnet fått modersmjölksersättning första levnadsveckan, men

inte med allergisk sensibilisering. Vad som utgör den ”antroposofiska effekten” är dock till stor del fortfarande oklar.

I **arbete IV** beskriver vi incidens och prevalens för födoämnes-, pälsdjurs- och pollensensibilisering upp till fem års ålder för 100 antroposofiska, 209 delvis antroposofiska och 165 icke-antroposofiska barn. Vi visar att sambandet mellan antroposofisk livsstil och sensibilisering till och med fem års ålder nästan uteslutande förklaras av en låg risk för födoämnessensibilisering före ett års ålder.

Sammanfattningsvis illustrerar denna avhandling den stora betydelsen av mycket tidiga livsstilsexponeringar för utvecklingen av allergi-relaterade utfall, men också komplexiteten i studier av samband mellan livsstil och sjukdomsutveckling. Att den antroposofiska livsstilen så övertygande visat sig vara kopplad till lägre risk för allergi-relaterade utfall, inte minst det objektiva utfallsmåttet allergisk sensibilisering, gör att ALADDIN-kohorten kan fungera som en modell för att studera hur livsstil påverkar utvecklingen av allergi-relaterad sjukdom. Detta oavsett vad som utgör den ”antroposofiska faktorn”.

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