Development of allergy, salivary IgA antibodies and gut microbiota in a Swedish birth cohort

Anna Sandin
Anna Sandin
Pediatrics, Department of Clinical Sciences
Umeå University
SE-901 87, Umeå, Sweden
E-mail: anna.sandin@jll.se

Illustrations:

Photo: Lars Sandin, cover

Drawing: Maria Bernholm, cover

Graphics: Print & Media

Printed in Sweden by Print & Media, Umeå 2008:2004292
To my family
Lars
Viktor, Arvid, Elvira
ABSTRACT

The increasing prevalence of allergic diseases in affluent societies has been associated with changes in microbial exposure early in life and a less diverse gut flora. The objective of this thesis was to assess the development of allergic sensitisation and symptoms during the first four years of life in a non-selected birth cohort in relation to environmental factors, family history, gut microbiota and salivary IgA antibodies.

The cohort comprised all 1,228 infants living in a Swedish county who were born over a one-year period. The parents replied to questionnaires, and 817 children (67%) were skin prick tested both at 1 and 4 years of age. Saliva (n=279), faecal (n=139) and blood (n=253) samples were collected at 1 year of age from children with a positive skin prick test at 1 year and from a sample of children with a negative skin prick test. Faecal samples were also obtained from 53 children at 4 years of age.

Dog keeping during infancy was associated with a decreased risk of sensitisation to pollen and late-onset wheezing at age 4, and the reduced odds ratios persisted after adjustment for heredity and avoidance measures, OR 0.3, 95% CI 0.1-0.9 and OR 0.5, 95% CI 0.2-1.0, respectively. In contrast, early dog keeping was associated with an increased risk of early-onset transient wheezing but only in children with parental asthma (OR 2.8, 95% CI 1.3-6.4).

Levels of short chain fatty acids (SCFAs) in faeces were assessed both at 1 and 4 years of age and related to the development of sensitisation and symptoms. The levels of acetic (p<.01) and propionic (p<.01) acids decreased from one to four years of age, whereas valeric acid (p<.001) increased which is in line with a more complex gut microbiota with age. Allergic children, compared with non-allergic children, had lower levels of i-butyric, i-valeric and valeric acid in faeces both at 1 and 4 years of age.

Low levels of secretory IgA (SIgA) in saliva were associated with wheezing but only in sensitised children. In children with positive SPT to at least one allergen both at 1 and 4 years of age and in children with circulating IgE antibodies to egg or cat at one year of age, those who developed late-onset wheezing had lower levels of SIgA than those who did not, p=.04 and p=.02 respectively. Of 9 children with levels of SIgA in the upper quartile and persistent sensitisation, none developed wheezing, compared with 10/20 children with lower levels, (p=.01). Having older siblings, more than three infections during infancy, at least one smoking parent and male gender were all associated with high levels (in the upper quartile) of total IgA and SIgA.

The findings in this thesis indicate that the microbial load early in life could affect the development of allergy. A functional assessment of the gut flora demonstrated differences between allergic and non-allergic children both at 1 and 4 years of age. Salivary IgA was associated with infections during infancy, and high levels of secretory IgA protected from symptoms in sensitised children. Finally, dog keeping in infancy may offer protection from allergy, but the mechanism is uncertain.

Key words: pet keeping, sensitisation, intestinal microflora, short chain fatty acids, allergy, salivary IgA, late-onset wheezing
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FTA</td>
<td>faecal tryptic activity</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>J chain</td>
<td>joining chain</td>
</tr>
<tr>
<td>MAC</td>
<td>microflora-associated characteristics</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>pIgR</td>
<td>poly-Ig receptor</td>
</tr>
<tr>
<td>SC</td>
<td>secretory component</td>
</tr>
<tr>
<td>SCFA</td>
<td>short chain fatty acid</td>
</tr>
<tr>
<td>SIgA</td>
<td>secretory IgA</td>
</tr>
<tr>
<td>SPT</td>
<td>skin prick test</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th cell</td>
<td>helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
</tbody>
</table>
PAPERS IN THIS THESIS


III. Anna Sandin, Lennart Bråbäck, Elisabeth Norin and Bengt Björkstén. Fecal short chain fatty acid pattern and allergy in early childhood. Submitted

IV. Anna Sandin, Bengt Björkstén, Malin Böttcher, Maria Jenmalm and Lennart Bråbäck. High salivary secretory IgA antibody levels are associated with less late-onset wheezing in IgE-sensitized infants. Submitted

Paper I and II are reprinted with kind permission from the publishers.
# TABLE OF CONTENTS

**ABSTRACT**

**ABBREVIATIONS**

**PAPERS IN THIS THESIS**

**INTRODUCTION** ........................................................................................................ 9

**BACKGROUND** ................................................................................................. 9

Allergy

**BASIC IMMUNE CONCEPTS** ........................................................................ 10

Innate immunity

Adaptive immunity

B-lymphocytes

IgA

T-lymphocytes

Th1 and Th2 cells

T regulatory cells

Th17

Tolerance

**THE HYGIENE HYPOTHESIS** ......................................................................... 16

Older siblings

Day care

Infections

Immunization

Antibiotics

Social class

Standard of living

**FARMING** ........................................................................................................ 19

**FURRED PETS** ............................................................................................. 20

**MICROBIOTA** ............................................................................................... 21

**GENETICAL ASPECTS** .................................................................................. 23

**AIMS OF THE THESIS** .................................................................................. 24

**SUBJECTS AND METHODS** ........................................................................... 25

**STUDY AREA**

**STUDY DESIGN AND STUDY POPULATION**

**STUDY METHODS**

**DEFINITIONS**

**STATISTICAL ANALYSES**

**ETHICAL ASPECTS**

**RESULTS** ......................................................................................................... 33

Paper I .............................................................................................................. 33

Paper II and III ............................................................................................... 36

Paper IV ............................................................................................................ 39

**DISCUSSION** ............................................................................................... 43

**CONCLUSIONS** .......................................................................................... 50

**SVENSK SAMMANFATTNING** ................................................................. 51

**ACKNOWLEDGEMENTS** .............................................................................. 53

**REFERENCES** .............................................................................................. 56

**PAPERS I-IV**
INTRODUCTION

BACKGROUND
The prevalence of allergic diseases is increasing in affluent societies. Different risk factors and protective factors have been put forward to present hypotheses that might explain the phenomenon from the perspectives of epidemiology, immunology, microbiology and genetics. Allergy has many nuances. The development of different, sometimes transient, age influenced symptoms in combination with environmental changes is best observed in longitudinal studies, preferably a prospective birth cohort study. Allergic diseases are often programmed early in life (1, 2). The immune system may be primed in infancy (3), with possible influences from maternal-foetal interaction (4).

Allergy
Allergic symptoms can be present already in the newborn, or can develop or change over time through immunological mechanisms originating from different organs.

Fig 1. (I) At the first contact with specific allergen, e.g. pollen, B cells differentiate into plasma cells and produce IgE antibodies to pollen that will attach to mast cells. (II) At the second contact, pollen allergens trigger IgE primed mast cells to secrete substances (e.g. histamine) causing allergic symptoms.
In classical allergy certain individuals start producing IgE antibodies in contact with specific common proteins or glycoproteins in our environment. These are called allergens. This reaction will result in the growth and differentiation of cells such as eosinophils and mast cells. If the individual is already sensitised with specific IgE antibodies bound to mast cells or basophils, a second allergen exposure can induce an allergic reaction (Fig 1).

**BASIC IMMUNE CONCEPTS**

To study the environmental and microbial impact behind allergic reactions it is important to understand basic immunological mechanisms. The defence against microbes and foreign particles can be divided into three different levels. The first line of defence is the mechanical and chemical barrier, which includes skin and mucous membranes with acidity in the ventricle, substances with antimicrobial effect, e.g. defensins in the gut and lysozymes in saliva and tears, but also the normal bacterial flora that competes with foreign microbes for attachment sites, nutrition and entrapment (5). The second and third lines of defence are innate and adaptive immunological mechanisms operating individually but highly interacting with each other.

**Innate immunity**

Innate immunity is unspecific, acts within minutes to hours, and can be described as cells e.g. monocytes, neutrophils, tissue macrophages and molecules that recognise and react to microbial alarms with endocytic, phagocytic and inflammatory activity (6). During the past decade the immunological defence role of Toll-Like Receptors (TLRs) recognising PAMPs (pathogen associated molecular patterns) has been expanded. TLR are expressed on various cells and recognise a number of microbial components presented in all microbial organisms e.g. lipopolysaccharide (LPS) on gram-negative bacteria. There is no need for phagocytosis as TLR4 will recognise a complex of LPS binding protein and LPS bound to a receptor on a macrophage. There is increasing evidence that the recognition of commensal microbes is involved in order to boost immunity or inhibit inflammation (7). For allergic diseases and possible therapies, TLR2 and TLR4 are of interest (7, 8).

**Adaptive immunity**

Adaptive immunity is more precise, recognising specific antigens, but takes days to weeks to develop. It involves antigen recognition by specific receptors presented on two cell types, T -
and B-lymphocytes. Foreign molecules will result in reactions characterised by four different entities: *specificity, diversity, immunological memory and self/non-self recognition*. Both B- and T lymphocytes originate in the bone marrow, where B-lymphocytes develop its self and non-self activity before leaving the bone marrow, migrate to the spleen for maturation and then spread in the body. T-lymphocytes migrate to the thymus to improve self and non-self recognition before further circulation.

**B-lymphocytes**

Each mature B cell presents its specific antibody on the surface as the B cell receptor (BCR). These antibodies have a common structure with two identical heavy and two identical light polypeptide chains bound together with disulfide bonds (Fig 2). The heavy and light chains form a cleft, which is the part for recognising and binding antigens. These, in turn, induce the clonal selection with B-cell division and the production of a large amount of antibodies against the antigen that is present. The constant region is presented in a limited number of variations that give rise to five different immunoglobulin classes: IgA, IgD, IgE, IgG and IgM.

![Fig 2. An immunoglobulin molecule presented as a monomer](image)

Immunoglobulin G, a monomer (Fig 2), is the most common Ig class in serum. It is an effective complement activator but also interacts with phagocytic cells for opsonisation. IgD has no identified biologically active function. IgE antibodies present an extremely low average serum concentration (0.3mg/L); most are bound to high affinity receptors on tissue mast cells just beneath epithelial surfaces and some are bound to membranes of blood basophils. When receptor bound IgE antibodies are cross-linked by an antigen, inflammatory mediators such as histamine, leukotrienes and prostaglandins are released. These account for
most of the signs and symptoms, which include hay fever, asthma and anaphylactic shock, of an early phase allergic reaction within minutes after exposure to allergens.

As opposed to these monomers, IgM is presented as a pentamer with ten antigen-binding sites (9). Because of its large size, IgM does not diffuse well and is found in low concentrations in intercellular fluid. However, it transports actively across epithelial linings to enter external secretions where it plays an accessory role as a secretory Ig, which is important in the neonate and in individuals with selective IgA deficiency (10).

IgA

The primary function of IgA antibodies is to provide a first line of defence against a wide variety of pathogens by preventing the attachment of bacteria or toxins to epithelial cells by absorbing foreign substances (11). In serum, IgA exists primarily as a monomer at a relatively low concentration, whereas the mucosal plasma cells secrete a huge amount of polymeric IgA connected with a single J chain (12). This J-chain polypeptide is capable of binding to the poly-Ig receptor (pIgR) on the basolateral surfaces of the epithelial cells, which are the lining of the digestive, respiratory and genital tracts, as well as on glandular epithelia in mammary, salivary and lacrimal glands (Fig 3).

Fig 3. The production of secretory IgA
The bound complex is then transported in a vesicle across the cell to the apical surface, where one part of the pIgR, called the secretory component (SC), is cleaved and released as a component of secretory IgA (SIgA). The SC masks the site’s susceptible to protease cleavage in the hinge region of SIgA, allowing these polymeric molecules to exist longer in the protease-rich mucosa, as they are well suited to surface protection (13). Because it is polymeric, SIgA can cross-link antigens, and these complexes are easily entrapped in mucous and eliminated by cilia on the epithelial cells of the respiratory tract or by peristalsis of the gut. As low concentration of salivary IgA has been associated with an increased risk of allergy (14), high levels could theoretically prevent allergen absorption.

**T lymphocytes**

T-lymphocytes migrate to the thymus for maturation where they express a unique membrane bound antigen-binding molecule, called the T cell receptor.

---

*Fig 4. Summary of adaptive immune responses*
These receptors can only recognise antigens that are bound to cell-membrane proteins, so-called major histocompatibility complex (MHC). There are two types: MHC I, expressed by nearly all nucleated cells, and MHC II, expressed only by antigen presenting cells (APCs) such as macrophages, B-lymphocytes and dendritic cells.

There are two well-defined subpopulations of T cells: T helper (Th) cells bearing CD4+ (CD=cluster of differentiation), which recognise only antigen bound to MHC II; and T cytotoxic (Tc) cells bearing CD8+, which recognise only antigen bound to MHC I (Fig 4).

The Th cell recognises and interacts with an antigen-MHC II complex, is activated and becomes an effector cell with secretion of various inflammatory messenger proteins, i.e. cytokines. These cytokines play an important role in activating other T cells, B cells and macrophages. To regulate activation and avoid autoimmune reactions, Th cells recognise only antigen displayed together with MHC II molecules on the surface of antigen presenting cells (APCs). APCs first internalise the allergen through phagocytosis or endocytosis and then display it bound to MHC II, where Th cells recognise them and interact with an additional co-stimulatory signal produced by APC. As a result, the Th cell is activated.

**Th1 and Th2 cells**

Th1 cells differentiate functionally (15) from naïve T cells under the influence of IL-12 and produce cytokine IFN-γ. IFN-γ plays an important roll in activating macrophages that kill intracellular bacteria and activate Tc cells to cytotoxic T lymphocytes (Fig 4).

T-helper cells play an important role in allergic inflammation and are initially activated by aeroallergens or food allergens. Th2 cells are differentiated from naïve Th cells in the presence of IL-4. Th2 cells produce cytokines that are responsible for allergic reactions and inflammation activities such as IgE antibody production (IL-4), eosinophil differentiation (IL-5), smooth muscle cell activity (IL-9) and epithelial cell secretion (IL-13) (17,18,16) (Fig 5).

There is a cross-regulation of the polarisation between Th1 and Th2 as IL-4 inhibits the production of IFN-γ, and IFN-γ inhibits the production of Th2 cytokines (19).
T regulatory cells

T regulatory cells (Treg), CD4+CD25+, are well characterised both in molecular and functional aspects as a functionally mature T-cell subpopulation (20). Treg cells prevent immune response, regulating activity from Th1, Th2 and Th17 cells as well as the APC function (Fig 5). The increased levels of IL-10 and transforming growth factor-beta (TGF-β) suppress IgE production, while IgG4 and IgA production is stimulated. In addition, Treg cells directly or indirectly suppress effector cells such as mast cells, basophils and eosinophils (21, 22) and inhibit the activation of cytotoxic T lymphocytes. If immune reactions to auto-allergens and allergens are not regulated, autoimmune disease and allergy can develop. A reduced capacity to suppress Th2 responses to allergens may lead to the development of allergic sensitisation (21). As Treg cells are highly dependent on IL-2 for their survival in the periphery, and IL-2 is produced from other activated T-cells, increased T cell activation enhances Treg activity. In healthy humans there is a balance between the number of activated T cells and Treg cells (20).
Th17
The discovery of the Th17 cells has added knowledge about how Th cells mediate inflammation in the tissue. As neutralisation of IL-17 reduces neutrophil infiltration while increasing that of eosinophil, it appears that Th17 neutrophil activation is inversely linked to Th2 eosinophil activation, which is similar to the known inverse relationship between Th1 and Th2 cell activity (16). The natural function of Th17 indicates a pro-inflammatory role (16). Impaired Th17 differentiation is a mechanism that underlies recurrent infections in hyper IgE syndrome (23).

Tolerance
Oral tolerance (OT) is defined as the specific immunological unresponsiveness to an previously fed antigen. It is a complex process involving suppression of some immune responses and the induction of others (24), and may be achieved in a number of ways. Exposure to high doses of antigen may favour anergy, with the deletion of antigen-specific T cells. This is expected to result in stable, long-lasting tolerance (25) with initial sensitisation prior to the appearance of suppression (and tolerance) (26). However, immunological tolerance cannot rely only on neonatal deletion events, but requires an active process that functions during the entire life. Low doses of antigen may favour the second form of oral tolerance, with the induction of cytokine activation related to Treg cells (IL-10, TGF-beta) and an active suppression (26, 27). As Treg cells inhibit signals to Th cells and APCs, there will be no differentiation and inflammation. Instead, tolerance develops. In addition to antigen dose, the nature of the antigen, the innate immune system, the genetic background and the immunological status of the host will influence the reaction following oral antigen administration (26).

THE HYGIENE HYPOTHESIS
In 1976, Gerrard originally hypothesised that relative freedom from infectious diseases underlay the increase in allergic diseases in affluent societies. He observed that allergic diseases were more likely in individuals in white families than in individuals from non-affluent Metis families living in the same area in northern Saskatchewan. In contrast, untreated infections were common in the Metis families (28). Fewer allergic symptoms in a society with an increased exposure to viral and bacterial infections could fit with the Th1/Th2 paradigm (29). With new knowledge of the impact of Treg cells in balancing immunological reactions and the development of tolerance, more interest is focused on the interaction and
regulation of immune responses not only from clinical but also from sub-clinical infections and total microbial load, including commensals. The “hygiene hypothesis” is not a single straightforward idea, but is part of a complex interplay between immune response, the characteristics of the invading micro-organism, the level and variety of environmental exposure, and the interactions between the genetic background and exposures (30).

Older siblings
Strachan demonstrated an inverse relationship between the risk of hay fever and the number of older siblings (31). This finding suggested that infections in early childhood, transmitted by unhygienic contacts with older siblings or infections acquired prenatally, reduced the risk of allergic disease. Since then, this relationship between sibship size and allergic manifestations has been supported by numerous studies. The protective effect of older siblings on allergic disease is stronger than the protective effect of younger siblings, indicating that contact with microbes early in life is important (32).

However, the association between birth order and the prevalence of allergic disease has not been consistent. Birth order appears to have different effects on asthma and allergic rhinitis (33) as the prevalence of hay fever was strongly associated with birth order and social class in two large British cohorts. In contrast, asthma had no association with birth order, suggesting that asthma is less closely associated with allergy (34). A large European study demonstrated that sharing a bedroom in childhood and having siblings provided protection from atopy in adulthood, but the number of siblings was more important than birth order (1). The number of pregnancies had an inverse relationship with cord IgE levels (35), suggesting that the sibling effect may have some of its origin already in utero (4, 36).

Day care
Attending day nursery at an early age has been associated with an increased risk of early wheezing (37) but a decreased risk of atopic dermatitis (38) and a reduced risk of positive skin prick test in German schoolchildren (39) and in Australian preschool children (40).

Infections
Some evidence indicates that certain infectious diseases may offer protection from allergic sensitisation and symptoms, but the findings from epidemiological studies have been contradictory (34).
Repeated viral infections may reduce the risk of developing asthma up to school age (41), and otitis media was negatively associated with allergic sensitisation in school children with atopic parents (42). Epidemiological studies from Italy and the US demonstrated an inverse relationship between sensitisation, allergic rhinitis and respiratory allergy and previous infection with hepatitis A virus, herpes simplex virus and Toxoplasma gondii (43, 44). Infections with hepatitis A are markers of poor hygiene and are often acquired early in life in non-affluent populations.

Other epidemiological studies do not indicate that infections during infancy could protect from allergy (45, 46). A positive serology to hepatitis A had no association with sensitisation in studies from Spain (47) and the UK (48). A prospective study failed to demonstrate any relation between the number of bacterial infections, including otitis media, and development of asthma or allergic symptoms (41). No association has been shown between allergic diseases and infections with rubella, mumps, measles, chicken pox, cytomegalovirus or herpes simplex virus (49). A large British study based on computerised patient records in general practice did not find any consistent association between hay fever and 30 different infectious diseases during infancy (50). Early attendance in day care and older siblings offered protection from atopic eczema at 18 months in a large Danish birth cohort study. However, infections during infancy did not affect the risk of allergic disease (38).

Respiratory infections early in life have been associated with an increased risk of asthma both at age 4 (51) and age 10 (52). Respiratory syncytial virus (RSV) infection in early childhood is a risk factor for atopic sensitisation (53) and persistent asthma (54, 55).

**Vaccination**

Several well-designed studies have been set up to elucidate the possible correlation between immunisation and allergic diseases, but so far immunisations have not been related to an increased risk of asthma (56) or other allergic symptoms (57).

**Antibiotics**

Use of antibiotics has been proposed as a risk factor for asthma in retrospective epidemiological studies (58). This association is likely to be explained by reverse causation as asthmatic symptoms in young children are often treated with antibiotics.
Antibiotics could change the gut flora, with a negative influence on the microbiota, and disturb normal immune reactions. A retrospective study detected a doubling of the risk of hay fever and eczema among children who had received oral antibiotics by the age of 2 (59). However, other studies have failed to find any association between the use of antibiotics in infancy and the development of allergic symptoms (60, 61).

**Social class**

An increased awareness of symptoms may explain a high prevalence of hay fever (62) and eczema (63) among children and adults in affluent families. However, an increased risk of atopy related to affluence has been verified with objective measurements in widely different countries such as the UK (63), USA (64), Eastern Germany (65) and urban Ghana (66).

**Standard of living**

Studies in the 1990s demonstrated a lower prevalence of allergic diseases and sensitisation in Eastern Europe than in Western Europe (67, 68, 69). The low prevalence of allergic diseases has been associated with the low standard of living (67). Factors related to domestic crowding provided protection against atopic sensitisation in Estonia and Poland (70). The ISAAC PHASE I Study demonstrated a very low prevalence of wheezing in poor countries such as Uzbekistan and Georgia (71).

**FARMING**

Several studies from different parts of the world have demonstrated that children living on farms with animal stock have a reduced prevalence of asthma, rhino-conjunctivitis and sensitisation compared with neighbouring children (72, 73, 74, 75, 76). This association persists into adulthood (77, 78). Animal sheds, hay lofts and the consumption of unpasteurised cow’s milk have been identified as protective exposures (75,79). Farming environments provided protection from allergic rhinitis in a birth cohort born in the 1950s whereas the protective effect of asthma appeared to be a recent phenomenon (80). Some studies have not found any differences in sensitisation between children of farmers and non-farmers (81, 82). The underlying protective mechanism has not yet been found, but contact with livestock is essential and has been related to the possible effects of bacteria (83). There is limited technology to assess microbial exposure, but endotoxin, a lipopolysaccharide (LPS) in the gram-negative bacterial wall, and muramic acid, a peptidoglycan in all bacteria, are
found in higher levels in the mattresses of farm children (84, 85). Farm children are also exposed to higher levels of mould components (86). Furthermore, high levels of endotoxin in house dust have been correlated with furred pets at home (87, 88). In the PARSIFAL study the asthma-protective effect was associated with pigs, feeding silage, involvement in haying, farm milk consumption, and regular stays in animal sheds (89). Not all exposures were found to be beneficial for all phenotypes (30). Exposure to endotoxin has been related to a reduced risk of atopic sensitisation, hay fever and atopic asthma. In contrast, however, high levels of endotoxin have been associated with an increased risk of non-atopic wheeze, airway hyperresponsiveness and low lung function (84, 90). Endotoxin levels in dust are associated with the development of tolerance towards allergens in natural environments. The recognition of these compounds by the immune system and the regulation of the inflammatory response are likely to be of key importance for the development of allergy (84). Levels of endotoxin and extracellular polysaccharides have recently been shown related to health outcomes independently of farm exposure (89). This supports the notion that non-viable products in the environment can stimulate immune responses in ways that offer protection from allergies (91), but it also relates protective factors to higher expression levels of innate immunity genes (89).

FURRED PETS
Avoidance of pets has not been a successful strategy to prevent atopic diseases as many studies have shown an inverse relationship between pet ownership and atopy both in children (92, 93, 94, 95) and in adults (96, 1). This association could be confounded by socio-economic status (97) and avoidance of pets because of allergies in the family (92, 98, 99). Current ownership among schoolchildren has been inversely related to positive skin prick test to cat as well as to asthma, especially among families with asthma heredity (100). Early exposure to cat has been associated with sensitisation to cat but not to asthma, whereas dog ownership has been associated with lower risk of sensitisation to airborne allergens and asthma (101).

High levels of endotoxin in house dust have been correlated with furred pets at home (88) both for dogs and cats individually (87). An inverse relationship has been shown between the level of indoor endotoxin and sensitisation in infants, and a direct relation has been shown between exposure to endotoxin and the proportion of T-helper cells producing IFN-γ (102).
Exposure to endotoxin during sensitisation could block the IgE response (103). In contrast, endotoxin exposure days after allergen exposure may enhance the IgE-mediated response (104), thereby suggesting that the effect of exposure depends on the context in which it occurs (105).

It has been proposed that repeated airborne endotoxin exposures have proinflammatory effects (106). In contrast, exposures to high levels of endotoxin could balance the T helper cell response and suppress inflammatory reactions (83).

A negative association between wheezing and exposure to dog and cat allergens has been found independent of the endotoxin load (107). High levels of pet allergen may induce a modified Th2 immune response characterised by an increased production of IL-10 and IL-4, high levels of IgG4 and IgG1 and suppression of the IgE response (108). It has been suggested that the association between exposure to increasing levels of cat allergen and development of sensitisation has a bell-shaped dose-response curve. Increasing levels of allergen may increase the risk of sensitisation. In contrast, however, very high levels of exposure could provide protection from sensitisation (109).

Exposure to pets in the community may affect the prevalence of sensitisation to animal dander and the prevalence of current wheezing (92). A large proportion of cat-sensitised children have never lived in a house with a cat (98). Adults reporting a family history of atopy were less likely to have IgE antibodies to cat as adults, and this sensitisation was negatively associated with childhood exposure to cat (96).

**MICROBIOTA**

The healthy child was born germfree. The newborn child is immediately in contact with microbes in the environment. The colonisation of the gut is influenced by e.g. the route of delivery, standard of living, hygiene, gestational age, and diet (25). In general the gut flora is established before two years of age (110). The gut flora is a complex ecosystem composed of numerous genera, species and strains of bacteria (111). Pioneer bacteria can modulate expression of genes in host epithelial cells (112), and create a favourable habitat for themselves and the host, thus preventing the growth of other bacteria introduced later. This makes the initial colonisation very relevant to the final composition of the gut flora in the
adult (113). The colonisation starts with aerobic bacteria. The aerobes consume oxygen, mucous and nutrients and are soon outnumbered by anaerobic bacteria. It is claimed that the bifidobacteria and lactobacilli are the most important health-beneficial bacteria (114), but the gut hosts hundreds of different other commensal bacteria living in peaceful partnership with, and involved in the development of, the immune system and different metabolic cross-talks between the host and the flora (115). The gut flora has three main functions: 1) metabolic, in terms of the metabolism of non-digestible dietary residues, contribution of energy as short chain fatty acids, production of vitamin K and absorption of ions, 2) trophic, with control of epithelial cell proliferation and the differentiation and development of homeostasis of the immune system, and 3) protective against pathogens through a barrier effect (colonisation resistance)(113).

The mucosal interface seems to play an important part in the development of a competent immune system (113). There are successively built up dynamic, reciprocal and complex interactions between intestinal microbes, the epithelium and gut-associated lymph tissue (GALT)(116). It has been shown that germfree animals have smaller Peyers patches, fewer intraepithelial lymphocytes and lower levels of secretory IgA and are resistant to the induction of oral tolerance to food antigens (117, 118). Inoculation of germfree mice with intestinal bacteria – conventionalisation – can restore the ability to generate oral tolerance, but this is only effective in neonates and not in older mice (117). The presence of a microbial flora favours the development of a fully functional Treg population, as Treg cells from germfree mice are not as potent suppressors as those from conventional mice (119).

Alterations in life styles in affluent societies, including standard of living, hygiene, diet and microbial exposure, have led to a less diverse flora. Instead of enterobacteria, infants are colonised by other bacteria that are regularly part of the normal flora of the skin (120). Contemporary Swedish infants are colonised with staphylococci at a very early age, whereas colonisation by enterobacteria is delayed (121). It has been hypothesised that perinatal colonisation with Staphylococcus aureus may influence the development of the infantile immune system and the risk of allergy (122).

Changes in the composition of the microbial flora with reduced numbers of traditional intestinal microbes could result in aberrant immune responses to harmless antigens later in life. Disturbance in gastrointestinal microbiota composition may disrupt mechanisms for
immunological tolerance (25). Recent data from three ongoing European birth cohorts showed delayed colonisation by *Clostridium species* in infants with older siblings but failed to find any association between specific bacteria and sensitisation (123). However, the diversity in gut flora at one week of age differed significantly between children with and without atopic eczema at 18 months (124).

**GENETIC ASPECTS**

Although heredity is important for the risk of sensitisation to allergens and the development of allergic symptoms, these are not inherited in simple Mendelian fashion. Twin studies have shown that at least 50% of the susceptibility to the development of asthma is determined by inherited predisposition (125). Epidemiological studies verify that atopy with its related symptoms is a highly complex disorder suggesting a substantial genetic component where at least two independent genes are thought to come together with epidemiological factors to result in allergic inflammation in a particular tissue (126). A number of different regions in the human genomes are evaluated as harbouring atopic genes (127). Studies have shown that genetic susceptibility modifies the effect of an exposure (128) e.g. polymorphisms in genes for TLR (129). Only subjects with genes conferring sensitivity to the exposure are associated with a protective effect whereas other polymorphisms are not.
AIMS OF THE THESIS

The overall goal of this thesis was to assess the development of allergic sensitisation and symptoms during the first 4 years of life in a non-selected birth cohort in relation to environmental factors, family history, gut microbiota and salivary IgA antibodies.

Specific aims were to assess

# The role of pet keeping and family history in the development of allergic sensitisation and wheezing phenotypes during early childhood

# Microflora-associated biochemical markers in allergic and non-allergic children at 1 year of age

# Whether levels of SCFAs at 1 year of age could predict sensitisation and allergic symptoms at 4 years of age

# Whether functional changes of the gut flora over time, as assessed by the analysis of SCFAs, were related to sensitisation and allergic symptoms at 4 years of age.

# Whether high levels of salivary IgA antibodies at 1 year offered protection from the development of allergic symptoms in sensitised children.

# Potential determinants of salivary IgA antibodies
SUBJECTS AND METHODS

STUDY AREA
Jämtland, a county in Northern Sweden, is 50% forested, 25% mountainous, and only 2% is left for farming areas and villages. Fifty per cent of the children live in Östersund, the largest town in the area, which has 43,500 inhabitants, few industrial plants and low levels of outdoor air pollutants. The other half of the population is distributed over the county, living in small towns with 1,000-4,000 inhabitants or in rural areas over an area of 49,400 sq. km. It has previously been demonstrated that sensitivity to house dust mite is rare in this area (92).

STUDY DESIGN AND STUDY POPULATION

Birth cohort
The study was designed to assess the development of allergy in a birth cohort of 1,231 children, all living in Jämtland and born during the period February 1996 – January 1997 at Östersund Hospital (Table 1). The families were enrolled either at the first visit to the antenatal clinic or at the birth of the child. Three babies died during the first year of life, leaving 1,228 infants. At birth, the parents of 857 children replied to the first questionnaire about living conditions, smoking habits, the keeping of pets and symptoms of asthma and allergy in the family. When the children were 1 year old, the study nurse had a standardised interview with the parents of 1,043 children concerning breast-feeding, infectious diseases, antibiotic courses, changes in home environment and allergic symptoms during the first year of life. At 4 years, the parents of 801 children responded to a mailed questionnaire concerning home environment, symptoms of allergic diseases, food intake and pet ownership. The parents of 736 children replied to questionnaires on symptoms at both 1 and 4 years of age. All children were offered skin prick tests (SPTs) both at 1 and 4 years of age (Table 1).

Table 1. Birth cohort and follow up at 1 and 4 years.

<table>
<thead>
<tr>
<th></th>
<th>Birth n (%)</th>
<th>12 months n (%)</th>
<th>4 years n (%)</th>
<th>Both 12 months and 4 years n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study objects</td>
<td>1,231</td>
<td>1,228</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Questionnaire</td>
<td>857 (70%)</td>
<td>1,043 (85%)</td>
<td>801 (65%)</td>
<td>736 (60%)</td>
</tr>
<tr>
<td>SPT</td>
<td>1,040 (85%)</td>
<td>879 (72%)</td>
<td>817 (67%)</td>
<td></td>
</tr>
</tbody>
</table>
**Subgroup**
At 1 year of age a subgroup of children was invited to a more detailed clinical examination. The subgroup comprised 71 children with a positive SPT (defined as an allergen wheal with a diameter of at least 3 mm), 68 children with a negative SPT but at least one allergen wheal of at least 1 mm, and 156 age-matched children with negative SPT (all allergen wheals less than 1 mm). All children in the subgroup were examined by a doctor (AS), and samples of blood, saliva and faeces were delivered from 254, 279 and 139 children, respectively. At 4 years of age, 236/295 (80%) accepted a follow-up with SPT (n=232), a parental questionnaire (n=236) and faecal sampling (n=53).

**Paper I**
The study comprised all children in the birth cohort in which the children participated in SPT and the parents replied to questionnaires at 1 and 4 years of age (Table 1).

**Paper II**
Faecal samples at 1 year were analysed from 25 children with at least one positive SPT (>=3mm in diameter) and allergic symptoms diagnosed by a doctor (eczema, asthma and/or food allergy), and from 47 non-allergic children with negative SPT and no symptoms.

**Paper III**

![Flow chart](image)

Fig 6. Flow chart. Faecal samples at 1 year and follow up at 4 years.
The study population was all children from whom faecal samples were delivered at 1 year of age (n=139) (Fig 6). Of those, 28 children had a positive SPT and 111 had a negative SPT. At 4 years, 53/139 (38 %) children delivered a second faecal sample, 120/139 children participated in SPT, and the parents of 125/139 children replied to a questionnaire about allergic symptoms. The study group is summarized in table 2.

Table 2. Characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Invited children n=295 (%)</th>
<th>Faeces at 1 year n=139 (%)</th>
<th>Faeces both at 1 and 4 years n=53 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Older siblings</td>
<td>195/292 (67%)</td>
<td>90/139 (65%)</td>
<td>33/53 (62%)</td>
</tr>
<tr>
<td>Female gender</td>
<td>127/295 (43%)</td>
<td>57/139 (41%)</td>
<td>20/53 (38%)</td>
</tr>
<tr>
<td>Exclusive breastfeeding at four months</td>
<td>208/284 (73%)</td>
<td>98/125 (73%)</td>
<td>38/52 (73%)</td>
</tr>
<tr>
<td>Heredity for asthma</td>
<td>84/294 (28%)</td>
<td>42/139 (30%)</td>
<td>17/53 (32%)</td>
</tr>
<tr>
<td>Heredity for rhinit/asthma</td>
<td>159/294 (54%)</td>
<td>73/139 (53%)</td>
<td>30/53 (57%)</td>
</tr>
<tr>
<td>Antibiotics in infancy</td>
<td>90/295 (31%)</td>
<td>36/139 (26%)</td>
<td>17/53 (32%)</td>
</tr>
</tbody>
</table>

**Paper IV**

Saliva samples were obtained from 67 children with a positive skin prick test (SPT) at 1 year and 212 children with negative SPT in all 279 children.

Fig 7. Flow chart. Saliva samples at 1 year and follow up at 1 and 4 years.
Of these, 200 children participated in a clinical examination at 1 year of age, their parents responded to questionnaires at 4 years, and 183 were also skin prick tested at 4 years of age (Fig 7). The rates of breastfeeding for more than 4 months, number of older siblings, passive smoking and family history of allergic disease were similar in the 200 participants and the 79 dropouts.

**STUDY METHODS**

**Clinical examination**
At 1 year of age all children (n=291) in the subgroup (see above) replied to an invitation to undergo a clinical examination by the study doctor (AS).

**Skin prick test**
Skin prick tests were performed at 1 year of age in 1,040 children (85%) and at 4 years of age in 879 children (72%). In all, 817 of the 1,228 children (67%) participated at both 1 and 4 years. The tests were performed on the volar side of the lower arm, and standardised extracts of five allergens, i.e. egg, milk, birch, timothy and cat (Solu-Prick SQ, ALK-Abeló, Denmark) were applied with lancets from ALK. The potency of the inhalant allergens was 10 histamine equivalent prick units (HEP). Histamine dihydrochloride, 10mg/mL, was used as a positive control. The test was considered positive for a particular allergen if the mean wheal diameter was at least 3 mm in diameter after 15 minutes. The mean diameter of the histamine wheal was 4.4 mm (SD 0.6) at 1 year and 4.0 mm (SD 0.7) at 4 years. Children were instructed to avoid antihistamines for 72 hours before the test. The same study nurse carried out all the tests. The reproducibility of the skin prick test technique was checked repeatedly according to recommendations in the ISAAC phase II manual (130).

**Blood sampling and analyses**
The serum samples were stored at –20°C and analysed for specific IgE to egg and cat (Pharmacia CAP System Specific IgE FEIA, Pharmacia Diagnostics, Uppsala, Sweden).

**Faecal sampling and analyses**
Faecal samples were collected at home by the parents, immediately put in a plastic tube at
–20°C, delivered frozen to the hospital and stored frozen until analysed. The samples were thawed at room temperature. Aliquots of the faeces (0.5-1.0g) were mixed with 0.9% NaCl in sterile deionised water and centrifuged. The sediment was used to determine cholesterol conversion, and the supernatants were analysed for faecal tryptic activity (FTA).

**Determination of conversion of cholesterol to coprostanol**

The sediment was extracted and analysed as described by Midtvedt at al (131) on a gas chromatograph (Hewlett Packard 5880A Gas Chromatograph). The values were expressed as percent coprostanol of the total amount of cholesterol and coprostanol present.

**Determination of faecal tryptic activity (FTA)**

The supernatants were added to Tris buffer, pH 8.2, and then N-bensoyl-DL-arginin-4 nitroanilide hydrochloride (BAPNA, Sigma Aldrich, Stockholm, Sweden) was added. The reaction was performed for 10 minutes. A bovine pancreas trypsin type III (Sigma Aldrich, Stockholm Sweden) diluted in 2 mM HCl was used to construct a standard curve. All samples and standards were run in duplicate and analysed spectrophotometrically (HITACHI 150-20 Spectrophotometer) in parallel with blanks at 450 nm. FTA was calculated and expressed as mg tryptic activity/kg faeces (132)

**Determination of SCFAs in faeces**

Aliquots (0.4-0.6g) were homogenised in 2 mL of distilled water containing 3 mmol/L of 2-ethylbutyric acid as an international standard and 0.5 mL of H2SO4 (0.5 mmol/mL). The homogenate was vacuum distilled and analysed for SCFAs as described by Zijlstra (133) with modifications by Høverstad (134) on a gas chromatograph (Perkin Elemer Autosystem XL). Chromatograms were recorded, peak areas determined and concentrations calculated with the Shimadzu Data Processor Chromatopac C-R3A. Each series of analyses (10-15 samples) started and ended with the injection of a standard solution. From these standard chromatograms, the relative response factors for that batch were obtained. The concentrations of the different SCFAs were expressed as mmol/kg. Eight SCFAs were analysed in the faecal samples i.e. acetic, propionic, butyric, i-butyric, valeric, i-valeric, caproic and i-caproic acid. The detection level was 0.2 mmol/kg faeces.
Saliva sampling and analyses

Saliva samples were collected in a standardised way from the buccal cavity using a vacuum-pump connected to a thin plastic tube and immediately frozen at -20°C. Long-term storage has been reported not to have any effect on the antibody affinity (135), nor do long-term storage and repeated cycles of freezing and thawing alter the molecular weight of IgA (136). The levels of salivary total and SIgA, as well as salivary IgA antibodies to egg and cat were determined by ELISA (137). For analysis of total IgA, an anti-human IgA antibody directed against the alpha chain (Dakopatts AB, Täby, Sweden) was used as coating antibody, enabling the detection of all IgA, including monomeric, polymeric and secretory IgA. For detection of SIgA, the plates were coated with an anti-human secretory component antibody (Dakopatts AB), detecting only SIgA.

DEFINITIONS

Atopy
Atopy is a personal and/or familial tendency, usually in childhood, to become sensitised and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins.

Sensitisation
IgE antibodies in blood samples >0.35kU/L and/or skin prick test with a mean wheal diameter >=3mm.

Eczema
Eczema is a pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distributions (bend of the arm and knee, ankle joint, on the back side of the thigh or on the neck, around the eyes or ears).

Allergic rhinitis
Allergic rhinitis is defined as itching, sneezing, blocked or runny nose appearing at least twice after exposure to a particular allergen and not related to infection.

Asthma
The diagnosis at 1 year of age was based on three or more episodes of bronchial obstruction or on the first occasion with bronchial obstruction if the child has any other allergic
symptoms. At 4 years of age, the diagnosis was based on an affirmative response to the question: “Has your child asthma diagnosed by a doctor?”

*Early-onset transient wheezing* was defined as reported wheezing only during the first year of life.

*Early-onset persistent wheezing* was defined as reported wheezing both at 1 and 4 years of age.

*Late-onset wheezing* was defined as reported wheezing between the ages of 3 and 4, but no wheezing during the first year of life.

*Food allergy*

Food allergy at 1 year of age was diagnosed by a doctor (AS) and based on a typical history of immediate and repeated reactions, at least twice, after intake of the offending food. Food allergy at 4 years was based on questionnaire-reported doctor-diagnosed food allergy.

*Allergic children*

At least one positive skin prick test in combination with allergic symptoms.

*Non-allergic children*

Negative skin prick test and no symptoms.

*Heredity*

Heredity for asthma or allergic rhino-conjunctivitis was defined as an affirmative answer to the question: “Has the child’s mother or father ever had asthma” or “allergic symptoms from the eye or the nose?”, respectively.

*Pet keeping*

Pet keeping was defined as either cat or dog ownership. Avoidance of pet keeping was defined as an affirmative answer to the question: “Have you actively avoided pet keeping (already at the birth of the index child) because of allergies or asthma in other family members?”
STATISTICAL ANALYSES
All the data were coded and entered into the database module of the SPSS statistics software (SPSS Inc, Chicago, IL), and the subsequent analyses were performed using this package, versions 10.0 – 14.0. Chi-square test was used to assess the association between categorical variables. Fischer’s exact test was used when the expected frequency for any cell was less than five. A p-value < .05 was considered significant. Multivariate logistic regression analyses were used (Paper I) to obtain estimates of odds ratios for sensitisation and reported symptoms, with adjustments for significant co-variables and (Paper IV) to obtain estimates of determinants for high levels of secretory and total IgA (in the upper quartile), with adjustments for significant co-variables. The association between pet keeping and sensitisation and symptoms was assessed after excluding children whose parents had avoided pet keeping already at the birth of the index child because of asthma and allergies in other family members. As the SCFAs in faeces (Papers II and III) and concentrations of antibodies (Paper IV) were not normally distributed, comparisons between unpaired groups were analysed with the Mann-Whitney U-test and between paired groups with the Wilcoxon signed ranks-test. Correlations were analysed with the Pearson Correlation.

ETHICAL ASPECTS
The study was approved by the Regional Ethical Committee for Human Research at the University Hospital, Umeå. Parental consent was obtained separately for each part of the study.
RESULTS

Sensitisation and wheezing in relation to heredity and pet keeping (Paper I)

The number of children with at least one positive skin prick test was doubled between 1 and 4 years. Among children who participated in the skin prick test (SPT) both at 1 and 4 years of age, 7% (56/817) had at least one positive SPT at 1 year, compared with 13% (110/817) at 4 years. In all, 4.5% (37/817) were skin prick test positive at both ages. (Table 3)

Table 3. Positive skin prick tests in 817 children tested both at 1 and 4 years of age

<table>
<thead>
<tr>
<th>Allergens</th>
<th>1 year</th>
<th>4 years</th>
<th>1 and 4 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg</td>
<td>36 (4%)</td>
<td>11 (1%)</td>
<td>8 (1%)</td>
</tr>
<tr>
<td>Milk</td>
<td>13 (2%)</td>
<td>6 (1%)</td>
<td>6 (1%)</td>
</tr>
<tr>
<td>Timothy</td>
<td>1</td>
<td>17 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>Birch</td>
<td>2 (0.2%)</td>
<td>49 (6%)</td>
<td>2 (0.2%)</td>
</tr>
<tr>
<td>Cat</td>
<td>21 (3%)</td>
<td>72 (9%)</td>
<td>16 (2%)</td>
</tr>
</tbody>
</table>

The 12-month prevalence of wheezing was 24% (174/736) at 1 year and 17% (124/736) at 4 years among those replying to questionnaires at both ages. Corresponding rates among children participating on only one occasion were 20% (61/304) and 13% (9/68), respectively.

The prevalence rates of wheezing only at 1 year (early-onset transient wheezing), both at 1 and 4 years (early-onset persistent wheezing), and only at 4 years (late-onset wheezing) were 16 % (119/736), 7.5 % (55/736) and 9.4 % (69/736), respectively. In all, 7% (50/736) were reported to have doctor-diagnosed asthma at 4 years.

Children with early-onset, persistent wheezing and late-onset wheezing were more likely to have positive skin prick tests both at 1 and 4 years of age (Table 4). Early-onset transient wheezing had a linear association with the number of older siblings (p=.02), but had no association with positive SPT.
Table 4. Crude odds ratios (95% CI) for positive SPT at 1 and 4 year of age in children with defined wheezing sub-types.

<table>
<thead>
<tr>
<th>At least one positive SPT</th>
<th>1 year</th>
<th>4 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-onset transient wheezing</td>
<td>0.4 (0.1-1.1)</td>
<td>0.5 (0.3-1.1)</td>
</tr>
<tr>
<td>Early-onset, persistent wheezing</td>
<td>3.2 (1.5-6.8)</td>
<td>2.8 (1.5-5.4)</td>
</tr>
<tr>
<td>Late-onset wheezing</td>
<td>5.0 (2.6-9.5)</td>
<td>3.1 (1.7-5.7)</td>
</tr>
</tbody>
</table>

During the first year of life, 205/1040 (19%) children were reported to have a dog at home and 153/1040 (14%) were reported to have a cat. Avoidance of pet keeping was often undertaken because of allergies in other family members. When the index child was born, 20% of our families had actively avoided pets. Among families with early pet keeping, 13 out of 14 families got rid of the pet if the SPT at 1 year was positive to cat. In addition, 48 children became pet owners after infancy. None had a positive test to cat allergen at 1 year and, as a consequence, a positive SPT to cat at 4 years was inversely related to current cat (odds ratio, OR 0.4, 95% CI 0.1-0.8) and dog (OR 0.4, 95% CI 0.1–0.8) ownership.

Sensitisation to cat allergen at 1 year was not associated with wheezing at 1 year (OR 1.0, 95% CI 0.4-2.4), but was associated with an increased risk of wheezing at 4 years (OR, 5.3, 95% CI, 2.0– 13.5). Infants who were exposed to cats at home during their first year of life had an increased risk of positive SPT to cat at 1 year (OR, 2.1, 95% CI, 0.9-4.9), but there was no association with wheezing at 1 or 4 year of age.

Dog keeping during the first year of life was associated with a decreased risk of sensitisation to pollen allergen at 4 years, and the reduced risk persisted after adjustments for older siblings, maternal smoking, parental hay fever and asthma and the avoidance of pet keeping because of allergies in other family members (Table 5).
Table 5. Adjusted odds ratios (95% CI) for at least one positive SPT to an inhalant allergen at 4 years in relation to dog ownership

<table>
<thead>
<tr>
<th></th>
<th>% Sensitised to cat</th>
<th>% Sensitised to pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog never</td>
<td>7.4</td>
<td>7.9</td>
</tr>
<tr>
<td>Dog during first year</td>
<td>7.1 0.9 (0.4-1.8)</td>
<td>2.5 0.3 (0.1-0.9)</td>
</tr>
<tr>
<td>Dog after first year</td>
<td>2.1 0.3 (0.0-2.4)</td>
<td>4.3 0.7 (0.2-3.1)</td>
</tr>
</tbody>
</table>

Dog keeping during the first year of life was associated with a decreased risk of late-onset wheezing, but was associated with a slightly increased risk of early-onset transient wheezing (Table 6).

Table 6. Crude and adjusted odds ratios for wheezing at 1 and 4 years of age in relation to dog ownership

<table>
<thead>
<tr>
<th></th>
<th>Early-onset transient wheezing</th>
<th>Late-onset wheezing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude OR (95% CI)</td>
<td>Adjusted OR (95% CI)</td>
</tr>
<tr>
<td>Dog during first year</td>
<td>1.5 (1.0-2.4)</td>
<td>1.5 (0.9-2.6)</td>
</tr>
<tr>
<td>Cat during first year</td>
<td>1.1 (0.6-1.9)</td>
<td>1.1 (0.6-1.9)</td>
</tr>
</tbody>
</table>

Parental hay fever and asthma were independent determinants of sensitisation and symptoms in the child, with direct association between paternal and maternal hay fever and sensitisation to inhalant allergens at 4 years. Maternal asthma had a strong relationship with early-onset persistent wheezing and was also a risk factor for late-onset wheezing. The positive association between dog keeping and early-onset transient wheezing was only observed in children with parental asthma (OR 2.8, 95% CI 1.3-6.4) and not in children without parental asthma (OR 1.0, 95% CI 0.6-1.7). This association was even stronger after excluding families who had avoided pet keeping because of allergies or asthma in other family members (adjusted OR 4.3, 95% CI 1.5-12.1).
Microflora–associated characteristics in faeces and allergy (Papers II and III)

Paper II demonstrated that allergic children had lower levels of propionic, i-butyric, butyric, i-valeric and valeric acids than non-allergic children (Fig 8). Levels of i-caproic acid were detected in only seven children among whom six were allergic. The relative distribution of i-caproic and acetic acid was higher in allergic than in non-allergic infants.

![Box plot of SCFAs from allergic and non-allergic infants](image)

Fig 8. Median concentration (mmol/kg faeces) of SCFAs from 25 allergic and 47 non-allergic infants

The cholesterol/coprostanol ratio and the faecal tryptic activity levels were similar in faecal samples from 15 allergic and 31 non-allergic infants.
The composition of SCFAs changed between 1 and 4 years, paper III. Faecal acetic (p<.01) and propionic (p<.01) acids decreased from 1 to 4 years of age, whereas valeric acid (p<.001) increased (Table 7) in children with faeces samples both at 1 and 4 years (n=53).

Table 7. Short chain fatty acids concentration (mmol/kg) in faeces from 53 children who delivered faeces both at 1 and 4 years

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Detected year 1</th>
<th>Median (range)</th>
<th>Detected year 4</th>
<th>Median (range)</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetic</td>
<td>53</td>
<td>53</td>
<td>63 (9-176)</td>
<td>53</td>
<td>45 (12-126)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>propionic</td>
<td>53</td>
<td>53</td>
<td>16 (4-51)</td>
<td>53</td>
<td>13 (4-45)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>i-butyric</td>
<td>53</td>
<td>52</td>
<td>1.4 (&lt;0.2-5.3)</td>
<td>53</td>
<td>1.9 (0.3-6.8)</td>
<td>p=.08</td>
</tr>
<tr>
<td>butyric</td>
<td>53</td>
<td>53</td>
<td>15 (4-57)</td>
<td>53</td>
<td>14 (4.3-61)</td>
<td></td>
</tr>
<tr>
<td>i-valeric</td>
<td>53</td>
<td>51</td>
<td>1.5 (&lt;0.2-7.7)</td>
<td>53</td>
<td>2.5 (0.2-10)</td>
<td>p=.06</td>
</tr>
<tr>
<td>valeric</td>
<td>53</td>
<td>38</td>
<td>0.6 (&lt;0.2-4.8)</td>
<td>53</td>
<td>1.7 (0.6-6.6)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>i-caproic</td>
<td>53</td>
<td>16</td>
<td>&lt;0.2 (&lt;0.2-3.8)</td>
<td>17</td>
<td>&lt;0.2 (&lt;0.2-0.4)</td>
<td></td>
</tr>
<tr>
<td>caproic</td>
<td>53</td>
<td>7</td>
<td>&lt;0.2 (&lt;0.2-2.8)</td>
<td>24</td>
<td>&lt;0.2 (&lt;0.2-4.7)</td>
<td></td>
</tr>
</tbody>
</table>

Levels of SCFAs at 1 year did not predict sensitisation at 4 years of age. However, low levels of i-butyric, i-valeric and valeric acids in faeces at 1 year of age were associated with questionnaire-reported symptoms of food allergy at 4 years. This association remained after excluding all children (n=17) with doctor-diagnosed food allergy or positive SPT to food allergen already at 1 year (Fig 9).
Fig 9. Concentrations (mmol/kg faeces) of SCFAs from 1 year old children, 17 with reported food allergy and 103 with no reported food allergy at 4 years. Filled circles: doctor-diagnosed food allergy or positive SPT to food allergen already at 1 year.

Fig 10. Concentrations (mmol/kg faeces) in faeces from children at 4 years in 10 allergic children and 19 non-allergic children.
At 4 years of age, 10 children were classified as allergic (positive SPT in combination with eczema, asthma or food allergy) and 19 as non-allergic (negative SPT and no symptoms) (Fig 6). Allergic compared with non-allergic children had higher levels of acetic acid but lower levels of i-butyric, i-valeric and valeric acids in their faeces (Fig 10). After exclusion of children with diagnosed food allergy at 1 year of age, the levels of acetic acid were similar in allergic and non-allergic children whereas the levels of i-butyric, i-valeric and valeric acids remained lower in the allergic children, though significantly so only for valeric acid (p=.02).

Family history of allergy, gender, antibiotic use during first year of life and exclusive breast-feeding up to 4 months did not significantly affect the median levels of the various SCFAs in faeces at either 1 or 4 years.

At 1 year of age infants with older siblings had higher median levels of valeric acid (0.7 mmol/kg, range <0.2 – 5.6, vs. 0.2 mmol/kg, range <0.2 – 4.7, p=.04) that did infants without older siblings. Caproic acid was detected in 90% of children with older siblings and in 60% of those without older siblings (p=.007). There was no relationship between sibling size and faecal SCFAs at 4 years.

**Salivary SIgA antibody levels and the development of late-onset wheezing (Paper IV)**

The median levels of total IgA and secretory IgA (SIgA) in the saliva were 60 mg/L (ranges 9-3770, upper quartile above 88) and 45 mg/L (ranges 5-197, upper quartile above 67), respectively. Total IgA levels had no association with sensitisation or wheezing, either at 1 or at 4 years. In contrast, high levels of SIgA (in the upper quartile) were associated with less late-onset wheezing, but only in sensitised children. Thus, among children with a positive SPT at 1 year, late-onset wheezing was reported in 1/13 children with high levels of SIgA, compared with 14/37 children with lower levels (p=.08). Likewise, in children with a positive SPT at 4 years, none of 14 children with high levels of SIgA had late-onset wheezing compared with 12/39 children with lower levels (p=.02). In children with positive SPT both at 1 and 4 years, SIgA levels were lower in those who developed late-onset wheezing than in those who did not (p=.04, Fig 11). Among children with persistent sensitisation, none of 9 children with high levels of SIgA developed wheezing, compared with 10/20 children with lower SIgA levels (p=.01).
Fig 11. Salivary SIgA levels in children who were SPT positive at both 1 and 4 years of age.

Among children with circulating IgE antibodies to egg and/or cat at 1 year, those who developed late-onset wheezing had lower levels of SIgA than those who did not (p=.02, Fig 12). None of 13 infants with high levels (in the upper quartile) of SIgA and circulating IgE antibodies to egg or cat developed late-onset wheezing at 4 years, compared with 11/39 similarly sensitised infants with lower levels of SIgA (p=.05). There was no association between SIgA levels and early-onset wheezing.

Fig 12. Salivary SIgA levels in children with circulating IgE antibodies to egg and/or cat at 1 year of age.
There was no relationship between wheezing symptoms at 1 or 4 years and IgA antibody levels to egg or cat. High cat-specific IgA levels were detected in infants with positive SPT to cat at 1 year, but had no relationship with any other sensitisation. Salivary IgA antibodies to egg were not associated with sensitisation.

Older siblings, more than three infections during infancy, at least one smoking parent and male gender were all associated with high levels (in the upper quartile) of total IgA and SIgA (Tables 8 and 9). The association between older siblings and total IgA was stronger after adjustment for infections during infancy and other significant variables. The findings were similar in sensitised and non-sensitised children.

Table 8. Determinants for total IgA levels in the upper quartile, crude odds ratios and odds ratios after logistic regression with adjustment for the other variables in the table

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Crude OR (95% C.I.)</th>
<th>Adjusted OR (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of older siblings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>105</td>
<td>1.1 (0.6-2.2)</td>
<td>1.3 (0.7-2.7)</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>1.9 (1.0-3.8)</td>
<td>3.2 (1.5-6.5)</td>
</tr>
<tr>
<td>&gt;1</td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>More than 3 infections during infancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes/no</td>
<td>177/95</td>
<td>1.9 (1.0-3.4)</td>
<td>1.8 (0.9-3.4)</td>
</tr>
<tr>
<td>At least one smoker in the family</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes/no</td>
<td>61/216</td>
<td>2.2 (1.2-4.0)</td>
<td>2.3 (1.2-4.4)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male/female</td>
<td>160/117</td>
<td>1.3 (0.8-2.3)</td>
<td>1.5 (0.8-2.7)</td>
</tr>
</tbody>
</table>
Table 9. Determinants for secretory IgA levels in the upper quartile, crude odds ratios and odds ratios (with 95% confidence intervals) after logistic regression with adjustment for the other variables in the table

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Crude OR (95% C.I.)</th>
<th>Adjusted OR (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of older siblings</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>105</td>
<td>1.1 (0.6-2.2)</td>
<td>1.1 (0.5-2.1)</td>
</tr>
<tr>
<td>1</td>
<td>102</td>
<td>1.9 (1.0-3.8)</td>
<td>1.9 (1.0-3.4)</td>
</tr>
<tr>
<td>&gt;1</td>
<td>71</td>
<td>1.1 (0.5-2.1)</td>
<td>2.2 (1.0-4.4)</td>
</tr>
<tr>
<td><strong>More than 3 infections during infancy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes/no</td>
<td>179/95</td>
<td>2.2 (1.2-4.2)</td>
<td>2.3 (1.2-4.4)</td>
</tr>
<tr>
<td><strong>At least one smoker in the family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes/no</td>
<td>61/218</td>
<td>1.8 (1.0-3.2)</td>
<td>1.8 (0.9-3.5)</td>
</tr>
<tr>
<td><strong>Gender (male/female)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male/female</td>
<td>162/117</td>
<td>1.8 (1.0-3.4)</td>
<td>1.9 (1.1-3.5)</td>
</tr>
</tbody>
</table>
**DISCUSSION**

Dog ownership during infancy was associated with a **decreased risk of sensitisation to pollen** and **decreased risk of late-onset wheezing** at 4 years of age. There has been controversy concerning whether early exposure to furred animals at home might increase or decrease the risk of sensitisation and allergic disease. Avoidance of pet keeping in allergic families may contribute to an inverse association between pet keeping and allergic disease (138)(99). In **Paper I** 20 % of the children had not been exposed to pets at home, as the families had avoided pet keeping due to allergic disease in other family members. Some families were also influenced by the outcome of the SPT at 1 year of age. Pets were introduced in the home after the first year in life only if the child had a negative SPT at 1 year of age. In families with early pet keeping, 13 out of 14 families got rid of the pet if the SPT was positive to cat. Some parents might have waited for the outcome of the SPT until deciding whether to keep a pet, but most children with a pet at home during their second year of life had been exposed to pets before the SPT.

The prospective study design has made it possible to adjust for selection bias in the analyses. The inverse association between early dog keeping and sensitisation to pollen and late-onset wheezing remained after adjustment for avoidance measures and a family history of asthma or hay fever. Such findings suggest that dog ownership in early life may offer protection from the development of allergic disease. This is consistent with a number of other studies showing that early pet keeping could provide protection from atopic eczema at 2 years (94), asthma and sensitisation to pollen at 4 years (101), hay fever and sensitisation to pollen in school children (139), and sensitisation to any inhalant allergen at 6 to 7 years (95).

The mechanism behind the protective effect of dog ownership is not known. Exposure to high levels of pet allergen may promote a modified Th2 response and facilitate tolerance (108). A lower prevalence of allergic disease in children with early pet keeping could also be consistent with the hygiene hypothesis. Living on a farm with livestock or poultry during childhood has been associated with a reduced risk of asthma and grass pollen sensitisation (75, 76, 77, 78). Exposure to high levels of endotoxin and other microbial compounds has been suggested as an explanation for the reduced risk of atopy in farm children (83).
Paper I endotoxin levels were not measured, but dog keeping has previously been related to an increased exposure to endotoxin (87, 140).

The skin prick test panel for this study did not include dog allergen. Therefore, it is not known whether early dog keeping affected sensitisation to dog allergen. However, early dog ownership had an inverse association with sensitisation to pollen, but no association with sensitisation to dog allergen up to age 6 in two German birth cohorts. In contrast, exposure to endotoxin did not affect the development of sensitisation. It was suggested that exposure to other microbial products related to dog keeping could explain the findings (141).

Family history is a major risk factor for the development of allergic disease in the child. Early-onset, persistent asthma and late-onset asthma were closely associated with maternal asthma but had no association with paternal asthma. In contrast, both maternal and paternal hay fever were important risk factors for sensitisation to pollen and cat. Several studies indicate that maternal heredity is more important than paternal history for asthma (142, 143) hay fever (144) or atopic sensitisation (145) due to influences on her offspring during foetal life and breastfeeding. However, the findings have not been consistent, and this parent-of-origin effect has been questioned for childhood atopic eczema (146) as well as for asthma in school children (147).

Persistent and late-onset wheezing were closely related only to maternal asthma in our study, a finding that could be related to misclassification if, for example, the mothers underreport symptoms in the fathers. However, most questionnaires were completed by both parents and the prevalence rates of asthma and hay fever did not differ between mothers and fathers. Furthermore, parental hay fever was related to an increased risk of sensitisation to inhalant allergens at age 4 with similar odds ratios for maternal and paternal hay fever.

Exposure to pet allergen was not based on objective measurements. Therefore, some misclassification might have occurred. However, data on pet keeping year by year was based on detailed reports from the parents. Moreover, children with pets at home are exposed to much higher levels of pet allergens than children without pets (101, 148), and objective measurements also have limitations (149).
**Paper I** uses a wide definition of *early-onset, transient wheezing* that includes all children with at least one wheezing episode during the first year of life. Transient wheezing in infancy had no association with atopy. Having older siblings was related to an increased risk of early-onset transient wheezing, suggesting that the wheezing symptoms were induced by respiratory infections. Dog keeping during the first year of life was associated with an increased risk of transient wheezing but only in children with parental asthma. Exposure to endotoxin has in two previous studies been related to an increased risk of wheeze during the first year of life (88, 150). The effect of endotoxin was stronger in children with atopic heredity in one study (150); the other study comprised children recruited from families with a history of allergy or asthma (88). Endotoxin has strong inflammatory effects, and bronchitis, coughing and non-atopic asthma have been associated with exposure to endotoxin (151).

It was originally suggested that recurrent infections could explain a reduced risk of allergy in children with older siblings. However, it seems unlikely that stimuli that are potentially harmful to the host should be necessary for the postnatal maturation of a balanced immune system. Exposure to the huge amount of microbiota in the intestines could be much more important (152). The establishment of an intestinal microflora plays a role in the development of a balanced immune system (25) in which commensal aerobes are established first and thus open up for the sequential establishment of facultative and obligatory anaerobes (111). Successively, the intestinal flora reaches equilibrium mainly with anaerobes (110). The commensal bacteria in the gut comprise a huge number of species, less than half of which can be detected with methods available today (153).

A holistic approach to studying microbial ecology is to assess functional aspects of the microbiota. Microflora-associated characteristics (MACs), measured in **Papers II and III**, are defined as any anatomical structure and/or physiological, immunological and biochemical functions in an organism that have been influenced by the microbes present and reflect the establishment of a functionally active gut flora. However, they are seldom markers of any specific micro-organisms. Germfree animal characteristics (GACs) are at hand when no live microbes are present as, for example, in newborn babies (154).

There are many differences between germfree and conventional organisms. Germfree animals require a higher caloric intake. They have no capacity to digest cellulose into short chain fatty acids (SCFAs) as they lack a normal microflora. Faecal SCFAs are intermediates and end
products of microbial action on dietary and host-derived components (155), representing the net sum of production, absorption and possible secretion of SCFAs. Butyric acid is almost completely consumed by the colonic epithelium as a major source of energy for colonocytes. Acetic and propionic acids are metabolised in the liver or muscles and act as modulators of glucose metabolism. All these three major SCFAs stimulate intestinal epithelial cell proliferation and differentiation, and it is shown that intraluminal bacteria stimulate cell proliferation in the colon (113, 156). Alterations in faecal SCFAs caused by changes in gut flora have been found after treatment with antibiotics (157) and dietary changes (158).

A number of studies have demonstrated differences in gut flora composition and function between allergic and non-allergic children (153). Differences in SCFAs between allergic and non-allergic children were demonstrated at both 1 and 4 years. Allergic 1-year-old infants had lower levels of propionic, i-butyric, butyric, i-valeric and valeric acids than did non-allergic children. Likewise, faecal samples at 4 years demonstrated that levels of i-butyric, i-valeric and valeric acids remained lower in the allergic children. Thus, a more complex flora result in “shift to the right” regarding the pattern of the SCFAs. Levels of SCFAs at 1 year did not predict sensitisation at 4 years. However, levels of i-butyric, i-valeric and valeric acids at 1 year of age were lower in children who were reported to have food allergy 3 years later compared with children without food allergy. The associations remained after exclusion of children with food allergy at 1 year of age.

In newborn children as well as in germfree animals, acetic acid is the only SCFA present. The pattern of SCFAs is altered as the microbes are established in the gut and an adult type of SCFA pattern is in children established already before 2 years of age (110). Allergic 4-year-old children had higher levels of acetic acid than non-allergic children, but this association disappeared after excluding children with diagnosed food allergy at 1 year of age. As there is no endogenous production (159), acetic acid is highly dependent on intestinal microbial metabolism and could thus be affected more by the content of food products than by desquamation and inflammatory cell turnover. The maturation of the intestinal flora is associated with the production of SCFAs with an increasing number of carbon atoms (110). Decreasing levels of acetic and propionic acids and increasing levels of acids with increasing carbon atoms may be a consequence of a switch towards an anaerobic gut flora with age (160). Consistent in Paper II and III, allergic children had lower levels of i-butyric, i-valeric
and valeric acids as compared to the non-allergic children. These findings suggest a reduced microbial complexity in allergic children, at least during the first years of life.

Interestingly, infants with older siblings had higher median concentrations of valeric acid at one year of age and caproic acid was more often detected in faeces. This could indicate a more rapid maturation of the intestinal flora with older siblings. Children in Paper II presented lower levels of valeric if allergic compared to non-allergic at one year of age.

The SCFAs are usually not markers of specific bacteria. In Paper II, however, the possible association between SCFAs and certain bacteria is discussed. The presence of i-caproic acid could be an indicator of *Clostridium difficile* (155), whereas high levels of propionic acid have been associated with high counts of lactobacilli (161). Colonisation with *Clostridium difficile* at 1 month preceded the development of sensitisation and allergic symptoms in a recent birth cohort study (162). In contrast, other commensals, such as lactobacilli, are beneficial and induce T regulatory cells (163). Clinical improvement of eczema has been observed in infants treated with extensively hydrolysed whey formula fortified with a probiotica, Lactobacillus GG (164). Perinatal supplementation of lactobacilli may prevent atopic eczema up to 7 years of age (165). However, these and similar clinical studies have not been able to present any association between treatment with lactobacillus or other probiotica and sensitisation but are suggested to modulate other immune responses e.g. vaccination (166). The total load and the diversity of the microbial flora could be more important than single strains of specific bacteria in the prevention of allergic disease.

**Paper I** demonstrates a 5-fold increased risk of late-onset wheezing in children who were sensitised at 1 year of age. **Paper IV** shows that high levels of SIgA, but not total IgA, at 1 year of age might reduce the risk of late-onset wheezing in sensitised infants (defined as a positive SPT to at least one of five allergens or circulating IgE antibodies to egg or cat). This finding is in line with a previous study in which allergic symptoms were less likely in sensitised infants with high levels of SIgA (137). It is also consistent with an Icelandic study showing high levels of salivary IgA in children who recovered from atopic eczema (167).

In **Paper IV**, salivary IgA antibodies did not affect the development of sensitisation. The findings from other studies have been contradictory. Secretory IgA prevents the adherence and penetration of antigens. High levels of SIgA could theoretically prevent allergen
absorption, whereas low levels of salivary IgA and transient IgA deficiency have been associated with an increased risk of allergy (14, 168, 167). In contrast, transient absence of salivary IgA was associated with a reduced risk of sensitisation to inhalant allergens (169), and high levels of total IgA as well as specific IgA antibodies to inhalant allergens have been associated with an increased risk of sensitisation (137). Inconsistent findings on the role of salivary IgA might be explained by different study designs, age groups, status of sensitisation and differences between total IgA and SIgA.

Salivary IgA antibodies seem to interfere with the development of allergic symptoms in sensitised children (Fig 13). As the secretory component (SC) is the extracellular binding region of poly Ig receptor (pIgR) and one molecule of pIgR is consumed for every molecule of secretory IgA (SIgA) released into the lumen, the level of pIgR expression controls the rate of epithelial transcytosis of SIgA. Regulation of pIgR is thus essential for such transport (12,170). Bacterial, inclusive commensals, and viral stimuli play a key role in upregulating pIgR expression (170, 171). IgA antibodies are able to suppress chemotaxis of inflammatory cells and inhibit IgE-induced histamine release (172). This might explain the reduced risk of late-onset wheezing in sensitised children with high levels of SIgA.

Fig 13. Possible relationship between sensitisation, recurrent infections, SIgA and late onset wheezing
A recent study demonstrated that SIgA comprised almost all of the total salivary IgA in Estonian preschool children. The ratio between SIgA and total IgA was much lower in Swedish children of the same age (Tomicic et al, submitted). Previous studies of infants in Australia (136) and Sweden (137) have demonstrated that SIgA comprised only a moderate proportion of total IgA. A low SIgA/total IgA ratio could indicate that Western lifestyle, with a decreased microbial load, is associated with a less mature mucosal immune system during infancy.

Older siblings, infections and passive smoking during infancy were potential risk factors for salivary IgA antibodies. Maternal smoking could have a direct effect on the immune system (173) and has recently been associated with a higher level of salivary total IgA in 1-year-old children (174). Older siblings and infectious diseases could hypothetically increase the total microbial load, thus increasing the expression of mucosal pIgR, and induce a more mature SIgA transport with higher salivary IgA levels. From what I can find in the literature, the association between older siblings and high secretory IgA has not been presented previously.

**Limitations and strengths**

The strength of the study is the longitudinal design. Many of the relevant exposures were assessed already around birth. All children were skin prick tested by the same nurse on both occasions, and the clinical examinations at 1 year were done by one paediatrician (AS).

A prospective birth cohort is expensive and time-consuming, however. Much effort (and the support of the local media) was needed to motivate the families to participate in the follow-up examinations. Of all the children in the targeted cohort, about 60 % participated in the follow-up after 4 years. Those participating may not be representative of the whole cohort. However, parental reported symptoms or exposures did not differ significantly between participants and non-participants.

The SCFA pattern differed between allergic and non-allergic children both at 1 and 4 years. However, the faecal samples were collected at the same time as the symptoms were reported. Therefore, we cannot fully exclude reverse causation. The SCFA pattern could be an effect of the allergic disease or an allergic predisposition, and some of our findings could be the outcome of dietary changes or other treatment.
CONCLUSIONS

# Pet keeping during the first year of life was not associated with an increased risk of sensitisation or allergic symptoms at 4 years of age. Dog keeping during infancy could provide some protection from sensitisation to pollen and late-onset wheezing.

# Parental (maternal or paternal) hay fever was associated with sensitisation to inhalant allergens at 4 years of age, whereas maternal, but not paternal, asthma had a strong relationship with early-onset persistent wheezing and was a risk factor for late-onset wheezing.

# A functional assessment of the gut flora demonstrated differences between allergic and non-allergic children both at 1 and 4 years of age. None of the SCFAs at 1 year predicted sensitisation at 4 years of age. Low levels of i-butyric, i-valeric and valeric acids at 1 year were, however, associated with questionnaire-reported symptoms of food allergy at 4 years.

# The changes in levels and composition of SCFAs between 1 and 4 years were in line with a more complex gut microbiota at 4 years.

# Caproic acid was more often detected in infants with older siblings, who also had higher levels of valeric acid at 1 year of age, indicating a more mature gut flora in children with older siblings.

# High levels of salivary IgA antibodies in sensitised infants were associated with less late-onset wheezing at 4 years of age, supporting the protective role of salivary IgA antibodies against the development of asthma in sensitised infants.

# Older siblings, recurrent infections during infancy and/or smoking parent were associated with high levels of salivary IgA and are of particular interest in the light of the relationship between these environmental exposures and wheezing.
Utveckling av allergi, IgA antikroppar i saliv och tarmens bakterieflora i en svensk födelsekohort


Syftet med avhandlingens första studie var att studera sambandet mellan hereditet, pälsdjur i hemmet under första levnadsåret och utveckling av sensibilisering och astma under småbarnsåren. Syftet med de två följande studierna var att studera sambandet mellan korta fettsyror i avföring och förekomst av allergi vid ett och fyra års ålder. Syftet med den fjärde studien var att studera om höga halten av IgA-antikroppar i saliv kunde skydda mot utveckling av allergisk sjukdom.

Studien är en prospektiv födelsekohort och omfattar alla barn i Jämtland födda under perioden februari 1996 till jan 1997 vid Östersunds sjukhus, totalt 1228 barn. Vid födelsen svarade 857 föräldrar på enkät med frågor om omgivningseffektorer, rökning, husdjur och allergiska symptombärförande. Vid ett års ålder blev samtliga barn inbjudna till uppföljning med pricktest och enkät om ändringar i hemförhållanden och allergiska symptom hos barnet och då deltog 1040 (85%) barn. Vid 4 år genomfördes en ny uppföljning med pricktest och ny pricktest. 817 (67%) barn deltog i pricktest vid både ett och fyra års ålder. Vid ett års ålder blev 79% av barnen pricktestpositiva (minst en allergenkvaddel >=3mm), 68 barn med positiv pricktest men svag allergenkvaddel (1-2 mm) och 156 åldersmatchade barn med helt negativ pricktest (alla allergenkvaddlar <1mm) att delta i läkarundersökning och samtidigt lämna prover från blod (n=254), saliv (n=279) och avföring (n=139) för analys. Vid fyra års ålder accepterade 236/295 (80%) barn pricktest, frågeformulär och 53 barn lämnade nytt avföringsprov.

Antalet pricktestpositiva barn fördubblades mellan ett år (7%) och fyra års (13%) ålder och 4,5% var pricktestpositiva vid både undersökningarna. Sensibilisering ökade kraftigt risken för astma. Hund i hemmet under första levnadsåret innebar minskad risk för utveckling av astma och sensibilisering mot pollen vid fyra års ålder. Astma hos modern medförde ökad risk för astma hos barnet. Hösnuva hos modern liksom hösnuva hos fadern ökade risken för sensibilisering mot pälsdjur och pollen vid fyra års ålder. Astmaliknande besvär vid ett års ålder men inte vid fyra år var vanligare i familjer med hund i hemmet under första levnadsåret men bara hos barn med hereditet för astma.
Vid ett års ålder hade allergiska barn lägre koncentration i avföringen av vissa korta fettsyror (SCFAs) jämfört med icke allergiska barn. Detta gällde propion-, i-smör-, smör-, i-valerian- och valeriansyra. Det fanns inget samband mellan SCFA-nivåer vid ett års ålder och sensibilisering vid fyra års ålder men barn som uppgavs ha födoämnesallergi vid fyra års ålder hade lägre nivåer av i-smör-, i-valerian och valerian-syra vid ett års ålder. Fettsyremönstret i avföringen förändrades mellan ett och fyra års ålder. Halten av attäktsyra (p<0.01) och propionsyra (p<0.01) sjönk medan halten av valeriansyra (p<0.001) ökade. Förändringarna innebar förskjutning mot fettsyror med ökat antal kolatomer som ett tecken till mer mogen tarmflora. Barn med äldre syskon hade högre nivå av valeriansyra jämfört med barn utan äldre syskon.

Totala mängden IgA i saliv uppvisade inte något samband med sensibilisering eller astma vid vare sig ett eller fyra års ålder medan däremot höga nivåer (i övre kvartilen) av sekretoriskt IgA (SIgA) i saliv innebar visst skydd mot utveckling av astma vid fyra år men endast bland sensibiliserade barn. Bland barn med positiv pricktest både vid ett och fyra års ålder var halterna av SIgA lägre vid ett års ålder för de barn som utvecklade astma vid fyra års ålder jämfört med de barn som inte utvecklade astma. Av nio barn med höga nivåer av SIgA utvecklade inget barn astma. Av 20 barn med lägre nivåer av SIgA hade emellertid 10 barn utvecklat astma vid fyra års ålder. Äldre syskon, fler än tre infektioner under spädbarnsåret, minst en rökande förälder och manligt kön var alla oberoende riskfaktorer för högt totalt IgA och SIgA.

**Sammanfattning**

# Pälsdjur i hemmet under spädbarnsåret förefaller ge ett viss skydd mot astma och sensibilisering för pollen vid 4 års ålder.

# Det finns en skillnad i sammansättningen av korta fettsyror i faeces mellan allergiska och icke-allergiska barn både vid 1 och 4 års ålder vilket tyder på en skillnad i tarmflorans sammansättning. Förändring av de olika korta fettsyrons mängd från 1 till 4 års ålder indikerar en mer komplex tarmflora med stigande ålder.

# Höga nivåer av SIgA i saliv vid ett års ålder skyddar mot utveckling av astma tre år senare men detta gäller endast sensibiliserade barn.

52
ACKNOWLEDGEMENTS

I wish to express my warmest gratitude and appreciation to everyone involved in this work, and in particular I would like to thank:

All the children and the families who took part in this study; without your contribution with answers and samples this work would not have been possible.

My supervisor, Lennart Bråbäck, for his excellent and supportive guidance in epidemiology and allergology and for his invaluable practical support. For sharing his knowledge and enthusiasm for research and for his never-ending patience.

My co-supervisor, Bengt Björkstén, for professional guidance in research and immunology. For sharing his superb knowledge in the area of allergology and in scientific thinking.

My co-supervisor, Olle Hernell, for friendly support and discussions.

My friend and research nurse, Anna Bernholm for her interest in the field of allergology and her ability to structure all the practical part of this work. Without her enthusiasm, planning and careful handling, most of the time on her own, this work would never have been done.

Östersund Hospital, with gratitude to the antenatal clinic, delivery ward and maternity ward for excellent help with the questionnaire and blood sampling and to the clinical chemical laboratory for patients with test samples and storage space for a lot of different research material.

BVC nurses, for showing interest in and friendly co-operation during time for follow-up of the children.

Anna-Karin Persson, Ann-Marie Fornander and Ulrika Bengtsson, for their skilful technical assistance.

Hans Stenlund and Erling Englund for clarifying discussions concerning epidemiology and most helpful statistical support throughout this work.

All collaborators involved in my parallel research work during ISAAC phase II and especially to Kristina Fluur Hedman, Karin Helgesson, Ing-Marie Sandberg, Lena Lindell and all school nurses in Östersund.

The Research and Development unit of the County Councils:
In Jämtland, to Ragnar Asplund and Christina Reuterwall for great support in providing grants for this study and especially to Susanne Johansson, for always being at hand for administrative questions, computer problem or room for research and study.
In Västernorrland, to Hans Malker for support, Vivan Rönqvist, for immediate administrative help and layout and Jeanette Sundberg-Granlund, for excellent help with all the financial procedures.
In Norrbotten, for financial support for my thesis.
My co-author, Elisabeth Norin, at Karolinska Institutet for her enthusiastic support, interesting discussions and excellent guidance within the field of microbiology.

My co-authors at Linköping University, Maria Jenmalm and Malin Fagerås-Böttcher, for their immediate support and valuable explanations within the field of immunology.

My friend and colleague, Päivi Söderman, for enthusiastic help and support in starting up, turning the research idea into a working study and stressing the need for, and providing the name of, a supervisor.

My friends and colleagues at the Children’s Ward, Östersund Hospital, Anna-Lena Nilsson, Agneta Smedsaas-Löfvenberg, Kristina Wallström and Charlotte Blank, for warm friendship and never-ending support.

Frösö BVC, Inger Persson, for unbelievable flexibility in working hours and support in research. Per Hedman, Anette Grangert, Eva Johnsson and Gunnel Holmberg, for interest in my work and constant willingness to reschedule.

My friends and colleagues at the Children’s Ward, Sunderby Hospital, everyone remembered, none forgotten. Thank you for support, cooperation and flexibility in working hours, friendship and a great working atmosphere. Special thanks to the head of the paediatric clinic, Per Fahlesson, for interest in my research and for providing me the opportunity to fulfil my thesis; Sara Mannesson, Svetlana Hortin and Agneta Brännström for support and sharing knowledge in clinical discussions; and Ingela Heimdahl for making me finish the papers and finally write this thesis.

My friends at the Children’s Ward at Kalix Hospital, everyone remembered, none forgotten. Special thanks to Anki Kemi, for support, cooperation and flexibility.

My friends and colleagues at the Children’s Ward, Umeå University Hospital, for discussions and seminars. Ulf Hjalmars for practically proving that research is possible, though not easy, at a distance from the university, and Aijas Farooki, for friendly and supportive remarks at all times.

Ivars Bil, Ivar Ericsson, for sponsoring the study with access to a new car for all our travelling throughout the county. Without this support it would not have been possible to begin the study.

Nemkon AB, Stig Halvarsson and Bernt-Åke Andersson, for understanding and patience with their partner who has a wife involved in research.

My friends in Ås, CISV and SK Ägir, everyone remembered none forgotten, for still being there.

Vår dagmamma, Solveig Nilsson och Allan, för fantastiskt omhändertagande och trygghet till barnen och våra grannar Anna och Ingmar Hansson för att alltid vara nära och villiga att hjälpa till.

Min mamma, Eivor Näslund, för praktiskt visat att ingenting är omöjligt och min pappa, Olaus Näslund, för hans koncept att alltid slutföra det han påbörjat.
My sister, Eva Hettinger, for great support through telephone chat and for reminding me that there are other things in life besides work and research. My brother, Björne Näslund and my niece, Karolina Hettinger, for accommodation in Stockholm at times.

My children, Viktor, Arvid, and Elvira, for being able to take great care of themselves, at school and in swimming and dancing. For all the support, journeys and discussions about what is worthwhile in life and for putting positive into practice these past few months.

Finally, my husband and best friend, Lars. Thank you for all your practical assistance and for standing by my side all these years, especially these last unbelievably intense months. Without your love and patience I would never have made it.

Who said hard work wasn´t fun!

My six-word memoir

Funding source
This study was financially supported by grant from the Swedish Medical Research Council, The Wårdal Foundation, The National Swedish Association against Allergic Diseases, the Foundation for Strategic Research, the National Heart and Lung Association, GlaxoWellcome, the Swedish Foundation for Health Care Science, the Swedish Environmental Protection Agency, the Research and Development Unit of the County Council in Jämtland, the County Councils in Northern Sweden and Queen Silvia’s Jubilee Foundation.
REFERENCES


60. Celedón JC, Litonjua AA, Ryan L, Weiss ST, Gold DR. Lack of association between antibiotic use in the first year of life and asthma, allergic rhinitis, or eczema at age 5 years. Am J Respir Crit Care Med 2002;166:72-5.


Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. Clin Exp Allergy 1999;29:28-34.


74. Downs SH, Marks GB, Mitakakis TZ, Lêuppi JD, Car NG, Peat JK. Having lived on a farm and protection against allergic diseases in Australia. Clin Exp Allergy 2001;31:570-5.


95. Ownby DR, Johnson CC, Peterson EL. Exposure to dogs and cats in the first year of life and risk of allergic sensitization at 6 to 7 years of age. Jama 2002;288:963-72.


105. Patiño CM, Martinez FD. Interactions between genes and environment in the development of asthma. Allergy 2001;56:279-86.


148. Munir AK, Einarsson R, Björkstén B. Mite (Der p 1, Der f 1) and cat (Fel d 1) allergens in the homes of babies with a family history of allergy. Allergy 1993;48:158-63.

149. Apter AJ. Early exposure to allergen: is this the cat's meow, or are we barking up the wrong tree? J Allergy Clin Immunol 2003;111:938-46.


152. Wold AE. The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? Allergy 1998;53:20-5.


