The Development of Immune Responses and Allergy in Children from Farming and Non-farming Environments

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ACADEMIC DISSERTATION

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ABSTRACT

Allergies have become a major public health problem in recent decades. Approximately 60 million people in Europe, and one billion worldwide, are affected by allergies. In Europe, approximately 30% of the population suffers from allergic rhinoconjunctivitis, approximately 20% from asthma, and 15% from allergic skin conditions. The farming environment has been shown to protect against allergies. In the present thesis, we studied the development of Immunoglobulin E (IgE) sensitization, allergic diseases, gut permeability and inflammation in children from farming and non-farming environments who participated in the PASTURE project (Protection against allergy: study in rural environments).

Our results showed that early-age inflammation, measured with serum high-sensitivity C-reactive protein (hs-CRP) at the age of 1 year, was associated with decreased risk of IgE-sensitization at the age of 4.5 years. Our findings suggest that poor inflammatory response at infancy may predispose to IgE-sensitization.

The allergy-protective effect of breastfeeding is debated. Our findings illustrated that increased breast milk soluble immunoglobulin A (sIgA) levels were associated with decreased risk of atopic dermatitis (AD) up to the age of 2 years. When the ingested dose of sIgA during the first year of life was considered, it was associated with decreased risk of AD up to ages of 2 and 4 years. Elevated sIgA levels in breast milk were associated
with environmental factors indicating increased environmental microbial load.

Serum immunoglobulin A (IgA) and immunoglobulin G (IgG) against cow’s milk β-lactoglobulin (BLG) and wheat gliadin are induced by the antigen exposure, but the antibody levels are also affected by the gut permeability and inflammation. Elevated serum IgA or IgG antibody levels against BLG at the age of 1 year increased the risk of sensitization to at least one of the measured allergens or food allergens at the age of 6 years. Elevated levels of IgG against gliadin at the age of 1 year increased the risk of sensitization to any, at least one inhalant, or at least one food allergen at the age of 6 years. Our findings suggest that an enhanced antibody response to food antigens reflects alterations in mucosal tolerance, such as altered increased gut permeability and/or microbiota, which is later seen as sensitization to allergens.

Fecal calprotectin is a marker of intestinal inflammation. We studied the association of early-infancy fecal calprotectin levels to the later development of allergic diseases in children from farming and non-farming environments, and further evaluated the effect of gut microbes on the levels of fecal calprotectin. Infants from a farming environment had higher levels of fecal calprotectin at the age of 2 months when compared to the infants from a non-farming environment. However, farming did not explain the very high levels of calprotectin, i.e. the levels above the 90th percentile. The infants with remarkably high fecal calprotectin levels at the age of 2 months, i.e. levels above the 90th percentile, had an increased risk of developing AD and asthma/asthmatic bronchitis by the age of 6
years. Infants who had high fecal calprotectin concentrations had less *Escherichia* in their feces. Our findings suggest that strong intestinal inflammation at early infancy represents a risk of allergic diseases, and the colonization with *Escherichia coli* is an important regulator of the inflammation implicating long-term health effects mediated by early intestinal colonization.

In conclusion, the findings in this thesis show that the environmental microbial load plays an important role in the development of the immune system in infants and ultimately may affect the risk of developing allergic diseases. In addition to the direct effects on the infant, the effects of the environmental microbial load are also mediated indirectly through alterations in maternal milk composition. Interestingly, we found that an early intestinal inflammation is associated with the later risk of allergic diseases and gut colonization indicating a crucial role of gut colonization in the development of allergy.

Keywords: Allergy, antibody, asthma, atopic dermatitis, farming environment, fecal calprotectin, IgE-sensitization, inflammation.
TIIVISTELMÄ


Tuloksemme osoittivat, että varhaisen iän tulehdusvaste, joka määritettiin seerumista herkkä C-reaktiivisen proteiinin (hs-CRP) - mittauksella yhden vuoden iässä, liittyi lapsen alentuneeseen riskiin olla IgE- herkistynyt 4.5 vuoden iässä. Tuloksemme viittaavat siihen, että heikko tulehdusvaste imeväisiässä voi altistaa IgE- herkistymiselle.

Rintaruokinnan allergioilta suojaava vaikutus on herättänyt paljon keskustelua. Tuloksemme osoittavat äidinmaidon kohonneiden liukoisen immunoglobuliini A (sIgA)- pitoisuksien laskevan atooppisen ihottuman riskiä 2 vuoden ikään mennessä, kun taas lapsen ensimmäisenä elinvuotena rintamaidon sIgA:n kohonnut kokonaismäärä vähensi atooppisen ihottuman riskiä sekä 2 ja 4 vuoden ikään mennessä.
Kohonneet sIgA- pitoisuudet äidinmaidossa liittyivät ympäristötekijöihin, jotka viittaavat korkeisiin mikrobimääriin.


Ulosteesta mitattu kalprotektiini on suolitulehduksen merkkiaine. Tutkimme varhaisen iän ulosteen kalprotektiinin yhteyttä myöhempään allergioiden kehittymiseen lapsilla, jotka asuivat maatilaympäristöissä ja lapsilla, jotka eivät asuneet maatilaympäristössä. Tutkimme myös suoliston mikrobienvaikutusta ulosteen kalprotektiliipitosuksiin. Maatilaympäristössä asuvilla lapsilla oli korkeammat kalprotektiiniipitosuudet verrattuna lapsiin, jotka eivät asuneet maatilaympäristössä. Maatilaympäristö ei kuitenkaan selittänyt erittäin
korkeita kalprotekiinipitoisuksia, eli 90 prosenttipisteestä ylittäviä tasoja. Niillä lapsilla, joilla oli korkeat ulosteen kalprotekiinipitoisuudet 2kk iässä, eli pitoisuus yli 90 prosenttipisteestä, oli kohonnut riski sairastua atooppiseen ihottumaan ja astmaan/ahtauttavaan keuhkoputkitulehdukseen 6 vuoden ikään mennessä. Korkea ulosteen kalprotekiinipitoisuus oli yhteydessä ulosteen *Escherichia*-bakteerien alhaiseen määrään. Tuloksemme viittaavat siihen, että varhaislapsuuden korkea suolitulehdus aiheuttaa kohonneen allergisten sairauksien riskin ja että varhaisella suoliston mikrobikolonisaatiolla on pitkääikaisia terveysvaikutuksia.

Johtopäätös tämän väitöskirjan tuloksista on, että mikrobikuorma varhaislapsuudessa muokkaa lapsen immuunivasteita ja allergisten sairauksien kehittymistä. Mikrobiáltistus vaikuttaa suoraan lapsen immuunijärjestelmän kehittymiseen, mutta myös epäsuorasti äidinmaidon koostumusta, kuten liukoista IgA-pitoisuutta, muuttaen. Mielenkiintoista oli havaita, että varhainen suolitulehdus imevääisiässä näyttää liittyvän allergisiin sairauksiin ja suolen kolonisaatiolla voi olla olennainen vaikutus allergioiden kehittymiseen.

Avainsanat: Allergia, astma, atooppinen ihottuma, IgE-herkistyminen, maatilaympäristö, tulehdus, ulosteen kalprotektiini, vasta-aine.
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:


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¹ This article was published earlier in Mustonen K (2014): The role of inflammation and its environmental triggers in allergic diseases.

* Both authors equally contributed to this study.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptides</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BLG</td>
<td>β- lactoglobulin</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EAACI</td>
<td>European Academy of Allergy and Clinical Immunology</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
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<tr>
<td>GF</td>
<td>Germ- free</td>
</tr>
<tr>
<td>HBD</td>
<td>Human β- defensin</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>High-sensitivity C-reactive protein</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M cell</td>
<td>Microfold cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OPD</td>
<td>Ortho-phenylenediamine</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PASTURE</td>
<td>Protection against allergy: study in rural environments</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short-chain fatty acids</td>
</tr>
<tr>
<td>SCORAD</td>
<td>Scoring atopic dermatitis</td>
</tr>
<tr>
<td>SFF</td>
<td>Standard flowgram format</td>
</tr>
<tr>
<td>sIgA</td>
<td>Soluble immunoglobulin A</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor- α</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>Treg</td>
<td>Regulatory T cell</td>
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1 INTRODUCTION

Allergies have become a major public health problem in the last 50 years, now affecting the lives of more than 60 million Europeans, and approximately one billion people worldwide. Of the European population, approximately 30% suffer from allergic rhinoconjunctivitis, 20% from asthma, and 15% from allergic skin conditions. Many allergies only manifest themselves during certain periods of life. For example, milk and egg allergies are common in children but often diminish with age, whereas allergic rhinitis has been estimated to affect up to 45% of 20-40 year old Europeans. Importantly, the European Academy of Allergy and Clinical Immunology (EAACI) estimates that in less than 15 years more than 50% of Europeans will suffer from at least one allergy.

Hygiene hypothesis states that excess environmental hygiene associates with allergic diseases. Decreased contact to environmental microbes, i.e. less childhood infections, can lead to alterations in the normal immune and inflammatory responses that cause allergic reactions to non-harmful environmental antigens. Children who grow up on farms have been shown to be at a decreased risk of hay fever, asthma, and allergic sensitization. The most allergy-protective environmental exposures are the consumption of unprocessed farm milk and early-life contact with livestock and their feed. Also, maternal contact with increasing number of farm animals, the time spent in stables while pregnant, and having siblings decrease the risk of allergies in the child. Environmental exposures which decrease the risk of allergies are the most effective when
encountered before or soon after birth, and they are often associated with increased environmental microbial load.

This thesis aimed to study the role of the farm environment and other environmental factors associated with elevated microbial load in the development of gut immune system and allergies with the goal of identifying risk and protective factors of allergies.
2 REVIEW OF THE LITERATURE

2.1 Allergy
Allergy is an adverse immunological reaction to a harmless environmental factor, most often a protein. The protein causing allergic response is called an allergen. Less frequently, allergens can be carbohydrates or small chemical compounds, such as nickel. Allergic reactions can be immediate or delayed. Atopy is a characteristic of a person prone to developing immunoglobulin (Ig) E antibodies against allergens, which is also called sensitization. Atopic reactivity is a risk factor for allergic diseases, but not all atopic individuals develop allergic diseases. While allergies can be treated with medication and sometimes immunotherapy, no cure has been discovered.

Allergic rhinoconjunctivitis is a major allergic health problem, but the symptoms vary in duration and can be difficult to define. The EAACI estimates that many people with allergic rhinitis are undiagnosed. Food allergies are common in children but often diminish with age. Anaphylaxis is a severe, potentially life-threatening systemic hypersensitivity reaction, which is characterized by life-threatening airway, breathing, or circulatory problems and can be associated with skin and mucosal changes. The onset of anaphylaxis is rapid and needs to be treated with epinephrine (1). The allergic diseases discussed in this thesis in more detail are asthma and atopic dermatitis, which have high incidence and relatively well established criteria of diagnosis.
2.1.1 Mechanisms of allergy

In the 1960’s, Gell and Coombs classified hypersensitivity reactions into four classes, which is suitable for an overview of the allergic mechanisms (2). Allergic reaction can be immediate (most often IgE-mediated) or delayed (non-IgE-mediated).

Hay fever and allergic asthma are examples of type 1 hypersensitivity in which the reaction is immediate and can be anaphylactic. Allergic symptoms in susceptible individuals occur after a receptor-bound IgE molecule on the mast cell surface has recognized a cognate allergen and the mast cells degranulate, releasing inflammatory mediators such as histamine and leukotrienes. Less often, an immediate allergic reaction can occur without IgE (type II reaction). IgM or IgG class antibodies recognize a microbe or antigens on the target cell and the complement is activated. This leads to the release of histamine, and leukocyte activation. The destruction of cells and subsequent enzyme release leads to tissue destruction and symptoms such as the vasculitides and certain drug allergies.

Delayed immunocomplex-mediated allergic reaction (type III) can occur when the allergen is bound to IgM or IgG antibodies in the serum. This complex activates the complement system leading to histamine release. The complement can also form complexes with IgM or IgG, which attach to vessel endothelium causing inflammation and urticaria. Contact allergies are delayed cell-mediated allergies (type IV), which may develop after several weeks or years of exposure to the allergen. Contact allergies are often caused by a small molecule called a hapten. Although
haptens can bind to IgM on B cells, they cannot be presented to T cells and therefore do not directly trigger allergic reactions. To turn into an allergen, the hapten must first bind to a protein. The hapten-protein complex is then attached to a major histocompatibility complex (MHC) on the surface of a Langerhans cell that then migrates to a lymph node where the protein in the hapten-protein complex can induce a T cell response. If tolerance does not develop, T cells are activated and relocated to the site of contact, and allergic eczema may occur (3,4).

2.1.2 IgE-sensitization

The function of IgE is to defend the host against parasite worms and certain protozoan parasites (5). IgE is the least abundant of the antibody classes, constituting only 0.004% of total immunoglobulins in serum. Moreover, IgE concentrations are age-dependent with very low levels in young children that increase towards adolescence (6).

In atopic individuals, production of IgE antibodies against environmental antigens such as animal dandruff or pollen is increased. The class switch to IgE in B cells is induced in a T helper (Th) 2 cytokine environment. The location of allergen-specific IgE production is not entirely clear, but it has been suggested to occur in the particular allergy effector organ, such as the lung (7). Specific IgE against an allergen can be measured in serum samples. Skin prick tests using suspected allergens show immediate response in IgE-sensitized patients or patients with IgE-mediated allergy while non-IgE-mediated allergies may be detected as delayed skin reactions.
Polysensitization can be divided into cross-sensitization, in which the same IgE molecule binds to several allergens with common structural features, and co-sensitization, where different IgE molecules bind to allergens that do not necessarily have similar structures. Monosensitized people are sensitized to only one allergen. Monosensitized children are likely to developed polysensitization and co-sensitization later in childhood (8). There is an overall association between sensitization in skin prick tests and total IgE values but it is not known if the levels of IgE can be used in defining the presence of clinical symptoms. The clinical relevance of IgE-sensitization is established when the patient reports having clear symptoms, or the symptoms are demonstrated in a direct allergen challenge.

In most allergies, the synthesis of an allergen-specific IgE is needed. Nevertheless, many people with allergen-specific IgE do not develop allergic symptoms. For example, one Dutch study found that 43% of the study subjects with serum-specific IgE to inhalant allergens did not develop respiratory symptoms (9). There may be several factors causing the differences in IgE-sensitized people who develop clinical allergies and IgE-sensitized people who do not develop symptoms. The factors affecting the course of clinical allergy may be genetic predisposition, total serum IgE and specific IgE or IgG levels, mono vs. polysensitization, or the balance of Th1/Th2 or T regulatory cells (Treg) (10).

Several studies have shown that growing up on a farm is associated with a decreased risk of IgE-sensitization. Of the farm-related factors, early-life
and consistent contact to stables and the consumption of farm milk have been associated with decreased risk of IgE-sensitization (11).

2.1.3 Tolerance
The development of immune tolerance is necessary for the maintenance of homeostasis. The human body encounters numerous antigens that are recognized by the immune system. Normally, the stimuli lead to unresponsiveness, i.e. tolerance, which is maintained by alterations in memory and allergen-specific T and B cell responses and raised thresholds for basophil and mast cell activation. If the development of tolerance fails, initially harmless environmental antigens can trigger a hypersensitivity reaction, or lead to an allergic disease. Intolerance to self-antigens can lead to autoimmune disorder. In turn, excessive tolerance can lead to infection or cancer. Induction of tolerance with repeatedly introduced doses of antigen has been performed in allergen-specific immunotherapy (12).

2.1.4 Asthma
Asthma is a chronic inflammation of the respiratory system, causing increased hyperreactivity to environmental stimuli. Long-term inflammation can induce coughing, wheezing, mucus production, and shortness of breath. Asthma is a multifactorial disease (13). Furthermore, asthma can be atopic or non-atopic. A viral infection can lead to airway obstruction in infants with transient wheezing and in small children with non-atopic wheezing, while children who are IgE-sensitized and have wheezing are more prone to developing chronic asthma. Stein et al. (14) described three different childhood wheezing phenotypes that can co-exist.
during childhood: “transient early wheezing” can occur in children up to the age of 3 years; “non-atopic wheezing” can occur in toddlers and during early school years with the highest prevalence of wheezing between the ages of 3 and 6 years; “IgE-associated wheeze/asthma” may exist at any age during childhood and is characterized by persistent wheezing.

The diagnosis of asthma is based on airway obstruction measured with altered airflow in pulmonary function tests, a physical examination, and a medical history of the patient. In epidemiological studies, the data on the diagnosis of asthma is usually received from questionnaires in which the participant or guardian has answered whether a doctor has diagnosed asthma or if they have had symptoms of asthma.

2.1.4.1 Risk and protective factors of asthma

The rapid increase in the prevalence of asthma cannot be explained by a change in population genetics given the slow rate of genetic drift. Certain environmental factors have been suggested to play a role in the development of asthma. In order to affect the development of asthma, the exposure has to occur before the symptoms and affect the onset of disease in conjunction with the genetic predisposition.

Microbial exposure plays a complex role in the development of asthma. There is evidence showing that frequent infections related to low-hygiene in childhood, having siblings, and day care attendance decrease the risk allergies and asthma. The infectious agents that modulate disease pathogenesis are thought to include respiratory viruses and non-
pathogenic environmental microbes, which support the Th1 type immunity (15). The farming environment is thought to decrease the risk of asthma due to the higher microbial load of environmental microbes present on the farms (16). However, respiratory viruses are also implicated in triggering asthma exacerbations in patients with established asthma. Furthermore, inhaled endotoxin produced by Gram-negative bacteria can cause strong pro-inflammatory reactions. Endotoxin has been shown to be a risk factor of wheezing in the first year of life (17). The severity of asthma has been shown to correlate with endotoxin levels in the homes of patients with asthma (18). Interestingly, endotoxin levels have been shown to be inversely associated with atopic sensitization and atopic asthma, but not with non-atopic asthma (17). Therefore, endotoxin seems to play two roles in asthma, with an atopy-protective effect while still posing a risk for non-atopic asthma and wheezing (13).

There are several findings that suggest that parental smoking is associated with childhood asthma and wheezing and this association is stronger with wheezing among the non-atopic children than in the asthmatic children. The severity of asthma is increased in children whose parents smoke (19). However, it has been suggested that tobacco smoke is a co-factor in triggering asthma symptoms (wheezing), rather than the cause of asthma (13). Active smoking has been associated with the onset of asthma in teenagers and adults (20,21). Studies performed in both animals and humans have shown that tobacco smoke during pregnancy results in reduced lung function in the newborn (22).
Air pollutants can reduce lung function and increase hospitalization rates for asthma patients. Although air pollutants can worsen the symptoms, there is no clear evidence showing that air pollutants lie behind the initial development asthma (23).

The association of obesity and development of asthma is not entirely clear. Body mass index has been shown to associate with asthma risk whereas weight loss has been shown to improve lung function (24). On the other hand, increased body mass index did not explain the increase in the asthma incidence in a British population from 1982 to 1994 (25). However, modern diet and lifestyle changes may influence both body mass index and asthma, causing parallel increased trends in both conditions (13).

2.1.5 Atopic dermatitis

Atopic dermatitis is a chronic inflammatory skin disease in persons most often showing IgE-sensitization. Eczematous skin lesions and relapsing progress characterize AD. Half of all AD cases begin in infancy. Approximately 20% of infants in developed countries are affected by AD (26). Approximately 25% of them continue to have symptoms throughout their lives (3). It has been estimated that 30% of children with AD also develop asthma. Patients with AD are prone to skin infections, which are often caused by *Staphylococcus aureus* or herpex simplex viruses (27,28).

Ceramides are epidermal lipids that maintain the barrier function of the skin. Ceramide levels are reduced in patients with AD, which leads to
increased transepidermal water loss (29). Altered tight junction formation affects the skin barrier function in AD. In addition, mast cell activation and the release of histamine may contribute to the defects of skin barrier in AD.

Eczema is a chronic atopic or non-atopic inflammatory skin disease. In the general population, including children and adults, up to 2/3 of eczema patients do not show IgE-sensitization (30).

2.1.5.1 Risk and protective factors of AD

The etiology of AD is affected by the interaction of genetic predisposition, environmental exposures and the immune system. The lack of environmental microbial exposure and the altered composition of skin microbiota may affect the development of the disease.

Results from the PASTURE study show that maternal contact to farm animals and cats during pregnancy decreased the child’s risk of AD until the age of 2 years. Also, elevated cord blood expression of innate immunity toll-like receptors (TLR) 5 and 9 was associated with decreased doctor diagnosis of AD (26). In a cross-sectional German study including 17 641 children aged 0 – 17 the prevalence of eczema diagnosis ever was 13.2%. The prevalence was positively associated with parental allergies, infection, or jaundice after birth. Having a dog as a pet or being a migrant decreased the risk of eczema (31).

Skin microbiota composition is associated with the skin barrier function (32). One factor that can affect the composition of skin microbiota is aberrant cytokine production, which can lead to alterations in the
production of antimicrobial peptides (AMP) in the skin of AD patients. This can contribute to the shift in the balance of skin microbial communities and affect the onset of disease (33). The commensal skin bacterium *S. epidermis* is capable of inducing TLR- mediated inflammatory responses which suppress inflammation after skin injury (34). In addition, *S. epidermis* can induce the production of AMPs from keratinocytes and affect the functions of skin resident T lymphocytes (35).

It has also been shown that gut microbial composition is associated with the risk of AD, possibly by interacting indirectly via the gut immune system. Early gut colonization with Clostridium cluster I was related to increased risk of AD by the age of 2 years (36).

Filaggrin protein is involved in cornification. Mutations in the gene coding for filaggrin associate with impaired skin barrier function and have been associated with AD (37).

**2.1.6 Breastfeeding and allergy development**

Breast milk contains antimicrobial and anti-inflammatory components, such as cytokines and antibodies. In addition, breast milk contains bacteria and antigens derived from food. These components of breast milk protect the infant from infections and educate the infant’s immune system towards tolerance. There are contradictory findings on the effect of breastfeeding to the development of allergic diseases in childhood.

Currently, there seems to be insufficient evidence to suggest that breastfeeding protects from food allergy. There are both studies that show
that breastfeeding can protect from the development of AD and studies that show no association (38,39). However, there is consistent evidence that breastfeeding protects from childhood wheezing, possibly due to breast milk components protecting from respiratory tract infections. Yet, the effect of breastfeeding on asthma is more complex since wheezing and childhood infections can affect the asthma risk. Breastfeeding has been reported to associate with increased risk of asthma in late childhood and adulthood (40). Nevertheless, the complex nature of asthma and wheezing suggest that such associations are cautiously examined. Conflicting findings in different studies can be due to differences in the assessment of breastfeeding (e.g. exclusive vs. non-exclusive, or different cut-offs used for the duration of breastfeeding), outcome definitions, or not taking the genetic predisposition into account (41). The composition of breast milk has been shown to differ between mothers with and without allergies (42,43), which can also affect the findings.

Breast milk factors that have been suggested to associate with the protective breastfeeding effect on allergic diseases are soluble IgA (sIgA), soluble CD14, and transforming growth factor-β (TGF-β) (44-46). Soluble IgA is the predominant immunoglobulin in breast milk. It provides passive antimicrobial protection in the infant’s gut and can protect from excess antigen intake and, thereby lower the risk of allergies. TGF-β has an immunosuppressive role, such as the inhibition of interferon-γ (IFN-γ) expression in CD4+ T cells, which may decrease the risk of allergies. Soluble CD14 is a co-receptor for TLR4, which has a significant role in lipopolysaccharide (LPS) recognition. TLR4 activation
may be important in infancy to strengthen the Th1 type immune responses. However, not all allergy-protective findings of breastfeeding and breast milk components are consistent, and the mechanisms remain obscure. It is possible that the decreased risk of developing an allergic disease associated with living in farming environment is mediated through breast milk constituents, which have been shown to be different in farm and non-farm mothers (47). Also, Estonian and Swedish mothers have been shown to have differences in breast milk composition (48), suggesting that environmental factors associated with elevated microbial load affect the breast milk composition.

2.2 The hygiene hypothesis

Strachan introduced the concept of the hygiene hypothesis in 1989 when he reported that allergic rhinitis and family size were inversely correlated (49). It was speculated that contact to other children increased infections in early life, which led to a decreased risk of allergy. The theory has been supported by several subsequent findings showing, e.g., that children who were exposed to other children by attending day care at early age or had older siblings had lower prevalence of atopy, asthma, and frequent wheezing later in childhood (50,51). Although the associations of environmental exposures and allergic diseases have been widely studied, the specific factor or mechanism explaining the hygiene hypothesis remains unknown. Yet, growing up in farming environment has been repeatedly shown to be an allergy-protective factor (52).
The original message of the hygiene hypothesis was that decreased exposure to environmental viruses, bacteria, and parasites leads to insufficient activation of the Th1 immune response and the balance is shifted towards Th2 response. More recent studies have shown that Th1/Th2 imbalance is not the only reason for the increase in allergic diseases in developed countries. For example, Th1 cytokines are also involved in asthma and AD. There is also evidence showing that defects in the Th1 pathway do not lead to increased risk of allergies, which implies that Th1 pathway does not directly regulate Th2 responses in allergy (53). Also, down-regulation of Th2-mediated inflammation does not occur when Th1 polarized cells are applied to a site of inflammation in an animal model. On the contrary, inflammation increases (54). In addition, Th1/Th17-associated inflammatory diseases such as type 1 diabetes, multiple sclerosis, and Crohn’s disease have become more prevalent in the same areas where allergies have increased (55,56) which can be a signal of a defect in the patients’ ability to regulate immune responses, leading to allergies or autoimmune diseases, rather than a mere Th1/Th2 imbalance (57). The underlying cause behind such a defect, whether it is the change in the environmental microbial load, a lack of childhood infections, or some unknown factor in the western life style, is yet to be defined.

The hygiene hypothesis can explain differences between the prevalence of allergic diseases in developed and developing countries. Yet, the impact of immunological responses is complex and not fully understood. Parasite infections are common in developing countries where the prevalence of
allergies is low. It has been estimated that 2.5 billion people worldwide are infected with helminth parasites (58). Helminths are able to modulate the human immune system towards type 2 immune responses, such as the secretion of Th2 type anti-inflammatory cytokines, but helminthes also are strong inducers of Treg cells, which down-regulate the host immunity against helminths. Epidemiological studies have shown that infections with helminths are associated with low incidence of asthma, allergy and autoimmunity. Animal studies have shown that helminths induce regulatory immune responses which can suppress Th2 and Th1/Th17 responses that mediate allergy and autoimmunity, respectively (59).

The present immunological knowledge has changed the view of the hygiene hypothesis so that the decreased microbial load is believed to result in impaired activation of regulatory mechanisms, such as Tregs, which further results in the impaired regulation of Th2 immunity and allergic reactivity.

2.3 Farming environment

During the last decades, traditional agricultural lifestyle has become rare in developed countries. While an urban life style demanded societal changes, such as industrialized agriculture, the environmental biodiversity and the microbial diversity decreased (60). It has been suggested that the decrease in the environmental biodiversity has led to an increase in allergic and autoimmune diseases in advanced societies (53).
Growing up in a farming environment has been shown to decrease the risk of hay fever, asthma, and atopic sensitization (52). The exact farm-associated factors behind the allergy-protective farm effect are unclear. Nevertheless, it seems evident that prenatal or early-age exposures are important. There are several separate farm-related factors, such as contact to livestock and animal feed, and the consumption of unprocessed cow's milk, that have been shown to associate with decreased risk of an allergic disease or atopic sensitization in children (61). In addition, prenatal exposure to animal sheds and hay has been reported to be inversely associated with cord blood IgE levels against seasonal allergens (62).

In a study conducted in Eastern Finland, children who lived in families with dogs, cats, or livestock had a lower risk of atopy, measured with skin prick test at school age (63). Although, the farmers’ children had a smaller risk of atopy, the effect of animal contact was independent of farming status. Also, a dose-response effect was found: the risk of atopy decreased with more frequent animal contact.

Farmers’ children are reported to use butter more often than margarine when compared to non-farmers’ children. Also, the daily consumption of farm milk or full milk is higher among farmers’ children. National recommendations may affect the usage of raw milk products. In the Finnish study (63), no association between atopy and farm milk consumption was found, possibly due to the small number of children consuming unprocessed milk. However, farm milk consumption has been associated with decreased risk of allergies in several studies, and is considered a distinctive farm exposure in decreasing the allergy risk.
A cross-sectional study by Perkin and Strachan showed that current unpasteurized milk consumption was associated with less current eczema symptoms, a 59% reduction in total IgE levels, and a 70% decrease in skin prick test positivity in school-aged children (65). The finding was also seen in children who did not live on farms.

Although there may be a wide range of factors that make up the allergy-protective farm effect, microbial antigen contact in early life may be the key factor in the modulation of the developing immune system. Microbes derived from the environment, such as household pets, livestock and their feed can modulate the normal flora and immunological responses via the respiratory tract, the digestive tract, and the skin. Elevated bacterial endotoxin levels measured in the mattress dust of school aged children have been shown to associate with living on a farm, decreased risk of asthma, atopic sensitization, and hay fever (66). In addition to a higher fat and protein composition, raw milk contains a significantly higher total bacteria count, more coliforms, *E. coli*, and coagulase-positive staphylococci than pasteurized milk (67). Digestion of the milk derived bacteria or their components can alter the gut normal flora and lead to beneficial modulation of the immune system.

### 2.4 Immune system

#### 2.4.1 Innate immune system

The innate immune system consists of non-specific mechanisms that enable an immediate response against pathogens. The mechanisms
include epithelial cell barrier and tight junctions, the secretion of mucus, intestinal peristalsis, digestive enzymes, fever, and pH values not suitable for microbes. Monocytes, neutrophils, macrophages, natural killer cells, acute phase proteins, and the complement are a major part of the innate immune response.

The innate immune response can be activated via signaling or non-signaling pattern recognition receptors (PRRs). Signaling PRRs include TLRs (68), which and are expressed on antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs), and on the surface of epithelial cells, monocytes and T cells. Microbial structures, such as LPS, are recognized by TLRs. Signaling via TLRs can induce the production of pro-inflammatory cytokines and chemokines (69). Non-signaling, soluble PRRs include acute phase proteins and transmembrane proteins. The acute phase protein CRP is produced in liver and released to circulation where it can bind to invading pathogens leading to phagocytosis or complement recognition. High levels of CRP measured in serum or plasma is used as a marker of inflammation. Low-grade inflammation, characterized by moderately increased levels of hs-CRP, has been associated with decreased risk of eczema (70).

2.4.2 Adaptive immune system

Antigen-specific T and B lymphocytes mediate the adaptive immune responses that are established in the secondary lymphoid tissues - spleen, lymph nodes, and mucosa-associated lymphoid tissue. Adaptive immune responses are specific to a particular pathogen and help to form long-
lasting immunologic memory against antigen structures. B cells are activated to secrete immunoglobulins, whereas T cells are involved in cell-mediated immune responses. T cells are divided into two broad categories: CD4+ and CD8+ T cells. CD8+ cells are important in the recognizing and killing of cells that are invaded by pathogens, whereas CD4+ cells are important helper cells for the function of B cells, CD8+ cells, and macrophages. It may take days or weeks to develop an effective adaptive immune response with antigen specific T and B cell populations. T cell receptors are expressed on the surface of T cells. On CD4+ Th cells, these receptors recognize antigens presented by class II major histocompatibility complex (MHC) molecules, which are expressed on APCs. The MHC in humans is called human leukocyte antigen (HLA). CD4+ Th cells activate and regulate immune responses both by secreting cytokines and by direct cell-cell interaction. Th cells mediate a broad spectrum of immunological functions, including activating cytotoxic CD8+ T cells, enhancing pathogen killing by macrophages, enhancing B cell antibody secretion, and affecting apoptosis. Cytotoxic CD8+ T cells recognize antigens presented by class I MHC molecules, which are expressed on all somatic cells. After recognition of a presented pathogen on the surface of a cell, cytotoxic CD8+ T cells proceed to kill the human cells that have been infected by intracellular pathogens. For destruction, cytotoxic CD8+ T cells use effector proteins such as perforin, granzyme B, and apoptosis-inducing cytokines (71).

CD4+ Th cells can be divided into at least four subpopulations: Th1, Th2, Th17, and Tregs (72-75). The composition of cytokines present at the
time of clonal expansion plays a large role in defining the polarization of CD4+ Th cells into their subtypes. The integral cytokines regulating the Th1 and Th2 polarization are IL-12 and IL-4, respectively. Th1 cells are characterized by the release of IFN-γ, IL-2, tumor necrosis factor-α (TNF-α), and lymphotoxin, which enhance cell-mediated immunity and macrophage activation. Th1 cells are needed to mobilize CD8+ cells against intracellular pathogens and have been linked to the development of tissue destruction in autoimmune disease. Th2 cells mainly secrete B cell activating cytokines such as IL-4, IL-5, and IL-13 (Figure 1) and play a role in protecting against extracellular pathogens, such as parasites. Dysregulated Th2 immunity is associated with the development of atopy and allergic diseases (76).

Th17 cells secrete IL-17 and IL-22 cytokines and are important in mucosal defence against fungi and bacterial infections. Activation of Th17 cells leads to neutrophil recruitment and secretion of AMPs, such as human β-defensin (HBD) 2, from epithelial cells. While Th17 cells are the main source of IL-17, cytotoxic CD8+ T cells are also known to secrete IL-17. Intriguingly, Th17 immunity is related to autoimmune and inflammatory diseases such as type 1 diabetes, rheumatoid arthritis, Crohn’s disease, and ulcerative colitis (77). The role of Th17 cells in allergies is not entirely defined, however, up-regulated Th17 responses have been detected in patients with allergic rhinitis or asthma (78). Furthermore, Th17 mediated inflammation has been reported in AD, which may play a role in tissue remodeling (79).
Tregs play a significant role in maintaining immunological homeostasis. Tregs can be classified into subtypes that share some common features including expression of Foxp3 and secretion of inhibitory cytokine IL-10 and/or TGF-β. Tregs regulate immune responses to allergens, self-antigens, commensal microbes, pathogens, and tumors. Suppression of effector T cells by Tregs can occur in two ways. In non-inflammatory states, suppression aims to keep T cells in a naïve state and maintain self-tolerance. This occurs by Treg-dependent deprivation of activation signals from responder T cells. In inflammatory states, Tregs work to restore local immune homeostasis by inactivating and killing responder T cells and APCs, a process that can be mediated via IL-10 secretion (80). Tregs may affect the pathogenesis of allergy, especially in the sensitization phase. In healthy individuals, Th2 responses to allergens may be actively prevented by Tregs, whereas in allergic patients, Treg functions may be impaired (81).

In order to recognize antigens, B cells use antibodies as receptors. In their naïve state, B cells express IgM and IgD on their surfaces. After maturation, IgA, IgG, or IgE are expressed. Activated B cells proliferate and develop into antibody-secreting plasma cells and can further differentiate into memory cells. Both T and B cell mediated responses are important, and presumably cross-regulated, in order to maintain a functional adaptive immune response (82).
2.5 Gut immune system

2.5.1 Gut anatomy

The gut forms a significant barrier between the outside world and the human body. The gut epithelium is often in direct contact with pathogens and environmental antigens, which have the potential to provoke a detrimental immune response. In order to function normally, the gut must maintain both a robust immunity against pathogens as well as a tolerance for the commensal flora and food antigens. In addition, the intestinal mucosa has to allow fluid exchange and maintain a selective permeability for the absorption of nutrients and secretion of waste (83).

Environmental agents and the cells of the immune system are in close contact in the gut since the epithelium is generally only one cell layer thick. On the lumen side, the epithelial cells have membrane protrusions called microvilli, which increase the surface area of the cells and thereby increase the surface area of the gut as well.

The goblet cells (Figure 1) are specialized epithelial cells that produce mucin 2, a mucus forming molecule essential for colonic protection. Mucus restricts relatively large particles, such as bacteria, from directly contacting the epithelia. However, small constituents can penetrate the mucus layer more easily. Animal studies have shown that mice with mucin gene deficiency will develop intestinal inflammation and eventually colitis (84). Microfold cells contain microfolds instead of microvilli, and they have functions in antigen presentation.
The intestinal epithelium is protected by physical (mucus) and chemical innate defence mechanisms which cooperate with the adaptive immune system (83). In addition, the low pH of the stomach and proteolytic enzymes secreted from the pancreas decrease the number of microbes entering the gut, and also digest proteins to peptides, which are less prone to activate immune responses.

The transepithelial transport of particles is made up of transcellular and paracellular transport pathways. Paracellular pathway allows passive movement of solutes across the epithelium. The solute flux is regulated by tight junctions, which are the intercellular junctions between epithelial cells. The tight junctions regulate the passage of solutes based on size. Therefore, relatively small particles may passively pass across a tight junction by a leaky pathway. The flux may be increased by cytokines (85). Furthermore, small pores, possibly defined by claudin proteins in the tight junction, allow the transport of particles based on their charge (86). Claudin expression may also be regulated by cytokines. The transcellular transport across the epithelium can be passive or active, and it is often enabled by specific transport channels. Transcellular transport creates a transepithelial concentration gradient that is sustained by tight junctions. Without an intact tight junction barrier, diffusion would balance the concentration between the two sides of the epithelium (83).

The intestinal epithelium plays an important role for both the innate and adaptive immune systems, yet the mechanisms how the epithelium and the mucosal immune system interact have not been fully elucidated.
Nevertheless, in response to infection, the tightly regulated nuclear factor κB pathway becomes activated and further activates DCs, T cells, and enterocytes (87).
Figure 1. The interaction between intestinal epithelial cells, antigen-presenting dendritic cells, and Th cell responses. Dendritic cells react to pathogens and support the secretion of IL-12, IL-4, and IL-23 which stimulate Th1, Th2, and Th17 responses, respectively. The secretion of calprotectin and/or HBD2 are induced during infection. (Adapted from Hundorfean et al. 2012).
2.5.2 Mucosal immune system and oral tolerance

The lamina propria (LP) is located between the surface epithelium and muscularis mucosa of the gut. The gut-associated lymphoid tissue (GALT) is located in the LP and it belongs to the mucosa-associated lymphoid tissue. GALT consists of mesenteric lymph nodes, solitary lymph follicles in ileum and colon, and Peyer’s patches, which are mainly located in the submucosa of the small intestine and the appendix. In the healthy gut, CD4+ and CD8+ T cells and IgA-producing plasma cells are the most abundant cells in GALT. In addition, naïve B cells, macrophages, and DCs can be found in GALT. As GALT is covered by an epithelial cell layer, interaction with the environment is controlled by barrier mechanisms (88).

Microfold cells (M cells) are specialized epithelial cells that reside in isolated lymphoid follicles. Within the lymphoid follicles, they actively transport foreign antigens and commensal bacteria to mucosal lymphoid tissues in order to maintain the protective immune responses. Certain pathogens use the microfold cells to enter the mucosa. It is suggested that immature DCs endocytose antigens and pathogens after they have been transported through M cells. DCs then present the antigens locally, or migrate to the adjacent interfollicular region or to mesenteric lymph nodes, mature, and present the antigens to T cells (88). DCs can also catch bacteria by extending their dendrites into the intestinal lumen (Figure 1).
The gastrointestinal tract is important for the development of immune system, which has to recognize harmless antigens and tolerate them without inducing severe inflammation, while being able to mount immune responses against invading pathogens. Tolerance to food antigens is induced via the small intestine and it generally affects systemic immune responses. Failure to establish such a tolerance may lead to allergy, celiac disease, or other immune-mediated diseases. Tolerance towards normal flora occurs more locally in the large intestine. Failure to tolerate normal microbiota may lead to inflammatory bowel disease (IBD) (89). Mechanisms of tolerance include limiting the exposure to microbes with mucus and IgA secretion (from plasma cells), and actively down-regulating the immune responses through the control of PRR expression, the secretion of inhibitory mediators, and the modulation of transcription factors in intracellular signaling pathways (90).

Oral tolerance is mediated through the induction of regulatory T cells, T cell anergy and clonal deletion. The effects of oral tolerance can be seen in reductions in T cell proliferation and cytokine production. Antibody levels in serum (such as IgE) in addition to mucosal IgA and T cell responses may also be suppressed (89). The constitutive factor determining the type of tolerance development is the dose of antigen exposure. High doses of antigen favor the T cell anergy or deletion in the gut and systemic antigen presentation, which can induce unresponsive T cells. Low doses of antigen lead to active suppression. In the case of a tolerogenic environment, antigen presentation by APCs results in the
production of antigen-specific regulatory T cells producing anti-inflammatory IL-10 and/or TGF-β (91).

Animal studies have shown that tolerance is dependent on commensal gut flora which can promote T cell hyporesponsiveness and down-regulate antibody responses (92). The number of T cells in GALT of germ-free (GF) mice has been shown to be lower when compared with the T cell count in specific pathogen free mice, in which the situation corresponds to an unsuccessful oral tolerance induction. Colonization of GF mice with *Bifidobacterium infantis* and *Escherichia coli* restored the number of T cells (93).

**2.5.3 Fecal biomarkers of inflammation**

Anti-microbial peptides (AMP) are important part of the mucosal innate defence mechanisms. Defensins, lactoferrin, and cathelicidins are common AMPs. In addition, microbial products such as short-chain fatty acids (SCFA) and inflammatory proteins such as calprotectin are essential in the shaping the gut inflammation (94).

AMPs distract microbes by forming micropores into their membranes. Defensins and cathelicidins have chemotactic properties which they use to attract cells such as T cells to the cite of microbial invasion. Lactoferrin can inhibit pro-inflammatory cytokines and interact with bacteria by binding iron. The production of SCFAs, such as butyrate, occurs when microbes ferment carbohydrates. Butyrate has anti-inflammatory properties and is an important factor in the regulation of gut permeability (95). These regulators of intestinal inflammation can be used as fecal
biomarkers of gut inflammation and some of them can help to delay the need for biopsy.

The most prominent human defensins are HBD1 in the colonic intestine and α-defensin in the small intestine. The secretion of additional defensins, such as HBD2 are increased during infection. Levels of fecal HBD2 have been reported to be elevated in ulcerative colitis (96) yet decreased in Crohn’s disease which can be due to the different mechanisms of regulating the mucosal innate defence system in the two diseases (97). The levels of fecal calprotectin and lactoferrin are increased in IBD (98). Of the markers mentioned above, all but SCFA can be measured with enzyme-linked immunosorbent assay (ELISA) (94). In this thesis, calprotectin was used as a biomarker for intestinal inflammation.

In addition to IBD, increased fecal calprotectin levels have been found in celiac disease, rheumatoid arthritis (99-101) and in children with cow’s milk allergy (102). Calprotectin is a cytosolic protein with the ability to bind calcium and zinc ions (103-105). It is released during neutrophil activation and monocyte-endothelial cell adhesion. The levels of fecal calprotectin correlate with neutrophil migration into the gut lumen (101). Monocytes and mucosal epithelial cells can also express calprotectin (106).

Infants have higher fecal calprotectin levels when compared to children and adults (104,107,108). This is likely due to the high gut permeability in infancy (109), which leads to a high antigenic load and stimulation of the inflammatory response in the gut immune system. The decrease in the fecal calprotectin levels after infancy may reflect the maturation of the gut
(“gut closure”) and also the alleviation of inflammation. Colonization of the gut by microbial flora may also contribute to intestinal inflammation during infancy. The influence of bacteria on calprotectin levels was reported in a study where antibiotic treatment correlated negatively with calprotectin levels in low birth weight infants (110).

2.6 Gut normal flora

The co-evolution of humans and their commensal microbes has resulted in humans’ dependence on their microbiota for normal immune function. It seems that the normal function of the immune system and metabolism are both strongly affected by the gut microbiota. The body has a 10-fold number of microbial cells compared to eukaryotic cells. The gut is specifically rich in normal bacterial flora. The number of microbial cells in the human intestine can reach up to $10^{14}$ (111). In addition to affecting the immune system, the gut bacteria use nutrients and poorly digestive carbohydrates from food to produce metabolites (such as butyrate) which can be used by the human host.

The composition of the gut microbiome is unique in each person and dependent on lifestyle factors such as dietary habits. The phylogenetic microbial composition is different when compared between people living in different countries (112). Nevertheless, the most prominent bacterial phyla in adults are the Gram-positive *Firmicutes* and *Actinobacteria*, and the Gram-negative *Proteobacteria* and *Bacteroidetes* (113). A 16S ribosomal RNA gene (rRNA) sequence-based method showed that a 90%
The majority of the gut bacteria belong to phylas *Firmicutes* or *Bacteroidetes* (114).

At birth, the infant’s gastrointestinal tract is inhabited by bacteria derived from the birth canal of the mother, including anaerobic bacteria. Infants born with cesarean section acquire their first microbes from the hospital environment in which anaerobes do not survive. They are also colonized by bacteria from the skin, such as *Staphylococci*. In general, facultative (aerotolerant) bacteria such as coliforms, enterobacteria, and lactobacilli dominate the gut microbiota for the first years until anaerobes such as *Bifidobacterium, Bacteroides, Eubacterium* and *Clostridium* establish their colonization in the gut (114). Nevertheless, it was shown that infants born by cesarean section have a lower ratio of anaerobic to facultative gut bacteria when measured by one year of age (115). The *Bacteroides*, bifidobacteria and *E. coli* colonization occurred later and in less frequency in children who were born by caesarean section (116). After delivery, skin-to-skin contact, breastfeeding, introduction of solid foods, and the environmental conditions begin to modulate the microflora, which is established during approximately the 3 first years of life and then begins to resemble the adult microbiota. In a study comprised of more than 500 individuals, children were shown to have a greater variety in interpersonal composition of gut microbes than adults. Bacterial diversity has been shown to increase with age (112).

Aberrancies in the ratio of microbial groups have been associated to the development of certain disorders such as obesity, allergies, and type 1 diabetes (95,117). *Bacteroidetes* have been shown to be more abundant in
diabetic children, whereas the microbiota of healthy children seems to be more in balance and contain more butyrate-producing bacteria (118). In obesity, the relative proportion of *Bacteroidetes* is decreased, and the proportion of *Firmicutes* is increased (117).

**2.6.1 Gut microbes in allergic diseases**

Similar to the environmental bacteria diversity, the diversity of the gut microbial flora has altered and become poorer in the developed countries. The diversity of gut microbiota was lower in children who later become allergic (119). In addition to a higher diversity of gut microbes, children from developing countries have been shown to be colonized earlier by *E. coli* and have a higher turnover of *E. coli* strains (120) which may push the development of the immune system towards a phenotype which is tolerogenic against potential allergens.

Several epidemiological studies have shown differences of gut microbes between atopic and healthy children. The quantity and colonization of bifidobacteria has been shown to be lower in allergic children than in their healthy peers. In addition, allergic infants are colonized by *Bifidobacterium* species normally associated with adults, such as *B. adolescentis* (114). It can be questioned which comes first, the allergic predisposition altering the microbiota, or the alteration in microbiota affecting the development of allergies. However, it has been shown that skin-prick positive children had more clostridia at 12 months of age and tended to have less bifidobacteria in feces at 3 weeks of age, when compared to non-atopic children (121).
Species of *Lactobacillus* and *Bifidocterium* are commonly used as probiotics. When probiotic bacteria has been given to the infants or their mothers, the colonization seems to not be persistent after the end of supplementation. Regardless of that, several studies have shown probiotics to possess allergy-preventive effects, although, in most cases only during the first 2 years of life. The incidence of atopic eczema was decreased in high risk populations when probiotics were given to the mothers before delivery and to the infants immediately after birth (122,123). Also, probiotics were effective in eczema prevention in high-risk children when the supplementation was started during pregnancy and continued during breastfeeding (124). It has also been shown that the altered bacterial colonization in children born with caesarean section could be modified by probiotics, which led to fewer IgE- associated allergies by the age of 5 years (125). It appears that the early timing and extensive use of probiotic supplementation, and possibly the broader range of bacteria, are essential since many studies have also failed to show allergy-prevention in the child. There are also several studies in which no effect of the probiotic treatment in the prevention or treatment of allergies has been observed (126,127) and there is often high heterogeneity between studies. However, the immunological changes induced by probiotics in the infants, such as increase in hs-CRP or changes in the cytokine profile, support the view that gut microbiota is an important regulator of the immune system.
2.6.2 Breastfeeding and gut microbes in infants

Gut normal flora composition is altered by diet. Breast milk contains a high concentration of fat, nutrients, anti-microbial agents, anti-inflammatory agents, and bacteria, such as bifidobacteria and lactic acid bacteria (114,128). It has been suggested that some of the bacteria in breast milk may be derived from the mother’s gut via dendritic cells and macrophages. In animal models it has been shown that bacterial translocation from the gut to mesenteric lymph nodes and mammary glands happened during late pregnancy and lactation (129). Therefore, alteration of the mother's gut microbiota may be beneficial to the infant, as did the above mentioned findings show, maternal probiotic supplementation in addition to the supplementation given to the infant prevented atopic eczema.

Several studies have shown that bifidobacteria dominate the gut microbiota in breastfed babies during the first year of life (112). Although formula milk is prepared to resemble the nutritional composition of breast milk, it lacks maternal antibodies such as IgA as well as active cytokines. Pre- and probiotics are often added to formula for the modification and establishment of beneficial normal flora. Nevertheless, the gut microbiota is different between formulafed and breastfed infants. Formulafed infants have higher levels and frequencies of facultative bacteria such as Bacteroides and clostridia, and lower numbers and frequencies of bifidobacteria than breastfed infants. Formulafed infants have been reported to have higher levels of fecal SCFAs when compared to breastfed infants. It may be due to the different
bacterial composition, and therefore the different ability to ferment carbohydrates (130).

2.6.3 Measurement of gut microbes

Measuring microbes in feces is the most straightforward way to define the gut microflora. Only approximately half of the gut microbes can be cultured (116). Modern techniques used for the microbial definition include quantitative PCR, DNA fingerprinting, metagenomics, phylogenetic microarrays and sequencing. These methods give different degrees of taxonomic information. The use of different methods can also cause difficulties in comparing the results of studies (131). It can be questioned how well microbes excreted into feces represent the actual microenvironments in different parts of the gut. Nevertheless, fecal samples are widely used for microbial and inflammation marker analysis since they are relatively easy to collect and process for laboratory measurements.
3 AIMS OF THE STUDY

Allergies are a growing health burden in Western societies. The incidence of allergic diseases, such as asthma, has also been increasing in developing countries in recent years (132). The increased incidence of allergic diseases and atopy is believed to associate with the more hygienic environment and the lack of certain infections – mainly bacterial infections. The aim of the studies presented in this thesis was to examine the development of immune responses, IgE-sensitization, and allergic disease in children from farming and non-farming environments, and the study subjects were participants of the PASTURE birth cohort study.

1. The aim of the first study was to determine whether low-grade inflammation, detected as elevated hs-CRP levels at early age, protects from IgE-sensitization or allergic diseases later in life. The association of farming factors related to microbial exposure and hs-CRP levels were also investigated.

2. The aim of the second study was to characterize whether breast milk sIgA or TGF-β1 levels associated with asthma, AD, or IgE-sensitization in children. The duration of breastfeeding and the association of farm related factors to the levels of breast milk components were also evaluated.
3. The aim of the third study was to determine whether a farming environment affected the development of mucosal tolerance defined by the levels of IgA and IgG antibodies against cow’s milk BLG and whey gliadin, and whether these antibodies to food antigens associated with allergic diseases or IgE-sensitization later in life.

4. The aim of the fourth study was to evaluate the association of early-age intestinal inflammation, detected as fecal calprotectin, with the later development of allergic diseases in children from farming and non-farming environments. In addition, we studied the effect of gut microbes on the fecal calprotectin concentrations.
4 SUBJECTS AND METHODS

4.1 Subjects

4.1.1 Families in the PASTURE study

Samples for the studies presented in this thesis were acquired from children and their mothers who participated in the PASTURE birth cohort study conducted in rural areas of Austria, Finland, France, Germany, and Switzerland. Women were recruited to the study during pregnancy. They were defined as farmers if they worked or lived on family-run farms where any livestock was kept. The reference group consisted of women from the same rural areas who did not live on a farm. Exclusion criteria were maternal age less than 18 years, no livestock at the farm, premature delivery, a genetic disorder in the infant, an insufficient knowledge of the language spoken in the country, and no telephone connection.

Originally, 1133 mothers agreed to participate. Questionnaires were administered by interview to the mother of the child within the third trimester of pregnancy. Questionnaires were answered again when the child was 2, 12, 18, and 24 months of age and then every year up to the age of 6 years. Questions were based on previous studies (133-136) and assessed respiratory and other health conditions of the mother, environmental exposures and potential confounders. In addition, the mothers kept a weekly diary from the third month of the child’s life to their first birthday. The duration of breastfeeding and the introduction of complementary foods were also recorded.
4.1.2 Health outcomes
Specific immunoglobulin E (sIgE) against 19 common allergens was measured in sera at the ages of 1, 4, and 6 years using the Allergy Screen Test Panel for Atopy (Mediwiss Analytic, Moers, Germany) (137). The inhalation allergens measured were house dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*), pollen (alder, birch, European hazel, grass pollen mixture, mugwort, rye, and plantain), horse, cat, and dog dander, and the mold *Alternaria alternata*. The food allergens measured were cow’s milk, hen’s egg, peanut, carrot, hazelnut, and wheat. Any allergic sensitization was defined as a positive result to at least one of the measured allergens. The cut-offs used for sensitization at different age-points were sIgE concentration of 0.35 kU/L or greater at the age of 1 year, 0.70 kU/L or greater at the age of 4 years, or 0.70 or alternatively 3.50 kU/L or greater at the age of 6 years. Inhalation or food atopy was defined as a positive result to any of the 13 inhalation or 6 food allergens tested, respectively.

Data on doctor-diagnosed AD and asthma (also defined as more than one episode of asthmatic, obstructive, or wheezy bronchitis which describe the same condition) were obtained from questionnaires and used in defining the diseases at specific age points in each study.

**Study I:** Parents reported if a doctor had diagnosed AD or asthma at least once, or asthmatic bronchitis at least twice during the follow-up. Children with no report of a doctor’s diagnosis of AD or asthma/had only one or no report of asthmatic bronchitis in the 1 year questionnaire but who developed the disease between ages 1 and 4 years were defined as having
AD or asthma, respectively. Children who had AD or asthma/more than one report of asthmatic bronchitis before the measurement of hs-CRP values (at the age of 1 year) were excluded from analyses. Atopic sensitization was defined at ages 1 and 4 years.

**Study II:** Children were defined as having cumulative AD up to the age of 2 or 4 years if the parents reported that the child had AD diagnosed by a doctor at least once up to 2 or 4 years of age, respectively, or if the child had a positive scoring index result for AD, i.e. SCORAD score (> 0) at the age of 1 year. Atopy at age 4 and 6 was defined as having sIgE antibodies against at least one of the measured allergens. Asthma at the age of 4 years was defined as a doctor’s diagnosis of asthma or wheezy bronchitis more than once during the last 12 months. Asthma at the age of 6 years was defined as doctor’s diagnosis of asthma or wheezy bronchitis after between the ages of 3 and 6 years.

**Study III:** AD was defined as the first doctor diagnosis of the disease during the 6 years of follow-up. Asthma was defined as the first doctor’s diagnosis of asthma and/or second doctor’s diagnosis of asthmatic (obstructive) bronchitis during the follow-up. Children with no reports of diagnosis in the 1 year questionnaire but who developed AD or asthma between the ages of 1 and 6 years were defined as having the disease. Children who already had AD or asthma/asthmatic bronchitis (more than once) before the measurement of IgA and IgG antibodies at the age of 1 year were excluded from analyses. Specific IgE was measured at the ages of 1 and 6 year.
Study IV: Children were defined as having AD up to the age of 6 years when the parents reported in the questionnaires that the child had at least one AD diagnosis during the 6-year follow-up. Atopy at 6 years of age was defined as a positive result for specific IgE antibodies (cut-off 0.70kU/L) against at least one of the measured allergens. Asthma by the age of 6 years was defined as a doctor’s diagnosis of asthma at least once or asthmatic bronchitis more than once during the 6 years.

4.1.3 Samples in Study I
Serum samples were collected at the age of 1 year for measurement of hs-CRP. Both hs-CRP and sIgE values at the age of 1 year were available from 634 children. Both hs-CRP values at the age of 1 year and sIgE at the age of 4 years were available from 448 children (Figure 2).

4.1.4 Samples in Study II
Breast milk samples were collected from 622 mothers two months after delivery. Austrian samples were not applicable since they were not sent to Finland for measurements. In total, 610 samples were analysed for both sIgA and TGF-β1 (Figure 2).

4.1.5 Samples in Study III
Serum samples were collected at the age of 1 year for measurement of IgA and IgG against cows’ milk BLG (N=639) and IgA and IgG against wheat gliadin (N=636) (Figure 2).

4.1.6 Samples in Study IV
Fecal samples were collected at the age of 2 months and calprotectin was measured in 758 samples. French samples had been stored improperly and
were therefore excluded. Furthermore, French samples were collected using different tubes and collection sticks than in other countries. Since the sample material was powder-like and clearly appeared to be different from the samples from other countries, a decision was made not to use them.

Gut microbiota was analysed in a subgroup of 120 infants. The children were included for the selection if they were exclusively or partially breastfed at the age 2 months, if the fecal calprotectin level was above the detection limit, if there was data available for AD and asthma at the age of 6 years, and if the amount of fecal sample was sufficient for extraction of DNA. Case samples (N=40) were selected starting from the first applicable sample with fecal calprotectin in the 90th percentile (fecal calprotectin values ranging from 517.6 to 1542.0 µg/g). Control samples were defined as samples with fecal calprotectin concentration below the 90th percentile. For each study centre, two control samples were randomly selected for each case sample (N=80). To study the correlation between fecal and breast milk calprotectin levels, calprotectin concentrations were measured in 115 breast milk samples from the Finnish mothers 2 months after giving birth (Figure 2).

The ethical committees of all study centers approved the study, and written informed consent was obtained from the children’s parents for questionnaires and samples.
Figure 2. Samples in the PASTURE study and in studies I, II, III, and IV of this thesis.
4.2 Methods

4.2.1 Measurement of hs-CRP levels in serum (Study I)

Serum samples were stored at -20° C. They were thawed for analysis in Study I. High-sensitivity CRP concentrations in serum samples were measured using ELISA (BenderMedSystems human CRP, Vienna, Austria) according to the manufacturer’s instructions. The hs-CRP ELISA is used for research purposes to measure low levels of CRP, not for diagnostic procedures. The sensitivity of the assay is 3.0x10⁻⁶ mg/l.

4.2.2 Measurement of TGF-β1 and sIgA in breast milk (Study II)

Breast milk samples were stored at -20° C. The samples were thawed and centrifuged at 10 000 g for 10 minutes at +4° C. The fat layer was removed and the clear supernatants were stored at -75° C. The supernatants were thawed for analysis in Study II.

TGF-β1 values were measured from breast milk samples (supernatants) using a commercial ELISA method (Quantikine Human TGF-β1 Immunoassay, Abingdon, UK). Prior to the measurements, latent TGF-β1 was activated using hydrochloric acid. The samples were subsequently neutralized with sodium hydroxide containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

Values of sIgA were measured using ELISA modified from Lehtonen et al. (138). The primary coating antibody was rabbit antihuman IgA (DakoCytomation, Glostrup, Denmark) diluted 1:1000 in carbonate buffer. The plates were incubated at +4° C overnight and blocked with
1% BSA in PBS for 1 h at +37° C. The plates were washed with 0.5% Tween 20 in PBS. The conjugate was peroxidase-conjugated rabbit anti-human IgA (DakoCytomation) 1:5000 in PBS. The substrate was Ortho-Phenylenediamine (OPD) (Kem-En-Tec-laboratories, Taastrup, Denmark). The reaction was stopped using 1 molar H2SO4 and optical density was read at 492 nm. A standard with known amounts of human IgA (Caltag laboratories, Burlingame, CA, USA) was used to create a control curve in each plate, from which the immunoglobulin concentrations were calculated.

### 4.2.3 Measurement of IgA an IgG antibodies against cow’s milk β-lactoglobulin and wheat gliadin in serum (Study III)

Serum samples were stored at -20° C. They were thawed for analysis in Study III. IgA and IgG antibodies against cow's milk BLG were measured using enhanced binding microtitre plates (Thermo Scientific, Roskilde, Denmark) which were coated with 0.1 μg of BLG/well (Sigma, St Louis, MO, USA). The antibody used for measuring IgA was biotinylated anti-human IgA (Vector Lab, Burlingame, CA, USA) and the conjugate was AP-streptavidin (Zymed, San Francisco, CA, USA). Fc fragment-specific alkaline phosphatase-conjugated AffiniPure rabbit anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) and Fast p-nitrophenyl phosphate tablet (Sigma) were used for measuring IgG.

IgA and IgG against wheat gliadin were measured using ELISA modified from Ludvigsson et al. (139). The coating of ELISA plates (MaxiSorp Surface; Nunc-Immunoplate, Roskilde, Denmark) was performed with 1
µg of gliadin/well (Sigma). Biotinylated polyclonal goat anti-human IgA (α-chain 62-8440) or IgG (γ-chain 62-7440) (Sigma) was used as antibody, and AP-streptavidin (Zymed) as conjugate.

Residual coatings were performed with 1% HSA, serum samples were diluted in 0.2% HSA-0.05% TWEEN20-PBS, and 0.05% TWEEN20/PBS was used for washing the plates. Optical densities were read at 405 nm. Standard curves were created using serial dilutions of pooled plasma obtained from children with high levels of IgA or IgG antibodies against BLG or gliadin. The results were presented as arbitrary units, which were calculated from the standard curve on each plate. Detection limits were 2.5 or 15 arbitrary units for IgA or IgG antibodies against BLG, respectively, and 10 or 6 units for IgA or IgG antibodies against gliadin, respectively.

4.2.4 Measurement of fecal and breast milk calprotectin (Study IV)

Fecal samples in Study IV were stored at -20° C. The Calpro Extraction Device (Calpro AS, Lysaker, Norway) was used for extracting samples. A beaker in the Calpro Extraction Device was filled with thawed and homogenized stool sample (~100 mg). The extraction tube was filled with 4.9 mL extraction buffer and vortexed for 30 s. The samples were mixed using a shaker at 1000 rpm for 3 min or until only solid particles remained, and centrifuged for 10 minutes at 10 000 g at room temperature. Supernatants were collected, stored at -20° C, and subsequently thawed for analysis in Study IV.
Fecal calprotectin levels were determined using ELISA (Calpro AS, Lysaker, Norway) according to the manufacturer’s instructions. Fecal extracts were diluted 1:50 in sample dilution buffer. The optical density was read at 405 nm. Samples below the lowest measurable concentration (39.2 μg/g) were given an arbitrary value of 19.6 μg/g.

The breast milk samples from the Finnish mothers were previously processed in Study II. The supernatants were diluted 1:10 and analyzed for calprotectin using ELISA (Calpro AS, Lysaker, Norway).

**4.2.5 Pyrosequencing (Study IV)**

In Study IV, total DNA was extracted from approximately 0.25 g fecal sample using the repeated bead beating method modified from Yu *et al.* (140). A Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France) was used for bead beating at 5.5 ms\(^{-1}\) in three rounds of 1 min. The incubation temperature after the bead beating was increased to 95°C. Protein precipitation with 260 μL ammonium acetate and elution of DNA from the purification columns were conducted twice. Columns from the QiaAmp Stool kit were replaced by columns from the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

16S rRNA genes were amplified from each sample using a primer set corresponding to primers 27F-degS (141) and 534-R (142) that targets the V1, V2, and V3 hypervariable regions of the 16S rRNA. 27-degS was chosen because it seems to provide a more complete assessment of the abundance of actinobacteria (141). Pyrosequencing of the PCR products
was performed with Roche FLX Genome Sequencer at DNAvision (Liège, Belgium) according to their standard protocol (143). Pyrosequencing produced 1,766,995 reads of 16S rDNA. Sequences were assigned to samples according to sample-specific barcodes. SFF files were converted into FASTA files and FASTA quality files, which contained an average (± SD) of 14,725 ± 5,018 reads per sample. The RDP pyrosequencing pipeline (144) (RDP 10 database, update 17) was then used to check the FASTA sequence files for the same criteria as previously described (143) and to confirm that the average experimental quality score was at least 20. After the quality check, the FASTA files contained an average (± SD) of 11,285 ± 4,102 high-quality reads.

The RDP classifier 2.01 (145) was used for the taxonomy assignment (phylum, family, and genus level). ARB software and SSU reference database (SSURef_111_SILVA_04_08_12) were used to identify the species level (146,147). Only sequences of cultured and identified isolates were used from the database. A “PT-server” database was built from these sequences, and used to find the closest match for each of our high-quality sequences imported from the FASTA files. Sequences from different strains of the same species were grouped together. All identified sequences, together representing 99.1 ± 1.04% of all high-quality FASTA reads per sample, were included for statistical analysis.

4.2.6 Detection of IL-10 secreting cells (Study IV)

To study the effect of LPS on IL-10 secretion, peripheral blood mononuclear cells (PBMC) from 3 healthy adult blood donors were isolated using Ficoll-Paque solution (GE Healthcare, Uppsala, Sweden)
and stimulated with LPS from \textit{E. coli} 0128:B12 (1\textmu g/ml) for 12 hours in CO\textsubscript{2} at 37\textdegree C. The concentration used was \(10^6\) cells/ml in 200 \textmu l of media. A negative control was PBMCs incubated in media (RPMI 1640 + 5% human AB-serum+ 2mM glutamine+ Gentamycin). PBMCs were then collected and washed with 0.5% BSA-PBS containing 2mM EDTA. Identification of IL-10 secreting cells was performed using IL-10 secretion assay (Miltenyi Biotech, Germany). In short, washed PBMCs were labelled with a catch reagent consisting of a cell surface specific antibody (anti-CD45) conjugated with an anti-IL-10 antibody. The cells were incubated in warm medium for the cytokine secretion to occur. Secreted IL-10 was detected with a secondary PE-labelled anti-IL-10 antibody. FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used for the detection of IL-10 secreting cells and their characterization by side and forward scatter properties.

\textbf{4.2.7 Statistical analyses}

In Study I, statistical analyses were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). The hs-CRP values (or the Log-transformed values) were not normally distributed. The risk of AD and asthma, and the risk of developing IgE-sensitization were analyzed by logistic multivariate regression. The hs-CRP values were categorized into quartiles. Odds ratios (OR) were adjusted for farming status, study center, mother's history of allergic disease (No/Yes), and potential confounders, which were at least marginally significantly (\(P<0.10\)) associated with hs-CRP values in the univariate analysis, that is gender, number of siblings, and day care with other children and siblings (No/Yes).
In the association analyses between hs-CRP values and environmental factors, results were presented as median values with interquartile ranges. Mann–Whitney U-test and the Kruskal–Wallis test were used for the calculations of P-values for the comparisons between different categories in the univariate analysis. For logistic multivariate regression analysis, the hs-CRP values were divided into two groups using the 75th percentile cut-off (0.19 mg/l). ORs were adjusted for farming status, study center, and potential confounders.

In Study II, statistical analyses were performed using STATA/SE 12.1 (STATACorp, College Station, TX, USA), and P-values <0.05 were considered statistically significant.

The concentrations of TGF-β1 and sIgA were categorized into quintiles (Q1-Q5). Differences in maternal variables and the duration of breastfeeding were tested using Pearson's chi-square test and the results were expressed as P-values. Pearson's coefficient was used to express correlations of continuous variables. TGF-β1 and sIgA values were log-transformed for showing approximate normal distribution, and expressed as geometric means and 95% confidence intervals. Environmental exposures occurring up to the age of 2 months were related to TGF-β1 and sIgA levels by linear regression and expressed as geometric mean ratios and 95% confidence intervals (CI). Factors that showed a significant association with TGF-β1 or sIgA levels in univariate models were entered in multivariate linear regression models.
In Study III, statistical analyses were performed with SAS 9.3 for Windows (SAS, Cary, NC, USA). The association between levels of IgA and IgG antibodies against BLG or gliadin and AD or asthma between the ages of 1 and 6 years were analyzed by discrete time hazard models. Logistic regression was used for analyzing the association between levels of antibodies and IgE-sensitization.

The levels of IgA and IgG antibodies against BLG and IgG antibodies against gliadin were categorized into quartiles (Q1-Q4) using 25th, 50th, and 75th percentile cut-off points. IgA antibodies against gliadin were categorized into three groups since the distribution was skewed.

The results were presented as ORs (95% CI) adjusted for farming status, study centre, gender, mother's history of allergic disease, farm milk consumption, and potential confounders, which were at least marginally significantly (P<0.10) associated with BLG or gliadin concentrations in the univariate analysis. In addition, the associations between antibody concentrations and IgE-sensitization at the age of 6 years were also adjusted for IgE-sensitization at the age of 1 year.

The results were given as median values with interquartile ranges in the analyses of associations between IgA and IgG antibodies and environmental and farming factors. P-values were calculated using the Mann–Whitney U and the Kruskal–Wallis tests, and were considered statistically significant below the value 0.05. For logistic multivariate regression analysis, the 75th percentile cut-off point was used. ORs were adjusted for farming status, study center, gender, farm milk consumption,
and potential confounders, which were at least marginally significantly (P<0.10) associated with the antibody levels in the univariate analysis, i.e., maternal smoking during pregnancy, duration of breastfeeding, age of introduction of formula milk, and pets at home at the age of 1 year.

In Study IV, statistical analyses considering the calprotectin data were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). The calprotectin values (or the Log-transformed values) were not normally distributed. The results were presented as median values with interquartile ranges. P-values for the comparisons between the categories in univariate analysis were calculated with the Mann-Whitney U- and the Kruskal-Wallis- tests. P-values <0.05 were considered statistically significant.

Logistic multivariate regression was used to analyze the risk of AD and asthma until the age of 6 years and the risk of IgE-sensitization at the age of 6 years. A 90\textsuperscript{th} percentile cut-off point was used to categorize the calprotectin concentrations into two groups. All ORs were adjusted for farming status, gender, and mother’s history of allergic disease, and with potential confounders, which were at least marginally significantly (P<0.10) associated with calprotectin in the univariate analysis.

Pyrosequencing data was analyzed with SPSS 20 (SPSS Inc, Chicago, IL, USA). Principal component analysis was performed to visualize the difference between atypical (diarrhea) samples and normal feces samples. FlowJo software (Tree Star, Inc., Ashland, OR, USA) was used to analyze flow cytometry data.
5 RESULTS AND DISCUSSION

5.1 Early-age inflammatory response and development of IgE-sensitization (Study I)

The acute-phase protein CRP is a marker of inflammation. Low-grade inflammation can therefore be measured using a hs-CRP ELISA method, which was used in Study I. Previous studies have shown that treatment with probiotics induced increased hs-CRP levels and this was associated with a decreased risk of allergic diseases (148,149). These findings suggest that inflammation triggered by environmental microbes could mediate protection from the development of allergic immune responses in children. Based on these findings, our aim was to test whether low-grade inflammation, measured as elevated levels of hs-CRP at the age of 1 year, provides protection from IgE-sensitization and allergic diseases later in childhood. Because the presence of atopy at the age of 1 year could affect hs-CRP levels, we stratified the children into IgE-sensitized and non-sensitized children at the age of 1 year.

All children had hs-CRP values below 5 mg/l. Among the children who were non-sensitized at the age of 1 year, elevated hs-CRP values associated with decreased risk of IgE-sensitization at the age of 4.5 years. That is, children who had the concentration of hs-CRP in the highest quartile had a significantly lower risk of sensitization to any allergen.
when compared to those with hs-CRP concentrations in the lowest quartile (aOR 95% CI: 0.48 (0.24-0.95), also presented in Figure 4).

There was an inverse, dose-dependent trend among the children who were non-sensitized at the age of 1 year, i.e. the higher the hs-CRP value at the age of 1 year, the lower the risk for IgE-sensitization at the age of 4.5 years (P=0.060). There was a similar trend in the whole cohort of children (P=0.077). No association of hs-CRP and later sensitization was seen in the children who were already sensitized at the age of 1 year.

No significant association of hs-CRP and allergic diseases was found either in the stratified group or in the whole cohort. However, the number of children who developed AD and asthma was low, making it difficult to draw conclusions regarding the relationship between hs-CRP values and allergic diseases.

Since there is evidence that a farming environment protects against allergic diseases (52,150), we evaluated whether farm-associated environmental factors indicating increased microbial load were associated with low-grade inflammation as measured by hs-CRP levels. No associations were seen between environmental factors and hs-CRP levels among non-sensitized children (Table 1). Therefore, this study found that a farming environment did not show significant effects in inducing systemic low-grade inflammation.

The early IgE-response to allergens does not necessarily imply allergic disease, but may signal a physiological response to environmental antigens in infants (151). When sensitization was observed separately
from hs-CRP levels, the children who had developed IgE-sensitization at the age of 1 year had a significantly higher risk of allergic sensitization at the age of 4.5 years (aOR 95% CI: 2.01 (1.39–2.90), P<0.001) and tended to have a higher risk of AD and asthma/asthmatic bronchitis (aOR 95% CI: 1.57 (0.96–2.56), P=0.072 and aOR 95% CI 1.55 (0.97–2.48), P=0.069, respectively) between the ages of 1 and 4 years when compared to the non-sensitized children.

Thus, the children who had developed IgE-sensitization at the age of 1 year represented the high allergy risk population. In these IgE-sensitized children, having dogs or both dogs and cats at home, drinking boiled or both unboiled and boiled farm milk, or having a mother who had visited stables weekly during pregnancy or during the last 10 months implied significantly lower hs-CRP values compared with children who did not encounter these exposures. These exposures are considered to associate with high microbial load. Thus, it was interesting that an association with low, rather than the expected high, hs-CRP values was found in the IgE-sensitized children.
### Table 1. The associations between environmental exposures and hs-CRP levels measured at the age of 1 year in non-sensitized and IgE-sensitized children.

<table>
<thead>
<tr>
<th></th>
<th>Non-sensitized at age 1 year</th>
<th></th>
<th></th>
<th>IgE-sensitized at age 1 year</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>hs-CRP*</td>
<td>aOR#</td>
<td>N</td>
<td>hs-CRP*</td>
<td>aOR#</td>
</tr>
<tr>
<td><strong>Farm milk consumption of the child</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>No</td>
<td>311</td>
<td>0.06 (0.04/0.14)</td>
<td>1</td>
<td>114</td>
<td>0.07 (0.04/0.30)</td>
<td>1</td>
</tr>
<tr>
<td>Only boiled</td>
<td>67</td>
<td>0.06 (0.04/0.26)</td>
<td>1.14 (0.58-2.22)</td>
<td>29</td>
<td>0.06 (0.04/0.08)</td>
<td><strong>0.16 (0.04-0.60)</strong></td>
</tr>
<tr>
<td>Both unboiled and boiled</td>
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<td>0.06 (0.03/0.37)</td>
<td>1.16 (0.48-2.81)</td>
<td>18</td>
<td>0.06 (0.04/0.14)</td>
<td><strong>0.18 (0.04-0.85)</strong></td>
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<td>Only unboiled</td>
<td>41</td>
<td>0.07 (0.05/0.20)</td>
<td>1.29 (0.57-2.91)</td>
<td>12</td>
<td>0.05 (0.03/0.18)</td>
<td>0.41 (0.09-1.97)</td>
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<td>0.581</td>
<td></td>
<td>0.283</td>
<td><strong>0.026</strong></td>
</tr>
<tr>
<td><strong>Mother staying in stable (weekly) during pregnancy</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>180</td>
<td>0.06 (0.04/0.13)</td>
<td>1</td>
<td>60</td>
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<td>1</td>
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<td>1.27 (0.69-2.33)</td>
<td>115</td>
<td>0.06 (0.04/0.29)</td>
<td><strong>0.24 (0.07-0.79)</strong></td>
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<tr>
<td>P-value</td>
<td></td>
<td>0.183</td>
<td>0.444</td>
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<td>0.129</td>
<td><strong>0.019</strong></td>
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<tr>
<td><strong>Mother's staying in stable weekly in last 10 months</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>257</td>
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<td>86</td>
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<td>Yes</td>
<td>202</td>
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<td>0.88 (0.32-2.46)</td>
<td>89</td>
<td>0.06 (0.04/0.20)</td>
<td><strong>0.13 (0.02-0.96)</strong></td>
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<tr>
<td>P-value</td>
<td></td>
<td>0.322</td>
<td>0.808</td>
<td></td>
<td>0.675</td>
<td><strong>0.045</strong></td>
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<tr>
<td><strong>Having pets at home (1 year of age)</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No cats or dogs</td>
<td>197</td>
<td>0.06 (0.04/0.13)</td>
<td>1</td>
<td>64</td>
<td>0.07 (0.04/0.40)</td>
<td>1</td>
</tr>
<tr>
<td>Only cat/cats</td>
<td>123</td>
<td>0.08 (0.04/0.23)</td>
<td>1.40 (0.76-2.58)</td>
<td>44</td>
<td>0.07 (0.04/0.30)</td>
<td>0.68 (0.24-1.93)</td>
</tr>
<tr>
<td>Only dog/dogs</td>
<td>43</td>
<td>0.06 (0.05/0.10)</td>
<td>1.11 (0.44-2.76)</td>
<td>21</td>
<td>0.06 (0.05/0.10)</td>
<td><strong>0.15 (0.04-0.62)</strong></td>
</tr>
<tr>
<td>Both cat(s) and dog(s)</td>
<td>89</td>
<td>0.07 (0.04/0.19)</td>
<td>1.33 (0.65-2.74)</td>
<td>44</td>
<td>0.06 (0.03/0.12)</td>
<td><strong>0.22 (0.07-0.72)</strong></td>
</tr>
<tr>
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<td></td>
<td>0.22</td>
<td>0.548</td>
<td></td>
<td>0.336</td>
<td><strong>0.003</strong></td>
</tr>
</tbody>
</table>

*hs-CRP values are presented as median values with interquartile range. P-value estimated by Mann-Whitney or Kruskal-Wallis test.

# hs-CRP values are presented in two groups according to 75th cut-off points. The comparison between groups are shown values as odds ratios (OR) with their 95% confidence intervals (CI). P-values are obtained from the trend test (Wald) in logistic regression models. Odds ratios are adjusted for gender, country, farming, siblings and day care with other children than siblings.

Statistically significant adjusted aORs are marked in bold.
Microbial exposure may play an important role in the establishment of effective regulatory networks during the development of the immune system in infancy. In environments with a higher microbial load, episodes of inflammation are more common (Figure 3). Nevertheless, adults living in developing countries have lower baseline hs-CRP levels when compared to adults living in more hygienic countries (152). Repeated activation and deactivation of inflammation may promote the development of more efficient regulatory pathways, which can effectively turn inflammation on and off when needed. In the case of a successful establishment of the regulatory pathways, inflammatory stimuli in adulthood are processed similarly, i.e. inflammatory responses increase quickly, and anti-inflammatory processes control the responses. In hygienic environments, the low microbial exposure can cause the inflammatory reactions in infancy to be less often activated and deactivated which may lead to a more pro-inflammatory phenotype. When inflammatory stimuli are encountered later in adulthood, inflammation can be activated but the deactivation by anti-inflammatory regulatory mechanisms is not sufficient to prevent the development of excessive or chronic inflammation (153). Similarly, in IgE-sensitized children, it is possible that continuous exposure to microbial stimuli has resulted in more tightly regulated inflammatory pathways and this is seen in the low hs-CRP values in the children exposed to high microbial load.
Figure 3. A hypothesis of the association between environmental microbial exposure in infancy and regulation of inflammation in adulthood. The arrow from infancy through adulthood represents time. The upper section represents environments with higher microbial exposures; the lower describes highly hygienic environments and low infection. (Adapted from McDade 2012.)

It is also possible that the level of hs-CRP reflects not only the microbial exposure, but also the individual ability to respond to diverse inflammatory stimuli. The children with early IgE-sensitization, who also showed a high risk of allergies later in their life, may not have developed regulation of the inflammatory response due to the repeated stimuli but originally reacted to the microbial exposure by an impaired inflammatory response. In the absence of proper inflammatory response, which would
be protective from the IgE-sensitization, they were more susceptible to IgE-sensitization. It is intriguing to hypothesize that a proper inflammatory response to microbial stimuli is needed for the regulation of allergic immune response and IgE-production. This view is supported by our findings showing that 1) increased hs-CRP levels at one year of life decreased the risk of IgE-sensitization in the children who were not sensitized at the time of hs-CRP measurement, i.e. 1 year of life; and 2) in the IgE-sensitized children decreased hs-CRP values are seen in those children exposed to microbial stimuli.

Therefore, the hs-CRP concentration as a marker of low-grade inflammation could at least be partially explained by diverse responders (good vs. poor, Figure 4), and not necessarily only by environmental microbial exposure. An association between impaired epithelial cell response to environmental antigens and asthma has been reported (154). Therefore, the poor response of the innate immune system, which is also reflected in an impaired induction of hs-CRP, could be a determinant for allergen-specific immune response later in life. Our findings are in accordance with these observations and suggest that the induction of inflammation plays a significant role in the controlling of IgE-responses. However, the people with poor inflammatory responses may benefit from treatments that enhance inflammation, such as shown for certain probiotic preparations (148), especially in environments where the inflammatory pressure is low. The impaired capability for inflammatory response may be sufficient protection against allergies in environments with high
microbial load, but could lead to allergic immune responses in highly hygienic environments.

The impaired inflammatory response to environmental triggers of inflammation may be a fundamental characteristic of the hosts' immune response, which may predispose to allergy development. In the children who had an allergic response at 1 year of age, it could be a factor associated with genetic or epigenetic variation. The production of acute phase proteins, such as CRP, is controlled by several cytokines released during the inflammatory process such as IL-1 and IL-6, which involve STAT3 and NF-κB signaling (155).

The poor response to known inflammatory determinants of the host at an early age appeared to be a key factor in controlling IgE-sensitization later in life. Our results suggest that the role of innate immunity and inflammation in the regulation of allergic immune responses and thus predisposition to IgE-mediated allergy is important. Our results support the earlier findings of the protective effect of low-grade inflammation in the control of IgE-mediated allergic diseases (148).
Figure 4. Summary of findings and discussion in Study I. Non-sensitized and IgE-sensitized children at the age of 1 year show divergent associations between hs-CRP concentrations at the age of 1 year and IgE-sensitization at the age of 4.5 years. “Good responders” indicate children who can, as response to microbial load, induce hs-CRP production which associates with decreased risk of IgE-sensitization. “Poor responders” indicate children who have impaired ability to produce hs-CRP.

5.2 Soluble immunoglobulin A in breast milk decreases the risk of atopic dermatitis in the child (Study II)

The previous studies of the effects of breastfeeding on allergy development have shown discrepant findings. The aim of our study was to observe the effect of breast milk components sIgA and TGF-β1, the duration of breastfeeding, and the estimated total amount of sIgA or TGF-β1 (termed the “dose” of ingested component) during the first year of life to the development of IgE-sensitization, asthma, and AD in the child. The main immunoglobulin in breast milk is sIgA, which protects the infant from infections and may also prevent excessive uptake of foreign antigens across the mucosa, therefore possibly decreasing the risk of allergic sensitization (156). The cytokine TGF-β1 regulates cell growth through
exerting both stimulatory and inhibitory effects on a variety of cell types. TGF-β activates Tregs by inducing the expression of forkhead box protein 3 (157). TGF-β increases the secretion of IgA in B cells by inducing class switch (158,159). IgA works in conjunction with TGF-β and other cytokines which are suggested to affect oral tolerance development (160). In addition, it has been shown in animal models that mice with TGF-β1 deficiency develop inflammatory autoimmune diseases (161).

Our results showed that sIgA levels in breast milk measured 2 months after delivery were inversely related to AD up to the ages of 2 and 4 years (Table 2). The association was strongest up to the age of 2 years, and followed a dose response (P-value 0.005 for adjusted linear trend). After adjusting for all potential confounders, the findings remained significant. The infants who were breastfed for a shorter period of time tended to have an increased risk of AD.
Table 2. The adjusted association of sIgA concentrations in breast milk 2 months after delivery and atopic dermatitis in the child up to the ages of 2 and 4 years. Quantile 5 represents the highest levels of sIgA.

<table>
<thead>
<tr>
<th>IgA in Quantiles (Q)</th>
<th>N</th>
<th>Up to age 2 years aOR (95% CI)</th>
<th>Up to age 4 years aOR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>124</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Q2</td>
<td>123</td>
<td>0.67 (0.35-1.29)</td>
<td>0.69 (0.37-1.31)</td>
</tr>
<tr>
<td>Q3</td>
<td>123</td>
<td>0.44 (0.22-0.88)</td>
<td>0.62 (0.32-1.18)</td>
</tr>
<tr>
<td>Q4</td>
<td>123</td>
<td>0.43 (0.22-0.85)</td>
<td>0.46 (0.24-0.88)</td>
</tr>
<tr>
<td>Q5</td>
<td>117</td>
<td>0.41 (0.20-0.85)</td>
<td>0.60 (0.31-1.17)</td>
</tr>
</tbody>
</table>

Statistically significant adjusted aORs are marked in bold. Logistic regression models adjusted for center, sex, farming, maternal history of allergies, and food score in the first year of life.

The dose of breast milk sIgA or TGF-β1 ingested by an infant during the first year of life was estimated by multiplying the breast milk sIgA or TGF-β1 level by the duration of total breastfeeding. The dose of sIgA was inversely associated with AD up to the ages of 2 and 4 years (aOR 95% CI: 0.74 (0.55–0.99) and 0.73 (0.55–0.96), respectively). Figure 5 shows the adjusted inverse association of sIgA dose and AD up to the age of 2 years. The dose of TGF-β1 was not significantly associated with AD up to the ages of 2 or 4 years (aOR 95% CI: 0.86 (0.65–1.14) or 0.83 (0.63–1.08), respectively).
Although we only had one measurement at 2 months, the concentrations are unlikely to change during lactation. It has been shown that the values of breast milk sIgA drop 10 days after birth and then remain relatively stable (162). The levels of TGF-β have been shown to be relatively constant at least for the first 3 months after birth (163). The effect of sIgA dose to AD remained significant despite the possible variations in the number of feeding times per day. Soluble IgA or TGF-β1 levels in breast milk were not related to asthma or IgE-sensitization.

Figure 5. Association of the dose of sIgA during the first year of life and the probability for atopic dermatitis up to 2 years of age. Adjusted association adjusted for center, sex, farming, maternal history of allergies, and food score in the first year of life.
The duration of breastfeeding was not associated with farming or any other environmental factor tested. Mothers with predisposition for allergic diseases tended to breastfeed for a longer period of time (over 6 months) when compared to mother with no predisposition. Children who had not been breastfed did not have an increased risk of developing AD, asthma or IgE-sensitization. The children who had maternal history of allergic diseases were at a higher risk of asthma at 6 years of age if they were breastfed (exclusively or partially) for an extended period of time. This was evident when the lowest and the highest quartile of breastfeeding duration were compared (aOR for the interquartile range of duration of breastfeeding; 95% CI: 2.34 (1.05–5.21)). The levels of TGF-β1 and sIgA were significantly higher in the breast milk of mothers who went on to breastfeed for a shorter duration of time (P-value for adjusted linear trend <0.001).

Although there was no association with farming, the levels of breast milk sIgA were associated with several environmental factors in unadjusted analyses including time spent in a barn (more than 5 hours per week; aOR 95% CI: 1.21 (1.06–1.39)) and contact to one or two species of farm animals (aOR 95% CI: 1.10 (1.01–1.20)) during pregnancy. The association was regarded as strong when it remained significant in adjusted analyses. After mutual adjustment, sIgA levels were elevated in the breast milk of mothers who had been in contact with cats during pregnancy (aOR 95% CI: 1.01 (1.01–1.20), smoked during pregnancy (1.22 (1.04-1.43)) or at the time of sample collection (1.40 (1.17-1.69)), and in the mothers who already had one child (1.11 (1.01-1.22)). These
factors could represent increased microbial exposure in the environment leading to the stimulation of the maternal immune system and increased sIgA in the breast milk. It is therefore possible that sIgA levels in breast milk are a signal of the environmental microbial load, which modifies the development of allergic diseases, such as AD. Asthma and breast milk sIgA did not show any association, suggesting a specific effect on AD.

The mechanism mediating the association of sIgA and AD is not known. Soluble IgA-mediated passive antimicrobial protection could influence gut normal flora colonization or affect antigen transport across the mucosa (40). Soluble IgA might also be a signal of other milk components that affect the development of adaptive immune responses or are involved in creating a microenvironment that promotes Treg development (164).

While we reported an inverse association with AD and sIgA in breast milk, there are other studies that did not find IgA levels in breast milk to be associated with atopic diseases such as AD (165,166). It has been shown that elevated levels of breast milk sIgA, measured three weeks after delivery, were associated with a reduced risk of asthmatic symptoms in the first year of life (45). A study by Savilahti et al. showed that low cow’s milk specific IgA levels in the colostrum associated with a significant risk of developing atopic symptoms and IgE-sensitization at the age of 4 years if the infant was exclusively breastfed for longer than 3.5 months (44). Interestingly, we observed that long breastfeeding was associated with an increased risk of asthma in children of mothers with allergic history. Matheson et al. also reported of studies where
breastfeeding was associated with an increased risk of asthma later in childhood (40), as discussed earlier in this thesis. Nevertheless, there are studies showing prolonged breastfeeding to be either protective or a risk factor of AD (39,167). We did not see any associations between TGF-β1 and atopic outcomes in the child which can be due to the low concentrations of breast milk TGF-β1 in this cohort (168).

Mothers who smoked during pregnancy had increased levels of breast milk sIgA and TGF-β1. Cigarette smoke contains toxins and microbial cell components, which can promote inflammation at mucosal surfaces, promote airway remodeling, and influence immunity through unknown mechanisms. It has been suggested that cigarette smoke promotes allergic inflammation by enhancing the activation of DCs, which transport inhaled antigens to lymph nodes (169). In addition, smokers may have more infections (170), which can contribute to the increased microbial load, thereby increasing the levels of sIgA in breast milk. Similar mechanisms might be behind the increased levels of breast milk TGF-β1 in mothers who smoke.

We found that elevated levels of sIgA or TGF-β1 in breast milk indicated a short duration of breastfeeding, which has previously been reported in colostrum by Savilahti et al. (171). Animal studies have shown bacteria-induced mastitis to increase the levels of TGF-β1 in breast milk (172,173). TGF-β has also been shown to regulate mammary gland development by inhibiting alveolar and ductal development in breast tissue (174). Mastitis is often caused by Staphylococci. The subclinical infection is common in dairy cows, with estimated prevalence of 5.5-
27.1% (175). Acute mastitis is reported to occur approximately in 10% of breastfeeding mothers (176). As the subclinical mastitis can occur without symptoms, the prevalence in humans is not well known. In the present study, the increased TGF-β1 and sIgA levels in breast milk can indicate microbial stimulus and the association with short breastfeeding may be due to subclinical inflammation in the breast tissue. Interestingly, an inverse association of sIgA and AD was observed regardless of the simultaneous association of elevated sIgA and shorter duration of breastfeeding.

Our results suggest that high sIgA levels in breast milk reduce the risk of developing AD at early age and, therefore support the protective effects of breastfeeding on atopic diseases and emphasize that the effect is dependent on breast milk composition.

5.3 Early-age antibody response to food antigens associates with IgE-sensitization later in life (Study III)

Dietary antigen exposure is necessary for the development of a functional immune response and the development of oral tolerance in the gut. Intestinal permeability is higher in infants when compared to adults and children, which can be necessary for the development of tolerance. Excessive intestinal permeability or inflammation may interfere with the development of oral tolerance and lead to increased antibody response. Cow's milk, hen’s egg, and wheat are among the most common allergens in children. We used serum IgA and IgG antibodies against cow’s milk
BLG and wheat gliadin (at the age of 1 year) as markers of mucosal tolerance development and studied their association with environmental factors, IgE-sensitization, and the development of asthma and AD.

Our results show that increased levels of IgA or IgG antibodies against BLG were associated with IgE-sensitization at 1 year of age (Table 3). Children who had IgA antibody concentrations against BLG in the highest quartile or who had increased IgG antibodies against BLG were at a significantly increased risk of being sensitized to at least one of the measured allergens (Fig. 6 A and B) and food allergens at the age of 6 year (Table 4).

Increased levels of IgA or IgG antibodies against gliadin were associated with IgE-sensitization at the age of 1 year (Table 3). Children who had increased IgG antibody levels against gliadin were at a higher risk of being sensitized to at least one of the allergens (Fig. 6 C), inhalant allergens (IgG in Q4: aOR 95% CI: 2.15 (1.16–3.98)), and food allergens at the age of 6 years (Table 4).

We did not find consistent associations between the antibody levels and asthma or AD. No association with hs-CRP at the age of 1 year and IgA or IgG against BLG or gliadin was found in 640 sample pairs (data not shown).
Figure 6. A) The association of IgA levels against cow’s milk β-lactoglobulin at 1 year of age and IgE-sensitization to any allergen at 6 years of age. B) The levels of IgG against β-lactoglobulin at 1 year of age and the risk of IgE-sensitization to any allergen at 6 years of age. C) The levels of IgG against gliadin at 1 year of age and IgE-sensitization to any allergen at 6 years of age. Antibody levels expressed as quartiles, Q4 being the highest.
Table 3. Association of IgA and IgG antibodies against milk β-lactoglobulin or wheat gliadin at the age of 1 year and IgE-sensitization at the age of 1 year.

<table>
<thead>
<tr>
<th>IgA (BLG)</th>
<th>Sensitization to at least one allergen at age 1y N(%)</th>
<th>aOR (95% CI)$^1$</th>
<th>IgG (BLG)</th>
<th>Sensitization to at least one allergen at age 1y N(%)</th>
<th>aOR (95% CI)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>29 (18.2)</td>
<td>1</td>
<td>Q1</td>
<td>36 (22.6)</td>
<td>1</td>
</tr>
<tr>
<td>Q2</td>
<td>44 (27.5)</td>
<td><strong>1.80 (1.03-3.16)</strong></td>
<td>Q2</td>
<td>38 (23.8)</td>
<td>1.09 (0.63-1.87)</td>
</tr>
<tr>
<td>Q3</td>
<td>47 (29.4)</td>
<td><strong>2.28 (1.28-4.06)</strong></td>
<td>Q3</td>
<td>43 (27.0)</td>
<td>1.38 (0.79-2.40)</td>
</tr>
<tr>
<td>Q4</td>
<td>56 (35.2)</td>
<td><strong>2.97 (1.64-5.37)</strong></td>
<td>Q4</td>
<td>59 (36.9)</td>
<td><strong>2.36 (1.33-4.19)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IgA (Gliadin)</th>
<th>Sensitization to at least one allergen at age 1y N(%)</th>
<th>IgG (Gliadin)</th>
<th>Sensitization to at least one allergen at age 1y N(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1+Q2</td>
<td>74 (22.8)</td>
<td>Q1</td>
<td>35 (22.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q2</td>
<td>40 (25.2)</td>
</tr>
<tr>
<td>Q3</td>
<td>45 (29.4)</td>
<td>Q3</td>
<td>42 (26.4)</td>
</tr>
<tr>
<td>Q4</td>
<td>56 (35.4)</td>
<td>Q4</td>
<td>58 (36.7)</td>
</tr>
</tbody>
</table>
Table 4. Predictive value of IgA and IgG antibodies against milk β-lactoglobulin or wheat gliadin at the age of 1 year and IgE-sensitization to at least one food allergen at the age of 6 years.

<table>
<thead>
<tr>
<th>IgA (BLG) Sensitization to at least food allergen at age 6y N(%)</th>
<th>aOR (95% CI)</th>
<th>IgG (BLG) Sensitization to at least one food allergen at age 6y N(%)</th>
<th>aOR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1 31 (24.8)</td>
<td>1</td>
<td>Q1 26 (22.0)</td>
<td>1</td>
</tr>
<tr>
<td>Q2 37 (30.8) 1.58 (0.85-2.91)</td>
<td>Q2 42 (34.7) 2.07 (1.12-3.82)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q3 38 (34.2) 1.71 (0.90-3.25)</td>
<td>Q3 35 (31.8) 1.95 (1.00-3.78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q4 51 (49.5) 3.64 (1.84-7.17)</td>
<td>Q4 54 (49.1) 4.85 (2.41-9.76)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3 and 4: Statistically significant adjusted ORs (aOR) are marked in bold.

Results are presented as odds ratios (OR) and their 95% confidence intervals (CI). ORs are adjusted for farmer, center, gender, maternal smoking during pregnancy, breastfeeding, farm milk consumption, pets at home, mother's history of allergic disease, atopy at the age of 1 year, and age of formula milk introduction.
Specific immunoglobulin E (sIgE) was measured against 19 common allergens. There were some possible weaknesses in the test panel. Some allergens may not be feasible for the screening of atopy since the level of exposure varies between persons, i.e. farmers’ children have more contact to cows when compared to children who live in towns. Specific IgE against cow was not measured in this study. However, IgE against horse was measured in the test panel even though horse is a common farm animal. In addition, variations in the quantity of certain allergens, such as plant pollen and house dust mite, in different parts of Europe might also affect the findings. It has been suggested that epidemiological studies should use allergens that all of the study subjects are exposed to in order to measure the genetic predisposition to develop IgE-sensitization (177). Nevertheless, the panel of tested allergens was large and the country of the child was taken into account in adjusted analyses. In addition to BLG and gliadin, it would have been interesting to measure IgA and IgG antibodies against hen’s egg ovalbumin, which is a common food allergen in children.

The levels of IgA and IgG antibodies against BLG varied between the study centers (P<0.001). Children who were introduced to formula milk before 14.6 weeks had elevated levels of IgA or IgG against BLG at the age of 1 year. (Table 5). The infants who were breastfed for longer than 6 months tended to have lower IgA and IgG antibody levels against BLG than children who were not breastfed or who were breastfed for less than 6 months. A farming environment did not show association with antibody levels. If the child consumed unboiled farm milk, the family had cats or
dogs at home, or if the mother smoked during pregnancy, there was a tendency of association with increased levels of IgA and IgG antibodies against BLG.

There are discrepant findings on breast milk promoting gut barrier maturation (178,179). Breastfeeding may affect tolerance induction in the infant by delivering antibodies and antigens derived from the maternal diet. Long breastfeeding can lead to decreased antibody production against BLG either by improving gut maturation or by postponing introduction of cow's milk to the diet. Interestingly, children who consumed formula milk during their first year of life had lower levels of IgA against gliadin when compared to children who did not receive formula milk (Table 6). This may be due to differences in the composition of breast milk and formula milk.

There is evidence that increased intestinal permeability can result from allergic inflammation in the gut (180,181). It has also been shown that altered intestinal permeability can remain while the patient is on an elimination diet, and that the symptoms correlate with measured gut permeability (182). It is possible that environmental factors affect gut permeability by causing intestinal inflammation, which can lead to food allergies in people at high risk of allergies. Therefore, gut permeability would not be the primary cause of allergies (183). Animal models have shown that increased intestinal permeability can lead to increased antigen uptake and IgE-sensitization (184). Therefore, our reported association of increased IgA and IgG antibody response to gliadin and BLG with IgE-
sensitization can be an indication of increased intestinal inflammation and permeability.

Table 5. Association between serum IgA and IgG antibodies against milk β-lactoglobulin at the age of 1 year and the age of formula introduction to diet.

<table>
<thead>
<tr>
<th>Age of formula introduction to diet (wk)</th>
<th>N</th>
<th>IgA (AU) (BLG)</th>
<th>IgA in Q4</th>
<th>aOR (95% CI)</th>
<th>N</th>
<th>IgG (AU) (BLG)</th>
<th>IgG in Q4</th>
<th>aOR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No milk formula</td>
<td>48</td>
<td>10.60 (2.50–16.60)</td>
<td>4 (8.3)</td>
<td>1</td>
<td>48</td>
<td>46.76 (29.93–88.31)</td>
<td>5 (10.4)</td>
<td>1</td>
</tr>
<tr>
<td>&lt;14.6</td>
<td>280</td>
<td>36.66 (9.87–90.03)</td>
<td>106 (37.9)</td>
<td>5.16 (1.65–16.12)</td>
<td>280</td>
<td>325.35 (103.83–747.59)</td>
<td>112 (40.0)</td>
<td>3.68 (1.27–10.68)</td>
</tr>
<tr>
<td>14.6 ≤ and &lt;22.1</td>
<td>112</td>
<td>14.79 (4.20–49.92)</td>
<td>24 (21.4)</td>
<td>2.96 (0.94–9.29)</td>
<td>112</td>
<td>145.70 (50.65–298.57)</td>
<td>17 (15.2)</td>
<td>1.32 (0.44–4.02)</td>
</tr>
<tr>
<td>≥22.1</td>
<td>191</td>
<td>7.87 (3.90–27.80)</td>
<td>23 (12.0)</td>
<td>1.41 (0.45–4.37)</td>
<td>191</td>
<td>72.86 (31.51–230.00)</td>
<td>24 (12.6)</td>
<td>1.16 (0.40–3.33)</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Association between serum IgA and IgG antibodies against wheat gliadin at the age of 1 year and the age of formula introduction to diet.

<table>
<thead>
<tr>
<th>Age of formula introduction to diet (wk)</th>
<th>N</th>
<th>IgA (AU) (BLG)</th>
<th>IgA in Q4</th>
<th>aOR (95% CI)</th>
<th>N</th>
<th>IgG (AU) (BLG)</th>
<th>IgG in Q4</th>
<th>aOR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No milk formula</td>
<td>48</td>
<td>10.60 (2.50–43.41)</td>
<td>4 (8.3)</td>
<td>1</td>
<td>48</td>
<td>46.76 (29.93–88.31)</td>
<td>5 (10.4)</td>
<td>1</td>
</tr>
<tr>
<td>&lt;14.6</td>
<td>279</td>
<td>10.00 (10.00–18.55)</td>
<td>60 (21.5)</td>
<td>0.39 (0.18–0.86)</td>
<td>279</td>
<td>108.27 (22.68–227.16)</td>
<td>65 (23.3)</td>
<td>0.86 (0.39–1.92)</td>
</tr>
<tr>
<td>14.6 ≤ and &lt;22.1</td>
<td>112</td>
<td>10.00 (10.00–19.19)</td>
<td>24 (21.4)</td>
<td>0.36 (0.17–0.78)</td>
<td>112</td>
<td>94.79 (28.96–226.64)</td>
<td>26 (23.2)</td>
<td>0.78 (0.36–1.72)</td>
</tr>
<tr>
<td>≥22.1</td>
<td>191</td>
<td>10.00 (10.00–22.70)</td>
<td>51 (26.8)</td>
<td>0.46 (0.23–0.90)</td>
<td>191</td>
<td>86.68 (26.80–275.85)</td>
<td>49 (25.8)</td>
<td>0.79 (0.38–1.62)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.032</td>
<td>0.905</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Tables 5 and 6: Statistically significant adjusted ORs (aOR) are marked in bold. Results are presented with median values and interquartile ranges. P-values are estimated by Kruskal–Wallis test. P-values are estimated by Pearson chi-square test. IgA and IgG values are presented in two groups according to their 75th cut-off points (Q4), and comparison between two groups are shown values as ORs and their 95% confidence intervals (CI). Odds ratios are adjusted for farmer, center, gender, maternal smoking during pregnancy, breastfeeding, farm milk consumption, pets at home, and age of formula introduction.
We were not able to measure gut permeability in children who were 1 year of age. Therefore, we cannot correlate antibody levels and gut permeability in this cohort. However, we reported that having pets at home and drinking of unboiled milk were associated with increased levels of dietary antibodies. Unboiled milk and animal contact associate with high environmental bacterial load, which can indicate that intestinal microbes enhance the antibody response.

IgG antibodies against foods have been shown to associate with IgE antibody development against inhalant allergens in children with and without allergy risk (185,186). Our results are in agreement with these previous studies and show that enhanced antibody response to cow's milk or wheat antigens can reflect altered intestinal immune response, which associates with later sensitization to allergens. The findings in our study suggest that mucosal antibody response in early life and IgE-sensitization later in childhood are regulated by similar factors such as gut microbiota and/or permeability. Since we did not see associations with mucosal antibody response and allergic diseases later in life, it seems that other factors are more significant in regulating the development of clinical allergy.

5.4 High level of fecal calprotectin at 2 months as a marker of intestinal inflammation predicts atopic dermatitis and asthma by age 6 (Study IV).

Alterations in gut microbiota and intestinal inflammation have been linked to immune-mediated diseases (187). We used fecal calprotectin as
a marker of early intestinal inflammation. We observed an association between fecal calprotectin and asthma or AD development by the age of 6 years. There were 758 fecal samples from 2 month old children available for the measurement of fecal calprotectin. The composition of fecal microbiota using 16S rRNA pyrosequencing was analyzed in 120 infants. In addition, the effect of *E. coli* LPS on the anti-inflammatory response in human monocytes was also studied in three adults outside the PASTURE cohort.

Farmers’ children had higher fecal calprotectin levels when compared to non-farmer’s children (P=0.003). Also, increased levels of fecal calprotectin were found in children with one or more siblings when compared to the children with no siblings (P<0.001), and in breastfed children (exclusively and partially breastfed) when compared to non-breastfed children (P<0.001). Infants of non-smoking mothers had higher levels of fecal calprotectin when compared to the infants of mothers who smoked during pregnancy or who abstained from smoking during pregnancy (P=0.003). Because fecal calprotectin was associated with breastfeeding, we measured the levels of calprotectin in breast milk samples from 115 Finnish mothers. There was no correlation between levels of calprotectin in breast milk and feces (R=0.104 and P-value 0.271).

Since the distribution of fecal calprotectin levels was skewed, we studied the importance of very high fecal calprotectin levels (above the 90\textsuperscript{th} percentile, i.e. 517.6 µg/g), which indicate a high degree of intestinal inflammation. The children who had very high fecal calprotectin levels
were at an increased risk of developing AD and asthma/asthmatic bronchitis by the age of 6 years when compared to the children who had fecal calprotectin levels below the 90\textsuperscript{th} percentile (Table 7).

We analyzed the composition of the gut microbiota in a selected subgroup of children with high fecal calprotectin (above the 90\textsuperscript{th} percentile) and lower fecal calprotectin (below the 90\textsuperscript{th} percentile) in order to study the associations of fecal microbiota and fecal calprotectin. The selection criteria are described in the study methods. Infants with very high fecal calprotectin levels (above the 90\textsuperscript{th} percentile) had a significantly lower percentage of \textit{Escherichia} in their fecal microbiota when compared to infants with fecal calprotectin levels below the 90\textsuperscript{th} percentile (P=0.019) (Figure 8). Children with fecal calprotectin levels below 200µg/g had significantly lower \textit{Lactobacillaceae} numbers than children with fecal calprotectin levels in between 200µg/g and the 90\textsuperscript{th} percentile (517µg/g) (P=0.016) or than children with fecal calprotectin above the 90\textsuperscript{th} percentile (P=0.011). However, the amount of \textit{Lactobacillaceae} did not significantly associate with the very high levels of fecal calprotectin (the 90\textsuperscript{th} percentile) when compared to fecal calprotectin values below the 90\textsuperscript{th} percentile (P=0.125) (Figure 9).
Table 7. Predictive value of fecal calprotectin at 2 months of age and development of atopic dermatitis, asthma/asthmatic bronchitis, and IgE-sensitization by the age of 6 years.

<table>
<thead>
<tr>
<th>Fecal calprotectin (percentile)</th>
<th>Atopic dermatitis N (%)</th>
<th>aOR (95% CI)</th>
<th>Asthma/asthmatic bronchitis N (%)</th>
<th>aOR (95% CI)</th>
<th>Sensitization to at least one allergen at age 6y N (%)</th>
<th>aOR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;90</td>
<td>125</td>
<td>1</td>
<td>106</td>
<td>1</td>
<td>217</td>
<td>1</td>
</tr>
<tr>
<td>&gt;90</td>
<td>19</td>
<td>2.02 (1.06-3.85)</td>
<td>18</td>
<td>2.41 (1.25-4.64)</td>
<td>29</td>
<td>1.50 (0.81-2.77)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.066</td>
<td>0.033</td>
<td>0.022</td>
<td>0.009</td>
<td>0.182</td>
<td>0.197</td>
</tr>
</tbody>
</table>
Figure 8. The association between fecal calprotectin concentrations and the abundance of *Escherichia* in feces. Both parameters were measured at 2 months of age.

Figure 9. The association between fecal calprotectin concentrations and the abundance of *Lactobacilliceae* in feces. Both parameters were measured at 2 months of age.
The percentage of IL-10 secreting cells from monocytes was increased after stimulation with LPS from *E. coli* (Figure 10). In person 1, the frequency of IL-10 secreting cells from monocytes was 3.7 times higher after LPS stimulation; in person 2, 6.9 times higher; and in person 3, 2.7 times higher when compared to the non-stimulated control sample.

![Figure 10. The effect of *E. coli* derived LPS on the percentage of IL-10 secreting cells in 3 adult blood donors.](image)

Although there was no linear association of fecal calprotectin levels and allergic disease, we found remarkably high levels of fecal calprotectin to predict asthma and AD by the age of 6 years. These results indicate long term effects of early gut immune system alterations on allergic diseases. Our results suggest that a farming environment induces intestinal inflammation, which can be caused by increased environmental microbial load. However, the very high fecal calprotectin levels were not explained by farming. Therefore, our finding does not contradict studies that show farming to be protective against allergies. In addition, we showed that fecal calprotectin did not directly derive from breast milk. Yet, the
children who were exclusively breastfed had the highest levels of fecal calprotectin, which implies that breastfeeding leads to a higher microbial exposure than bottlefeeding. Breast milk bacteria (188) may lead to increased fecal calprotectin levels by inducing neutrophil infiltration due to the stimulation of the gut immune system. The growth of intestinal microbes can also be modulated by breast milk factors such as oligosaccharides.

Gut permeability and fecal calprotectin levels are high in infants. Until recently, there have not been established cut-off levels to define elevated fecal calprotectin levels as a marker of disease in infants and young children. In 2014, Oord et al. (189) defined three cut-off levels based on the 97.5% percentiles of fecal calprotectin in different age groups: 538 µg/g (1 < 6 months), 214 µg/g (6 months < 3 years) and 75 µg/g (3 < 4 years). The 90th percentile cut-off (517.6 µg/g) used in our study for 2 month old infants is comparable to the limits defined by Oord et al. A reference value of 50 µg/g calprotectin in feces is used for children from 4 to 17 years of age and healthy adults in commercial ELISA tests (190). In un-published data, we found that the PASTURE cohort children had significantly lower levels of fecal calprotectin at 1 year of age when compared to the levels at the age of 2 months. Other studies have shown similar results (104,108).

In Study IV, the distribution of fecal calprotectin levels was skewed and some of the infants had very high levels of calprotectin without a known history of intestinal disease. Therefore, we decided to use the 90th percentile cut-off, and found an association between high fecal
calprotectin and elevated risk of developing asthma/asthmatic bronchitis and AD by the age of 6 years.

The gut microbiota findings in Study IV suggest down-regulation of intestinal inflammation in infants who are colonized with *Escherichia*. Continuous exposure to LPS has been shown to cause tolerance and down-regulation of the TLR-4 signaling pathway (191). Therefore, the early colonization of the gut with *Escherichia* may result in a down-regulation of TLR-4 signaling and alleviation of the inflammatory response, which was detected as lower levels of fecal calprotectin in our study. TLR-4 is also expressed by Tregs (192), and early colonization with *Escherichia* could alleviate inflammation via the initiation of regulatory mechanisms. In addition, several studies demonstrate that TLR-4 signaling attenuates allergic diseases and asthma (193-195). Our findings in three adult blood donors showed that the production of IL-10 in monocytes was triggered by *E. coli* derived LPS and are in agreement with previous studies (196). Thus, LPS induced IL-10 production could offer an explanation for the decreased intestinal inflammation associated with early *Escherichia* colonization in Study IV.

We reported that lower fecal calprotectin levels in infants were associated with low numbers of *Lactobacillaceae*, which implies that early colonization with *Lactobacillaceae* contributes to the levels of intestinal inflammation. Lactobacilli have been shown to induce the pro-inflammatory IL-12 and IFN-γ production in murine DC cultures (197) and in human blood mononuclear cells (198,199). In addition, some Lactobacilli can produce hydrogen-peroxide which may contribute to the
induction of intestinal inflammation (200). Although it has been shown that the low-grade inflammation caused by a mixture of probiotics (including *L. rhamnosus* GG and *L. rhamnosus* LC705) could be beneficial against allergies (70), we observed an association between early high degree intestinal inflammation and later development of asthma. However, we did not find the fecal calprotectin values above the 90th percentile to significantly associate with *Lactobacillaceae* abundances, suggesting that *Lactobacillaceae* alone do not cause the very high inflammation.

Our findings suggest that early intestinal inflammation can indicate both skin related inflammation and respiratory tract related inflammation later in life. The colonization pattern appeared to be an important regulator of the intestinal inflammation. Our finding that early colonization with *Escherichia* alleviated the intestinal inflammation may have implications for the design of probiotic treatments for infants. Our findings also suggest that early colonization is associated with long-term health effects.
6 CONCLUSIONS

The following conclusion can be drawn from the results presented in this thesis:

I. The findings suggest that low-grade inflammation at an early age associates with decreased allergic sensitization later in life and that poor inflammatory response could predispose for IgE-sensitization.

II. The study showed an inverse association between breast milk sIgA levels and the risk of AD later in childhood. The results support the protective effects of breastfeeding on AD development and suggest that the effect is dependent on breast milk composition.

III. The results suggest that enhanced antibody responses to wheat or cow’s milk antigens reflect aberrancies in mucosal tolerance such as altered microbiota and/or increased gut permeability, which is later seen as sensitization to allergens.

IV. The present study showed that very high intestinal inflammation in infancy is a risk factor for the development of allergic diseases later in life. The results also suggest that early intestinal colonization regulates intestinal inflammation.

Children who live in a farming environment are generally exposed to a higher number and diversity of environmental microbes, and they have been reported to have fewer allergies. The results presented in this thesis
showed that farmers’ children had increased levels of fecal calprotectin indicating increased intestinal inflammation. Although a farm environment did not constantly associate with altered immune response in the present studies, we reported several associations between factors reflecting elevated environmental microbial load and altered immune response in the child or in the mother. In addition, low-grade inflammation was shown to have a protective role against IgE-sensitization.

The findings presented in this thesis suggest that gut alterations and bacterial colonization play a significant role in the development of the immune system. Our study provides new a perspective on allergy-development by showing that very high intestinal inflammation in early infancy associates with increased risk of allergic diseases. These results contribute to the growing understanding of the interaction between early life microbial exposure and immune system developments. Further developments in this field might shed new light on potential avenues for the prevention or treatment of allergic diseases.
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Helsinki, July 2015

Laura Orivuori
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