

Kirsi Mustonen

The role of inflammation and its environmental triggers in allergic diseases



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To my husband

Abstract

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Systemic low-grade inflammation, typically defined as the increased production of C-reactive protein (CRP) below the levels seen in clinical infections, plays a role not only in the immune-mediated diseases, but also in other chronic diseases, such as in atherosclerosis and in metabolic syndrome, obesity and asthma. On the other hand, low-grade inflammation may also be beneficial and contribute to the development of healthy immune system as suggested by the studies showing that low-grade inflammation may protect from allergic diseases. Farming environment in early life has been suggested to protect from atopy and allergic diseases but the mechanisms behind the protective effect are still unrevealed. Microbial exposure in our environment, e.g. non-pathogenic microbes in the farming environment, could cause detectable systemic low-grade inflammation. On the other hand, moisture damage and mould in buildings are associated with adverse health effects, but again the mediators of the harmful effects are not known yet.

The main aim of this thesis was to investigate if there is an association between systemic low-grade inflammation and the development of allergic diseases in children and could environmental exposures cause systemic low-grade inflammation in children. In addition, other markers of inflammation were explored.

The research was based on two birth cohorts, the international PASTURE study and the Finnish LUKAS study. Inflammatory markers were collected via blood or faecal samples at the ages of two months, one, 4.5 and six years and the data on the children's health, confounding factors and potential environmental microbial and farming exposures were collected by questionnaires up to the age of six years.

Among the children who were non-atopic at the age of one, the risk of atopy at the age of 4.5 years was significantly lower among those with a concentration of CRP in the highest quartile (aOR 0.48, 95 % CI 0.24–0.95) when compared with those with CRP in the lowest quartile. There were no associations of CRP values with farming-related environmental factors.

When faecal calprotectin levels were used as a marker of intestinal inflammation, the children who had very high calprotectin levels at the age of two months (in the 90th percentile) had an increased risk of developing atopic dermatitis (aOR 2.02, 95 % CI 1.06–3.85) and asthma/asthmatic bronchitis (aOR 2.41, 95 % CI 1.25–4.64) by the age of six years when compared to the children who had calprotectin levels below the 90th percentile.

Major moisture damage in the child's main living areas was associated with high levels of CRP, enhanced interleukin (IL)-1 β response in phorbol 12-myristate 13-

acetate and ionomycin (PI) stimulated whole blood and increased secretion of combined levels of IL-1 β , tumor necrosis factor (TNF)- α and IL-6 cytokines in lipopolysaccharide (LPS) stimulated whole blood at the age of six years. Also mould in the bathroom showed similar associations.

In conclusion, the results suggest that increased CRP may protect from atopic deviation among non-atopic children. In the present study farming environment did not significantly induce systemic low-grade inflammation but associations were observed with moisture damage in home. In contrast to the low-grade inflammation, a high degree of intestinal inflammation during the first months of life increased the risk of asthma and atopic dermatitis, which indicates a long term effect of early changes in the gut immune system on the development of allergic diseases. Taken together, the results support the importance of systemic low-grade inflammation in the development of allergic diseases.

Keywords: Asthma, atopic dermatitis, atopy, calprotectin, children, cohort, CRP, cytokines, environmental factors, exposure, IL-1 β , IL-6, inflammation, low-grade inflammation, TNF- α , specific IgE

Tiivistelmä

Kirsi Mustonen, The role of inflammation and its environmental triggers in allergic diseases [Tulehduksen merkitys ja ympäristötekijöiden aiheuttamana tulehdus allergisten sairauksien kehittymisessä]. Terveyden ja hyvinvoinnin laitos. Tutkimus 140. 258 sivua. Kuopio, Finland 2014.

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Elimistön matala-asteinen tulehdus, joka tyypillisesti määritellään kohonneeksi C-reaktiiviseksi proteiinipitoisuudeksi (CRP) joka on kuitenkin matalampi kuin infektio-tauteissa, on yhdistetty immuunivälitteisiin sairauksiin, mutta myös muihin kroonisiin sairauksiin, kuten valtimokovettumatautiin, metaboliseen oireyhtymään, liikalihavuuteen ja astmaan. Matala-asteinen tulehdus voi toisaalta olla myös hyödyllinen ja edistää terveen immuunijärjestelmän kehittymistä, kuten on esitetty aikaisemmissa tutkimuksissa, joissa on osoitettu, että matala-asteinen tulehdus voi suojata allergisilta sairauksilta. Aikaisemmissa tutkimuksissa on ehdotettu, että maatilaympäristö suojaa atopialta ja allergisilta sairauksilta varhaislapsuudessa mutta mekanismit suojaavan vaikutuksen takana ovat yhä tuntemattomia. Mikrobialtistus ympäristössämme, kuten esimerkiksi ei-patogeeniset mikrobit maatilaympäristössä, voivat aiheuttaa matala-asteista tulehdusta. Toisaalta kosteusvauriot ja home on yhdistetty haitallisiin terveysvaikutuksiin, mutta näitä välittävät tekijät ovat yhä löytämättä.

Tämän väitöskirjan tarkoituksena oli tutkia onko elimistön matala-asteisella tulehduksella yhteyttä allergisten sairauksien syntyyn lapsuudessa ja voivatko ympäristöaltisteet aiheuttaa elimistön matala-asteista tulehdusta lapsilla. Myös muita tulehduksen merkkiaineita tutkittiin.

Tutkimus perustui kahteen syntymäkohorttiin: kansainväliseen PASTURE-tutkimukseen ja suomalaiseen LUKAS-tutkimukseen. Tulehduksen merkkiaineet kerättiin veri- tai ulostenäytteillä kahden kuukauden, yhden, 4,5 ja kuuden vuoden iässä. Aineisto lasten terveydentilasta, sekoittavista tekijöistä ja ympäristön mikrobi- ja maatala-altisteista kerättiin kyselylomakkeilla kuuden ikävuoden aikana.

Kun lapsella ei ollut atopiaa yhden vuoden iässä, atopian riski 4,5 vuoden iässä oli pienempi lapsilla, joilla CRP-pitoisuus oli korkeimmassa neljänneksessä (vOR 0.48, 95 % LV 0.24–0.95), kuin niillä, joilla CRP-pitoisuus oli matalimmassa neljänneksessä. CRP-tasoilla ja maatala-altistukseen liitettyillä ympäristöaltisteilla ei ollut tilastollisesti merkitsevää yhteyttä.

Kun ulosteen kalprotektiinitasoa käytettiin suoliston tulehduksen osoittajana, havaittiin että lapsilla, joilla oli korkea kalprotektiinipitoisuus (korkeampi kuin 90. prosenttipiste) oli lisääntynyt riski sairastua kuuteen ikävuoteen mennessä atooppisen ihottumaan (vOR 2.02, 95 % LV 1.06–3.85) ja astmaan/ahtauttavaan keuhkoputkentulehdukseen (vOR 2.41, 95 % LV 1.25–4.64) verrattuna lapsiin, joiden kalprotektiinipitoisuudet olivat alle 90. prosenttipisteen.

Merkittävä kosteusvaurio lapsen pääasiallisissa asuintiloissa oli yhteydessä korkeisiin CRP-pitoisuuksiin, lisääntyneeseen interleukiini (IL)-1 β vasteeseen PI-stimuloidussa veressä ja lisääntyneeseen IL-1 β , tuumorinekroositekijä (TNF)- α ja IL-6 sytokiinien yhdistelmämuuttujan pitoisuuksiin LPS-stimuloidussa veressä kuuden vuoden iässä. Myös kylpyhuoneen homeella oli vastaavia yhteyksiä sytokiinipitoisuuksien kanssa.

Tutkimustulokset viittaavat siihen, että koholla oleva CRP-pitoisuus voi suojata atopialta ei-atoppisilla lapsilla. Tässä tutkimuksessa maatilaympäristö ei aiheuttanut elimistön matala-asteista tulehdusta merkittävästi, mutta matala-asteinen tulehdus oli yhteydessä kodissa todettuun kosteusvaurioon. Sitä vastoin korkea-asteinen suolistotulehdus ensimmäisten elinkuukausien aikana lisäsi riskiä sairastua astmaan ja atooppiseen ihottumaan, mikä viittaa siihen että varhaisilla muutoksilla suolen immuunijärjestelmässä on pitkäkestoisia vaikutuksia allergisten sairauksien kehitymisessä. Yhteenvetona voidaan todeta, että tulokset tukevat käsitystä elimistön matala-asteisen tulehduksen tärkeästä merkityksestä allergisten sairauksien kehityksessä.

Avainsanat: Altiste, astma, atooppinen ihottuma, atopia, CRP, IL-1 β , IL-6, kalprotektiini, kohortti, lapset, matala-asteinen tulehdus, sytokiinit, spesifinen IgE, TNF- α , tulehdus, ympäristötekijät

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List of original papers

- I Mustonen K, Keski-Nisula L, Vaarala O, Pfefferle PI, Renz H, Riedler J, Dalphin JC, Buechele G, Lauener R, Braun-Fahrländer C, von Mutius E, Pekkanen J and the PASTURE Study Group. Few associations between high-sensitivity C-reactive protein and environmental factors in 4.5-year-old children. *Pediatric Allergy and Immunology*. 2012; 23 (6): 522–528.
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- IV Orivuori L*, Mustonen K*, Goffau M, Hakala S, Paasela M, Braun-Fahrländer C, Dalphin J-C, Genuneit J, Lauener R, Riedler J, Weber J, von Mutius E, Pekkanen J, Harmsen H, Vaarala O & the PASTURE Study Group. Increased fecal calprotectin at age 2 months associates with early colonization and predicts atopic dermatitis and asthma by age 6. *Submitted*. * Authors had equal contribution to this study.

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Abbreviations

AD	Atopic dermatitis
aOR	Adjusted odds ratio
BMI	Body mass index
CI	Confidence interval
CRP	C-reactive protein
FeNO	Fractional exhaled nitric oxide
IL	Interleukin
IFN- γ	Interferon gamma
kU/L	Kilo units per litre
LPS	Lipopolysaccharide
OR	Odds ratio
PASTURE	Protection against Allergy Study in Rural Environments
PI	Phorbol myristate acetate and ionomycin
PPG	Peptidoglycan
PRR	Pattern recognition receptor
sIgE	Specific immunoglobulin E
Th1	T-helper type 1 cell
Th2	T-helper type 2 cell
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor α
Treg	Regulatory T cell

1 INTRODUCTION

Inflammation is a key feature in many diseases such as cardiometabolic diseases (Emerging Risk Factors Collaboration 2012, Zhu et al. 2000) and chronic noncommunicable diseases such as autoimmune diseases and asthma (Kim et al. 2011, Prescott 2013). It is possible that some chronic inflammatory diseases may be due to dysfunctions in the regulation of the inflammatory response (Naik and Wala 2013). On the other hand, it has been suggested that low-grade inflammation may protect individuals from allergic diseases (Marschan et al. 2008, Viljanen et al. 2005).

The hygiene hypothesis was introduced in 1989. The original hypothesis claimed that the increase in the prevalence of allergic diseases during childhood might be due to declining family sizes, improvement in household amenities and higher standards of personal cleanliness, leading to fewer incidents of clinical and subclinical infections (Strachan 1989). Later, the focus of the hygiene hypothesis has been shifted towards contact with non-pathogenic microbes in early life rather than clinical infections (Martinez 2001).

There are different microbial agents in our environment that can cause systemically or locally measurable inflammation, e.g. microbes in the farming environment, which has been shown to protect from allergic diseases (Ege et al. 2011). Also the microbiota of the gastrointestinal tract is an important regulator of inflammation and immune responses. Gut immune system is a major part of the immune system. It has been newly discovered that disturbances in gut microbiota are associated with the development of allergic diseases (West 2014). On the other hand, moisture damage or mould in buildings can cause asthma (Cox-Ganser et al. 2009, Jaakkola et al. 2005, Park et al. 2008, Pekkanen et al. 2007).

Mechanisms behind inflammatory responses and the nature of the inflammation induced by these different microbial and other stimuli are still unknown and more research is needed to understand these associations. There might also be exposures which cause downregulation of inflammation and thus cause health effects.

In addition, there is limited amount of information on the role of inflammation and the environmental exposures causing inflammation in the development of allergic diseases in children.

2 REVIEW OF THE LITERATURE

2.1 Inflammation

The innate immune system deals infections, tissue injuries or stress by stimulating local and/or systemic inflammation (Medzhitov 2008). Inflammation is a process characterized by the recruitment of leucocytes and plasma proteins from the blood into tissues where the inflammatory response is activated, in order to defend the host against infections, repair tissue and adapt to stress and restore a homeostatic state (Medzhitov 2008). A persistent or aberrant dysregulated inflammation tends to initiate tissue destruction rather than tissue restoration.

Several factors are involved in an inflammatory process: cellular components (mast cells, eosinophils, basophils, lymphocytes, dendritic cells), inflammatory mediators (cytokines, chemokines, lipid mediators), and structural cells (epithelial cells, airway smooth muscle cells, keratinocytes, endothelial cells, mucus-secreting cells). The nature of the inflammatory response is determined by the profile of the inflammatory mediators and cellular components involved. As an example, the secretion of interleukin (IL) -4, IL-5, IL-9 and IL-13 cytokines by T-helper 2 (Th2) lymphocytes and proinflammatory IL-1 β , IL-6, tumor necrosis factor- α (TNF- α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokines secreted by a variety of cells are the key factors mediating inflammation in allergic reactions (Barnes 2011).

C-reactive protein (CRP) is a marker of systemic inflammation in the plasma produced by liver after the stimulation of cytokines IL-6, IL-1 β and TNF- α which are produced by dendritic cells, macrophages and other types of cells of the innate immune system. CRP binds to several different species of bacteria, fungi and parasites and CRP recognizes molecular ligands found on bacterial membranes and on apoptotic cells, such as phosphorylcholine and phosphatidylethanolamine (Pepys and Hirschfield 2003). CRP activates the classical complement pathway by binding a plasma protein called C1q. Elevated levels of CRP are seen in several allergic and non-allergic conditions (Bottazzi et al. 2010, Deraz et al. 2012, Lopresti et al. 2014, Rhodes et al. 2011, Vermeire et al. 2004). The plasma concentrations of CRP are very low in healthy individuals and therefore in the clinic CRP is mainly used as a marker of acute infection and during such CRP level can increase up to 1000-fold (Pepys and Hirschfield 2003).

In recent years, inflammatory mediators have been studied as potential markers of more subtle and chronic systemic inflammatory state that is called low-grade inflammation (Medzhitov 2008). In this thesis a low-grade inflammation is defined

as subclinical systemic inflammation characterised by the increased production of acute-phase proteins (CRP) below the levels seen in clinical infections.

One of the earliest responses of the innate immune system to any stimulation is the secretion of cytokines by tissue cells, which is crucial for the acute inflammatory response. The three most important proinflammatory cytokines IL-1 β , IL-6 and TNF- α have systemic effects and are responsible for many clinical signs of infection and inflammatory disease. In this study, IL-1 β , IL-6, TNF- α , number of leucocytes and exhaled nitric oxide are used as a way of measuring inflammatory processes (Kharitonov and Barnes 2001, Sabato et al. 2006, Schroder and Tschopp 2010, Sims and Smith 2010).

Noncommunicable inflammatory or immune-mediated diseases, including allergic diseases, have drastically increased over the last few decades. Chronic inflammation is a common factor of these diseases, indicating the central role of the immune system (Renz et al. 2011). Many environmental factors, such as dietary factors (Dunstan et al. 2003, Field et al. 2010), maternal smoking during pregnancy (Noakes et al. 2003), environmental pollutants (Liu et al. 2008), microbial patterns (Prescott 2011) and stress (Mattes et al. 2009) have been shown to be able to affect the cytokine levels and thus are able to cause changes in the immune system and affect inflammatory processes in humans, even in children in utero. Thus, it has been suggested that dysregulation of inflammation, partly due to environmental effects, leads to the onset of noncommunicable diseases, including asthma and allergies (Prescott 2013).

It has been shown that low-grade inflammation is a relevant and an independent predictor of coronary heart diseases (Danesh et al. 2000, Willerson and Ridker 2004) and strongly related to obesity and type 2 diabetes (Pickup 2004, Sjöholm and Nystrom 2005). In obesity, inflammation of the fat tissue is considered as one of the causes of increased CRP levels. High levels of CRP in adolescence predicted the metabolic syndrome in adulthood (Mattsson et al. 2008) suggesting that low-grade inflammation may predict adverse health effects.

Although low-grade inflammation may be involved in pathogenesis and exacerbation of many diseases, it has also been hypothesised that low-grade inflammation might predict a decreased risk of allergic diseases (Marschan et al. 2008, Viljanen et al. 2005). This has been explained by the beneficial effects of microbial exposure to the maturation of the immune system according to the principals of the hygiene hypothesis and in this model CRP is a marker of microbial load.

In contrast, the up-regulation of the inflammatory mediators has been seen in subjects exposed to moisture damage and related microbial load (Punsmann et al. 2013a). Altogether, the role of inflammation and the nature of the inflammatory response in the development of allergic diseases appears to be crucial but the present knowledge of these factors is still limited and thus the interpretation of the studies on inflammation in allergic diseases is challenging.

2.2 Definitions and risk factors of allergic diseases

2.2.1 Definitions of atopy, asthma and atopic dermatitis

Atopy is defined by an individual's capacity to produce a specific IgE antibody responses or a positive skin prick reaction to common environmental or food protein antigens (Johansson et al. 2004). Atopic people have a greater tendency to develop typical symptoms of allergic diseases such as asthma, rhinoconjunctivitis or dermatitis (Johansson et al. 2004).

According to Global Initiative for Asthma report (GINA 2012), asthma is defined as a chronic inflammatory disease of the airways and lungs which are obstructive due to bronchoconstriction, mucus plugs and inflammation. Symptoms of asthma are recurring episodes of wheezing, breathlessness, chest tightness and coughing, especially at night or early in the morning. Due the several phenotypes the characterisation of different asthma is challenging (GINA 2012). There have been identified at least six different wheezing phenotypes in children: never wheeze, early transient wheeze, prolonged early wheeze, intermediate-onset wheeze, late-onset wheeze and persistent wheeze (Henderson et al. 2008, Martinez et al. 1995). Asthma is difficult to define as a single disease and therefore there is no consistent classification for asthma phenotypes.

Atopic dermatitis (AD, atopic eczema) is an inflammatory, relapsing and pruritic skin disease which occurs usually among families with atopic diseases and affects especially infants and small children (Darsow et al. 2010). AD is a non-contagious inflammation of epidermis and dermis and its characteristic clinical signs are itch, erythema, papule, seropapule, vesicle, squames, crusts, lichenification, in synchronous or metachronous polymorphy and dermatopathological signs are spongiosis, acanthosis, hyper- and parakeratosis, lymphocytic infiltrates and exocytosis and eosinophils (Bieber 2010, Darsow et al. 2010). AD is associated with asthma and allergic rhinitis and patients with AD often have allergic symptoms to various food and inhalant allergens (Hon et al. 2008, Ponyai et al. 2008).

Atopy has shown to be strongly associated with AD (Leung 1995). Atopy does not imply that a person has an allergy, but the risk for develop allergies is increased among atopics. On the other hand, asthma and atopy are not that strongly correlated. It has been reviewed that less than a half of the asthmatics are attributable to atopic sensitisation (Pearce et al. 1999). A systematic review showed that on average, one of three young children with AD develops asthma later during his/her childhood (van der Hulst et al. 2007). Atopic dermatitis is dependent on the Th1/Th2 lymphocyte imbalance, with a tendency to produce IgE (Bieber 2010) and the risk of food allergy, asthma and allergic rhinitis is defined as 'the atopic march' (Dharmage et al. 2013).

2.2.2 Hygiene hypothesis and allergic diseases

David Strachan postulated the hygiene hypothesis in 1989, and suggested that infectious agents might protect us from allergic disease (Strachan 1989). The hygiene hypothesis suggests that frequent confrontation with proinflammatory microbial agents in childhood induces the maturation of Th1-immunity and downregulation of Th2 immune response which might protect from allergy prone reactions. The original observation was that the risk of hay fever decreased with the increasing number of siblings (Strachan 1989).

It has later been reviewed that these original observations explain only part of the story (Fuleihan 2002, Kumar 2008) and the focus of the hypothesis has shifted since. The present knowledge is that not the clinical infections but the lack of appropriate immune stimulation during early childhood with the consequence of disturbed alignment in the encountering antigens and microbes might account in the rise of allergic diseases (Martinez 2001). Moreover, the pregnant mother's exposure to farm-related microbial agents, which cause the production of cytokines and regulatory T cells and expression of Toll-like receptors, has been hypothesised to provide immunotherapy to the child and thus protects the child from allergic diseases later (Eder et al. 2006, Pfefferle et al. 2010, Schaub et al. 2009, von Mutius and Vercelli 2010).

The risk of asthma is inversely correlated with increasing diversity of bacterial and fungal taxa in home-dust samples (Ege et al. 2011). In addition altered microbial flora in the gut is associated with food allergy (Rodriguez et al. 2011), whereas colonisation in the neonatal gut by certain bacterial species is thought to be protective against the development of asthma (Vael et al. 2011).

Varicella zoster infection has been shown to associate with the protection against eczema (Silverberg et al. 2010) supporting the view that some infections might protect from allergic diseases. Several studies have shown the relationship between bacterial/viral infection and asthma but it is still unknown whether infections are risk factors for developing asthma (Callaway and Kim 2011, Fuchs and von Mutius 2013, Sly et al. 2010). Children with asthma may be more sensitive to viral and bacterial infections due to impaired mucosal and systemic immune responses (Fuchs and von Mutius 2013). Furthermore, previous infections seem to shape the immune system as compared with more recent illnesses (Yang and Gao 2011).

The mechanisms that protect from the development of allergic diseases include effects on the innate immune system, development of regulatory T cells and Th1 skewing. Also, the gene–environment interaction is crucial in predisposing to allergic diseases. It is not likely that there is one specific cause as the trigger for allergic diseases, and even more unlikely that one specific infection can prevent or cause atopy (Fishbein and Fuleihan 2012).

Regardless of whether the environmental microbes are primarily exposed to the respiratory, the gastrointestinal or the skin mucosal surface, these microorganisms

may closely interact with the epithelial barrier and the innate immune system. It has recently been shown that these barrier microbes play a pivotal role in shaping immune responses, particularly early in life. These microbes apparently play a decisive role in either generating (mucosal) tolerance or leading up to the development of allergic diseases (Shreiner et al. 2008).

2.2.3 Other risk factors of atopy, asthma and atopic dermatitis

2.2.3.1 Atopy

The complex interactions between genetic predisposition and environmental exposures are the factors that mainly determine whether a child develops atopy (Wright 2004). Maternal history of atopy is the strongest risk factor for the development of atopy (Liu et al. 2003, Ruiz et al. 1992). Maternal atopy and infections during pregnancy may act as immunomodulators in the development of atopy, but it is unknown whether these factors can enhance or inhibit the development of atopy (Illi et al. 2014). Moreover, exposure to allergens in utero may influence the capability of the infant to respond immunologically to allergen exposure (Hagendorens et al. 2004, Miller et al. 2001, Peters et al. 2009).

2.2.3.2 Asthma

Both environmental stimuli and genetic predisposition contribute to the development of asthma (Murphy and O'Byrne 2010). Various stimuli, such as chemokines, cytokines and lipid mediators can cause membrane fibrosis, smooth muscle hyperplasia, new vessel formation and glandular hyperplasia that are referred to as airway remodeling and lung inflammation and largely contribute to asthma pathogenesis (Naik and Wala 2013).

In addition to familiar disposition, passive or active smoking seems to be the most consistent risk factor for asthma. It is shown that parental smoking is associated with wheezing in early life and with wheezing and asthma in childhood (Strachan and Cook 1998). Active smoking is also shown to be associated with the onset of asthma in adolescents and adults (Larsson 1995, Strachan et al. 1996). In addition, maternal home second-hand smoke exposure during pregnancy is associated with childhood asthma, even if the mother herself does not smoke actively during pregnancy (Simons et al. 2014).

Exposure to outdoor air pollution has been shown to associate with an increase in asthma symptoms and health care use (Tatum and Shapiro 2005). However, it is still uncertain whether pollutants, such as ozone and particulate matter, contribute to the development of asthma (Eder et al. 2006).

Exposure to environmental allergens has controversial effects on the development of asthma. There is evidence that exposure to pets, especially to dogs, perinatally and at an early age protects persons from asthma (Lodge et al. 2012).

Furthermore, early atopic sensitisation to inhalant allergens and food allergy are risk factors for developing asthma (Guilbert et al. 2004).

Dampness and mould in buildings have been stated to cause adverse respiratory symptoms, including asthma (Cox-Ganser et al. 2009, Jaakkola et al. 2005, Park et al. 2008, Pekkanen et al. 2007). There is however no consensus on what are the organisms and compounds which cause the adverse effects are and through which mechanisms they work. In addition obesity from infants to young adults predicts increased incidences of asthma diagnosis or symptoms in the future, especially for girls (Liu et al. 2013). There is also some weak evidence that deficiencies of the nutrients, such as vitamins A, D and E, zinc, fruit and vegetables may be associated with the development of asthma (Nurmatov et al. 2011).

2.2.3.3 Atopic dermatitis

It seems that a defect in the cutaneous barrier function is an important factor for the development of AD. This view is supported by studies showing abnormalities in filaggrin and in the proteins of the epidermic differentiation complex (Hoffjan and Stemmler 2007). Also dysfunction of the innate and/or adaptive immune response is seen in AD (Eyerich and Novak 2013). The pathogenesis of the disease is not fully understood.

The etiology is multifactorial and different types of genetic predisposition and environmental factors, such as mutations of filaggrin gene, the exposure to allergens and the expression of thymic stromal lymphopoietin, have been suggested to be involved in the initiation of the disease (Baurecht et al. 2007, Capristo et al. 2004, Wilson et al. 2013).

Risk factors for AD are family's higher socioeconomic status (DaVeiga 2012), family's higher level of education (DaVeiga 2012, Harris et al. 2001), a smaller family size (DaVeiga 2012, Harris et al. 2001), urban environment (Schram et al. 2010), "Western" diet (Ellwood et al. 2013), broad-spectrum antibiotic exposure (Schmitt et al. 2010) and reduced diversity in the bacterial gut flora (Wang et al. 2008). Environmental factors such as climate, outdoor air pollution and tobacco smoke, have been studied but the relationship between these factors and atopic dermatitis are complex (Flohr and Mann 2014).

2.3 Farming, immune system and allergic diseases

2.3.1 Farming and allergic diseases

Among adults, there is evidence that large animal feeding operations, organic dusts and bacteria in farming environments cause upper and lower respiratory tract disorders, including asthma (Charavaryamath and Singh 2006, Malmberg et al. 1993, May et al. 2012).

In contrast, since over a decade, numerous studies have shown evidence that farming environment in childhood protects children from atopy, allergic diseases

and asthma (Braun-Fahrlander et al. 1999, Downs et al. 2001, Leynaert et al. 2001, Remes et al. 2003, Riedler et al. 2001, Von Ehrenstein et al. 2000). First it seemed that the protective effect was mainly against atopy and only partly against asthma (Naleway 2004). Later, systematic meta-analysis concluded that the prevalence of asthma may be 25 % lower in the children who live in farming environments (Genuneit 2012). The estimated relative risk of atopy among farm-living children has been stated to be 41 % lower than among nonfarm-living children (Perkin and Strachan 2006). However, there is no consistent evidence that farming environment during childhood protects from the development of AD and the estimated risk for AD is 0.97 (Perkin and Strachan 2006). It has been difficult to find consensus on the protective farm effect due to the different phenotypes of asthma and allergic diseases (Fuchs et al. 2012).

The protection from the diseases is strongest if the exposure to farms occurs in utero or during the early years of life (Illi et al. 2012, Riedler et al. 2001). However, farms and farming environments are not homogenous and thus diverse exposures are characteristic for the farm effect. In addition, it has been shown that different exposures protect individuals from distinct diseases, not from all atopic or allergic diseases (Ege et al. 2007, Illi et al. 2012). Protective effects for atopy have been identified to be agriculture, pig and poultry farming and barns, whereas agriculture, pig farming, silage, haying, farm milk, animal sheds and barns have been identified to protect individuals from asthma (Ege et al. 2007).

After the findings of the protective farm effect, it has been intensively investigated what the protective exposures on the farms are. Identified exposures that have been contributed to the reduced risk of asthma and allergies in farm children are contact with cattle, pigs and poultry, contact with hay, grain, straw and silage and the consumption of unprocessed cow milk (Douwes et al. 2008, Ege et al. 2007, Riedler et al. 2000, Riedler et al. 2001, Von Ehrenstein et al. 2000). Other farm-related lifestyle factors such as duration of breast feeding, family size, daycare attendance, other dietary factors or parental education were not decisive factors for the protective farm effect (von Mutius and Vercelli 2010).

Farmers have been reported to own more pets than non-farmers and there is reviewed evidence of the protective pet ownership (Naleway 2004). However, the protective effect may only occur during early life and later on it may actually worsen the symptoms in children who already have asthma or allergic disease (Johnson and Alford 2002, Wickens et al. 2002). The farms with livestock have been reviewed to protect individuals from asthma and allergic disease (Naleway 2004). The farm animal effect seems to be rather strong and the protective effect of early life livestock contact against atopy, asthma and atopic dermatitis has been shown to remain until adulthood (Lampi et al. 2011).

It has been recently reviewed that a pregnant mother's and a child's exposure to cows and straw on farms and the consumption of farm milk are identified as protectors from asthma but the protection from atopy seems to be less significant

(Illi et al. 2012, Wlasiuk and Vercelli 2012). Furthermore, these three exposures have been described to be responsible for the overall farm effect considering asthma (Illi et al. 2012). On the other hand, staying in a fodder storage room (Illi et al. 2012), helping with haying (Karadag et al. 2007) and maternal contact with farm animals and cats during pregnancy (Roduit et al. 2011) proved to be protective factors against atopic dermatitis. High microbial diversity in the farm environment is reported to protect from asthma but its association with atopy seems to be less significant (Ege et al. 2011, Wlasiuk and Vercelli 2012).

Farm milk consumption is one of the most discussed farming exposures. There is reviewed evidence that the consumption of raw cow milk protects from the development of asthma and allergic diseases (Braun-Fahrlander and von Mutius 2011). It has also been reviewed that maternal consumption of unprocessed cow milk protects from the development of childhood asthma and atopy (von Mutius 2012). However, raw milk contains different pathogens which can cause severe infections and thus it can not be recommended as a preventive agent against allergic diseases (Braun-Fahrlander and von Mutius 2011, von Mutius 2012).

Also, in Finland farming environment in childhood, especially contact with cattle, reduces the occurrence of asthma and atopy in children (Remes et al. 2005) and in adults (Lampi et al. 2011), but the consumption of farm milk was not associated with the reduced risk of atopy and allergic diseases (Remes et al. 2003).

In conclusion, the main protective farming exposures against allergic diseases could be livestock contact, farm milk consumption and early contact to cats and dogs.

2.3.2 Farming environment and its effects on the immune system

Earlier studies suggested that microbial markers such as endotoxin (lipopolysaccharides, LPS), muramic acid and beta-glucans are responsible for the protective effect of the farm exposure on asthma and allergic diseases and there is evidence that these factors are at least partly involved (Braun-Fahrlander et al. 2002, Karadag et al. 2007, Schram-Bijkerk et al. 2005, van Strien et al. 2004). Bacterial components engage with antigen-presenting cells eliciting cytokine inducing response (Braun-Fahrlander 2003). It has been shown that endotoxin levels in home correlated with interferon gamma (IFN- γ) T-helper type 1 cells but not with IL-4, IL-5 or IL-13 producing T-helper type 2 cells in infants (Braun-Fahrlander 2003, Braun-Fahrlander et al. 2002, Gereda et al. 2000). In addition, another study showed that IFN- γ producing capacity during first months of life was positively associated with endotoxin levels in house dust (Roponen et al. 2005). On the other hand, among school age children greater endotoxin exposure was associated with the reduced production of TNF- α , IL-10, IL-12 and IFN- γ cytokines (Braun-Fahrlander et al. 2002). One interpretation is that endotoxin exposure may enhance Th1 immunity (Gereda et al. 2000). On the other hand, endotoxin exposure can suppress

the cytokine production and the phenomenon is called LPS tolerance and furthermore LPS tolerance may contribute to the protective effect of a farming environment (Stiehm et al. 2013, West and Heagy 2002).

Low IFN- γ expression levels at birth have been shown to associate with an increased risk for the later development of allergic symptoms and atopic disease and furthermore low IFN- γ in the first year of life is known to be a strong predictor of airway obstruction during childhood (Stern et al. 2007, Vuillermin et al. 2009, Wright 2004).

Pathogens and damage-associated molecules are recognized by pattern recognition receptors (PRR) of the toll-like receptor (TLR) -family, which are the major triggers of the innate immunity response. Farmers' children blood cells expressed higher amounts of CD14 and TLR2 than non-farmers' (Lauener et al. 2002). Furthermore, the higher the number of different farm animal species the pregnant mother had contacted, the higher the levels of TLR2, TLR4 and CD14 were at school age (Ege et al. 2006). These studies indicated that the exposure to the ample microbial environment on farms produces an upregulation of innate immunity receptors (von Mutius and Vercelli 2010). Furthermore, enhanced expression of genes interleukin-1 receptor-associated kinase 4 (IRAK-4) and receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIPK1) of innate immunity and enhanced expression of IL-10, transforming growth factor beta (TGF- β), suppressor of cytokine signaling 4 (SOCS4) and IRAK-2 regulatory molecules have been found in farmers' school-aged children suggesting that these factors contribute to the mechanisms of the hygiene hypothesis (Frei et al. 2014).

Many studies have tried to identify specific bacteria and fungi from dust samples taken from children's mattresses on farms. No specific protective microbes have been characterised but there is evidence that higher microbial diversity is inversely associated with asthma (Ege et al. 2011, Normand et al. 2011).

The protective effect of farm milk has been tried to be explained by its concentration of pathogenic bacteria and whey proteins (Lluis and Schaub 2012). However, bacterial endotoxin has not been detected to be the protective agent in farm milk (Gehring et al. 2008). It has been newly discovered that farm milk exposure was associated with increased number of regulatory T cells (Tregs) in children and Tregs were inversely associated with asthma and atopy (Lluis et al. 2013). The authors suggest that Tregs play a major role by transmitting the protective effect of farm milk consumption (Lluis et al. 2013).

There are a limited number of studies reporting the association between environmental microbial exposure and stimulated peripheral blood cytokine production. One study has shown that Gram-positive and negative bacteria, measured from house dust, were associated with stimulated peripheral blood cytokine production in the mothers of the study (Lappalainen et al. 2008). Gram-negative bacteria were associated with the down regulation of proinflammatory cytokines (TNF- α and IL-6) and Gram-positive bacteria had the opposite effect

(Lappalainen et al. 2008). In addition, high level of Gram-positive bacteria or *Mycobacterium* species in house dust was associated with decrease IFN- γ production at the age of one year (Lappalainen et al. 2012).

It has been shown that pig farmers had higher levels of circulating neutrophils, IL-13 and IL-4 (Th2 responses) cytokine concentrations compared to the control subjects (Sahlander et al. 2010) and this study suggests that after the exposure to organic dust there might be a systemic inflammatory response. A similar response was also seen among smokers and the researchers suggest that this phenomenon is related to adverse respiratory disorders in these groups (Sahlander et al. 2010). It has also been shown that occupational exposure to farm animals associated with serum IL-8, IL-10 and TNF- α responses compared to non-exposed controls (Tabibi et al. 2012). These studies show that farming environment may influence proinflammatory cytokine production during adulthood.

It has been recently reviewed that the exposure to the organic dust modulates innate immunity, involving toll-like receptors and nucleotide oligomerization domain 2 (NOD2) mediating inflammatory consequences, and these alterations might protect from allergic response (Poole and Romberger 2012). Furthermore, there is evidence that farming exposure can affect innate immunity in children (Poole and Romberger 2012).

Moreover, a model of the immunobiology of farming has been presented (von Mutius and Vercelli 2010). In this model, different key effector mechanisms of allergic diseases are inhibited by the immunoregulatory characteristic of farm-related microbial exposures. Contact with several animal species and consumption of farm milk together, results in strong microbial exposure of women living on a farm during pregnancy. Authors conclude that these exposures, occurring at a time when immune responses are programmed, affect regulatory T cells and cytokine production at birth, which enhance innate immune responses, and suppress T-helper 2 cell-dependent allergic inflammation in early childhood (von Mutius and Vercelli 2010).

There is, however, still a limited number of studies concerning which environmental farming factors are capable of inducing inflammation in humans and what the best markers to detect such inflammation are. Moreover, it is still unsolved whether the induced inflammation is capable of protecting individuals from allergic diseases.

2.4 Moisture damage, inflammation, allergic diseases

2.4.1 Moisture damage and allergic diseases

It has been concluded that moisture damage, mould problems and dampness in buildings cause adverse health effects in humans (Institute of Medicine (IOM) 2004, World Health Organization 2009). The strongest evidence of adverse health effects

of moisture damage, mould or dampness are for respiratory symptoms such as wheezing, cough and asthma exacerbation (Bornehag et al. 2001, Bornehag et al. 2004). There are limited amount of studies that have showed the relationship between moisture damage and mould and the development of new asthma but few studies have shown the positive association (Cox-Ganser et al. 2009, Jaakkola et al. 2005, Park et al. 2008, Pekkanen et al. 2007). Later on, the WHO has stated that there is an association between moisture damage and mould in buildings and the development of asthma in children (World Health Organization 2009). In addition, a recent meta-analysis of 16 studies concluded that dampness and mould in the home are determinants of developing asthma (Quansah et al. 2012). There is also some evidence of the association between dampness or mould and allergic rhinitis, eczema and atopy (Mendell et al. 2011).

Suggested exposures behind the adverse health effects in buildings with moisture damage, mould or dampness, are micro-organisms or their fragments, toxins, allergens, other volatile organic compounds (World Health Organization 2009) or non-biologic emissions such as formaldehyde and 2-ethyl-1-hexanol (Mendell et al. 2011). Despite a large number of studies investigating moisture damage, mould or dampness, the mechanisms or causal relations of the adverse health effects are still unknown.

There is a large number of fungal and bacterial species that have been isolated from moisture damaged buildings (Andersson et al. 1997, Samson et al. 1994) but the species that cause the adverse health effects have not been identified yet. Many factors, such as the diversity and quantity of species, relations between different microbes, severity of the moisture, temperature, relative humidity, air flows and pressure and availability of nutrients, influence the conditions of the microbial growth and the microbes' capability to produce toxins or microbial volatile compounds.

It has been reported that in moisture damaged schools there is a larger amount of microbial metabolites than in schools without moisture damage (Peitzsch et al. 2012). For example the mycotoxins, likely to originate from indoor fungal growth, were found in the moisture damaged schools in Finland (Peitzsch et al. 2012). Furthermore, it has been shown that in moisture damaged homes bacterial compounds monactin, nonactin, staurosporin and valinomycin were detected in building materials from moist structures (Taubel et al. 2011). These compounds are produced by *Streptomyces* species which is one of the indicator markers of moisture damage indoors. These findings suggest that bacterial and fungal metabolites are a part of microbial exposure in moisture damaged buildings and they may be at least partly responsible for the observed adverse health effects.

2.4.2 Moisture damage and inflammation

The associations between moisture damage or microbes originating from moisture damaged building and airway inflammatory responses have been studied in murine models and in the nasal lavage samples in humans (Hirvonen et al. 1999, Jussila et al. 1999, Jussila et al. 2003, Purokivi et al. 2001, Roponen et al. 2013, Stark et al. 2006). A study showed that proinflammatory IL-1 β and TNF- α cytokine levels were increased in nasal lavage after exposure to the indicator microbe for moisture damage (Stark et al. 2006). Another study showed that IL-4 concentration was lower and IL-6 concentration tended to be lower in the nasal lavage fluid samples of those subjects who were in the buildings after the renovation of the moisture damage and higher before the renovation (Roponen et al. 2013). Personnel who were exposed to a mould-contaminated school had higher levels of TNF- α , IL-6 and nitric oxide (NO) in their nasal lavage samples than their control subjects (Hirvonen et al. 1999). Furthermore, a study by Purokivi et al. (2001) showed that concentration of IL-1 was higher in nasal lavage samples of personnel of the moisture damaged school compared to the subjects from the control school and concentrations of TNF- α and IL-6 in nasal lavage of exposed workers were significantly higher during exposure to the moisture damaged school than after vacation. In addition moisture damage has been suggested to be capable of inducing immunostimulation and systemic immunotoxicity in mice (Jussila et al. 2003).

There are numerous microbes that have been found to associate with moisture damage but some microbes have been detected to induce cytokine production. Bacteria *Streptomyces anulatus*, *Streptomyces californicus*, *Bacillus cereus*, *Pseudomonas fluorescens*, *Mycobacterium Mycobacterium avium complex* and *Mycobacterium terrae* and fungi *Stachybotrys chartarum* and *Aspergillus versicolor* have been able to induce the production of IL-1, IL-4, IL-6 and TNF- α cytokines and nitric oxide in mouse and human cell line models (Hirvonen et al. 2005, Huttunen et al. 2001, Huttunen et al. 2003, Jussila et al. 1999, Jussila et al. 2001, Murtoniemi et al. 2005, Penttinen et al. 2005, Roponen et al. 2001). On the other hand, it has been shown that fungal spores did not cause inflammatory changes in nasal lavage samples among sawmill workers whose fungal spore exposure at work is much higher than the exposure of people to fungal spores in mould-damaged buildings (Roponen et al. 2002).

In addition, particle samples, high number of viable bacteria and fungi and high microbial total amount from moisture damage sources have also been found to induce inflammatory mediators in murine models and in human nasal lavage fluid samples (Huttunen et al. 2008, Roponen et al. 2003).

It has been suggested that bacterial and fungal components derived from moisture damage can stimulate systemic production of proinflammatory cytokines in humans (Beijer et al. 2003, Punsmann et al. 2013b). Furthermore, the production of cytokines IL-8 and IL-1 β have been shown to be increased in vitro after exposure to

moisture damage or dampness (Punsmann et al. 2013a, Zhang et al. 2012). On the other hand, two studies found inverse association between building dampness and cytokine IL-6 (Sahlberg et al. 2012, Zhang et al. 2012). In addition, among humans who had high levels of β -(1,3)-glucan at home, the secretion of TNF- α cytokine was higher in vitro than among the group of individuals with low levels of β -(1,3)-glucan (Beijer et al. 2003). Furthermore, no significant differences were found in the secretion of IFN- γ and IL-4 levels (Beijer et al. 2003).

There are three studies which have investigated the relationship between moisture damage and CRP values in humans. Purokivi and her colleagues compared CRP values between adults who were working in moisture damaged and control school buildings (Purokivi et al. 2001). A longitudinal study investigated the association between moisture damage or mould at workplace during ten year follow-up and CRP values at the time of the follow-up (Zhang et al. 2012). These studies showed no significant association between moisture damage or mould and CRP values (Purokivi et al. 2001, Zhang et al. 2012). On the other hand, another study from the same study cohort as Zhang et al. (2012) found a positive association between dampness at home at the beginning of the study and CRP values at the time of the follow-up (Sahlberg et al. 2012). Limited amount of reported studies between the association of moisture damage and CRP values makes the conclusions difficult.

2.5 Gut inflammation and allergic diseases

The mucosal immune system including airways, gut, conjunctiva, urinary and genital tracts provides a first defense line of the inner body surfaces (Brandtzaeg 2009). There are three ways of the mucosal tissue to handle different antigens: initiate an immune response, ignore antigens or induce tolerance to it. Mucosal tolerance is systemic unresponsiveness where in the absence of proinflammatory signals regulatory T cells in the mesenteric lymph nodes mediate systemic antigen-specific tolerance (Fukaya et al. 2010, Hadis et al. 2011). Mucosally induced tolerance to antigens probably involves also other suppressive mechanisms and tolerance initiation needs microbe-associated molecular signals sensed by innate immunity (Slack et al. 2009).

The gastrointestinal tract contains a major part of the immune system, with several immunologic cellular and humoral components that defend the host against foreign antigens (Fouhy et al. 2012, Lozupone et al. 2012). The influence of ingested micro-organisms together with the host's features modifies the gut microbiome and thus may influence the immune response of the host (Wlasiuk and Vercelli 2012).

There are environmental exposures, genetic, epigenetic and microbe-associated factors that influence the infant's gut colonisation (Fouhy et al. 2012, Lozupone et al. 2012). Numerous factors are involved in inducing tolerance to large quantities and varieties of food and bacterial antigens. The composition of intestinal microbiota,

gut permeability and exposure to food antigens affect the development of the gut immune system and tolerance during the first months of life. Factors that mostly influence gut microbiota profiles in infancy are the mode of the delivery (Azad et al. 2013, Isolauri 2012, Jakobsson et al. 2014, Penders et al. 2014), the quality of breastmilk (Azad et al. 2013, Isolauri 2012) and the number of older siblings (Penders et al. 2014).

An aberrant composition of the gut microbiota has been shown to associate with the onset of inflammatory bowel disease and necrotising enterocolitis (Carlisle and Morowitz 2013, Leone et al. 2013). Also, early studies showed that infants in Estonia and infants in Sweden were different with respect to microflora, with a low prevalence of allergies in the former and a high prevalence of allergies in the latter (Bjorksten et al. 1999, Sepp et al. 1997) and after this pioneer observation the association between gut microflora and allergic diseases has keenly been investigated. It has been stated that disturbances in gut colonisation patterns and reduced microbial diversity are associated with the development of allergic diseases (West 2014). However, it is still unknown what the content of a healthy human gut microbiota which protects from allergic diseases later on is like.

There are several microbial species in the gut which have been found to associate with atopic diseases (Penders et al. 2007). It has been newly discovered by using pyrosequencing that lower abundance of the genus *Bacteroides* and lower diversity of *Bacteroidetes* in gut microbiota have been associated with birth by Caesarean (Azad et al. 2013, Jakobsson et al. 2014) and thus may explain the findings that children who have been born by Caesarean delivery have an increased risk of asthma and atopy (Neu and Rushing 2011). In addition gut colonisation by *Clostridia* in early infancy increased the risk for atopic dermatitis (Penders et al. 2014). Furthermore, it has been discovered that *Bifidobacterium longum* was more abundant in the faecal samples of the nonwheezing children who were exposed to pets in infancy than in the samples of those children who were wheezing and nonexposed to pets (Nermes et al. 2013). There was also more *Bifidobacterium breve* in the samples of the nonexposed children (Nermes et al. 2013).

Calprotectin is a 36 kDa cytosolic protein complex composed of 8 kDa and 14 kDa subunits (Fagerhol et al. 1980). It is produced by neutrophilic granulocytes and monocytes, and can bind zinc and calcium ions (Fagerhol et al. 1980). Calprotectin is released via neutrophil activation or monocyte-endothelial cell adhesion after stimulation of various factors such as IL-1 β , IL-6 and TNF- α cytokines (Mahnke et al. 1995, Striz and Trebichavsky 2004) under conditions of cellular stress, and it can be detected in faeces, urine, plasma, and other body fluids as an inflammatory marker (Foell et al. 2007). Faecal calprotectin levels have been shown to be relatively high during the first year of life (Kapel et al. 2010).

Increased levels of calprotectin indicate for instance bacterial infections, rheumatoid arthritis, inflammatory bowel diseases, celiac disease and other inflammatory conditions where the permeability of the gut is increased (Sidler et al.

2008). Interestingly, it has been newly suggested that calprotectin could be a useful marker in predicting the course of atherosclerotic process, coronary artery disease and the outcomes of acute coronary syndrome (Kruzliak et al. 2014). Only few studies have investigated the association between faecal calprotectin and allergic diseases. In a French study allergic infants had higher faecal calprotectin values than healthy ones but the difference was not statistically significant (Waligora-Dupriet et al. 2011). Another study did not find any association between faecal calprotectin values during infancy and development of allergic diseases by the age of two years (Baldassarre et al. 2011). More research of the association between faecal calprotectin and allergic diseases is needed.

3 AIMS OF THE STUDY

The aim of the current thesis was to investigate the role of inflammation and the environmental exposures causing inflammation in the development of allergic diseases in children. The specific aims were:

1. To examine the cross-sectional association between different environmental factors and low-grade inflammation measured with serum high-sensitivity C-reactive protein (Study I)
2. To examine the association between low-grade inflammation measured with serum high-sensitivity C-reactive protein at the age of one and the development of atopy, asthma and atopic dermatitis by the age of 4.5 years (Study II)
3. To investigate the cross-sectional association between moisture damage and systemic inflammation in children at the age of six years (Study III)
4. To investigate the relation between gut inflammation at the age of two months and atopy, asthma and atopic dermatitis by the age of six years (Study IV)

3.1 The study question

The main study questions of the present thesis can be summarized as in Figure 1.

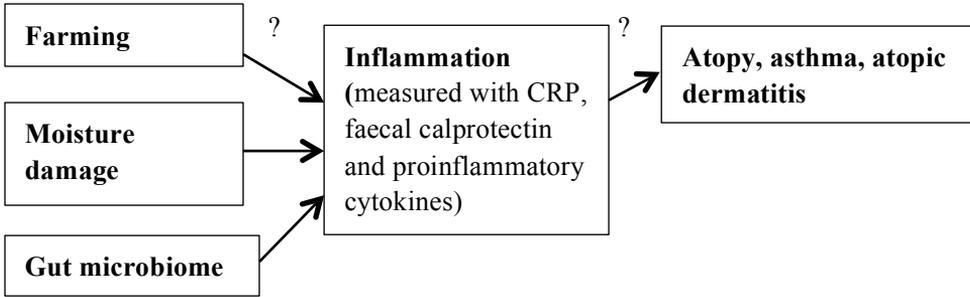


Figure 1. The illustration of the study questions.

4 MATERIALS AND METHODS

4.1 Study population—PASTURE (I, II and IV)

The study population consisted of the Protection against Allergy Study in Rural Environments (PASTURE) birth cohort (von Mutius et al. 2006) which is an ongoing birth cohort study in Austria, Finland, France, Germany and Switzerland. Between years 2002–2005, pregnant women who lived on farms and pregnant women who lived in other rural areas than farms were invited to the study.

Altogether 1133 children were included in the study between August 2002 and March 2005 (Figure 2). The inclusion criteria for the mothers were as follows: living on a farm with livestock or in rural areas, a maternal age of ≥ 18 years, a singleton pregnancy, delivery in one of the study hospitals, no plans of moving from the study area, the native language Finnish and siblings of the study child not participating in the study. The mothers were excluded from the study after the delivery if there was a parturition at less than 37 weeks of gestation, home delivery, infants' congenital abnormalities or a failure to obtain cord blood samples. The families who did not have a phone were not recruited.

The study protocol was approved by the Research Ethics Committees in every study centre and written consents were obtained from the parents of the participating children.

CRP values (I, II) and specific IgE-values (I, II, IV) were obtained from venous blood samples at three time points: when the children were one, 4.5 and six years old. Faecal samples were collected at the age of two months (IV).

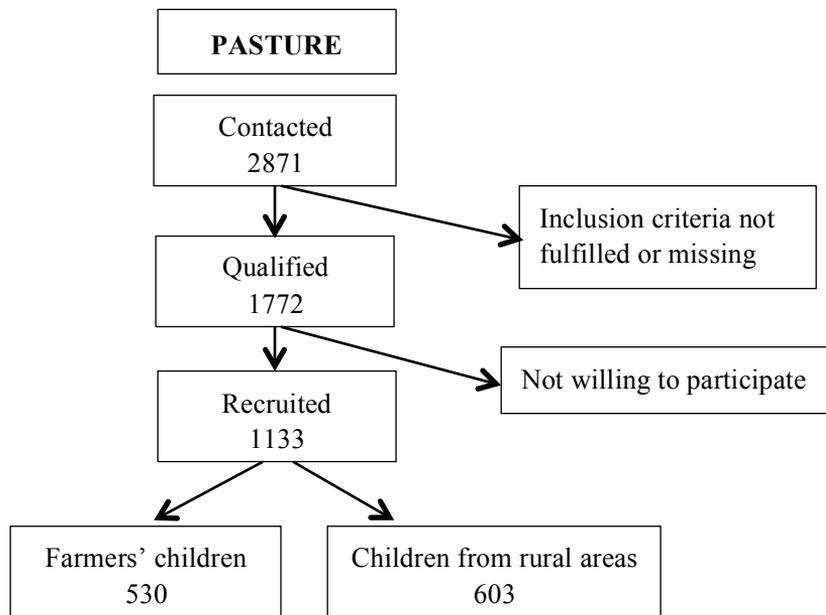


Figure 2. Recruitment of the PASTURE study population.
(Modified from Ege et al. 2008)

4.2 Study population—LUKAS (III)

The LUKAS (“Lapsuuden kasvuympäristö ja allergiat”) study consists of two Finnish birth cohort studies (Karvonen et al. 2009): the Finnish part of the PASTURE cohort (LUKAS1) and an extended cohort LUKAS2 (Figure 3). 214 children were enrolled in the LUKAS1 study between September 2002 and May 2004. All pregnant women who lived on farms with livestock and an equally-sized group of non-farming women who lived in rural areas were invited to the study during the third trimester of pregnancy in the areas of Kuopio, Jyväskylä, Joensuu and Iisalmi hospitals in Eastern and Middle Finland.

228 children were recruited in the LUKAS2 study. The inclusion and exclusion criteria of the LUKAS2 were otherwise similar to those in the PASTURE study; only the parental occupation and the living area were excluded from the criteria this time. All the mothers who were to give birth in the Kuopio University Hospital between May 2004 and May 2005 and who lived in non-apartment households were invited to participate in the study. For laboratory logistic reasons, the women delivering between Thursday afternoon and Sunday morning were excluded from the extended cohort.

The ethical permission of the LUKAS study was granted by the Research Ethics Committee of the Hospital District of Northern Savo, Kuopio, Finland. Written consents were acquired from the parents of the participating children.

Venous blood samples for leucocyte, cytokine and CRP analyses and fractional exhaled nitric oxide samples were collected at the age of six years (III).

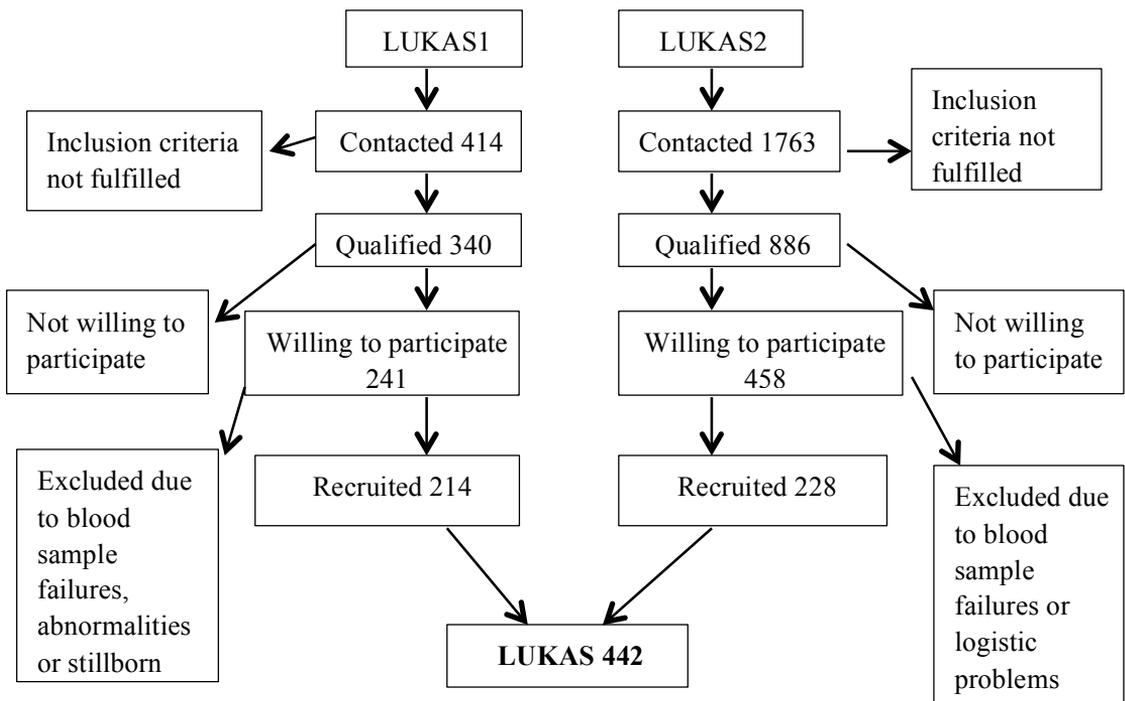


Figure 3. Recruitment of the LUKAS study population.
(Modified from Karvonen et al. 2009)

4.3 Questionnaires

The questionnaires were self-administered by parents during the third trimester of pregnancy, and when the children were 2, 12, 18, 24, 36, 48, 60 and 72 months old. Study nurses administered the two-month questionnaire in the PASTURE study (and in the LUKAS1 study) by interviewing parents during a home visit. The questionnaires enquired information on the end of the pregnancy, the mode of delivery, the child's weight and height, the use of medication by the mother or the child, breastfeeding, dog and cat ownership, mother working in or child staying in a cow shed or hay barn passive smoking, the symptoms and diseases of the children, the consumption of food and farm milk and day care attendance.

4.3.1 Health outcomes

Asthma was defined as parent-reported doctor-diagnosed asthma and/or asthmatic (obstructive) bronchitis more than once during his/her first four years of life (II). Asthmatic (obstructive) bronchitis more than once is related to the condition often termed recurrent wheezing, but is more severe, as it requires contact with a doctor. Children who already had asthma or had had asthmatic bronchitis more than once before the measurement of the CRP values (at the age of one year) were excluded

from the analyses (II). In study IV asthma was defined as parent-reported doctor-diagnosed asthma and/or asthmatic (or obstructive) bronchitis more than once during the six-year follow-up.

‘Atopic dermatitis’ was defined as parent-reported doctor-diagnosed atopic dermatitis at least once during the child’s first four years (II) or during the first six years (IV) of life. Children who already had had atopic dermatitis before the measurement of the CRP values (at the age of one year) were excluded from the analyses (II).

Any wheezing, wheezing apart from cold, cough apart from cold, nocturnal cough, rhinitis apart from cold or rhinoconjunctivitis apart from cold was defined as a parent-reported symptom, which occurred less than once a month or more frequently between five and six years of age (III). Children who already had asthma diagnosis were excluded from the analyses of these symptoms (III).

Otitis media, laryngitis and diarrhea (for at least two days) were defined as a positive answer for the question about having a given infection between the ages of five and six years (III). A common cold with a fever ($>38.5^{\circ}\text{C}$) was defined as at least two episodes of common cold with a fever between the ages of five and six years (III).

4.3.2 Confounders

All models were adjusted for study centre (I, II and IV) or study cohort (III), living on a farm and gender. Other confounding factors were the maternal history of allergic diseases (asthma, atopic dermatitis or allergic rhinitis, II, III and IV), the number of older siblings (II, III and IV), day care with other children than siblings (I), smoking during pregnancy (III and IV), breastfeeding (IV), BMI (I and III), the season of the CRP sampling at the age of 4.5 years (I) and the recent nitrate consumption of the child (III).

The selection of adjusting confounders was performed by two different methods: the adjusting confounders were at least marginally significantly ($p < 0.10$) associated with CRP values in the univariate analysis (I, II and IV) or a confounder changed the estimates more than 10 % (tested with the relationship between exposures to moisture damage or mould and CRP values, III). Other factors tested were the paternal history of allergic diseases, parental educations, the age of mother, birth weight, the mode of delivery, second-hand smoke, dog and cat ownership, mother working in a cow shed and hay barn during pregnancy, the presence of a dung hill near the residence and the consumption of farm milk. For analyses on FeNO values the season of the measurement, recent eating, eating of nitrates, exercise and passive smoking exposure before the FeNO measurements were tested and recent nitrate consumption was included in the final model (III). There was neither clinical examination by a doctor nor viral sample collection from the children at the time of the blood extractions.

4.4 CRP values

The CRP values at the age of one year (II) were measured using ELISA method (BenderMedSystems human CRP, Vienna, Austria). The sensitivity of the assay was $3.0 \cdot 10^{-6}$ mg/l. The CRP values at the ages 4.5 (I) and six (III) years were measured by SYNCHRON® System(s) (Beckman Coulter Inc., Fullerton, CA, USA) in Marburg, Germany, according to the manufacturer's instructions. The method is based on the highly sensitive Near Infrared Particle Immunoassay rate. The detection limit was 0.20 mg/l. Children who had CRP values at any time point more than 5 mg/l were excluded from the analyses to exclude the effect of acute infection on inflammatory markers.

4.5 Specific IgE values

Specific immunoglobulin E (sIgE) to 19 common allergens were analysed using the Allergy Screen Test Panel for Atopy (Mediwiss Analytic, Moers, Germany) (Herzum et al. 2005). The 13 inhaled allergens determined were two house dust mites (*Dermatophagoides pteronyssinus* and *D.farinae*), seven types of pollen (alder, birch, European hazel, grass pollen mixture, rye, mugwort and plantain), cat, horse and dog dander, and the mould *Alternaria alternata*. The six food allergens tested were hen's egg, cow's milk, peanut, hazelnut, carrot and wheat. The IgE analyses were done in Marburg, Germany.

Atopy was defined as any sIgE concentration at least 0.35 kU/L at the age of one year (II) and at least 0.70 kU/L at the age of 4.5 (I, II) and six years (IV). Atopic sensitisation to inhalant allergens was defined as at least one sIgE concentration to any of the 13 inhalant allergens at least 0.35 kU/L, and sensitisation to food allergens as at least one sIgE concentration to any of the six food allergens at least 0.35 kU/L at the age of one year and at least 0.70 kU/L at the age of 4.5 and six years. The following eight inhalation allergens were considered as seasonal: alder, birch, hazel or rye pollen, grass pollen mix, mugwort, plantain, and *Alternaria* species. *Dermatophagoides pteronyssinus* and *farinae*, cat, horse, and dog dander were considered as perennial allergens (Ege et al. 2008).

4.6 Cytokines

Processing of the blood started within 27 hours after blood drawing. Diluted blood samples (1:8) were incubated for 24 hours with the following stimuli: a combination of phorbol 12-myristate 13-acetate (5 ng/mL) and ionomycin (1 µg/mL, PI), lipopolysaccharide (LPS 0.1 µg/mL) or peptidoglycan (PPG 10 µg/ml) alone and analysed for the following proinflammatory cytokines: Interleukins 1β and 6 (IL-1β, IL-6) and tumor necrosis factor (TNF-α). All stimulants were from Sigma, Deisenhofen, Germany. The concentrations of all cytokines were measured by using multiplexed cytometric bead array (CBA) according to the manufacturer's instructions (BD) in Marburg, Germany. All the cytokines were standardized by the

white blood cell counts. The detection limits were 2.3 pg/ml for IL-1 β , 0.7 pg/ml for TNF- α and 1.6 pg/ml for IL-6 cytokines. Due to product mislabeling by the manufacturer (PAA Laboratories) the whole blood cultures of 50 study children contained fetal bovine serum supplement (FBS) with significant impurities and thus had to be excluded from the statistical analyses.

4.7 Fractional exhaled nitric oxide (FeNO)

The fraction of nitric oxide in the expiratory air was analysed by Offline Sampling using OfflineKits (EcoMedics AG, Dürnten, Switzerland) and Mylar Bags® (Quintron, Cedar Rapids, Iowa). Children breathed into the Mylar Bags® three times using the mobile offline device and the exhaled nitric oxide was measured by the NO-analyser CLD 88sp (EcoMedics AG) within 12 hours after sampling. The mean of the FeNO values (ppb) was used in the analyses. 12 participants with reported intake of inhaled steroids in the week before the FeNO sampling were excluded from the analyses.

4.8 Moisture damage and mould in home

In the LUKAS study, a trained civil engineer of the study group inspected the homes for moisture damage and visible mould when the child was approximately six years old. The engineer had years of experience in the use of a standard protocol (Nevalainen et al. 1998).

The homes were inspected for signs of moisture on the surfaces and the structures using a pre-designed checklist without opening the structures (Pekkanen et al. 2007, Nevalainen et al. 1998). Moisture damage and mould in the homes were categorised according to their locations: kitchen, living room, bathroom, child's bedroom and other living area. Due to small number of moisture damage and mould in homes, moisture damage in the child's main living areas were combined and analysed together: living room, kitchen and child's bedroom.

Moisture damage was classified into three classes, i.e. no damage, minor damage and major damage, based on a six-point "need for repair" estimation scale (Nevalainen et al. 1998) and the area of the damage (Pekkanen et al. 2007). Classes 0 and 1 meant damage with no need for repair or only cosmetic repair; class 2 meant a repair of surface materials needed; class 3 meant a repair of structural components needed; and classes 4 and 5 meant more extensive repair needed. "No damage" was defined as need-for-repair classes 0 or 1. "Major damage" was defined as either a need-for-repair class 2 with the area of damage ≥ 1 m² or a need-for-repair class 3 with the area of damage ≥ 0.1 m², or a need-for-repair class 4 or 5 (Karvonen et al. 2009, Pekkanen et al. 2007). Other damage was classified as "minor damage". During the home inspection, the observed mould was also categorised into three classes: no mould, spots of mould and visible mould. Moisture damage, mould spots

or visible mould on silicone sealants in the kitchen were excluded from the definition of moisture damage or mould.

4.9 Calprotectin

Faecal samples were stored at -75°C . A beaker in the bottom cap of the Calpro Extraction Device (Calpro AS, Lysaker, Norway) was filled with thawed and homogenized stool sample, avoiding seeds and grains (~ 100 mg). The extraction tube was filled with 4.9 mL extraction buffer and vortexed for 30 s. The mixing was continued in a shaker at 1000 rpm for 3 min or until only solid particles remained. The samples were centrifuged for 10 minutes at 10 000 g at room temperature. Supernatant was collected and stored at -20°C .

Calprotectin levels were determined using Calprolab calprotectin ELISA tests (Calpro AS, Lysaker, Norway). 20 μL of extract was mixed with 980 μL of sample dilution buffer. Standard and positive controls were performed in duplicate. The optical density was read at 405 nm using an ELISA reader. Samples below the lowest measurable concentration (39.2 $\mu\text{g/g}$) were given an arbitrary value of 19.6 $\mu\text{g/g}$.

4.10 Selection of the subgroup of 120 children for faecal microbial determination

In the subgroup of the cohort the composition of faecal microbiota was analysed using 16S RNA pyrosequencing. The children were included for the selection of the subgroup if they were breast-fed at the age two months, the concentration of calprotectin was above the detection limit, data was available for atopic dermatitis and asthma at the age of six years, and the amount of faecal sample was sufficient for DNA extraction.

A total of 40 case samples were selected (starting from the first applicable sample with calprotectin in the 90th percentile), calprotectin values ranging from 517.6 to 1542.0 $\mu\text{g/g}$. The control samples were randomly selected from the applicable samples with calprotectin value below the 90th percentile, two per case sample from the same center (N=80).

4.11 Pyrosequencing

From each sample, the 16S rRNA genes were amplified using a primer set corresponding to primers 27F-degS (van den Bogert et al. 2011) and 534-R (Wu et al. 2010). These PCR primers target the V1, V2, and V3 hypervariable regions of the 16 S rRNA; 27-degS was chosen in particular because it appears to provide a more complete assessment of actinobacterial abundance (van den Bogert et al. 2011). Pyrosequencing was performed using a Roche FLX Genome Sequencer at DNAvision (Liège, Belgium) using their standard protocol (De Filippo et al. 2010).

4.12 Statistical analyses

The CRP values at any time points, calprotectin values or cytokine concentrations were not normally distributed, and were therefore categorised into two or four groups using the 25th, 50th and 75th percentile cut points (I—IV). In addition the 90th percentile cut point was used in study IV. Also, leucocyte and FeNO values were divided into two groups using the 75th percentile cut point (III).

Different cytokines stimulated with same stimulant correlated positively and therefore the combination variables of IL-1 β , IL-6 and TNF- α were calculated by taking an average of the ranks of the three cytokines within each stimulation. Also these combination variables were categorised into two classes based on the 75th percentile cut point.

The results were given as median values with interquartile ranges. P-values for the comparisons between different categories in univariate analyses were calculated by the Mann–Whitney U-test and the Kruskal–Wallis tests (I, II, IV). Bivariate analyses were conducted by Pearson χ^2 – test or Fisher’s exact test in categorised variables (I—IV). Correlations between nonparametric variables were calculated as Spearman’s correlation coefficients.

Logistic regression was used to analyse the associations between CRP values, calprotectin values and health outcomes and atopy (I, II, IV). Logistic regression was also used to analyse the associations between moisture damage/mould problems and cytokine, CRP, leucocyte and FeNO values and health outcomes (III) and to analyse the associations between CRP values and environmental determinants (I, II). Furthermore, the associations between CRP levels and environmental factors were evaluated among atopic and non-atopic children separately (I, II). The results were presented as adjusted odds ratios (aORs) with their 95 % confidence intervals (95 % CI). P-values lower than 0.05 were considered statistically significant. Statistical analyses were performed with either PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA, I, II and IV) or with SAS 9.3 for Windows (III).

5 RESULTS

5.1 Low-grade inflammation, environmental factors and health outcomes (I, II)

At the age of one year there were 636 study children who had CRP values available and all of them had CRP value lower than 5 mg/l and the median value was 0.06 mg/l with interquartile range 0.04–0.19 mg/l. At the age of 4.5 years CRP values were available for 694 children and some children ($n = 41$, 5.9 %) had CRP values over 5 mg/l and were therefore excluded from the statistical analyses. Median value was 0.22 mg/l with interquartile range 0.00–0.75 mg/l.

5.1.1 Environmental factors

The girls had higher CRP levels at the age of one and 4.5 years than boys (p -value=0.045 and p -value<0.001 respectively) and the levels were higher in children who were obese or very obese at the age of 4.5 years compared to healthy weight children (aOR 4.47, 95 % CI 1.94–10.31). Values measured in summer at the age of 4.5 years were lower than in other seasons (aOR 0.44, 95 % CI 0.24–0.80). The French children had higher CRP values compared with the other children in a univariate test at the age of 4.5 years (medians: 0.35 vs. 0.00 mg/l; $p = 0.003$) and the values also tended to be higher if a child was in a day-care with 11 or more children at the age of 4.5 years (medians: 0.29 vs 0.00 mg/l; $p = 0.069$). The CRP values did not differ significantly between farmers' and non-farmers' children at the age of 4.5 years and there were no association between the CRP levels and the number of siblings (data not shown).

The CRP levels at the age of 4.5 years tended to be higher in children who had been breastfed for less than 2 months, but the adjusted association was not significant (Table S1, I). Furthermore, no significant associations were detected between environmental and farming factors and the levels of CRP at the age of 4.5 years after adjustments (data not shown). In addition, no significant associations were detected between the children who did or did not drink farm milk (data not shown).

After dividing children into two groups by atopy at the age of 4.5 years, non-atopic children who were visiting stables weekly had significantly lower CRP levels at the age of 4.5 years compared to those who did not spend time in stables at all (Table 1). This was not seen in atopic children (Table 1). Further, atopic children whose mothers had been smoking recent years had significantly higher CRP levels and in addition, they also tended to have higher levels of CRP if the mother had smoked during pregnancy compared to the children of the non-smoking mothers (Table 1). There were no associations between CRP values at the age of 4.5 years

and parental farming, farm milk consumption, duration of breastfeeding, contact with cats or dogs at home or duration of time spent in hay barns.

In univariate analyses, CRP values at the age of one year were higher among children who had siblings (p-value=0.017) and were in contact with other children at day-care (p-value=0.004). In multivariate analyses, lower CRP values at the age of one year were detected among atopic children if there had been farm milk consumption of a child, dogs or both dogs and cats at home (Table 3, II) or a mother visiting stables weekly during pregnancy or recently compared with the children who did not have these exposures (Table 2). These associations were not seen among non-atopic children at the age of one year (Table 2).

Table 1. Associations between CRP and different environmental and farming factors by in relation to any atopic sensitisation at the age of 4.5 years.

	Non-sensitised			Sensitised		
	n	CRP*	aOR# (95th CI)	n	CRP*	aOR# (95th CI)
Duration of breastfeeding (months)						
No breastfeeding	35	0.28 (0.00-0.88)	1	20	0.11 (0.00-0.45)	1
0.5-2	39	0.36 (0.00-1.24)	1.36 (0.49-3.81)	28	0.34 (0.00-0.99)	9.44 (1.01-88.55)
2-6	95	0.23 (0.00-0.77)	0.84 (0.33-2.12)	73	0.00 (0.00-0.53)	4.40 (0.51-38.39)
≥6	188	0.24 (0.00-0.75)	0.77 (0.32-1.88)	174	0.00 (0.00-0.73)	8.40 (0.98-72.33)
<i>p-value</i>		0.218	0.593		0.282	0.106
Child's staying in stable weekly in last 12 months						
No (<15min)	164	0.27 (0.00-0.93)	1	118	0.00 (0.00-0.51)	1
15 min-10h	157	0.24 (0.00-0.78)	0.40 (0.16-0.96)	149	0.00 (0.00-0.77)	1.23 (0.48-3.11)
≥ 10 hours	36	0.00 (0.00-0.63)	0.25 (0.07-0.90)	28	0.22 (0.00-0.70)	0.80 (0.19-3.36)
<i>p-value</i>		0.411	0.072		0.849	0.674
Child's farm milk consumption (dL-day) in last 12 months						
0	200	0.27 (0.00-0.77)	1	154	0.00 (0.00-0.51)	1
0-2	65	0.00 (0.00-0.78)	1.21 (0.55-2.64)	47	0.00 (0.00-0.95)	1.88 (0.77-4.61)
> 2	92	0.27 (0.00-0.98)	1.48 (0.74-2.96)	94	0.00 (0.00-0.63)	1.24 (0.55-2.79)
<i>p-value</i>		0.584	0.535		0.916	0.370
Maternal smoking during pregnancy						
Never smoked	227	0.24 (0.00-0.76)	1	182	0.10 (0.00-0.68)	1
Not during pregnancy	90	0.34 (0.00-0.97)	1.41 (0.79-2.52)	72	0.00 (0.00-0.40)	0.52 (0.22-1.20)
During pregnancy	40	0.25 (0.00-0.83)	1.24 (0.55-2.79)	41	0.26 (0.00-1.53)	1.91 (0.83-4.41)
<i>p-value</i>	357	0.272	0.491	295	0.179	0.034
Maternal smoking (during last 2 years)						
No	319	0.26 (0.00-0.83)	1	255	0.00 (0.00-0.58)	1
Yes	38	0.10 (0.00-0.75)	0.94 (0.41-2.17)	40	0.00 (0.00-1.20)	2.51 (1.12-5.59)
<i>p-value</i>		0.637	0.882		0.694	0.025

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

CRP values are presented in two groups according to 75th cut-off points (≥0.75mg/l and < 0.75mg/l) and comparison between two 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 parental farming, country, season of measurement, gender, BMI and day care with other children than siblings. *P*-values are obtained from the trend test (Wald).

Table 2. Associations between different environmental and farming factors and CRP at the age of 1 year in children who have no signs of IgE sensitisation and in children who have developed allergen specific IgE response at the age of one year.

	Non-sensitised			Sensitised		
	n	CRP*	aOR#	n	CRP*	aOR#
Duration of breastfeeding (months)						
No breastfeeding	23	0.08 (0.04/0.26)	1	19	0.08 (0.04/0.64)	1
0.5-2	40	0.05 (0.03/0.15)	0.82 (0.25-2.74)	11	0.06 (0.03/0.14)	0.56 (0.08-4.06)
2-6	114	0.06 (0.05/0.17)	0.90(0.32-2.58)	45	0.06 (0.04/0.12)	0.66 (0.16-2.70)
6 or more	276	0.06 (0.04/0.15)	1.02 (0.38-2.77)	98	0.07 (0.05/0.28)	1.19 (0.31-4.51)
<i>p-value</i>		0.212	0.942		0.247	0.594
Child's staying in stable weekly in last 10 months						
No	246	0.06 (0.04/0.13)	1	91	0.07 (0.05/0.29)	1
Yes	213	0.07 (0.04/0.19)	0.87 (0.46-1.66)	84	0.06 (0.04/0.19)	0.36 (0.12-1.05)
<i>p-value</i>		0.586	0.673		0.355	0.060
Child's farm milk consumption						
No	311	0.06 (0.04/0.14)	1	114	0.07 (0.04/0.30)	1
Only boiled	67	0.06 (0.04/0.26)	1.14 (0.58-2.22)	29	0.06 (0.04/0.08)	0.16 (0.04-0.60)
Both unboiled and boiled	34	0.06 (0.03/0.37)	1.16 (0.48-2.81)	18	0.06 (0.04/0.14)	0.18 (0.04-0.85)
Only unboiled	41	0.07 (0.05/0.20)	1.29 (0.57-2.91)	12	0.05 (0.03/0.18)	0.41 (0.09-1.97)
<i>p-value</i>		0.928	0.581		0.283	0.026
Maternal smoking (during last 10 months)						
No	395	0.06 (0.04/0.15)	1	151	0.06 (0.04/0.20)	1
Yes	58	0.06 (0.04/0.20)	1.31 (0.68-2.55)	22	0.08 (0.05/0.37)	2.21 (0.76-6.47)
<i>p-value</i>		0.715	0.420		0.387	0.147
Mother's staying in stable weekly during pregnancy						
No	180	0.06 (0.04/0.13)	1	60	0.07 (0.05/0.40)	1
Yes	279	0.07 (0.04/0.20)	1.27 (0.69-2.33)	115	0.06 (0.04/0.29)	0.24 (0.07-0.79)
<i>p-value</i>		0.183	0.444		0.129	0.019
Mother's staying in stable weekly in last 10 months						
No	257	0.06 (0.04/0.14)	1	86	0.07 (0.04/0.28)	1
Yes	202	0.07 (0.04/0.19)	0.88 (0.32-2.46)	89	0.06 (0.04/0.20)	0.13 (0.02-0.96)
<i>p-value</i>		0.322	0.808		0.675	0.045

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

CRP values are presented in two groups according to 75th cut-off points ($\geq 0.19\text{mg/l}$ and $< 0.19\text{mg/l}$) and comparison between two 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 country, gender, farmer, siblings and day care with other children than siblings.

5.1.2 Atopy, asthma and atopic dermatitis

At the age of 4.5 years the prevalence of atopy was 45.2 % (295/652), and the prevalence of atopic sensitisation against any inhalant or against any food allergens was 32.7 % (213/652) and 27.3 % (178/652), respectively. The prevalence of atopic sensitisation against perennial or seasonal allergens was 21.9 % (143/652) and 18.7 % (122/652), respectively. If a child had a CRP level lower than the 75th percentile there was a lower risk of sensitisation to inhaled and seasonal allergens (aOR 0.57, 95 % CI 0.37–0.88 and aOR 0.58, 95 % CI 0.35–0.98 respectively) compared to the non-detected levels of CRP but the associations was not seen if the CRP levels were over the 75th percentile or when the CRP was used as a continuous variable (data not shown).

At the age of one year, 27.6 % (N = 175) of the study children were atopic. Between ages one and 4.5 years 60 (12.4 %) of the study children developed atopic dermatitis, 68 (13.1 %) developed asthma/asthmatic bronchitis and at the age of 4.5 years 215 (48.0 %) were atopic.

Among non-atopic children at the age of one year, the children whose CRP levels were over the 75th percentile, had a significantly lower risk of atopy at the age of 4.5 years (aOR 0.48, 95 % CI 0.24–0.95) compared with those children whose CRP values were in the lowest quartile. There was a significant inverse dose-dependent trend: the higher the CRP value at the age of one year, the lower the risk for atopy later on (p-value=0.014). There were no significant associations between the CRP levels at the age of one year and the risk of atopic dermatitis or asthma/asthmatic bronchitis between ages one and four years (Table 1, II).

Furthermore, there was a significant inverse dose dependent trend between CRP values and sensitisation to inhalant allergens (p-value=0.017). In addition, among atopic children at the age of one year, the risk of atopic dermatitis later on tended to be associated with low CRP values: the risk was significantly lower if the CRP value was between the 50–75th percentile compared with values in the lowest quartile (aOR 0.11, 95 % CI 0.02–0.77).

There were no significant associations between CRP values at the age of one year and the development of atopic dermatitis or asthma/asthmatic bronchitis between ages one and four years in the total study population (Table 2, II). A tendency of an inverse dose dependent trend was seen: the higher the CRP value at the age of one year, the lower the risk of atopy at the age of 4.5 years (p-value=0.077).

5.2 Moisture damage and mould in the home and inflammatory markers (III)

5.2.1 CRP, leucocytes, FeNO

At the age of six years there were 291 children who had CRP values available in the LUKAS study, but 10 (3.4 %) of them had a value over 5 mg/l and they were excluded from the analyses. The median value was 0.25 mg/l and interquartile range 0.00–0.66 mg/l. Leucocyte values were available from 284 children: the median value being $7.00 \times 10^3/\text{nL}$ with the interquartile range 5.90–8.10 $\times 10^3/\text{nL}$. FeNO values were available from 292 children and the median value was 5.99 ppb with an interquartile range 4.54–7.81 ppb.

Major moisture damage in child's main living areas (living room, kitchen or child's bedroom) was associated with high levels (>75th percentile) of CRP (Table 3). There were no significant associations between moisture damage/mould problems and leucocyte or FeNO values (data not shown).

5.2.2 Cytokines

Major moisture damage in child's main living areas (living room, kitchen or child's bedroom) tended to associate with high levels (>75th percentile) of most of the analysed proinflammatory cytokines. These associations reached statistical significance for IL-1 β in PI-stimulated whole blood (Table 3) and for the combined variable of IL-1 β , TNF- α and IL-6 cytokines in LPS-stimulated whole blood (aOR 3.10, 95 % CI 1.01–9.55).

In addition, moisture damage with mould in the bathroom was associated with high levels of IL-1 β (in PI-stimulated whole blood), TNF- α (in LPS-stimulated whole blood) and IL-6 (PI-stimulated whole blood) cytokines (Table 3). Furthermore, moisture damage with mould in the bathroom was associated with high levels of combined variable of IL-1 β , TNF- α and IL-6 cytokines in PI-stimulated whole blood (aOR 6.02, 95 % CI 1.06–34.12). There were no statistically significant associations between the presence of mould in child's main living areas or in other living areas and any of the inflammatory markers (data not shown).

Although several positive associations were observed between moisture damage and mould and symptoms, the associations were mostly non-significant and in many places conflicting. Moisture damage with mould in the bathroom increased the risk of rhinitis apart from cold (aOR 5.45, 95 % CI 1.03–28.73) and rhinoconjunctivitis apart from cold (aOR 21.17, 95 % CI 2.57–174.58) when asthmatic children were excluded from the analyses. On the contrary, moisture damage in child's main living areas and moisture damage with mould in the bathroom had an inverse association with rhinitis apart from cold (aOR 0.20, 95 % CI 0.04–0.93 and aOR 0.20, 95 % CI 0.05–0.75 respectively) when asthmatic children were excluded. There were no significant associations between moisture damage/mould problems in home and common cold with fever, otitis, laryngitis or diarrhea (Table S4, III).

Table 3. Association between moisture damage/mould problems at the age of 6 years and CRP values and cytokines at the age of 6 years in different locations of the home: multivariate analysis

Moisture damage in child's main living areas ^a	CRP			IL-1 β (PI stimulated)			TNF- α (LPS stimulated)			IL-6 (PI stimulated)		
	N	n (%) of hsCRP \geq 75th percentile	aOR (95 % CI)	N	n (%) of IL1 β \geq 75th percentile	aOR (95 % CI)	N	n (%) of TNF \geq 75th percentile	aOR (95 % CI)	N	n (%) of IL6 \geq 75th percentile	aOR (95 % CI)
None	184	41 (22.3)	1	166	38 (22.9)	1	166	34 (20.5)	1	166	40 (24.1)	1
Minor	31	8 (25.8)	1.35 (0.53-3.43)	26	5 (19.2)	0.72 (0.22-2.37)	26	9 (34.6)	1.94 (0.71-5.32)	26	7 (26.9)	0.89 (0.31-2.54)
Major	18	8 (44.4)	3.11 (1.05-9.17)	18	9 (50.0)	3.85 (1.30-11.41)	18	6 (33.3)	2.41 (0.77-7.53)	18	7 (38.9)	1.68 (0.57-4.96)
Moisture damage with mould in the bathroom												
No mold	218	52 (23.9)	1	193	45 (23.3)	1	193	43 (22.3)	1	193	47 (24.4)	1
Only spots	8	3 (37.5)	2.03 (0.41-10.06)	8	0	- ^b	8	5 (62.5)	6.67 (1.22-36.44)	8	0	- ^b
Visible mold	7	2 (28.6)	1.80 (0.31-10.58)	9	7 (77.8)	8.18 (1.42-47.01)	9	1 (11.1)	0.64 (0.07-5.79)	9	7 (77.8)	5.93 (1.05-33.46)

^aIncludes living room, kitchen and child's bedroom

Odds ratios (aORs) are adjusted for farmer status, gender, number of older siblings, BMI, maternal smoking during pregnancy, maternal allergic diseases and study cohort. CI indicates confidence interval.

^bCan not be estimated

5.3 Intestinal inflammation, environmental factors and health outcomes (IV)

At the age of two months there were 758 faecal calprotectin samples available and the median value was 163.50 $\mu\text{g/g}$ with the interquartile range 88.59–283.24 $\mu\text{g/g}$. Due to the association of calprotectin with breast-feeding, the levels of calprotectin in breast milk samples from 115 Finnish mothers were analysed, the levels of calprotectin in breast milk and faeces did not correlate significantly with each other ($R=0.104$ and $p\text{-value}=0.271$).

5.3.1 Environmental factors

In univariate analyses, faecal calprotectin levels varied between the study centres, German children having significantly higher levels of calprotectin at the age of two months compared to the other centres ($p\text{-value}=0.001$). The farmers' children had increased levels of faecal calprotectin when compared to the non-farmers' children ($p\text{-values}=0.003$). Also, increased levels of faecal calprotectin were seen among children with one or more siblings when compared to the children without siblings ($p\text{-value}<0.001$) and among exclusively and partially breast-fed children when compared to non-breast fed children ($p\text{-value}<0.001$). The infants of the mothers who did not smoke had higher levels of faecal calprotectin when compared to the infants of the mothers who smoked during pregnancy or who had been smoking before pregnancy ($p\text{-value}=0.003$).

5.3.2 Atopy, asthma and atopic dermatitis

When the levels of faecal calprotectin were divided into four groups using quartiles, there was no significant dose-dependent association with the risk of allergic diseases or atopy at the age of six years, although, there tended to be a trend for the association with asthma ($p\text{-value}=0.093$).

The 90th percentile of calprotectin levels in the whole cohort was used as a cut-off for the significantly increased calprotectin levels indicating a high level of intestinal inflammation, and the children who had very high calprotectin levels (in the 90th percentile) had an increased risk of developing atopic dermatitis and asthma/asthmatic bronchitis by the age of six years when compared to the children who had calprotectin levels below the 90th percentile (Table 4).

There were no significant associations between calprotectin values and atopy at the age of six years (Table 4).

5.3.3 Bacterial associations

For gut microbiota analyses, the composition of the gut microbiota in a selected subgroup with faecal calprotectin below and above the 90th percentile were performed. The infants who had faecal calprotectin levels above the 90th percentile

had significantly lower amount of *Escherichia* in their faecal microbiota when compared to the infants who had faecal calprotectin levels below the 90th percentile (p-value=0.019).

Children with faecal calprotectin levels lower than 200µg/g had significantly lower amount of *Lactobacilleae* in faeces than children with faecal calprotectin levels in between 200 µg/g and 90th percentile (p-value=0.016) or than children with calprotectin levels above the 90th percentile (p-value=0.011). However, the amount of *Lactobacilleae* did not significantly associate with the very high levels of faecal calprotectin (the 90th percentile) when compared to the faecal calprotectin values below the 90th percentile (p-value=0.125).

Table 4. Predictive value of faecal calprotectin at the age of two months and development of allergic diseases between ages one and six years

Calprotectin	Atopic dermatitis		Asthma/asthmatic bronchitis		Atopy at age of 6 years	
	n (%)	aOR (95% CI)	n (%)	aOR (95% CI)	n (%)	aOR (95% CI)
<90 percentile	125 (22.7)	1	106 (19.5)	1	217 (47.7)	1
> 90 percentile	19 (34.5)	2.02 (1.06-3.85)	18 (33.3)	2.41 (1.25-4.64)	29 (58.0)	1.50 (0.81-2.77)
p-value	0.066	0.033	0.022	0.009	0.182	0.197

Results are presented as odds ratios (OR) and their 95% confidence intervals (CI). Odds ratios are adjusted for farmer, centre, gender, siblings, breastfeeding, maternal smoking and mother's history of allergic disease.

6 DISCUSSION

6.1 Environmental factors inducing inflammation

6.1.1 Early environment and low-grade inflammation

Earlier studies have suggested that there may be factors in an early environment that could induce low-grade inflammation in children and the present study investigated the environmental and farming factors that could possibly induce low-grade inflammation and thereby give protection from allergies (Marschan et al. 2008, Viljanen et al. 2005). To my knowledge, there are no previous studies investigating the association between environmental factors and CRP values in children the same as in the present study. The present study showed no association between farming exposures and CRP values suggesting that the protective effect of farming is not related to increased production of CRP. However, the study confirmed the earlier findings of associations between low-grade inflammation and female gender, higher BMI and the season of the measurement (Chiriboga et al. 2009, Ford et al. 2001).

In contrast, among non-atopic children at the age of 4.5 years, those who spent more time in stables had significantly lower levels of CRP, which is the opposite of the original hypothesis. It is always possible that children with higher CRP levels are recovering from infections and thus stay more inside their homes. Also, among atopic children at the age of 4.5 years, maternal smoking during the last two years seemed to associate with the low-grade inflammation at the age of 4.5 years. These results are in line with earlier findings in a mouse model study where microbes from damp building modulated the inflammatory reaction depending on the allergic status of the exposed mice (Leino et al. 2006). The results of the present study need to be confirmed but they do suggest that some environmental factors may associate with the low-grade inflammation in children, but the factors may differ between atopic and non-atopic children. More research is needed.

At the age of one year, somewhat surprisingly, in the children who had already developed atopy by the age of one year, indicators of microbial exposure, i.e. farm milk consumption, exposure to dogs or both cats and dogs at home and mother's visiting stables during pregnancy and recently were associated with low rather than high CRP levels at the age of one year. Thus, these findings further suggest that there might be a down-regulation or other alteration of inflammation as a response to microbial load that could be characteristic for the children who develop early atopy. These results support previous findings of the down-regulation of proinflammatory cytokines after exposure to Gram-negative bacteria (Lappalainen et al. 2008). Furthermore, this suggests that CRP does not always directly reflect the

microbial load but individual's ability to respond to the inflammatory stimuli. This makes the interpretation of results challenging.

The results do not exclude the possibility that the individuals with poor inflammatory response could get benefit from treatment that stimulates inflammation, such as suggested for some probiotic preparations (Marschan et al. 2008), especially in environments where inflammatory pressure is low. The individuals with aberrant inflammatory response may be protected from allergic diseases in an environment where microbial load is high, but may be at risk of allergic immune reactions in an environment of high hygiene.

The study proposes that the down-regulation of inflammatory response to environmental triggers of inflammation is a fundamental characteristic of the hosts' immune response, which may predispose to the development of atopy. The ability of environmental factors to induce long term low-grade inflammation and the effects of long term low-grade inflammation on individuals remain still unknown.

In conclusion, farming environment does not induce low-grade inflammation in children but some environmental factors associate with the low-grade inflammation in atopic and non-atopic children differently.

The children who had siblings at the age of one year had higher CRP levels than the children who did not have siblings. In addition, the children who were in day care with children other than siblings at the age of one and 4.5 years tended to have higher CRP levels than children who were not in day care. These results support the findings of the original hygiene hypothesis (Strachan 1989). The contact with other children may provide different microbial exposure which could induce low-grade inflammation.

6.1.2 Environmental factors and gut inflammation

Faecal calprotectin levels associated with background factors indicating elevated environmental microbial load, and faecal calprotectin levels were significantly higher in the farmers' children. These associations further suggest that farming environment induces intestinal inflammation likely due to increased microbial load. In addition to farming, consumption of breast milk at the age of two months associated with the levels of faecal calprotectin. The children who were exclusively breast-fed had the highest levels of faecal calprotectin. This implies that breastfeeding provides higher microbial exposure than bottle feeding. Bacteria in breast milk (Gueimonde et al. 2007) may result in elevated levels of faecal calprotectin by neutrophil infiltration due the stimulation of the gut immune system of the child.

To evaluate the bacterial source that could induce the intestinal inflammation, the composition of gut microbiota were studied using 16S DNA pyrosequencing method of the 120 children of the study cohort. Relatively low faecal calprotectin levels in infants were associated with low amount of *Lactobacillae* and this implies that

early colonisation with *Lactobacilleae* may lead to intestinal inflammation. The species of *Lactobacillus* have been shown to be capable of inducing the pro-inflammatory IL-12 and IFN- γ production in murine dendritic cell cultures (Christensen et al. 2002) and IL-12 from human blood mononuclear cells (Hessle et al. 1999). Among infants with cow's milk allergy and in infants with IgE-associated dermatitis, *Lactobacillus rhamnosus* GG (LGG) has been shown to increase IFN- γ production of stimulated human peripheral blood mononuclear cells (Pohjavuori et al. 2004). Some lactobacilli are able produce hydrogen-peroxide which may contribute intestinal inflammation (Hertzberger et al. 2014). It has been shown that the low-grade inflammation induced by probiotics could protect from allergies (Marschan et al. 2008). Instead, in this study the association between early intestinal inflammation and development of asthma in childhood was observed and intestinal inflammation associated with colonisation with *Lactobacilleae*.

The infants who had very high faecal calprotectin levels, had low abundance of *Escherichia* in faeces. This result suggests that early *Escherichia* colonisation associates with down-regulation of intestinal inflammation.

Different cells of the innate immune system, such epithelial cells, monocytes, macrophages and dendritic cells and in addition T-cells express toll-like receptors on their surfaces. Lipopolysaccharides (LPS) of Gram-negative bacteria, such as *Escherichia*, causes signalling via TLR-4 and activation of myeloid differentiation primary response 88 (MyD88) mediated pathway of cytokine production. It has been previously shown that continuous exposure to LPS causes tolerance and contributes to a down-regulation of TLR-4 signalling pathway (Hornef et al. 2002). That is why the early colonisation of the gut with *Escherichia* may contribute to a down-regulation of TLR-4 signalling and moderations of inflammatory response which, in this study, was seen as low faecal calprotectin levels. It has been shown that also regulatory T-cells express TLR-4 (Caramalho et al. 2003), and an early colonisation with *Escherichia* could moderate inflammation via initiation of intestinal regulatory mechanisms.

6.1.3 Moisture damage and induction of inflammation

Moisture damage, mould and dampness have been reported to have adverse health effects (World Health Organization 2009) and suggested mechanism behind this phenomenon is induction of inflammation (Wolff 2011). The present study shows the association between moisture damage and mould in home and proinflammatory type responsiveness and systemic inflammation in children. Associations were assessed from the LUKAS participants for either CRP values, PI or LPS stimulated peripheral blood IL-1 β , IL6 and TNF- α cytokines.

Major moisture damage in child's main living areas (living room, kitchen or child's bedroom) was associated with high CRP levels and increased stimulated production of IL-1 β at the age of six years. In addition, moisture damage with mould

in the bathroom associated with increased levels of proinflammatory cytokines in stimulated blood samples of six-year-old children. The results suggest that moisture damage or mould in home is capable of inducing systemic inflammation measured with CRP and further, causing the hyperresponsiveness of innate immunity measured with proinflammatory cytokines. These results support the earlier findings of moisture damage being able to induce systemic inflammation (Frankel et al. 2014, Punsmann et al. 2013a, Zhang et al. 2012).

Few studies have tested inflammatory effects in association with moisture-damaged buildings. One study found inflammatory markers in nasal lavage and induced sputum to be increased in subjects from moisture-damaged buildings compared with control buildings (Purokivi et al. 2001) and the present results are in line with this observation. Another study found that subjects who were exposed to moisture damage had altered receptor expression and inflammatory mediators released (Punsmann et al. 2013a) and similarly in the present study differences in cytokine production capacity was seen between children exposed and non-exposed to moisture damage or mould. However, there was no association between moisture damage and number of leucocytes in the present study, as seen in the previous study (Punsmann et al. 2013a). The number of leucocytes is a very rough estimate of inflammation in the body and consists of several subtypes such as lymphocytes, neutrophils, monocytes, eosinophils and basophils. Lymphocytes have been reported to increase due to moisture damage or mould (Leino et al. 2003), but this may not be reflected in the total number of leucocytes used in the present study.

Despite various *in vitro* and mouse model studies of moisture damage and inflammation, our study is the first to show that damp homes induced systemic inflammation in children. The results are in line with the findings that moisture damage related agents can cause the induction of proinflammatory cytokines (Roponen et al. 2013, Wolff 2011). Cytokines IL-1 β , IL-6 and TNF- α are known to be proinflammatory and in the study the levels of these cytokines were elevated possibly due to irritation associated with moisture damage and mould in home.

Although several positive associations were observed between moisture damage and mould and respiratory symptoms, the associations were mostly non-significant and in many places conflicting. This was especially true for nasal symptoms, where conflicting significant associations, both positive and negative, were observed. The small sample size of the present study compared to most of the earlier studies on symptoms (Bornehag et al. 2001, Bornehag et al. 2004) may partly explain the observed results.

This study showed that moisture damage and/or mould in home was associated with increased up-regulation of proinflammatory cytokines and increased levels of CRP in children. These results support the view that moisture damage and mould in home may be able to induce systemic inflammation and enhanced innate immunity responsiveness. The results are novel and need to be confirmed in future studies.

6.2 The association between inflammatory markers and allergic diseases

Atopy

Among non-atopic children increased CRP levels at age of one year were associated with a decreased risk for the development of atopy between the age of one and 4.5 years, which supports the earlier findings of the protective effect of low-grade inflammation in the control of atopy (Viljanen et al. 2005). However, as discussed above, farm exposure was not associated with elevated CRP levels in the present study. In the previous studies of CRP as a marker of low-grade inflammation, the use of probiotics induced an increase in the values of CRP (Marschan et al. 2008, Viljanen et al. 2005), which suggests that environmental microbial load might be an important factor in the induction of inflammation-related protection from an allergic immune response. The clinical importance of early sensitisation to environmental allergens has been discussed, and it does not necessarily implicate allergic disease, instead it may be a normal physiological response to environmental antigens in infants (Rowe et al. 2007). The present results provide new insights in the hygiene hypothesis and give some support to the link between microbial load and allergic diseases but emphasizing the importance of adequate inflammatory response to the stimuli of microbial exposure in the regulation of the allergic reactivity.

On the other hand, there was no strong evidence for an association between low-grade inflammation at the age of 4.5 years and the prevalence of any atopy at the same age. However, the results suggest that CRP levels between the detection limit and the 75th percentile were associated with a decreased risk of atopic sensitisation to inhaled and seasonal allergens.

It should be noted that in the study I, both CRP and specific IgE levels were measured at the same time. Earlier studies have found that low-grade intestinal inflammation at early infancy reduces the risk of allergic diseases later (Marschan et al. 2008, Viljanen et al. 2005) and the study II supports these findings. It might be that there is a window of opportunity in the early programming of the immune system, and the value of the CRP at the age of 4.5 years in the prediction of atopy may not be relevant anymore.

These results are the very earliest findings on the associations between low-grade inflammation, measured with CRP values, and atopy in children and should be confirmed in the future.

The study did not find an association between faecal calprotectin levels at the age of two months and atopy at the age of six years. The result is in line with the previous cross-sectional study that investigated the association between serum calprotectin levels and atopy (Cobanoglu et al. 2013). It might be that high faecal calprotectin and neutrophil infiltration in the intestine during the early life modulates the factors affecting the development of allergic diseases but not factors regulating IgE response such as IL-4 and IL-13 cytokines.

Asthma and atopic dermatitis

There were no significant associations between the CRP values at the age of one year and the development of atopic dermatitis or asthma/asthmatic bronchitis between ages one and four years. There are no previous studies to compare these results. The low number of children developing atopic dermatitis and asthma or asthmatic bronchitis between ages one and four years is the fact that makes the interpretation of the results difficult in the present study.

The study revealed that high levels of faecal calprotectin at the age of two months predicts asthma and atopic dermatitis by the age of six years thus indicating long term effects of early intestinal inflammation on the development of allergic diseases. There were no linear association between levels of faecal calprotectin and allergic diseases, only high levels of faecal calprotectin were associated with asthma and atopic dermatitis at later age.

The results are different from earlier findings that faecal calprotectin levels in first months of life did not associate with the allergic diseases at the age of two years (Baldassarre et al. 2011). In the present study the association between faecal calprotectin and allergic diseases were evaluated up to six years of age, while the age of two years might be too young for allergic diseases to be manifested as seen in the previous study.

6.3 Methodological considerations

Study population

The major strengths in the study populations are the prospective study designs which provide large amounts of information on children's prenatal, infant, and childhood environments. In the PASTURE study there were 1133 and in the LUKAS study 442 children included. The number of the children in the study is sufficient to observe environmental factors and health effects. As single observational studies can provide only limited support for causal relationships, the findings should be confirmed by new observational, mechanistic and finally experimental studies.

There are always difficulties to follow the whole study population in cohort studies through the years. The amount of drop-outs was not a problem in the PASTURE study, 934 (82 %) of the children participated in the six-year follow-up. However, in the LUKAS study there were data on home inspections only for 309 (69.9 %) homes at six year follow-up. In addition, the blood samples were not available from the total study populations.

In the PASTURE study, half of the children are from rural areas and half from farms. This allowed for the comparisons of farmer and nonfarmer effects with a wide range of collected data on the early living environment. The farming environment provides a good opportunity to evaluate the effect of specific environmental exposures to the immune system and children's allergic diseases.

However, farming environment differs from country to country (von Mutius and Radon 2008) which might be one reason for the differences between countries. Even though multi-center studies like the PASTURE study are carried out with identical protocols in all countries, the use of different languages and different diagnostic criteria of allergic diseases and asthma inevitably lead to some incoherence in the data. The study cohort or the study centre and farming were always included as confounding factors in the statistical models because there were differences between the LUKAS1 and LUKAS2 cohorts as well as between countries in the PASTURE cohort and in addition between farmers and non-farmers.

Some of the exposure assessments of environmental determinants were mainly based on self-reported questionnaires but exposure assessments were carried out via questionnaires at each time point, which is one of the strengths of the study. The exposures were assessed during early childhood, which is an important time for the development of the immune system (von Mutius and Vercelli 2010). In addition, some information was available from the time of pregnancy and two months after the delivery and that provides evidence of the environmental exposures *in utero*.

Asthma determination

Asthma is not a single disease and the characterisation of different asthma is challenging due to the several phenotypes (GINA 2012). In addition, asthma is diagnosed somewhat differently in different countries. Especially, the determination of asthmatic or obstructive bronchitis varies greatly between nations. It should also be noted that the information of the asthma diagnosis was collected via parent reported questionnaires instead of clinical examination. This might cause information bias in the study. Also, the definition of asthma used in the study was cumulative over time meaning that the definition inevitably includes also some children with transient wheezing.

Moisture damage

The fact that a trained civil engineer made the home inspections is one of the main strengths of the LUKAS study. Moisture damage or mould were recorded and allocated in each room separately (Karvonen et al. 2009).

The results of this study show that the location of the moisture damage or mould in the home is relevant as was suggested previously (Pekkanen et al. 2007). The significant associations with proinflammatory cytokines were seen for moisture damage or mould in the child's main living areas (in the kitchen, livingroom or child's bedroom) and in the bathroom.

The results of the home inspections were informed to the parents. It might be that the families with asthmatic children in homes with moisture damage/mould problems pay more attention to respiratory symptoms and that is why the children who had asthma diagnosis were excluded from the analyses of moisture damage and respiratory symptoms.

Inflammatory markers

There are multiple factors affecting the expression of inflammatory markers in the system. It is possible that a one-time measurement of the inflammatory markers is not sufficiently stable marker of the status of the immune system. However, to reduce the possible effect of background factors on inflammatory markers, all relevant confounders were intended to include in the statistical models. Viruses are often present in nasopharyngeal samples taken from healthy children. In the study there was neither clinical examination by a doctor nor viral sample collection of the children during the time of the blood extractions at any time point and thus there is a possibility that some children were suffering viral infection which on the other hand could have affected the cytokine levels and other inflammatory markers. It may also be that atopic children are more susceptible to viral infections which could affect the inflammatory markers. To reduce the possible effect of infections the children who had elevated CRP values indicating acute infection were excluded from the analyses.

The strength of the study is that there were results of various proinflammatory cytokines induced by different stimulations and CRP values at the same time point available. This provides the possibility to investigate the association between moisture damage and inflammation extensively and this study is the first one to investigate this association in children. The cytokine responses were assessed using whole blood samples. Whole blood culture contains circulating plasma components which may have an effect on specific stimulants. When using whole blood culture the precise number of leucocytes in different stimulation is not identical between individuals. Individual variation was controlled by standardising the measured cytokine concentration by the number of leucocytes.

Faecal calprotectin levels were measured at the age of two months. At this age, the immune system is immature and the gut is permeable. Moreover, the normal flora is not fully established yet. In normal state calprotectin levels are high in infancy and there are no accurate level for the elevated calprotectin that indicates a disease in the gut. A reference value of 50 µg/g calprotectin in faeces is used for healthy adults and children aged from four to 17 years according to the previous findings (Fagerberg et al. 2003). Levels of calprotectin were shown to be remarkably higher in infants under the age one month (Baldassarre et al. 2007) and, in general, approximately five times higher during the neonatal period than in adults or children over four years of age (Kapel et al. 2010).

6.4. Future perspectives

The LUKAS and PASTURE birth cohorts are ongoing studies and future follow-ups will give more information on the association between early environment, immune system and allergic diseases during childhood. Longer follow-ups will also provide the possibility to investigate the long-term effects of moisture damage and mould problems in home and immune system and allergic diseases later in life.

In the future there is a possibility to investigate the effect of the low-grade inflammation on the immune system later in life and the long term effects of the low-grade inflammation on the allergic diseases. Also, the association between moisture damage in infancy and inflammatory markers at the age of six years will be investigated.

7 CONCLUSIONS

Based on the results presented in this thesis, following conclusions can be drawn:

1. The present study was able to show associations between environmental factors and serum CRP levels. However, the results suggest that the role played by environmental factor in low-grade inflammation may differ between atopic and non-atopic children and be different at different ages. Farming environment did not significantly induce systemic low-grade inflammation but was associated with intestinal inflammation in children.
2. The study suggests that moisture damage or mould at home may be capable of increasing the levels of proinflammatory cytokines in peripheral blood of children.
3. Systemic low-grade inflammation measured with serum CRP at early age may protect from atopy later in life. This raises the hypothesis that the down-regulation of inflammatory response to environmental triggers of inflammation may be a fundamental characteristic of the immune response of a host, predisposed to the development of atopy.
4. The results suggest that intestinal inflammation at early life predicts the development of allergic diseases later in life. The results also suggest that the early microbial colonization is an important regulator of the intestinal inflammation.

The present study shows the protective role of low-grade inflammation from atopy. The study provides new perspective to the development of allergic diseases by showing that intestinal inflammation during the first months of life, predicts asthma and atopic dermatitis. Furthermore, the present study shows the association between moisture damage and mould in home and hyperresponsiveness of the innate immunity in children. These results are novel and need to be confirmed in the future.

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