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MICROORGANISMS IN INFANCY AND DEVELOPMENT OF ALLERGY: COMPARISON OF ESTONIAN AND SWEDISH CHILDREN

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications and some unpublished data:

I Voor T, Julge K, Böttcher MF, Jenmalm MC, Duchén K, Björkstén B. Atopic sensitization and atopic dermatitis in Estonian and Swedish infants. Clin Exp Allergy. 2005; 35: 153–9.

II Voor T, Julge K. Atoopilise sensibiliseerumise ja allergiahaiguste kujunemine Eesti ning Rootsi väikelastel ja seda mõjutavad tegurid. Eesti Arst. 2004; 160–7.

III Björkstén B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol. 2001; 108: 516–20.

IV Sepp E, Naaber P, Voor T, Mikelsaar M, Björkstén B. Development of intestinal microflora during the first month of life in Estonian and Swedish infants. Microbial Ecology in Health and Disease. 2000; 12: 22–6.

V Sepp E, Voor T, Julge K, Lõivukene K, Björkstén B, Mikelsaar M. Is intestinal microbiota bound up with changing lifestyle? (submitted)

VI Böttcher MF, Björkstén B, Gustafson S, Voor T, Jenmalm MC. Endotoxin level in Estonian and Swedish house dust and atopy in infancy. Clin Exp Allergy. 2003; 33: 295–300.

DEFINITIONS AND ABBREVIATIONS

Allergen is an antigen with the capacity to elicit immunoglobulin (Ig) E antibody formation

Allergy is a hypersensitivity reaction initiated by immunologic mechanisms involving IgE antibodies.

Atopy is a tendency to become sensitised and produce IgE antibodies in response to exposure to allergens. In this thesis, atopy was documented by the presence of allergen-specific IgE antibodies in plasma and/or by a positive skin prick test.

A manifestation of IgE-mediated **allergic diseases** includes atopic eczema, bronchial asthma, allergic rhinoconjunctivitis, urticaria, anaphylaxis, and food allergy.

Atopic eczema is typically located pruritic eczema with a chronic or chronically relapsing course. In this thesis, the term **atopic dermatitis** has been used instead and it was defined as pruritic, chronic, or chronically relapsing non-infectious dermatitis with typical features and distribution, as suggested by Hanifin and Rajka.

Bronchial asthma is a chronic inflammatory disorder of the airways involving reversible airflow limitation and increased airway responsiveness to stimuli resulting in recurrent episodes of symptoms, such as wheezing, breathlessness, chest tightness, and cough.

Allergic rhinoconjunctivitis is an IgE mediated inflammation and hypersensitivity symptoms of the nose and eyes e.g. itching, sneezing, blockage, increased secretion, and watery eyes.

β-LG	beta lactoglobulin
Bla g 1	*Blatella germanica* (cockroach allergen)
CD	cluster of differentiation
CM	cow's milk
CONS	coagulase negative staphylococci
CFU	colony forming unit
Der f 1	*Dermatophagoides farinae* 1 (house dust mite allergen)
Der p 1	*Dermatophagoides pteronyssinus* 1 (house dust mite allergen)
Est	Estonian
EU	endotoxin unit
EW	egg white

Fel d 1	*Felis domesticus* (cat allergen)
Can f 1	*Canis familiaris 1* (dog allergen)
HAV	hepatitis A virus
ISAAC	the International Study of Asthma and Allergies in Childhood
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IU	international unit
kU_A	kilounit, where A represents allergen specific antibodies
LAL	Limulus Amebocyte Lysate
LPS	lipopolysaccharide (endotoxin)
RSV	respiratory syncytial virus
SPT	skin prick test
SU	standardised unit
Swe	Swedish
TGF	transforming growth factor
Th	T-helper
TLR	toll-like receptor
TNF	tumour necrosis factor
Tr	T-regulatory

1. INTRODUCTION

The prevalence of allergic diseases has increased worldwide during the last decades and particularly among children in industrialised countries with a market economy (Åberg N *et al* 1995; Butland BK *et al* 1997; Devenny A *et al* 2004). To become allergic, both a genetic predisposition and repeated contact with allergens are required. The role of adjuvant factors in the development of allergy has been highlighted during the last years, and it has been suggested that microorganisms play a crucial role (Rook GA *et al* 1998; Yazdanbakhsh M *et al* 2002).

Estonia is a country with a low prevalence of allergies whereas in Sweden the prevalence of allergies and atopy is high. Comparative data about the prevalence of allergic diseases and atopy between Estonian and Swedish adults (Jõgi R *et al* 1998) and schoolchildren (Bråbäck L *et al* 1995) were available, but at the time of present study, there were no comparative studies about the development of immune responses and allergies from birth through early childhood in these two countries. However, a previous study by Julge *et al* (2001) found a relatively high prevalence of circulating IgE antibodies, but a low prevalence of positive skin prick tests in Estonian children during the first five years of life. These findings contrast to reports from Sweden, showing a good correlation between positive skin prick test and determination of circulating IgE antibodies (Jenmalm MC *et al* 1999). Significant differences in the intestinal microflora of Estonian and Swedish children (Sepp E *et al* 1997) and atopic and non-atopic children (Björkstén B *et al* 1999) were also established.

Estonia was culturally and geographically largely similar to Sweden before the Second World War. As a consequence of Soviet occupation for 50 years, however, the life style in Estonia did not change after the war to the same extent as in Sweden. Shortly before initiating this study Estonia regained independence providing an opportunity to study prospectively the impact of different environmental factors, particularly microorganisms, on the development of immune responses, atopic sensitisation and allergic diseases from birth up to five years of life in children in two neighbouring countries with low and high prevalence of allergic diseases.

2. REVIEW OF LITERATURE

2.1. EPIDEMIOLOGICAL ASPECTS

Several studies suggest that the prevalence of allergic diseases has increased during the second half of the 20th century in many countries, especially among children and young adults of industrialised and high-income countries (Burr M *et al* 1989; Ninan TK *et al* 1992; Åberg N *et al* 1995; Devenny A *et al* 2004). There are considerable geographical differences in the prevalence of allergy in children (ISAAC Steering Committee 1998) as well as adults (Janson C *et al* 2001). However, among older adults the differences are not as obvious as in younger age groups (Heinrich J *et al* 1998; Jõgi R *et al* 1998). According to the phase I study of International Study of Asthma and Allergies in Childhood (ISAAC), the highest prevalence was recorded in the United Kingdom, Australia, New Zealand, and the Republic of Ireland, followed by most centres in North, Central, and South America while the lowest prevalence was observed in the centres of several Eastern European countries, Indonesia, Greece, China, Taiwan, Uzbekistan, India, and Ethiopia (ISAAC Steering Committee 1998). A low prevalence of atopy has been reported in low-income countries (Riikjärv MA *et al* 1995; ISAAC Steering Committee 1998; Björkstén B *et al* 1998). It has been hypothesised that since the 1960s some factors have been related to the lifestyle in high-income but not in low-income countries, which has led to the higher prevalence of allergies in the Western populations.

The reunification of Germany provided a unique opportunity to examine the influence of different environmental factors on the development of allergic diseases and atopy in genetically similar populations with a different lifestyle. Living conditions in East Germany had changed rapidly since unification in 1990, and the East German population had adopted a Western lifestyle in quite a short period. Immediately after unification, the prevalence of asthma and allergic rhinitis was lower in children living in East as compared to West Germany (von Mutius E *et al* 1992, 1994). After five years the prevalence of allergic rhinitis and positive skin prick tests (SPT) had increased among children in East Germany to the same level as in West Germany (von Mutius E *et al* 1998) while bronchial hyperresponsiveness and flexural dermatitis were more common among children of the previous East Germany. The level of total immunoglobulin (Ig) E was higher in the children living in East Germany and circulating IgE antibodies against inhalants and foods were equally common in both countries (Weiland SK *et al* 1999). In another study the overall prevalence of circulating IgE antibodies against inhalant allergens did not increase, whereas the prevalence of strong sensitisation (radioallergosorbent test ≥17.5 kilo units (kU)/l) increased in East Germany (Heinrich J *et al* 2002).

A comparative study conducted at the beginning of the 1990s showed that symptoms of asthma and hay fever were less common in Estonian than in

Swedish adults, but other respiratory problems were more common (Jõgi R *et al* 1996). Atopy, defined by the presence of IgE antibodies, was more prevalent in Swedish as compared to Estonian adults, except in the age group of 20–24 years, where the prevalence of atopy in Estonia and Sweden was not significantly different (Jõgi R *et al* 1998). There is evidence that in recent years the prevalence of atopic sensitisation has increased among Estonian young adults (Raukas-Kivioja A *et al* 2003).

The first study about the prevalence of allergic diseases comparing Estonian children with children of other countries was conducted in 1992/1993. According to this study, allergic diseases and positive SPT were less common among Estonian as compared to Swedish schoolchildren (Bråbäck L *et al* 1995; Riikjärv MA *et al* 1995). The results were confirmed by ISAAC done in 1993/94, indicating a higher prevalence of atopy-related disorders among schoolchildren in Scandinavia than in Estonia (Björkstén B *et al* 1998), but the reasons for these differences were not clarified. During the research period from 1993/1994 to 2001/2002 a slight increase in the prevalence of flexural dermatitis was found among Estonian 6–7 and 13–14 years-old children (Annus T *et al* 2005). In the age group of 13–14 years also rhinoconjunctivitis during the pollen season became more frequent.

Interestingly, very recent studies have shown that the prevalence of bronchial asthma has remained stable or even slightly decreased in some developed countries in recent years (Robertson CF *et al* 2004; Lee SL *et al* 2004). However, this was not the case for flexural eczema and allergic rhinoconjunctivitis (Lee SL *et al* 2004; Anderson HR *et al* 2004).

Conclusion: The prevalence of atopy and clinical allergy has increased during the last decades. These changes are obviously caused by environmental factors in countries with a Western lifestyle.

2.2. GENETIC ASPECTS

Genetic predisposition is essential for the development of allergic diseases. In children without atopic heredity, around 10–20% develop allergic disease, whereas about one third of children with single atopic heredity and about 50–80% of infants with double parental heredity have been shown to develop atopic disease (Kjellman NI 1998). The stronger concordance of atopic diseases in homozygotic than dizygotic twins supports the hereditary basis of allergic diseases (Hopp RJ *et al* 1984). The risk for development of allergic diseases is higher in the case of maternal than paternal allergy and atopy (Litonjua AA *et al* 1998; Moffatt M *et al* 1998). Different chromosomes have been connected with allergy and/or atopy in genetic studies. Allergy and atopy susceptibility has been linked to at least 15 chromosomes (Cookson W 2002), and results of

genome screens provide evidence for at least 18 genes contributing to asthma/atopy (Hoffjan S *et al* 2002). However, no single chromosome or gene, which is solely responsible for allergies, has been identified. There is a general consensus that allergic diseases are polygenic, and there is a complex interaction between host susceptibility and environmental factors (Rosenwasser L 1996). More likely, multiple genes acting either alone or in combination with other genes increase the risk for atopic conditions after exposure to environmental triggers. Different combinations of allergy susceptibility genes interacting with varying external factors also regulate the onset of allergic diseases.

The importance of environmental factors in the development of allergies has been demonstrated in studies of immigrants. Immigration from developing to Western countries enhances allergic diseases, and atopy and clinical manifestation of allergy is correlated with adaptation and duration of residence in an industrialised country (Waite DA *et al* 1980; Leung RC *et al* 1994; Grüber C *et al* 2002). One reason for relationship between duration of residence and manifestation of allergic diseases might be lack of allergy-protective microbial agent due to improved hygienic life conditions in affluent countries, which might favour the appearance of allergy in genetically predisposed subjects (Ventura MT *et al* 2004).

Conclusion: although genetic factors are important for the development of allergic diseases, the increase of allergic diseases is too rapid to be explained by mutation in genetic factors.

2.3. DEVELOPMENT OF IMMUNE RESPONSES TO ALLERGENS

T-helper (Th) cells play a crucial role in the regulation of immune responses to allergens. The two subsets of Th cells – Th1 and Th2 are characterized by unique patterns of cytokine production and immune responses. Th1 cells play an important role in host defence against viral, bacterial, and fungal antigens (Lucey DR *et al* 1996). They produce interleukin (IL)-2, interferon (IFN)-γ, and tumour necrosis factor (TNF)-β and cooperate with B cells in the production of IgG1, IgG3 and IgM antibodies and activate phagocytic cells and $CD8^+$ T cells, thus promoting cell-mediated immunity and cytotoxic T-cell responses. Th2 cells play a critical role in the defence of helminthic infections (Lucey DR et al 1996). This type of infection requires recruitment and activation of eosinophils, as well as formation of IgE. Th2 cells produce IL-4, IL-5, IL-9, and IL-13 in the absence of IFN-γ and TNF-β production. Cytokines of Th2 cells induce B cells to produce IgG4 and IgE antibodies (IL-4) and promote the differentiation and growth of mast cells (IL-9) and eosinophils (IL-5) and inhibit several phagocytic functions and increase mucus production and induce airway

hyperreactivity (IL-13). IL-4 inhibits the development of Th1 cells, and IFN-γ inhibits the development of Th2 cells (Romagnani S 2004).

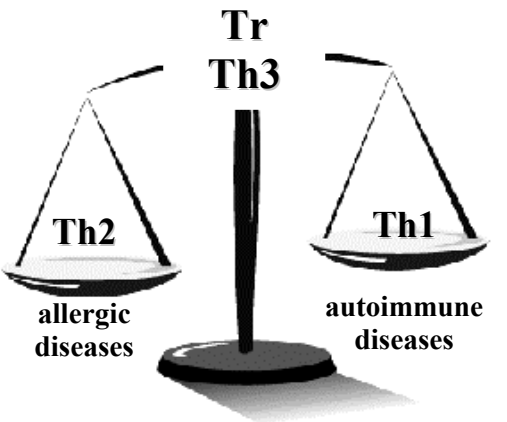
Allergic diseases are associated with allergen-specific Th2 responses. The wheal size of SPT with allergen is positively associated with in-vitro IL-5 and INF-γ responses and negatively associated with IL-10 (Heaton T *et al* 2005). However, cytokines produced by Th1 cells are pro-inflammatory, and the Th1-associated inflammatory response could exacerbate allergic disease (Hansen G *et al* 1999). Furthermore, IFN-γ is often present at sites of allergic inflammation, e.g. atopic dermatitis (Grewe M *et al* 1994; Thepen T *et al* 1996).

At birth, the production of cytokines by lymphocytes in response to allergens is polarised towards a Th2 response. Many children, regardless of whether they will become atopic or non-atopic, transiently produce IgE antibodies in infancy (Hattevig G *et al* 1984; 1993). Infants who either exhibited symptoms of atopic disease, or had a positive skin test at one year of age, produced significantly less IFN-γ at birth as compared to non-atopic infants (Prescott SL *et al* 1999). During the first year of life, non-atopic children develop a Th1 pattern similar to adults and by one year of age there is an increase in the production of IFN-γ and a decrease in the production of IL-10 and Th2 cytokines by peripheral blood mononuclear cells. In contrast, atopic children produce lower concentrations of both IFN-γ and Th2 cytokines at birth and over the next two years the increase in the production of IFN-γ is less than in non-atopic children while the production of Th2 cytokines does not decrease to the same extent. These findings suggest that the ability to produce Th1-cytokines in early life may predict a child's susceptibility to later atopic disease. Studies have suggested that initial sensitisation or events that modulate asthma or atopy occur in very early life. Cytokine production profiles reflecting the Th1 and Th2 cell balance in atopic and non-atopic children become similar at 4 to 5 years of age, suggesting that the early years of life, when Th2 responses may dominate in the atopic child, may be critical for atopic sensitisation.

However, the pattern of immune responses is not so clearly polarised. As was shown by Renz et al (2002), individuals who grew up in East Germany had a marked bias towards Th0 responsiveness regardless of whether they were atopic or not. In contrast, children living in West Germany, particularly when they were atopic showed Th2 polarisation (Renz H *et al* 2002). Thus, Th2-type immune responses might be attributable to some environmental influences prevalent in Westernised populations, and in the absence of these factors immune responses follow the Th0 rather Th2 pattern. Cytokine studies carried out in Estonian and Swedish children indicate Th0 type immune responses in Estonian infants (unpublished data). The Estonian infants had a lower prevalence of positive SPT but a higher prevalence of circulating IgE antibodies (Julge K *et al* 1997). Studies of atopy and delayed type hypersensitivity are also consistent with recent observation that Th1 and Th2 responses to allergens are enhanced in atopic Estonian children (Julge K *et al* 2002).

Developed countries have witnessed a simultaneous increase in both allergies and Th1-type immune diseases, such as inflammatory bowel diseases, type 1 diabetes, and multiple sclerosis (Lindberg E *et al* 2000; Sawczenko A *et al* 2001; Stene LC *et al* 2001). There was reported a very recent increase in the prevalence of type 1 diabetes among children under five years of age in Estonia (Podar T *et al* 2001). Therefore, the key role in immune responses has been ascribed to regulatory cells, including Th3 cells, T regulatory (Tr) 1 cells, $CD4^+CD25^+$ cells (Umetsu DT *et al* 2003). Th3 cells mainly produce transforming growth factor (TGF)-β, and their regulatory function is attributable to a TGF-β-dependent mechanism, whereas Tr1 cells are mainly able to produce IL-10, with or without TGF-β (Romagnani S 2004). The functions of $CD4^+CD25^+$ T cells may induce the differentiation of IL-10 and TGF-β-producing Tr1 cells (Akbari O *et al* 2003). Tr1 cells inhibit Th2 responses, as well as Th1 responses (Cottrez F *et al* 2000). Regulatory T cells may suppress the development of allergy and asthma by providing anti-inflammatory responses.

Conclusion: allergic diseases and atopy are associated with an imbalance in the immune system, as shown in the picture, and Tr and Th3 cells play crucial role in the regulation of balance of the immune responses.



2.4. ENVIRONMENTAL FACTORS AND ALLERGIC DISEASES

In addition to genetic predisposition, the development of allergic diseases requires the contact with an allergen for the development of sensitisation and repeated contact with the same allergen for the manifestation of allergic diseases. Besides this an important role has been ascribed to adjuvant factors that offer protection or promote allergy.

Until the end of the last century, exposure to an allergen in infancy was considered to be a major risk factor for atopic sensitisation and allergy. Recent studies have shown, however, that exposure to the allergen is not a risk factor by itself for the development of allergy. Conversely, high allergen exposure in infancy might even be protective against allergic diseases.

2.4.1. Exposure to indoor allergens

In 1999 Hesselmar and co-workers reported that pet exposure during the first year of life was associated with a lower prevalence of allergic rhinitis and asthma in schoolchildren (Hesselmar B *et al* 1999).

Several subsequent studies carried out in Western Europe also showed that close contact with animals and growing up in the farming environment offered some protection against allergic diseases and sensitisation (Braun-Fahrländer C *et al* 1999; Riedler J *et al* 2000; Ownby DR *et al* 2002; Perzanowski MS *et al* 2002; Almqvist C *et al* 2003; Oryszczyn MP *et al* 2003). Having a dog in infancy is associated with higher IL-10 and IL-13 cytokine secretion and reduced allergic sensitisation and atopic dermatitis, suggesting that postnatal exposure to dogs can influence immune reactions (Gern JE *et al* 2004).

The immune system produces IgG and IgG4 antibodies without IgE synthesis in response to exposure to a high level of pet allergens, which has been considered to be an allergy-protective immune response (Platts-Mills TA *et al* 2004). However, this might be the case for animal allergen but not for the house dust mite, and the cockroach (Sporik R *et al* 1999; Platts-Mills TA *et al* 2001; Custovic A *et al* 2001; Ownby DR *et al* 2002). It has been suggested that the protective effect of pets may to some extent be explained by a healthy worker effect as parents with symptoms of asthma or allergy tend to remove pets from the home for controlling their condition (Apelberg BJ *et al* 2001; Bornehag CG *et al* 2003). In the study of Hesselmar *et al* (1999), however, this possibility was assessed and found to be a minor significance.

On the other hand, some studies show that exposure to pets is a risk factor for the development of allergy and especially when pet keeping is delayed until adulthood (Wickens K *et al* 2002; de Meer G *et al* 2004). Also, pet keeping was a risk factor for the development of allergic diseases in individuals growing up in areas with a low community prevalence of pets (Svanes C *et al* 2003; Pescollderungg L *et al* 2000).

A threshold level for the induction of sensitisation has been proposed for the main household allergens (house dust mites - *Der p 1* and *Der f 1* and German cockroach - *Bla g 1*) (Sporik R *et al* 1990; Sarpong SB *et al* 1996). However, there is no general exposure threshold for any allergen. The outcome after the exposure depends on the genetic predisposition of the person and environmental factors. In children with a positive family history of allergy, very low levels of allergen exposure might already be sufficient for sensitisation (Munir AK *et al* 1997; Wahn U *et al* 1997; Cullinan P *et al* 2004).

2.4.2. Exposure to pollen

Exposure to high levels of birch pollen in infancy increases the risk of sensitisation to the same allergen and the risk of airway allergies (Björkstén F *et al* 1976; Zwick H *et al* 1991; Guerra S *et al* 2002; Kihlström A *et al* 2002). Some other studies have shown that children born shortly before or during the pollen season develop allergic rhinoconjunctivitis less often, and they are less often sensitised to pollen than those born in other times of the year (Nilsson L *et al* 1997; Hesselmar B *et al* 2001). ISAAC data show that exposure to allergenic pollen in early life does not increase the risk of respiratory allergy and may even offer some protection (Burr ML *at al* 2003).

Immune responses to pollen have been demonstrated in children regardless of their atopic status (Hattevig G *et al* 1993), and a transient early Th2-like response is down regulated, except in children who develop clinical allergy to the particular allergen (Böttcher MF *et al* 2002).

2.4.3. Diet

About 6% of infants suffer from food allergy, and usually they grow out of this problem, as the prevalence of food allergy in adults is only 1–2% (Sampson HA 2003). The phenomenon called oral tolerance plays the key role in the development of tolerance to food allergen. Only few studies describe the relationship between the amount of food allergen and the development of allergic diseases. An animal study by Strid J *et al* (2004) showed that oral administration of a high dose of allergen induced hyporesponsiveness, whereas administration of a low dose of allergen caused an allergic reaction. However, this phenomenon is strictly allergen-specific and has not been proved in humans yet.

Breast milk is the first and optimal food for the baby. There are studies suggesting that breastfeeding decreases the risk of asthma and allergic diseases (Oddy WH *et al* 2002; Kerkhof M *et al* 2003) and the protective effect appears stronger in children with atopic heredity (van Odijk J *et al* 2003). However, some studies failed to confirm the protective effect of breastfeeding (Rust G *et al* 2001) or even suggest an increased risk of asthma and eczema associated

with breastfeeding, particularly if the mother has allergy (Wright AL *et al* 2001; Bergmann RL *et al* 2002; Sears MR *et al* 2002). Allergy preventive effect of breastfeeding is contradicted by the fact that in many countries breastfeeding has been become more popular, and the duration of breastfeeding has increased (Callen J *et al* 2004; Ryan AS *et al* 2002; Ministry of Social Affairs of Estonia), at the same time allergies are becoming increasingly common.

It is unclear how breastfeeding could prevent allergy. Suggested mechanisms include low content of allergen and immune modulation (Böttcher MF 2002). Recently it has been suggested that breastfeeding may offer some protection against allergy by modifying the microflora of the gut (Böttcher MF *et al* 2002). *Bifidobacteria* and *Lactobacilli* dominate in the gut microflora of the breastfed infant, whereas the gut flora of a formula-fed infant contains more *Bacteroides*, *Clostridia* and *Enterobacteriaceae* (Edwards CA *et al* 2002). Intestinal microbes such as *Bifidobacteria* and *Lactobacilli* are considered to exert a beneficial response on the immune system. High levels of iso-caproic acid, which are associated with *Clostridium difficile*, were found in the stool samples of children who developed atopic disease (Böttcher MF *et al* 2000). Atopic children had lower numbers of *Bifidobacteria* in their faeces than healthy children (Kalliomäki M *et al* 2001). In addition, compared to formula breast milk contains lower ratios of n-6/n-3 fatty acids, which reduce allergic inflammatory mediators.

There are studies showing the importance of home-grown and home-made food. Among children who grew up on a farm, the drinking of raw milk was related to less atopy and allergy (Riedler J *et al* 2001). Raw milk contains more bacteria and lipopolysaccharide (LPS) than pasteurised milk. Therefore, the protective factor associated with the consumption of farm milk could be associated with ingestion of non-infectious microbial components resulting in changes in the commensal gut flora (Riedler J *et al* 2001). Swedish children attending an anthroposophical school had a lower prevalence of atopy and allergic diseases as compared to children of conventional schools. One reason for this difference could be the diet associated with an anthroposophical lifestyle, which includes a lot of fermented vegetables and organic or biodynamic food (Alm JS *et al* 1999, 2002).

Conclusion: The influence of early exposure to animal and pollen allergen on the development of allergies and sensitisation are contradictional. Diet may influence bacterial flora of the gut and thereby maturation of the immune response.

2.5. MICROORGANISMS AND ALLERGY

In 1976 John Gerrard claimed that "Atopic disease is the price paid by some members of the white community for their relative freedom from diseases due to viruses, bacteria and helminths" (Gerrard JW *et al* 1976). Several years later, David Strachan observed that children from large families suffered less from allergic diseases and suggested that the risk of allergy was reduced by infectious diseases in infancy transmitted by older siblings (Strachan DP 1989). This formed the basis for the so-called hygiene hypothesis.

Since then the understanding of the hygiene hypothesis has been extended to postulate that overall microbial pressure early in life protects against atopy and allergy by stimulating Th1 and inhibiting Th2 immune responses or/and induction of Tr cells.

2.5.1. Infections

Respiratory infections

The influence of respiratory infections on allergy is controversial. A lower prevalence of positive SPTs was reported in tuberculin-positive than in tuberculin-negative children, suggesting that *Mycobacteria* could inhibit sensitisation (Shirakawa T *et al* 1997). However, Swedish and German studies did not support this hypothesis (Strannegård IL *et al* 1998; Grüber C *et al* 2001). The reason for contradictory results could be explained by the different immune responses in atopic and non-atopic children. The cutaneous induration in tuberculin testing is a Th1-type dependent reaction. Individuals who develop asthma and atopy have Th2-type immune responses to antigens, including *M. tuberculosis.* Possibly, therefore Japanese atopic children tended to be tuberculin-negative (Grüber C *et al* 2001).

Some studies suggest a protective effect of measles on atopy and allergy (Shaheen SO *et al* 1996; Lewis SA *et al* 1998; Alm JS *et al* 1999); other studies do not confirm this or even show an increased risk for atopy after measles (Paunio M *et al* 2000; Matricardi PM e*t al* 2000; Bager P *et al* 2002).

In theory, the greater is the exposure to other children, the greater is the likelihood of exposure to infectious agents. The Tucson Children's Respiratory Study revealed that children with older siblings or who attended day-care centres in infancy were more likely to have recurrent wheezing at 2 years of age (Ball TM *et al* 2000). However, these children were less likely to suffer from wheezing from 6 to 13 years of age, and they had lower total serum immunoglobulin E levels, and a lower prevalence of atopy at the age of six. Respiratory syncytial virus (RSV), parainfluenza virus, adenovirus, and influenza virus were identified as the most common causes of lower respiratory tract diseases in infants and children. RSV infection in infancy is a risk factor for wheezing and subsequent development of bronchial asthma (Pullan CR *et al* 1982; Sigurs N *et*

al 2005). The role of RSV in the development of atopy is unclear, however. RSV infection can increase airway hyperresponsiveness associated with increased numbers of eosinophils in the airways (Dakhama A *et al* 1999) and also enhance the sensitisation to allergens (Sigurs *et al* 1995, Schwarze J *et al* 1997). Some studies do not confirm the relationship between RSV and allergy (Stein RT *et al* 1999). However, there are studies consistent with the hygiene hypothesis showing that repeated viral infections in the upper respiratory tract, but not infections in the lower respiratory tract in early life, may reduce the risk of developing asthma up to school age (Illi S *et al* 2001).

Despite conflicting data about respiratory infections and development of allergic diseases, probably respiratory infections are not protective against allergy. Moreover, there is also some doubt about the hygiene hypothesis, and it has been suggested that allergic individuals are less susceptible to infections due to the Th2-type immune response (Varner AE 2002). A recently published large Danish cohort study confirmed an inverse association between atopic dermatitis and the number of siblings, early day care, pet keeping, and farm residence while infections were associated with atopic dermatitis, suggesting that these effects are mediated early in life and independently of clinically apparent infectious diseases (Benn CS *et al* 2004).

Gastrointestinal infections

There is evidence that gastrointestinal infections or related factors may influence the development of allergies. Cullinan *et al* (2003) found an inverse relationship between the number of gastrointestinal infections during the first five years of life and the likelihood of atopy, recorded as positive skin prick tests in adults. In contrast, there was a positive association between atopy and the number of respiratory infections. In comprehensive epidemiological studies Matricardi PM e*t al* (2000, 2002) found that atopy and respiratory allergies were inversely related to exposure to orofaecal or food-borne infections such as *Toxoplasma gondii*, *Helicobacter pylori*, hepatitis A virus [HAV] but not to viruses transmitted through other routes such as measles, mumps, rubella, chickenpox, cytomegalovirus, herpes simplex virus type 1. A Danish study revealed that different groups of food-borne and orofaecal microorganisms could have different effects on the risk of atopy. Seropositivity to markers of poor hygiene (*Toxoplasma gondi, Helicobacter pylori,* HAV) was associated with a lower prevalence of atopy whereas seropositivity to intestinal bacterial pathogens (*Clostridium difficile, Campylobacter jejuni,* and *Yersinia entero-colitica*) was associated with a higher prevalence of atopy (Linneberg A *et al* 2003). In Finland, the prevalence of IgE antibodies increased 3.5-fold from 1973 to 1994, and was seen mainly in a subpopulation without *Helicobacter pylori* antibodies (Kosunen TU *et al* 2002). Seropositivity to HAV, *Helico-bacter pylori*, and *Toxoplasma gondii* is a marker of a less hygienic lifestyle (including consumption of foods without preservatives and unpasteurised products and close contact with animals) with a high exposure to microbes,

which stimulates the immune system and could thus protect against atopy (Linneberg A *et al* 2003).

There is at least one more explanation beside a less hygienic environment how infection could protect against atopy. Before 1970 the seroprevalence of antibodies against HAV was high in Western countries. The improvement of hygiene and living conditions in less crowded homes caused a drop in HAV prevalence (Böttiger M *et al* 1997; Briem H *et al* 1982), and at the same time the prevalence of atopy increased. Chromosome 5q23–35 has been linked to atopy and asthma, and the T cell membrane protein (TIM)-1 is a highly polymorphic gene in this region (McIntire JJ *et al* 2001). TIM-1 is expressed by activated $CD4^+$ T cells during the development of Th2 responses and regulates cytokine production. Since TIM-1 functions as the cellular receptor for HAV, activation of T cells through TIM-1 by HAV or by its natural ligand may affect T cell differentiation and down-regulate the Th2-driven allergic inflammatory responses (McIntire JJ *et al* 2003).

2.5.2. Antibacterial treatment

Several studies demonstrate a relationship between antibacterial treatment in infancy and allergy in later life (Farooqi IS *et al* 1998; Alm JS *et* al 1999; von Mutius E *et al* 1999; Wickens K *et al* 1999; Droste JH *et al* 2000; Illi S *et al* 2001; McKeever TM *et al* 2002). The risk of allergic diseases was especially obvious when antibiotics were given before the age of 2 years (Farooqi IS *et al* 1998; Droste JH *et al* 2000; von Mutius E *et al* 1999; Wickens K *et al* 1999) and broad-spectrum antibiotics were used (Farooqi IS *et al* 1998; McKeever TM *et al* 2002).

Even prenatal exposure to antibiotics may increase the risk of atopic disease (McKeever TM *et al* 2002, Benn CS *et al* 2002). A possible explanation for these findings could be that antibiotics may inhibit immune modulation or disturb the normal gut microflora. For example, antibiotic treatment may decrease the counts of anaerobic organisms (*Bifidobacteria*, *Lactobacilli* and *Bacteroides*) and may increase the counts of potentially harmful microbes, such as *Clostridium difficile* and the yeast *Candida albicans* (Sullivan A *et al* 2001). When antibiotics alter the composition of the bacterial flora, toll-like receptor (TLR) 4 wild-type mice become as susceptible to the induction of allergy as their TLR4-mutant counterparts (Bashir ME *et al* 2004). Both allergen-specific IgE and Th2 cytokine responses were reduced in antibiotic-treated mice where the microflora had been allowed to repopulate. TLR4-dependent signals provided by the intestinal commensal flora inhibited the development of allergic responses to food antigens.

However, some studies do not confirm the claim that antibacterial treatment might be a risk factor for the development of allergic diseases. The authors hypothesise that lower airway infections are similar to the first asthma symptoms and therefore patients with asthma have been treated repeatedly with

antibiotics before they are diagnosed as asthmatics (Cullinan P *et al* 2004; Celedon JC *et al* 2002).

2.5.3. Microbial products from the environment

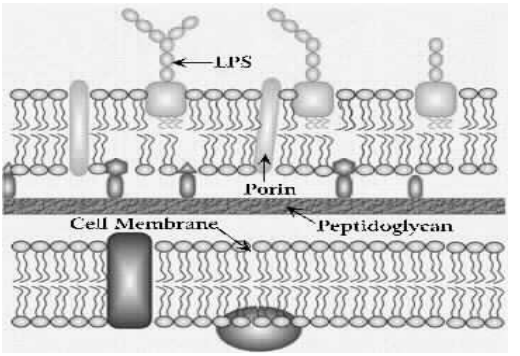


Figure 1. Schematic picture of cell wall of Gram-negative bacteria

Endotoxin

Bacterial LPS (endotoxin) are major outer surface membrane components that are present in almost all Gram-negative bacteria (figure 1). Endotoxin can cause various clinical symptoms from fever to septic shock (McCartney AC *et al* 1983). Contact with endotoxin stimulates the production of mediators such as TNF-α and IL-1, IL-12 and IFN-γ (Verhasselt V *et al* 1997). IL-12 is an obligatory signal for the maturation of naive T cells into Th1-type cells. The allergy-protective effect of endotoxin might be modified by variations in the gene encoding TLR4, an important LPS receptor on antigen-presenting cells, macrophages, and monocytes (Eder W *et al* 2004). Until now, ten mammalian TLRs and several of their ligands have been identified. TLR2 interacts with peptidoglycan, lipopeptides and other products from Gram-positive bacteria, acid-fast bacteria and fungi, as well as LPS from selected bacterial species, whereas TLR4 recognises LPS produced by most Gram-negative bacteria (Takeda K *et al* 2003).

Animal studies have shown that endotoxin might protect against atopy and asthma. In a rat study, inhalation of endotoxin during primary allergen exposure in the presence of a Th2-promoting adjuvant factor prevented allergic sensitisation, whereas inhalation of endotoxin after sensitisation aggravated the inflammatory airway responses (Tulic MK *et al* 2000, 2002; Watanabe J *et al* 2003).

Human studies have shown that exposure to high levels of house dust endotoxin was associated with a lower prevalence of allergic sensitisation in children (Gereda JE *et al* 2000; Gehring U *et al* 2002). Contact with animals increased concentrations of endotoxin at home (Park JH *et al* 2001; Heinrich J *et al* 2001). Children who grew up in farms and had close contact with livestock

had less atopy and asthma than children from other environments, and it has been suggested that exposure to endotoxin plays a crucial role for this (Riedler J *et al* 2001; Gereda JE *et al* 2001). The risk of developing atopic eczema was lower during the first six months, but not during the first year of life in German infants exposed to high endotoxin levels (Gehring U *et al* 2001).

On the other hand, exposure to endotoxins may induce wheezing (Litonjua AA *et al* 2002; Eduard W *et al* 2004). In children with atopic parents, exposure to high levels of endotoxin was associated with an increased risk of wheezing in early life. However, the risk was reduced in older children. Exposure to microbial products, such as endotoxin, can induce strong neutrophilic airway inflammation and subsequent non-IgE mediated reversible airflow obstruction (Douwes J *et al* 2002).

Muramic acid

A major component in the peptidoglycan of bacterial cell wall is muramic acid, which is not found elsewhere in nature (Black GE et al 1994). Gram-positive bacteria have a thick peptidoglycan layer with no outer membrane, whereas Gram-negative type has inner and outer membranes with a thin peptidoglycan layer (figure 1). Muramic acid constitutes a major part of the cell wall of Gram-positive bacteria and occurs also in the cell wall of Gram-negative bacteria, but in much smaller amounts. Therefore, muramic acid may be more representative than endotoxin in estimation of total microbial exposure. Peptidoglycan activates innate immunity via TLR-2, which induces a functionally different cellular response than TLR-4, which is the endotoxin receptor. Stimulation of TLR-2 enhances the production of TNF-α, IL-1, IL-6, and IL-10 by human monocytes although the necessary dose of muramic acid is very high compared to lipopolysaccharides (van Strien RT *et al* 2004). Farm children are exposured to higher levels of muramic acid than non-farm children and high concentrations of muramic acid in mattress dust were associated with a lower frequency of wheezing among rural school children (van Strien RT *et al* 2004).

2.5.4. Intestinal microflora and oral tolerance

The commensal microflora (normal microflora, indigenous microflora) consists of microorganisms present on body surfaces covered by epithelial cells and are exposed to the external environment (gastrointestinal and respiratory tract, vagina, skin, etc.). The mucosa covers in an adult human an area about 300 m^2 while skin covers approximately 2 m^2 and the number of bacteria colonising mucosal surfaces exceeds the number of cells in the human body (Holzapfel WH *et al* 1998). The normal human microflora comprises mainly bacteria, but viruses, fungi, and protozoa are also present. Commensal bacteria include more than 400 species (Moore WE *et al* 1974); however, about 50% of the bacteria of

the intestinal microflora are non-cultivable. Therefore, molecular methods are increasingly used to analyse gut microflora (Tannock GW 2001). Obligate anaerobes constitute more than 90% of the intestinal bacterial population. Predominant species include *Bacteroides*, *Eubacteria, Bifidobacteria, Fusobacteria*, *Peptostreptococci* and others (Savage DC *et al* 1999). *Escherichia coli*, *Enterobacteria,* and *Lactobacilli* are also regularly present.

The composition of the intestinal microflora is variable and depends on different factors such as residence of the person (Adlerberth I *et al* 1991) and diet (Drasar BS *et al* 1973; Alm JS *et al* 2002). The intestine is the largest immune organ containing around 80% of all antibody-producing cells (Tlaskalová-Hogenová H *et al* 2002). The intestinal microflora plays a crucial role in the postnatal development of the immune system. Colonisation of the child with microorganisms begins immediately after birth and a newborn receives the first microorganisms from the mother's birth canal and the environment. The bacterial flora of a newborn's intestinal tract is usually heterogeneous during the first days of life; however, the initial colonisation includes aerobic or facultatively anaerobic bacteria such as *Enterobacteria*, *Lactobacilli,* and *Streptococci* (Rotimi VO *et al* 1981). The colonisation pattern changes over time and the amount of such anaerobes as *Bacteroides*, *Bifibobacteria*, *Clostridia* and anaerobic cocci increases (Rotimi VO *et al* 1981; Fanaro S *et al* 2003). During the early postnatal period intestinal microflora is essential for the maturation of both local and systemic immunity. Later on, the microflora keeps both mucosal and systemic immunity in balance by regulatory mechanisms (Tlaskalová-Hogenová H *et al* 2002). It has been shown that infants who were colonised with *Bacteroides* after birth had elevated numbers of IgA-secreting and IgM-secreting cells in peripheral blood. Thus, microbes of the normal gut microflora stimulated the maturation of IgA-secreting cells, the first line immune protection against foreign antigens at the mucosal membranes (Grönlund MM *et al* 2000).

A comparative study revealed differences in the gut microflora in Estonian and Swedish 1-year-old children (Sepp E *et al* 1997). High counts of *Lactobacilli* and *Eubacteria* were established in the Estonian and high numbers of C*lostridia* in the Swedish infants, indicating a disturbed microbial balance in Swedish infants.

Studies have shown an association between the composition of the gut microflora and the development of allergic diseases. For example, allergic children had in stool fewer *Bifidobacteria* and more *Clostridia* (Kalliomäki M *et al* 2001) and more often isocaproic acid, an indicator of *Clostridium difficile,* were deteced (Böttcher M *et al* 2000) as compared to healthy infants. Later, in two and five years of age allergic and non-allergic children still maintained the differences in the composition of gut microflora (Björkstén B *et al* 1999, Sepp E *et al* 2005).

Studies with probiotics, defined as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-

being of the host, support the relationship between intestinal microbes and allergic diseases. Some strains of *Lactobacilli* decrease the production of IgE in animals (Matsuzaki T *et al* 1998; Ishida Y *et al* 2003) and also reduce clinical symptoms of atopic dermatitis and food allergy in children (Majamaa H *et al* 1997; Isolauri E *et al* 2000; Rosenfeldt V *et al* 2003; Pohjavuori E *et al* 2004).

Oral tolerance

Humans ingest large amounts of different food antigens, but only a small percentage of people suffer from food allergy. This is due to the development of oral tolerance, that is, inhibition of immune responses to ingested antigens.

Oral tolerance after exposure to low concentrations of antigens is mediated through suppression of Th1 cells by IL-4 and IL-10 (Smith KM *et al* 2000). Exposure to high doses of antigen promotes clonal anergy or deletion mediated by TGF-β, which turns T cells into a state of cellular unresponsiveness (Melamed D *et al* 1993). Alternatively, regulatory cells or mediators modifying the immune response may be induced. A number of cytokines, including INF-γ and IL-10, have been identified as important for the development of oral tolerance (Kweon MN *et al* 1998). Animal studies have shown that in the condition of tolerance the mice have a higher number of IgA-secreting cells in Peyer´s patches (Frossard CP *et al* 2004). TNF-α knockout mice, lacking Peyer's patches and mesenteric lymph nodes, are not able to develop oral tolerance (Spahn TW *et al* 2001). On the other hand, the administration of flt3L, a growth factor for dendritic cells, promotes the development of oral tolerance (Viney JL *et al* 1998).

Sudo *et al* (1997) demonstrated the importance of intestinal flora for the induction of oral tolerance. Neonatal germ-free mice became tolerant to food antigens only in the presence of intestinal microflora. This was possible only if normal gut microflora was added during the neonatal period and not later.

Conclusion: it is reasonable to suppose that infectious agents do not protect against allergies, but the harmless microorganisms might be crucial for immune regulation. In this respect the intestinal microflora could play a critical role in the development of atopy and allergies. Factors that affect the colonisation of the gut may have an impact on the development of atopic sensitisation and allergic diseases.

3. AIMS OF THE STUDY

1) to compare clinical manifestations of allergy during the first five years of life in Estonian and Swedish children (I, II);
2) to examine the development of immune responses to allergens in Estonian and Swedish children (I, II);
3) to evaluate the relationship between the development of allergic diseases and gut microflora (III, IV, V);
4) to estimate the influence of exposure to microbial components in early life and the development of sensitisation and allergic diseases (I, II, VI).

4. SUBJECTS AND METHODS

4.1. STUDY GROUPS (I–VI)

Two study groups, one in Estonia and the other in Sweden, were recruited from the general population. The midwives of the maternity clinics in Tartu, Estonia, and Linköping, Sweden, informed pregnant women and their families about the study.

The inclusion criteria were: normal pregnancy, delivery at term and without complications, uneventful early neonatal period and parents´ agreement for participation.

123 Estonian and 150 Swedish families were invited to participate in the study, and 115 Estonian and 138 Swedish children met the inclusion criteria, and parents agreed to participate in the study. The Estonian children were born at Tartu University Women's Clinic during the period February 1997 – June 1998, and the Swedish children were born between March 1996 and March 2000 at Linköping University Hospital. The number of children participating in the different follow-ups is given in table 1. The main reason for dropping out was moving to another district.

Table 1. Number of participating children in the different study phases

Study group	invated to the study	entered into study	1yr follow-up	2 yrs follow-up	5 yrs follow-up
Estonian	123	115	109	104	102
Swedish	150	138	130	123	110

The mother/parents of the child were contacted after the birth of the baby, and a family history of allergy was obtained according to a questionnaire. A positive family history was defined as a history of allergic rhinitis, asthma, or flexural, itching dermatitis in the parents.

The children were followed during the first 5 years of life according to the study plan provided in table 2.

Table 2. The study plan for the prospective studies in Estonia and Sweden

	Birth	1 mo	3 mo	6 mo	1 yr	2 yrs	5 yrs
Questionnaires	+		+	+	+	+	+
Clinical examination			+	+	+	+	+
Skin prick tests			+	+	+	+	+
Blood sample			+	+	+	+	+
Stool sample	+	+	+	+	+	+	+
Dust samples	were collected during the first winter season.						

4.2. QUESTIONNAIRES (I–III, VI)

Before each follow-up visit the parents completed questionnaires. The data analyses in present study were based on the following questions:

- about symptoms of allergic diseases
  - Has the child had skin rash? Mark the location of the rash in the picture? Does the rash itch? Is the rash connected with something (infection, food, pet, etc)?
  - Has the child wheezed? How many times? Is wheezing connected with something (infection, food, pet, pollen, etc)?
  - Has the child sneezed and/or had watery eyes after exposure to pet, pollen?
  - Has the child often had blocked nose?
- diet
  - Are you breastfeeding the child? If no, how old was the child when you finished breastfeeding?
  - Did the child get infant formula? If yes, when was the first time?
  - Did the child get solid food? If yes, when was the first time?
  - Has the child got cow's milk? If yes, when was the first time?
  - Has the child eaten hen's egg? If yes, when was the first time?
- infections and the use of antibiotics
  - Has the child had any infections (inflammation of ear, eye, flu)? How many times?
  - Has the child received any medication? Name of the medication? How many times?
- living conditions
  - How many rooms and m^2 of living area does your family have?
  - How many people live in your home? Adults? Children?
  - Do you have a pet at home? If yes, what kind of pet?
  - Is there a fitted carpet in the child's room?

The questionnaires were translated from Swedish into Estonian and then back into Swedish by other persons in order to confirm consistency.

4.3. CLINICAL EXAMINATION AND DIAGNOSIS OF ALLERGIC DISEASES (I–III, VI)

Clinical examinations were performed at 3, 6 months and 1, 2, and 5 years of age. In Estonia, all clinical investigations were performed by the author (T.V). In Sweden, the clinical investigations were performed at three, six months and one year by a trained research nurse and at two and five years by a paediatrician.

Atopic dermatitis was defined as pruritic, chronic, or chronically relapsing non-infectious dermatitis with typical features and distribution, as suggested by

Hanifin and Rajka (Hanifin JM *et al* 1980). Asthma was defined as three or more episodes of bronchial obstruction, with at least once verified by a physician. Allergic rhinitis/rhinoconjunctivitis was defined as rhinitis and/or conjunctivitis appearing at least twice after exposure to a particular allergen and not related to infection. Respiratory infections were defined as cold symptoms with fever and with or without wheezing.

In order to confirm consistency between the two study sites, the Estonian investigator (T.V.) visited the Clinical Research Centre in Linköping several times, and participated in the follow-up examinations of the Swedish children. Diagnosis of allergic diseases was based on the findings of clinical investigations and data from the questionnaires.

4.4. ASSESSMENT OF ATOPIC SENSITISATION

Atopic sensitisation was verified by positive skin prick tests and/or presence of allergen-specific IgE antibodies in plasma.

4.4.1. Skin prick tests (I–III, VI)

Skin prick tests were done at all follow-ups in Estonia, whereas in the Swedish babies SPTs were done either at 3 or 6 months and then at 1, 2, and 5 years of age using 8 allergens (table 3).

The tests were performed in duplicate on the volar aspects of the forearms, using lancets from ALK (Hørsholm, Denmark). Histamine hydrochloride, 10 mg/ml, was used as a positive and glycerol as a negative control. Natural food and standardised inhalant allergen extracts (Solu-Prick SQ™, ALK) were used, and the cockroach allergen extract was from Bayer (Spokane, WA, USA). The prick-prick test with natural food is considered to be more accurate than extracts of commercial allergens (Norgaard A *et al* 1992). Egg white (EW) and cow´s milk (CM) are the most important food allergens in infancy and with increasing of age inhalant allergens are becoming important (Hattevig G *et al* 1987).

Table 3. Allergens used for SPT at different ages

Age / Allergen	3 months	6 months	1 year	2 years	5 years
Cow's milk	+	+	+	+	+
Egg white	+	+	+	+	+
Cat			+	+	+
Dog			+	+	+
Birch			+	+	+
Timothy				+	+
House dust mite*			+	+	+
Cockroach*				+	+

* only in Estonia

In Estonia house dust mite and cockroach allergens were added to the panel because they are widely common and cause sensitisation among older children and adults (Riikjärv MA *et al* 1995; Raukas-Kivioja A *et al* 2003). The SPT was regarded as positive if the mean of the longest and right angle diameters of one of the wheals was at least 3 mm. The same allergen extracts and lancets were used in the two study groups, and persons who made SPT compared their technique carefully before the study, as described in the manual of ISAAC (ISAAC 1998).

4.4.2. IgE measurements (I, II)

Venous blood samples were drawn into heparinised vacutainers at 3, 6 months and 1, 2 and 5 years of age. In the Swedish babies blood samples were collected either at three or six months, while in Estonia blood samples were obtained at all ages. Plasma and peripheral blood mononuclear cells were isolated. The plasma samples were frozen immediately and kept at –20°C until analysed.

Total IgE levels and IgE antibodies to egg white and *β-lactoglobulin (*β-LG) were determined at all ages, and the levels of IgE antibodies to cat and birch allergens were analysed at 1, 2, and 5 years. Samples with total IgE levels above 100 international unit (IU)/mL at two years were additionally analysed for IgE antibodies against ascaris. A commercial chemiluminescence method (Magic Lite™, ALK) was used to analyse plasma up to two years of age, and at five years UniCAP was explored according to the recommendations of the manufacturer Pharmacia & Upjohn Diagnostics AB. The test results are given in classes corresponding to the concentration of allergen-specific IgE antibodies, i.e. class 1: 0.35–0.7 standardised unit (SU)/mL and class ≥2: above 0.7 SU/mL in the case of Magic Lite, and class 1: 0.35–0.7 KU_A/l and class ≥2: above 0.7 KU_A/l for UniCAP. The Estonian and the Swedish samples for a particular test were analysed in the same laboratory, that is the measurements of circulating IgE antibodies to β-LG and EW and total IgE up to two years were done at the Allergy Research Laboratory of Tartu University Hospital and IgE antibodies to cat and birch were done at the Research Laboratory of Linköping University. All the IgE measurements at five years were done at the Allergy Research Laboratory of Tartu University Hospital. Certain samples were analysed in both laboratories in order to confirm consistency.

4.5. INVESTIGATIONS OF MICROORGANISMS

In order to analyse the influence of different microbes on the development of allergy, stool and dust samples were collected and data about respiratory infection and antibacterial treatment were obtained.

4.5.1. Infections and antibacterial treatment (I–III)

Data about respiratory infections and antibacterial treatment were taken from the questionnaires. Parents completed questionnaires before the visit to the research centre. Only data about respiratory infections were used, as diarrhoea in infancy could be due to food intolerance and therefore mislead the results. Respiratory infections and antibacterial treatment were analysed in relation to the development of atopic sensitisation and allergic diseases.

4.5.2. Intestinal microflora (III–V)

Stool samples were analysed to estimate the differences between early gut colonisation in Estonian and Swedish as well as in allergic and non-allergic children.

Early gut colonisation during the first month of life was analysed in 20 Estonian (12 male and 8 female) and 20 Swedish (13 male and 7 female) babies (Paper IV). The selection criterion was availability of stool samples collected 5 to 6 days after birth and at one month of age.

To establish the relationship between gut colonisation and the development of allergy at two years of age 24 Estonian (15 non-allergic and 9 allergic) and 20 Swedish (11 non-allergic and 9 allergic) children were investigated (Paper III). The children were selected for this part of the study based on the diagnosis of allergy at 2 years of age and availability of stool samples at all follow-up ages.

At five years the intestinal microflora in 7 Estonian and 8 Swedish children were compared and the results were compared with gut microflora of Estonian childen born in 1993/94 (Paper V).

Approximately 1 to 2 g of voided stool was collected into sterile plastic containers. Samples collected at home were kept in a refrigerator at 4°C for no more than 2 hours before transportation to the laboratory, where they were frozen at –70°C until analysis. The samples from Swedish children were transported to Estonia in dry ice for bacterial analyses. All bacterial analyses were performed at the Institute of Microbiology of Tartu University. Weighed samples of faeces were serially diluted in pre-reduced phosphate buffer (pH 7.2) and were cultivated on 11 freshly prepared media. Colonies that differed morphologically and were growing on the plate with the highest dilution of bacteria were Gram-stained and subjected to microscopy. The microorganisms

were identified at the genus level (coagulase negative staphylococci (CONS), *Enterococci, Streptococci, Acinetobacteria*, *Candida*, *Bifidobacteria, Bacteroides, Eubacteria, Clostridia*) and the species level (*Lactobacilli, β-hemolytic streptococci, Enterobacteria*, *Staphylococcus aureus*, *Clostridium difficile*). The detection level of various microorganisms was 3 log10 colony forming unit (CFU)/g. The total count (log10 CFU/g) of microorganisms and the counts of various genus and species were calculated for each stool sample. In addition, the relative amount of each particular microbe was expressed as a percentage of the total microbial count in that sample.

4.5.3. Endotoxin level in house dust (II, VI)

Dust samples were collected from 108 Estonian and 111 Swedish homes during the first winter season of the infant, although, 14 Swedish samples were collected during the summer. Two dust samples were collected from each home, one from a carpet and the other from the child's mattress. Dust collectors with a 6-µm pore size filter (ALK, Hørsholm, Denmark) were used. Two m^2 of mattress and 2 m^2 of carpet were vacuum-cleaned for 4 min. In Estonia, the study staff collected the samples, as all homes did not have a high-power vacuum cleaner. Swedish parents collected the dust samples themselves. The dust samples were kept at −20 °C until analysis.

The dust was extracted with vigorous shaking for 2 h in pyrogen-free glass test tubes (Bio Whittaker, Walkersville, ND, USA) with pyrogen-free Limulus Amebocyte Lysate (LAL) water (1 mL/100 mg dust; Bio Whittaker) with 0.05% Tween-20 (Sigma-Aldrich, Stockholm, Sweden). The supernatants were stored in pyrogen-free test tubes at −20°C until analysis. The endotoxin levels were analysed with the same lot of a chromogenic LAL assay (QCL-1000®, Bio Whittaker) according to the manufacturer's instructions. The endotoxin analyses were done at the Research Laboratory of Linköping University.

4.6. HOME ENVIRONMENT AND ALLERGEN LEVELS IN HOUSE DUST (II, VI)

Data about living conditions and home environment were obtained from the questionnaires. The allergen levels were measured from the same dust samples as endotoxin (see Methods section Endotoxin level in house dust).

The levels of cat (*Fel d 1*), dog (*Can f 1*), *Dermatophagoides farinae* (*Der f 1*) and German cockroach (*Bla g 1*) allergens were analysed by enzyme-linked immunosorbent assay according to the instructions of the manufacturer (Indoor Biotechnologies, Cardiff, UK) in the Allergy Research Laboratory of Tartu University Hospital.

4.7. STATISTICAL METHODS

Statistical analyses were performed with different statistical packages: StatView 5.0 (SAS Insitute Inc.), SAS 8.2 (SAS Insitute Inc.) (I, II, VI), Statgraphics (Statistical Graphics Corp, Rockville, Md) (III, IV, V).

χ^2-test was used to test differences in the prevalence of clinical allergy, SPT results, and presence of circulating IgE antibodies and infections between the Estonian and Swedish children. As the number of prescribed antibiotics was not normally distributed among the children, the Wilcoxon test was employed for these comparisons. The association between the development of atopic diseases and the use of antibiotics was analysed by logistic regression (I, II). The Fisher exact test was employed to compare allergic and non-allergic children with respect to the prevalence of microbial colonisation at different ages. The counts of various microbial species were compared with the Mann-Whitney rank sum test and the proportions of different microorganisms were compared with Students´ t-test (III, IV, V). For the analysis of endotoxin levels paired analyses were performed with the Wilcoxon signed-rank test, unpaired analyses with the Mann-Whitney U-test, and correlations with Spearman's rank order correlation coefficient test. The χ^2-test was employed for categorical variables (VI).

A probability (P) level of <0.05 was considered to be statistically significant.

4.8. ETHICAL ASPECTS

The study was approved by the Ethical Committee on Human Research of the University of Tartu and The Regional Ethics Committee for Human Research at the University of Linköping. The parents of all children gave written informed consent for participation in the study.

5. RESULTS AND DISCUSSION

5.1. PREVALENCE OF ALLERGIC DISEASES

The cumulative prevalence of at least one allergic disease was 27% in Estonian and 42% in Swedish children (p=0.03) during the first five years of life. The cumulative prevalence in Estonian children was similar to a previous cohort (32%) reported by Julge K *et al* (2001). A slightly lower prevalence of allergic diseases had been reported in previous Swedish studies (Hattevig G *et al* 1987; Nilsson L *et al* 1998; Alm JS *et al* 1999).

The increase of the prevalence of allergies between two and five years of life was more pronounced in Swedish children (table 4). This increase was predictable as it is a part of the so-called atopic march (Bergmann RL *et al* 1994).

Table 4. Prevalence (%) of allergic diseases in Estonian (Est) and Swedish (Sw) children during the first five years of life

	3 months		6 months		1 year		2 years		5 years	
	Est n=110	Sw n=75	Est n=110	Sw n=80	Est n=109	Sw n=129	Est n=104	Sw n=123	Est n=102	Sw n=110
Atopic dermatitis	4	16*	5	13	7	15	12	28*	8	20*
Asthma	0	0	0	0	3	0	6	4	7	16*
Allergic rhino-conjunctivitis	0	0	0	0	0	0	1	0	6	14*
Total	4	16*	5	13	10	15	14	31*	18	30*

*P<0.05

Atopic dermatitis was the most common manifestation of allergy. The prevalence of atopic dermatitis was higher among Swedish children, significantly at three months and at two and five years. The prevalence of atopic dermatitis in Estonian and Swedish children was similar to previous studies (Julge K *et al* 2001; Broberg A *et al* 2000). In the Swedish children airway allergies were diagnosed later as compared to Estonian children. However, the prevalence of asthma in five-year-old Swedish children was higher than reported previously (6.3%) by Gustafsson D *et al* (2004). There was an increase in allergic rhinoconjunctivitis in Swedish children at the age of five, however, the prevalence of AR was similar to the data reported in an earlier study (Kihlström A *et al* 2003). In Estonian children the prevalence of respiratory allergies was only half of the prevalence among Swedish children (table 4).

5.2. PREVALENCE OF ATOPIC SENSITISATION

5.2.1. Skin prick tests

The prevalence of a positive SPT to any allergen was lower in the Estonian than in the Swedish children at all ages, except at three months (table 5). The lack of a statistically significant difference at three months could be explained by the small Swedish group size because the Swedish children attended follow-ups at three or six months but Estonian children attended at both ages.

Table 5. Prevalence (%) of any positive SPT result in Estonian and Swedish children

Study group	3 mo	6 mo	1 yr	2 yrs	5 yrs	Cumu-lative
Estonian	4.5	6.4	7.3	6	8.9	20
Swedish	9	17.8	20	20	26	34
p	0.3	0.027	0.005	0.04	0.05	0.02

The comparison for particular allergens revealed that Estonian children had less positive SPT results to the food allergens than the Swedish children (table 6). None of the children was SPT positive to dog allergen. The differences in the first year of life could not be explained by diet. The duration of breastfeeding was shorter in the Estonian than in the Swedish infants (median 6 [range 0.5–24] vs. 8 [range 0.25–24] months; p=0.04, respectively). Also, the duration of exclusive breastfeeding was shorter in the Estonian than in the Swedish infants (median 2.5 [range 0.25–6.5] vs. 4 [range 0–8] months; p<0.001, respectively). Furthermore, the Estonian children were exposed to cow's milk and egg white at a younger age, and therefore the skin test reactions might become positive at an earlier age, but this was not the case.

Table 6. Prevalence (%) of positive SPT results to any of egg white, cow's milk, cat, birch and timothy allergen in Estonian and Swedish children

Age	3 months		6 months		1 year		2 years		5 years	
	Est	Sw	Est	Sw	Est	Sw	Est	Sw	Est	Sw
Allergen	n=109	n=67	n=108	n=73	n=109	n=129	n=103	n=123	n=101	n=100
CM	0	3	0	6.8*	1	3.8	0	2.3	0	1
EW	4.5	9	6	16.4	6.4	19.3*	3.8	11.6*	1	8*
Cat					1	1	3.8	2.3	6	8
Birch							0	5.4*	4	16*[a]
Timothy							0	0	1	11* [a]

* p<0.05 comparison between Estonian and Swedish children

[a] p<0.05 comparison between two and five years of age in Swedish children

The prevalence of positive SPT to pollens was significantly higher in the Swedish than in the Estonian children (18% vs. 5%; p=0.006, respectively). There was a significant increase in the prevalence of positive SPT against pollens in the Swedish children at the age of five years (table 6) and at the same time the prevalence of airways allergies increased in the Swedish study group (table 4). The exposure to birch allergens is comparable in the two countries (Ekebom A *et al* 1998), and therefore allergen exposure does not explain the significant difference in birch sensitisation between Estonian and Swedish children. There are no comparative data about timothy pollen exposure in Estonia and Sweden. However, it is unlikely that different exposure to timothy allergen in two countries is reason for the different prevalences of positive SPT results against timothy.

Similarly to our data, a lower prevalence of positive SPTs was reported previously in Estonian than Swedish schoolchildren (Riikjärv MA *et al* 1995; Björkstén B *et al* 1998) as well as preschool children (Julge *et al* 2001; Kihlström A *et al* 2003).

At five years, the most common allergen in Estonian children beside cat was house dust mite (*D. pteronyssinus*) with a 6% prevalence of positive SPTs. Additionally, in Estonian children the prevalence of positive SPT to the cockroach was at the age of two years 1% and 2% at five years. The house dust mite and cockroach allergen were not used in the Swedish cohort as house dust mite allergy is uncommon (Riikjärv MA *et al* 1995; Bråbäck L *et al* 2001), and exposure to cockroach is exceptional in Sweden (Munir AK *et al* 1994). The prevalence of positive SPT against any allergens (included house dust mite and cockroach) was 7.7% at two years and 14.8% at five years of age in Estonian children.

In Estonia, the sensitivity of SPTs to clinical allergy was low during the first two years of life (40%) and increased to 61% at the age of five years. However, the specificity remained high i.e. 95% and 96%, respectively. In Sweden, the sensitivity of SPTs was higher (68%), whereas specificity was slightly lower (89%) during the first two years, as compared to the Estonian results. At five years of age, the sensitivity and specificity of SPT to clinical allergy in Swedish children were 80% and 82%, respectively.

During the first two years of life most Swedish children with atopic dermatitis had positive SPT, but this was not the case in Estonia. Similar observations were reported in cross-sectional studies in the German pre-school children showing that eczema in Eastern Germany was not related to atopy while most children with eczema were sensitised in Western Germany (Schafer T *et al* 2000). Furthermore, more Swedish children (n=12) had positive SPT results without clinical allergies, as compared to the Estonian children (n=5). Positive SPT results had a stronger predictable value for allergic disease in the Estonian than in the Swedish study group.

In a previous study was demonstrated a down-regulation of skin reactivity in Estonian children between 2 and 5 years (Julge K *et al* 2001). The prevalence

of positive SPT during the first two years of life was similar in the present study and the Estonian cohort born 4–5 years earlier (Julge K *et al* 1997). However, there was now a prominent increase in positive SPT results in Estonian children at the age of five years i.e. 14.8% vs. 3% in the previous cohort, p=0.002. The prevalence of positive SPT results was also somewhat higher in the Swedish children as compared to the other Swedish studies (Kihlström A *et al* 2003, Sandin A *et al* 2004), which can partly be explained by the smaller study group in the present study.

The results of the present study demonstrate that the previously observed down-regulation of skin reactivity in Estonian children has been disappeared. The increase in the prevalence of positive SPT in Estonian children could be due to diminishing allergy-protective mechanisms in Estonia.

5.2.2. IgE antibodies

The total IgE levels were similar in the Estonian and the Swedish children, except for higher level at 12 months in Sweden (table 7). Levels of total IgE were not measured at the age of five years.

Table 7. Geometric mean (its standard deviation) of total IgE levels in Estonian and Swedish children up to two years of age

Age Study group	3 months	6 months	1 year	2 years
Estonian	0.76 (5.52)	1.70 (8.98)	3.53 (10.99)	13.24 (10.62)
Swedish	1.31 (5.77)	1.93 (6.72)	9.50 (6.53)	14.47 (7.83)
p	0.06	0.7	<0.001	0.4

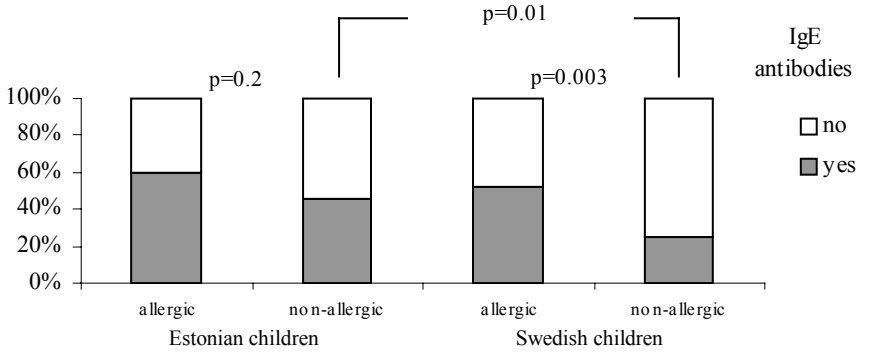


Figure 2. Presence of IgE antibodies in Estonian and Swedish allergic and non-allergic children during the first five years of life.

Despite the lower prevalence of positive SPT at all ages, circulating IgE antibodies to EW, β-LG, cat, and birch were more common in the Estonian than in the Swedish children (50% vs 35%; p=0.03, respectively). Moreover, Estonian non-allergic children had circulating IgE antibodies more often than Swedish children (p=0.01) (figure 2).

The prevalence of IgE antibodies against EW tended to increase after 1 year of age and remained higher at five years in Estonian, as compared to Swedish children (table 8). We can speculate that the shorter duration of breastfeeding and earlier introduction of solid foods in Estonian infants could explain the higher prevalence of IgE antibodies to EW. However, the time of introduction of cow's milk and hen's eggs was unrelated to the presence of IgE antibodies against these foods. The prevalence of IgE antibodies to inhalant allergens increased significantly in Swedish children from two to five years of age (table 8).

Table 8. Prevalence of allergen-specific IgE antibodies in Estonian (Est) and Swedish (Sw) children

Age	3 months		6 months		1 year		2 years		5 years	
	Est	Sw	Est	Sw	Est	Sw	Est	Sw	Est	Sw
Allergen	**n=104**	**n=57**	**n=101**	**n=53**	**n=100**	**n=122**	**n=96**	**n=119**	**n=101**	**n=92**
β-LG										
All	9	8	9	13	6	7	10	5	6	9
≥class 2	2	4	1	8*	0	3	1	2	2	4
EW										
All	11	12	15	13	10	17	18	14	13	8
≥class 2	0	2	6	7	6	11	2	8	3	5
Cat										
All					6	0	7	2	5	7
≥class 2					3	0	2	1	2	4
Birch										
All					4	1	3	3	7	14
≥class 2					1	0	1	2	3	12*

The sensitivity of IgE antibodies to allergic diseases was low in Estonia and Sweden (50% and 58%) during the first two years of life. The specificity was lower in the Estonian, than in the Swedish children (64 % vs. 83%, respectively) during the first two years of life. The sensitivity of IgE antibodies to allergic diseases at five years of life was almost the same as at two years in Estonian children but decreased in Swedish children (47% and 44%, respectively). The specificity increased in Estonian children (78%) and remained stable in the Swedish children (82%). However, after including IgE antibodies against house dust mite in the comparisons, sensitivity of IgE antibodies to

allergic diseases increases from 47% to 64% in the Estonian children. During the follow-up period the Estonian children had eight positive SPT without corresponding IgE antibodies, whereas the Swedish children had 35 positive SPT results without corresponding circulating IgE antibodies ($p<0.001$). Moreover, the Estonian children had circulating IgE antibodies without positive SPTs more often than the Swedish ones (101 vs. 41 times, $p<0.001$).

Studies in Western countries have reported a good correlation between positive SPT results and the presence of circulating IgE antibodies. Skin tests are considered to be more sensitive (Kelso JM *et al* 1991; Pastorello EA *et al* 1995; Schuetze G *et al* 1999) and circulating IgE antibodies to be more specific (Dekker FW *et al* 1990). Transient low-level IgE antibody responses to foods have been previously reported in young non-allergic children (Hattevig G *et al* 1993). The findings of the present study concerning SPT and circulating IgE antibodies showed a better correlation in the Swedish than in the Estonian children. However, more positive SPT without IgE antibodies demonstrated a higher prevalence of non-specific skin reactivity in the Swedish children. The data regarding Estonian children showed that the prevalence of circulating IgE antibodies was higher than in the Swedish children and there was a poor correlation between circulating IgE antibodies and positive SPTs.

The low prevalence of positive SPT results in the Estonian children cannot be explained by the technical factors as the same allergen extracts and lancets were used in both study groups, and the persons who made SPT were carefully trained, and their techniques were compared before the beginning of the study.

A poor correlation between circulating IgE antibodies to pollen and positive SPT results had been observed previously in Estonian schoolchildren (Riikjärv MA *et al* 1995). It was initially suspected that the reason for the poor correlation was a seasonal influence (Riikjärv MA *et al* 1995). Further studies in Estonian children assured that there was no technical error and Estonian children often had circulating IgE antibodies without positive SPTs and allergic diseases (Julge K *et al* 2001). The present study confirmed once again the discrepancy between the presence of a positive SPT and circulating IgE antibodies in Estonian children.

Lower IgE affinity could possibly be one explanation for the low incidence of SPT with circulating IgE antibodies in the Estonian children. Most low-affinity IgE antibodies were seen in young children, which could suggest a critical period of time during early life when the affinity responses mature (Pierson-Mullany LK *et al* 2002). Hypothetically, this critical time could be different in Estonian and Swedish children, and one cannot exclude influence of different environmental factors during this period.

In a study from Gabon, urinary schistosomiasis was associated with a low prevalence of positive SPTs to house dust mites (van den Biggelaar AH *et al* 2000). The authors suggested that the anti-inflammatory cytokine IL-10, which is induced in chronic schistosomiasis, could suppress atopy. The same phenomena have been described among children of low socio-economic class in

Venezuela, where the majority had low levels of specific IgE antibodies against a variety of inhalant allergens, but relatively few had positive SPTs (Lynch NR *et al* 1987). A recent study from Ethiopia revealed that intestinal parasitic infections, especially *Trichuris*, were risk factors for atopic dermatitis while *Ascaris* and hookworm were not (Haileamlak A *et al* 2005). As only two Estonian children of 96 had IgE antibodies to *Ascaris*, and the levels of total IgE (table 7) were similar in the Estonian and the Swedish children, parasitic infections cannot explain negative SPTs in the Estonian children with circulating IgE antibodies. The poor agreement between SPT and circulating IgE antibodies in Estonia would suggest that not only parasites but also other factors are responsible for the findings.

The disagreement between the two methods of detecting IgE antibodies in Estonia might be due to the down-regulation of skin reactivity, which was revealed already in the previous Estonian prospective study (Julge K *et al* 2001). In the present study the prevalence of positive SPT results increased with age, although, Estonian children had more IgE antibodies without SPT as compared to Swedish children even at 5 years. Possibly, there were factors in the Estonian environment, which might down-regulate skin reactivity, whereas some environmental factors associated with a Western lifestyle could enhance skin reactivity to the allergens. The results of this study are in accordance with the data by Ronchetti *et al* (2005) who observed that the diameter of histamine wheals was larger in Libyan, than in Italian and Polish schoolchildren. However, Libyan children had less often positive SPTs against allergens.

5.3. FAMILY HISTORY OF ALLERGIC DISEASES

A history of allergic diseases was less common among the Estonian than the Swedish parents, i.e. 22/107 (21%) vs. 50/123 (41%) of the mothers (p=0.001) and 18/107 (17%) vs. 47/ 123 (38%) of the fathers (p=0.001). There was no correlation between parental allergy and prevalence of allergic diseases or sensitisation in Estonian and Swedish children during the first two years of life.

In the Estonian children allergic diseases at five years of life were related to the family history of allergy (allergic mother and/or father) (p=0.007), and paternal allergy was related to positive SPTs (p=0.006).

The Swedish children revealed a relationship between maternal allergy and the presence of IgE antibodies in plasma at five years (p=0.038). There was no correlation between parental allergy and allergic diseases or positive SPT in Swedish children at the age of five.

The lack of correlation between parental allergy and allergic diseases and sensitisation of children could be due to small size of the present study.

The lower number of infants with a positive family history of allergy in Estonia would at first sight be suspected as a confounding factor. However, family history in populations living in countries with a low and on the other hand with a increasing prevalence of allergies might not be informative since the genotype is not phenotypically expressed.

5.4. INFECTIONS AND ANTIBACTERIAL TREATMENT

According to the questionnaires, reported episodes of respiratory illnesses were more common in the Estonian than in the Swedish infants (median 6.2 [range 1–12] and 3.6 [range 0–15] episodes, respectively; $p<0.001$) during the first two years of life. All the Estonian children had at least one episode of respiratory infection, while 18% of the Swedish children did not have any infections during the first two years of life. The Swedish figure is similar to that reported from West Germany where 14% of infants did not have any viral infection during the first year of life (Lau S *et al* 2002). However, there was no correlation between the number of infection episodes and allergic diseases or atopic sensitisation in either country in the present study. Orofaecal transmitted or some viral infections have been reported to protect against atopy (Illi S *et al* 2001; Matricardi PM *et al* 2000; Kosunen TU *et al* 2002; Linneberg A *et al* 2003). The lack of correlation could be explained by the fact that the study focused on viral respiratory infections, which are considered to be non-protective against allergy. Data were not collected about orofaecal infections, which have been considered to be allergy protective infections (Matricardi PM *et al* 2000; Linneberg A *et al* 2003) because infants do not suffer from them. One can speculate that perhaps any allergy-protective effect of infections will be manifested in later life. Therefore, children will be followed until ten years, and this speculation can be answered after the 10-year follow-up.

Data about the treatment of infections during the first two years of life were collected from questionnaires. The results of the present study showed that antibiotics were more frequently prescribed to the Estonian than the Swedish infants (mean 2.1 (SD 0–3.9) vs. 1.2 (SD 0–2.6) times; $p<0.001$, respectively). Twenty-three (21%) Estonian and 40 (33%) Swedish children did not receive any antibiotics during the first two years of life ($p=0.04$) (figure 3). Already by the age of one year, 25% (28/109) of the Estonian children had received antibacterial therapy at least twice, as compared to 9% (12/129) in Sweden ($p<0.001$). In Sweden, the most common antibiotics were beta-lactams, mostly penicillin, and only six children received erythromycin, cephalosporins, or trimethoprim-sulfamethoxasol. In Estonia 17 children were prescribed only beta-lactams, whereas 70 children received also erythromycin, cephalosporins or, trimethoprim-sulfamethoxasol.

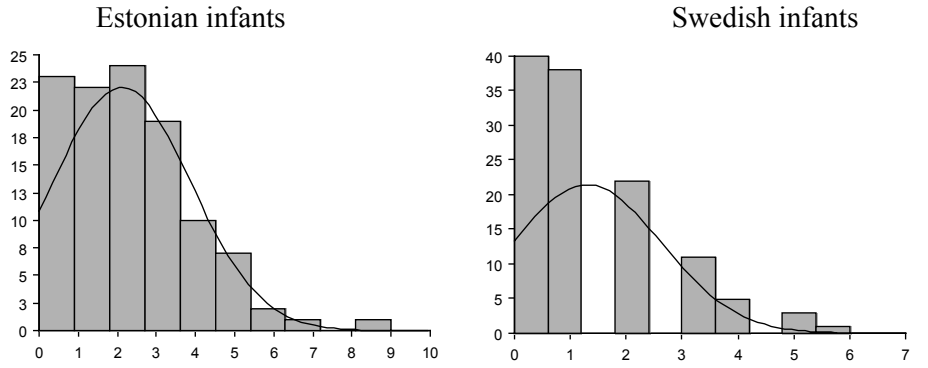


Figure 3. Use of antibiotics during the first two years of life in Estonian and Swedish infants. The x-axis indicates the number of children and the y-axis the number of antibacterial courses

In Estonia SPT-positive children received more antibiotics during the first year of life as compared to the SPT-negative children (mean 1.8 (SD 0.4–3.2) vs. 0.8 (SD 0–1.9) courses; p=0.007). The use of antibiotics during the first year of life increased the risk on positive SPT in Estonia (odds ratio (OR)=1.7, 95% confidence interval (CI) 1.1–2.5), but this was not the case in the Swedish infants (OR=0.8, 95%CI 0.5–1.4). There was no correlation between the use of antibiotics during the second year of life and SPT results and allergic diseases in either country.

According to the present study, respiratory infections apparently did not offer protection against allergic diseases and atopic sensitisation. However, treatment with antibiotics during the first year of life enhanced the risk of sensitisation. The risk was pronounced in Estonia probably due to wide use of broad-spectrum antibiotics. Therefore, antibiotics in infants should be used only in the case of very restricted indications.

5.5. HOME ENVIRONMENT AND MICROBES

It has been shown previously that living conditions may influence the development of sensitisation and allergies. Growing up in crowded houses (Strachan DP 1989), close contact with pets (Hesselmar B *et al* 1999), and a higher endotoxin level in house dust (von Mutius E *et al* 2000) were all associated with fewer allergies later in life.

In order to examine the role of living conditions for the development of allergies, dust samples and data about living conditions were collected from 107 Estonian and 104 Swedish homes. The Estonian homes were smaller, and the families were bigger than in Sweden (table 9). Furthermore, the Estonian homes more often harboured pets at the time of dust collection.

Table 9. Living conditions in Estonian and Swedish homes at the time when house dust samples were collected

Living conditions Country	Dwelling space, (m^2/family member) median and range	Number of family members, median and range	Pets at home (%)
Estonia	14.3 (3.6–75)	4 (2–9)	39%
Sweden	28.3 (16.3–91.7)	3 (2–8)	15%
p	< 0.001	0.03	< 0.001

The median levels of endotoxin were higher in dust samples collected from the Estonian than from the Swedish households, in mattresses (29 [range 0.25–275] endotoxin unit (EU)/mg dust vs. 19 [range 0.25–99] EU/mg dust; p<0.001, respectively) and in carpets (28 [0.5–358] EU/mg dust vs. 19 [0.5–90] EU/mg dust; p<0.001, respectively). The differences in endotoxin levels between the two countries, however, were unrelated to dwelling area, presence of pets, or family size. Dust from homes with allergic infants contained less endotoxin than the dust from homes with non-allergic infants in Sweden (figure 4), but this was not the case in Estonia. Surprisingly, the median endotoxin level was higher in Estonian homes with allergic than non-allergic infant, but the difference was not statistically significant.

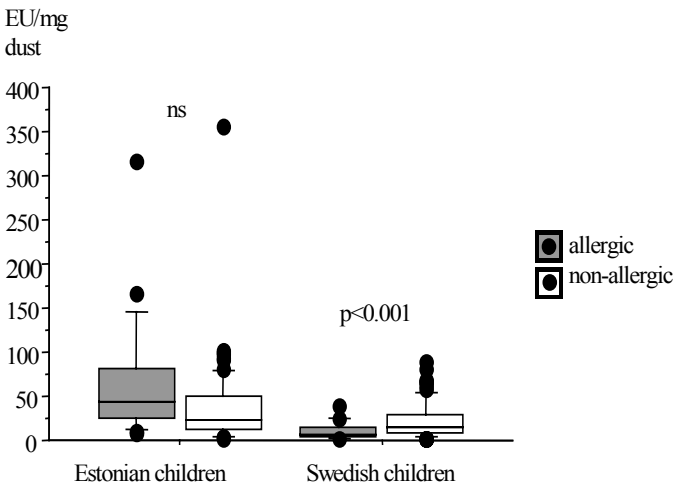


Figure 4. Endotoxin levels of house dust (endotoxin unit [EU] per mg dust) and allergic symptoms in Estonian and Swedish children during the first two years of life

Allergic symptoms of the Swedish children at two years of age were associated with low levels of endotoxin in dust samples collected from carpets but not from mattresses. Morover, endotoxin levels were lower in carpet dust from homes of SPT-positive than in SPT-negative children in Sweden and not in Estonia (figure 5).

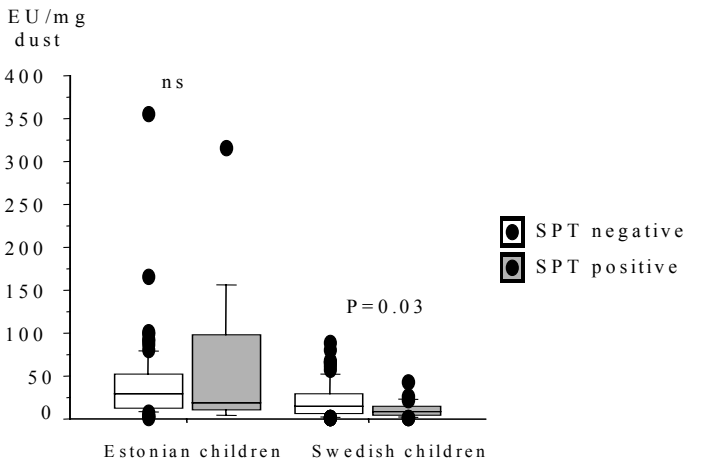


Figure 5. Endotoxin levels in carpet (EU/mg dust) and SPT results of Estonian and Swedish children at two years of life

However, the allergy-protective effect of higher level of endotoxin in infancy did not manifest any more in five-year-old Swedish children. Although, allergic five-year-old Swedish children had lower endotoxin levels in house dust in their infancy (figure 6).

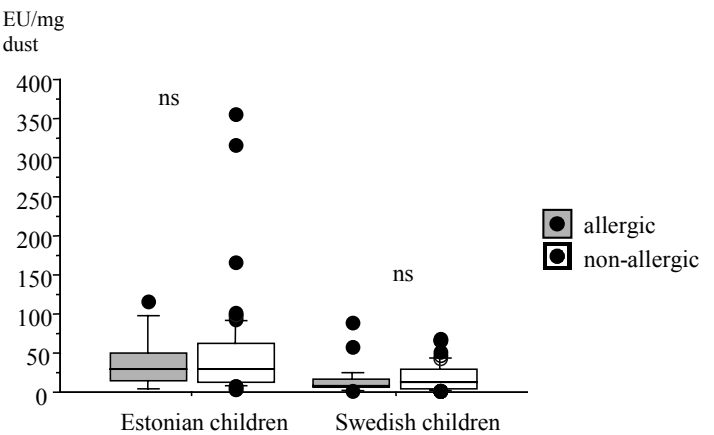


Figure 6. Endotoxin levels of house dust (EU/mg dust) in infancy and allergic symptoms in Estonian and Swedish children at five years of life

Furthermore, the endotoxin levels were lower in carpet dust from the Swedish families with atopic parents than in the families without atopic heredity (median levels 10.3 EU/mg dust [range 0.25–47.7] and 19.1 EU/mg dust [range 1.4–90]; $p < 0.001$, respectively).

Endotoxin itself might only be a robust marker of a general microbial contamination. In Estonian homes, the levels of endotoxin were higher than in Swedish homes, and this finding supports the idea that a Western lifestyle is associated with a decreased overall exposure to microbial stimulation. In Sweden, low endotoxin levels in carpets, but not in mattresses, were associated with atopic disease and sensitisation in infancy. The endotoxin levels in carpets reflect exposure to general household endotoxin better than the levels in mattresses. These findings support the hypothesis that exposure to endotoxin during early life may stimulate the maturation of the immune system and thus protect against the development of atopic disease.

It has been suggested that the immune system needs to be stimulated persistently, and continuously by microbes in order to function properly. Animal studies demonstrated that absence of stimulation of the immune system by the microorganisms resulted in Th2-type immunity (Holt PG *et al* 1997). Higher house dust endotoxin levels correlate with increased proportions of IFN-γ producing T-helper cells in the peripheral blood of infants, suggesting that natural endotoxin exposure may promote Th1-type immune development (Gereda JE *et al* 2000). However, there was no difference in endotoxin levels between atopic and non-atopic children in Estonia. It could be explained by the generally high endotoxin levels in Estonia, providing a maximum effect, concealing the influence of other unknown environmental factors.

Animal studies have confirmed the importance of the timing of endotoxin exposure. Endotoxin inhalation during primary allergen exposure in the presence of a Th2-promoting adjuvant prevented allergic sensitisation, whereas inhalation of endotoxin after sensitisation aggravated the inflammatory airway responses (Tulic MK *et al* 2000, 2002; Watanabe J *et al* 2003). Furthermore, a high dose of inhaled endotoxin induces an immediate and prolonged bronchoconstriction in most people and aggravates the allergic inflammation of airways. High endotoxin levels in house dust correlate with increased asthma symptoms and with infant wheezing in the first year of life (Park JH *et al* 2001; Bolte G *et al* 2003). The high prevalence of wheezing episodes (44%) in the Estonian children during the first two years of life in the present study could be partially explained by the high endotoxin level in house dust. However, there was no correlation between endotoxin levels and wheezing in the Estonian children. The lack of correlation was probably due to the overall high endotoxin level in Estonian homes.

High domestic endotoxin levels have been reported in the farming environment, and one reason for this are animals (von Mutius *et al* 2000). In urban homes, higher endotoxin levels have been found in homes with pets (Gereda JE *et al* 2001; Park JH *et al* 2001). The present study found no

correlation between endotoxin levels and ownership of pet in Estonia, and only a weak correlation between endotoxin and pet allergen levels in Sweden.

Probably, the significant differences in the endotoxin level in Estonian and Swedish homes were due to different microbial load in the environment and different cleaning habits. In 1998, when most dust samples were collected most Estonian families did not have powerful vacuum cleaners and chemicals were not used in home cleaning.

Allergen exposure at home

The allergens' levels were higher in the Estonian than in the Swedish homes (table 10).

The most significant differences between Estonian and Swedish houses concern the level of house dust mite allergens ($p<0.001$). House dust mite allergens resided in all Estonian mattresses and in 93% of carpets, whereas in Sweden the corresponding figures were only 34% and 9%. The difference in the level of house dust mites cannot be explained by climate, as it is similar in Tartu and Linköping. One reason for the higher level of house dust mite allergen in the Estonian homes may be the use of old mattresses. In Estonia mattresses are often passed on from older to younger relatives, and the mean age of Estonian mattresses was 7 (0–35) years, while in Sweden every child usually received a new mattress. It has been demonstrated that the level of the house dust mite allergen increases with the age of mattress (Van Strien RT *et al* 1994; Mihrshahi S *et al* 2002).

Table 10. Allergen level in house dust (ng/g) in Estonian and Swedish homes. Results are given in median (range) values — log10

Allergen	***House dust mite (Der f 1)***		***Cat (Fel d 1)***		***Dog (Can f 1)***	
	mattress	**carpet**	**mattress**	**carpet**	**mattress**	**carpet**
Estonian homes	2.5 (0.7–4.9)	1.9 (0–4.2)	3.1 (1.8–5.6)	2.7 (1.6–5.5)	2.9 (1.8–4.9)	2.9 (1.4–5.8)
Swedish homes	0 (0–1.7)	0 (0–1.2)	2.5 (0–5.0)	2.5 (0.3–5.2)	2.5 (0–5.2)	2.4 (0–5.5)
p	<0.001	<0.001	=0.001	=0.005	<0.001	=0.001

Cat and dog allergens were detected in all Estonian homes. In Sweden, cat allergen was detected in 97% and dog allergen in 98% of the homes. There was no correlation between pet allergen and the endotoxin levels in the Estonian house dust samples. However, in Sweden there was a weak correlation between *Fel d 1* and endotoxin levels in mattresses ($\rho=0.21$, $p=0.04$), between *Fel d 1* and endotoxin levels in carpets ($\rho=0.21$, $p=0.03$), and between *Can f 1* and

endotoxin levels in carpets (ρ=0.21, p=0.03). At the time of dust collection more Estonian families had pets at home (table 9; p<0.001), and during the whole follow-up period also more families in Estonia had pets at home, as compared to Sweden (43% vs 24%; p=0.004, respectively). Some authors (Hesselmar B *et al* 1999; Ownby DR *et al* 2002) have demonstrated that pet keeping in infancy reduces the risk of allergy and atopy in later. At least two different mechanisms have been proposed how pets can protect against allergy. Firstly, pet keeping is associated with a higher endotoxin level (Gehring U *et al* 2001), which enhances immune deviation towards Th1 type. Secondly, high allergen exposure may induce the so-called modified Th2 responses and tolerance (Platts-Mills *et al* 2001). However, the present study was unable to find any correlation between sensitisation and pet allergen levels in house dust. One reason for the lack of correlation could be the small number of children sensitised to cat. Furthermore, the lack of protection by pets may be due to the short follow-up period. Thus in the study of Hesselmar *et al* (1999) pet keeping during the first year of life was associated with less sensitisation and allergy at 12 years but not at 7 years. In addition, pet allergens are widespread in the environment (Custovic A *et al* 1994; Berge M *et al* 1998), and probably children had contact with pet allergens also outside home.

None of the Swedish home dust samples contained cockroach allergen (*Bla g 1*), whereas 56% of the Estonian dust samples contained cockroach allergen. The mean levels of cockroach allergen in Estonian homes were 1±8 IU/g in mattress dust and 9±31 IU/g in carpet dust.

Threshold doses for the induction of sensitisation have been proposed for some of the main indoor allergens (house dust mites and cockroaches) (Munir AK *et al* 1995; Eggleston PA *et al* 1998). However, there is no exposure threshold for any allergen that is relevant for all children, as children with a genetic risk can respond to very low levels (Munir AK *et al* 1997). In the present study *Der f 1* levels were measured in house dust, whereas the SPTs were done with *Der p1* allergen extract. Therefore, it was impossible to establish any correlation between exposure to house dust allergen and sensitisation. Five of six Estonian children who were sensitised to house dust mite also had allergic symptoms. There were only three Estonian children who were sensitised against cockroach and therefore no correlation could be assessed.

5.6. INTESTINAL MICROFLORA AND ALLERGY

According to the study of Sepp et al (1997) gut microflora of the Estonian and Swedish one-year-old children was different. Early colonisation was studied in order to establish differences in intestinal colonisation during the first months of life in the Estonian and the Swedish children.

The early colonisation pattern differed in the Estonian and the Swedish children. For example, the counts of aerobic bacteria were higher in the Estonian than in the Swedish newborns in the first week of life (median 11.5 [range 8.4–12.3] vs. 10.4 [range 8.4–12]; $p<0.001$). Estonian newborns had higher counts of coagulase negative staphylococci (CONS), *Enterococci, Enterobacteria* than the Swedish babies (table 11). The counts of *Candida* were also higher in the Estonian than in the Swedish newborns (table 11). During the first month of life the Estonian newborns were colonised more frequently with lactobacilli than the Swedish newborn babies (80% vs. 30%; $p<0.05$) and the counts were higher (table 11). There were no differences in the colonisation of two species of aerobes – *Staphylococcus aureus* and S*treptococci* nor of anaerobes (anaerobic cocci, *Bifidobacteria, Eubacteria*, *Bacteroides, Clostridia*) between the Estonian and the Swedish infants during the first month of life. The microflora of the Estonian infants was in many aspects similar to the flora prevailing in infants of Western Europe in the 1960s (Grütte FK *et al* 1979) indicating that the gut microflora has changed in Sweden, including later and less colonisation with *Escherichia coli*, other *Enterobacteria*, *Enterococci*, *Bifidobacteria*, and *Lactobacilli,* and an increased prevalence of colonisation with *Clostridium difficile* and *Staphylococcus aureus*.

The present study confirms that the differences in colonisation are apparent soon after birth. The gut microflora has an important role in the maturation of the immune system, and it is essential for the development of tolerance (Sudo N *et al* 1997). The Estonian children were colonised with higher counts and more frequently with lactobacilli than the Swedish infants during the first month of life. The overall counts of aerobes were higher in the Estonian than in the Swedish infants. A high number of aerobic microorganisms during the neonatal period could facilitate the induction of tolerance (Moreau MC *et al* 1988). The differences in the colonisation of the gut flora between Estonian and Swedish infants may therefore be relevant for the lower prevalence of allergy in Estonian than Swedish children. Another reason for the low prevalence of allergic diseases in Estonia could be early intensive colonisation of the gut with *Lactobacilli*, as it is known that oral feeding of *Lactobacillus casei* inhibits IgE production of IgE in mice (Matsuzaki T *et al* 1998). Previous studies have shown that gut colonisation of the children in developing countries is intensive and rapid, and more *Enterobacteria* reside in gut microflora (Adlerberth I *et al* 1991; Bennet R *et al* 1991). The findings of the present study show that the intestinal microflora of Estonian children is similar to the gut microflora in developing countries (e.g. more *Enterococci* and *Enterobacteria*). Furthermore, the importance of the initial colonisation for the subsequent development of allergies has been supported by an intervention study, where colonisation of the intestine with *Escherichia coli* after birth decreased the incidence of allergies and repeated infections at 10 and 20 years of age (Lodinová-Zádníková R *et al* 2003).

Table 11. Counts of faecal aerobes of Estonian and Swedish infants at the age of 1 week and 1 month. The results are given in median (range) values–log CFU/g

Age/group	*CONS*	*Enterococci*	*Enterobacteria*	*Lactobacilli*	*Candida*
1 week					
Estonian	10.5 (0–11.1)	10.7 (0–11.8)	10.9 (4.8–11.8)	<3 (0–10)	3 (0–7.3)
Swedish	7 (0–10.3)	8.9 (0–10.6)	9.9 (0–12)	<3 (0–8.6)	3 (0–6.6)
p	<0.001	<0.01	<0.01	ns	ns
1 month					
Estonian	6.5 (0–11.4)	9.9 (0–12.2)	11 (0–11.9)	7.7 (0–10.8)	5.9 (0–10.3)
Swedish	<3 (0–10.8)	10.3 (0–11.7)	10.4 (0–11.9)	3 (0–10.8)	3 (0–10.9)
p	ns	ns	ns	<0.01	<0.05

As all Estonian and Swedish newborn babies in the present study were born vaginally, the mode of delivery did not account for the difference in early colonisation. One possible explanation for different colonisation patterns in Estonian and Swedish newborn babies could be a disturbed maternal microflora in Swedish mothers as the maternal intestinal and vaginal flora is a source of bacteria for the neonatal gut. The importance of the maternal flora in the development of allergic diseases was shown by Benn CS et al (2002). They indicated that the presence of *Staphylococcus aureus* in the maternal vaginal microflora during pregnancy was the risk of asthma during the fifth year of life.

The composition of gut microflora is heterogeneous during the first few days of life and is largely independent of feeding habits (Hall MA *et al* 1990). After the first week of life a stable bacterial flora is gradually established and the feeding of a newborn baby plays a role in the pattern of gut colonisation. It has been shown that the microflora of breastfed infants is rich in *Bifidobacteria* species, whereas in the microflora of bottle-fed babies a bifidus flora is uncommon (Yoshioka H *et al* 1983; Harmsen HJ *et al* 2000). Some studies do not demonstrate any differences in the counts of *Bifidobacteria* between infants born in large urban hospitals, regardless of whether the babies were bottle-fed or exclusively breastfed (Fanaro S *et al* 2003). In the present study, all the Estonian and the Swedish children received breast milk as their first food, and there was no difference between breastfeeding prevalence during the first month of life. The findings of the present study do not support studies reporting that the feeding of newborn babies plays a role in the gut colonisation underlining the possibility that environmental factors may be more important than breastfeeding for gut colonisation during the neonatal period.

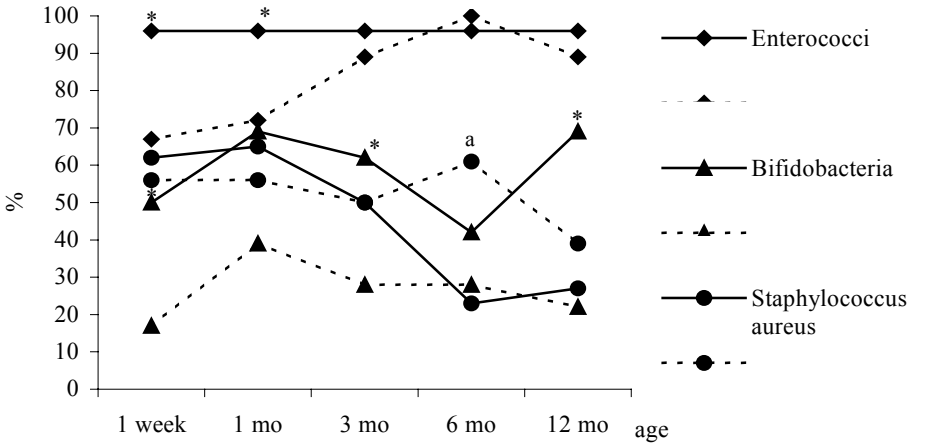
Surprisingly, the intestinal microflora changed similarly in the Estonian and the Swedish children at five years of age. There were no any more statistically significant differences between bacteria counts in the Estonian and the Swedish children born in 1996–1998. By comparison, Estonian children born in 1993/94 and in 1997/98 revealed that the counts of anaerobes (p=0.001), as anaerobic

cocci (p=0.02) and *Bacteroides* (p=0.008) were decreased in later-born children. The data indicate that shifts have occurred in the composition of gut microflora in the Estonian children. This finding refers that the Estonian lifestyle has become more similar to Western countries during the last years.

The reasons for the changed microflora in countries with a Western lifestyle are unknown, but one explanation is related to food. Typical Western diet does not offer symbiotic conditions for microbes, and gut pH is low, which represses Gram-positive bacilli and favours the growth of *Clostridia* (Benno Y *et al* 1986). Colonisation of the gut with *Clostridium difficile* is probably more frequent in persons living in a environment with less microbes as *Clostridium difficile* often colonises the gut after antibiotic treatment (Klingler PJ *et al* 2000). Linneberg *et al* (2003) proposed that *Clostridium difficile* is a marker of a certain gut flora that is associated with exposure to a sterile environment or diet. In Estonia, the hygiene and food standards have improved, and the Estonian standards conform to the directives of the European Union. At the beginning of the 1990 when Estonia regained independence, few food additives were used. The use of food additives has become more common in recent years. Changed food and wide use of home chemicals might be reasons for the shift of intestinal microflora in Estonian children.

Previous studies have shown that the composition of gut microflora at one and two years of life was related to allergy. For example, allergic children more often harboured *Clostridia* and *Staphylococcus aureus* and less often *Bacteroides* and *Bifidobacteria* (Björkstén B *et al* 1999; Böttcher MF *et al* 2000). The findings of present study showed that the infants with allergic diseases were less often colonised with *Enterococci* during the first week (p=0.02) and the first month of life (p=0.03) and with *Bifidobacteria* through the first year of life. The prevalence of *Lactobacilli* was lower in non-allergic (8%) as compared to allergic (36%) newborns (p=0.02) (figure 7). The counts of *Lactobacilli* increased more rapidly in non-allergic children during the first month of life (5.8 (4.3–7.3) vs. 8.6 (6.4–10.8); p=0.02) and there were no anymore differences in the prevalence of *Lactobacilli* between allergic and non-allergic children.

At six months in allergic children, the prevalence of *Staphylococcus aureus* was higher than in healthy infants (p=0.02). Allergic, as compared to non-allergic infants, had higher counts of *Clostridia* at three months (10.3 (7.8–12) vs. 7.2 (3.6–10.8) CFU log/g; p=0.01, respectively) and lower counts of *Bacteroides* at 12 months (9.9 (5.3–11.6) vs. 10.6 (8.3–11.8) CFU log/g; p=0.03, respectively). In non-allergic children the proportion of anaerobic *Bifidobacteria* increased from 15% at first month to 40% at three months (p=0.01), and the proportion of aerobic CONS in the microflora decreased from 20% at first month to 2% at three months of age (p=0.02).



* p<0.05 higher prevalence in non-allergic as compared to allergic children at the same age
[a] p<0.05 higher prevalence in allergic as compared to non-allergic children at the same age

Figure 7. Different prevalence of gut microbes in allergic and non-allergic children during the first year of life. Allergic children are marked with fragmentary line.

The findings of the present study indicate that the intestinal microflora of allergic infants was disturbed already in the first week of life and that the differences remained through the first year of life. Allergic children were less colonised with *Enterococci* and *Bifidobacteria* and more with *Staphylococcus aureus*, and they had higher counts of *Clostridia* and lower counts of *Bacteroides*. Similar results were found in a Finnish study where atopic infants had fewer *Bifidobacteria* and higher counts of *Clostridia* in faeces (Kalliomäki M *et al* 2001). A shift to *Clostridia* in the gut microflora is related to the Western diet and lifestyle (Linneberg A *et al* 2003). The increased prevalence of a positive SPT in Estonian children at the age of five as compared to the earlier Estonian cohort (Julge K *et al* 2001) may perhaps be explained by a changed gut microflora. It has been shown that early colonisation with *Bacteroides* and *Bifidobacterium*-like bacteria was associated with a higher number of IgA and IgG secreting cells in peripheral venous blood (Grönlund MM *et al* 2000), which highlights the importance of microorganisms in the development of the immune response. An early and more extensive colonisation with aerobic bacteria in healthy infants could conceivably induce a strong stimulation of the immune system, including stimulation of IL-12 by Gram-positive bacteria (Hessle C *et al* 2000). The higher prevalence of *Bifidobacteria* in healthy children in early life is also of interest because these microorganisms are known to elicit a Th1-type immune response (Pochard P *et al* 2002).

The importance of gut microflora in the development of allergies is also confirmed by intervention studies with probiotics. Probiotics reduce the symptoms of atopic dermatitis (Majamaa H *et al* 1997; Kalliomäki M *et al* 2001; Rosenfeldt V *et al* 2003). However, there is no evidence that *Lactobacilli* can prevent atopic sensitisation (Kalliomäki M *et al* 2003). Atopic dermatitis in infancy is related to food allergy. It has been demonstrated that gut permeability is increased in children with atopic dermatitis (Pike MG *et al* 1986). Probiotic bacteria may reduce intestinal inflammation and promote endogenous barrier mechanisms and thereby reduce symptoms of atopic dermatitis (Isolauri E *et al* 2000).

The Estonian children were more often prescribed antibiotics by family doctors, and the use of antibiotics was associated with positive SPT results in the Estonian children. The lack of association between antibiotic treatment and sensitisation in Sweden could be explained by the fact that mostly penicillin was used. This compound does not affect the intestinal microflora to the same extent as broad-spectrum antibiotics. Treatment with antibiotics with a broad microbial spectrum could affect a putatively allergy-protective microflora in the Estonian children. Previous clinical studies showed that antibacterial treatment often resulted in long-term decreases in *Bifidobacteria*, *Lactobacilli* and *Bacteroides* and increases of such anaerobic pathogens as *Clostridium difficile*. Animal studies have shown that the use of antibiotics during infancy may enhance Th2-immunity and prevent postnatal Th1 cell maturation, thus resulting in Th2 polarised immune deviation (Oyama N *et al* 2001). Moreover, it has been shown that administration of probiotics after antibiotic treatment improves the intestinal ecosystem and abolishes Th2-shifted immunity induced by the use of antibiotic (Sudo N *et al* 2002). An alternative explanation could be that atopic children are more likely to develop severe infections in early childhood and are therefore more likely to receive antibacterial treatment. However, this explanation is not supported by the present study. Furthermore, atopic children were not admitted more frequently to hospital than non-allergic children.

Recently it has been suggested that the inverse relationship between allergy and a high pathogen load is caused by the stimulation of an immune regulatory network (Yazdanbakhsh M *et al* 2002). The reduced microbial burden in childhood results in the missing immune deviation and reduction in the activity of Tr cells (Romagnani S 2004). The overly hygienic lifestyle in modern Western societies has altered the normal intestinal colonisation pattern in infancy, and this has led to a failure to induce and maintain tolerance of antigens.

The higher prevalence of positive SPT in Estonia shown in the present study as compared to an earlier study (Julge K *et al* 2001) might be partly explained by changes in intestinal microflora. Lifestyle changes may have been modified intestinal microflora, towards becoming more similar to that of Western countries. To support this, a significant decrease in the prevalence of *H. pylori* infection among Estonian children during the last years has been demonstrated (Oona M *et al* 2004). The present study confirms that the gut microflora of Estonian children now resembles to that of Swedish children.

6. CONCLUSIONS

1. Allergic diseases were more common in the Swedish than in the Estonian children. Atopic dermatitis was the most frequent allergic disease in both countries, but manifestation started earlier in the Swedish infants. The Swedish children had more respiratory allergies at the age of five.

2. The Swedish children had more often positive SPTs, whereas the Estonian children had more often circulating IgE antibodies in plasma during the first five years of life. Circulating IgE antibodies in the Estonian children were poorly related to allergic diseases. In the Swedish children circulating IgE antibodies were more common in allergic children.

3. The different responses to allergens in the Estonian and the Swedish children might be due to higher allergen exposure in early life in Estonia, which could lead to higher IgE production. Alternatively, factors that down-regulate clinical allergy and skin reactivity in the presence of IgE antibodies are more commonly present in Estonia than in Sweden.

4. Early colonisation with *Enterococci* and *Bifidobacteria* and lower prevalence of colonisation with lower counts of *Clostridia and Staphylococcus aureus* were associated with the lower prevalence of allergy.

5. A possible protective factor against allergy in Estonian children could be a higher exposure to different microorganisms and microbial products, as shown by the higher endotoxin level in house dust and differences in early gut colonisation. However, the gut microflora of Estonian children has become more similar to that of Swedish children in the recent years.

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SUMMARY IN ESTONIAN

ALLERGIAHAIGUSTE KUJUNEMISE SÕLTUVUS EESTI JA ROOTSI LASTE KOKKUPUUTEST MIKROORGANISMIDEGA VARAJASES EAS

Epidemioloogilised uuringud näitavad, et viimastel aastakümnetel on allergiahaigusjuhtumid kõrge elatustasemega riikide laste ja noorukite seas oluliselt sagenenud (Åberg jt. 1995, Devenny jt. 2004). Pärast Saksamaa taasühinemist tehtud uuringutest selgus, et Ida-Saksamaa lastel oli allergiahaigusi ja atoopilist sensibiliseerumist oluliselt harvem kui Lääne-Saksamaa eakaaslastel (von Mutius jt. 1992). Viis aastat hiljem oli allergiahaiguste ja atoopilise sensibiliseerumise levimus Ida-Saksamaa laste hulgas sagenenud ning muutunud võrdseks Lääne-Saksamaa vastavate näitajatega (von Mutius jt. 1998). Need uuringud tõestasid, et keskkonnateguritel on allergiahaiguste sagenemisel väga oluline roll.

Kümme aastat tagasi Eesti koolilaste hulgas korraldatud uuringud näitasid, et atoopiline sensibiliseerumine ja allergiahaigused olid Eestis sel ajal vähe levinud (Björkstén jt. 1998), viimasel ajal on nad aga sagenenud (Annus jt. 2005). Ka täiskasvanute seas oli siis allergiat harvem kui Rootsis (Jõgi jt. 1998), nüüd aga sageneb atoopiline sensibiliseerumine eriti noorte täiskasvanute hulgas (Raukas-Kivioja jt. 2003).

Allergia tekkimises on olulised geneetiline eelsoodumus, kokkupuude allergeeniga ja keskkonnategurid, mis võivad nii soodustada kui ka takistada allergiahaiguste kujunemist. Geneetilised mutatsioonid ei saa põhjustada allergiahaiguste sagenemist, sest nad on sagenenud lühikese aja jooksul. Ilmselt ei ole süüdi ka suurenenud kokkupuude allergeenidega, sest kui see juhtub varases lapseeas, võib allergiahaigus hoopis kujunemata jääda (Hesselmar jt. 1999, Riedler jt. 2000, Burr jt. 2003).

Allergiahaiguste sagenemise üheks põhjuseks laste ja noorukite hulgas peetakse muutunud eluviisi ja parenenud hügieenitingimusi (Strachan 1989), mille tõttu lapsed puutuvad vähem kokku mitmesuguste mikroorganismidega. Seetõttu ei kaldu Th2-tüüpi immuunsus pärast sündi Th1-tüüpi immuunsuse suunas, mis hoiaks ära atoopilise immuunvastuse tekke. Kokkupuude suure hulga endotoksiinidega varases lapseeas (Gereda jt. 2000) ning fekaaloraalsel teel levivate nakkushaiguste põdemine (Matricardi jt. 2000) võivad kaitsta allergia tekkimise eest hilisemas elus.

Olulist rolli allergiahaiguste vältimisel etendab immuunsüsteemi kujunemist mõjutav soolemikrofloora (Sudo jt. 1997). Sooletrakti mikrofloora tähtsust allergiahaiguste tekkimises näitavad uuringud probiootikumidega, mille manustamine vähendab immuunglobuliin (Ig) E produktsiooni (Matsuzaki jt. 1998) ja leevendab allergiahaiguste sümptomeid (Majamaa jt. 1997).

Uurimuse eesmärk

1. Võrrelda allergiahaiguste esinemissagedust ja immuunvastuse kujunemist allergeenide suhtes Eesti ja Rootsi lastel esimese viie eluaasta jooksul.
2. Hinnata soolemikrofloora mõju allergiahaiguste tekkimisele väikelapseeas.
3. Uurida keskkonnas leiduvate mikroorganismidega kokkupuutumise toimet sensibiliseerumisele ja allergiahaiguste kujunemisele.

Uuringugrupid ja -meetodid

Uuring korraldati ühtse programmi alusel Tartus ja Linköpingis. Vaatluse all olnud Eesti lapsed sündisid SA TÜK naistekliinikus veebruarist 1997 juunini 1998 ning Rootsi lapsed märtsist 1996 märtsini 2000 Linköpingi Ülikooli haiglas. Uuringugruppides oli 115 eesti ja 138 rootsi last, kellest 102 eesti ja 110 rootsi last osales uuringus 5 aastaseks saamiseni.

Lapsed käisid Eestis arsti ning Rootsis õe vastuvõtul kolmandal ja kuuendal elukuul ning ühe-, kahe- ja viieaastaselt. Kahe ja viie aasta vanused Rootsi lapsed vaatas läbi ka arst. Vastuvõttudel hinnati allergiasümptomite olemasolu, tehti nahatorketestid, võeti vereproov ning vanemad täitsid küsimustiku lapse dieedi, läbipõetud haiguste, tarvitatud ravimite ja elutingimuste kohta.

Igal läbivaatusel tehti nahatorketestid naturaalsete toiduainetega (kanamunavalge, lehmapiim), ühe aasta vanuses lisati kassi-, koera- ja kaseallergeen ning kahe aasta vanuses timutiallergeen (firma ALK standardiseeritud allergeenilahused). Ühe, kahe ja viie aasta vanustele Eesti lastele tehti lisaks testid toatolmulesta allergeeniga (*Dermatophagoides pteronyssinus*) ning kahe ja viie aasta vanustele prussakaallergeeniga (*Blatella germanica* – firma Bayer standardiseeritud allergeenilahus).

SA TÜKi allergoloogia laboris määrati kemoluminestsentsmeetodil (Magic Lite) ja fluoroensüümimmuunmeetodil (UniCap) plasma üld-IgE ning allergeenspetsiifiliste IgE antikehade hulk.

Mikroorganismide osatähtsuse hindamiseks allergiahaiguste tekkimises tehti järgmised uuringud:

- Soole mikrofloora uurimiseks koguti roojaproovid lapse esimesel elunädalal, esimesel, kolmandal ja kuuendal elukuul ning ühe ja viie aasta vanuses. Proove hoiti analüüsimiseni –80º juures ning analüüsid tehti Tartu Ülikooli mikrobioloogia instituudis.
- Lapse esimesel elutalvel koguti kodudest tolmuproovid, mille endotoksiinisisaldus määrati USA firma *Bio Whittaker* juhendi järgi Linköpingi Ülikoolis, ning kassi-, koera-, toatolmulesta- ja prussakaallergeenide sisaldus ensüümkaudse immunosorptsiooni meetodil Suurbritannia firma *Indoor Biotechnologies* juhendi kohaselt SA TÜKi allergoloogia laboris.

Uurimuse peamised tulemused ja järeldused

Esimesel viiel eluaastal oli Eesti lastel allergiahaigusi harvem kui Rootsi lastel (27% *vs* 42%, p=0,03). Nahatorketestid näitasid, et atoopilist sensibiliseerumist oli sagedamini Rootsi (34%) kui Eesti (20%) lastel (p=0,02). Rootsi lapsed olid sagedamini ülitundlikud kanamunavalge ja õietolmude suhtes. Nahatorketestide järgi olid viie aasta vanustele Eesti lastele olulised allergeenid kass ja toatolmulest ning mõlema allergeeni puhul oli positiivsete nahatorketestide esinemissagedus 6%. Vaatamata sellele, et Eesti lastel diagnoositi vähem allergiahaigusi ning nahatorketestide tulemused oli harvem positiivsed, leiti Eesti laste vereplasmast IgE antikehi sagedamini kui Rootsi lastel (50% *vs* 35%, p=0,03). Rootsi lastel oli allergeenspetsiifiliste IgE antikehade olemasolu seotud allergiahaigustega, Eesti lastel oli see seos aga väga nõrk. Eesti laste positiivsed nahatorketesti tulemused olid ilma allergeenspetsiifiliste IgE antikehadeta harvemad (8 juhtu) kui Rootsi lastel, kellel nahatorketest oli 35 korral positiivne ilma IgE antikehadeta vereplasmas. Rootsi varasemad uuringud on näidanud, et madala klassi allergeenspetsiifilised IgE antikehad ringlevad nii allergiliste kui ka mitteallergiliste imikute vereseerumis ning mõne aja pärast kaovad (Hattevig jt. 1993). Atoopilise sensibiliseerumise kindlakstegemisel peetakse nahatorketesti väga tundlikuks ja IgE antikehade määramist spetsiifiliseks meetodiks. Meie uuringu tulemused olid Rootsi laste suhtes eelöelduga kooskõlas. Varasemates Eesti laste uuringutes on näidatud, et nahatorketesti tulemused ei langenud kokku allergeenspetsiifiliste IgE antikehade olemasoluga (Riikjärv jt. 1995, Julge jt. 2001). Käesolev uuring kinnitas veel kord, et Eesti lastel on allergeenspetsiifiliste IgE antikehade olemasolu ja nahotorketestide tulemuste kokkulangevus madal.

Eesti ja Rootsi laste immuunvastuse erinevuse põhjuseks võis olla nende erinev kokkupuude mikroorganismidega varajases eas. Uurimusest selgus, et Rootsi lapsed põdesid esimese kahe eluaasta jooksul hingamisteede nakkushaigusi vähem kui Eesti lapsed, mõlemal maal ei olnud aga seost hingamisteede infektsioonide ja allergiahaiguste ning atoopilise sensibiliseerumise vahel. Võrreldes Rootsi lastega määrati Eesti lastele sagedamini antibakteriaalset ravi ning kasutati sagedamini laia toimespektriga antibiootikume. Seda, et antibiootikumide kasutamine esimesel eluaastal suurendas positiivsete nahatorketestide riski Eesti, mitte aga Rootsi lastel, võiks seletada laia toimespektriga antibiootikumide kasutamisega Eestis.

Kodutolmu endotoksiini- ja allergeenisisaldus oli suurem Eestis kui Rootsis. Rootsis oli allergiliste ja positiivse nahatorketestiga laste kodude tolmu endotoksiinisisaldus väiksem kui allergiat mittepõdevate laste kodudes, Eestis aga selline seos puudus. Kodutolmu allergeenisisaldus ei olnud allergiahaiguste ega ka atoopilise sensibiliseerumisega seotud.

Soolemikrofloora uuringutest selgus, et Rootsi lastega võrreldes koloniseerus Eesti laste soolestik suurema hulga bakteritega esimesel elukuul ning Eesti laste soolemikroflooras leidus rohkem koagulaas-negatiivseid stafülokokke,

enterokokke, enterobaktereid, pärmiseeni ja laktobatsille. Eesti laste soolemikrofloora oli sarnane 1960-ndate aastate Rootsi laste omaga. Viiendaks eluaastaks olid Eesti ja Rootsi laste soolemikrofloora erinevused kadunud. Neli aastat varem sündinud lastega võrreldes oli Eesti laste soolefloora viiendaks eluaastaks muutunud selliseks, nagu on kõrge elatustasemega riikides.

Allergiahaiguste ja soolemikrofloora seose võrdlusest selgus, et allergiliste laste soolestik oli esimesel elukuul enterokokkidega ning esimesel eluaastal bifidobakteritega vähem koloniseeritud kui allergiata laste oma. Allergilistel lastel esines seevastu kolmandal elukuul rohkem klostriide, kuuendal elukuul sagedamini *Staphylococcus aureus´*t ning ühe aasta vanuses vähem bakteroide. Soolemikrofloora erinevused annavad vastuse küsimusele, miks antibiootikumravi suurendas Eesti lastel atoopilise sensibiliseerumise riski, mitte aga Rootsi lastel. See võib tuleneda sellest, et Eestis kasutati laia toimespektriga antibiootikume, mis mõjutavad sooletrakti mikrofloorat, Rootsis aga peamiselt penitsilliini, mille mõju sooletrakti mikrofloorale on oluliselt nõrgem.

Kokkuvõtteks: Eesti lastel oli allergiahaiguste ja positiivsete nahatorketestide esinemissagedus väiksem kui Rootsi lastel, seevastu esines Eesti laste vereseerumis oluliselt rohkem IgE antikehi. Tänu suuremale kokkupuutele mitmesuguste mikroorganismidega (kodutolmu suurem endotoksiinisisaldus, sooletrakti intensiivsem koloniseerumine) stimuleeritakse Eesti laste immuunsüsteemi esimestel eluaastatel intensiivsemalt kui Rootsi lastel. Immuunsüsteemi varajane ja tugev stimuleerimine mikroorganismide poolt võiks kaitsta Eesti lapsi allergiahaiguste eest edaspidises elus.

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2002	Põhjamaade suvekool nakkushaiguste epidemioloogiast, Skjoldenæsholm
2002	Voolutsütomeetria ja allergia, Bilbao

Erialane teenistuskäik

2000–2002	Sihtasutus Tartu Ülikooli Kliinikumi lastekliinik, pediaater
1996–1999	Tartu Ülikooli lastekliinik, pediaater
1995–1996	Tartu Ülikooli lastekliinik, vanem-laborant

Teadustöö

Peamine uurimisvaldkond: Allergeenidevastase immuunvastuse ja allergihaiguste kujunemise sõltuvus mikroorganismidest 20 teaduslikku publikatsiooni, kaheksa ettekannet rahvusvahelistel konverentsidel Euroopa Allergoloogia ja Kliinilise Immunoloogia Akadeemia, Eesti Immunoloogide ja Allergoloogide Seltsi ning Eesti Lastearstide Seltsi liige