

# FORALLVENT

## Synoptic report highlighting the gaps in existing knowledge

### Introduction

Until the late nineteenth century the main causes of infant and adult mortality were infectious diseases such as tuberculosis, smallpox, dysentery, pneumonia, typhoid fever and diphtheria (1). Eighty percent or more of children died from these diseases before reaching adulthood. At that time epidemiological studies were mostly concerned with the observation of the spread of infectious diseases among populations. Since the beginning of the twentieth century improvements in housing conditions, sanitation, water supply, nutrition and medical treatment have virtually eliminated infectious diseases as major causes of death in developed countries such as the U.S., and increased the average life expectancy from about 50 years in 1900 to nearly 74 years in 1984 (1). This change has been paralleled by the emergence of chronic illnesses such as heart disease and cancer as the main causes of mortality in developed nations.

As many other chronic disorders asthma, atopic dermatitis and hay fever are likely to be determined by multiple factors, some of which constitute host characteristics such as gender, race, genetic predisposition and atopy, and some of which consist of extrinsic, environmental influences. These factors are likely to interact on many different levels. In addition, certain windows of opportunity for different exposures may exist and differ between types of atopic conditions. Therefore, the timing of exposure may play a crucial role for a certain factor to affect either the onset or the progression of a certain allergic illness. Potential gene-environment interactions must become a focus of attention since complex diseases such as asthma and allergic conditions are likely to be determined by interactions between multiple major and minor genes, and involve important environmental factors for their expression. Both, the search for genes and for environmental determinants relies on increasing knowledge about the mutual cofactors. The role of genetic factors will be discussed in another chapter, therefore the focus of this discussion is on environmental factors related to modern life styles.

## **Prevalence of allergies with respect to traditional or less developed life styles.**

In general, the prevalence of asthma and allergic diseases are higher in affluent, western countries with a high degree of industrialisation than in developing countries with a large rural population. In the large scale International Study of Asthma and Allergy in Childhood (ISAAC) the world wide prevalence of allergic diseases was assessed in the 1990s (2). More than 450.000 school-aged children from 56 countries participated in this survey. Identical methods in all study centres were used in an effort to draw a picture of present-day asthma prevalence around the globe. A 20-fold difference between study centres was found for childhood asthma, while the variation for other allergic diseases was between 20 and 60 fold. The highest prevalence of current asthma symptoms (assessed by questionnaire as wheezing in the last 12 months) was found in the UK, New Zealand and Australia while most developing countries showed comparatively low prevalence rates. In general, centres with low asthma rates also showed low levels of other atopic diseases. However, countries with the highest prevalence for allergic rhinitis and atopic eczema were not identical to those with the highest asthma rates but showed a distribution different from that of childhood asthma.

Furthermore, within European countries a west-east gradient in the prevalence of childhood asthma is apparent. In Germany, the fall of the Berlin wall in 1989 opened a window of opportunity to study a genetically homogeneous population that lived under very different economical and environmental circumstances. In 1992, shortly after the German reunification the prevalence of asthma, allergic rhinitis and atopic sensitisation was significantly higher in West Germany than in the East (3) Likewise, in a study from the Baltic area of northern Europe Swedish children showed a higher prevalence of atopic sensitisation and asthma than did those from Poland and Estonia (4, 5). When a cross-sectional survey was repeated in 1996 in East Germany atopic sensitisation measured by skin prick tests had increased significantly in these children though not as much as to attain prevalence rates of atopy previously observed among school children in Munich (6). The children from East Germany participating in this repeated survey were born 3 years before the downfall of communism and were therefore only exposed to Western living conditions after their third birthday. This may indicate that factors early in life are particularly important for the development of asthma, while environmental factors beyond infancy may influence the development of other allergic disorders. Yet, it remains to be seen whether the prevalence of asthma will increase in the

countries of the former communist Eastern Europe, once children are born and raised in a more "western" lifestyle.

In some developing and developed countries a lower prevalence of childhood asthma in rural as compared to urban areas has been reported, but the evidence is not conclusive. Airway challenges performed in urban and rural areas of Africa suggested that bronchial hyperresponsiveness is almost non-existent in rural areas in the late 80s and early 90s (7, 8). In turn, among the more affluent urban populations of South Africa and Zimbabwe BHR reached a prevalence of 3.2% and 5.9%, respectively. Similar results have been reported from other studies in Africa. However, over the last decade BHR seems to have increased also in rural Africa (9).

Differences in childhood asthma between rural and urban populations in Western countries are less pronounced. In a large British study only marginal differences in the prevalence of childhood asthma between rural and urban areas were observed (10). However, children from rural Scotland tended to show a lower prevalence of severe asthma symptoms (11). Data from Sweden indicated a higher prevalence of atopic sensitisation to aeroallergens in children from urban centres as compared to rural areas, but no information was available for childhood asthma (5). The increased risk for children to develop an atopic disease in an urban environment was largely attributed to increased levels of air pollution, particularly related to heavy car traffic exposure. However, recent findings from Switzerland (12), Austria (13) and Southern Germany (14) gave evidence that lower prevalences of atopic diseases in rural populations may rather be attributable to the presence of protective factors in a farm environment than due to the absence of urban risk factors.

### **The role of microbial exposure for the development of allergic illnesses.**

Strachan first reported that sibship size is inversely related to the prevalence of childhood atopic diseases (15). This observation was confirmed by numerous studies ever since. Consequently, it was shown that with increasing numbers of siblings children had less hay fever (16), atopic eczema (17) and showed a lower prevalence of positive skin prick tests (18) and lower levels of allergen-specific IgE (16). In large enough studies it was furthermore possible to distinguish between the effects of older as compared to younger siblings. The majority of these analyses found a stronger protective effect when older siblings were present.

However, the relation between family size and childhood asthma and BHR is less clear. Studies from New Zealand (19) and Australia (20) suggested that having more than one older sibling is a strong and independent protective effect for the development of childhood asthma. A similar association between asthma and the presence of younger siblings was observed in a large British study (21). Conversely, a number of earlier studies did not see a protective effect of a large family size for the development of asthma and BHR (22).

Several hypotheses have been proposed in an effort to explain the intriguing association between sibling numbers and the occurrence of atopic diseases. For one, it has been consistently shown that the effect of a larger family size is independent from that of parental socio-economic status. Furthermore, it was proposed that maternal age at birth may be responsible for the protective effects of having older siblings. Nevertheless, a lower prevalence of atopic diseases was still observed after adjustment for maternal age and some studies have even linked increasing maternal age to an increase in the prevalence of atopy (23). Another potential explanation for the effects of family size on childhood atopic diseases may be that multiple pregnancies alter the immune status of the mother in a way that protects a child from developing atopic diseases. However, the findings from recent studies do not support this notion. Another potential explanation for the effects of family size on childhood atopic diseases may be the fact that having more siblings increases the exposure to infections passed on between children as proposed by Strachan. Therefore, having older siblings may contribute to a higher infectious burden thereby directing the development of the immune system in a non-atopic direction.

In recent years, widespread attention has been given to the advancement of this field in allergy research which investigates the potential link between exposures to microbial sources and the development of allergic illnesses. The theory attempting to catch the various elements of this complex relation has been coined the 'hygiene hypothesis'. A large scientific and laic audience has been confronted with these ideas over the last years and in the course of numerous deliberations new angles and aspects of the hypothesis have been proposed. At least three distinct claims on the true nature of the 'hygiene hypothesis' have been brought forward. These contentions relate to the potential role of overt and unapparent infections of human subjects with viruses and bacteria; the relevance of non invasive microbial exposures

in the environment; and the influence of such exposures and infections on a subject's innate and adaptive immune response.

Before attempting to address these various aspects of the 'hygiene hypothesis' it seems justified to highlight the complex nature of the problem. The grid in which the pieces of this jigsaw must be assembled is at least four dimensional comprising the affected phenotype, time, the environment and the genetic susceptibility. Although in clinical practice manifestations of allergic illnesses sometimes appear rather uniform, the underlying mechanisms and causes may be manifold. For example, urticaria is an easily recognisable skin condition. But the variety of factors eliciting these appearances ranges from infectious stimuli to allergic mechanisms to neoplastic illnesses. It seems reasonable to assume that not a single cause but many will underlie the clinical manifestation. Likewise, there is increasing evidence over recent years to support the notion of a heterogeneous nature of the asthma syndrome.

A number of studies have clearly shown that the effect of a given exposure depends on the timing. At least over childhood and adolescence the human organism is in a constant stage of development and maturation. It is conceivable that these predefined processes display windows of accessibility and vulnerability to extrinsic influences only at certain stages of development. Moreover, prenatal factors may play a significant role either through mechanisms acting *in utero* or as epigenetic modulation of subsequent developmental trajectories. Our journey into the discovery of the relevant genes for allergic diseases has just begun and we are at the very beginning of a fascinating field of research. The first glimpses into this novel field suggest that no single gene will be responsible for the clinical manifestation of allergic illnesses. Rather alterations in many genes interacting with environmental influences at various time points of development are expected to contribute to the mechanisms underlying the various atopic conditions.

An interesting debate about the immunological mechanisms potentially underlying the protection against allergies mediated by living in a "less hygienic" environment is ongoing. One mechanism frequently associated with the 'hygiene hypothesis' is the skewing of the Th1/Th2 balance away from allergy-promoting Th2 towards Th1 cells (24) The link between Th1/Th2 balance and allergic diseases is mediated in part by IgE: Th2 cells, by secreting IL-4 and IL-13, promote immunoglobulin class switch recombination to IgE (for review, see (25)).

However, there are some conflicting data which cannot be disregarded. Not only Th2-related diseases such as allergies are on the rise over the last decades, but also inflammatory diseases such as Crohn's disease and diabetes mellitus (26-27). Populations with high incidence of helminthic infections favouring a Th2 type of immune response are protected from allergic diseases (28). Furthermore, *in vitro* and animal data show that activation of the innate immune system does not necessarily promote a Th1 response; depending on the experimental conditions also Th2 responses may result (29). Finally, upon *in vitro* restimulation with LPS of peripheral blood leukocytes of children living in an environment with high microbial load both Th1- as well as Th2-related cytokines were found to be downregulated (30).

Thus, while mechanisms related to Th1/Th2 balance undoubtedly are of primordial importance for the development of allergies, they may not suffice to explain the effects related to the 'hygiene hypothesis'. Mechanisms operative at other steps of allergy development have to be considered when trying to understand the effects of microbial exposures. Serum levels of IgE, as of any other immunoglobulin isotype, are not only determined by class switch recombination to this isotype but also by factors regulating terminal differentiation of already switched B cells and the rate of secretion of IgE. Interleukin-6, a pro-inflammatory cytokine, is one prominent factor governing these processes. Furthermore, T regulatory cells (Tregs) in interaction with dendritic cells occupy a central role in controlling immune responses, and their importance for the development of allergies has been well documented (31-32). Finally, also mechanisms inherent to the innate immune system related to endotoxin tolerance may contribute to the effects elicited by exposure to microbes, as will be discussed later.

## **Infections as intermediary of the 'hygiene hypothesis'**

### Viral infections and asthma

Before examining the role of various viral infections for the inception of asthma and wheeze we must define the phenotype as precisely as possible. On a chronological scale the first delimitable phenotype relates to the transient wheeze of infancy which is manifest in the first 1-2-3 years of life and then disappears (33). Antenatally acquired decrements in lung function, exposure to tobacco smoke through the mother *in utero* and low birth weight are likely to causally contribute to disease expression (34-35). Viral infections of the upper

respiratory tract are the most prevalent and potent triggers of transient wheezing in infancy (36), but are unlikely to be causal determinants of this condition.

Beyond infancy wheezing phenotypes are much harder to unequivocally classify. Persistence of symptoms from infancy to school age has been proposed (33), while others have classified wheeze at school age either as current symptoms or as a diagnosis of asthma by parental report. Conflicting results have emerged from these analyses. While infections of the lower respiratory tract early in life have been identified as risk factors for persistent wheeze and asthma (37-38), nasal symptoms and day care attendance early in life have been inversely related to the same outcomes at school age (39 – 41).

The inconsistency may in part be attributable to the disregard of an important discriminating feature, namely atopy. Children with persistent wheeze into school age can have signs of atopy or lack any specific IgE antibody production. In a general population birth cohort in the UK, non-atopic wheeze was as prevalent as atopic wheeze at age 10 years and each phenotype affected around 10 percent of enrolled children (42). The risk factor profile differed significantly between both conditions. Non atopic wheeze was mainly associated with recurrent chest infections at age 2 years, whereas atopic wheeze was related to allergic illness in the child and the family. It has been known for long that viral associated wheeze has a milder course and a better prognosis than allergic asthma (43). Thus, the inverse association between frequent wheeze at school age and day care or nasal symptoms early in life might be attributable to the strong link of viral infections with the milder form of non-atopic wheeze thereby falsely suggesting a protection for all forms of wheeze.

It is, however, also conceivable that a child's increased exposure to viral infections through day care or contact with other children can influence the phenotypic expression. In cross-sectional surveys recurrent respiratory tract infections during the first 3 years of life have been shown to be negatively associated with atopy among asthmatic children at school age (44-45). Thus increased exposure to viruses in a child's environment may foster a milder form of wheezing by suppressing the atopic component. This notion is further supported by the studies investigating the effects of day care and rhinitis exposure early in life which all showed a protection from atopy in the exposed children (39, 40, 46).

These epidemiologic observations rather crudely assessed the exposure to respiratory viruses without taking any detail such as the type of infecting virus, its virulence, the severity of the infection and the viral load into account. Among respiratory viruses some may exert more deleterious effects than others. The ensuing discussion has mainly centred around two viruses that have been associated with asthma and wheeze in a large number of studies, namely rhinovirus and respiratory syncytial virus.

Rhinovirus has been detected in 80% of nasal aspirates of school age asthmatic children within 4 days after parents reported episodes of wheezing (47). A recent birth cohort of high risk infants (COAST Study) has extended this work to lower age groups and confirmed that also in infants and toddlers up to the age of 3 years rhinovirus was the main isolate from nasal lavages taken during symptomatic periods (48). Infantile rhinovirus illnesses were the ones most significantly associated with the prevalence of wheezing in the 3rd year of life. Interestingly, infants with wheezing rhinovirus illnesses were 2 to 3 times more likely to wheeze in year 3 compared with infants who wheezed with RSV infections. Furthermore, 3rd year wheezers were not infected more often during the 1st year of life, but clearly developed more severe symptoms of illness suggesting that it is not the repeated infectious insult which elicits the wheeze, but rather the viral infection unmasks the underlying disposition.

These data put previous findings regarding RSV infections and their relation to asthma development into perspective. Only half of the high risk infants of the COAST study who were infected with RSV wheezed, again suggesting that host factors are likely to play a significant role. Once a RSV infection has been accompanied by wheeze and bronchiolitis the risk of subsequent wheeze is increased until school and early adolescent age (49-50). Most studies did not find an association between RSV infection and the development of atopy. Furthermore, in the Tucson Children's Respiratory Study confirmed RSV had a similar impact on subsequent wheeze as other confirmed viral infections (49). It is therefore plausible that RSV is just one of many respiratory viruses which are associated with the non-atopic persistent wheeze phenotype as discussed above. The effect of each particular virus will depend on its own characteristics, the elicited immune response and the interacting host factors. In addition, various genetic factors are likely to significantly modulate the immune responses to viruses as recently shown in the COAST Study (51).

It has been suggested that viruses influence the differentiation of T cells upregulating Th1 responses (52). Viruses may also stimulate IL-10 production in T regulatory cells (53). But viruses differ with respect to their invasive properties and the elicited immune response. Rhinovirus for example infects only small areas of the epithelial layer with little or no mucosal damage. Even with large inoculating doses of virus, less than 10% of cells become infected. In contrast RSV damages large groups of epithelial cells resulting in edema, shedding of death cells and increased mucosal permeability (54).

The response to an invading virus depends on a host's immunological set up. Deficiency in IFN- $\gamma$  responses before the onset of bronchiolitis has been shown in men and mice, suggesting an impaired viral defence prior to infection (55-56). Infants at risk of allergies may be particularly deficient in IFN- $\gamma$  responses. Although initial studies have supported this concept (57), long term follow up of such infants into school age has not confirmed the initial observations (58). In fact, none of the early immunological parameters including Th1 and Th2 cytokines were significantly predictive of allergic disease at age 6 years (58). In turn neonatal antigen presenting cells fail to upregulate MHC class II and costimulatory molecules (59) thereby providing insufficient stimuli to responding cells. Whether this deficiency differs between infants at risk and controls, remains to be elucidated. Finally, it is unknown whether differences in antibody concentrations and classes exist between these groups.

In children at risk of allergy development very little is known about the immunological predictors of virus induced wheeze. Gern and colleagues have recently shown that in infants with a family history of allergies and/or asthma, mononuclear cell phytohemagglutinin-induced IL-13 and virus-induced IFN- $\gamma$  responses at birth were indicative of the risk for wheezing in the first year of life (60).

In older children viral infections might interact with airway inflammation in several ways. Damage to the airway epithelium and consequently enhanced absorption of aeroallergens might lead to increased airway inflammation (61). Furthermore, induction of pro-inflammatory cytokines (IL-6, IL-1- $\beta$ , TNF- $\alpha$ ), chemokines (IL-8, MCP-1, MIP-1 $\beta$ ), adhesion molecules (ICAM-1) and leukotrienes enhance cellular recruitment, activation and inflammatory responses (62- 63). This scenario is likely to occur among atopic and non-atopic wheezers. Finally, in asthmatic individuals viral clearance might be inhibited thereby leading

to more severe infections. However, studies in humans and mice do not support this notion (64,-65).

Differences in specific characteristics of each virus resulting in distinct immunological changes may play a role. The studies to date present a multifaceted story, demonstrating either Th2 or Th1 responses following RSV infection in humans and murine models (66-67). For rhinovirus infections, increases in IL-10 indicating a role for regulatory mechanism through e.g. regulatory T cells were proposed which might lead to an atopy-protective effect by this virus (68-69). Further, a deficiency in interferon  $\beta$ , impaired apoptosis and increased virus replication was demonstrated in rhinovirus in vitro models (70), opening the possibility for type I interferon's in the treatment or prevention of virus-induced asthma exacerbations. Some studies point out epidemiologic and immunologic similarities between bronchiolitis caused by influenza and RSV, and suggest that host factors are more important than the nature of the infecting virus in the development of severe forms of bronchiolitis caused by influenza and RSV (63).

#### Other viral infections and allergic illnesses

There is conflicting evidence relating results of serological studies investigating hepatitis A and relating these findings to the prevalence of hay fever and atopic sensitization in population based studies. A number of surveys have shown protection from allergy with a positive serology to hepatitis A (71–73) whereas others could not confirm these results (74 – 75). A positive serology to hepatitis A may thus be a marker of other unhygienic environmental exposures rather than a true culprit of the association, although the immunological characteristics of hepatitis A may suggest a modulating effect. The receptor for the hepatitis A virus is TIM-1. This receptor and its ligand TIM-4 belong to a family of proteins which are involved in the regulation of CD4 T cell differentiation, airway inflammation and airway hyperresponsiveness (76).

Few other viruses have been specifically investigated in the context of epidemiological studies investigating the determinants of allergic diseases. Two reports have suggested a protective role for herpes infections (39, 71), but confirmation in other populations is awaited. Also infections with Epstein Barr and cytomegalie virus have been inversely related to specific IgE levels (77).

### Bacterial infections

There are few studies investigating the association between the occurrence of bacterial infections and the development of asthma and allergies. While in a large Norwegian survey otitis media in infancy was negatively associated with allergic sensitisation in school age children of atopic parents (78), no relation between a number of bacterial infections including otitis media and asthma was seen in the prospective MAS cohort (39). In Sardinia, children who had been hospitalized with salmonellosis had a lower prevalence of allergic rhinoconjunctivitis and asthma than children who had been hospitalized with non bacterial enteritis (79), but these findings need confirmation in other populations. In turn a number of studies have investigated the potential protective effect of infection with *helicobacter pylori* by measuring IgG antibodies towards this pathogen. Inverse associations with sensitisation towards aeroallergens were seen in 2 studies (74, 80), and in a composite score of seropositivity to *hepatitis A*, *toxoplasma gondii* and *helicobacter pylori*, the latter also contributed to an inverse relation with atopic sensitisation, allergic rhinitis and asthma (73, 81). Interestingly, a dose response relation was observed in these studies: the more infections these subjects had encountered, the lower the observed prevalence of atopy, allergic rhinitis and asthma was. These findings clearly suggest it is not one microorganism which accounts for the observed protection but most likely a number of agents.

The potential beneficiary role of *mycobacteria* exposure has been heavily discussed. The deliberations were grounded on literature suggesting a strong inverse association between BCG vaccination and the prevalence of allergic illnesses in Japan (82). Subsequent work has however refuted such a role of BCG immunization for the development of asthma and allergies in western populations (83 – 86). The interest in *mycobacteria* is however continuing as this microorganism shows some remarkable potentially immunomodulatory characteristics. In murine models of allergic asthma treatment with mycobacteria resulted in a suppression of several allergic features. The precise mechanisms of this suppressive effect are under investigation. Mycobacteria have been associated with increased Th1 immune responses, primarily an augmented IFN- $\gamma$  secretion (87-88), but others were unable to reproduce these findings (89-90). Therefore, other mechanisms such as the induction of regulatory T cells and IL-10 dependent mechanism may be responsible for the observed effects (91- 92).

## Parasites

It seems unlikely that in westernized societies parasitic infections will play a major role for the protection from asthma and allergies. There is however good evidence to suggest that in endemic areas such as in Africa or Latin America parasitic infections are strongly inversely related to the development of atopy (see reviews in (93-94).

## **Environmental exposures as intermediary of the 'hygiene hypothesis'**

The strongest claim for a critical role of protective environmental exposures in the inception of asthma and allergies arises from studies investigating environmental factors in rural areas of Europe, particularly in the farming community.

### The epidemiological evidence

Since 1999 fifteen studies have been performed in rural areas in Europe, namely in Switzerland( 12, 30, 95) Germany (30, 95, 96) Austria (30, 95, 97) France (98), Sweden (95, 99, 100), Denmark (101, 102), Finland (103 – 105) and Britain(106). Almost all studies reported a decreased prevalence of hay fever and allergic rhinoconjunctivitis and all surveys that included objective measures of specific IgE antibodies either by skin prick tests or by serum measurements demonstrated a significantly decreased prevalence of atopic sensitisation among farm children compared with non farm children (107). Findings relating to asthma and wheeze are less consistent. While in a large survey including over 10,000 schoolchildren in Germany (96), children of farmers had a lower prevalence of asthma (OR=0.65 [95% CI: 0.39-1.09]) and wheeze (OR=0.55 [95% CI: 0.36-0.86]), other surveys in Switzerland (12), France (98) and Finland (103) did not find significant differences between groups. Only two studies included measures of airway hyperresponsiveness and one of these using wood and coal heating as a proxy for farming (101, 108 – 109). In Denmark and Germany the prevalence of airway hyperresponsiveness was significantly reduced among farm children compared with non farm children (101, 108). Another Canadian survey confirmed these findings (110). To my knowledge, no study has shown an increased prevalence of airway hyper-responsiveness among children raised on farms.

Children raised on farms in Europe seem to retain their protection from allergy at least into young adulthood (98, 102, 111 - 112). In a comparison of Danish farming students and, as

controls, conscripts from the same rural areas, the prevalence of atopic sensitisation to common allergens was lowest in farmers who in childhood had lived on a farm, intermediate in farmers without a farm childhood and controls with a farm childhood, and highest in controls without a farm childhood (102). Likewise, in a German study of adult farmers aged 18 – 44 years (111 – 112), farm animal contact in childhood was associated with a decreased risk of atopic sensitization. Continued exposure to farm animals in adulthood further decreased the odds ratio of atopic sensitisation associated with symptoms of allergic illnesses (OR= 0.2 [95% CI: 0.1-0.4]). However, starting farm animal contact in adulthood increased the odds of asymptomatic atopic sensitization (OR=2.4 [95% CI: 1.1-5.2]). Whether the increased protection of continued exposure from infancy into adulthood is attributable to sustained exposure or whether a ‘healthy worker effect’ in part explains these findings remains to be elucidated.

Farming practices vary between farms and between countries and this diversity may contribute to the heterogeneity of farm effects on asthma across studies and countries. Some investigators have attempted to identify individual exposures in farm surroundings contributing to the reduction in risk of asthma and allergic diseases. Initial observations from Germany and Switzerland reported that children from full-time farmers had lower risk of atopic disease than children of part time farmers (12, 96) suggesting a dose-response effect. Two recent studies conducted outside Europe (113 – 114) seem to suggest that an important component of the farm environment is livestock exposure, since no protective effect of farming was observed among children living in a primarily crop farming region in Australia. This notion is supported by the finding of the European studies where exposure to livestock has been identified as an important contributor to the protective farm effect (31, 96, 103, 106). Interestingly in the Austrian study children who did not live on a farm but who had regular contact with farm animals also had a lower prevalence of allergic sensitization (13.5 % versus 34.8%) (97). Another source of protection has been identified in a number of studies: the consumption of unpasteurized milk (30, 106, 114). As with livestock exposure, this effect was not restricted to children living on a farm, but was also seen among non farm populations consuming unpasteurized milk (106). Recent work has transferred this observation into experimental studies and has shown that treatment of BALBc mice with extracts of stable dust during a conventional sensitization protocol with ovalbumin inhibits the development of airway hyperresponsiveness and eosinophilia upon ovalbumin challenge(115). These findings suggest that dust from stables of animal farms contains strong immune modulating substances

and that these as yet unknown substances suppress allergic sensitization, airway inflammation and airway hyperresponsiveness in a murine model of allergic asthma.

Few studies have investigated the role of the timing of the various farm exposures. Both in the ALEX and the PARSIFAL study there is strong evidence pointing towards the importance of early life exposures either in the first year of life or even in pregnancy for the development of protection from atopic sensitization and asthma (30, 116). Exposures occurring after the first year of life had no or much weaker effects (30). Although these studies admittedly are cross-sectional in design and therefore the exposure has been assessed retrospectively, recall bias is unlikely to significantly confound the findings since infantile exposures are closely linked to maternal exposures, reflecting a continuing pattern of maternal tasks on the farm.

### Microbial exposures

If livestock exposure is indeed associated with a decreased prevalence of asthma and atopy, then underlying exposures should be investigated. Children exposed to livestock may be exposed to more allergens, bacteria, viruses and fungi than children without exposure to livestock. Yet, only few out of the many microbial exposures have been measured in farming environments so far. Bacterial substances such as endotoxin from gram negative species and muramic acid, a component of peptidoglycan from the cell wall of all types of bacteria, have been found to be more abundant in mattress dust from farm children than non farm children (117). Likewise, extracellular polysaccharide (EPS) from *Penicillium* and *Aspergillus spp.* is more prevalent in farming households than non farm households (117).

In the ALEX study, the multicenter study conducted in Austria, Switzerland and Germany, endotoxin levels in samples of dust from the children's mattresses were inversely related to the occurrence of hay fever, atopic asthma, and atopic sensitization (118). However, nonatopic wheeze was not significantly associated with the endotoxin level. In turn, independently of the endotoxin concentration, increasing mattress dust muramic acid concentrations were associated with a lower frequency of wheezing and possibly asthma among rural school children in the ALEX study (119).

The inverse association found in the ALEX study between endotoxin and atopic wheeze was confirmed in the larger multicenter PARSIFAL survey performed in Sweden, the

Netherlands, Germany, Austria and Switzerland (120). When restricting the PARSIFAL analyses to similar groups of children as in the ALEX study, farm and farm-reference children in Germany, Austria and Switzerland, an adjusted odds ratio (aOR) of 0.56 (95 %CI: 0.35-0.90) was observed. After adjustment for glucans and EPS, the effect of endotoxin was however no longer significant. Since exposures to endotoxin, EPS and glucans were moderately, but significantly correlated, a firm conclusion on the degree to which specific agents contributed to the observed effect is precluded. Moreover, these microbial agents might be markers of a much broader spectrum of microbial agents. Muramic acid was not measured in the PARSIFAL study. It is furthermore noteworthy that in this study all measured microbial compounds (endotoxin, glucans and EPS) did not explain the protective ‘farming effect’ suggesting that other yet unknown microbial exposures confer the protection seen in the farming environments. The results do however also suggest that mould components might modulate immune responses and thereby protect against allergic diseases, as previously suggested for endotoxin (121 – 122). This is in line with a study on the effects of endotoxin and fungal spores on atopy and asthma in adult farmers, in which fungal spores, rather than endotoxin, were inversely related to atopic wheeze (123).

#### Protective immune responses in farming environments

Few studies have investigated immune responses in children raised on farms and have compared them to immune responses in children raised in rural but non farming environments. Since farming is associated with elevated exposure to microbes and/or microbial components, the Swiss arm of the ALEX study investigated whether growing up on a farm affects the expression of receptors for microbial compounds such as the monocytic antigen CD14 (involved in recognition of Gram-positive and Gram-negative products), TLR2 (a receptor for bacterial lipoproteins), and TLR4 (the receptor for lipopolysaccharide, LPS). Peripheral blood leukocytes from children of the ALEX population living on a farm were found to display increased expression of the genes for CD14, TLR2, and TLR4 (124).

The impact of farming on the expression of innate immunity genes was then further examined by the Swiss research team in 125 children of farmers and 127 children of non-farmers from the PARSIFAL study (). RNA was extracted from EDTA-treated blood, and expression of *TLR2*, *TLR4*, and *CD14* was determined by quantitative PCR (TaqMan®). The results confirmed the initial observation suggesting that environmental exposures, in particular to

microbial components affects the expression of genes encoding microbial ligand receptors (116).

The Swiss research team then assessed whether changes in gene expression correlated with prenatal or postnatal exposure to farm factors. After adjusting for age, sex, family history of atopy, parental education, environmental tobacco smoke, maternal smoking during pregnancy, number of older siblings, contact with pets ever, study center and current farm exposures, maternal exposure to stables during pregnancy (i.e., prenatal exposure of the child) significantly correlated with an increase in the expression of *TLR2* [1.44 (1.04-1.98), geometric means ratio and 95% confidence interval], *TLR4* [1.40 (1.07-1.83)] and *CD14* [1.66 (1.18-2.33)]. Interestingly, a dose response relation was seen. Expression of *TLR2*, *TLR4* and *CD14* increased with the number of different farm animal species the mother had contact with during her pregnancy (116). Overall, these findings suggest that environmental exposures very early in life may lead to epigenetic changes, which in turn may alter gene expression.

These findings also suggest a role for innate immunity in response to these environmental exposures. Pathogen-associated molecular patterns (PAMPs), evolutionarily highly conserved structural components of microbes, are recognized by similarly conserved receptors of host innate immune systems, the pattern recognition receptors (PRRs). Examples for PAMPs are the bacterial compounds cited above, lipopolysaccharide (LPS, endotoxin), and muramic acid, a component of peptidoglycan which is part of the cell wall of most bacteria. Examples for human PRRs are the human Toll-like receptors (TLRs) and CD14. To date ten functional TLRs have been described in humans. The cellular signalling cascade ensuing engagement of TLRs initiates innate host defence mechanisms (125), but it also provides signals required for initiating and modulating the adaptive immune response (126). In the context of the development of asthma and allergies, TLR4 as receptor for LPS, and TLR2 which recognizes peptidoglycan of gram-positive bacteria, have received the most attention. Polymorphisms in the genes for TLR4 and TLR2 have furthermore been shown to interact with the farm environment modulating the allergy protective effect (127).

Engagement of TLRs triggers a signalling cascade resulting in activation of host defence mechanisms, inflammatory responses and signals for initiating adaptive immune responses. Exposure to microbial compounds may thereby skew the Th1/Th2 balance towards Th1 responses. However, in the ALEX study no skewing towards Th1 responses was observed among school-aged farm children (118). Production of both Th1 and Th2 cytokines (TNF- $\alpha$ ,

IFN-g, IL-10, IL12) by leucocytes stimulated with LPS was inversely related to the endotoxin level in the bedding, indicating a marked down-regulation of adaptive immune responses in exposed children. These findings point towards a phenomenon described as endotoxin tolerance, reflecting an old observation that repeated or prolonged exposure to endotoxin results in a refractory state (128). The potential role of regulatory T cells has so far not been explored in the context of farm exposures.

With respect to sensitisation to common aeroallergens, the underlying mechanisms may be even more complex. In the ALEX study allergen-dependent switching patterns were reconstructed *in vivo* to identify the level(s) at which farm exposure acts to protect against atopy by assessing serum IgG1 to IgG4 and IgE levels to grass, cat, and mite allergens (129). Farm exposure had complex allergen-specific effects on IgG1, IgG4, and IgE levels. Exposure protected against grass-specific responses at every step along the IgG1/IgG4/IgE switching pathway. Protection from cat responses was concentrated at the IgG1 level. For all allergens, failure to express IgG1 was associated with low prevalence of IgG4 or IgE responses. These findings suggest that the protective effects of farm exposure on atopic sensitisation are allergen and switch stage specific. No counterbalancing, protective effect of IgG4 production as proposed in the context of urban cat exposure (130) has been seen in these farming environments.

In the absence of farm animal contact a number of studies have investigated the role of cat and dog exposure. A review of the associations between pet exposure and asthma and asthma like symptoms was undertaken by Apelberg in 2001 who concluded that in children over 6 years of age a significant increase in the risk for asthma or wheezing was seen, whereas in children younger than 6 years of age exposure to pets had a protective effect (131). Since then, a number of prospective studies showed a reduced risk of allergic diseases or allergic sensitization in children exposed to cats or dogs in infancy, but consistency among these studies is lacking (132-140). Parental, in particular maternal history of asthma and atopy is likely to exert significant effect modification with pet exposure being a risk factor among high risk children and a protective factor among low risk children (136, 138, 132, 137). Additionally, marked differences between different animal species are likely to exist, with exposure to dogs showing a more consistent pattern of protection than exposure to cats (136, 132). These results must however be regarded with caution since reverse causation due to already implemented avoidance strategies in families with atopic heredity cannot be excluded

with certainty. As of today, the available literature does not provide conclusive guidance regarding the effects of pet exposure early in life and thereby on the potential preventive aspects of dog or cat ownership.

### **Practical implications of the ‘hygiene hypothesis’.**

To date there are no practical implications which can be deduced from the ‘hygiene hypothesis’ for the prevention of asthma and allergies, as discussed in part above. Many authors have been concerned about a potential harm associated with the prescription of antibiotics. Antibiotics, vaccinations and paracetamol have been postulated to interfere with the immune response in the developing immune system and thus to enhance the development of allergic diseases. These associations may however be attributable to reverse causation. Because in many countries asthma, particularly at young age, is still treated with antibiotics and paracetamol, an association between the use of these drugs and asthma must become positive. Only surveys taking the indication of antibiotic and paracetamol prescription or the antedating of drug usage before the beginning of asthma into account will therefore be able to adequately address this question. From such prospective studies there is little evidence to suggest that antibiotics administered early in life before any wheezing has become manifest indeed increase the risk of disease (141-143). Antibiotics and paracetamol administered during late pregnancy or during lactation may however confer a risk. A number of studies have shown an association between antibiotics administered to the mother during pregnancy or lactation with the development of allergic diseases in their offspring in prospective birth cohorts (141-143). Likewise, the Longitudinal Study of Parents and Children (ALSPAC) study found an association between paracetamol use during late pregnancy and the development of asthma, wheeze and total IgE by the age of 6-7 years in the offspring (144).

As discussed above observational studies have found a correlation between the colonization of the gastrointestinal tract with lactobacillus as well as eubacteria and a decrease in allergic diseases. Interventional birth cohorts have examined the effect of different doses of probiotics (live microbial food ingredients) on the development of allergic diseases in the last years. All studies were conducted on high risk infants and each studied a different probiotic bacterial strain. After administering either the probiotics Lactobacillus GG, Lactobacillus reuteri or a mixture of four probiotics and one prebiotic to pregnant women and subsequently to their offspring for the first 6 (147-148) and 12 (149) months, three studies have seen a protective

effect for the incidence of eczema (147) and atopic eczema (148-149) up to the age of 2 (148-149) and 7 years (147). One survey administered *Lactobacillus acidophilus* to infants during the first 6 months, but not to their mothers during pregnancy (150). The authors found an increase in atopic eczema but did not see an effect on the cumulative incidence of eczema at the age of 12 months. Whether these contrasting results are attributable to a different bacterial strain or to the postnatal administration remains unclear. Moro et al. showed a beneficial effect of a mixture of prebiotic oligosaccharides (GOS/FOS) added to bottle fed infants in reducing the incidence of atopic eczema during the first six months of age (151). Taken together, the current evidence suggests that probiotics might have a preventive effect on the development of eczema. However, in our opinion a number of issues remain unanswered which preclude firm recommendations on the use of probiotics for the prevention of allergies: which is the best probiotic strain? What is the optimal dose? Is there any effect among children from the general population without a family history of atopic diseases in first degree relatives? Is this preventive effect prolonged into childhood years? Are other allergic illnesses such as asthma and hay fever also amenable to prevention by the administration of probiotics?

Numerous research groups are working on other future applications of other immunomodulatory compounds for example derived from *Mycobacteria* (152), helminths (93) and bacteria (CpGs) (153-154). These are promising avenues into the future which are awaited with great interest. The aim of the FORALLVENT project is therefore the creation of a forum for the development of strategies enabling to close the gap between the science on allergic disease and the practical application of its results in prevention, clinical treatment and in the formulation of specialized foods as described in the previous paragraphs.

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