

EU forum

The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe

Since January 2002, the European Commission is funding a large project, 'Protection against Allergy – Study in Rural Environments' (PASTURE; contract no. QLK4-2001-00250), under the Fifth Framework Program in the field of epidemiology of allergic diseases. The aim of this paper was to describe the background and design as well as the aims of the project. Asthma and allergic disorders are a major public health problem in many Western countries. The aetiology of asthma and allergic disease remains poorly understood despite considerable research. Epidemiology has the potential to add greatly to the understanding by elucidating the risk factors for asthma and allergic disease and thereby suggesting productive avenues for research into causation and prevention. Several risk factors for the development of asthma and atopic disease in children such as passive smoke exposure during pregnancy and infancy, low birth weight or high body mass index later in life have been described. Furthermore, there is consistent evidence that the prevalence of atopy increases with higher socio-economic status. Levels of air pollution such as ozone, NO₂, SO₂ and particles are likely to provoke acute exacerbations of pre-existent respiratory disease. Their role in the inception of asthma and allergies remains to be clarified. Allergen exposure has been linked to the development of atopic sensitization to that particular allergen in children as well as in adults with occupational exposures. Exposure to house dust mite or cat allergen is, however, unlikely to contribute to the development of childhood asthma. In turn, pet keeping in the first year of life, particularly, dog keeping, has been inversely related to the development of wheeze and atopic illnesses. Several prospective birth cohort studies found a decreased prevalence of atopic disease in children having daily contact to pets, in particular to cats and dogs, during early infancy (1, 2). The protective effect might be attributable to allergen or other exposures associated with pet ownership, but may also in part be because of the removal of pets in families with sensitized or symptomatic children or in families with a positive history for atopy at the time the child was born.

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Introduction

Protective factors – the hygiene hypothesis

In the last decade, the so-called hygiene hypothesis proposed by Strachan (3) in 1989 has found increasing support. This hypothesis states that in young families cross infection between older siblings and the infant as well as between the mother (infected by her older children) and the infant may provide protection against the development of atopic disease. Several studies confirmed Strachan's finding that the prevalence of hay fever and atopy decreased with an increasing number of older siblings. Furthermore, it was shown that the number of

subjects living in the same household (crowding) was positively associated with the incidence of infections of the respiratory tract and negatively associated with the incidence of asthma and atopy (4). These findings suggested that in fact infections early in life might protect from developing asthma and allergies. If this notion was correct, then children in day care centres with an increased exposure to infections through their peers should be at lower risk of asthma and allergies. Indeed, several studies demonstrated that children visiting day care centres in early infancy developed wheezing less frequently later in life (5). Other studies have found that children with recurrent infections of the upper respiratory

tract in early life had a lower prevalence of asthma and allergies at school age (6–8).

However, not all study findings support the ‘hygiene hypothesis’. Infections of the respiratory tract have also been identified as risk factors for wheeze and asthma. This discrepancy may in part be attributable to the phenotype of wheeze under study, early transient wheeze being positively associated with infections of all kind, whereas the atopic phenotype may be protected from increased infectious exposure. Furthermore, the type of infection may play a role.

Controversial role of viral infections

It has been known for long that viral infections are potent triggers of asthma exacerbations. The debate about viral infections as a cause of asthma inception is still ongoing. Particularly, the role of respiratory syncytial virus (RSV) is under scrutiny. The RSV is one of the most frequently identified viruses in young infants with acute respiratory disease, while in older children picornaviruses such as rhinovirus and enteroviruses predominate. Several investigators studied the relationship between RSV infection in early infancy and the development of wheezing disease or atopy later in life. The results of these studies are conflicting. Higher rates of wheezing and/or lung function decrements were found in children and adolescents having suffered from hospitalization because of RSV infection early in life (9–12). Children hospitalized for RSV bronchiolitis are, however, a minority of all children infected with RSV. Most infants have unapparent infections or mild symptoms only. Therefore, hospital-based follow-ups are likely to be biased by the severe end of the clinical spectrum. It is, therefore, not surprising that population-based surveys such as the Tucson Children’s Respiratory Study came to other conclusions. Infections with RSV in the first 3 years of life were associated with an increased risk of frequent wheezing at school age, but this risk vanished up to the age of 13 years (13). Recent work by Gern & Busse (14) furthermore demonstrates that a number of host factors predispose certain children to develop recurrent wheeze in the aftermath of a viral infection, particularly RSV infection of the respiratory tract.

Infections and colonization of the gastrointestinal tract

It has, therefore, been proposed that not infections of the respiratory tract but rather those affecting the gastrointestinal tract, acquired through the orofaecal route, may provide protection from atopy and asthma. In fact, some studies have shown that subjects with a positive serology for hepatitis A, *Helicobacter pylori* or *Toxoplasma gondii* suffered less frequently from atopic disease (15). Apart from invasive infections, other sources of microbial exposures might be of importance such as the commensal intestinal flora (16). A prospective study performed in

newborns in Sweden and Estonia found significant associations between gut colonization with certain bacteria during the first year of life and the development of atopic dermatitis or allergic sensitization (17). In particular, infants developing atopic disease were less often colonized with enterococci and bifidobacteria, while they had higher counts of clostridia and staphylococcus in their faeces.

These findings led to the idea that microbiota might be used for the prevention of allergy. First experiments in animal models gave promising results (18). Kalliomäki et al. (19) in a Finnish study performed an interventional study using *Lactobacillus rhamnosus* in pregnant mothers and their offspring. At the age of 4 years, the incidence of atopic dermatitis was still significantly lower for the study subjects on the active arm of the study when compared with children having received placebo.

All these findings support the notion that the protective effect of a decreased level of hygiene might not only be provided through clinically manifest infections, but may also in part be conferred by exposure to nonviable microorganisms in a child’s environment.

Farmers’ and nonfarmers’ children

Cross-sectional studies not only in various European countries, but also in the United States, have repeatedly shown that children growing up on a farm have a significantly lower prevalence of asthma, hay fever and atopic sensitization than their peers (6, 20–25). This protective effect was primarily observed in dairy farms, especially in small and traditional ones where contact of family members to animals is close and frequent. The effect of growing up on a farm as a child was still seen later in adolescence. Likewise, in a Norwegian cross-sectional survey, a lower prevalence of asthma was found among adult farmers, which may, however, also be attributable to a ‘healthy-worker effect’ (26).

Limited information is available on the specific factors conferring protection in farming environments. Findings from cross-sectional studies in school children in Bavaria, Austria and Finland suggest that exposure to livestock and unpasteurized milk are relevant exposures, which may induce tolerance towards environmental allergens (23). These exposures must, however, occur early in life to confer protection (22). In the ALlergy and EndotoXin study (ALEX) study, a multicentre study in rural environments in Bavaria, Switzerland and Austria, exposure to stables and unpasteurized milk during pregnancy and the first year of life resulted in a significantly lower prevalence of asthma (0.8% vs 11.8%), hay fever (0.8% vs 16.0%) and atopic sensitization (8.2% vs 32.9%). These effects remained significant after adjusting for potential confounders: adjusted odds ratio (aOR) = 0.14 (95% CI 0.04–0.48) for asthma, aOR = 0.20 (95% CI 0.08–0.50) for hay fever and aOR = 0.32 (95% CI 0.17–0.62) for atopic sensitization. The strong protective effect was seen

for atopic as well as for nonatopic asthma. However, so far all the projects investigating the so-called farm effect have used cross-sectional designs, assessing (early) exposure retrospectively. Therefore, prospective studies such as the 'Protection against Allergy – Study in Rural Environments' (PASTURE) cohort are necessary to investigate the temporal sequence of events, relating early life exposures to the maturation of the immune response and the development of tolerance and allergies respectively.

As of today, it remains unclear, which exposures within stables are beneficial against the development of asthma and allergies. Exposure to microbial compounds such as endotoxin and peptidoglycan from bacteria is highly prevalent in stables and other farming environments. Endotoxin is a component of the cell wall of gram-negative bacteria with recognized immunostimulatory capacities. Muramic acid in turn is a component of peptidoglycan in the cell wall of all bacteria. In the ALEX study, elevated concentrations of both, endotoxin and muramic acid, were found in dust from the child's mattress among farming children as well as nonfarming children with regular contact to stables (27, 28). Furthermore, endotoxin levels in these indoor dust samples were inversely related to the occurrence of hay fever, atopic asthma and atopic sensitization, while nonatopic wheeze was significantly negatively associated with muramic acid levels in indoor dusts.

Several other studies corroborated these findings (29–31). For example, in a nonrural environment in Germany, an inverse association between endotoxin levels and atopic sensitization was found in children (32). Endotoxin has also been inversely associated with the development of atopic dermatitis in high-risk children (30). The effect modification of atopy on endotoxin exposures was also confirmed in other studies. Park et al. (33) found a positive association between endotoxin concentrations in house dust and the incidence of wheeze in the first year of life in a high-risk birth cohort. Furthermore, a recent study in adult farmers found an inverse association between endotoxin and atopic asthma, whereas for nonatopic asthma a positive relation was seen (34).

In the ALEX study, the effect of endotoxin exposure was independent of stable contact and did not explain the protective stable exposure (29). Therefore, other microbial exposures from other sources, e.g. fungi, mycobacteria or other microbes are likely to contribute to the observed protective effect. It would be the aim of the PASTURE cohort to further identify other relevant exposures.

Potential mechanisms

But how does exposure to microbial compounds result in protection from atopic disease? Much is unknown about the changes in immune responsiveness following microbial stimulation and resulting in tolerance towards environmental allergens. In whole blood stimulation

assays, cytokine production of tumour necrosis factor- α , interferon (IFN)- γ , interleukin (IL)-10, IL-4 and IL-12 after lipopolysaccharides (LPS) and staphylococcal enterotoxin B (SEB) stimulation were all negatively associated with environmental levels of endotoxin in mattress dust (27). These findings may point towards an overall down-regulation of immune responses, rather than a shift in the Th1-/Th2 balance. Other mechanisms are, however, also plausible such as the involvement of T-regulatory cells or phenomena associated with signalling processes of receptors recognizing microbial compounds on cell surfaces such as the toll-like receptors (TLR). Interestingly, an increased expression of TLR2 and another receptor of innate immunity, CD14, has been observed among farmer's children (35) suggesting an activation of the immune response through innate immunity mechanisms.

It seems, however, likely that complex mechanisms, where genetic and environmental factors interact to influence critical components in the recognition of bacterial products, affect the development of atopic disease in humans. A polymorphism in one of the suspected genes, namely the TLR4 gene, has been related to atopic sensitization among endotoxin-exposed individuals (36) suggesting a strong gene-by-environment interaction. Likewise, significant gene-by-environment interactions have been observed between a polymorphism in the TLR2 gene and the growing up on a farm (36).

Many unsolved questions remain from the findings of cross-sectional surveys in farmers' and nonfarmers' children. Therefore, a consortium of investigators all with previous experience in conducting large, multicentre studies came together to develop and run the PASTURE birth cohort as described below.

The PASTURE study

Aims of the PASTURE project

The overall aim of the project was to assess the role of indoor exposure to various microbial products for the development of childhood asthma and allergies in a prospective design in rural environments across Europe. Furthermore, the project aim to investigate the immunological and genetic mechanisms involved in the determination of individual responses to these environmental influences.

The PASTURE study group

The PASTURE brings together a highly multidisciplinary team of clinicians, epidemiologists, immunologists, experts in genetics and microbiologists. All partners and their main contributions are listed in Table 1. The broad range of experience was chosen as it was felt that many interactions exist between the various aspects of the development – or the protection from the development – of atopic disease.

Table 1. PASTURE partners

Partner	City	Country	Contribution
E. von Mutius	Munich	Germany	Co-ordinator, German field centre, Analyses of DNA (M. Kabesch)
C. Braun-Fahrlander	Basel	Switzerland	Swiss field centre; in particular: exposure assessment based on questionnaires
J. Riedler	Schwarzach/Salzburg	Austria	Austrian field centre; in particular: assessment of health effects
J. Pekkanen	Kuopio	Finland	Finnish field centre
B. Brunekreef	Utrecht	Netherlands	Analyses of dust samples
H. Renz	Marburg	Germany	Analyses of proteoms and metaboloms (including cytokines and immunoglobulin E)
R. Lauener	Zurich	Switzerland	Analyses of RNA
S. Weiland	Ulm	Germany	Data monitoring, Quality control
J.-C. Dalphin	Besançon	France	Additional field centre with own funding

Design of the project

The PASTURE is a prospective birth cohort study located in rural areas in four European countries: Upper Bavaria (Munich, Germany), Salzburg area (Austria), Eastern Switzerland (e.g. St Gallen) and Central Finland (Kuopio). Furthermore, a fifth group joined the project with its own funding (Besançon, Doubs/France). The aim of the study team was to complete follow up of participants till the age of 6 years, and prolongation of the initial study period (up to age of 1 year) seem to find support by the European Commission.

The work programme covered by the European Union grant spans 46 months from January 2002 to October 2005, with a 6-month no-cost extension till April 2006. It was divided into seven work packages listed in Table 2.

Methods

Questionnaires. Initially, farming and nonfarming pregnant women living in these rural areas were identified in the third trimester of pregnancy. Lifestyle factors of these women were assessed via questionnaires. The questionnaires have been developed within the PASTURE study group, using questions about different exposures from the International Study of Allergy and Asthma in Childhood (ISAAC), ALEX and Prevention of Allergy – Risk factors for Sensitisation in children related to farm and anthroposophic (PARSIFAL) studies, as well as questions derived from the American Thoracic Society (ATS) questionnaire (37) for the assessment of respiratory

health in the parents. Questionnaires were administered by interview to the mother of the child during a home visit at the end of pregnancy. Furthermore, at the age of 2 months of the study, a personal interview through the local fieldworker was performed. At the age of 1 year and 18 months, a self-administered questionnaire is applied and at the age of 24 months a telephone interview is performed. Moreover, the father received a self-administered questionnaire during the child's first months of life.

Questionnaires were designed to assess respiratory and other health issues of the child and the parents, as well as a broad range of agricultural exposures. Furthermore, questions about common potential confounders such as passive smoking, parental education, family size, breast-feeding, etc. were included in all questionnaires.

From the age of 2 months to 1 year, the children are followed via a detailed diary, which is completed by the mother. The diary assesses in weekly intervals the child's health as well as nutritional factors and farming exposures.

The PASTURE cohort has so far only been funded until the completion of the first year visit. We hope to get funding for a further follow up at least until age 6 years. If we are successful in recruiting new funds, we intend to use yearly self-administered questionnaires (i.e. at 36, 48 and 60 months) and a clinical visit with blood sampling at age 6 years.

Blood Sampling. Venous blood samples were collected from both parents and from the child at birth and age of 1 year. In the blood samples, the following analyses are performed.

Specific IgE in serum samples. Under the responsibility of partner 7 in Marburg, analyses of specific immunoglobulin E (IgE) for 20 common inhalant and food allergens were performed in parental blood samples and cord blood as well as in the serum samples from the 1-year-old children.

DNA. Under the responsibility of partner 1 in Munich, DNA is extracted from the blood samples and genotyping of all children and their parents for relevant

Table 2. Pasture work packages

Workpackage number	Contents
WP1	Epidemiological study design and data collection
WP2	Assessment of health effects
WP3	Exposure assessment based on questionnaires
WP4	Assessment of exposure to microbial contaminants in house dust and cow's milk
WP5	Measurement of T-cell effector status
WP6	Determination of gene-by-environment interactions
WP7	Co-ordination, quality control, data management and combined analyses

polymorphisms in the TLR2, TLR4 and TLR9 genes is performed. Given sufficient funding we will also include other interesting candidate genes.

RNA. Under the responsibility of partner 8 in Zurich, RNA extraction for gene expression studies of all genes included in genotyping is performed in all cord blood samples. For this purpose, mRNA is retro-transcribed into cDNA and gene expression levels for TLR2, TLR4 and TLR9 are measured using quantitative realtime polymerase chain reaction (TaqMan®; Applied Biosystems, Rotkreuz, Switzerland).

Cytokines. Under the responsibility of partner 7 in Marburg, analyses of cytokines are performed in cord blood as well as in blood samples from the 1-year-old children, in order to characterize T-cell effector status. In the local study centres, full blood samples are stimulated with various stimuli (LPS, SEB, phorbolmyristate acetate [PI]/ionomycin) within 24 h after blood sampling, and supernatants are collected at 24 and 48 h. At the central laboratory in Marburg, culture supernatants are analysed by automated and manual enzyme-linked immunosorbent assay techniques. The following cytokines are measured quantitatively to assess the T-cell effector status at birth and at 1 year of age: IL-2 and IFN- γ (reflecting Th1 immunity), IL-4, IL-5, IL-9, IL-13 (reflecting Th2 immune status), tumour growth factor (TGF)- β , IL-10 (reflecting regulatory T cells, Th3 T-cells) and IL-12 representing an important promoter of Th1 effector cell development. This panel provides important insight into the overall status of T-cell effector function in farm- and nonfarm-exposed newborn and infants. The follow up will allow an investigation of the maturation of the immune response under various environmental conditions.

Dust samples. At the age of 2 months, during a home visit to the family, dust samples are collected by the fieldworker from the living-room floor as well as from the child's and mother's mattress following a standardized protocol using nylon dust sampling socks. For this sampling, exactly the same type of vacuum cleaner is used in all study centres. As additional funding has so far not been secured to cover exposure assessment for the future follow up of the children, less cost intensive dust sampling procedures are performed at the age of 1 and 3 years. The dust samples are collected in identical way, but by the parents themselves using their own vacuum cleaner. At the age of 1 year, samples are taken from the living-room floor and the child's mattress only, while at 3 years of age samples are collected from the child's mattress and the living-room floor as well as from the floor in the child's bedroom.

Dust samples are analysed under the responsibility of partner 6 in Utrecht. Samples are analysed for endotoxin and other microbial components as well as for allergens

such as house dust mites and cat allergen. Endotoxin is measured with the Limulus Amebocyte Lysate test, which is well established for measuring endotoxin in environmental samples. Allergens (DerP1, Derf1, and Feld1) in house dust extracts are analysed by using standardized commercially available enzyme immunoassays. Furthermore, dust samples are analysed for extracellular polysaccharides, with a sandwich enzyme immunoassays. The methods of this test have been described by Douwes et al. (38).

Cow's milk samples. During the home visit at the age of 2 months, not only dust samples, but also samples from the cow's milk that is usually consumed in the family are collected following a standardized protocol. Under the responsibility of partner 6 in Utrecht, these cow's milk samples are analysed for endotoxin, using the same methods as for the dust samples.

Breast milk sampling. Furthermore, during the 2-month home visit, the mother is asked for a small amount of breast milk collected in standardized way. The breast milk samples are stored for future analyses.

Clinical visit. At the age of 1 year, a clinical visit is performed paying particular attention to the examination of the skin to detect atopic dermatitis. In case of clinical signs of atopic dermatitis, the SCORAD index is applied for a standardized assessment of severity. At the clinical visit, the completeness of the self-administered questionnaire is furthermore checked by the study staff.

Quality control. Extensive quality control measures have been incorporated in the PASTURE cohort study particularly for laboratory work, and also for the fieldwork. In this context, the reproducibility and variability of procedures previously standardized in detailed standard operating procedures (SOPs) were assessed. For example, technicians from all laboratories convened and performed the blood stimulation procedures under identical conditions. Results were then compared between individual technicians and centres.

Checks for completeness of (questionnaire) data and plausibility checks are primarily performed under the responsibility of partner 9 in Ulm. Likewise, apart from the Finnish questionnaires where for linguistic reasons data entry is performed locally, partner 9 performs all the data entry for the questionnaires in a centralized and standardized manner.

Status of activities

The recruitment of participants was between August 2002 and March 2005, with the last recruited children born in May 2005. In total, more than 1000 participants could be included in the five study centres. Apart from Finland, it took more time than anticipated to recruit sufficient

numbers of farming families into the study, as the number of active farmers is continuously decreasing in Central Europe.

The first analyses of cord blood cytokines and IgE have been completed and the measurement of specific serum IgE in the parental blood samples is also nearing completion. The genotyping and RNA studies will be performed in summer 2005 when all cord blood and parental samples will be available at the respective analysing laboratories in Munich and Zurich. The first dust samples, collected at home visits at the age of 2 months, have been analysed for allergens and microbial components. Data entry has also been completed for a number of study tools.

We expect to have a complete database for the analysis of the pregnancy and first year results at the beginning of 2006. The PASTURE team hopes that the results of the

study will elucidate the nature of the relevant protective exposures and will contribute to the understanding of the mechanisms resulting in the development of allergic immune responses in the context of certain environmental and genetic conditions. We hope that such an interdisciplinary effort, combining expertise with *in vivo* and *in vitro* studies, will greatly enhance the insight into the observed strong protective effects and will allow the identification of necessary steps in these pathways for future development of preventive strategies for asthma and allergies in childhood.

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