EFRAIM

MECHANISMS OF EARLY PROTECTIVE EXPOSURES ON ALLERGY

Description of scientific results and foregrounds by the end of the project in January 2012

Field work - the Birth Cohorts

The heart of the EFRAIM project was the follow up of the PASTURE cohorts at age 6. As a result of effective centre specific retention strategies like editing cohort newspapers, and sending Christmas cards or birthday presents the contact to the study families was maintained resulting in very good participation rates. Only 12% of the initial cohort had dropped out before school age, another 7% quit participation at age 6. Due to the long recruitment period between September 2002 and May 2005, the field work phase of the 6 year follow up extended to a period of 25 months (Jan 09 – Jan 11). A questionnaire assessment at school age was designed to continue the yearly collection of information since birth on health outcomes and environmental exposures. Prior to contacting the study families for the follow up study at age 6, all five study centres had sought ethical approval at their respective local authorities. Written informed consent by at least one parent had been obtained before the start of the assessment.

Among the 934 study participants of the 6 year follow up 450 (48.2%) were farmers' children. By age six, 253 children (28.8%) had developed atopic dermatitis, 94 children (10.3%) had reported rhinoconjunctivitis, 521 children (55.1%) had wheezed ever since birth and 72 (8.4%) reported that a doctor had diagnosed asthma at least once or spastic bronchitis at least twice in their life. The numbers express cumulative prevalences since birth. To increase the population we invited the participants' siblings for questionnaire assessment and allergy tests. From a majority of the siblings we already had questionnaire data from 3 years before.

A clinical examination complemented the assessment of allergic diseases. Comparable to a routine clinical assessment a lung function test in the field determined respiratory

parameters. A validated mobile spirometry device (EasyOne®, ndd, Switzerland) was used according to latest ERS/ATS guidelines in all five study centres after standardized training and under constant quality control. Forced expiratory flows (FEF_x) and volumes (FEV_x), as well as peak expiratory flows (PEF) and forced vital capacity (FVC) were measured by forced expiratory manoeuvres before and after inhalation of salbutamol under heart rate control. The measurements allow determination of the overall lung growth and development (FVC), of size and development of the airways (PEF, FEF_{25-75}) and of the reactivity of the airways (FEV_x) as an objective measure of asthmatic airway disease with reversible obstruction. We obtained complete data from 687 subjects (before and after application of the bronchodilator). In addition to spirometry the study participants measured PEF and FEV1 twice daily during a period of four weeks at home. A portable PEF monitor (Piko-1®, nSpire, UK) was used according to ERS/ATS guidelines. The study participants were trained by the field workers. The PEF variability data provided information about the stability of the respiratory system, i.e. remodelling processes, bronchial (hyper)reactivity and dynamic properties of the respiratory system as e.g. airway tone regulation in comparison with respiratory symptoms documented by the study participants during the sampling period. 647 data sets were generated. We found that, as expected, asthmatics had worse lung function. Furthermore, asthmatic study participants had a higher bronchodilator response as compared to non-asthmatics defined by a relative increase of FEV1 of more than 12%.

Concentrations of exhaled nitric oxide (FeNO) identified inflammatory airway responses in the cohorts. The concentration of exhaled nitric oxide aimed at discriminating asthmatic from non-asthmatic children and between asthmatics with and without eosinophilic airway inflammation. The test was performed in all five study centres using the same equipment after standardized training and under constant quality control by the responsible study centre. To account for the field work situation a validated offline kit (Ecomedics®, Duernten, Switzerland) was used. Exhaled air was collected in Mylar bags (Quintron®, Milwaukee, USA). Within a maximum period of 12 hours FeNO was measured by a CLD 88 analyzer (Ecomedics®, Duernten, Switzerland) at each study centre. The data were transferred to the central data base electronically. As expected, atopic children had higher FeNO levels. Asthmatics displayed higher FeNo levels, especially if they were atopic. Overall the concentrations of exhaled nitric oxide was not different in farm children and their controls, this did not change when we stratified for atopic sensitization status. When stratifying by

study centre, only for the German arm of the study (Bavaria) a significant farm effect on FeNO levels was detected.

Atopic dermatitis was investigated not only by questionnaire, but also by skin examination according to the SCORAD (scoring atopic dermatitis) criteria, a standardized instrument for the evaluation of the severity of atopic dermatitis. The field workers had been instructed in a one- day-course how and where to inspect the skin and had to perform an online training at home prior to study start.

Atopic sensitization was determined as the presence of specific serum IgE antibodies against common inhalant and food allergens. The prevalence of IgE against any tested allergen had increased from 1 year of age to 6 years from 17.1% to 57.7% for a cut-off higher than 0.35 IU/ml. Farm children were more sensitized to food allergens and less sensitized to seasonal allergens than the children not living on a farm.

Field work was flanked by the analysis of blood and environmental samples collected between birth and school age. This work was undertaken in the EFRAIM consortium's own laboratories of high standards using standard and innovative methodology.

Since not all measurements could be performed on the whole sample, given financial and man power limitations, some investigations were performed in a nested case-control study, in order to find candidate exposures for asthma and allergy protection. The nested case-control subsample of 160 subjects was drawn from the whole population at random and matched by centre. It had been selected according to outcome information from the assessment at age 4 as follows

	n= 80 asthmatics (cases)	n= 80 controls
n=80 atopic	48.7 % farm children	48.4 % farm children
n=80 non atopic	39.0 % farm children	57.1 % farm children

Environmental and lifestyle factors

Several epidemiologic studies have shown that the allergy protective 'farm effect' can be explained by dietary habits, lifestyle and environmental exposures.

The nutritional factors identified in epidemiological studies as possibly affecting the development of asthma and allergic diseases include breastfeeding, certain dietary fatty acids early in life, dietary antioxidants, foods which affect the gut microflora, and obesity.

Previous epidemiological studies had connected the consumption of raw milk to a reduced risk of childhood allergic disorders. The cause and mechanism of the allergy-protective effect of raw milk is unknown. A factor suspected to confer the allergy-protective effect is the immunomodulatory effect of raw milk bacteria. We established a qualitative (fingerprinting) method (Polymerase Chain Reaction-Single Strand Conformation Polymorphism PCR-SSCP) for the analysis of bacterial diversity in milk samples provided by the study families. The PCR-SSCP was performed in the nested case-control subgroup to find candidate microorganisms. We sequenced four bacterial classes which were inversely associated with asthma and allergy, and one class which was positively associated. However, the consecutive quantitative genus specific PCR protocols failed to replicate the finding for one of the bacterial classes again in the nested case- control samples. PCR protocols for the other protective bacteria were established but none of them was able to amplify the two specific species. We therefore did not expand this study to all milk samples in favour of saving material for a broader and more up-do-date method which will be available in the near future, i.e. pyrosequencing.

Cow's milk and other dairy products are relevant nutritional sources of fatty acids. We concluded that monitoring the consumption of fatty acids through cow's milk may give further insight into the relationship between dietary fatty acid intake and allergic outcomes. We established a qualitative and quantitative method to analyse fatty acids in milk samples but also in serum samples in the EFRAIM project. This method was published by the responsible project partners: Böcking et al. in the Journal of Clinical Chemistry and Laboratory Medicine in December 2010. As many as 46 different fatty acids were separated by this high throughput gas chromatography in one sample. This investigation was performed in milk samples from the nested case-control selection collected at two different time points (age 2 months and 4.5 years). We found strong differences in the milk fatty acid content between asthmatic and non-asthmatic milk drinkers. Milk fatty acids were inversely

related to preschool asthma. Specific milk fatty acids linked to anti-inflammatory pathways via prostaglandin synthesis were also shown to be associated with preschool asthma, while no associations between preschool asthma and fatty acids were found for the fraction of milk fatty acids that enters β-oxidation. The fatty acid pattern in samples from both time points varied with season. Milk exhibiting fatty acid patterns which are typical for green feeding or pasturing might have acted protectively against allergy development. A publication on the milk fatty acids and how they associate with allergy is in preparation.

The Western diet over the last decades with the change in the ratio of ω3- and ω6-polyunsaturated fatty acids (PUFAs) has been related to the increase in the prevalence of allergic diseases. We were interested in knowing whether the ratio of ω3- and ω6-PUFAs was different in children with and without allergic diseases, and if the farm effect was mediated by different diets of farm versus non-farm families reflected in blood fatty acids. Furthermore, animal experiments suggest a role of maternal ω3-fatty acid intake on allergic immune responses in the offspring. We measured 46 different fatty acids in serum samples taken from the children at birth, and at 1, 4.5 and 6 years and in their mothers in the nested case-control subpopulation. We applied the gaschromatography method which had been established for this study. Milk fatty acids seemed to have contributed only to a minor extent to the nutrient fatty acids reflected in. These fatty acids might have resulted from other nutrients. Another explanation is that serum is not a suitable matrix to explore the effect of milk consumption on circulating fatty acids. Whereas serum fatty acids reflect the short term fatty acid intake (7-14 days), membrane-bound fatty acids as in the erythrocyte membrane reflect long-term dietary habits up to 120 days. We performed measurements of the fatty acid patterns in erythrocyte membranes, wherever we could harvest them from blood clot residues in the serum blood tubes. This investigation was undertaken with additional funding.

An imbalance between reactive oxygen species and antioxidants has been increasingly recognized as one of the major factors contributing to the chronic inflammatory process in asthma. Lack of free-radical scavengers in modern diets, including vitamins A, E, and C as a result of decreased consumption of fresh fruit and vegetables has been correlated with changes in the prevalence of asthma and allergies. Vitamin D and its receptor are important in immune function and development and, therefore, could potentially have a role in the development of asthma and allergies. To observe whether previous findings can be confirmed in the PASTURE cohorts, we analyzed levels of vitamin E and D in serum samples

collected when the children were one year old. Serum tocopherols (vitamin E) were determined by a method suitable for small sample volumes. Chromatographic analysis was performed with an Agilent 1200 HPLC system including a HP1046A fluorescent detector. The serum concentration of 25-OH D (vitamin D) is recognized to be the most reliable measure of the overall vitamin D status. The compound was assessed using IDS 25-Hydroxy Vitamin EIA kit (Immunodiagnosticsystems, REF AC-57F1/AC-57F2). Serum α- and γ-tocopherol concentrations were similar among children of farmers and non-farmers. Among those who were sensitized against any of the tested allergens α-tocopherol levels were significantly higher than among the non sensitized subjects. Vitamin D levels were lower in farmers' children than in the controls. As expected the vitamin D levels were highest during summer and autumn months. Vitamin D levels were also significantly higher in asthmatics compared to non-asthmatics. There was no association of vitamin levels with sensitization to aeroallergens. The vitamin analyses will in a next step be investigated in multivariate analyses to control for confounding factors. A paper on the association of the vitamins and wheeze asthma and allergic sensitization is in preparation.

The perinatal period represents an important window to initiate the development of allergic disorders. Breast milk contains anti-microbial and anti-inflammatory agents, which protect the child from infections and educate the infant's immunological system to tolerate food and microbial derived antigens. We examined whether the levels of transforming growth factor- β1 (TGF-β1) and total immunoglobulin A (IgA) in breast milk and the duration of breast feeding differed between farming and non-farming women, and if they were associated with the child's risk for atopy, atopic dermatitis or asthma. TGF-β1 values were measured by ELISA (Quantikine Human TGF-β1 Immunoassay). IgA values were analyzed using ELISA modified from Lehtonen et al. (Infect Immun 1984). TGF-β levels in breast milk showed significant associations with maternal smoking and the duration of breast feeding. IgA levels were inversely associated with the duration of breast feeding. We obtained evidence for a dose-response relationship showing a decreasing risk for atopic dermatitis with increasing levels of IgA in breast milk, this seemed to be influenced by the duration of breastfeeding. TGF-β levels tended to have a protective effect on atopic sensitization at age 6 irrespective of the duration of breast feeding. A paper will soon be submitted.

The protective farming environments are on one hand rich in microbes and on the other they protect from the development of allergic diseases. Common markers of microbial exposure are: endotoxin for gram negative bacteria; muramic acid predominantly for gram positive; ß(1,3)-glucans, and extracellular polysaccharides (EPS) from *Aspergillus* and *Penicillium spp*. for fungal exposures. Although all four indicators of microbial exposure have been shown to be inversely related to allergic diseases, neither one nor the combination of any of the four has yet been able to explain the 'farming effect' suggesting that yet unidentified exposures are effective. In the EFRAIM project biomarkers for microbes were measured in mattress dust samples collected between age 2 months and 3 years reflecting early microbial exposure. The assessment of chemical markers was complemented by the search for new environmental microbial exposure by using a microbial finger printing method.

Endotoxin was measured with the kinetic chromogenic Limulus Amebocyte Lysate (LAL) assay (Bio Wittaker, Walkersville, MD). Extracellular polysaccharides (EPS) were measured with a specific EPS sandwich enzyme immuno assay (EIA). Muramic acid analyses were performed with a PolarisQ ion trap mass spectrometer (MS–MS) from Thermo (Austin, TX, USA) equipped with a Trace GC-ultra gas chromatograph (GC) from (Milan, Italy).

Our preliminary results show that in dust samples from mattresses of asthmatics lower levels of endotoxin were detected than in those of non-asthmatics when endotoxin was expressed per square meter of the mattress. Endotoxin was also associated with sensitization to any of the investigated allergens and to inhalant allergens only with lower levels among the sensitized children. This association was significant when endotoxin was expressed per gram dust. These results replicate findings from many other epidemiological studies exploring the effects of house dust biomarkers on allergic diseases. Correlations between all markers and bacteria were calculated to see to which extent the concentrations of the various agents should be treated as strictly separate proxies of exposure, or might all represent different aspects of a general 'house dust–associated microbial exposure'. The results give reason to explore the construction of "combined house dust microbial agent" parameters, like a 'total score for house dust-associated microbial exposures', based on the results from the various analyses, as a potentially more robust exposure parameter to be used in further and more complex analyses of the EFRAIM exposure data.

Fingerprinting of the compositional characteristics of the microbial flora in mattress dust samples was carried out, which was determined by DGGE (denaturating gradient gel

electrophoresis). Fingerprints were compared with each other using the Bionumerics (Applied Maths, Gent, Belgium) software to identify the microbes showing up differently by disease or by exposure. The information was used for subsequent development of qualitative PCR assays targeting specifically the identified bacterial groups to be applied to the whole cohort of children for quantitative assessment of exposure. The main emphasis of this project was to identify protective candidates. Only one bacterial signal was found to have a protective association with sensitization to any of the investigated allergens and to inhalant allergens only, and it was selected to be further investigated. In addition, several other signals that appeared as risk factors for the development of allergies will also be further analyzed. The identification of microbial groups (bacteria and fungi) was not completed at the end of the project, and hence at this stage one microbial group and one fungal group were selected based on an earlier farm study (GABRIEL) for qualitative analysis. Exposure to the two selected groups (*Aspergillus/Penicillium/Paecilomyces variotii* and *Mycobacteria)* was assessed in all mattress dust samples collected when the study subjects were 3 years old. The results indicate that higher concentrations of Aspergillus/Penicillium/Paecilomyces variotii and Mycobacteria conferred protection against atopic sensitization and asthma. The results are highly interesting and further steps with microbes deriving from the EFRAIM dust samples will continue and will be supplemented by more protective candidate microorganisms from the GABRIEL and the PARSIFAL studies. This microbial exposure will eventually be associated with the exceptional volume of environmental and immunological data this project provides.

A number of scientific publications focusing on the importance of environment and life style on the development of allergies and the immune system have been accepted by high ranking journals during the EFRAIM project period.

Rochat et al., Maternal vitamin D intake during pregnancy increases gene expression of ILT 3 and ILT4 in cord blood. Clin Exp Allergy 2010 describe that maternal vitamin D intake induced tolerogenic immune responses.

Pfefferle et al. Cord blood cytokines are modulated by maternal farming activities and consumption of farm dairy products during pregnancy. J Allergy Clin Immunol 2010:125:108-15.e1-3 show that maternal exposure to farming activities and farm dairy products during pregnancy modulated cytokine production patterns of the offspring at birth.

Roduit et al. (under revision at Journal of Allergy and Clinical Immunology) found protective dietary components ingested in the first year of life on the development of atopic dermatitis. The same author shows in the *Journal of Allergy and Clinical Immunology 2011, Prenatal animal contact and gene expression of innate immunity receptors at birth are associated with atopic dermatitis* that maternal contact with farm animals and cats during pregnancy significantly protected from atopic dermatitis in the first 2 years of life.

One EFRAIM group found differential effects of several dietary and farm exposures on gene expression of the innate immune system. The publication is currently under revision at the *Journal of Allergy and Clinical Immunology.*

A paper analyzing the association of major explanatory factors of the farming effect with markers of low grade systemic inflammation in allergic and non-allergic children has been accepted by *Pediatric Allergy and Immunology.* Two further manuscripts about pet exposure and respiratory problems, wheeze and atopy are in preparation.

Levels of airborne moulds and actinomycetes were higher in bedrooms of the farm children than in bedrooms of non-farm children. This effect strongly related to feeding cattle in cowsheds and other agricultural activities. This is the result of a publication by *Roussel et al. 2011, Exposure to moulds and actinomycetes in Alpine farms: a nested environmental study of the PASTURE cohort.* in *Environmental Research*.

The conclusion of *Karvonen et al., Confirmed Moisture Damage at Home, Respiratory Symptoms and Atopy in Early Life: A Birth-Cohort Study. Pediatrics 2009;124;e329-e338* is that moisture and mold problems in the kitchen and in the main living area increased the risk for wheezing in early childhood. Further work by the same author exploring microbial markers, its determinants and its effect on allergy is under revision at the *Journal of Clinical and Experimental Allergy.*

Maturation of the immune system

We aimed at investigating innate and adaptive immune mechanisms underlying the protective effect of environment and life style on the development of allergic diseases. We assessed the maturation of immune responses at different time points in a longitudinal follow-up of the PASTURE birth cohort with investigations on the innate immune system and dendritic cell responses, on T helper cell differentiation (Th1, Th2, T regulatory cells, Th17 cells) and on B cell responses.

The innate immunity is crucial in the development of allergic disease. We investigated the influence of farm-related exposures associated with contact to microbes on the expression of relevant genes of the innate immunity and their development over time. In the EFRAIM project investigations in blood samples collected at age 4.5 and 6 complemented the data we already had from cord blood and from samples from the first year of life on the expression of genes of receptors, of the signaling molecules, of the regulatory molecules, and of molecules influencing the function of the adaptive immune response of the innate immunity. We used quantitative real-time PCR. Furthermore, we assessed the levels of the soluble molecules CD14 and ST2 in serum samples. We found that the expression of crucial genes of the innate immunity are up-regulated by farm-related exposures and might be involved in the protection of farmers' children against the development of allergies. A paper on the effect of prenatal and early life exposures on the expression of innate immunity genes has been submitted.

It had been proposed that an increased microbial burden in the environment leads to a shift in T helper cell differentiation towards the Th1 phenotype; alternatively, the development of regulatory T cells might be promoted by environmental exposure to microbes. Other factors have been reported to be upregulated on monocytes/macrophages by microbial compounds and to promote immuno-globulin isotype class switch in B cells to IgA and IgE in a T cell independent way. Our experiments investigated possible effects of environmental exposures on the differentiation of T helper cells, regulatory T cells and Th17 cells. We assessed the gene expression of marker genes associated with different T helper cell phenotypes (T-bet (Th1), GATA-3 (Th2), STAT6 (Th2), Foxp-3 (T regulatory cells), RORγt (Th17), and CD40L) to complement the results we had obtained in blood samples from the first year of life in the

PASTURE project. We did not observe a clear shift in T helper cell 1 or 2 development at any age in farmers' children compared to their controls.

A balanced immune response is closely related to the presence and activity of anti-inflammatory T-cell subsets which are named regulatory T-cells. To investigated possible effects of environmental exposures on the differentiation of regulatory T cells in fresh blood samples collected from the children from the German and the French arm of the study, we established the methods and measured surface and intracellular expression of regulatory T cell markers (CD4, CD25, Foxp3, CD127) using flow cytometry. For staining the following markers were used: For surface staining: CD4, CD25, CD127 and combinations of these. For intracellular staining: additional Foxp3 staining. We detected significant higher expression of several surface markers of activated T cells and regulatory T cells in farm children. Compared to non-exposed children, expression of intracellular markers of activated T cells and regulatory T cells was higher in children exposed to farm milk.

We assessed the expression of the sterile ε transcripts as a marker for isotype class switch in B cells towards IgE production by quantitative real-time PCR. The gene expression of sterile ε transcripts showed a tendency to be decreased among the farmer population and especially with the consumption of farm milk at almost all ages.

In the literature dendritic cells (DCs) have been identified as key players linking the innate and adaptive immune response. The frequency and function of dendritic cells in the children of the Finnish birth cohort were assessed by flow cytometry using frozen peripheral blood mononuclear cells (PBMCs) from samples collected at age 4.5 years. We analysed DC phenotypes and monocytes and the expression of TLR4 and CD86 on mDC1's and monocytes and stimulated PBMCs and analysed intracellular levels of IL-6 and TNFα as well as surface expression of CD80 and CD86 on the mDC1 population (DCs which had responded to stimulation) and monocytes. Frequencies and functional properties of dendritic cells and monocytes could be linked to both farm exposure and atopy. Associations between farm exposure and DC phenotype and function were mostly detected among non-atopic children. Hence, in further analyses, the effect of specific farm or microbial exposures on DCs needs to be studied among non-atopic children. Associations between DCs and atopic sensitization depended on the type of sensitisation and the selected cut-off concentration. Hence, the definition of the specific atopy is crucial when associations between DCs and atopy are studied.

The investigations of the maturation and differentiation of T helper cells also comprised measurements of cytokine expression of the different T cell lineages in stimulated blood samples. We had undertaken similar experiments in the cord and year one blood samples of the cohorts in the PASTURE project. We complemented these data with stimulation experiments in blood samples collected at 4.5 and 6 years of age. We had incubated the cord and the year one blood samples with 3 different microbial stimuli and added another 3 stimuli for the experiments with the year 4.5 and the year 6 samples. Five different cytokines had been measured following stimulation in the samples from the first year of life by ELISA, another five were added to the measurements in the stimulated samples from age 4.5 and 6 years to account for increasing knowledge in the field of immunology. Since more stimuli and more cytokines had been added to these studies within the EFRAIM project, ELISA was not longer a suitable method with respect to volume and throughput. A more effective analytic method had to be validated and implemented (Multiplex). Cytokine-production was calculated by using the FACSArray Bioanalyzer software. Cytokines provided interesting insights into the status of the innate and adaptive immune system. Differential cytokine expression in farmers' and non-farmers' children indicated a strong differentiation of cells of the immune system and that this process is strongly influenced by exposures. The observation that in the 4.5 years samples, farm children had a lower pro-inflammatory status compared to non-farm children was even more pronounced in the 6 years samples. This might indicate that the farming environment protects against these chronic processes on the T-cell as well as on the PBMC-cell level.

While the DNA sequence of genes is predetermined in the genome of an individual from conception, genetic variations (polymorphisms) occur within a population over time and gradually expand and change the capability of a population to cope with the environment during evolution. We were interested how genetic variation of immunity genes related to the development of allergy and asthma in our cohort. Many single nucleotide polymorphisms (SNPs) had been found to be associated with asthma in candidate-gene approaches or nowadays more often in genome-wide association studies within large cohorts. The EFRAIM cohort is a unique farming population for gene by environment analyses. We investigated if and how genetic variation affected the susceptibility to early protective exposure, and if there were farm specific genetic associations. SNPs were genotyped and their effects were analyzed. The iPLEX® Gold technology a MALDI-TOF

(Matrix Assisted Laser Desorption-Ionisation-Time Of Flight) system from SEQUENOM was used for genotyping. The DNA for the genotyping had been extracted according to the QIAGEN FlexiGene protocol from the cord blood samples, and was replaced by DNA from the 4.5 year blood collection if the yield was insufficient.

The selection of the SNPs to be investigated was based on different criteria. Firstly, SNPs identified as top hits of the large genome wide GABRIEL study were selected to replicate the findings on asthma and asthma related phenotypes in the PASTURE cohort. In this bundle, 18 SNPs located in the following ten genes or gene regions were successfully genotyped. Another 38 SNPs were selected from a set of 18 asthma candidate genes based on association with asthma in the literature or in the MAGICS/ISAAC cohort. Three SNPs were selected to investigate the influence of the genotype on epigenetic patterns.

39 SNPs were successfully genotyped with a call-rate of 98.5%. In the EFRAIM study several SNPs were significantly associated with disease status. Important findings of the GABRIEL study were replicated. A publication on the genotyping data is in preparation.

Mechanisms of gene regulation, which allow for a rapid change in the genetically determined response to the environment in one individual, are called epigenetic mechanisms. Epigenetic regulation of gene expression has been proposed to be important in allergic diseases by influencing the transcription of genes through methylation. Methylation status of specific genes can change over time.

To analyse DNA methylation patterns in a genome-wide manner high density microarray chips were used. Immunoprecipitated methylated DNA (MeDIP) was processed in an automated way on 385K promoter and CpG island tiling arrays. A subgroup of EFRAIM children from the nested case-control population was selected to pilot the detection of differing methylation patterns between the four groups of farm/non-farm families and asthmatics/non-asthmatics, as well as between the two time points birth and age 4. The gene network analyses failed due to exaggerated type 2 errors in more complex phenotypes. Hence, we developed a new method based on novel algorithms, a sliding window approach that captured the signals from five consecutive probes in a tiled gene region within one phenotypic group and calculated the difference of the fluorescent signals to the respective probes in the other group. We performed a gene network analysis to investigate if differentially methylated genes were overrepresented in certain functional biological networks. The comparison of non-asthmatic farmers to asthmatic non-farmers in both, cord blood and 4.5 year samples, resulted for both time points in a network that was strongly

enriched for genes involved in inflammatory response. Interestingly, patterns of methylation changed between cord blood and 4.5 year samples, indicating that there might be differing DNA methylation patterns in cord blood and 4.5 year samples influencing inflammatory responses. Further development of the sliding window approach is ongoing, and a publically available software tool is developed with the publication of the method in preparation.

To study the PASTURE cohorts if epigenetic patterns in asthma candidate genes are influenced by farm exposure in general, if they change over the first years of life, and if these changes contributed to the development of asthma, we selected genes that had been associated with asthma in genome-wide association studies, genes involved in T helper cell2 development, and genes involved in T regulatory effects. Cord blood cells from farmers' and non- farmers' children showed significant differences in methylation in three of the selected genes, whereas in samples from the 4.5 year collection one significant finding was observed. Almost no differences in methylation of the investigated genes were observed when children with asthma were compared to those without asthma. In cord blood, only one gene was hypermethylated in asthmatics compared to non-asthmatics and in 4.5 year samples the same gene was hypomethylated. Strong and significant differences in methylation were found within strata comparing the two blood collection time points (birth and age 4.5). Interestingly, the difference was not the same over all analysed regions and for some no difference at all was detectable, showing that the methylation patterns in those regions are flexible and methylation events associated with asthma or farming status can occur any time. A publication is in preparation summarizing the results herewith reported.

In humans a number of studies suggest that the gastrointestinal microbiota composition may play a role for allergy development. Clinical studies indicate that children with atopic dermatitis have increased gut permeability that could lead to enhanced antigenic load and immune stimulation contributing to the development of allergies. Gut colonisation can further influence mucosal barrier mechanisms by affecting gut permeability and the induction of Immunoglobulin A (IgA) in the faeces. The role of IgA in mucosal surfaces is to neutralise the antigens and decrease their immunogenicity in the intestinum. Gut leakage can furthermore be caused by intestinal inflammation and enhanced immune activation.

We aimed to identify specific microbial colonization of the gut that is associated with immune maturation and protection from allergic disease. To find candidate microorganisms associated with allergy, we performed a qualitative fingerprinting method (denaturating

gradient gel electrophoresis PCR-DGGE) in faecal samples collected at age 1 in the children selected for the nested case-control population. We did not see any differences in the diversity of predominant bacteria in faecal samples of children with and without allergic problems. Hence, no protective candidate microorganism was detected. This finding may have been confounded by the broad use of antibiotics even before the first birthday in the participating European countries. We also did not see differences between children living on farms compared to those not living on farms. Instead, we selected five gut microbes described as being allergy protective in the literature, namely *Bifidobacterium, Atopobium, Bacteroides spp., Clostridium leptum, Eubacterium rectale-Blautia coccoides*, which were analyzed quantitatively in the faecal samples by qPCR. The Atopobium bacteria group was much more prevalent in children from farming environments than in their controls. This finding was not however associated with the atopic sensitization to a specific allergen or with allergic symptoms.

We evaluated gut inflammation by assessing IgA and Calprotectin as markers in faecal samples collected when the PASTURE children were 2 months old. Calprotectin and IgA were analyzed by ELISA according to the manufacturer's manual (for the Calprotectin test Calpro AS, Norway, for IgA rabbit anti-human IgA DakoCytomation, Glostrup, Denmark). In raw data analysis Calprotectin levels were significantly increased in the faeces of farm children as compared to their controls, suggesting that environmental factors, such as microbes, in the farming environment had caused mild intestinal inflammation. No effect was seen for faecal IgA.

The activation status of the gut immune system leucocytes was assessed by measuring the expression of a selection of genes of interest in this context in the farm and the non-farm population. We used real-time PCR in total RNA isolated from whole blood of the 4.5 and the 6 year old children. We did not observe a clear pattern in the expression of genes related to lymphocyte activation that was associated with farm exposures.

We tested the gut permeability in the Finnish and the German cohort using the Mannitol - Lactulose Test. Lactulose is a sugar molecule which is normally poorly absorbed in the gut, whereas mannitol is easily absorbed and excreted in the urine. The ratio of these two molecules was defined in urine samples after ingestion of a solution of both sugars. An enzymatic assay for measuring urinary lactulose and urinary mannitol was applied. High concentration of lactulose in relation to mannitol indicated increased gut permeability, which can be a marker for disease. We were interested to see if the farmers' children had

increased permeability due to the mild gut inflammation caused by the microbial load in the environment. However, neither farming environment nor allergic disease was significantly associated with gut permeability in this experiment.
Wheat gliadin and cow's milk beta-lactoglobulin are antibodies to food proteins and can be used as indirect markers of gut permeability. The levels of these markers were measured in serum samples by ELISA. Subjects sensitized at age 6 to at least one of the specific allergens we had tested in the blood had higher levels of IgA and IgG to wheat gliadin and milk beta-lactoglobulin. Our results indicate that in sensitized children the tolerance to wheat gliadin and milk beta-lactoglobulin was decreased, and therefore, the production of antibodies to wheat and milk antigens was increased. Submission of several publications on the gut bacteria and mucosal barrier are expected in the next months.

Altogether, the availability of large numbers of consecutive samples covering immune responses at different levels (receptors, RNA, signalling and modulating proteins, regulating cytokines, immunologically active cells) and genetic information in the five rural birth cohorts enables analysis of immune system development under well-defined environmental conditions in utero up to school age.
We are still facing a major challenge and the main reward resulting from this project: the integration of how the different environmental exposures and life styles act on the variety of players of the immune system, and what it means for allergy development, taking into account genetic and epigenetic predisposition. Unraveling the complex mesh of exposures, immune system and underlying conditions (genetic, gut barrier, timing) leading to disease in our rich data set requires profound experience in the linking fields of epidemiology, biometrics and statistics, genetics and immunology, which is present in the EFRAIM consortium. During the EFRAIM project preconditions for the more profound data analysis have been created. The consortium has defined variables for our outcomes of interest to be used by all partners. We have preliminary analyses for all our data and have an overview of their character and quality. The cohesion of the consortium is outstanding. We have scheduled conference calls and meetings to discuss the progress in data analysis, which will continue way beyond the end of the project.

The EFRAIM project addressed two routes of preventive interventions in animal models and in vitro studies: the development of an allergy protective milk formula and the development

of an allergy preventive application. Both approaches were based on knowledge gained in the human studies.

The first approach took advantage of a gene by environment interaction which had been found in two independent populations for children consuming unprocessed cow's milk early in life. A polymorphism in the CD14 gene determined, whether a child consuming raw milk was protected. Protection from asthma in carriers of the particular polymorphism was also associated with increased expression of the CD14 gene. CD14 is a pattern-recognition receptor of the innate immune system for a wide spectrum of microbial compounds, but also for non-microbial compounds, such as phospholipids. In the EFRAIM project transfection based bioassays to detect immuno-modulatory activity of raw versus pasteurised milk, and of milk components have been set up to test the effects on CD14 expression and global genetic response in human cells. Active compounds analysis of milk samples from different milk production centres was undertaken with a view to comparing raw and pasteurised milk. A number of genes were up- and other where down-regulated in the cell based assays. However, as a consequence of non-repeatability of the results between different tests, a definitive conclusion on the global genetic response of the used human cell line to raw versus pasteurised milk cannot be made from this data. The expression of several cytokines of the cell lines after exposure to raw versus pasteurized milk was measured using a multiplex electrochemiluminesence-based assay from Meso Scale Discovery (Gaithersburg, Maryland USA). The cytokine data generated in this study demonstrated a significant difference in the immunological state between cells treated with raw compared to pasteurised milk samples, i.e. the increase in IL-2 secretion can be interpreted as a result of T cell receptor activation.

Four different compounds in raw and pasteurized milk samples, namely the protein, the lipid, the carbohydrate fraction and the microbial action, were investigated to determine the nature of the compound, which is mediating the protective 'farm milk effect' observed in epidemiological studies. Only for the exposure of raw versus pasteurised casein fractions a differential response in cells was observed. This effect was gone when the casein fraction was further broken down and separated, indicating the loss of a synergistic effect of a number of components separated during the fractionation process. With respect to the microbes we saw that after pasteurisation many of the microorganisms detected in raw milk samples were still present in the pasteurised milk. While the proportions of many microorganisms decreased after milk pasteurisation, a number of others increased.

Interestingly, all bifidobacteria were seen to down regulate CD14 expression. A probiotic Lactobacillus strain control was shown to upregulate CD14 expression in this study. These effects demonstrate that the change in microbial load and population by pasteurisation can alter the immunomodulating capabilities of milk.

The second approach used two bacterial species isolated from farm cowsheds, which had shown to possess strong allergy-protective properties in mouse experiments, and one species which had been associated with allergy in an epidemiologic study. All three candidate microorganisms for allergy protection by farm exposure were evaluated for suitability for as allergy preventive substance. One candidate dropped out during the pre-screening phase because lack of pathogenicity could not be confirmed. The *in vivo* and *in vitro* analyses of the other two candidates have resulted in two strains ready as product candidate for an allergy-protective application for preclinical toxicology outside the EFRAIM project.