FACULTY OF HEALTH SCIENCES
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Intrauterine and Genetic Factors in Early Childhood Sensitization

A prospective cohort study of children at high risk of atopy

PhD Thesis

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The present thesis is based on the following papers referred to by their Roman numerals:

I. Bønnelykke K, Pipper CB, Bisgaard H. Sensitization does not develop in utero. J Allergy Clin Immunol 2008; 121(3):646-651.

II. Bønnelykke K, Pipper CB, Bisgaard H. Elevated IgE in cord blood is biased from materno-fetal transfer. Submitted manuscript.

III. Bønnelykke K, Pipper CB, Tavendale R, Palmer CNA, Bisgaard H. Filaggrin gene variants and asthma exacerbations in early childhood. Submitted manuscript.

Contents

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Abbreviations

ANOVA	ANalysis Of VAriance
COPSAC	COenhagen Prospective Studies on Asthma in Childhood
IgA	Immunoglobulin A
IgE	Immunoglobulin E
FLG	Filaggrin
IL	InterLeukin
IU	International Units
LLQ	Lower Limit of Quantification
SNP	Single Nucleotide Polymorphism
Th2	T-helper 2

1. Introduction

1.1. Allergy and atopic diseases in childhood

Disease burden and prevalence

The allergy-associated (atopic) diseases; asthma, eczema and rhinoconjunctivitis are the most common chronic diseases in childhood. There are large geographical differences in disease prevalence,[1] but in industrialized countries approximately 30% of preschool children have asthma-like symptoms and 8-10% of schoolchildren have asthma,[1;2] 15% of preschool children have eczema,[3;4] and 15-20% of schoolchildren have rhinoconjunctivitis.[1;5] The prevalence has been increasing in industrialized countries during the last decades in what has been called an "allergy and asthma epidemic".[6;7]

Severe, life threatening disease is rare but the diseases are associated with significant impairment of quality of life in affected children.[8] Due to the high prevalence, atopic diseases have major impact on health and quality of life of a large proportion of the population as well as general costs for the society through drainage of health care resources and loss of effective working hours by affected families.[9;10]

Definition and nomenclature

Allergy is a hypersensitivity disorder caused by specific immunological mechanisms. According to the revised nomenclature, atopic disease is classified as a subtype of allergic disease.[11;12] Atopy is defined by a tendency to become sensitized and produce immunoglobulin E antibodies (IgE) in response to allergens, and atopic disease is defined as allergic symptoms in an individual with documented atopy by serum IgE or skin prick test. The typical clinical manifestations are asthma, eczema and rhinoconjunctivitis.

In the literature, the term atopic disease is often used in a broader sense referring to the clinical diagnosis of asthma, eczema or rhinoconjunctivitis not restricted to cases with documented IgE sensitization. I have chosen to use this symptom-based definition of atopic disease in this thesis. When referring to the involvement of allergen-specific IgE, this is specifically stated as (IgE-) sensitization.

1.2. Etiologies

Environment

It is a general belief that atopic diseases are caused by complex interactions between environmental factors and genetic susceptibility.[13] The importance of environmental factors is stressed by the increasing disease prevalence during recent decades, which cannot be due to genetics. Tobacco smoke exposure is a well documented risk factor for asthma and probably also sensitization.[14] A number of other environmental factors are suspected to affect the risk of atopic diseases including a generally "clean environment" associated with few infections, [15;16] environmental microbial exposure,[17] intestinal and airway microflora,[18;19] viral infections,[20] allergen exposure,[21] breastfeeding,[22] and maternal nutritional status during pregnancy in forms of vitamin D[23] and n-3 fatty acids.[24] Unfortunately the responsible environmental factors are still largely unknown and except from avoiding tobacco smoke exposure, no effective preventive measure is available.

Genes

A strong hereditary component in atopic diseases has been known for many years from twin studies showing that more than 60% of the variation in disease risk is due to genetic factors.[25] A number of risk genes have been identified for asthma, eczema and allergy but generally the discovered risk variants have only conferred limited risk and results from different studies have been inconsistent.[26;27] One exception is the newly discovered risk variants in the gene coding for the skin barrier protein filaggrin (FLG), associated with eczema, asthma, rhinoconjunctivitis and allergic sensitization.[28;29] FLG variants have shown to be the strongest known genetic risk factor for eczema and one of the strongest genes ever discovered for a complex disease.[30] One potential explanation for the inconsistent results between studies are the complex effects of gene-environment interactions that may strongly effect expression of genetic susceptibility and is rarely accounted for in large scale studies.[31]

Understanding heterogeneity and disease mechanisms

Another limiting step for research and our understanding of disease etiology is the heterogeneous nature of atopic diseases.[32] Heterogeneity is very pronounced in early childhood as indicated by differences in temporal patterns, such as age at onset and persistence of disease, and the association with intermediate phenotypes, such as sensitization.[33;34] It is therefore essential that studies are performed in all age groups and that details on disease characteristics are obtained in order to dissect and understand the underlying disease entities. Atopic diseases are likely to represent several

specific endotypes (a contraction of endophenotype), subtypes of disease associated with distinct clinical features and defined functionally by the underlying molecular mechanisms or by treatment response.[35] Identifying these endotypes seems essential for establishing effective preventive measures and improved treatment. Genes are causally related to disease and discovery of genetic risk variants may therefore be one way of identifying new endotypes. One example of this is the genetic variants in the filaggrin gene associated with skin barrier dysfunction and atopic disease. [28] Importantly, filaggrin seems only to be expressed in the skin [36;37] suggesting that filaggrin may define a specific endotype of atopic disease initiated by impaired skin barrier function.
Still, our understanding of atopic diseases is limited and increased understanding of both environmental and genetic etiologies, their interaction and the involved mechanisms is needed in order to improve prevention, diagnoses and treatment.

1.3. Early immunological mechanisms

Established atopic disease is archetypically characterized by a T-helper 2 (Th2) deviated immune response involving cytokines such as IL-4 and IL-5, which promote eosinophilia and IgE-production. [38] The immune response of the infant is generally Th2-dominated and it has been suggested that development of atopic disease is caused by sustained Th2-dominance compared to a rapid Th1-deviation during the first year of life in normal infants.[39;40]However, the immunological changes associated with later atopy are not simple and are not easily explained by the Th1/Th2 paradigm, for example children who develop atopic disease have reduced Th2 responses at birth.[39]It should be noted, that the Th1/Th2 paradigm is probably oversimplified and that the value of this paradigm has been questioned as explanation for changes in disease prevalence [41] and for understanding of disease mechanisms.[35]
Allergen-specific sensitization is rare during the first years of life with increasing prevalence during childhood. Sensitization during the first years of life is typically against food allergens while sensitization to inhalant allergens dominate in later childhood.[42]

1.4. Sensitization and disease

Allergic sensitization with production of allergen-specific immunoglobulin E (IgE) is a hallmark of atopic disease and in classical cases these allergen-specific IgE-antibodies are directly involved in the pathogenesis of allergic symptoms.[11;12] Sensitization is a strong risk factor for atopic disease in all ages and sensitization early in life is associated with later development of sensitization and allergic symptoms.[43-45]

Total IgE levels, of which a proportion is not directed against specific allergens, is also statistically associated with disease. Individuals with atopic disease will on average have higher total IgE levels and after 5 years of age the total IgE level is strongly correlated to the level of specific IgE.[46] However, on the individual level total IgE provides little information about the allergic component of the disease and there is no known allergy-related biological function of non-specific IgE.[11] Also, genetic studies have shown that some genetic variants are associated with increased IgE production but not with disease,[47] suggesting that risk factors may affect total IgE levels without increasing the risk of disease. Specific IgE is therefore preferred to total IgE both in the clinical setting and research.

The relationship between sensitization and disease is not simple. First of all, sensitization is also present without symptoms.[48] Furthermore, the clinical pictures of atopic diseases also occur *without* sensitization. Sensitization is present in approximately 50% of children with asthma (less in preschool- and more in school-children),[49] 35% of children with eczema[50] and 60-80% of children with rhinitis.[51] Finally, even in patients with sensitization and atopic disease, symptoms are often driven by non-specific factors rather than allergy. For example viral infection is the most common trigger of asthma symptoms also in sensitized children.[52;53]

Nevertheless, sensitization represents an important pathogenetic step in a large proportion of asthma, eczema and rhinoconjunctivitis cases and is therefore one relevant research focus for improved understanding and prevention of disease.

1.5. Fetal programming and the intrauterine environment

Programming of disease in fetal and early postnatal life has been hypothesized to be an important mechanism for atopic diseases.[40] This is supported by debut in early life in many cases of disease and findings of birth anthropometrics[54] and immunological alterations at birth [39;55] predicting IgE production and development of disease.

Furthermore, a number of prenatal environmental risk factors have been identified suggesting that the intrauterine environment may play an important role.[14;23;24;56] An observation in line with this hypothesis is a stronger effect of maternal compared to paternal atopic disposition on risk of atopic disease and IgE production in the offspring,[57;58] which could be explained by intrauterine effects of factors related to maternal atopy. The role of genetic factors and confounding from environmental risk factors tracking from prenatal life into childhood is difficult to interpret from observational studies. However, randomized trials of nutritional intervention during pregnancy support the causal

relationship behind these associations.[24;59] This suggests that fetal life is a critical period for development of atopic diseases and may be an important "window of opportunity" for prevention of disease.

The concept of early programming of disease directs research focus towards the earliest stages of life and the first indication of disease development. The age at onset of sensitization and symptoms is important information if the aim is primary prevention before the disease course is programmed. As an example, the hypothesis that allergen-specific may take place *in utero* and has led to recommendations about allergen avoidance during pregnancy.[60;61] Putative disease programming also underlines the relevance of identifying early markers of disease to be able to intervene as early as possible in disease development and to identify risk factors working in prenatal life. Elevated levels of IgE in cord blood are supposed to be a product of the fetus and are used as such early marker of atopy in the newborn.[62;63] Genetic risk factors are obviously present already in fetal life but again the concept of early programming suggest that the temporal development of sensitization and symptoms associated with the risk variant may provide important information. This may help identifying the relevant timing of intervention and the environmental risk factors interacting with the genetic susceptibility.

1.6. Intrauterine sensitization

It has been suggested that atopic sensitization may occur in utero.[40] This hypothesis has led to a focus on prenatal exposure to allergens as a potential cause of atopic disease[62] and guidelines recommending peanut avoidance during pregnancy by atopic women,[60;61] though studies of inhalant [21] and food allergen [64;65] avoidance during pregnancy have shown no effect on sensitization in infancy.

The hypothesis of intrauterine sensitization was stimulated by findings of non-specific IgE in cord blood predicting atopic disease in children.[66-71] Later studies confirmed that *allergen-specific* reactions may develop in utero by demonstrating allergen-specific T-cell memory [72-74] in cord blood with association to atopic disease. Also, intrauterine exposure to allergens have been documented even to inhalant allergens such as house dust mite,[75] showing that intrauterine sensitization is a theoretical possibility.

Allergen-specific IgE is present in very low levels at birth and has therefore rarely been studied, but some have reported allergen-specific IgE of suspected maternal origin against both food and inhalant allergens in cord blood.[76-78] One of these studies showed association to atopic disease

suggesting that allergen-specific sensitization and IgE production may develop in fetal life and track into childhood.[77] We studied the existence and relevance of intrauterine sensitization measured by allergen-specific IgE in cord blood (Paper I).

1.7. Total IgE in cord blood as marker of atopy

IgE is a hallmark of atopic disease and elevated levels in cord blood is used as an indicator of fetal atopy. Total (non-specific) IgE is more easily measured than allergen-specific IgE due to higher levels in cord blood and has therefore been used in a number of studies through decades.[62;63;66-69;69-71;71;79-81]

As indicator of fetal atopy, cord blood IgE is of interest because it can provide information on the earliest stages of disease and the prenatal versus postnatal effects of environmental and genetic risk factors[62;63;69-71;79-81].

Another usage of cord blood IgE is as predictor to identify infants in high risk of atopic disease.[66-69;82] As such, it has been used in clinical practice as basis for decisions on preventive measures, although the reported predictive capacity has been smaller in recent than in the earliest studies and the clinical usefulness has been questioned.[71;83]

The fetal origin of IgE in cord blood is supported by studies showing that fetal cells are capable of producing IgE from the 2nd trimester [84;85] and that IgE does not traverse the human placenta.[86] It is well known that falsely elevated IgE levels may occur due to "contamination" of cord blood with maternal blood, but this is usually controlled for by measuring IgA in cord blood and is found to be an infrequent event using this method.[87]

In the study described in paper I, we found strong evidence that *allergen-specific* IgE in cord blood is the result of transfer of IgE from the mother to the feto-placental unit rather than fetal production.[88] This suggests that also *non-specific* (total) IgE in cord blood may primarily be a maternal product and hence, the validity of total IgE as a measure of fetal atopy may be biased and rather reflect maternal atopic status.

On the other hand, total IgE levels in cord blood are generally much higher than allergen-specific levels, and non-specific IgE can be detected in the majority of cord blood samples in contrast to allergen-specific IgE, which is rarely detected. Furthermore, the capability of the fetus to produce IgE has been documented.[84;85] It is therefore possible that the fetus produces significant amounts of non-specific IgE and that materno-fetal transfer is less of a problem in studies of total IgE.

In paper II we studied to which extent elevated levels of IgE in cord blood is the result of materno-fetal transfer of IgE rather than fetal production.

1.8. From genetically (filaggrin) defined skin barrier dysfunction to sensitization and asthma

FLG is the main protein component of the keratohyalin granules within the stratum corneum that provides a physical barrier which reduces water loss and protects the body from potentially harmful environmental exposures such as allergens, toxic chemicals and infectious organisms.[89] Two independent variants in the FLG gene (R510X and 2282del4), carried by ~9% of people of European origin, result in complete loss of functional FLG in the epidermis.[28] Homo- or heterozygotes for these FLG variants alleles have varying degrees of impaired skin barrier and homzygotes are in high risk of developing icthyosis.[90] This observation made FLG an obvious candidate gene for eczema, and the COPSAC (COpenhagen Prospective Studies on Asthma in Childhood) cohort was one of the founder cohorts in the discovery that variants in the gene encoding filaggrin (*FLG*) are major determinants of eczema.[28] This association has been consistently replicated since, making FLG the strongest known genetic risk factor for eczema and having changed the paradigm of eczema pathogenesis from a focus on immunological deviation to skin barrier defect.[30] The proportion of eczema cases attributed to FLG variants has been estimated at 11%.[91]

Interestingly, FLG gene variants are also associated with development of sensitization, asthma and allergic rhinitis even though FLG seems only to be expressed in the skin and not in respiratory epithelium.[36;37;92] Such association between skin barrier dysfunction, sensitization and airway disease is intriguing. It indicates skin barrier dysfunction as a causative and potentially modifiable mechanism in the pathogenesis of sensitization and asthma. In order to understand this mechanism there is a need for longitudinal studies in early life describing the temporal relationship in the development of FLG-associated atopic diseases. This may help identifying the environmental risk factors interacting with this genetic susceptibility and the age at which intervention should be initiated. Also, atopic diseases in early childhood differ from those at older ages and must be studied separately.

In paper III we analyzed FLG variants against the longitudinal clinical diagnoses of recurrent wheeze, acute severe exacerbations and asthma together with assessments of sensitization and

described the temporal relationship in development of the different FLG-associated atopic outcomes.

2. Aim and objectives

The aim of this thesis was to increase the understanding of sensitization in prenatal and early life. Such insight into the early phases of disease may increase our understanding of disease pathogenesis in general, direct future research and help developing relevant and correctly timed preventive measures.

The specific objectives were:

- To study the origin and relevance of allergen-specific IgE in cord blood.
- To study the origin of elevated levels of total IgE in cord blood.
- To study the effect of genetically (filaggrin) determined skin barrier dysfunction on development of sensitization and asthma in early life.

3. Design, setting and participants - COPSAC

All studies in this thesis are based on the Copenhagen Prospective Study on Asthma in Childhood (COPSAC). COPSAC is an ongoing prospective birth cohort study of 411 infants born to mothers with a history of asthma. Children were recruited between August 1998 and December 2001. The recruitment, baseline description of participants and design of the study were previously described in details.[93;94]

The aim of COPSAC is to investigate the gene-environmental interactions causing atopic diseases in children and identify early-life exposures that can be modified to prevent disease. A unique strength of COPSAC is the extensive objective assessments and detailed clinical phenotyping of atopic diseases with prospective data collection at visits to the clinical research unit every 6 months, as well as at acute symptomatic episodes. Children are diagnosed and treated for acute respiratory and skin symptoms by the doctors in the research unit following predefined algorithms. This minimizes the risk of misclassification of symptoms and diagnostic variation due to local diagnostic tradition. The objective of this approach is to minimize variability in the clinical data and characterize specific clinical features associated with underlying endotypes.

Data validity and quality control procedures follow "Good Clinical Practice" guidelines. History is collected on-line during visits to the COPSAC clinical research unit. Objective measurements are double checked against source data and the database subsequently locked. An audit trail is run routinely.

The study was approved by the Ethics Committee for Copenhagen (KF 01-289/96 and KF 11-107/02) and The Danish Data Protection Agency (2008-41-1754). Oral and written informed consent was obtained from both parents of participating children.

The specific methodologies used in this thesis are described in details together with the respective studies.

4. The origin of allergen-specific IgE in cord blood

Paper I: ***Sensitization does not develop in utero***

In this paper, we studied intrauterine sensitization measured by allergen-specific IgE in cord blood. We studied the clinical *relevance* of allergen-specific IgE in cord blood by comparing with allergen-specific IgE in the 6-month old infant. We then studied its *origin* hypothesizing materno-foetal transfer as the source of specific IgE in cord blood. Evidence in favor of this hypothesis would be if fetal specific IgE closely matched maternal specific IgE both with respect to allergen specificity, level of IgE and ratio of total/specific IgE and if there was a correlation between specific IgE and IgA in cord blood indicating maternal blood contamination.

4.1. Methods

Blood sampling

Midwifes received written information instructing them to collect cord blood by needle puncture of the umbilical cord vein. Blood was further collected from the infants at 6 months of age and from parents after birth of the child. Serum and plasma was stored at -80^{o}C until analysis.

IgE analyses

IgE antibody levels were determined via the ImmunoCAP assay [95] (Phadia AB, Uppsala, Sweden). Cord blood samples and infant blood at 6 months of age were analyzed for level of total IgE, specific IgE against milk and egg allergens and cumulative level of specific IgE against a panel of common inhalant and food allergens (Phadiatop Infant).[96] Samples positive for Phadiatop Infant were further analyzed for specific IgE against relevant single allergens from this panel (*D. pteronyssinus*, cat dander, dog dander, birch, timothy, mugwort and peanut). Detection limit for total and specific IgE was 0.1 IU/mL.[97] Specific IgE results in cord blood were double tested. IgE levels in parental blood were analyzed similarly after screening with Phadiatop. Detection limits for total and specific IgE in parental blood were 2 IU/mL and 0.35 IU/mL respectively.
Cord blood samples were analyzed for total IgA using a sensitive ELIA assay designed to measure very low levels of IgA (detection limit 0.1 mcg/L, analysed by Phadia AB, Uppsala, Sweden).

Statistical analyses:

The statistical analysis of specific IgE is complicated be the high frequency of samples with levels below the lower limit of quantification (LLQ). This problem is even more pronounced when studying cord blood, where levels are lower than later in life. Quantitative analyses are therefore limited to the samples with levels above LLQ or rely on assumptions about the level in samples below LQQ. Assuming a fixed level (e.g. the LLQ level) is not optimal, especially since IgE levels must usually be analyzed on log-transformed scales due to the distribution, and here the low values will have a large impact on results. In order to analyze IgE levels quantitatively without losing information from samples with levels below LQQ, we modeled the underlying association by linear regression and by assuming an underlying normal distribution. From this we extracted a model for the observed values accounting for detection limits. This method was used for analyzing the association between IgE and IgA in cord blood, specific IgE in cord blood and maternal blood for each level of cord blood IgA, and for the association between specific IgE in cord blood and paternal blood. Maximum likelihood estimates with asymptotic 95% Wald confidence intervals were calculated and likelihood ratio tests for hypotheses were performed. The association between ratios of total/specific IgE in cord blood and maternal blood was analyzed by simple linear regression analysis. All values were transformed on a logarithmic scale. The modeling of levels below detection limit is not available in standard statistical software. The standard analyses were made in SAS version 9.1.

4.2. Main results

Corresponding cord blood and maternal samples were available for 243 children.

Specific IgE against mixed-allergens (Phadiatop Infant) was found in 34 (14 %) of all cord blood samples. Twenty two samples had detectable levels of specific IgE against single allergens. Together these 22 samples had 36 positive single allergen tests, of which 35 were against inhalant allergens and 1 was against peanut. Specific IgE against milk or egg was not detectable in any of the 243 cord blood samples.

Specific IgE in infant blood at 6 months of age

None of the specific IgE against single allergens found in cord blood was reproduced in the infant's blood at 6 months of age.

Specific IgE in cord blood and mother's blood

Specific IgE in cord blood was only found in off-springs of mothers with corresponding specific IgE and not in any of the 92 infants of non-sensitized mothers. Similarly all 36 positive single allergen tests in cord blood corresponded to a positive test in maternal blood.

Comparing patterns of specific IgE against single allergens in the 22 individual pairs of cord blood and mother's blood showed that the cord blood pattern perfectly matched the maternal pattern both with respect to allergen specificity and relative levels of specific IgE. The specific IgE with highest levels in maternal blood was consistently found in cord blood. Complementary to this, specific IgE with lower maternal levels was not always detectable in cord blood as would be expected due to the detection limit in cord blood. An example of a mother-cord blood pair is shown in Figure 1, all of the 22 mother-cord blood comparisons of specific IgE are shown in Paper I.

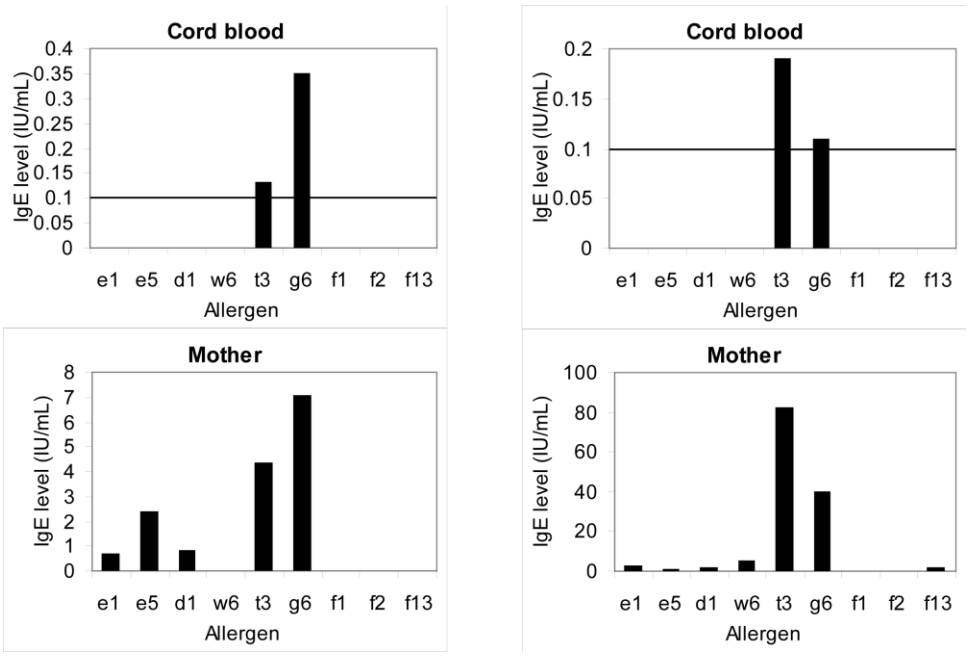


Fig 1. Two examples of patterns of specific IgE in individual mother-cord blood pairs. The horisontal line in the cord blood diagrams represents lower limit of IgE detection (0.1 IU/mL). Allergen abbreviations: e1=cat dander, e5=dog dander, d1= D. pteronyssinus, w6=mugwort, t3=birch, g6=grass, f1=egg white, f2=milk, f13=peanut.

The level of specific IgE in cord blood was also positively correlated to the maternal level of specific IgE. This correlation was related to cord blood-IgA levels ($P < .0001$) (Figure 2). With high levels of cord blood-IgA there was a strong correlation for all maternal levels. With low levels of cord blood-IgA specific IgE was only found in cord blood if mothers had high levels of specific

IgE. The maternal/cord blood specific IgE-ratio was approximately 1/10, 1/100 and 1/1000 in infants with high, intermediate and low levels of cord blood-IgA respectively.

The ratios of total/specific IgE in cord blood and maternal blood were highly significantly correlated ($P < .001$) with an approximately 1:1 relationship between the two (paper I, figure 6). There was no correlation between specific IgE in cord blood and *paternal* blood ($P = .19$).

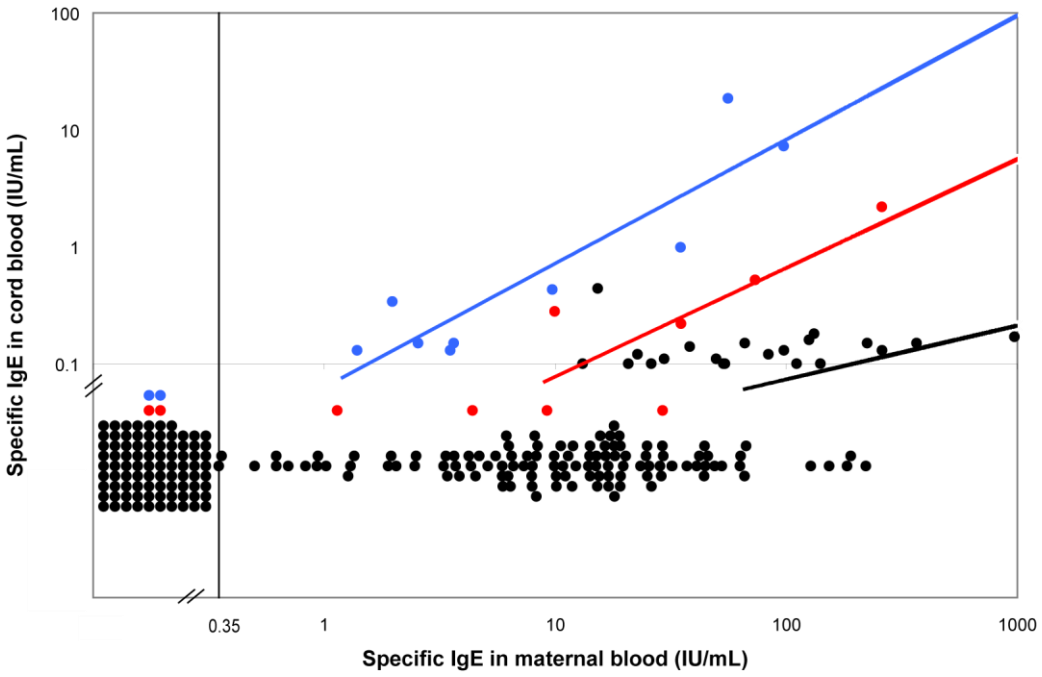


Fig 2. Relation between specific IgE (against mixed allergens) in cord blood and specific IgE in maternal blood, stratified for level of IgA in cord blood. Regression lines are shown for each level of cord blood-IgA.
— and ● Cord blood IgA > 100 mcg/L, — and ● Cord blood IgA = 50-100 mcg/L, — and ● Cord blood IgA < 50 mcg/L

Specific IgE and IgA in cord blood

The level of specific IgE in cord blood was positively correlated to the level of cord blood-IgA ($P < .0001$) (Figure 3). The correlation was evident with IgA-levels above 25-50 mcg/L (by visual inspection of Figure 3). Specific IgE in cord blood was also detected in samples with low IgA.

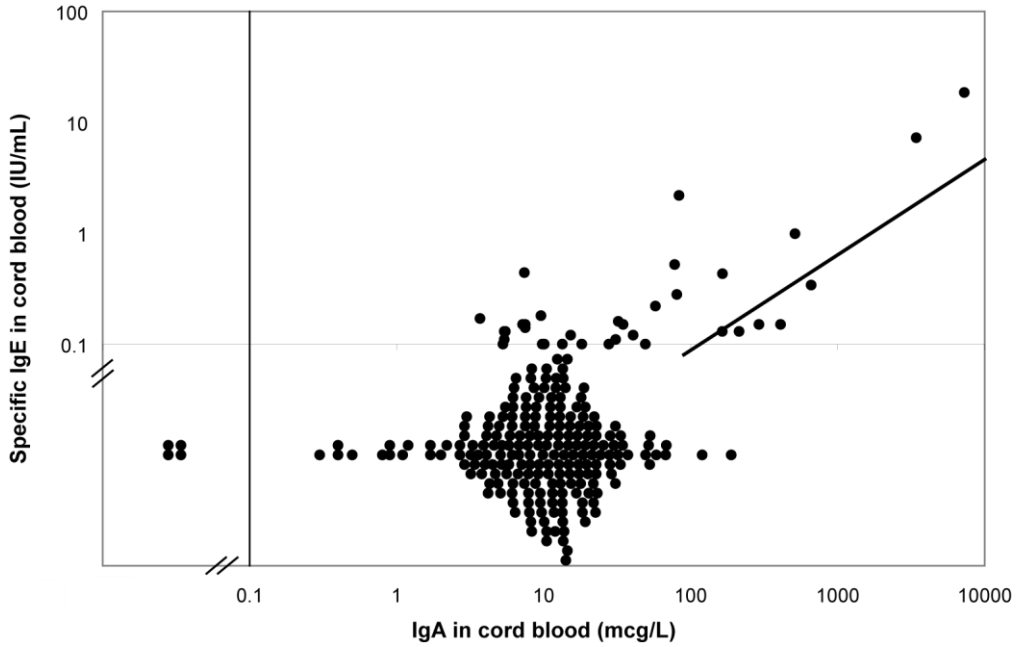


Fig 3. Relation between IgA and specific IgE (against mixed allergens) in cord blood. Regression line is shown.

4.3. Discussion

Principal findings

Allergen-specific IgE was found in 14 % of cord blood samples. However, this allergen-specific IgE was no longer detectable in infant blood at 6 months of age showing that it was not clinically relevant and did not indicate sensitization in the newborn. Furthermore this suggests that specific IgE in cord blood is not a product of the fetus but rather the result of transfer of maternal IgE to cord blood or fetal blood.

We found a close match between specific IgE in mothers blood and in cord blood: (i) Specific IgE was only found in newborns where the mother had the same specific IgE and never in mothers without specific IgE ; (ii) the pattern of specific IgE in cord blood consistently exhibited a "finger-print" match of the maternal pattern; (iii) the level of specific IgE in cord blood was closely correlated with the maternal level; (iiii) the ratio of total/specific IgE in cord blood showed approximately 1:1 correlation with the maternal ratio. In contrast there was no association between specific IgE in cord blood and *paternal* blood. Such close cord blood-mother match is in perfect agreement with materno-fetal transfer of IgE.

Specific IgE in cord blood correlated with cord blood-IgA. Since IgA does not cross the placental barrier and is not produced in utero in significant amounts this indicates maternal blood contamination of cord blood samples.[98] Furthermore cord blood-IgA interacted with the relationship between cord blood and maternal specific IgE as would be expected if maternal blood contamination was the causative mechanism. However, allergen specific IgE was also found in cord blood with low levels of IgA if the mother had very high levels of such IgE indicating both an IgA-associated and a non-IgA-associated mechanism behind specific IgE in cord blood. Importantly the close mother-cord blood match with respect to allergen specificity of IgE, level of specific IgE and total/specific IgE-ratio and the disappearance of specific IgE before 6 months of age was similar with high and low levels of IgA suggesting a passive mechanism as source of specific IgE in cord blood in both groups.

Together this evidence strongly suggests that allergen-specific IgE in cord blood is passively acquired from the mother and is not the result of intrauterine sensitization. A single case of specific IgE in cord blood without corresponding IgE in the mother or a correlation between cord blood and father's specific IgE would have indicated at least some intrauterine sensitization but this was not found.

Other studies

Because of the low levels of specific IgE against inhalants in cord blood this has rarely been detectable with the methods of analysis used in previous studies. There are therefore only few studies of allergen-specific IgE in cord blood and data are conflicting.

One previous study supports that cord blood specific IgE against inhalant allergens is caused by materno-fetal transfer of IgE since 2/3 of cord blood samples with such IgE showed markedly decreasing total IgE values from birth to 4-5 days of age.[99] Another study of low levels of specific IgE against cow milk proteins in cord blood is also in line with our findings.[100] Specific IgE was detected in 37% of samples by a sensitive chemiluminiscens method and there was a similar perfect match between maternal and cord blood specific IgE. Indirect support for IgE sensitization taking place postnatally rather than in utero comes from the finding that none of children with specific IgE against house dust mite at 6 months of age had detectable levels in cord blood.[101]

In contrast, a recent study by Pfefferle et al. conflicts with our findings.[76] In that study allergen-specific IgE against inhalant or food allergens was detected in 24% of cord blood samples and in 65% of cases this was without corresponding specific IgE in maternal blood. Furthermore there was no association between maternal and cord blood specific IgE levels for inhalant allergens in strong

contrast to our findings. The authors concluded from this, that allergen specific IgE was most likely of fetal origin. Another earlier study also contrast with our findings in detecting house dust mite specific IgE in cord blood in the absence of such IgE in maternal blood and finding no correlation between cord blood and maternal levels.[77] None of these studies investigated if specific IgE detected in cord blood was still present later in life.

There are no obvious explanations for these discrepancies between studies. In both of the studies conflicting with the present the prevalence and levels of allergen-specific IgE were much higher suggesting that methodological differences may explain the different results. Pfefferle et al. state that the IgE assay were tested to perform well also at low levels, but the published methodological paper does not provide evidence of specific and reproducible results at the lowest IgE levels.[102] Also none of the two studies report running the cord blood samples in duplicate which may be essential when analyzing a large number of samples for levels close to the detection limit. In our study, several falsely elevated samples, probably due to batch problems, were detected by double-testing of samples and these falsely elevated samples would otherwise have appeared to be of fetal origin. It is therefore possible that some of the specific IgE detected in these studies are false positive results. Such analytical problems may explain lack of association between maternal blood and cord blood but is unlikely to cause the close mother cord blood match observed in our study. A "false positive association" between maternal and cord blood in our study could be due to more frequent contamination by maternal blood but this is not in agreement with the significantly lower levels of specific IgE in our study.

Meaning of the study

Our data strongly suggest that allergen-specific IgE is the result of materno-fetal transfer rather than fetal production and show that no matter the source of such IgE it does not reflect clinically relevant lasting sensitization. This questions the existence of intrauterine sensitization and thereby the rationale for current recommendations on allergen avoidance during pregnancy. This is consistent with the lack of protective effect from allergen avoidance, both of inhalant [21] and food [64;65] allergens, during pregnancy on sensitization in infants.

The mechanism behind the suggested materno-fetal transfer of IgE is not clear from this study. Since maternal IgE levels are often more than a 1000 fold higher than IgE levels in cord blood, even low levels of transfer from maternal blood are sufficient to cause elevated levels in cord blood. Maternal blood contamination during cord blood sampling is one possible mechanism although the method of needle puncture of the umbilical cord vein used in the present study probably causes

minimal amount of contamination. Small placental bleedings during late pregnancy or delivery may be a more plausible mechanism. Finally, transplacental transfer of IgE is another potential mechanism and may be responsible in samples with low levels of IgA. It is generally supposed that IgE does not cross the placental barrier.[86] However, it has been shown that human IgE injected into the blood of a pregnant monkey can be detected in the blood of the offspring in a ratio similar to albumin.[103] This suggests that a small amount of maternal IgE is always transferred to the fetus, in relation to the maternal level of IgE, and is consistent with our finding that the majority of children of mothers with very high specific IgE had measurable specific IgE in cord blood while this was never seen if mothers had no specific IgE or low levels.

The alternative interpretation of our findings is that the fetus produces specific IgE by a mechanism strongly dependent on maternal sensitization. It has been speculated that intrauterine sensitization may occur as a result of trans-amniotic passage of maternal IgE to the fetus [104] where it might bind to IgE receptors on antigen-presenting cells in the fetal gastrointestinal tract.[105] Antigen focusing could then facilitate sensitization to allergens, which are also present in amniotic fluid.[75] This has been hypothesized to have evolved as a mechanism for protection of the fetus from parasites now causing sensitization to allergens.[40;104] This could explain shared allergen specificity between maternal and fetal IgE and could be argued to be the responsible mechanism, especially in cases with no detectable IgA. Although theoretically possible, this mechanism is highly speculative. The perfect match between maternal and cord blood IgE with respect to allergen specificity (not a single case of specific IgE in cord blood without matching maternal IgE), level of specific IgE, total/specific IgE-ratio, and the disappearance of specific IgE before 6 months of age seems more plausibly explained by passive transfer than a biological mechanism involving production of IgE. Furthermore, such mechanism of passive transfer is supported by animal models.[103]

Specific IgE against infectious agents such as parasites and HIV have also been found in cord blood.[106;107] It has been suggested that HIV-specific IgE in cord blood could be used as an indicator of infection in the infant under the assumption that such IgE is the result of intrauterine production.[106] However, the present study suggests that such IgE may be the result of materno-fetal transfer and we suggest that future studies on specific IgE in cord blood should focus on studying and excluding such materno-fetal transfer of IgE.

The above discussion relates specifically to the origin of detectable allergen-specific IgE in cord blood. Putative intrauterine sensitization of T-lymphocytes without production of specific IgE is not addressed by our study. Several studies have shown cytokine or proliferative responses in cord

blood mononuclear cells in response to inhalant and food allergens suggesting allergen-specific sensitization of lymphocytes in utero.[72-74] However, it has recently been proposed that such T-cell responses are not the result of allergen-specific priming but rather non-specific reactions [108] and that development of Th2-polarized allergen specific memory occurs postnatal rather than in utero.[101]

Strengths and limitations

It is a limitation of this study that cord blood was sampled by the midwife and not by trained research staff. This may have increased the frequency of samples with "contamination" from maternal blood. However, we find no reason to believe that the frequency of contamination was higher in our study than in earlier studies of cord blood IgE. Midwifes were instructed to sample cord blood by needle puncture of the umbilical vein, a method that has been shown to cause less contamination than collecting blood by letting it drip from the cut umbilical cord.[109] Furthermore the frequency of cord blood IgE against inhalant allergens is similar to a previous report of cord blood IgE against inhalant allergens in infants of atopic mothers.[99]

We did not exclude samples with high IgA-levels from the analysis. First, it is an important point of this study that materno-fetal transfer of specific IgE takes place at all levels of IgA, but to different degrees. Second, no samples had IgA-levels higher than 10 mg/L which has previously been the lowest cut-off level used to exclude contaminated cord blood samples.[87]

Parental IgE was measured after birth of the child. Probably the close mother-cord blood match of specific IgE would have been even closer if the maternal sample was taken during pregnancy.

We only studied prenatal sensitization measured by allergen-specific IgE and can therefore not draw any conclusions on allergen-specific sensitization of lymphocytes without production of specific IgE. Studies on specific IgE in cord blood seem to be hampered by materno-fetal transfer and such transfer may be a smaller problem in studies of mononuclear cells.

We cannot exclude that allergen-specific IgE may be produced in lower levels than we could detect with our method. Two previous studies found low levels of allergen-specific IgE against milk and egg antigens, which we did not find. On the other hand, even if allergen-specific IgE production should take place at lower levels, our data suggest that materno-fetal transfer seem to be the origin in many cases an must be accounted for before considering fetal origin.

We did not study specific IgE against other antigens, such as parasites or HIV. It is possible that other mechanisms may be involved in the host response to infective agents thereby enabling fetal production of IgE against these antigens. However, our data stresses the importance of considering materno-fetal transfer of IgE also in such studies.

The results of the present study should not be taken as evidence against the hypothesis of fetal programming and importance of the intrauterine environment. The intrauterine environment is likely to affect the fetal immune system by processes not involving allergen-specific mechanisms. Finally, this study only included children of asthmatic mothers. Since the level of specific IgE in cord blood is dependent on the maternal level the frequency of detectable cord blood specific IgE would be lower in an unselected population. It seems less likely that the origin of such IgE would be different in an unselected population.

4.4. Conclusions and perspectives

Allergen-specific IgE in cord blood did not represent lasting sensitization in the infant and seemed to be the result of materno-fetal transfer of IgE rather than intrauterine sensitization.

Previous findings of allergen-specific IgE in cord blood may similarly have been the result of materno-fetal transfer. These studies should be interpreted with caution and future studies should suspect maternal origin of specific IgE. This may also be relevant for IgE against other antigens than allergens.

Materno-fetal transfer of IgE seemed to occur frequently if the maternal level was high by a mechanism not involving IgA. This suggests that, in contrast to the general belief, IgE may traverse the placental barrier. It also indicates that measurement of cord blood IgA does not adequately control for materno-fetal transfer of IgE with potential implications for studies of total IgE in cord blood, which usually rely on this method.

Our results do not support the concept of intrauterine sensitization and thereby the rationale behind allergen avoidance during pregnancy. Furthermore, such recommendations are not supported by randomized clinical trials and should be withdrawn.

Future research

Our findings suggesting maternal origin of allergen-specific IgE in cord blood are in accordance with some but in contrast to other studies. Recent publications in high impact journals suggesting intrauterine production of allergen-specific IgE shows that this is an issue that needs to be studied further. This is important for the interpretation of future results and potentially to avoid irrelevant studies of specific IgE in cord blood if this is not a fetal product and therefore not a relevant marker of disease.

Our findings should therefore be replicated and the limitations of the present study should be addressed. A future study should be performed in mothers not selected for asthma or other atopic

disease to investigate whether these findings are specific for atopic mothers. Maternal blood samples should be taken in late pregnancy and cord blood should optimally be sampled be research staff. Different assays for IgE measurement could be applied in order to explain discrepancies between studies. Furthermore measurement of maternal IgA will allow optimization of cord blood IgA as marker of "contamination" by adjustment for maternal variation in IgA levels.
If our results are replicated it would strongly support maternal origin of allergen-specific IgE in cord blood. However, it would still be a theoretical possibility that specific IgE in samples with low IgA levels could be a result of fetal production rather than trans-placental transfer. Trans-placental transfer of IgE could be studied by perfusion studies in human placenta.

5. Elevated IgE in cord blood as a marker of atopy in the newborn

Paper II: ***Elevated IgE in cord blood is biased from materno-fetal transfer***

In the study described in paper I, we found strong evidence that allergen-specific IgE is the result of materno-fetal transfer of IgE rather than fetal production.[88] This suggests that also total IgE, which is the most frequently used measure of atopy in the newborn may be affected significantly by materno-fetal transfer of IgE.

On the other hand, production of non-specific IgE by the fetus is plausible and well documented and the majority of total IgE is non-specific. Also, total IgE-levels are much higher than allergen specific levels and can be detected in the majority of cord blood samples in contrast to specific IgE which is a rare finding. Total IgE levels may therefore predominantly be of fetal origin with materno-fetal transfer of IgE playing a small and insignificant role.

We investigated to which extent elevated levels of total IgE in cord blood was the result of materno-fetal transfer of IgE rather than fetal production. We studied this by high sensitivity analyses of cord blood IgA and IgE and comparison with IgE levels at 6 months of age and parental levels.

5.1. Methods

Blood sampling and IgE analyses

Cord blood, blood from the infant at 6 months of age and parental blood was sampled and analyzed for IgA, and total and specific IgE levels as described in chapter 4.

Statistical analyses:

We modeled the underlying association between total IgE in cord blood and maternal blood for each level of cord blood IgA and for the association between total IgE in cord blood and paternal blood, accounting for detection limits as described in chapter 4.

Association between total IgE and IgA in cord blood was tested by rank correlation. A smoothing curve (Lowess) was calculated based on levels above detection limit for the association between total IgE and IgA to estimate a relevant cut off level for association. The association between predicted and observed levels of total IgE in cord blood was analyzed by simple linear regression analysis. Associations of cord blood IgE with IgE at 6 months of age were analyzed similarly by using levels above detection limit only. IgE level at 6 months of age in children with elevated cord

blood samples with or without indication of transfer of IgE was compared by ANOVA statistics. All values were transformed on a logarithmic scale.

5.2. Main results

Pairs of cord blood-maternal, cord blood-paternal and cord blood-6 month samples were available for 243, 220 and 219 children respectively.

Detectable levels of total IgE (>=0.1 kIU/L) were found in 184 (74%) cord blood samples and elevated levels (>0,5 kIU/L) were found in 74 (30%) samples.

Total IgE in cord blood and parental blood

There was a strong correlation between cord blood and maternal levels of total IgE ($P < .0001$). (Figure 4) IgE was always detected in cord blood if the maternal level was high and was always elevated in the samples with highest maternal levels. None of the 21 cord blood samples with lowest maternal levels had elevated IgE. In contrast there was no correlation between cord blood and paternal levels of total IgE ($P = .70$). (Figure 5)

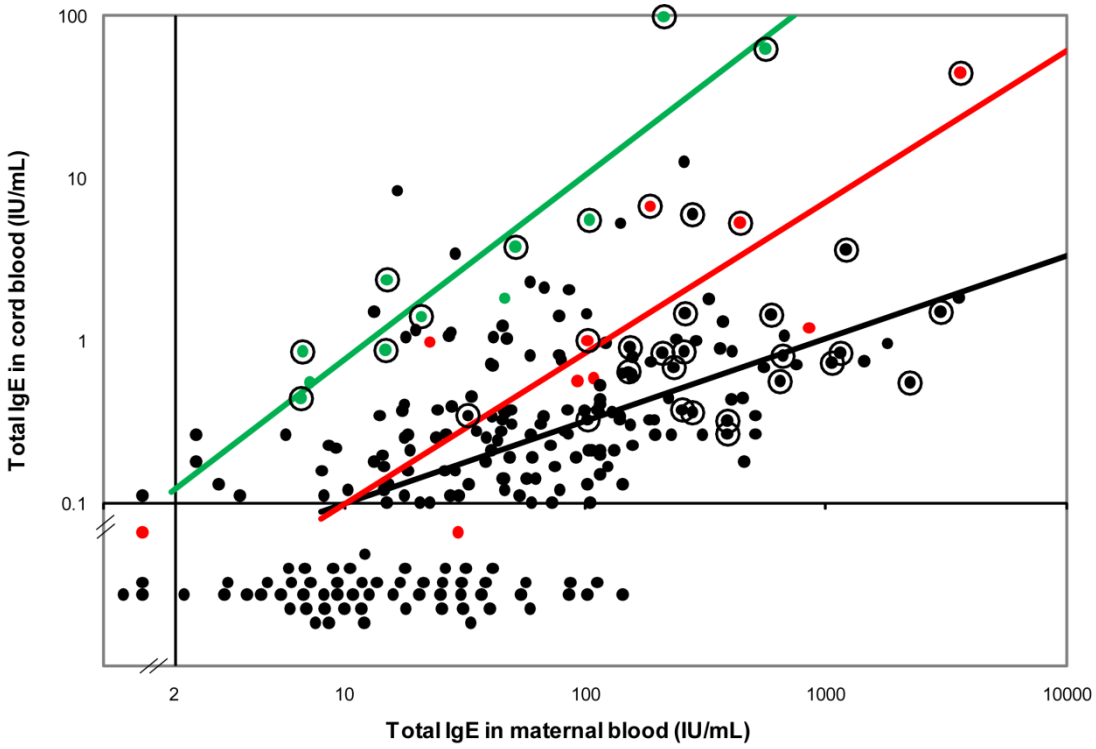


Fig 4. Relation between total IgE in cord blood and maternal blood, stratified for level of IgA in cord blood. Regression lines are shown for each level of cord blood-IgA. ▬ and ● Cord blood IgA > 100 mcg/L, ▬ and ● Cord blood IgA = 50-100 mcg/L, ▬ and ● Cord blood IgA < 50 mcg/L, O = detection of specific IgE in cord blood.

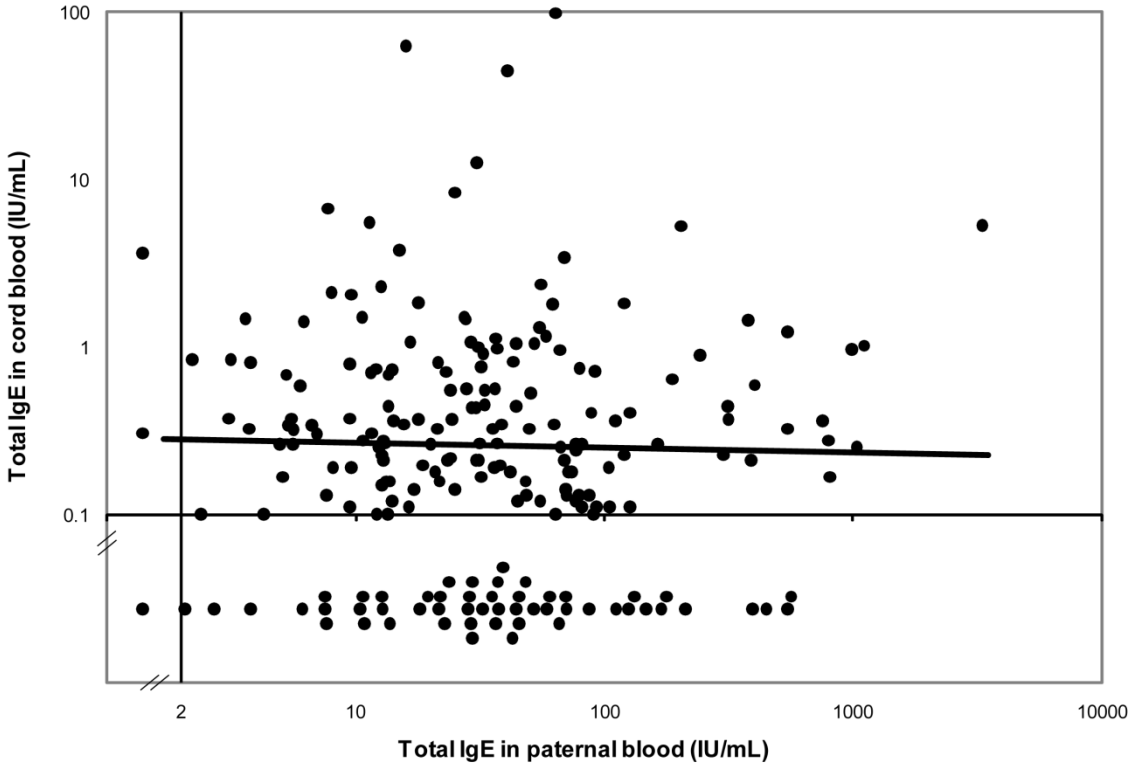


Fig 5. Relation between total IgE in cord blood and paternal blood. Regression line is shown.

IgA in cord blood as indicator of maternal transfer of total IgE

The level of total IgE in cord blood was positively correlated to the level of cord blood-IgA ($P <$ .0001) (Figure 6). The correlation was not linear as illustrated by the smoothing curve of average values. A correlation was evident for IgA-levels above 50 mcg/L (by visual inspection of Figure 6). This was supported by IgA-stratified analyses of cord blood and maternal IgE showing an upward shift of the association for higher IgA levels (Figure 4). These results are is in accordance with our previous findings for allergen-specific IgE in cord blood (Paper I)[88] and suggests that cord blood samples with IgA-levels higher than 50 mcg/L should be suspected to have falsely elevated IgE levels due to contamination of cord blood with maternal blood. This level was used as indicator of materno-fetal transfer of IgE in the following analyses.

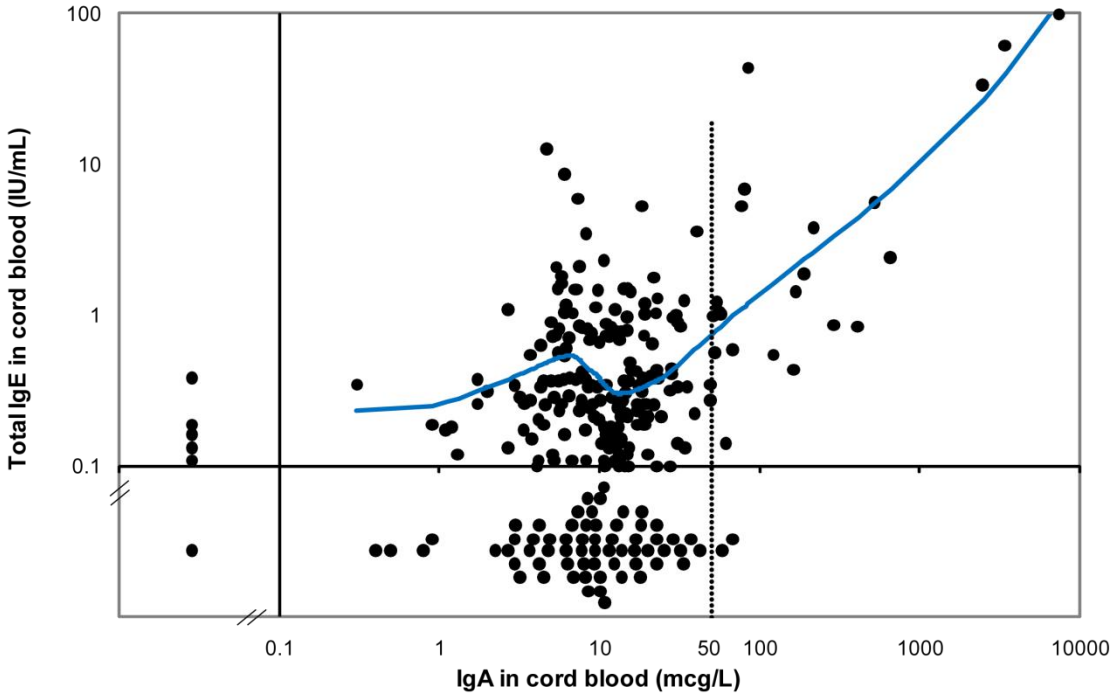
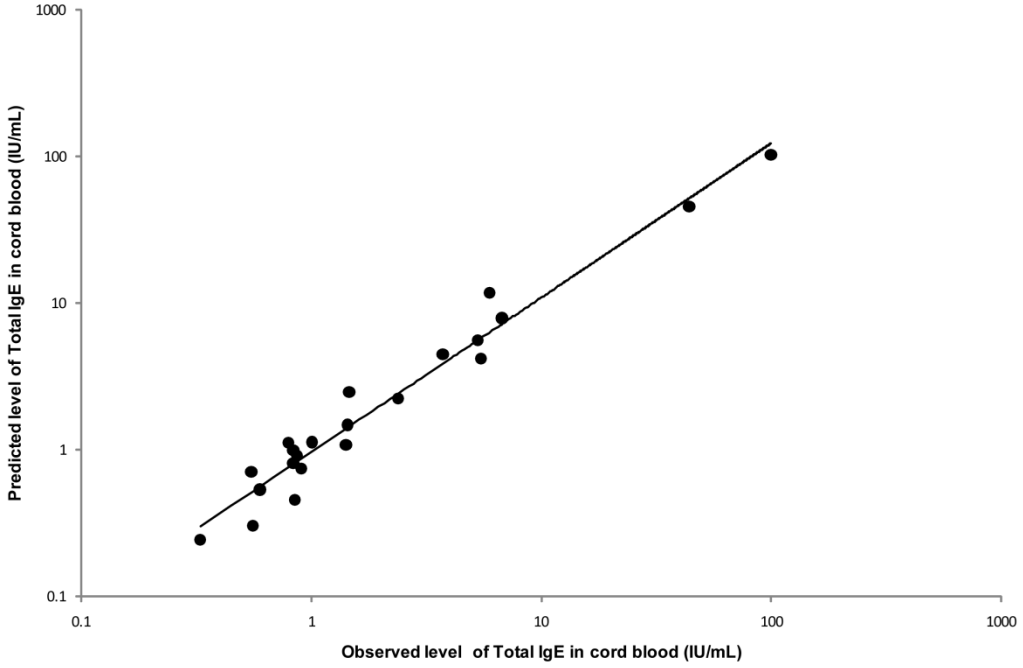


Fig 6. Relation between IgA and total IgE in cord blood. Smoothing curve (Lowell) is shown.

Allergen-specific IgE in cord blood as indicator of materno-fetal transfer of total IgE

We previously found strong evidence that allergen-specific IgE in cord blood was the result of materno-fetal transfer of IgE.[88] Assuming that such transfer is the only source of IgE in these samples, then the total level of IgE can be calculated on the basis of total/allergen-specific IgE in maternal blood and allergen-specific IgE in cord blood. We therefore calculated the predicted level and compared with the actual observed level of total IgE in cord blood. The predicted and observed levels of total IgE in cord blood were highly significantly correlated ($P < .0001$) with an approximately 1:1 relationship between the two (Figure 7.a). Accordingly, the observed level of cord blood IgE was generally close to 100% of the predicted level (Figure 7.b). This strongly suggests that also *total* IgE is mainly a product of the mother in samples with detectable allergen-specific IgE. Detection of allergen-specific IgE was therefore used, together with cord blood IgA, as indicator of maternal transfer of IgE in the following analyses.

7.a



7.b

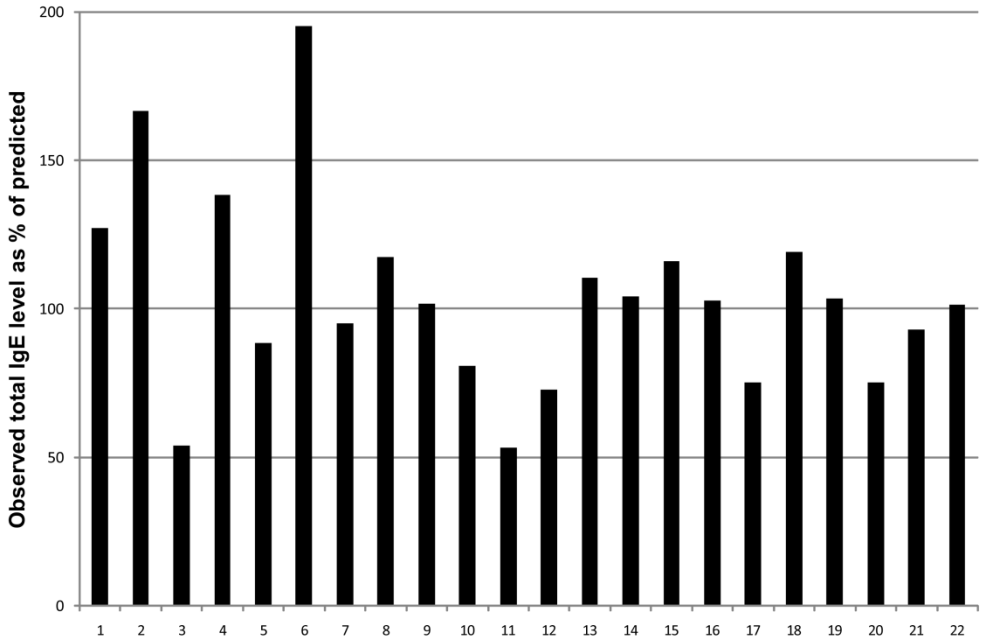


Fig 7. a) Relation between observed total IgE in cord blood and predicted (calculated) level in samples with detectable allergen-specific IgE against single allergens. Regression line is shown. b) Observed level of total IgE in cord blood as percentage of predicted level. Values are listed in order of increasing level of cord blood-IgA.

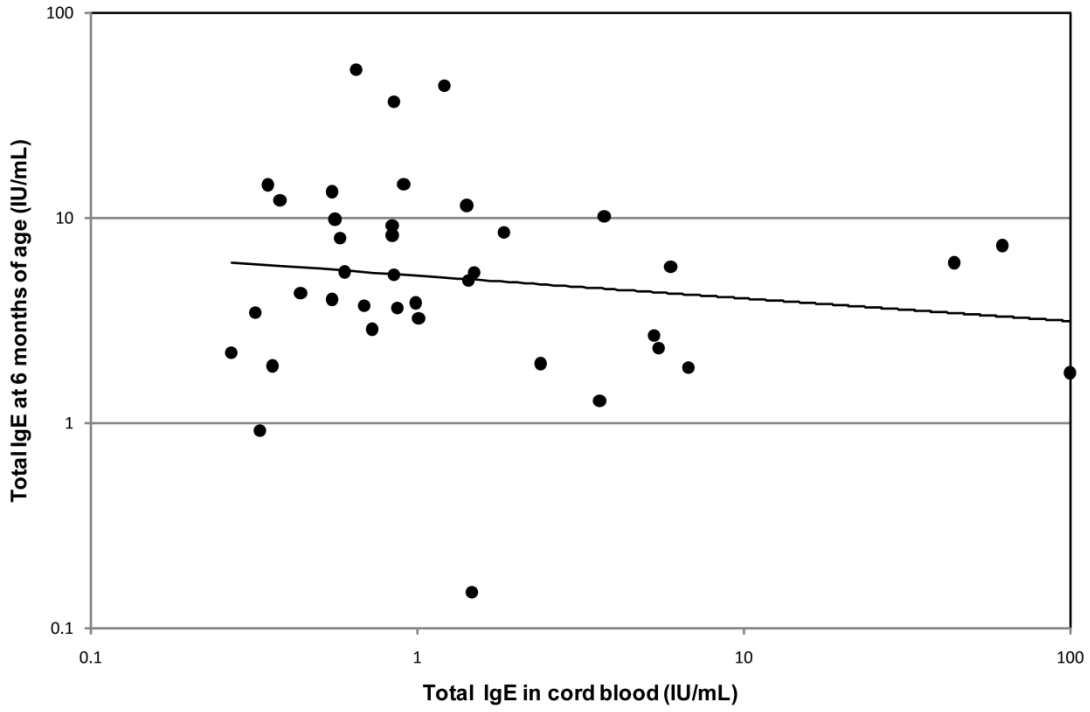
Frequency of materno-fetal transfer of IgE

There was indication of materno-fetal transfer of IgE in 34/74 (46%) of cord blood samples with elevated total IgE (>0.5 IU/mL). Of these, 35% were suspected 'contaminated' on the basis of both elevated IgA and detectable allergen-specific IgE, 47% on the basis of allergen-specific IgE only, and 18% on the basis of elevated IgA only. This distribution in relation to IgE and IgA levels is visualized in Figure 4.

Cord blood IgE and IgE at 6 months of age

Children with elevated cord blood IgE and indication of contamination (N=32) had significantly lower IgE levels at 6 months of age compared to children with elevated IgE and no indication of contamination (N=35), (geometric mean 5.4 IU/mL vs. 9.4 IU/mL, $P = .01$). In cord blood samples *with* indication of materno-fetal transfer of IgE there was no association with IgE level at 6 months of age ($P = .37$, Figure 8.a) in contrast to samples *without* indication of transfer ($P < 0.001$, Figure 8.b). These results were materially unchanged after adjustment for maternal IgE levels.

8.a



8.b

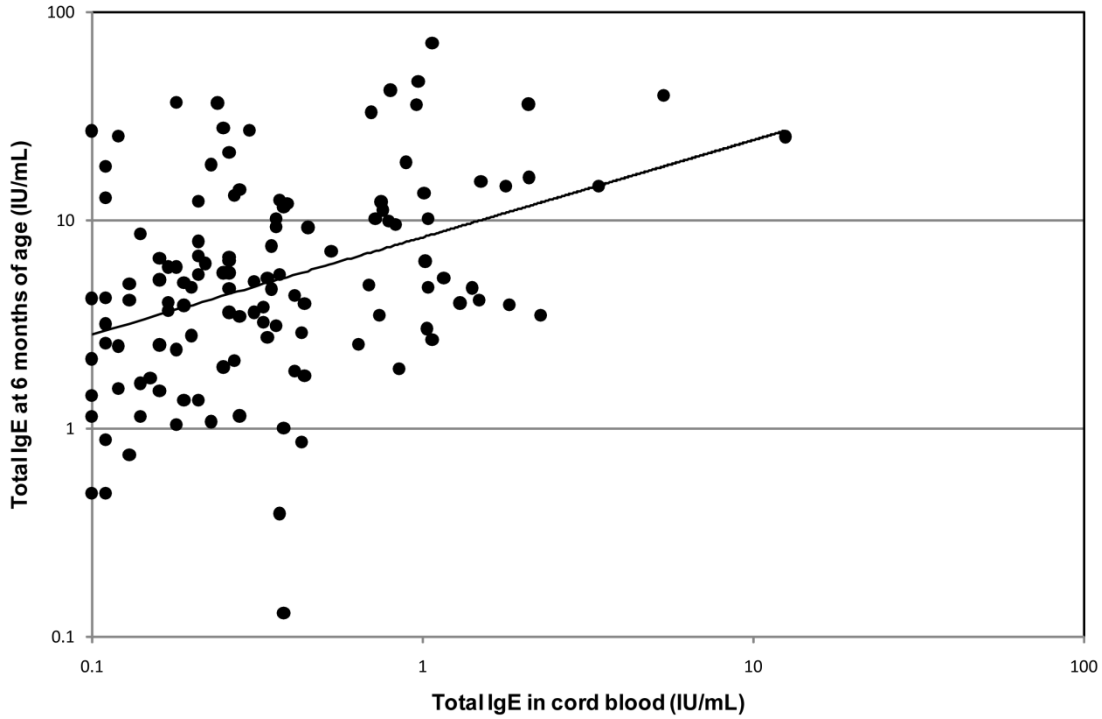


Fig 8. a) Relation between total IgE in cord blood and at 6 months of age for cord blood samples *with* indication of materno-fetal transfer of IgE. Regression line is shown. b) Relation between total IgE in cord blood and at 6 months of age for cord blood samples *without* indication of materno-fetal transfer of IgE. Regression line is shown.

5.3. Discussion

Principal findings

Approximately half of cord blood samples with elevated IgE levels showed indication of materno-fetal transfer of IgE. This frequency of suspected 'contamination' with maternal IgE is much higher than expected from previous studies with important implications for the value of elevated cord blood IgE as a marker of atopy in the newborn.

We investigated the extent of materno-fetal transfer of IgE in three subsequent steps.

First, we demonstrated a strong association between maternal and cord blood IgE, and IgE was always detected in cord blood if the maternal level was high. In contrast there was no association between paternal IgE and cord blood IgE. Transfer of IgE from the mother to the fetus could be one likely explanation for these findings.

We then used two different methods to detect materno-fetal transfer of IgE in individual samples. This first method was based on elevated levels of IgA in cord blood. Since IgA does not cross the

placental barrier and is not produced in utero in significant amounts,[98] elevated levels are supposed to reflect maternal blood contamination. We confirmed this by showing association between IgA and IgE levels in cord blood above a certain IgA level. The second method was based on the detection of allergen-specific IgE in cord blood. We previously provided strong evidence that such IgE is the result of materno-fetal transfer of IgE as demonstrated by a perfect, allergen-specific, match between specific IgE in maternal blood and cord blood and disappearance of cord blood specific IgE before 6 months of age.[88] In the present study we further demonstrated that in samples with detectable allergen-specific IgE, also total IgE levels seem to be the result of transfer from the mother. We hypothesized that if both total and allergen-specific IgE was the result of transfer then total IgE levels in cord blood could be predicted by the maternal total IgE/specific IgE ratio and the level of specific IgE in cord blood, which our data subsequently confirmed. This means that in these samples, the fetus does not seem to produce significant amounts of unspecific IgE in addition to the amount transferred from the mother, and therefore detection of allergen-specific IgE could be used as a marker of materno-fetal transfer of IgE.

Finally, we validated the hypothesis of maternal origin of IgE in samples with indication of materno-fetal transfer by analysing association with the child's IgE level at 6 months of age. Children with elevated cord blood IgE had significantly lower IgE levels at 6 months of age if the cord blood sample showed indication of materno-fetal transfer, and cord blood IgE was not associated with IgE at 6 months of age in such samples.

Meaning of the study

Our results suggest that elevated IgE in cord blood is the result of materno-fetal transfer in almost half of the cases. This frequency of suspected maternal origin of elevated IgE in 46% of samples is much higher than suspected in previous studies. Some have reported frequencies of contamination as low as 1% of cord blood samples with elevated total-IgE[71]or did not test for falsely elevated IgE-levels.[62;63] This suggests that previous studies using elevated IgE in cord blood as marker of atopic status included a number of cord blood samples with falsely elevated total IgE levels due to materno-foetal transfer of IgE. A number of prenatal factors including allergen exposure during pregnancy,[62] parity,[79] maternal age,[63] and sex of the infant[63] have been associated with elevated levels of IgE in cord blood and have therefore been interpreted as risk factors for atopy in the child. Since all of these prenatal factors have also been associated with maternal IgE levels,[62;63;110] the associations with cord blood IgE may be caused by materno-foetal transfer of IgE and therefore not reflect an effect on atopic development in the offspring but only an effect on maternal IgE levels. In

accordance with this a path-analysis suggested that the effect of parity on cord blood IgE was mediated through maternal levels of IgE [110] and similarly, other suggested risk factors were no longer significantly associated with cord blood IgE after adjustment for maternal IgE. [62;63] The results of such previous studies should therefore be interpreted with caution and future studies must control for potential materno-fetal transfer of IgE to assure that findings are related to fetal and not maternal IgE-production.

Frequent contamination of CB samples may also explain the low predictive values of CB total IgE for prediction of atopic disease that has been observed in most recent studies.[71;83] Future studies in the COPSAC cohort will show if a more restrictive exclusion of contaminated samples will enhance the predictive values. However, the high frequency of samples with falsely elevated IgE levels limits the usefulness of this method both in research and in clinical practice.

An alternative to cord blood IgE as marker of atopy in the newborn is IgE in capillary blood some time after birth where most IgE caused by materno-fetal transfer may be metabolized. However the half-life of IgE in neonates is unknown and one study did not find higher predictive values using capillary blood at 4-5 days of age compared to cord blood.[99]

The mechanism behind the suggested materno-fetal transfer of IgE is not clear from this study, and it is likely that several different mechanisms are responsible. As discussed in chapter 4, maternal blood contamination of cord blood is one possible mechanism and may explain transfer in samples with high IgA levels. Even low level of contamination with maternal blood may cause elevated levels in cord blood since IgE levels are often more than a 1000 fold higher in maternal blood. Maternal blood contamination may occur during cord blood sampling or by placental bleeding during late pregnancy or delivery. Finally, transplacental transfer of IgE is another potential mechanism and may be responsible in samples with low levels of IgA. Such mechanism was indicated by our previous finding of allergen-specific IgE in cord blood in perfect allergen-specific match with maternal IgE even at *low levels* of IgA. (Paper I) Trans-placental transfer is supported by a report of transfer of human IgE from a pregnant monkey to the offspring in a ratio similar to albumin.[103] This suggests that a small amount of maternal IgE is always transferred to the fetus in relation to the maternal level of IgE, and is consistent with our finding that all children of mothers with IgE above a certain level had measurable IgE in cord blood.

The association between IgE in cord blood and maternal, but not paternal, blood is in line with some previous studies.[111] One study also found association with paternal IgE, but in that study maternal and paternal IgE levels were correlated and it was not tested if the association with

paternal IgE was confounded by maternal IgE.[86] The association with maternal IgE was striking in the present study. All cord samples had detectable level of IgE if the maternal level was high, and all had elevated levels in the samples with highest maternal IgE. Complementary to this none of the samples where maternal IgE was very low had elevated IgE. As discussed, this association may be explained by transfer of IgE from the mother, and this seems to be the case in cord samples with elevated IgA or detection of allergen-specific IgE. However, cord blood IgE was also correlated to maternal IgE in samples not showing indication of transfer of maternal IgE. This may be due to limited sensitivity of our method not allowing detection of materno-fetal transfer of IgE in all cases. However, it is also possible that in addition to materno-fetal transfer of IgE, maternal but not paternal atopy may affect fetal IgE production through intrauterine mechanisms or maternal inheritance as has been shown for infant eczema and IgE levels.[57]

The standard method to detect maternal blood "contamination" of cord blood has been demonstration of elevated levels of cord blood IgA. We found elevated levels of IgA in 9% of all cord blood samples and in 24% of samples with elevated levels of IgE. Usually the reported fraction of samples with elevated IgA has been much lower, as low as 1% in samples with elevated IgE.[71] One explanation for this discrepancy may be that the level of IgA previously considered indicative of contamination has been too high. The usual cut off level has been above 10 mg/L[87] compared to our cut off level of 0.05 mg/L. Many studies apply IgA cut off levels from previous older studies and this may not be appropriate due to differences in analytical methods of IgA measurement and improvement of anti-IgA antibody specificity during recent years. The strength of our study is that we were able to determine an internally relevant cut off level by showing association with IgE levels, which has usually not been done. Our results therefore suggest that the IgA method used in previous studies have not been appropriate. Furthermore we find indication of transfer of maternal IgE even at low levels of IgA, suggesting that IgA-measurement on its own does not appropriately exclude samples with falsely elevated IgE. Almost half of the samples (47%) with indication of materno-fetal transfer of IgE were detected by presence of allergen-specific IgE only. Previous studies by Lilja et al also suggested that the IgA-method do not control sufficiently for maternal blood contamination.[99] Their conclusion was based upon a similar observation of specific IgE against inhalant allergens in cord blood samples with low CB-IgA levels and was further supported by reduced levels of Total IgE at 4-5 days of age in samples suspected to be contaminated.

Several studies have documented that the fetus is capable of producing IgE[84;85] and this is supported by the present study. We only detected materno-fetal transfer of IgE in half of the samples and in

cord blood samples *without* indication of materno-fetal transfer, cord blood IgE was highly significantly associated with IgE at 6 months of age independent of maternal IgE. This supports that the fetus is capable of producing non-specific IgE and that the tendency to produce high levels at birth tracks into infancy.

5.4. Strengths and limitations

It is a strength of this study that we were able to establish an internally relevant cut off level for IgA as measure of maternal blood contamination.

As discussed in chapter 4, this study is limited by cord blood samples being taken by midwifes rather than research staff, although there is no reason to believe that the frequency of contamination was higher in our study than in earlier studies of cord blood IgE.

Maternal blood was sampled after birth. Mother-cord blood associations would most likely have been stronger, if maternal blood had been sampled during pregnancy rather than after birth.

Our method for detection of materno-fetal transfer of IgE based on allergen-specific IgE in cord blood is likely to underestimate the frequency of transfer, since it will only reveal materno-fetal transfer in cases of high maternal levels of specific IgE. Mothers with high total IgE but relatively low specific IgE levels could transfer IgE to the cord blood that would not be detected by this method.

This study addressed the origin of IgE in cord blood, which has implications for studies using cord blood IgE as outcome, since these are based on the assumption of cord blood IgE being a fetal product. However, we did *not* address the capacity of elevated IgE in cord blood as a *predictor* of atopic disease. Even if some cord blood samples have falsely elevated levels due to materno-fetal transfer of IgE and therefore are not independent risk factors for atopic disease, they may still have a predictive value as surrogate markers of maternal atopic status. The relevance of cord blood IgE as a predictor is not determined by the origin of IgE but by the predictive performance and the availability and costs of alternative predictors.

Finally, this study is limited by only including children of asthmatic mothers. Mothers with asthma and their newborn have higher IgE levels and the number of cord blood samples with elevated IgE would have been smaller in an unselected population. However, we focus on the relative frequency of transfer among samples with elevated IgE and this relative measure may be less affected by the high risk nature of the cohort.

5.5. Conclusions and perspectives

Elevated levels of IgE in cord blood seemed to be the result of materno-fetal transfer of IgE in approximately half of the cases. Our study of allergen-specific IgE suggested materno-fetal transfer of IgE by a non-IgA dependent mechanism and the present study indicated that this mechanism also results in significantly affects levels of total IgE. Such transfer is not accounted for by the standard method of IgA-meaurement. This suggests that previous studies using cord blood IgE as outcome probably included a number of samples with falsely elevated levels and the results from such studies should be interpreted with caution. This may also explain the low predictive value of elevated cord blood IgE found in recent studies. More strict exclusion of samples with indication of materno-fetal is warranted to provide a valid measure of fetal atopy and may increase the predictive value for atopic disease. However, the high frequency of samples that must be excluded seems to limit the value of cord blood IgE both in research and clinical practice.

Future research

The same limitations with respect to cord blood sampling and the high risk nature of the cohort described in chapter 4 apply to this study. Therefore, also this study should be replicated in a cohort of unselected mothers. Maternal blood sampling should be done during pregnancy and cord blood samples optimally taken by research staff.

Future studies in COPSAC will show if exclusion of samples with indication of materno-fetal transfer will increase the predictive capacity, not only for IgE production but also for disease and how it performs compared to other predictors.

Finally, the apparently frequent problem with materno-fetal transfer of IgE suggests the need for new, better markers of early atopy.

6. From genetically (filaggrin) determined skin barrier dysfunction to sensitization and atopic disease in early life

Paper III: ***Filaggrin gene variants and asthma exacerbations in early childhood***

Filaggrin (FLG) gene variants are well known strong risk factors for eczema. The association with sensitization and asthma is less well described and poorly understood. There is a need of studies describing development of FLG associated disease in early life and the temporal relatedness of the different atopic traits. This is important for understanding the pathway leading from skin barrier dysfunction to sensitization and asthma and may help identifying the environmental risk factors interacting with this genetic susceptibility and the age at which prevention or intervention should be initiated.

We therefore analyzed FLG variants against the longitudinal clinical diagnoses of recurrent wheeze, acute severe exacerbations and asthma together with assessments of sensitization assessed prospectively from birth through the first five years of life.

6.1. Methods

Design

The COPSAC clinical research unit provided regular as well as acute clinical assessments for all participating children, who attended this clinic instead of family practitioners or other health care providers for diagnosis and treatment of any respiratory or skin-related symptoms. Regular visits were scheduled at the COPSAC clinical research unit at six–monthly intervals and additional visits were arranged immediately upon the onset of symptoms. At every visit the child was seen by both doctor and nurses of the research clinic and a full physical examination and history was obtained using structured questions and closed response categories focusing on the child's airway and skin symptoms, as well as recent history of medication, healthcare utilization, lifestyle and home environment.

Clinical End-Points

Recurrent wheeze: Respiratory symptoms were recorded daily by the parents in diaries from birth. Wheeze was translated to the parents as wheeze or whistling sounds, breathlessness or recurrent troublesome cough severely affecting the wellbeing of the infant, and was recorded as composite dichotomized scores (yes/no) as previously described in details.[19;94] The doctor at the clinical research unit reviewed symptom definition and the diary entries with the parents at the six-monthly clinical sessions as well as at acute severe asthma exacerbations. Recurrent wheeze was pre-defined as five episodes within 6 months, each episode lasting at least three consecutive days, or daily symptoms for four consecutive weeks documented from the diaries.

Asthma was diagnosed according to the international guidelines as previously detailed[19] based on recurrent wheeze as defined above; symptom character judged by the clinical research unit doctor to be typical of asthma (e.g. exercise induced symptoms, prolonged nocturnal cough, recurrent cough outside common cold, symptoms causing wakening at night); in need of intermittent rescue use of inhaled β_2-agonist; and responding to a 3-month course of inhaled corticosteroids and relapsing when stopping treatment. Asthma was also diagnosed without a previous history of wheeze in case of acute severe asthma exacerbation as defined below.

Acute severe asthma exacerbations were defined from need of oral prednisolone or high-dose inhaled corticosteroid for wheezy symptoms prescribed at the discretion of the doctor at the clinical research unit or acute hospitalization for treatment for such symptoms at local hospital.

Debut of an asthma related phenotype was defined as onset of any of the above mentioned diagnoses and this measure was used in the survival analysis.

Eczema: Skin lesions were described at both scheduled and acute visits according to pre-defined morphology and localization; eczema was defined based on the Hanifin-Rajka criteria as previously detailed.[112;113]

Specific IgE at age 1½ and 4 years was determined by ImmunoCAP [95] (Phadia AB, Uppsala, Sweden) against the most common food (egg, milk, peanut, cod, wheat, and soya bean) and inhalant allergens (cat, dog, horse, birch, timothy grass, *Dermatophagoides pteronyssinus*, mugwort, molds). Values >= 0.35 kU/L were considered indicative of sensitization and was analyzed as the dichotomized index of any sensitization.

FLG Genotyping

Genotyping for FLG variants R501X and 2282del4 was performed as previously described.[28]

Statistical analyses

The association between FLG variants and end-points were analyzed by a two level dominant genetic model combining the two SNPs R501X and/or 2282del4.

The possible time-dependent association between FLG variants and age at onset of asthma related events was explored graphically by Kaplan-Meier plot and judged non-parametrically by a log-rank test. Quantification was done in terms of hazard ratios in a Cox regression model. P-values correspond to Wald tests and asymptotic 95% Wald confidence intervals were calculated for the log(hazard ratio) and back-transformed.

Incidences of the event of one or more acute severe asthma exacerbations per child were calculated in the age-spans (0-1, 1-2, 2-3, 3-4, 4-5) for each FLG level. Age adjusted incidence ratios for acute severe asthma exacerbations were analyzed by a log-linear model taking into account within child correlation by an independence working GEE approach. P-values correspond to robust Wald tests and asymptotic 95% robust Wald confidence intervals were calculated for the log(incidence ratios) and back-transformed.

The associations between FLG variants and the events current asthma at age 5, sensitization at age 4, and sensitization at age 1.5 were quantified in terms of odds ratios by logistic regression. P-values correspond to likelihood ratio tests and asymptotic 95% Wald confidence intervals were calculated for the log(odds ratios) and back-transformed.

For each outcome effect modification through eczema was examined by including an interaction in the model between FLG status and eczema status prior to registration of that outcome.

The proportion of the cross-sectional diagnosis (asthma by age 5 and sensitization by age 4) attributed to FLG mutations was calculated as

100*(population prevalence-prevalence in wildtype population)/population prevalence

All analyses were made in SAS version 9.1.

6.2. Main results

The clinical follow-up rate of the COPSAC cohort was 95% by age 1; 90% by age 2; 85% by age 3; 79% by age 4; and 76% by age 5.

Of 411 infants from the COPSAC cohort, 382 Caucasians were genotyped for the loss-of-function variants R501x, 2282del4.[28] The mutated alleles R501x and 2282del4 were present in 18 and 25 children.

Asthma related phenotypes

Age at onset of recurrent wheeze, asthma and acute severe asthma exacerbations all exhibited similar profiles (data on file) and diagnoses were closely correlated. We therefore analyzed the composite end-point asthma related phenotype in the survival analysis. 95 of 382 children developed an asthma related phenotype (paper III, figure 1). The overall hazard ratio due to FLG variants was 1.82 [1.06-3.12],p=0.03. The Kaplan Meier curve shows that differentiation in development of an asthma related phenotype was clearly present in the first 18 months of life 0-1.5 year hazard ratio 2.44 [1.17-4.71],p=0.02, where after it could no longer be recognized statistically; 1.5-5 year hazard ratio 1.33 [0.57-3.13],p=0.51.

Asthma exacerbations

Yearly incidences of acute severe asthma exacerbation are shown in Figure 9. Incidences were clearly elevated from infancy due to FLG variants and this elevation persisted throughout all five years. The overall age adjusted incidence ratio due to FLG variants was estimated to 2.40 [1.19-4.81],p-value=0.01.

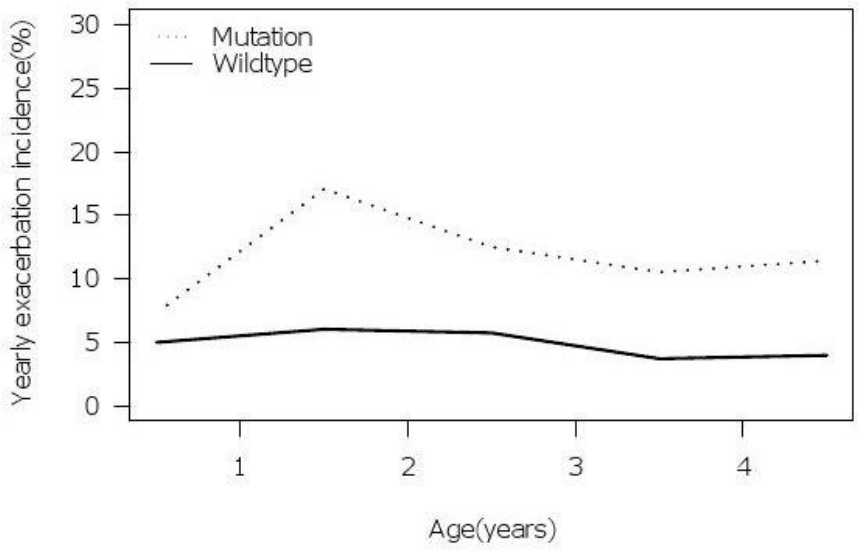


Fig 9. Acute severe asthma exacerbations: Yearly incidence of one or more acute severe asthma exacerbations requiring high-dose steroid intervention or hospitalization stratified on age and FLG variants. The overall age adjusted incidence ratio due to FLG variants was estimated to 2.40 [1.19-4.81],p-value=0.01.

Asthma

Yearly point-prevalence of asthma is shown in Figure 10. Incidences were elevated due to FLG variants and this elevation increased throughout all five years. The odds ratio of asthma by age 5 for mutated versus non mutated was 2.62 (1.12;6.11), p-value = 0.03. The proportion of current asthma attributed to FLG was 10.8%.

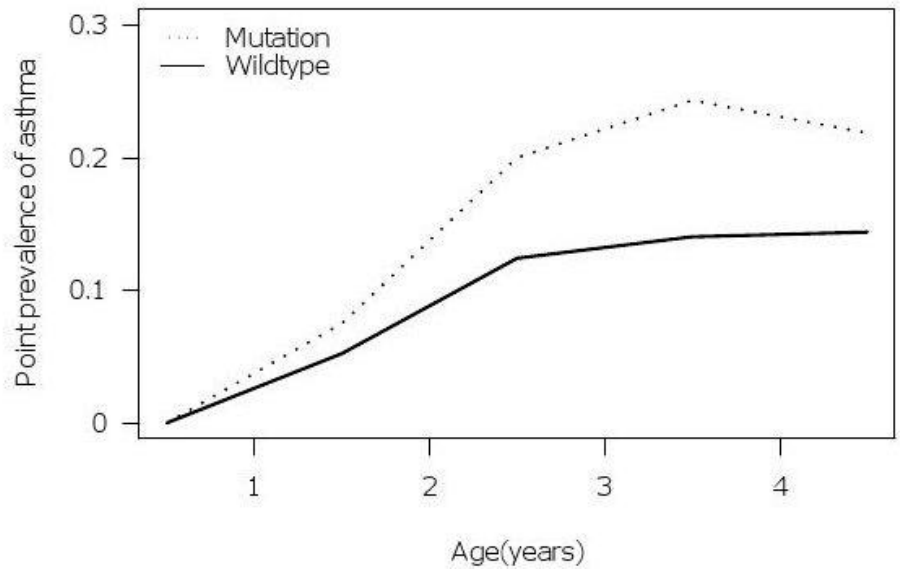


Fig 10. Asthma; point prevalence. The odds ratio of asthma by age 5 for mutated versus non mutated was 2.62 (1.12;6.11), p-value = 0.03.

Specific and total IgE

Point-prevalence of specific IgE is shown in Figure 11 for age ½, 1½ and 4. The effect from FLG mutations occurs later than the asthma and eczema phenotypes with no effect for the first 2 years, but by age 4 the odds ratio of sensitization for mutated versus non-mutated was 3.53 [1.72-7.25], p-value=0.0007. The proportion of current specific allergy attributed to FLG was 12.3%

There was a tendency towards increased levels of total IgE at age ½, 1½ and 4 years but this was not statistically significant. (Data not shown).

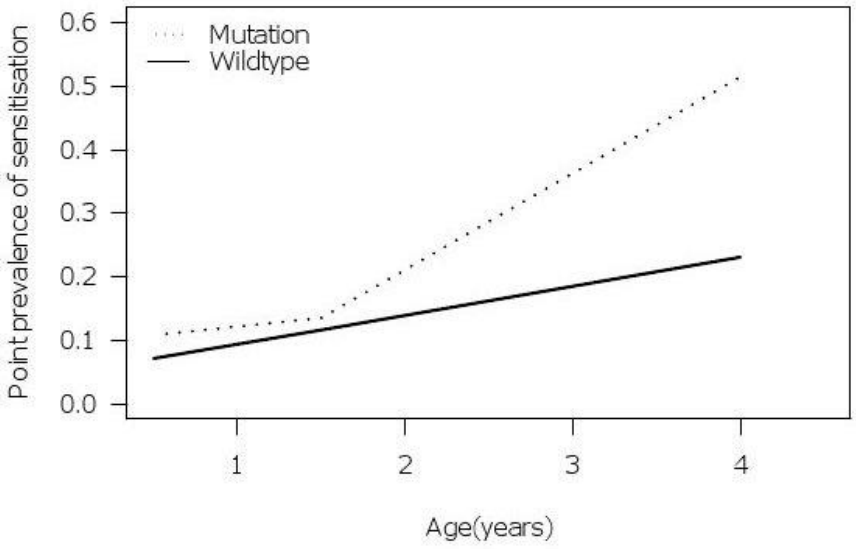


Fig 11. Sensitization; point prevalence. By age 4 the odds ratio of sensitization for mutated versus non-mutated was 3.53 [1.72-7.25], p-value=0.0007.

Eczema

FLG variants were strongly associated with the development of eczema manifesting fully in the first year of life as previously reported.[28;114] There was no evidence of effect modification through atopic dermatitis in any of the end-points. Direct effects adjusted for eczema status were largely comparable in size to the total effects.

6.3. Discussion

Principal findings

FLG variants conferred clinically significant risk of investigator-diagnosed early asthma phenotypes (recurrent wheeze, acute severe asthma exacerbations and asthma) and sensitization.

The temporal pattern of FLG associated atopic diseases was characterized by early onset of asthma symptoms and eczema, a persistent risk of acute severe asthma exacerbations, and later development of sensitization.

The association of FLG variants with asthma symptoms suggests skin barrier dysfunction as a novel, and potentially modifiable mechanism driving asthma symptoms in early childhood.

Other studies

FLG loss-of-function variants were associated with increased risk of acute severe asthma exacerbation in the first years of life. This has not previously been reported but is in line with a previous report of FLG-associated increased risk of exacerbations in older children and adults.[115] FLG variants increased the risk of recurrent wheeze in infancy and investigator diagnosed current asthma by age 5, contributing 10.8% of the cases. One study has shown association with history of preschool wheeze[116] and others showed association with asthma [36;91;116-118] while one study reported no association with asthma.[119] Recent meta-analyses conclude that FLG is a risk factor for asthma although not as strong as for eczema.[29;120]

FLG loss-of-function variants were a risk for sensitization contributing 12.3% of the cases in line with previous reports.[29;36;91;116] Interestingly the risk of sensitization increased after onset of asthma symptoms and eczema. This temporal relationship has not previously been reported.

We did not find evidence of eczema mediating the FLG-associated risk of asthma or sensitization. Due to the longitudinal diagnosis of eczema we could study the effect of eczema diagnosis prior to other atopic traits which has not been done previuosly. However, our study was not powered for such analyses, which can explain why we find no statistical significant interaction. Previous studies have only found increased risk of asthma in association with eczema [29;36;91;116;120;121]. The FLG associated risk of allergic rhinitis may be increased independently of eczema although less than in cases with eczema. [29;36]

Meaning of the study:

We previously demonstrated that FLG associated risk of eczema is specific for development of symptoms during the first year of life [114] and her extend the temporal description to asthma symptoms before 2 years of age and later sensitization. This temporal pattern suggests that gene-environmental research targeting FLG-deficient individuals should focus on infancy. In agreement with this we recently demonstrated interaction between FLG variants and cat at birth on development of eczema.[114] Relevant outcomes other than eczema will be early asthma symptoms and later sensitization. The strong effect of FLG mutation resulting in a large proportion of children with risk variants getting disease makes it a useful predictor for future mechanistic as well as interventional studies.

The association between FLG gene variants and sensitization, allergic rhinitis and asthma is intriguing, both due to the consistent replication and considerable effect size but also due to the

specific function and location in the skin pinpointing skin barrier dysfunction as an important pathogenetic step for at least a proportion of atopic disease.

The mechanism leading from skin barrier dysfunction to allergic sensitization may be increased skin permeability to allergens. Recent animal studies support this mechanism by showing increased uptake of intact allergen through the skin in FLG-deficient mice and resulting increased IgE sensitization and skin inflammation.[122] It is not clear to which extent FLG-mediated sensitization requires skin inflammation since association with allergic rhinitis has also been shown independent of eczema.[36]

The pathway from FLG defect to airway disease is not yet understood. FLG is expressed in the skin[28] and in the outer layers of the oral and nasal mucosa [92] but not in the respiratory epithelium of the nose [36;92] or the lower airways.[37;92] This suggests that FLG-associated asthma is mediated through a systemic, possibly immunological, mechanism stimulated through the impaired skin barrier. As discussed this could be due to increased allergen exposure and sensitization. However the late effect on sensitization compared to the early increase in risk of asthma symptoms in the present study as well as other studies showing increased risk asthma risk independent of sensitization [36;116] suggest that sensitization does not mediate the pathway from FLG deficiency to airway disease. Non-specific immunological mechanisms associated with eczema may be involved since previous studies only demonstrate increased risk of asthma in association with eczema.[29;36;91;116;120;121] The vast majority of acute wheezy episodes in children are triggered by viral infections,[123] and the link between FLG deficiency and early airway symptoms could therefore be an impaired response to respiratory viral infections. This could be mediated by non-specific immunological alterations caused by skin barrier dysfunction or eczema.

The observation of FLG associated risk of early severe asthma exacerbation is novel and important for this difficult to control disease entity. Asthma exacerbations in young children represent a severe disease burden with major impact on quality of life for patients and socioeconomic costs for the health care system.[10] The association with early asthma symptoms suggests skin barrier dysfunction as a novel endotype of preshool asthma. Asthma heterogeneity is pronounced in this age group as evidenced by observations of different temporal patterns and association with intermediate phenotypes.[34;124] Asthma symptoms in preschool age are often transient and unspecific. Often the term wheeze is used instead of asthma, reflecting the uncertainty about the diagnosis and persistence of symptoms into school-age and adulthood. Up to 6 different temporal phenotypes have been suggested.[124] This heterogeneity is probably a main cause for inadequate

management [35] and there is an urgent need for a better understanding of disease mechanisms to improve treatment and prevention of disease.[125] Genetic risk factors are causally related to disease and identification of these may therefore identify endotypes with different etiology and response to treatment. These FLG findings suggest that a subgroup of children with preschool asthma has disease initiated by skin barrier dysfunction which maybe can be prevented by targeting the skin defect. Another genetic variation on chromosome 17 was recently discovered as a risk factor for early asthma and we demonstrated that this gene variant is associated with a totally different phenotype characterized also by early asthma exacerbations but not with eczema or sensitization.[126] It is likely that these genetically defined phenotypes represent distinct endotypes with different pathogentic mechanisms, environmental triggers and treatment responses, and further understanding of the underlying mechansims may be a breakthrough for management of disease.

Strengths and limitations

This is the first longitudinal study describing the effect of FLG variants on the temporal clinical expressions of asthma, eczema and sensitization assessed objectively by clinical examinations from birth. The onset and programming of atopic disease occurs in early life and only through longitudinal studies from birth can the effect of genetic variants on the temporal relationship between the different atopic disease manifestations be understood and provide insight into the causal interrelation. The novel contribution of our study is the prospective day-by-day monitoring of disease onset. This provides detailed insight into the age-dependent disease presentations. Earlier studies relied on cross-sectional assessments of disease rather than exact age of disease debut and therefore could not appropriately account for temporal relationship between diseases. Also, cross-sectional studies relying on questionnaire-diagnosed eczema carry a risk of recall bias with parents of asthmatic children being more likely to recall early skin symptoms.

Accurate phenotyping is the major strength of our study and improves the power of the genetic association analyses. The meticulous prospective clinical monitoring, diagnoses and treatment of lung and skin symptoms through the first 5 years of life was carried out solely by the investigators. The cohort was seen regularly at 6 month intervals as well as for acute lung and skin symptoms by the doctors in the COPSAC clinic, who controlled diagnosis and treatment according to predefined algorithms, i.e. diagnoses and treatments were not made by doctors outside our research unit. The diagnostic accuracy from prospective clinical monitoring is the key-difference between this clinical study and traditional epidemiologic cohorts often based on questionnaires and parents history of diagnoses made by doctors in the community. This is of particular importance in the clinical

diagnosis of wheeze where evaluation and perceptions of the terms are variable among practitioners and caregivers[127-129] and eczema where inter-observer variation is a problem.[130] Asthma was diagnosed prospectively at the clinical research unit according to a rigid algorithm based on predefined recurrence of diary recorded wheezy episodes monitored since birth, symptoms typical of asthma, need of short-acting bronchodilator treatments, response to inhaled corticosteroids and relapse after stopping treatment.[19] The accuracy of such pragmatic diagnosis was strengthened by the history being reported by mothers all experienced with asthma.

Furthermore, the power of the statistics was improved from the longitudinal data-set with the time of onset clearly distinguishing these populations. Complex human diseases have variable ages of onset. Since the age of onset is likely to be genetically mediated, the subject's age of onset carries more information about the etiology of the disease than the case-control status.

The major limitation of this study is the limited power due to low number of cases with FLG variants. However, we were able to demonstrate statistically significant and biologically plausible effects.

The external validity of the conclusions of this study is limited by the cohort selection of newborns born of asthmatic mother's, with a gestational age above 35 weeks with no congenital abnormality, systemic illness, or history of mechanical ventilation or lower airway infection. The carrier frequency of FLG variants in COPSAC was 11% which is slightly higher than the reported frequencies in the background population between 8.8 % and 9.6 %.[28;131;132]

Several new variants within the FLG gene have been reported.[133] However, these new SNPs are substantially less prevalent and qualitatively different with some residual function as demonstrated by protein immunoreactivity and a significantly lower penetrance of eczema, and they were therefore not included in this study.

6.4. Conclusions and perspectives

This study describes a FLG-associated pattern of atopic diseases in early childhood characterized by early onset of eczema, early onset of asthma with severe exacerbations and later development of sensitization.

The association with early asthma symptoms and severe exacerbations suggests a specific endotype of preschool asthma initiated by skin barrier dysfunction. Early asthma seemed not to be driven by sensitization, which was a later phenomenon.

FLG provides a useful predictor for future targeted research and prevention of these diseases. Research and intervention should focus on infancy and relevant outcomes other than eczema will be early asthma symptoms and later sensitization.

Future research

Future research should study the pathway from FLG variants to eczema, sensitization and airway disease with the potential of increasing our understanding of the pathology. The focus should be on infancy and early childhood.

Clinical intervention studies, e.g. with intensive skin care and treatment of early eczema symptoms, should be performed in infants with FLG gene variants in order to prevent severe eczema and later asthma and sensitization.

The FLG associated effect on early asthma exacerbations should be replicated in an unselected cohort.

Finally, analyses of FLG-genotypes in clinical trials of asthma medication could test the hypothesis of FLG-associated asthma in early childhood as a separate endotype with a different treatment response. This may allow individualized and thereby more effective treatment.

Summary and conclusions

The allergy-associated (atopic) diseases; asthma, eczema and rhinoconjunctivitis, are the most common chronic diseases in childhood. A large number of environmental and genetic risk factors have been suggested, but still our understanding of the underlying disease mechanisms and etiologies is limited. One limiting step for research is the large heterogeneity of atopic diseases, especially in early childhood. The diseases are likely to represent several underlying subtypes, and identifying these is essential for improved treatment and prevention.

A hallmark of atopic disease is sensitization with production of specific IgE-antibodies against allergens. Sensitization may cause allergic symptoms, and sensitization early in life is a strong risk factor for later disease.

Fetal and early postnatal life seems to be a critical period for development of atopic disease and may be an important "window of opportunity" for prevention. The aim of this thesis was to increase the understanding of sensitization in early life. We studied indicators of sensitization in the newborn, and early development of sensitization and disease associated with a newly discovered genetic risk factor. Such insight may increase our understanding of disease pathogenesis in general, direct future research and help developing relevant and correctly timed preventive measures.

It has been suggested that sensitization may occur already in utero and this has led to guidelines recommending allergen avoidance during pregnancy. We studied intrauterine sensitization measured by allergen-specific IgE in cord blood. Allergen-specific IgE, primarily against inhalant allergens, was detected in 14 % of cord blood samples. However, corresponding specific IgE was not detectable in infant blood at 6 months of age. Furthermore, specific IgE in cord blood completely matched specific IgE in maternal blood both with respect to allergen specificity, level of specific IgE and ratio of total IgE/specific IgE. This suggests that allergen-specific IgE in cord blood does not reflect intrauterine sensitization but seems to be the result of transfer of maternal IgE to the fetus. Our results do not support the concept of intrauterine sensitization and thereby the rationale behind allergen avoidance during pregnancy. Furthermore, such recommendations are not supported by randomized clinical trials and should be withdrawn.

Elevated levels of non-specific (total) IgE is thought to be the result of fetal production and has been used as a marker of atopy through decades. It has been used both as an outcome in studies of prenatal risk factors and as a predictor of disease to decide on preventive measures in the infant. It is well known that falsely elevated IgE levels may occur due to "contamination" of cord blood with

maternal blood, but this is usually controlled for by measuring cord blood IgA and is found to be an infrequent event. Our previous study suggesting that allergen-specific IgE is the result of materno-fetal transfer of IgE suggests that also total IgE may be significantly affected by such transfer. On the other hand, production of non-specific IgE by the fetus is well documented, and materno-fetal transfer may be less of a problem in studies of total IgE. We found indication of materno-fetal transfer in 46% of cord blood samples with elevated IgE. Furthermore, detection of such transfer seemed not to be appropriately done by the standard method of IgA-measurement. This suggests that previous studies using total IgE as outcome probably included a number of samples with falsely elevated levels and should be interpreted with caution. This may also explain the low predictive value of elevated cord blood IgE found in recent studies. Future studies should control for materno-fetal transfer of IgE or preferably use other markers of atopy.

Variation in the gene coding for the skin barrier protein filaggrin (FLG) is the strongest known genetic risk factor for eczema. FLG seems only to be expressed in the skin but interestingly, FLG variants are also associated with increased risk of sensitization and asthma. Longitudinal studies in early life describing the FLG-associated development of sensitization and atopic diseases may help understanding disease mechanisms and identifying the environmental risk factors interacting with this genetic susceptibility and the age at which intervention should be initiated. We found a FLG-associated pattern of atopic disease in early childhood characterized by early onset of eczema, early onset of asthma with severe exacerbations and later development of sensitization. This suggests a specific subtype of preschool asthma initiated by skin barrier dysfunction. Early asthma seemed not to be driven by sensitization, which was a later phenomenon. Future research of FLG gene variants should focus on infancy and relevant outcomes other than eczema will be early asthma symptoms and later sensitization.

In conclusion, studies of sensitization and atopic disease in early life may be essential in order to improve understanding of disease pathogenesis in general and help developing relevant and correctly timed preventive measures. For this purpose, early markers of disease are important but the validity of such measures is essential. Our studies suggest that allergen-specific IgE in cord blood is entirely a maternal product while elevated total IgE is often caused by materno-fetal transfer of IgE . FLG gene variants seem to identify a subtype of disease where skin barrier dysfunction leads to early eczema, early asthma symptoms and later sensitization. Future FLG-targeted research has the potential of improving understanding prevention and treatment of atopic diseases in childhood.

Danish summary

De allergi-associerede (atopiske) sygdomme; astma, eksem og rhinoconjunctivitis, er de hyppigste kroniske sygdomme hos børn. Et stort antal potentielle miljømæssige og genetiske risikofaktorer er identificeret, men vores forståelse for de tilgrundliggende sygdomsmekanismer og årsager er stadigvæk mangelfuld. Én forklaring på dette er den store heterogenicitet i sygdommene, der er særligt udtalt i den tidlige barndom. Sygdommene repræsenterer formentlig en række forskellige undertyper, og identifikation af disse er essentielt for forbedret behandling og forebyggelse.
Et kendetegn for atopiske sygdomme er sensibilisering med produktion af specifikke IgE antistoffer mod allergener. Sensibilisering kan forårsage allergiske symptomer, og sensibilisering tidligt i livet er en kraftig risikofaktor for senere udvikling af atopisk sygdom.
Tiden før og lige efter fødslen synes at udgøre en kritisk periode for udvikling af atopisk sygdom og er måske en afgørende periode for forebyggelse. Formålet med denne afhandling var at øge vores forståelse af sensibilisering tidligt i livet. Vi undersøgte indikatorer for sensibilisering hos den nyfødte, samt tidlig sensibilisering og sygdom forbundet med en nyligt opdaget genetisk risikofaktor. En sådan viden kan øge vores forståelse af patogenesen, dirigere fremtidig forskning og bidrage til udvikling af relevante og rettidige forebyggende tiltag.
Det er en antagelse at allergisk sensibilisering allerede kan udvikles i fostertilværelsen, og dette har ført til anbefalinger af at undgå allergener under graviditeten. Vi undersøgte intrauterin sensibilisering målt som allergen-specifikt IgE i navlesnorsblod. Allergen-specifikt IgE, primært mod inhalations-allergener, blev fundet i 14% af navlesnorsprøverne men kunne ikke genfindes i blodet i 6 måneders alderen. Herudover var der et perfekt match mellem specifikt IgE i navlesnorsblod og moderens blod både hvad angik allergen-specificitet, IgE-niveau og total/specifik IgE-ratio. Dette tyder på, at allergen-specifikt IgE i navlesnorsblod ikke er et udtryk for intrauterin sensibilisering men skyldes overførsel af IgE fra moder til foster. Vores resultater støtter ikke hypotesen om intrauterin sensibilisering og dermed rationalet bag undgåelse af allergener under graviditeten. Ydermere er sådanne anbefalinger ikke understøttet af randomiserede kliniske studier og bør trækkes tilbage.
Forhøjede niveauer af ikke-specifikt (total) IgE antages at være et resultat af føtal produktion og er blevet anvendt som markør for atopi gennem årtier. Det har været brugt som outcome i studier af prænatale risikofaktorer og som prædiktor for sygdom som grundlag for beslutning om forebyggende tiltag hos spædbørn. Det er velkendt at falsk forhøjede niveauer kan forekomme som følge af ”kontaminering” med maternelt blod, men dette kontrolleres der typisk for ved måling af

navlesnors-IgA, og det anses for en sjælden begivenhed. Vores foregående studie tydede på, at specifikt IgE i navlesnoren skyldes overførsel fra moderen, og det antyder, at også total-IgE niveauet kan være påvirket betydeligt af en sådan overførsel. På den anden side er føtal produktion af ikke-specifikt IgE veldokumenteret, og overførsel fra mor til barn kan derfor spille en mindre rolle for studier af total IgE. Vi fandt tegn på materno-føtal overførsel af IgE i 46% af navlesnorsprøver med forhøjet IgE. Endvidere syntes måling af navlesnors-IgA ikke i tilstrækkeligt omfang at tage højde for dette. Dette indikerer at tidligere studier, der har brugt total IgE som outcome, har inkluderet en række prøver med falsk forhøjede niveauer, og de skal derfor tolkes varsomt. Dette kan også forklare de lave prædiktive værdier af forhøjet navlesnors IgE, der er fundet i nyere studier. Fremtidige studier bør kontrollere for materno-føtal overførsel af IgE eller om muligt anvende andre markører for atopi.

Variation i genet der koder for hudproteinet filaggrin (FLG) er den stærkeste kendte genetiske risikofaktor for eksem. FLG udtrykkes i huden, men alligevel er FLG varianter også associeret til udvikling af sensibilisering og astma. Longitudinelle studier af sammenhængen mellem FLG varianter og udvikling af sensibilisering og atopisk sygdom kan måske bidrage til forståelsen af disse mekanismer og til at identificere de miljøfaktorer der interagerer med genet og det rette tidspunkt for intervention. Vi fandt at FLG varianter var associeret til et mønster af atopisk sygdom i den tidlige barndom karakteriseret ved tidlig eksem, tidlige astmasymptomer med svære forværringer og senere udvikling af sensibilisering. Dette indikerer en særlig undertype af småbarnsastma, der er initieret af en defekt hudbarriere. Astmasymptomerne syntes ikke at være udløst af sensibilisering, der var et senere fænomen. Fremtidig forskning af FLG varianter bør fokusere på spædbarnsalderen, og udover eksem vil tidlige astmasymptomer og senere sensibilisering være relevante outcomes.

Den sammenfattende konklusion er, at studier af sensibilisering og atopisk sygdom tidligt i livet kan være afgørende for forståelsen af patogenesen for atopisk sygdom generelt og bidrage til udvikling af relevante og rettidige forebyggende tiltag. Til det formål er tidlige markører for sygdom vigtige men validiteten af disse markører er essentiel. Vores studier indikerer, at allergen-specifikt IgE i navlesnoren er et maternelt produkt, mens forhøjet total IgE ofte skyldes materno-føtal overførsel af IgE. FLG gen-varianter synes at identificere en undertype af atopisk sygdom, hvor defekt hud barriere medfører tidligt eksem, tidlige astmasymptomer og senere sensibilisering. Fremtidig forskning rettet mod FLG-varianter kan potentielt forbedre både forståelse, forebyggelse og behandling af atopiske sygdomme hos børn.

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Appendix

1. *Paper I*
2. *Paper II*
3. *Paper III*

Sensitization does not develop *in utero*

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Background: Intrauterine sensitization has been suggested to play a role in the development of atopic disease in children, and this has led to current guidelines recommending allergen avoidance during pregnancy.
Objective: To investigate the relevance of allergen-specific IgE in cord blood to sensitization in early infancy and the origin of such IgE.
Methods: Inhalant and food allergen-specific IgE in cord blood was analyzed and compared with specific IgE in infant blood at 6 months of age and in parental blood. Cord blood IgA was measured to detect maternal blood contamination of cord blood.
Results: Allergen-specific IgE, primarily against inhalant allergens, was detected in 14% of cord blood samples. However, corresponding specific IgE was not found in infant blood at 6 months of age. Specific IgE in cord blood completely matched specific IgE in maternal blood with respect to allergen specificity, level of specific IgE, and ratio of total IgE/specific IgE. Finally, there was a correlation between specific IgE and IgA in cord blood.
Conclusion: Allergen-specific IgE in cord blood does not reflect intrauterine sensitization but seems to be the result of transfer of maternal IgE to the fetus. (J Allergy Clin Immunol 2008;121:646-51.)

Key words: *Sensitization, cord blood, infant, intrauterine, atopy*

It has been suggested that atopic sensitization may occur *in utero*.[1] This idea finds some support from reports of fetal cells being capable of producing IgE from the second trimester[2] and some association between total IgE levels in cord blood and the development of atopic disease in children.[3-5] Likewise, allergen-specific IgE[6] and allergen-specific T-cell memory[7,8] in cord blood have been reported to predict atopic disease. Intrauterine allergen exposure has been documented in cord blood and amniotic fluid, making intrauterine sensitization a theoretical possibility.[9]

The concept of intrauterine sensitization has led to current guidelines recommending peanut avoidance during pregnancy by atopic women,[10,11] although studies of inhalant[12] and food allergen[13,14] avoidance during pregnancy have shown no effect on sensitization in infancy.

The aim of this study was to investigate intrauterine sensitization measured by allergen-specific IgE in cord blood. We studied the clinical relevance of allergen-specific IgE in cord blood by comparing with allergen-specific IgE in the 6-month old infant. We then studied its origin hypothesizing materno-fetal transfer as the source of specific IgE in cord blood. Evidence in favor of this hypothesis would be present if fetal specific IgE closely matched maternal specific IgE with respect to allergen specificity, level of IgE, and ratio of total/specific IgE, and if there was a correlation between specific IgE and IgA in cord blood indicating maternal blood contamination.

METHODS

The Copenhagen Prospective Study on Asthma in Childhood (COPSAC) is a prospective birth cohort study of 411 children born of mothers with verified asthma, the recruitment of whom was previously described in detail.[15] The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and approved by the Ethics Committee for Copenhagen (KF 02-118/98) and the Danish Data Protection Agency (1998-1200-359). Before enrollment, informed consent was obtained from parents. Data validity was assured by compliance with Good Clinical Practice guidelines and quality control procedures.

Midwives received written information instructing them to collect cord blood by needle puncture of the umbilical cord vein. Blood was further collected from the infants at 6 months of age and from parents after recruitment to the study. Serum and plasma were stored at –80°C until analysis.

IgE antibody levels were determined via the ImmunoCAP assay[16] (Phadia AB, Uppsala, Sweden). Cord blood samples and infant blood at 6 months of age were analyzed for level of total IgE, specific IgE against milk and egg allergens, and cumulative level of specific IgE against a panel of common inhalant and food allergens (Phadia AB, Uppsala, Sweden).[17] Samples positive for Phadiatop Infant were further analyzed for specific IgE against relevant single allergens from this panel (*Dermatophagoides pteronyssinus*, cat dander, dog dander, birch, timothy, mugwort, and peanut). The detection limit for total and specific IgE was 0.1 IU/mL.[18] Specific IgE results in cord blood were double-tested. IgE levels in parental blood were analyzed similarly after screening with Phadiatop. Detection limits for total and specific IgE in parental blood were 2 IU/mL and 0.35 IU/mL, respectively.

Cord blood samples were analyzed for total IgA by using a sensitive ELIA assay designed to measure very low levels of IgA (detection limit, 0.1 μg/L, analyzed by Phadia AB).

Statistical analyses

We modeled the underlying association between specific IgE and IgA in cord blood by linear regression and by assuming an underlying normal distribution. From this we extracted a model for the observed values

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accounting for detection limits. Similar models were analyzed for the association between specific IgE in cord blood and maternal blood for each level of cord blood IgA and for the association between specific IgE in cord blood and paternal blood. Maximum likelihood estimates with asymptotic 95% Wald CIs were calculated, and likelihood ratio tests for hypotheses were performed. The association between ratios of total/specific IgE in cord blood and maternal blood was analyzed by simple linear regression analysis. All values were transformed on a logarithmic scale.

RESULTS

Corresponding cord blood and maternal samples were available for 243 children. A paternal blood sample was available in 220 of these. Sixty-three percent of mothers and 36% of fathers were sensitized to inhalant allergens.

Specific IgE against mixed allergens (Phadiatop Infant) was found in 34 (14%) of all cord blood samples. Twenty-two samples (2 had insufficient plasma for further analyses) had detectable levels of specific IgE against single allergens. Together these 22 samples had 36 positive single-allergen tests, of which 35 were against inhalant allergens and 1 was against peanut. Specific IgE against milk or egg was not detectable in any of the 243 cord blood samples.

IgA was detectable in 239 (98%) of cord blood samples, and the median level (range) was 10.8 (0-7267) μg/L.

Specific IgE in infant blood at 6 months of age

Twenty-one of 22 infants with specific IgE against single allergens in cord blood also had a blood sample taken at 6 months of age. None of the specific IgE against single allergens found in cord blood was reproduced in the infant's blood at 6 months of age.

Specific IgE in cord blood and mother's blood

Cord blood IgE against mixed allergens was found only in offspring of mothers with corresponding specific IgE and not in any of the 92 infants of nonsensitized mothers. Similarly, all 36 positive single-allergen tests in cord blood corresponded to a positive test in maternal blood.

Comparing patterns of specific IgE against single allergens in the 22 individual pairs of cord blood and mother's blood showed that the cord blood pattern perfectly matched the maternal pattern with respect to both allergen specificity and relative levels of specific IgE (Fig 1). The specific IgE with highest levels in maternal blood was consistently found in cord blood. Complementary to this, specific IgE with lower maternal levels was not always detectable in cord blood as would be expected because of the detection limit in cord blood.

The level of specific IgE in cord blood (IgE against mixed allergens or sum of specific IgE against single allergens) was positively correlated to the level of cord blood IgA ($P < .0001$; Figs 2 and 3, respectively). However, with IgA levels below 25 μg/L, there seemed to be no such correlation (by visual inspection of Figs 2 and 3, respectively). Forty-four percent and 35% of cord blood samples with detectable specific IgE had IgA levels below 25 μg/L and median level (10.8 μg/L), respectively.

The level of specific IgE in cord blood (IgE against mixed allergens or sum of specific IgE against single allergens) was also positively correlated to the maternal level of specific IgE. This correlation was related to cord blood IgA levels ($P < .0001$; Figs 4 and 5, respectively) but was highly significant for all levels of cord blood IgA ($P < .001$). With high levels of cord blood IgA, there was a strong correlation between cord blood and maternal specific IgE for all maternal levels. With low levels of cord blood IgA, specific IgE was only found in cord blood if mothers had high levels of such specific IgE. The maternal/cord blood specific IgE ratio was approximately 1/10, 1/100, and 1/1000 in infants with high, intermediate, and low levels of cord blood IgA, respectively.

The ratios of total/specific IgE in cord blood and maternal blood were highly significantly correlated ($P < .001$), with an approximately 1:1 relationship between the 2 (Fig 6).

There was no correlation between specific IgE in cord blood and paternal blood ($P = .19$).

DISCUSSION

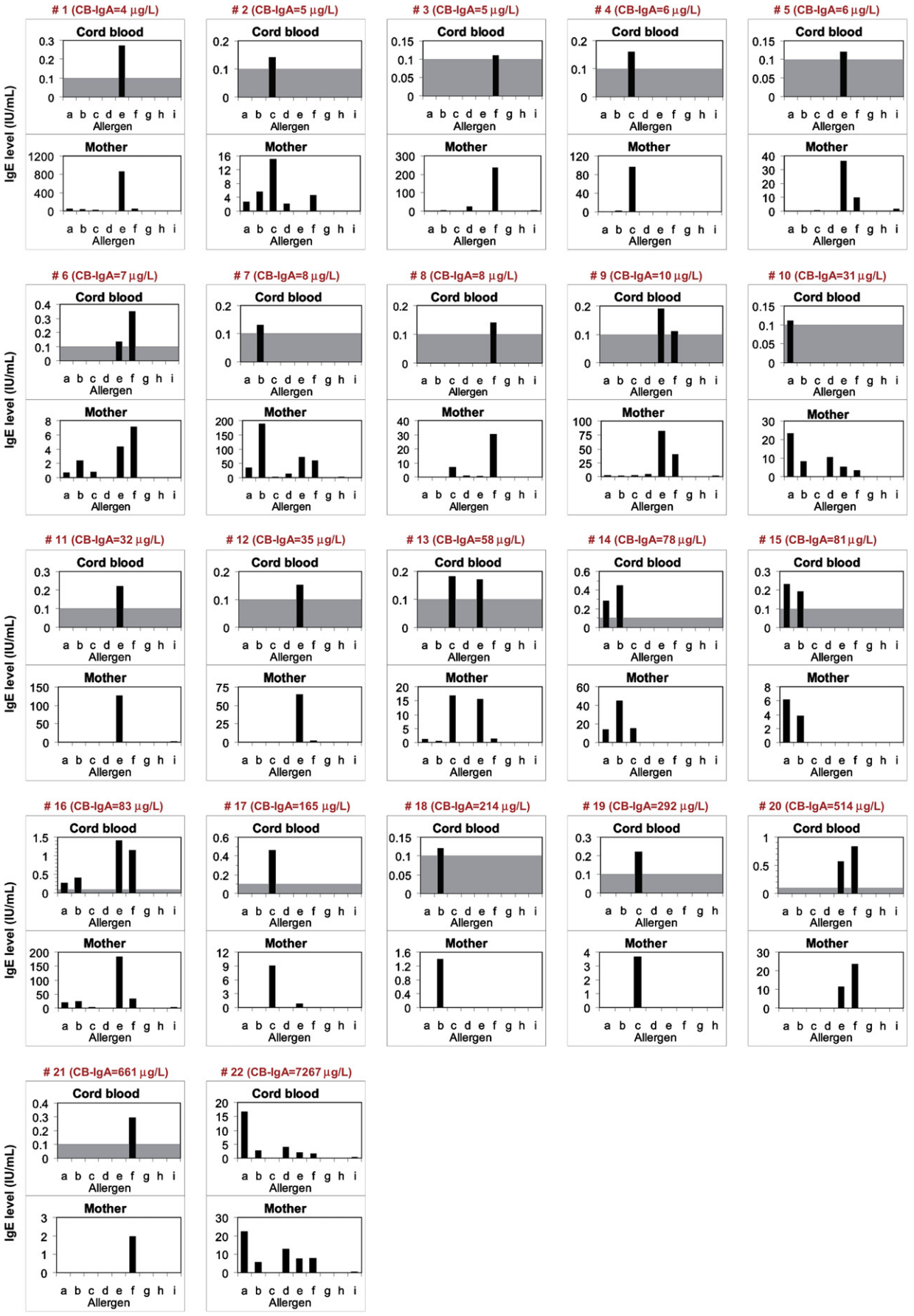
Allergen-specific IgE was found in 14% of cord blood samples. However, this allergen-specific IgE was no longer detectable in infant blood at 6 months of age, showing that it was not clinically relevant and did not indicate sensitization of the newborn. Furthermore, this suggests that specific IgE in cord blood is not a product of the fetus but rather the result of transfer of maternal IgE to cord blood or fetal blood.

We found a close match between specific IgE in mother's blood and in cord blood: (1) specific IgE was found only in newborns where the mother had the same specific IgE and never in mothers without specific IgE, (2) the pattern of specific IgE in cord blood consistently exhibited a fingerprint match of the maternal pattern, (3) the level of specific IgE in cord blood was closely correlated with the maternal level, and (4) the ratio of total/specific IgE in cord blood showed approximately 1:1 correlation with the maternal ratio. In contrast, there was no association between specific IgE in cord blood and paternal blood. Such close cord blood–mother match is in perfect agreement with materno-fetal transfer of IgE.

Specific IgE in cord blood correlated with cord blood-IgA. Because IgA does not cross the placental barrier and is not produced *in utero* in significant amounts, this indicates maternal blood contamination of cord blood samples.[19] Furthermore, cord blood IgA interacted with the relationship between cord blood and maternal specific IgE, as would be expected if maternal blood contamination was the causative mechanism. However, allergen specific IgE was also found in cord blood with low levels of IgA if the mother had very high levels of such IgE, indicating both an IgA-associated and a non–IgA-associated mechanism behind specific IgE in cord blood. Importantly, the close mother–cord blood match with respect to allergen specificity of IgE, level of specific IgE, and total/specific IgE ratio and the disappearance of specific IgE before 6 months of age was similar with high and low levels of IgA, suggesting a passive mechanism as a source of specific IgE in cord blood in both groups.

Together, this evidence strongly suggests that allergen-specific IgE in cord blood is passively acquired from the mother and is not the result of intrauterine sensitization. A single case of specific IgE in cord blood without corresponding IgE in the mother or a correlation between cord blood and father's specific IgE would have indicated at least some intrauterine sensitization, but this was not found.

Our data do not support fetal production of allergen-specific IgE and show that no matter the source of such IgE, it does not reflect clinically relevant lasting sensitization, which questions the existence of intrauterine sensitization and thereby the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6 (CB-IgA=7 µg/L)
7 (CB-IgA=8 µg/L)
8 (CB-IgA=8 µg/L)
9 (CB-IgA=10 µg/L)
10 (CB-IgA=31 µg/L)
11 (CB-IgA=32 µg/L)
12 (CB-IgA=35 µg/L)
13 (CB-IgA=58 µg/L)
14 (CB-IgA=78 µg/L)
15 (CB-IgA=81 µg/L)
16 (CB-IgA=83 µg/L)
17 (CB-IgA=165 µg/L)
18 (CB-IgA=214 µg/L)
19 (CB-IgA=292 µg/L)
20 (CB-IgA=514 µg/L)
21 (CB-IgA=661 µg/L)
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IgE level (IU/mL)
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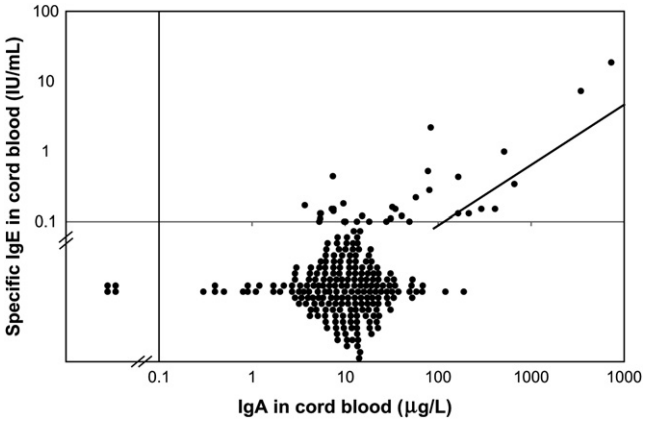

FIG 2. Relation between IgA and specific IgE (against mixed allergens) in cord blood. Regression line is shown.

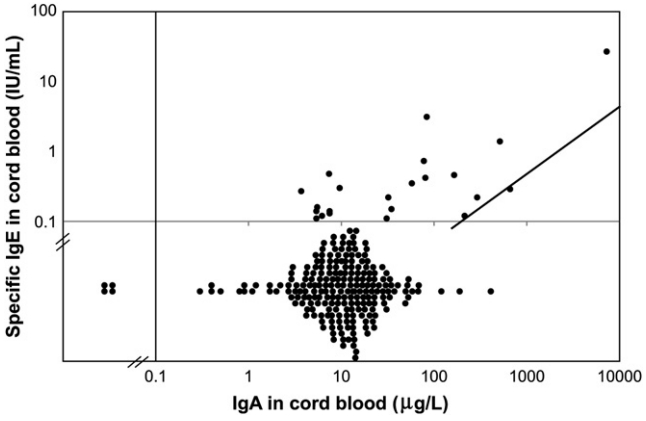

FIG 3. Relation between IgA and specific IgE (sum of specific IgE against single allergens) in cord blood. Regression line is shown.

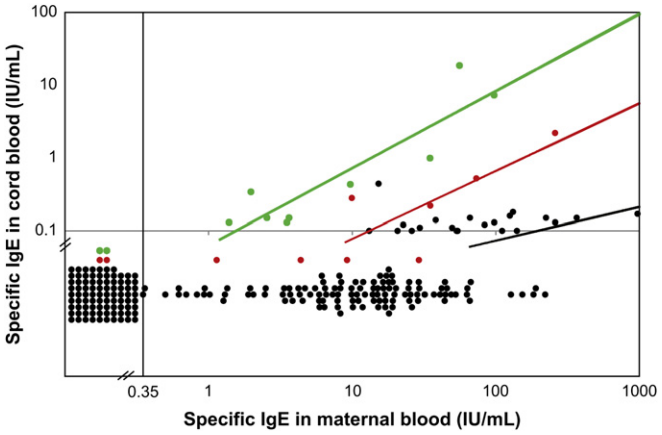

FIG 4. Relation between specific IgE (against mixed allergens) in cord blood and specific IgE in maternal blood, stratified for level of IgA in cord blood. Regression lines are shown for each level of cord blood IgA. — and ● Cord blood IgA > 100 μg/L, — and ● Cord blood IgA = 50-100 μg/L, — and ● Cord blood IgA < 50 μg/L.

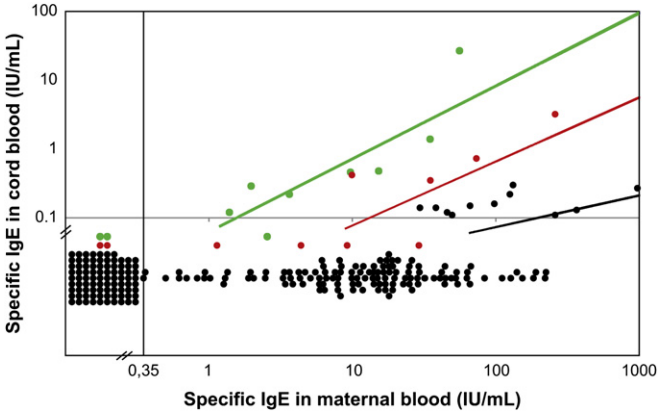

FIG 5. Relation between specific IgE in cord blood (sum of specific IgE against single allergens) and specific IgE in maternal blood, stratified for level of IgA in cord blood. Regression lines are shown for each level of cord blood IgA. — and ● Cord blood IgA > 100 μg/L, — and ● Cord blood IgA = 50-100 μg/L, — and ● Cord blood IgA < 50 μg/L.

rationale for current recommendations on allergen avoidance during pregnancy. This is consistent with the lack of protective effect from allergen avoidance, both of inhalant[12] and food[13] allergens, during pregnancy on sensitization in infants.

These results are in keeping with recent studies of cord blood mononuclear cells suggesting that putative T_H memory responses are not the result of allergen-specific priming but rather nonspecific reactions[20] and that development of T_H2-polarized allergen specific memory occurs postnatally rather than *in utero*.[21]

Because of the low levels of specific IgE against inhalants in cord blood, this has rarely been detectable with the methods of analysis used in previous studies. One previous study supports that cord blood specific IgE against inhalant allergens is caused by materno-fetal transfer of IgE because 2/3 of cord blood samples with such IgE showed markedly decreasing total IgE values from birth to 4 to 5 days of age.[22] Another earlier study contrasts with the current study in also detecting house dust mite specific IgE in cord blood in the absence of such IgE in maternal blood and finding no correlation between cord blood and maternal levels of specific IgE.[6] However, our results question the validity of those findings. The prevalence of house dust mite specific IgE in cord blood was 10 times higher in the previous study of unselected infants than in the current study of high-risk infants (20% vs 2% ≥0.1 IU/mL), suggesting that methodological differences may explain the different results.

We did not detect any specific IgE against milk or egg allergens in cord blood in the current study. A recent study reported low levels of specific IgE against cow milk proteins in 37% of cord blood samples by using a sensitive chemiluminescence method.[23] Interestingly, that study demonstrated a similar close match between maternal and cord blood specific IgE, in accordance

FIG 1. Patterns of specific IgE in individual mother–cord blood pairs. The *gray rectangle* in the cord blood diagrams represents lower limit of IgE detection (0.1 IU/mL). *CB,* Cord blood. Allergen abbreviations: *a,* cat dander; *b,* dog dander; *c, D pteronyssinus*; *d,* mugwort; *e,* birch; *f,* grass; *g,* egg white; *h,* milk; *i,* peanut. Pairs are listed in order of increasing level of cord blood IgA.

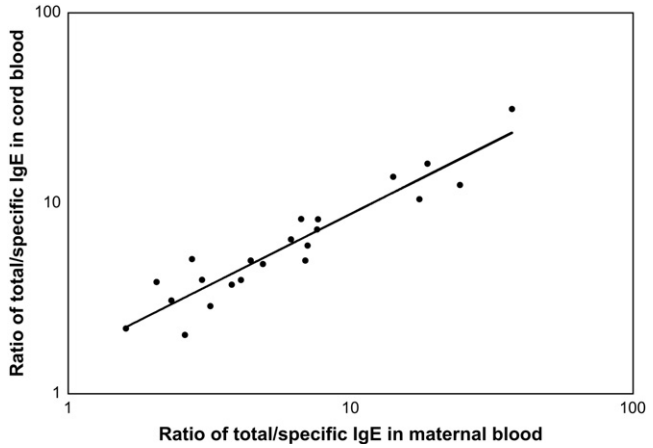

FIG 6. Relation between ratios of total/specific IgE in maternal blood and cord blood.

with materno-fetal transfer of IgE also being the responsible mechanism behind those findings.

Specific IgE against infectious agents such as parasites and HIV has also been found in cord blood.[24,25] It has been suggested that HIV-specific IgE in cord blood could be used as an indicator of infection in the infant under the assumption that such IgE is the result of intrauterine production.[25] However, the current study shows that such IgE is likely to be the result of materno-fetal transfer, and we suggest that future studies on specific IgE in cord blood should focus on studying and excluding such materno-fetal transfer of IgE.

We did not exclude samples with high IgA levels from the analysis. First, it is an important point of this study that materno-fetal transfer of specific IgE takes place at all levels of IgA, but to different degrees. Second, no samples had IgA levels higher than 10 mg/L, which has previously been the lowest cutoff level used to exclude contaminated cord blood samples.[26]

The mechanism behind materno-fetal transfer of IgE is not clear from this study. Because maternal IgE levels are often more than a 1000-fold higher than IgE levels in cord blood, even low levels of contamination with maternal IgE are sufficient to cause elevated levels in cord blood. Maternal blood contamination during cord blood sampling is one possible mechanism, although the method of needle puncture of the umbilical cord vein used in the current study probably causes a minimal amount of contamination. Small placental bleedings during late pregnancy or delivery may be a more plausible mechanism. Finally, transplacental transfer of IgE is another potential mechanism and may be responsible in samples with low levels of IgA. It is generally supposed that IgE does not cross the placental barrier,[27] but human IgE has been shown to pass through the placenta of monkeys in a ratio similar to albumin.[28]

There is no reason to believe that the frequency of contamination was higher in our study than in earlier studies of cord blood IgE. Midwives were instructed to sample cord blood by needle puncture of the umbilical vein, a method that has been shown to cause less contamination than collecting blood by letting it drip from the cut umbilical cord.[29] Furthermore, the frequency of cord blood IgE against inhalant allergens is similar to a previous report of cord blood IgE against inhalant allergens in infants of atopic mothers.[22]

This study included children of mothers with asthma. Because cord blood specific IgE is dependent on maternal specific IgE, the frequency of cord blood specific IgE would be lower in an unselected population. However, it seems unlikely that the origin of such IgE should be different in an unselected population.

Conclusion

Allergen-specific IgE in cord blood does not reflect sensitization *in utero* but seems to be the result of materno-fetal transfer of IgE. This challenges the concept of intrauterine sensitization and thereby the rationale behind allergen avoidance during pregnancy. Our results together with previous clinical studies give no support to recommendations about allergen avoidance during pregnancy, which should be withdrawn.

We thank the parents and children who took part in the study, the Copenhagen Study on Asthma in Childhood research team, and Bjarne Kristensen and Inger Pedersen for their dedicated work with the IgE analyses.

Clinical implications: These findings refute the concept of intrauterine sensitization and thereby the rationale behind allergen avoidance during pregnancy.

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Elevated cord blood IgE is biased from materno-fetal transfer

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Word count: 3,310

Abstract

Background: IgE in cord blood is thought to be a product of the fetus. A high level of total IgE is therefore used as a measure of atopy in the newborn. We recently found strong evidence that *allergen-specific* IgE in a cord blood is the result of transfer of IgE from the mother to the feto-placental unit rather than fetal production. This suggests that also *non-specific* (total) IgE in cord blood may primarily be a maternal product.

Objective: To determine to which extent elevated levels of total IgE in cord blood is the result of materno-fetal transfer of IgE.

Methods: Total IgE in cord blood was analyzed in a prospective birth cohort study. Materno-fetal transfer of IgE was detected by high sensitivity analyses of cord blood IgA and allergen-specific IgE and comparison of IgE in cord blood with parental levels and levels at 6 months of age.

Results: Indication of materno-fetal transfer of IgE was found in 46% of cord blood samples with elevated levels in forms of elevated IgA and detection of allergen-specific IgE. Maternal origin of IgE in these samples was validated by showing reduced levels of IgE at 6 months of age (geometric mean 9.4 vs. 5.4 IU/mL, $P = .01$). Materno-fetal transfer of IgE was not appropriately accounted for by measurement of cord blood IgA alone.

Conclusions: Previous studies of cord blood IgE probably included a number of samples with falsely elevated levels and should be interpreted with caution. Future studies must control for materno-fetal transfer of IgE or preferably use other markers of atopy.

Word count: 258

Key Messages:

- Elevated IgE in cord blood is frequently caused by materno-fetal transfer of IgE.
- Such transfer is not appropriately accounted for by measurement of cord blood IgA alone.
- This question the value of cord blood IgE as a marker of atopy in the newborn.

Capsule summary

This study suggests that elevated IgE in cord blood is often caused by materno-fetal transfer of IgE rather than fetal production. Future studies should control for such transfer or preferably use other markers of atopy.

Key words

Immunoglobulin E

Cord blood

Infant

Intrauterine

Atopy

Abbreviations

IgE: Immunoglobulin E

IgA: Immunoglobulin A

Introduction

IgE in cord blood is thought to be a product of the fetus. A high level of total IgE is therefore used as a measure of atopy in the newborn.[1-5]

In a recent study, we found strong evidence that *allergen-specific* IgE in a cord blood is the result of transfer of IgE from the mother to the feto-placental unit rather than fetal production of such *allergen-specific* IgE.[6] This suggests that also *non-specific* (total) IgE in cord blood may primarily be a maternal product and hence, the validity of total IgE as a measure of fetal atopy may be biased and rather reflect mothers atopic status.

On the other hand, total IgE levels in cord blood are generally much higher than allergen-specific levels, and non-specific IgE can be detected in the majority of cord blood samples in contrast to allergen-specific IgE, which is rarely detected. Furthermore, the fetus has been shown capable of producing IgE.[7] It is therefore possible that the fetus produces significant amounts of non-specific IgE and that "contamination" from maternal blood is less of a problem in studies of total IgE.

The aim of this study was to determine to which extent elevated levels of total IgE in cord blood is the result of materno-fetal transfer of IgE rather than fetal production. We studied this in the Copenhagen Prospective Study of Asthma in Childhood (COPSAC) birth cohort by high sensitivity analyses of cord blood IgA and total and allergen-specific IgE and comparison with IgE levels at 6 months of age and parental levels.

Methods

The Copenhagen Study on Asthma in Childhood (COPSAC) is a prospective birth cohort study of 411 children born of mothers with verified asthma, the recruitment of which was previously described in details.[8] The study was conducted in accordance with the guiding principles of the Declaration of Helsinki, and approved by the Ethics Committee for Copenhagen (KF 02-118/98) and The Danish Data Protection Agency (2008-41-1754). Before enrolment, informed consent was obtained from parents. Data validity was assured by compliance with "Good Clinical Practice" (GCP) guidelines and quality control procedures.

Midwifes received written information instructing them to collect cord blood by needle puncture of the umbilical cord vein. Blood was further collected from the infants at 6 months of age and from parents after recruitment to the study. Serum and plasma was stored at -80^{o}C until analysis.

IgE antibody levels were determined via the ImmunoCAP assay[9] (Phadia AB, Uppsala, Sweden). Cord blood, blood at 6 months of age and parental blood was analyzed for level of total IgE and specific IgE against common food and inhalant allergens as previously described.[6] Detection limit for IgE was 0.1 IU/mL in cord blood and at age 6 months.[10] In parental blood detection limits for total and specific IgE were 2 IU/mL and 0.35 IU/mL respectively.

Cord blood samples were analyzed for total IgA using a sensitive ELIA assay designed to measure low levels of IgA (detection limit 0.1 mcg/L, analysed by Phadia AB, Uppsala, Sweden).

Statistical analyses:

We modeled the underlying association between total IgE and IgA in cord blood by linear regression and by assuming an underlying normal distribution. From this we extracted a model for the observed values accounting for detection limits. Similar models were analyzed for

the association between total IgE in cord blood and maternal blood for each level of cord blood IgA and for the association between total IgE in cord blood and paternal blood. Maximum likelihood estimates with asymptotic 95% Wald confidence intervals were calculated and likelihood ratio tests for hypotheses were performed. A smoothing curve (Lowess) was calculated based on levels above detection limit for the association between total IgE and IgA to estimate a relevant cut off level for association. The association between predicted and observed levels of total IgE in cord blood was analyzed by simple linear regression analysis. Associations of cord blood IgE with IgE at 6 months of age were analyzed similarly by using levels above detection limit only. IgE level at 6 months of age in children with elevated cord blood samples with or without indication of transfer of IgE was compared by Students T-test. All values were transformed on a logarithmic scale.

Results

Pairs of cord blood-maternal, cord blood-paternal and cord blood-6 month samples were available for 243, 220 and 219 children respectively.

Detectable levels of total IgE (>=0.1 kIU/L) were found in 184 (74%) cord blood samples and elevated levels (>0,5 kIU/L) were found in 74 (30%) samples.

Total IgE in cord blood and parental blood

There was a strong correlation between cord blood and maternal levels of total IgE ($P <$ .0001). (Fig 1.a) This correlation was related to cord blood IgA levels but was significant for all levels of cord blood IgA. IgE was always detected in cord blood if the maternal level was above 145 IU/mL (55 samples) and all cord blood samples had elevated levels if the maternal level was above 516 IU/mL (17 samples). None of the 21 cord blood samples where the maternal level was below 6.7 IU/mL had elevated IgE. In contrast there was no correlation between cord blood and paternal levels of total IgE ($P = .70$). (Fig 1.b)

IgA in cord blood as indicator of maternal transfer of total IgE

The level of total IgE in cord blood was positively correlated to the level of cord blood-IgA ($P < .0001$) (Fig 2). The correlation was not linear as illustrated by the smoothing curve of average values. A correlation was evident for IgA-levels above 50 mcg/L (by visual inspection of Fig 2), and this was supported by IgA-stratified analyses of cord blood and maternal IgE showing an upward shift of the association line for IgA levels above 50 mcg/mL and a further shift for IgA levels above 100 mcg/L (Fig 1.a). These results are is in accordance with our previous findings for allergen-specific IgE in cord blood[6] and suggests that cord blood samples with IgA-levels higher than 50 mcg/L should be suspected to have falsely elevated IgE levels due to contamination of cord blood with maternal blood. This level was used as indicator of materno-fetal transfer of IgE in the following analyses.

Allergen-specific IgE in cord blood as indicator of materno-fetal transfer of total IgE

We previously found strong evidence that allergen-specific IgE in cord blood was the result of materno-fetal transfer of IgE.[6] Assuming that such transfer is the only source of IgE in these samples, then the total level of IgE can be calculated on the basis of total/allergen-specific IgE in maternal blood and allergen-specific IgE in cord blood. We therefore calculated the predicted level and compared with the actual observed level of total IgE in cord blood. The predicted and observed levels of total IgE in cord blood were highly significantly correlated ($P < .0001$) with an approximately 1:1 relationship between the two (Fig 3.a). Accordingly, the observed level of cord blood IgE was generally close to 100% of the predicted level (Fig 3.b). This strongly suggests that also *total* IgE is mainly a product of the mother in samples with detectable allergen-specific IgE. Detection of allergen-specific IgE was therefore used, together with cord blood IgA, as indicator of maternal transfer of IgE in the following analyses.

Frequency of materno-fetal transfer of IgE

There was indication of materno-fetal transfer of IgE in 34/74 (46%) of cord blood samples with elevated total IgE (>0.5 IU/mL). Of these, 35% were suspected 'contaminated' on the basis of both elevated IgA and detectable allergen-specific IgE, 47% on the basis of allergen-specific IgE only, and 18% on the basis of elevated IgA only.

Cord blood IgE and IgE at 6 months of age

Children with elevated cord blood IgE and indication of contamination (N=32) had significantly lower IgE levels at 6 months of age compared to children with elevated IgE and no indication of contamination (N=35), (geometric mean 5.4 IU/mL vs. 9.4 IU/mL, $P = .01$). In cord blood samples *with* indication of materno-fetal transfer of IgE there was no association with IgE level at 6 months of age ($P = .37$, Fig 4.a) in contrast to samples *without* indication of transfer ($P < 0.001$, Fig 4.b). Adjustment for maternal IgE level did not materially change these results.

Discussion

Approximately half of cord blood samples with elevated IgE levels showed indication of materno-fetal transfer of IgE. This frequency of suspected 'contamination' with maternal IgE is much higher than expected from previous studies with important implications for the value of elevated cord blood IgE as a marker of atopy in the newborn.

We investigated the extent of materno-fetal transfer of IgE in three subsequent steps.

First, we demonstrated a strong association between maternal and cord blood IgE and IgE was always detected in cord blood if the maternal level was high. In contrast there was no association between paternal IgE and cord blood IgE. Transfer of IgE from the mother to the fetus could be one likely explanation for these findings.

We then used two different methods to detect materno-fetal transfer of IgE in individual samples. This first method was based on elevated levels of IgA in cord blood. Since IgA does not cross the placental barrier and is not produced in utero in significant amounts,[11] elevated levels are supposed to reflect maternal blood contamination. We confirmed this by showing association between IgA and IgE levels in cord blood above a certain IgA level. The second method was based on the detection of allergen-specific IgE in cord blood. We previously provided strong evidence that such IgE is the result of materno-fetal transfer of IgE as demonstrated by a perfect, allergen-specific, match between specific IgE in maternal blood and cord blood and disappearance of cord blood specific IgE before 6 months of age.[6] In the present study we further demonstrated that in samples with detectable allergen-specific IgE, also total IgE levels seem to be the result of transfer from the mother. We hypothesized that if both total and allergen-specific IgE was the result of transfer then total IgE levels in cord blood could be predicted by the maternal total IgE/specific IgE ratio and the level of specific IgE in cord blood, which our data subsequently confirmed. This means that in these samples, the fetus does not seem to produce significant amounts of unspecific IgE in addition to the amount

transferred from the mother, and therefore detection of allergen-specific IgE could be used as a marker of materno-fetal transfer of IgE.

Finally, we validated the hypothesis of maternal origin of IgE in samples considered "contaminated" by analysing association with the child's IgE level at 6 months of age. Children with elevated cord blood IgE had significantly lower IgE levels at 6 months of age if the cord blood sample showed indication of contamination, and cord blood IgE was not associated with IgE at 6 months of age in samples with indication of contamination.

Our results strongly suggest that elevated IgE in cord blood is the result of materno-fetal transfer in almost half of the cases. This frequency of suspected maternal origin of elevated IgE is much higher than suspected in previous studies. Some have reported frequencies of contamination as low as 1% of cord blood samples with elevated total-IgE[12]or did not test for falsely elevated IgE-levels.[1;2] This suggests that previous studies using elevated IgE in cord blood as marker of atopic status included a number of cord blood samples with falsely elevated total IgE levels due to materno-foetal transfer of IgE. A number of prenatal factors including allergen exposure during pregnancy,[2] parity,[3] maternal age,[1] and sex of the infant[1] have been associated with elevated levels of IgE in cord blood and have therefore been interpreted as risk factors for atopy in the child. Since all of these prenatal factors are also associated with maternal IgE levels,[1;2;13] the associations with cord blood IgE may be caused by materno-foetal transfer of IgE and therefore not reflect an effect on atopic development in the offspring but only an effect on maternal IgE levels. In accordance with this a path-analysis suggested that the effect of parity on cord blood IgE is mediated through maternal levels of IgE,[13] and similarly, other suggested risk factors were no longer significantly associated with cord blood IgE after adjustment for maternal IgE.[1;2] The results of such previous studies should therefore be interpreted with caution and future studies must control for potential materno-fetal transfer of IgE to assure that findings are related to fetal and not maternal IgE-production.

Frequent contamination of CB samples may also explain the low predictive values of CB total IgE for prediction of atopic disease that has been observed in most recent studies.[12;14] Future studies in the COPSAC cohort will show if a more restrictive exclusion of contaminated samples will enhance the predictive values. However, the high frequency of samples with falsely elevated IgE levels limits the usefulness of this method both in research and in clinical practice.

An alternative to cord blood IgE as marker of atopy in the newborn is IgE in capillary blood some time after birth where most IgE caused by materno-fetal transfer may be metabolized. However, the half-life of IgE in neonates is unknown and one study did not find higher predictive values using capillary blood at 4-5 days of age compared to cord blood.[15]

The mechanism behind materno-fetal transfer of IgE is not clear from this study, and it is likely that several different mechanisms are responsible. Maternal blood contamination of cord blood is one possible mechanism and may explain transfer in sample with high IgA levels. Even low level of contamination with maternal blood is sufficient to cause elevated levels in cord blood since IgE levels are often more than a 1000 fold higher in maternal blood than in cord blood. Maternal blood contamination may occur during cord blood sampling although the method of needle puncture of the umbilical cord vein used in the present study probably causes minimal amount of contamination.[16] Small placental bleedings during late pregnancy or delivery may be a more plausible explanation. Finally, transplacental transfer of IgE is another potential mechanism and may be responsible in samples with low levels of IgA. It is generally supposed that IgE does not cross the placental barrier.[17] However, it has been shown that human IgE injected into the blood of pregnant monkey can be detected in the blood of the offspring in a ratio similar to albumin.[18] This suggests that a small amount of maternal IgE is always transferred to the fetus, in relation to the maternal level of IgE, and is consistent with our finding that all children of mothers with IgE above a certain level had measurable IgE in cord blood.

The association between IgE in cord blood and maternal, but not paternal, blood is in line with some previous studies.[19] One study also found association with paternal IgE, but in that study maternal and paternal IgE levels were correlated and it was not tested if the association with paternal IgE was confounded by maternal IgE.[17] The association with maternal IgE was striking in the present study. All cord samples had detectable level of IgE if the maternal level was high, and all had elevated levels in the samples with highest maternal IgE. Complementary to this none of the samples where maternal IgE was very low had elevated IgE. As discussed, this association may be explained by transfer of IgE from the mother, and this seems to be the case in cord samples with elevated IgA or detection of allergen-specific IgE. However, cord blood IgE was also correlated to maternal IgE in samples not showing indication of transfer of maternal IgE. This may be due to limited sensitivity of our method not allowing detection of materno-fetal transfer of IgE in all cases. It is also possible that, in addition to materno-fetal transfer of IgE, maternal but not paternal atopy may affect fetal IgE production through intrauterine mechanisms or maternal inheritance as has been shown for infant eczema and IgE levels.[20]

The standard method to detect maternal blood "contamination" of cord blood has been demonstration of elevated levels of cord blood IgA. We found elevated levels of IgA in 9% of all cord blood samples and in 24% of samples with elevated levels of IgE. Usually the reported fraction of samples with elevated IgA has been much lower, as low as 1% in samples with elevated IgE.[12] One explanation for this discrepancy may be that the level of IgA previously considered indicative of contamination was too high. The usual cut off level has been above 10 mg/L[21] compared to our cut off level of 0.05 mg/L. Many studies apply IgA cut off levels from previous older studies and this may not be appropriate due to differences in analytical methods of IgA measurement and improvement of anti-IgA antibody specificity during recent years. The strength of our study is that we were able to determine an internally relevant cut off level by showing association with IgE levels, which has usually not been done. Our results

therefore suggest that the IgA method used in previous studies have not been appropriate. Furthermore we find indication of transfer of maternal IgE even at low levels of IgA, suggesting that IgA-measurement on its own does not appropriately exclude samples with falsely elevated IgE. Almost half of the samples (47%) with indication of materno-fetal transfer of IgE were detected by presence of allergen-specific IgE only. Previous studies by Lilja et al also suggested that the IgA-method do not control sufficiently for maternal blood contamination.[15] Their conclusion was based upon a similar observation of specific IgE against inhalant allergens in cord blood samples with low CB-IgA levels and was further supported by reduced levels of Total IgE at 4-5 days of age in samples suspected to be contaminated

There is no reason to believe that the frequency of contamination was higher in our study than in earlier studies of cord blood IgE. Midwifes were instructed to sample cord blood by needle puncture of the umbilical vein, a method that has been shown to cause less contamination than collecting blood by letting it drip from the cut umbilical cord.[16] Furthermore the frequency of cord blood IgE against inhalant allergens is similar to a previous report of cord blood IgE against inhalant allergens in infants of atopic mothers.[15]

Our method for detection of materno-fetal transfer of IgE based on allergen-specific IgE in cord blood is likely to underestimate the frequency of transfer, since it will only reveal materno-fetal transfer in cases of high maternal levels of specific IgE. Mothers with high total IgE but relatively low specific IgE levels could transfer IgE to the cord blood that would not be detected by this method. Furthermore, mother-cord blood associations would most likely have been stronger, if maternal blood had been sampled during pregnancy rather than after birth. Finally, this study is limited by only including children of asthmatic mothers. Mothers with asthma have higher IgE levels and the number of cord blood samples with elevated IgE would have been smaller in an unselected population. However, we focus on the relative frequency of transfer among samples with elevated IgE and this relative measure may be less affected by the high risk nature of the cohort.

Conclusions

In conclusion our study highlights several problems using cord blood IgE as indicator of atopy in the newborn. First, a considerable fraction of samples with elevated IgE seems to be the result of transfer of IgE from the mother. Second, detection of such transfer is not appropriately done by the standard method of IgA-meaurement. This means that previous studies using cord blood IgE as outcome should be interpreted with caution and this may also explain the low predictive value of elevated cord blood IgE found in recent studies. Future studies must control for such transfer of IgE or preferably use other markers of atopy.

Acknowledgments:

We thank the parents and children who took part in the study, the COPSAC research team and Bjarne Kristensen and Inger Pedersen for their dedicated work with the IgE-analyses.

KB is responsible for data acquisition, analysis and interpretation of data and writing of the manuscript. HB is responsible for and designed the COPSAC study and contributed to interpretation of data and writing of the manuscript. CP contributed to the statistical analyses and interpretation of data. All co-authors have contributed substantially to the analyses and interpretation of the data, and have provided important intellectual input and approval of the final version of the manuscript.

Legends

Fig 1. a) Relation between total IgE in cord blood and maternal blood, stratified for level of IgA in cord blood. Regression lines are shown for each level of cord blood-IgA.

— and ● Cord blood IgA > 100 mcg/L

— and ● Cord blood IgA = 50-100 mcg/L

— and ● Cord blood IgA < 50 mcg/L

b) Relation between total IgE in cord blood and paternal blood. Regression line is shown.

Fig 2. Relation between IgA and total IgE in cord blood. Smoothing curve (Lowell) is shown.

Fig 3. a) Relation between observed total IgE in cord blood and predicted (calculated) level in samples with detectable allergen-specific IgE against single allergens. Regression line is shown.

b) Observed level of total IgE in cord blood as percentage of predicted level. Values are listed in order of increasing level of cord blood-IgA.

Fig 4. a) Relation between total IgE in cord blood and at 6 months of age for cord blood samples *with* indication of materno-fetal transfer of IgE. Regression line is shown.

b) Relation between total IgE in cord blood and at 6 months of age for cord blood samples *without* indication of materno-fetal transfer of IgE. Regression line is shown.

351 Fig 1.a

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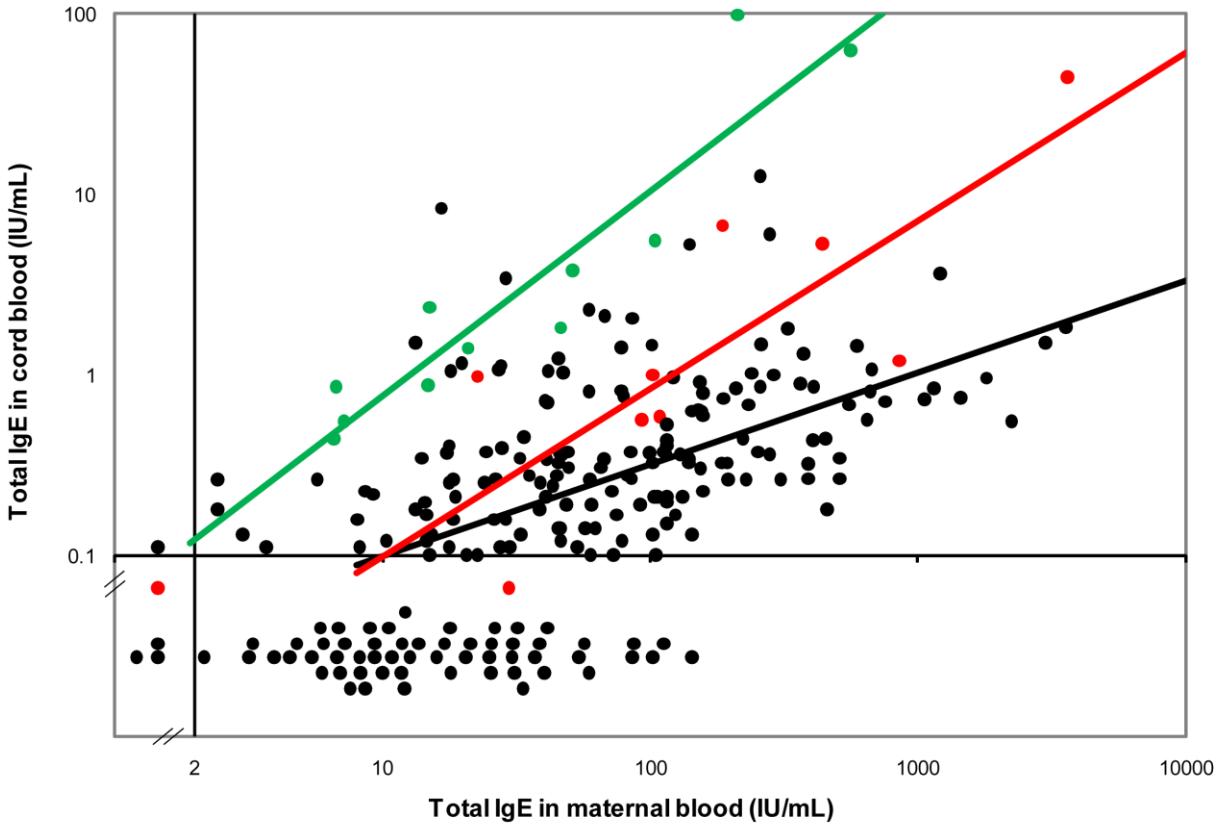

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355 Fig 1.b

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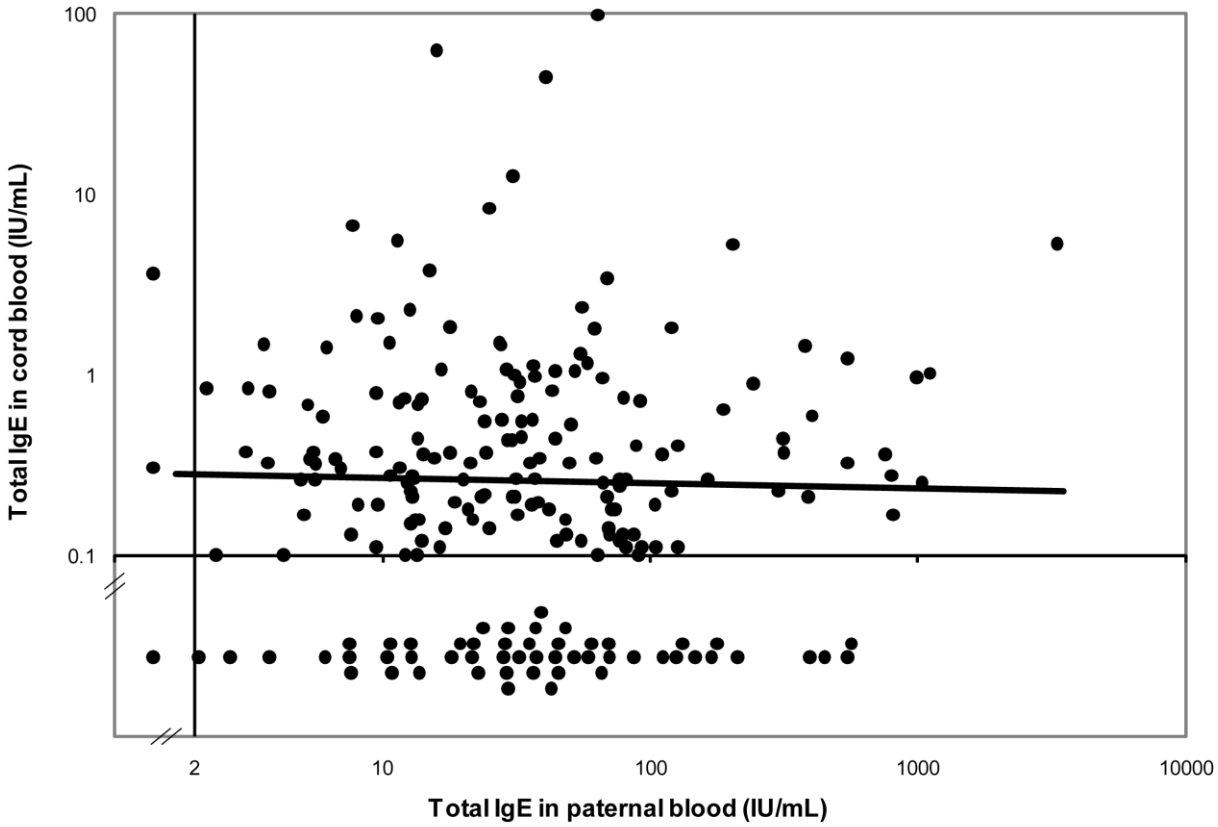

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358 Fig 2

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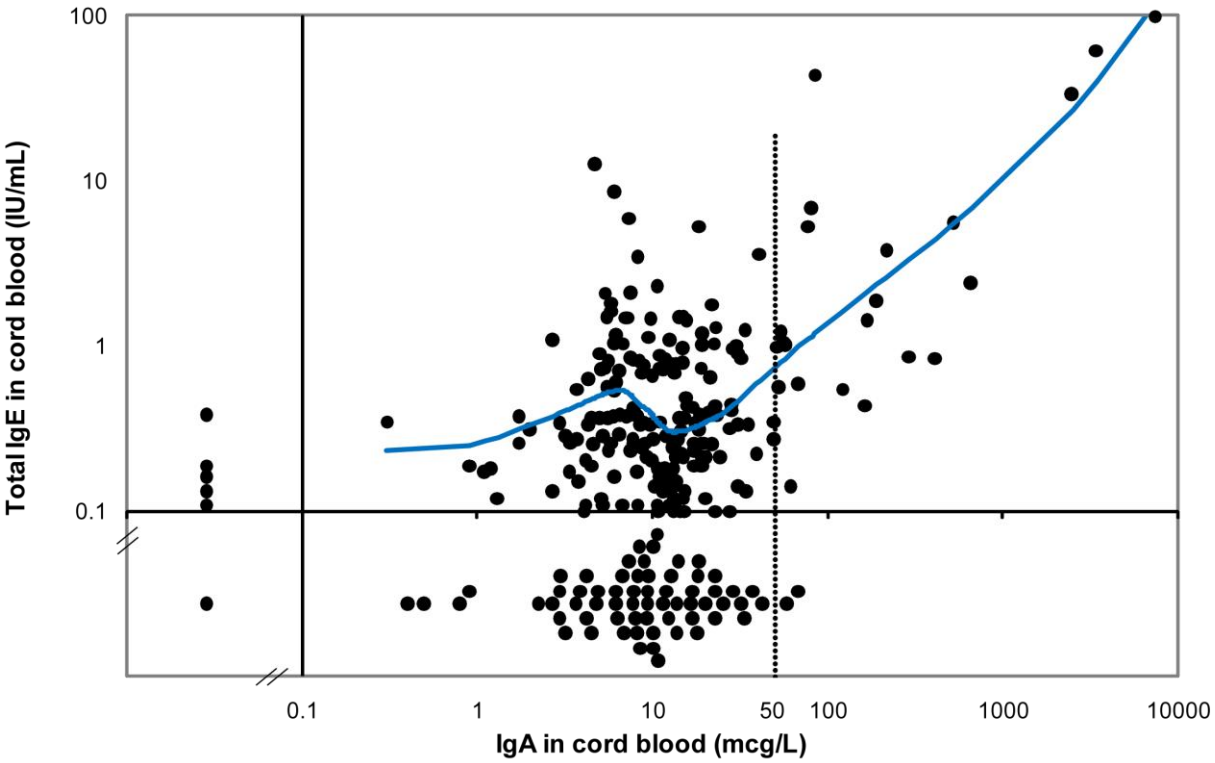

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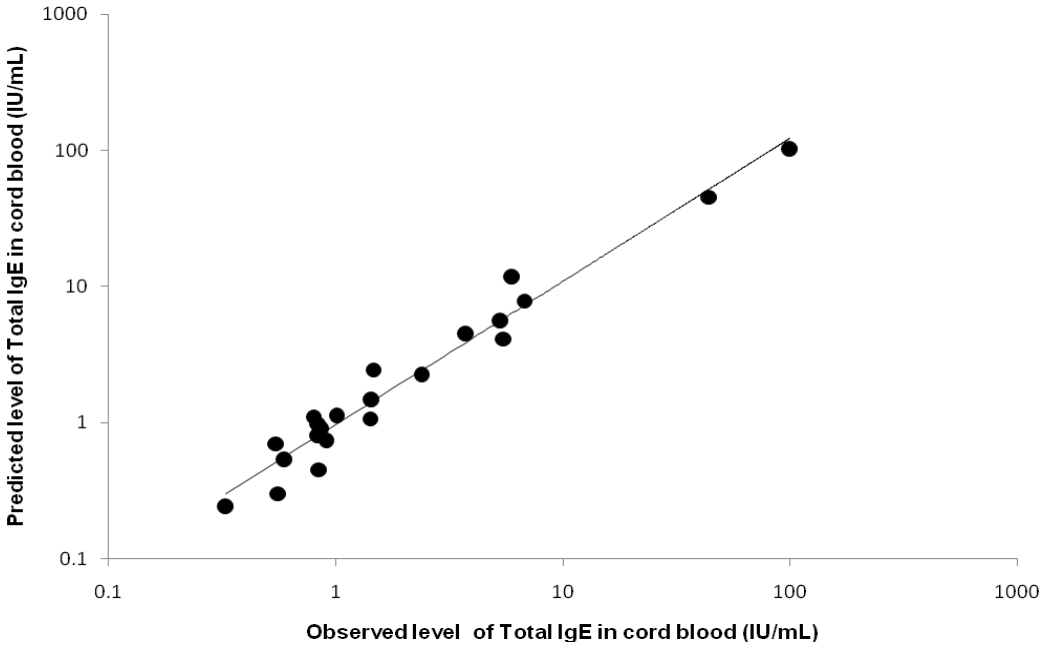
362 Fig 3.a



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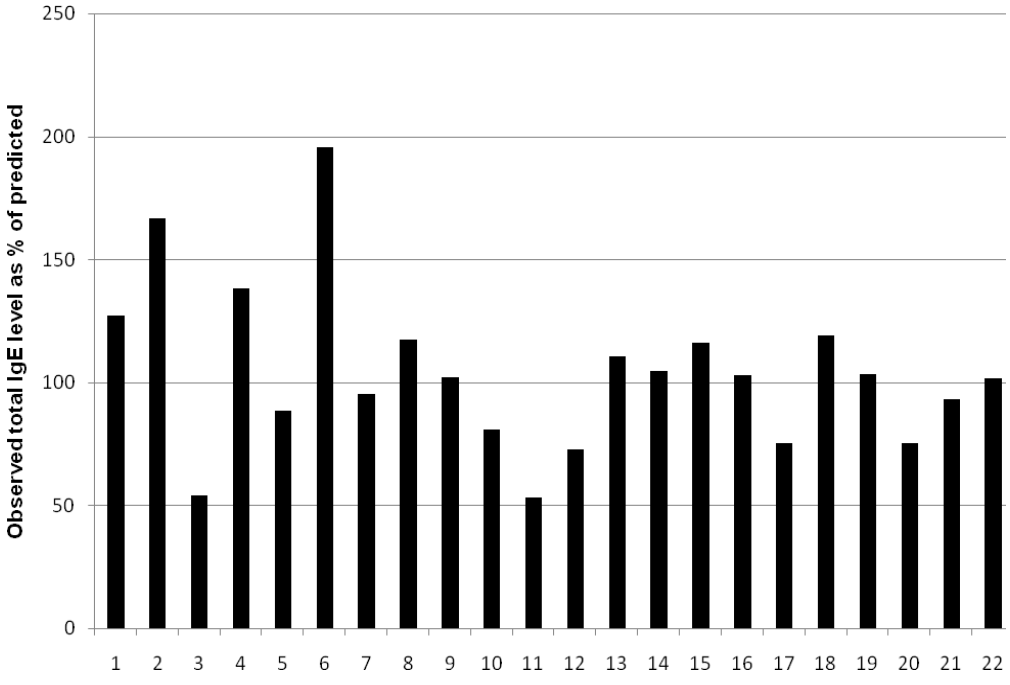
364 Fig 3.b



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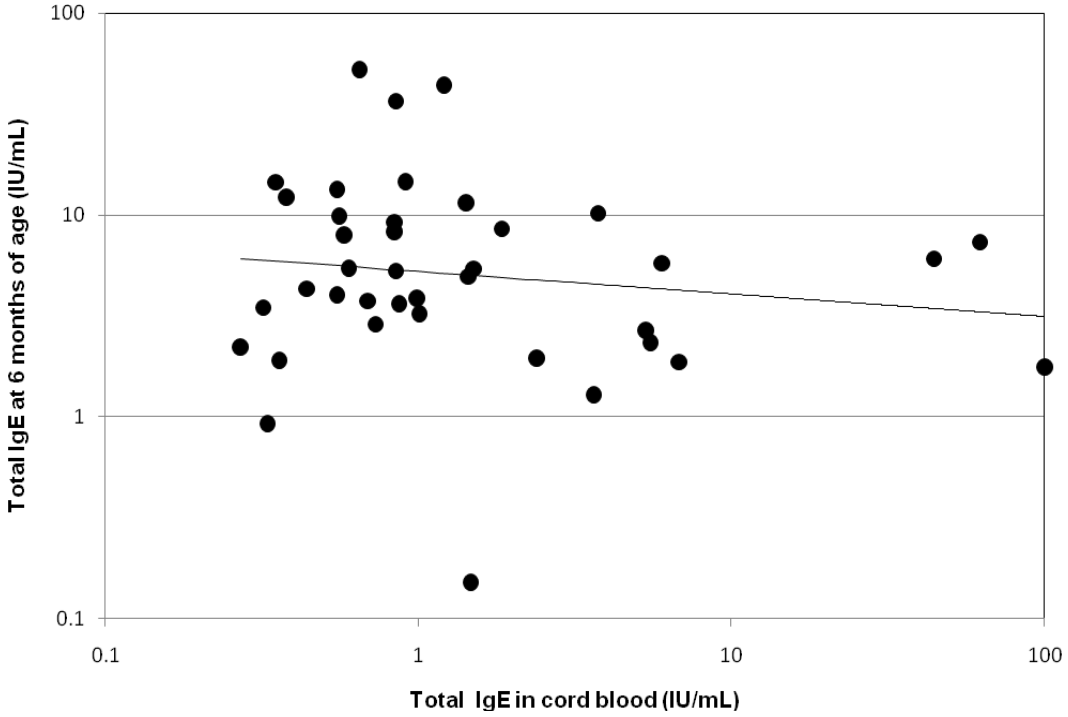
366 Fig 4.a



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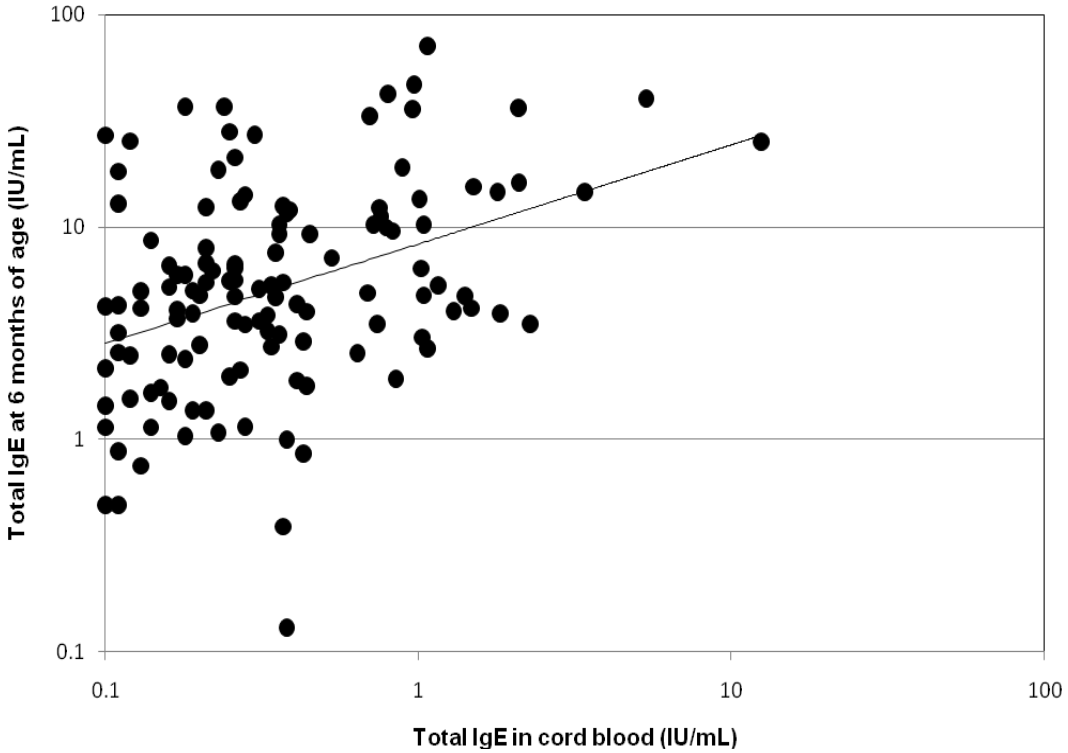
368 Fig 4.b



369

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Filaggrin gene variants and asthma exacerbations in early childhood

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Conflict of interest:

None of the authors report any conflict of interest relevant to the content of this report.

Short Title:

Filaggrin, early asthma and sensitization

Data from this manuscript has been presented as an abstract at the ATS congress 2009 but not elsewhere in abstract or any other form.

ABSTRACT

Background: We recently discovered the association between eczema and skin barrier dysfunction due to variants in the filaggrin (FLG) coding gene. Here we study the FLG-associated risk of asthma symptoms and sensitization in early life.

Methods: A high-risk cohort of 411 children was assessed in a prospective clinical study from birth to school age. Recurrent wheeze, acute severe exacerbations, asthma and sensitization were diagnosed prospectively by the investigators. FLG variants R501X and Del4 were determined in 382 Caucasians.

Results: Filaggrin variants increased risk of developing recurrent wheeze, asthma and asthma exacerbations (hazard ratio 1.82 [1.06-3.12], p=0.03), which was expressed within the first 1.5 years of life. Children with filaggrin variants had a marked and persistent increase in acute severe asthma exacerbations from one year of age (incidence ratio 2.40 [1.19-4.81], p=0.01) and increased risk of asthma by age 5 (odds ratio 2.62 [1.12;6.11],p=0.03). FLG-variants increased the risk of specific sensitization by age 4 (odds ratio 3.52 [1.72-7.25], p=0.0007) but not age 1.5.

Conclusions: FLG genetic variants were associated with early onset of asthma symptoms, particularly severe exacerbations, and subsequent development of sensitization. The association of filaggrin variants with asthma suggests skin barrier dysfunction as a novel, and potentially modifiable, mechanism driving early childhood asthma.

Number of words in the abstract: 200

Abbreviation list

COPSAC	Copenhagen Prospective Study on Asthma in Childhood
FLG	filaggrin coding gene

INTRODUCTION

We recently discovered that loss-of-function variants in the gene encoding filaggrin (*FLG*) are major determinants of eczema[1] and replicated this finding in a population based study[2] as have others.[3-8]

We also suggested an association with asthma in the discovery cohorts,[1] as confirmed in subsequent reports on asthma occurring in association with eczema[3,5-8] though one study reported no association with asthma.[9]

Such association between skin barrier dysfunction and airway disease is intriguing since it indicates a novel and potentially modifiable mechanism in asthma pathogenesis. In order to understand this mechanism there is a need for longitudinal studies in early life describing the temporal relationship in the development of FLG-associated atopic diseases.

Also, asthma in early childhood differs from asthma at older ages and must be studied separately. The majority of children with asthma symptoms in early life outgrow their symptoms before school-age, but the symptom burden in this age-group is high with severe exacerbations and hospitalization being more common than later in life.

Here, we therefore analyze FLG variants against the longitudinal clinical diagnoses of recurrent wheeze, acute severe exacerbations and asthma together with assessments of sensitization and describe the temporal relationship in the development of the different FLG-associated atopic outcomes assessed prospectively from birth through the first five years of life in the prospective clinical study of a high-risk birth-cohort "Copenhagen Prospective Study on Asthma in Childhood" (COPSAC).[10-12]

METHODS AND MATERIALS

Design

The Copenhagen Prospective Study on Asthma in Childhood (COPSAC) is a longitudinal clinical study of a birth-cohort of 411 infants born to mothers with a history of asthma; the recruitment of which was previously described in detail.[10-12] Data validity and quality control procedures follows "Good Clinical Practice" guidelines. History is collected on-line during visits to the COPSAC clinical research unit. Objective measurements are double checked against source data and the database subsequently locked. An audit trail is run routinely.

The COPSAC clinical research unit provided regular as well as acute clinical assessments for all participating children, who attended this clinic instead of family practitioners or other health care providers for diagnosis and treatment of any respiratory or skin-related symptoms. Regular visits were scheduled at the COPSAC clinical research unit at six–monthly intervals and additional visits were arranged immediately upon the onset of symptoms. At every visit the child was seen by both doctor and nurses of the research clinic and a full physical examination and history was obtained using structured questions and closed response categories focusing on the child's airway and skin symptoms, as well as recent history of medication, healthcare utilization, lifestyle and home environment.

The study was approved by the Ethics Committee for Copenhagen (KF 01-289/96) and The Danish Data Protection Agency (2008-41-1754). Informed consent was obtained from both parents. The motivations and perceptions of parents on the participation of their infants was recently reported.[13]

Clinical End-Point*s*

Recurrent wheeze: Respiratory symptoms were recorded daily by the parents in diaries from birth. Wheeze was translated to the parents as wheeze or whistling sounds, breathlessness or recurrent troublesome cough severely affecting the wellbeing of the infant, and was recorded as composite dichotomized scores (yes/no)

as previously described in details.[11,12] The doctor at the clinical research unit reviewed symptom definition and the diary entries with the parents at the six-monthly clinical sessions as well as at acute severe asthma exacerbations. Recurrent wheeze was pre-defined as five episodes within 6 months, each episode lasting at least three consecutive days, or daily symptoms for four consecutive weeks documented from the diaries.

Asthma was diagnosed according to the international guidelines as previously detailed[12] based on recurrent wheeze as defined above; symptom character judged by the clinical research unit doctor to be typical of asthma (e.g. exercise induced symptoms, prolonged nocturnal cough, recurrent cough outside common cold, symptoms causing wakening at night); in need of intermittent rescue use of inhaled β_2-agonist; and responding to a 3-month course of inhaled corticosteroids and relapsing when stopping treatment. Asthma was also diagnosed without a previous history of wheeze in case of acute severe asthma exacerbation as defined below.

Acute severe asthma exacerbations were defined from need of oral prednisolone or high-dose inhaled corticosteroid for wheezy symptoms prescribed at the discretion of the doctor at the clinical research unit or acute hospitalization for treatment for such symptoms at local hospital.

Debut of an asthma related phenotype was defined as onset of any of the above mentioned diagnoses and this measure was used in the survival analysis.

Eczema: Skin lesions were described at both scheduled and acute visits according to pre-defined morphology and localization; eczema was defined based on the Hanifin-Rajka criteria as previously detailed.[14,15]

Specific IgE at age 1½ and 4 years was determined by ImmunoCAP [16] (Phadia AB, Uppsala, Sweden) against the most common food (egg, milk, peanut, cod, wheat, and soya bean) and inhalant allergens (cat, dog, horse, birch, timothy grass, *Dermatophagoides pteronyssinus*, mugwort, molds). Values >= 0.35 kU/L were considered indicative of sensitization and was analyzed as the dichotomized index of any sensitization.

FLG Genotyping
Genotyping for FLG variants R501X and 2282del4 was performed as previously described.[1]

Statistical analyses
The association between FLG variants and end-points were analyzed by a two level dominant genetic model combing the two SNPs R501X and/or 2282del4.

The possible time-dependent association between FLG variants and age at onset of asthma related events was explored graphically by Kaplan-Meier plot and judged non-parametrically by a log-rank test. Quantification was done in terms of hazard ratios in a Cox regression. P-values correspond to Wald tests and asymptotic 95% Wald confidence intervals were calculated for the log(hazard ratio) and back-transformed.

Incidences of the event of one or more acute severe asthma exacerbations per child were calculated in the age-spans (0-1, 1-2, 2-3, 3-4, 4-5) for each FLG level. Age adjusted incidence ratios for acute severe asthma exacerbations were analyzed by a log-linear model taking into account within child correlation by an independence working GEE approach. P-values correspond to robust Wald tests and asymptotic 95% robust Wald confidence intervals were calculated for the log(incidence ratios) and back-transformed.

The associations between FLG variants and the events current asthma at age 5, sensitization at age 4, and sensitization at age 1.5 were quantified in terms of odds ratios by logistic regression. P-values correspond to likelihood ratio tests and asymptotic 95% Wald confidence intervals were calculated for the log(odds ratios) and back-transformed.

For each outcome effect modification through eczema was examined by including an interaction in the model between FLG status and eczema status prior to registration of that outcome.

The proportion of the cross-sectional diagnosis (asthma by age 5 and sensitization by age 4) attributed to FLG mutations was calculated as

100*(population prevalence-prevalence in wildtype population)/population prevalence

All analyses were made in SAS version 9.1.

RESULTS

The clinical follow-up rate of the COPSAC cohort was 95% by age 1; 90% by age 2; 85% by age 3; 79% by age 4; and 76% by age 5.

Of 411 infants from the COPSAC cohort, 382 Caucasians were genotyped for the loss-of-function variants R501x, 2282del4.[1] The mutated alleles R501x and 2282del4 were present in 18 and 25 children.

Asthma related phenotypes

Age at onset of recurrent wheeze, asthma and acute severe asthma exacerbations all exhibited similar profiles (data on file) and diagnoses were closely correlated. We therefore analyzed the composite end-point asthma related phenotype in the survival analysis. 95 of 382 children developed an asthma related phenotype (fig 1). The overall hazard ratio due to FLG variants was 1.82 [1.06-3.12],p=0.03. The Kaplan Meier curve shows that differentiation in development of an asthma related phenotype was clearly present in the first 18 months of life 0-1.5 year hazard ratio 2.44 [1.17-4.71],p=0.02, where after it could no longer be recognized statistically; 1.5-5 year hazard ratio 1.33 [0.57-3.13],p=0.51.

Asthma exacerbations

Yearly incidences of acute severe asthma exacerbation are shown in figure 2. Incidences were clearly elevated from infancy due to FLG variants and this elevation persisted throughout all five years. The overall age adjusted incidence ratio due to FLG variants was estimated to 2.40 [1.19-4.81],p-value=0.01.

Asthma

Yearly point-prevalence of asthma is shown in figure 3. Incidences were elevated due to FLG variants and this elevation increased throughout all five years. The odds ratio of asthma by age 5 for mutated versus non

mutated was 2.62 (1.12;6.11), p-value = 0.03. The proportion of current asthma attributed to FLG was 12.3%.

Specific IgE

Point-prevalence of specific IgE is shown in figure 4 for age ½, 1½ and 4. The effect from FLG mutations occurs later than the asthma and eczema phenotypes with no effect for the first 2 years, but by age 4 the odds ratio of sensitization for mutated versus non-mutated was 3.53 [1.72-7.25], p-value=0.0007. The proportion of current specific allergy attributed to FLG was 12.3%

Eczema

FLG variants were strongly associated with the development of eczema manifesting fully in the first year of life as previously reported.[1,17] There was no evidence of effect modification through atopic dermatitis in any of the end-points. Direct effects adjusted for eczema status were largely comparable in size to the total effects.

DISCUSSION

FLG variants conferred clinically significant risk of investigator-diagnosed early asthma phenotypes (recurrent wheeze, acute severe asthma exacerbations and asthma) and sensitization.

The temporal pattern of FLG associated atopic diseases was characterized by early onset of asthma symptoms and eczema, a persistent risk of acute severe asthma exacerbations, and later development of sensitization.

The association of FLG variants with asthma symptoms suggests skin barrier dysfunction as a novel, and potentially modifiable, mechanism driving asthma symptoms in early childhood.

This is the first longitudinal study describing the effect of FLG variants on the temporal clinical expressions of asthma, eczema and sensitization assessed objectively by clinical examinations from birth. The onset and programming of atopic disease occurs in early life and only through longitudinal studies from birth can the effect of genetic variants on the temporal relationship between the different atopic disease manifestations be understood and provide insight into the causal interrelation. The novel contribution of our study is the prospective day-by-day monitoring of disease onset. This provides detailed insight into the age-dependent disease presentations. Earlier studies relied on cross-sectional assessments of disease rather than exact age of disease debut and therefore could not appropriately account for temporal relationship between diseases. Also, cross-sectional studies relying on questionnaire-diagnosed eczema carry a risk of recall bias with parents of asthmatic children being more likely to recall early skin symptoms.

Accurate phenotyping is the major strength of our study and improves the power of the genetic association analyses. The meticulous prospective clinical monitoring, diagnoses and treatment of lung and skin symptoms through the first 5 years of life was carried out solely by the investigators. The cohort was seen regularly at 6 month intervals as well as for acute lung and skin symptoms by the doctors in the COPSAC clinic, who controlled diagnosis and treatment according to predefined algorithms, i.e. diagnoses and

treatments were not made by doctors outside our research unit. The diagnostic accuracy from prospective clinical monitoring is the key-difference between this clinical study and traditional epidemiologic cohorts often based on questionnaires and parents history of diagnoses made by doctors in the community.[18-20] This is of particular importance in the clinical diagnosis of wheeze where evaluation and perceptions of the terms are variable among practitioners and caregivers[21-24] and eczema where inter-observer variation is a problem.[25] Asthma was diagnosed prospectively at the clinical research unit according to a rigid algorithm based on predefined recurrence of diary recorded wheezy episodes monitored since birth, symptoms typical of asthma, need of short-acting bronchodilator treatments, response to inhaled corticosteroids and relapse after stopping treatment.[12] The accuracy of such pragmatic diagnosis was strengthened by the history being reported by mothers all experienced with asthma.

The validity of this analysis is further strengthened by the full disease continuum in the cohort.

Furthermore, the power of the statistics was improved from the longitudinal data-set with the time of onset clearly distinguishing these populations. Complex human diseases have variable ages of onset. Since the age of onset is likely to be genetically mediated, the subject's age of onset carries more information about the etiology of the disease than the case-control status. Therefore longitudinal data analysis provides more statistical power than cross-sectional case-control analyses.

The external validity of the conclusions of this study is limited by the cohort selection of newborns born of asthmatic mother's, with a gestational age above 35 weeks with no congenital abnormality, systemic illness, or history of mechanical ventilation or lower airway infection. The carrier frequency of FLG variants in COPSAC was 11% which is slightly higher than the reported frequencies in the background population between 8.8 % and 9.6 %.[1,26,27]

Several new variants within the FLG gene have been reported.[28] However, these new SNPs are substantially less prevalent and qualitatively different with some residual function as demonstrated by protein

immunoreactivity and a significantly lower penetrance of eczema, and they were therefore not included in this study.

FLG loss-of-function variants were associated with increased risk of acute severe asthma exacerbation in the first years of life. This observation is novel and important for this difficult to control disease entity. The clinical endpoints represent a severe disease burden with major impact on quality of life for patients and socioeconomic costs for the health care system and thus, our findings underline the clinical significance of FLG variants not only for eczema but for asthma and its exacerbations as well. Our finding is in line with a previous report of FLG-associated increased risk of exacerbations in older children and adults.[29] FLG variants increased the risk of recurrent wheeze in infancy and investigator diagnosed current asthma by age 5 contributing 10.8% of the cases. One study has shown association with history of preschool wheeze[7] and others showed association with asthma [3,5-8] while one study reported no association with asthma.[9]

FLG loss-of-function variants were a risk for sensitization contributing 12.3% of the cases in line with previous reports.[3,7,8] Interestingly the risk of sensitization increased after onset of asthma symptoms and eczema suggesting that sensitization does not mediate the pathway from FLG deficiency to skin or airway disease. This temporal relationship has not previously been reported.

The temporal pattern of the phenotypes suggests that gene-environmental research targeting FLG-deficient individuals should focus on infancy. In agreement with this we recently demonstrated interaction between FLG variants and cat and dog exposure at birth on development of eczema.[17]

The association of FLG variants with asthma symptoms suggests skin barrier dysfunction as a novel mechanism driving this particular asthma phenotype. *FLG* is the main protein component of the keratohyalin granules within the stratum corneum that provides a physical barrier which reduces water loss and protects the body from potentially harmful environmental exposures such as allergens, toxic chemicals and infectious organisms.[29] Two independent variants in the FLG gene (R510X and 2282del4), carried by ~9% of people of

European origin, result in complete loss of processed functional FLG in the epidermis.[1] Homo- or heterozygotes for these FLG variants alleles have varying degrees of impaired skin barrier.[31] This leads to increased risk of eczema, which is inherited as a semi-dominant trait with high penetrance in FLG null homozygotes or compound heterozygotes and reduced penetrance in heterozygotes.[1] FLG variants were statistically significantly associated with a more severe phenotype (SCORAD>31)[32] early onset[27] and persistence into adulthood.[26] The population attributable risk for eczema has been estimated at 11%, i.e. 11% of cases with eczema is attributed to this genetic variant.[3]

While the pathway from FLG deficiency to epidermal impairment and thereby increased risk of eczema is plausible the mechanism through which FLG deficiency contributes to airway disease and sensitization is not yet understood. FLG is expressed in the skin[1] and in the outer layers of the oral and nasal mucosae[33] but not in the respiratory epithelium of the nose[8,33] or the lower airways.[33,34] This suggests that FLG-associated asthma is mediated through a systemic, possibly immunological, mechanism stimulated through the impaired skin barrier, but our study suggests that this mechanism does not involve specific sensitization. Non-specific immunological mechanisms associated with eczema may be involved since most previous studies only demonstrate increased risk of asthma and sensitization in association with eczema,[3,7,8,35] although one study showed association with allergic rhinitis independent of eczema.[8] We could not confirm that the effect on asthma and sensitization was mediated through eczema in our longitudinal analyses, but our study was not powered for such analyses. The vast majority of acute wheezy episodes in children are triggered by viral infections,[36] and the link between FLG deficiency and early airway symptoms could therefore be an impaired response to respiratory viral infections. This could be mediated by immunological alterations caused by skin barrier dysfunction or eczema. Sensitization, even in the absence of allergic airway symptoms, was associated with an increased rate of viral-induced asthma episodes in children with viral-induced asthma.[37]

Future research should study the pathway from FLG variants to airway disease with the potential of increasing our understanding of the pathology of early wheeze and childhood asthma.

In conclusion, this study describes a FLG-associated pattern of atopic diseases in early childhood characterized by early onset of eczema and asthma with severe exacerbations and later development of sensitization. FLG provides a predictor useful for future targeted research into prevention of these diseases which should focus on infancy. Our findings proposes a common molecular mechanism underlying early asthma, eczema and sensitization and gives a surprising new direction to asthma research, which should consider the pathway leading from FLG variants in the skin to asthma. Together, the recognition of these common gene variants has the potential of being an important step for improvement in understanding, prevention and treatment of asthma, eczema and allergy.

LEGENDS:

Figure 1: Asthma related phenotype. Estimated cumulative risk of developing asthma related phenotype with and without FLG variants, log rank test p-value=0.03. Numbers at risk at are given below the graph.

Figure 2: Acute severe asthma exacerbations: Yearly incidence of one or more acute severe asthma exacerbations requiring high-dose steroid intervention or hospitalization stratified on age and FLG variants. The overall age adjusted incidence ratio due to FLG variants was estimated to 2.40 [1.19-4.81],p-value=0.01.

Figure 3: Asthma; point prevalence. The odds ratio of asthma by age 5 for mutated versus non mutated was 2.62 (1.12;6.11), p-value = 0.03.

Figure 4: Sensitization; point prevalence. By age 4 the odds ratio of sensitization for mutated versus non-mutated was 3.53 [1.72-7.25], p-value=0.0007.

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Figure 1

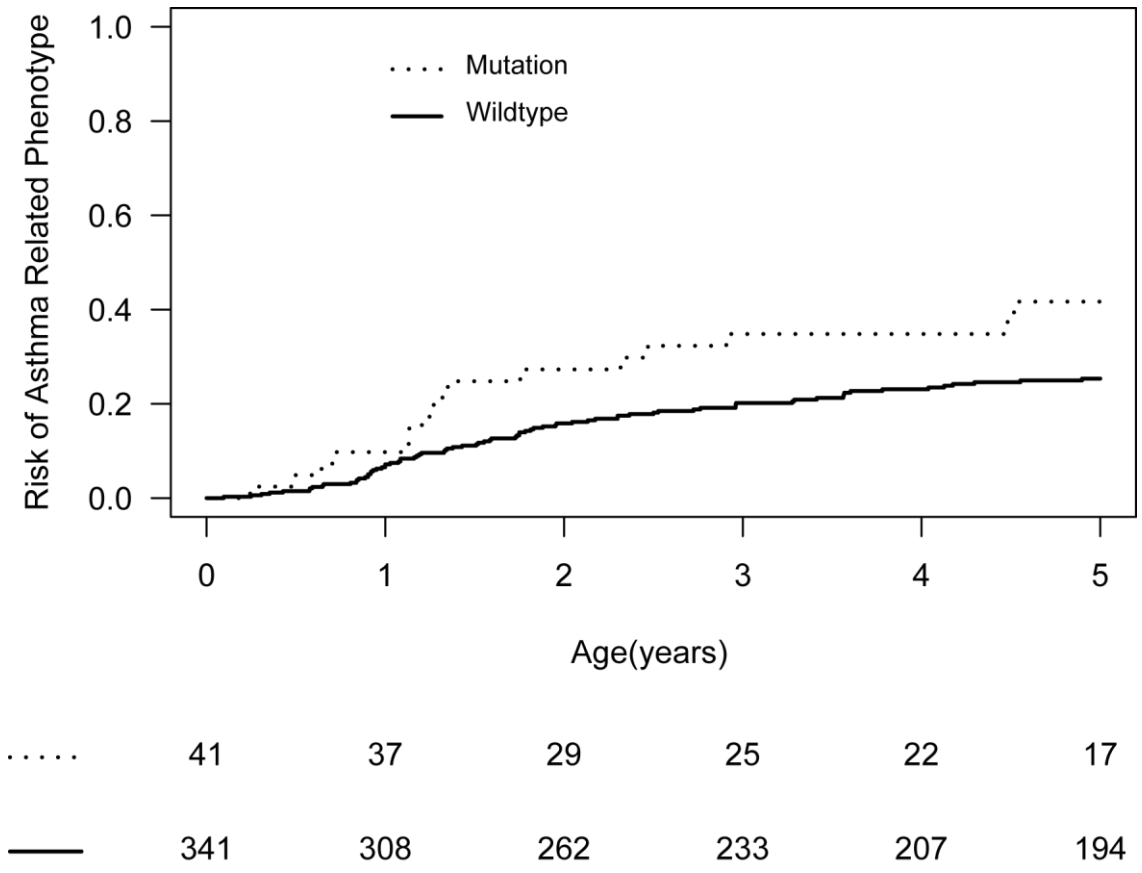

Risk of Asthma Related Phenotype
1.0
0.8
0.6
0.4
0.2
0.0
Mutation
Wildtype
0
1
2
3
4
5
Age(years)
41 37 29 25 22 17
341 308 262 233 207 194

Figure 2

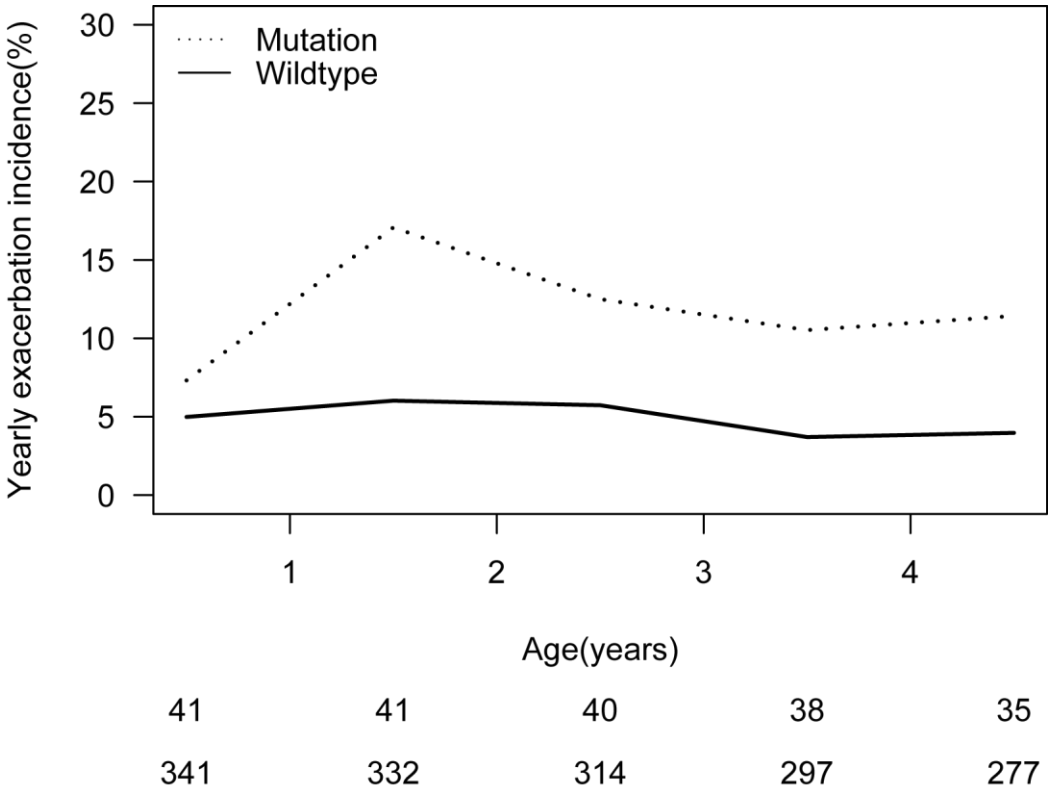

Mutation
Wildtype
Yearly exacerbation incidence(%)
30
25
20
15
10
5
0
1
2
3
4
Age(years)
41 41 40 38 35
341 332 314 297 277

Figure 3

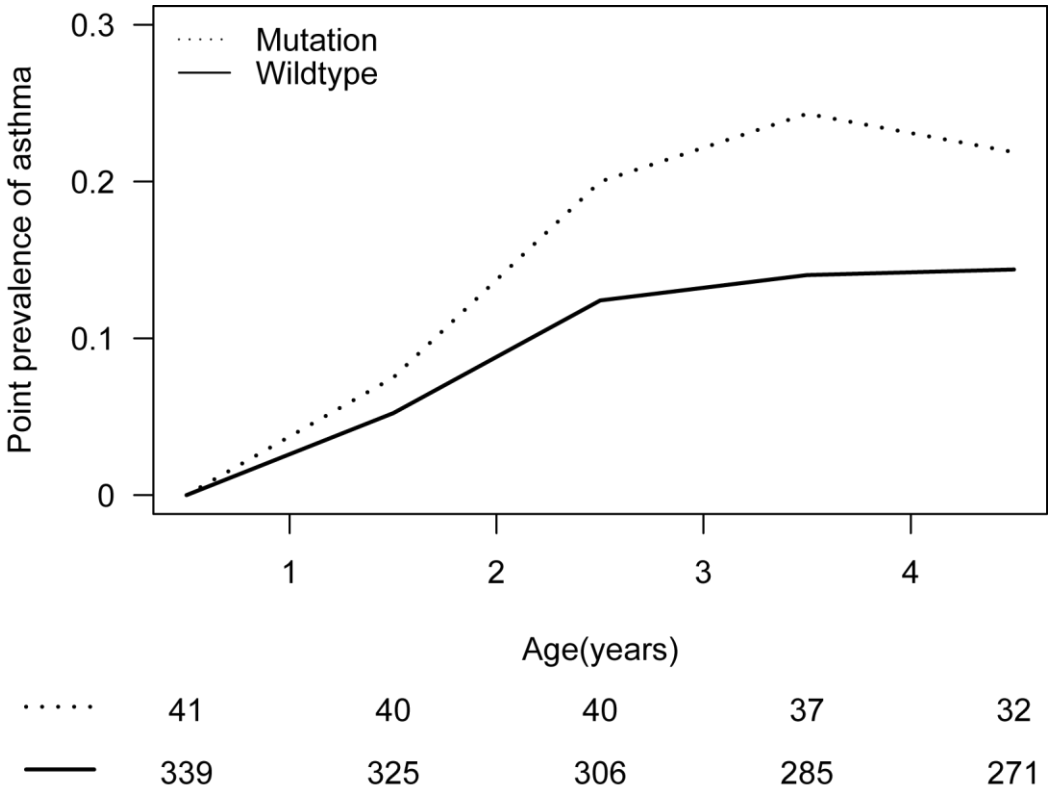
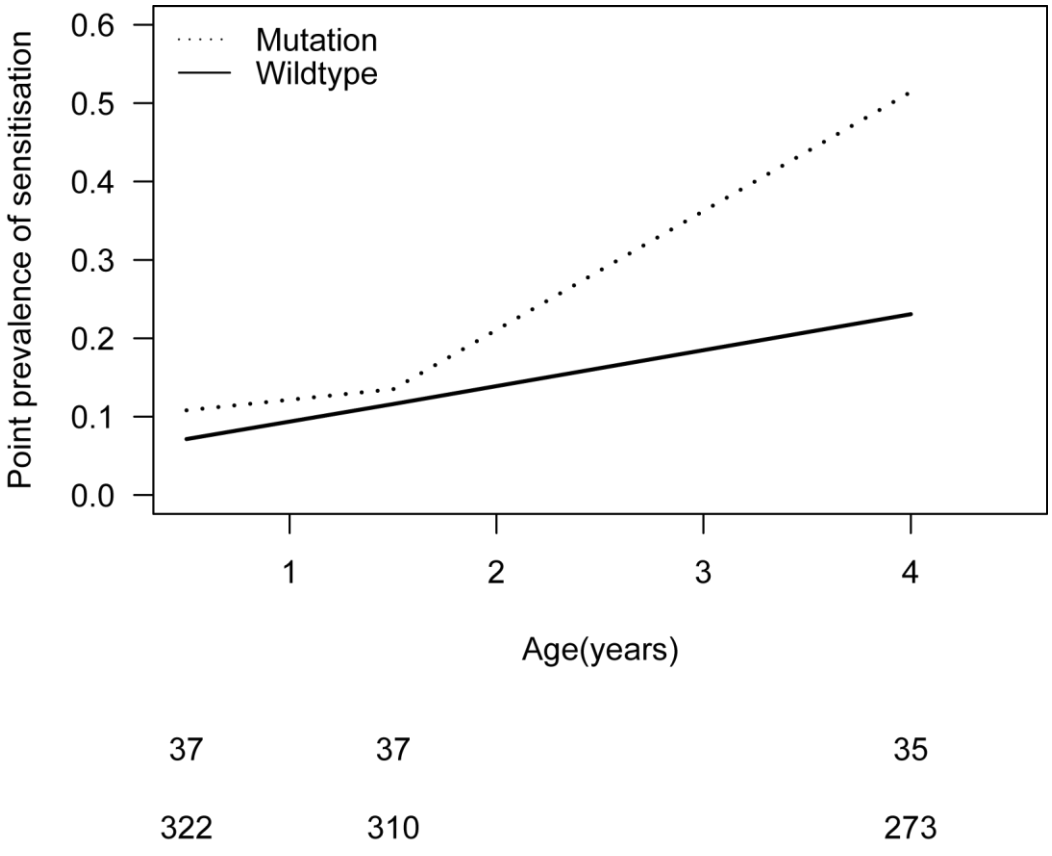

Mutation
Wildtype
Point prevalence of asthma
0.3
0.2
0.1
0
1
2
3
4
Age(years)
41 40 40 37 32
339 325 306 285 271

Figure 4


Point prevalence of sensitisation
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Mutation
Wildtype
1
2
3
4
Age(years)
37
37
35
322
310
273