

Filaggrin gene variants and atopic diseases in early childhood assessed longitudinally from birth

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Copenhagen Prospective Study on Asthma in Childhood (COPSAC) was one of the discovery cohorts of the association between eczema and variants in the filaggrin coding gene (FLG). Here, we study the FLG-associated risk of asthma symptoms in early life and describe the temporal relationship in the development of the different FLG-associated atopic outcomes: asthma, sensitization and eczema, assessed longitudinally from birth. A high-risk cohort of 411 children was assessed in a prospective clinical study from birth to school-age. Asthma, acute severe asthma exacerbations, sensitization and eczema were diagnosed prospectively by the investigators. FLG variants R501X and Del4 were determined in 382 Caucasians. 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 yr of life. Children with filaggrin variants had a marked and persistent increase in acute severe asthma exacerbations from 1 yr 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 eczema, manifesting fully in the first year of life (point prevalence ratio for age 0–5 was 1.75 [1.29–2.37]; p -value = 0.0003) contrasting the increased risk of specific sensitization by age 4 (odds ratio 3.52 [1.72–7.25], $p = 0.0007$) but not age 1.5. This study describes a FLG-associated pattern of atopic diseases characterized by the early onset of asthma symptoms and eczema and later 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.

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Copenhagen Prospective Study on Asthma in Childhood (COPSAC) was one of the discovery cohorts of loss-of-function variants in the gene encoding filaggrin (FLG) as major determinants of eczema (1) and this has been consistently replicated (2–10). An association with asthma was also suggested in the discovery cohorts (1) and confirmed in subsequent reports on asthma occurring in association with eczema (2–4, 6, 8–10) though one study reported no association with asthma (11).

Such association between skin barrier dysfunction and airway disease is intriguing as it indicates a novel and potentially modifiable mechanism in asthma pathogenesis. 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.

We therefore analyzed FLG variants against the longitudinal clinical diagnoses of recurrent wheeze, acute severe asthma exacerbations, asthma and eczema together with assessments of sensitization and describe the temporal relationship in the development of the different FLG-associated atopic outcomes through the first 5 yr of life in the prospective clinical study of a high-risk birth-cohort 'COPSAC' (12, 13).

Methods and materials

Design

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 (12, 13). Data validity and quality control procedures follow 'Good Clinical Practice' guidelines. History is collected online 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 6-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, health care 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.

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 well-being of the infant, and was recorded as composite dichotomized scores (yes/no) as previously described in details (13). The doctor at the clinical research unit reviewed symptom definition and the diary entries with the parents at the 6-monthly clinical sessions as well as at acute severe asthma exacerbations. Recurrent wheeze was predefined 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 (13) based on recurrent wheeze as defined previously; 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 later.

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 previously mentioned diagnoses and this measure was used in the survival analysis.

Eczema. Skin lesions were described at both scheduled and acute visits according to predefined morphology and localization; eczema was defined based on the Hanifin-Rajka criteria as previously detailed (14).

Specific IgE at age 1½ and 4 was determined by ImmunoCAP (15) (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 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 performed 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.

Point prevalence of eczema was calculated for ages (½, 1½, 2½, 3½ and 4½) for each FLG level. Age-adjusted prevalence ratio for eczema was 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(prevalence ratio) 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. In addition, analyses stratified on eczema status were performed.

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

$100 \times (\text{population prevalence-prevalence in wild-type population}) / \text{population prevalence}$

All analyses were made in SAS version 9.1. (SAS Institute, Cary, NC, USA).

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. Ninety-five of 382 children developed an asthma-related phenotype (Fig. 1). The overall hazard ratio owing to FLG variants was 1.82 [1.06–3.12], $p = 0.03$. The Kaplan–Meier curve shows that differentiation in the development of an asthma-related phenotype was clearly present in the first 18 months of life 0- to 1.5-yr hazard ratio 2.44 [1.17–4.71], $p = 0.02$, where after it could no longer be recognized statistically; 1.5- to 5-yr hazard ratio 1.33 [0.57–3.13], $p = 0.51$.

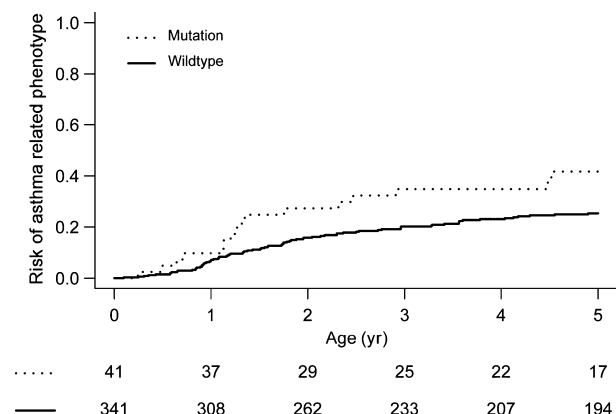


Fig. 1. Asthma-related phenotype. Estimated cumulative risk of developing asthma-related phenotype with and without filaggrin coding gene variants, log-rank test p -value = 0.03. Numbers at risk are given below the graph.

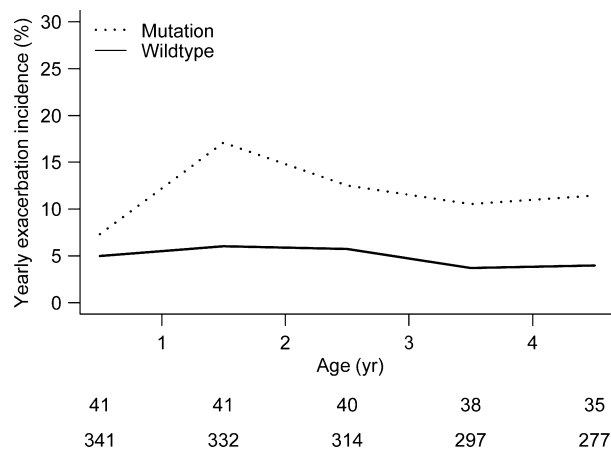


Fig. 2. Acute severe asthma exacerbations. Yearly incidence of one or more acute severe asthma exacerbations requiring high-dose steroid intervention or hospitalization stratified on age and filaggrin coding gene (FLG) variants. The overall age-adjusted incidence ratio owing to FLG variants was estimated to 2.40 [1.19–4.81], p -value = 0.01.

Asthma exacerbations

Yearly incidences of acute severe asthma exacerbation are shown in Fig. 2. Incidences were clearly elevated from infancy because of FLG variants and this elevation persisted throughout all 5 yr. The overall age-adjusted incidence ratio because of FLG variants was estimated to 2.40 [1.19–4.81], p -value = 0.01.

Asthma

Yearly point-prevalence of asthma is shown in Fig. 3. Incidences were elevated because of FLG variants, and this elevation increased throughout all 5 yr. 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 Fig. 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 yr, 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, 16).

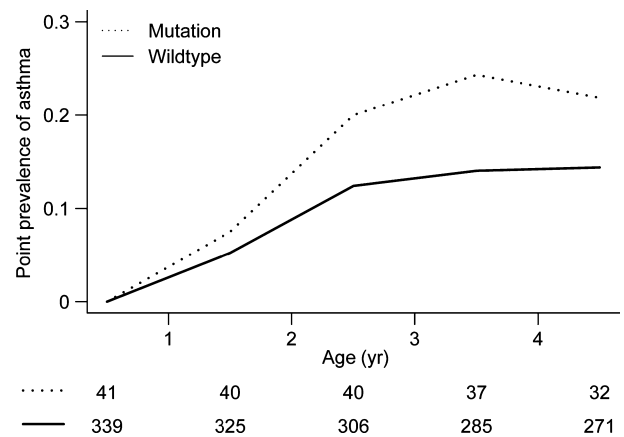


Fig. 3. Asthma; point-prevalence. The odds ratio of asthma by age 5 for mutated versus non-mutated was 2.62 (1.12;6.11), p -value = 0.03.

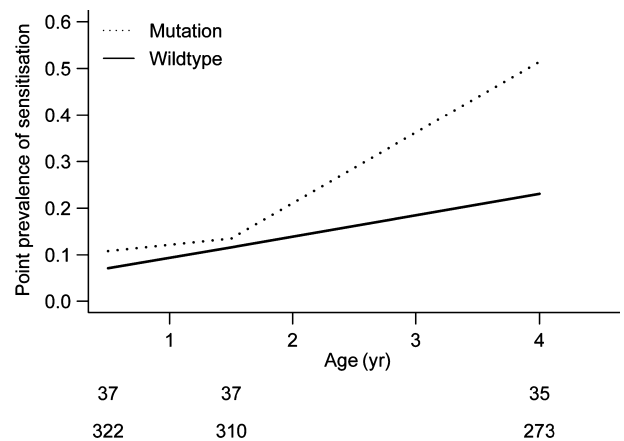


Fig. 4. Sensitization; point prevalence. By age 4, the odds ratio of sensitization for mutated versus non-mutated was 3.53 [1.72–7.25], p -value = 0.0007.

The profile of the yearly point prevalence of eczema suggested a similar profile after 6 months of age in children with and without FLG mutations (Fig. 5). The overall age-adjusted point-prevalence ratio owing to FLG variants was estimated to 1.75 [1.29–2.37], p -value = 0.0003.

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. Stratified analyses based on eczema status supported this conclusion except for sensitization where the effect size seemed larger in children with eczema (Table 1).

Discussion

FLG variants conferred clinically significant risk of investigator-diagnosed early asthma pheno-

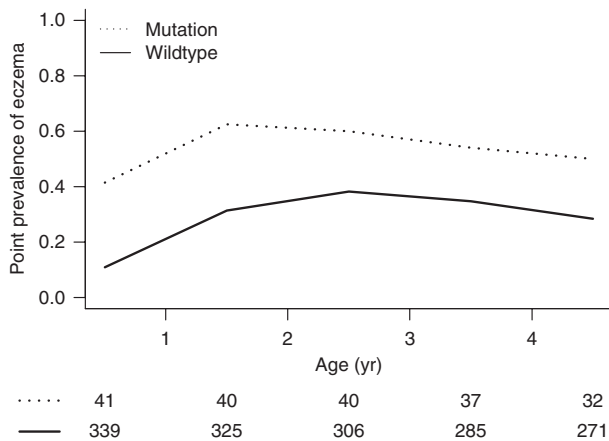


Fig. 5. Eczema; yearly point prevalence. The overall age-adjusted prevalence ratio owing to filaggrin coding gene variants was estimated to 1.75 [1.29–2.37], p -value = 0.0003.

types (recurrent wheeze, acute severe asthma exacerbations and asthma) and sensitization in addition to eczema. 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. This 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 longitudinal studies from birth can provide insight into the temporal relationship and potential causal interrelation of atopic manifestations of genetic variants. 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 yr 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. The diagnostic accuracy from prospective clinical monitoring is the key difference between this clinical study and traditional epidemiologic cohorts and is of particular importance in the clinical diagnosis of wheeze where evaluation and perceptions of the terms are variable among practitioners and caregivers and eczema where inter-observer variation is a problem.

The statistical power of the study is limited by the relatively low number of cases with FLG variants. However, we were able to demonstrate statistically significant and biologically plausible effects. The external validity of the study is limited by the high-risk nature of the cohort. A recent study reported no parental-of-origin effect of FLG, suggesting that the selection of high-risk mothers and not fathers only did not result in a specific maternal FLG effect (5). 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, 17, 18). Diagnosing asthma in early life is difficult with differences in diagnostic tradition between doctors and countries, so even though the risk of misclassification was minimized by the standardized diagnostic procedures, it cannot be ruled out.

Several new variants within the FLG gene have been reported (19). However, these new SNPs are substantially less prevalent and qualitatively different with some residual function as demonstrated by protein immunoreactivity and a

Table 1. Association of filaggrin gene variants with asthma outcomes and sensitization stratified by eczema status (eczema ever from 0 to 5 yr of age)

Outcome	Non-eczema population	Eczema population	Whole population
Asthma-related events (HR [CI])	2.00 [0.85–4.71]	1.65 [0.82–3.32]	1.82 [1.06–3.12]
Exacerbation incidence (IR [CI])	2.34 [0.82–6.69]	2.52 [0.97–6.55]	2.40 [1.19–4.81]
Asthma 5 yr (OR [CI])	3.78 [0.90–15.88]	1.82 [0.63–5.21]	2.62 [1.12–6.11]
Sensitization 4 yr (OR [CI])	1.98 [0.57–6.90]	4.25 [1.63–11.03]	3.52 [1.72–7.25]

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

FLG loss-of-function variants were associated with the 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 (20).

FLG variants increased the risk of recurrent wheeze in infancy and investigator-diagnosed current asthma by age 5, contributing 10.8% of the cases. Other studies have shown association with asthma symptoms in pre-school-age (3, 5, 21) and others showed association with asthma later in life (2–4, 6, 8), while one study reported no association with asthma (11). Recent meta-analyses conclude that FLG is a risk factor for asthma although not as strong as for eczema (9, 10).

FLG variants also increased the risk of sensitization contributing 12.3% of the cases in line with previous reports (3, 4, 8, 10). 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. Because of the longitudinal diagnosis of eczema, we could study the effect of eczema diagnosis prior to other atopic traits that has not been performed previously. 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 (3, 4, 8–10).

We previously demonstrated that FLG-associated risk of eczema is specific for the development of symptoms during the first year of life (16) and here extend the temporal description to asthma symptoms before 2 yr 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 (16). 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 atopic diseases is intriguing, both because of the consistent replication and considerable effect

size but also because of the specific function and location of FLG in the skin pinpointing skin barrier dysfunction as an important pathogenetic step for at least a proportion of atopic disease. *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 (22). Two independent variants in the *FLG* gene (R510X and 2282del4), carried by approximately 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 (23). 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). The association with eczema has been consistently replicated (2–10), 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.

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 (24). It is not clear to which extent FLG-mediated sensitization requires skin inflammation as association with allergic rhinitis has also been shown independent of eczema (8).

The pathway from FLG defect to airway disease is not yet understood. FLG is expressed in the skin (1) and in the outer layers of the oral and nasal mucosa (25) but not in the respiratory epithelium of the nose (8, 25) or the lower airways (25, 26). This suggests that FLG-associated asthma is mediated through a systemic, possibly immunological, mechanism stimulated through the impaired skin barrier. The late effect on sensitization compared to the early increased risk of asthma symptoms in this study suggests that sensitization does not mediate the pathway from FLG deficiency to airway disease. This is supported by a report of interaction between FLG variants and early sensitization on asthma risk suggesting that these represent 2 distinct mechanisms in the pathogenesis of asthma (27). One interpretation of our findings is that skin barrier defect may have independent effects on

the different atopic traits (eczema, asthma and sensitization). This is in line with studies showing that the eczema-asthma association is a relationship of co-existence, with asthma symptoms often debuting together with or before eczema, rather than a progressive 'atopic March' leading from eczema to asthma (28).

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. Asthma heterogeneity is pronounced in this age-group as evidenced by observations of different temporal patterns and association with intermediate phenotypes. Atopic diseases are likely to represent several specific endotypes, subtypes of disease associated with distinct clinical features and defined functionally by the underlying molecular mechanisms or by treatment response (29). This heterogeneity is probably a main cause for inadequate management and there is an urgent need for a better understanding of disease mechanisms to improve treatment and prevention of disease. 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 pre-school asthma has disease initiated by skin barrier dysfunction that maybe can be prevented by targeting the skin defect. Chromosome 17q21 (ORMDL3) gene variants were recently discovered as a risk factor for early asthma and we demonstrated that this genetic variation is associated with a totally different phenotype characterized also by early asthma exacerbations but not with eczema or sensitization (30). It is likely that such genetically defined phenotypes represent distinct endotypes with different pathogenic mechanisms, environmental triggers and treatment responses, and further understanding of the underlying mechanisms may be a breakthrough for the management of disease.

In conclusion, 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. Our findings suggest a specific endotype of pre-school asthma initiated by skin barrier dysfunction but not driven by sensitization. FLG provides a predictor useful for future targeted research into prevention of atopic diseases that should focus on infancy.

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Conflict of interest

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

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