

Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic march

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Background: Childhood eczema often precedes the development of asthma and allergic rhinitis in the so-called *atopic march*. Recently, 2 loss-of-function mutations in the gene encoding the epidermal barrier protein filaggrin were reported to be predisposing factors for eczema and concomitant asthma, suggesting a possible role in disease transition.

Objective: We aimed to assess the importance of filaggrin loss-of-function mutations in the susceptibility to eczema and associated clinical phenotypes.

Methods: The filaggrin mutations were genotyped and tested for association with allergic disorders in 2 large European populations including 1092 children with eczema.

Results: Highly significant association of the filaggrin null mutations with eczema and concomitant asthma was replicated. Moreover, we found that these mutations predispose to asthma, allergic rhinitis, and allergic sensitization only in the presence of eczema. We show that the presence of 2 filaggrin null alleles is an independent risk factor for asthma in children with eczema, and that the 2 investigated mutations account for about 11% of eczema cases in the German population.

Conclusion: These results lend strong support to the role of filaggrin in the pathogenesis of eczema and in the subsequent progression along the atopic march. The fact that previous expression of eczema is a prerequisite for the manifestation of allergic airways disease and specific sensitization highlights the importance of the epidermal barrier in the pathogenesis of these disorders.

Clinical implications: Our results suggest that the maintenance and repair of the epidermal barrier in infants with eczema may prevent the subsequent development of allergic airways disease. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Eczema, atopic march, asthma, allergic rhinitis, atopy, filaggrin, genetic association, skin barrier

Atopic disorders such as eczema, asthma, and allergic rhinitis are among the most common chronic diseases in childhood, with a prevalence of more than 20% in industrialized countries.¹ Eczema is typically the first clinical manifestation of allergic disease, presenting early in infancy, followed by the development of allergic airways disease in about 40% of cases. This so-called *atopic march* suggests a common etiology for the different atopic diseases.²

A positive family history of eczema or atopy has been shown to be predictive for the development of eczema, underlining the importance of genetic factors in the disease etiology.³ To date, most of the studies that aimed to unveil the genetic determinants of eczema have focused on immunologic mechanisms underlying inflammation, such as IgE-mediated sensitivity and T_H2 function, and positive associations have been notoriously difficult to replicate.⁴ A recent report by Palmer et al⁵ showed that loss-of-function mutations in the filaggrin gene (*FLG*) predispose to eczema and asthma that occurs in association with eczema. This study demonstrated that a primary defect in the skin barrier plays an important role in the pathophysiology of eczema and provided a molecular link with the epidermal barrier dysfunction that is clinically observed in patients with eczema.⁶

Filaggrin is a key protein of the epidermis that plays an essential role in the formation of the protective skin barrier. In the outer granular layer of the epidermis, filaggrin is associated with keratin intermediate filaments and aids their packing into bundles. In terminally differentiated keratinocytes, filaggrin is crosslinked to the cornified cell envelope, which constitutes an insoluble barrier in the stratum corneum, protecting the organism against environmental agents and preventing epidermal water loss.⁷

A possible role of filaggrin in eczema susceptibility is supported by several lines of evidence. Loss-of-function mutations in the filaggrin gene predispose to ichthyosis,⁸ which in its mild form shows many overlapping characteristics with eczema such as dryness and hyperlinearity of the skin. About 8% of patients with eczema have

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Abbreviations used

FLG: Filaggrin gene
 GENUFAD: GENetic studies in NUClear Families
 with Atopic Dermatitis
 MAS: Multicenter Allergy Study
 OR: Odds ratio
 PARF: Population attributable risk fraction
 TDT: Transmission disequilibrium test

ichthyosis,⁹ and Palmer et al⁵ have demonstrated that eczema and ichthyosis cosegregate in some families. Furthermore, reduced expression levels of filaggrin in atopic skin lesions have been reported,¹⁰ as well as the genetic association of the *FLG* gene number polymorphism with the phenotype dry skin.¹¹ Finally, the *FLG* is located within the epidermal differentiation complex on human chromosome 1q21. The epidermal differentiation complex is a gene complex composed of more than 30 genes of several structurally and evolutionarily related gene families that are all involved in terminal differentiation of the epidermis.¹² Genetic linkage of childhood atopic eczema to this region has previously been reported.¹³

In the current study, we aimed to replicate the association between the *FLG* mutations and eczema in 2 large European cohorts. Moreover, we evaluated the association of the *FLG* mutations with other atopic phenotypes accompanying eczema such as asthma, allergic rhinitis, and elevated specific IgE levels.

METHODS

Study populations

We evaluated 2 study groups. The extended GENetic studies in NUClear Families with Atopic Dermatitis (GENUFAD) cohort consists of 490 nuclear families including 903 children with eczema. All families were of European descent and were recruited through at least 1 child having moderate to severe eczema with an age of onset younger than 2 years. The mean age of the affected children at the time of recruitment was 7.9 years. The majority of the families originated from Germany, with others from Italy (8.2%), Sweden (9.6%), The Netherlands (2.2%), and Poland (1%). The study sample consisted of 366 multiplex (at least 2 affected children) and 124 simplex (1 affected child) families. As previously described,¹⁴ the diagnosis of eczema was made according to standard criteria^{15,16} in the presence of a chronic or chronically relapsing (>3 months) pruritic dermatitis with the typical morphology and distribution. Disease severity was assessed using the scoring atopic dermatitis (SCORAD) system.¹⁷ Inclusion was limited to severe to moderate disease as defined by an objective SCORAD of >15 or involvement of >20% of the body surface.¹⁶ Total IgE levels and levels of specific IgE against 12 common environmental allergens were determined using a fluorescent enzyme immunoassay (CAP-RAST-FEIA; Pharmacia, Freiburg, Germany). All children whose parents reported that a doctor diagnosed asthma or hay fever were defined as having asthma or allergic rhinitis, respectively.

The German Multicenter Allergy Study (MAS) cohort has previously been described in detail.^{18,19} Briefly, this prospective cohort

TABLE I. Characterization of the GENUFAD and MAS cohorts

GENUFAD families		MAS birth cohort	
Families	490	Individuals	871
Ethnic origin	White	Ethnic origin	European
Multiplex	366	Female	47.8%
Simplex	124	Controls	321
Children with eczema	903	Cases	
Female	48.5%	Eczema	189
Mean age	7.9 y	Asthma	118
Additional phenotypes		AR	123
Asthma	220 (24.4%)	Elevated specific IgE	383
AR	248 (27.5%)		
Asthma and AR	135 (15%)		
Elevated specific IgE	582 (64.5%)		

AR, Allergic rhinitis.

consisted of 1314 children born in 1990. All children were followed up at the ages of 1, 3, 6, 12, 18, and 24 months, and at yearly intervals thereafter up to the age of 10 years. Clinical assessment included standardized interviews, questionnaires, and physical examinations. Total and specific IgE antibodies against 9 common food and inhalant allergens were determined at the ages of 1, 2, 3, 5, 7, and 10 years. DNA samples of 871 children of German descent were available for this study. Of this subset, 189 individuals had eczema as previously defined by the presence of (1) the reported physician's diagnosis, (2) parental report of eczema symptoms, or (3) visible eczema at the time of follow-up.²⁰ Children with eczema and allergic sensitization or no allergic sensitization were defined as having atopic and nonatopic eczema, respectively. A total of 118 children had asthma, defined as the presence of 1 or more wheezing episodes during the previous 12 months at the age of 7 and/or 10 years.²¹ We selected 321 control children who had no wheezing, itchy nose, or itching rash ever at the age of 7 or 10 years. All parents had indicated their history of eczema before the child's birth. As expected, children with eczema were more likely to have a positive family history of eczema (28.6%) than the controls (17.4%; $P = .019$).

In all probands, allergic sensitization was defined as the presence of specific IgE to at least 1 tested allergen of ≥ 0.70 kU/L (CAP class II). In the MAS cohort, the absence of specific sensitization was declared only if measurements from at least 2 time points were available. A total of 459 children were thus defined as nonatopic, of whom 92% had at least 1 measurement of specific IgE within the first 3 years of life. The institutional review boards of all participating centers approved the study protocol, and informed consent was obtained from all probands or their legal guardians.

Genotyping

Genomic DNA was prepared from whole blood by standard methods. All individuals were genotyped for the mutations R501X and 2282del4 in the filaggrin gene. Genotyping of R501X was performed by TaqMan allelic discrimination using an assay designed and tested by the manufacturer (Assay-by-design; Applied Biosystems, Foster City, Calif). The primers were FLG1-501F (5'-gcactggaggaagacaaggat) and FLG1-501R (5'-ctcttgggacgctgaatgc) and the probes FLG1-501V2 (5'-VIC-ctggagctgtctctg) and FLG1-501M2 (5'-FAM-tggagctgtctcatg). Fluorescence-based semiautomated genotyping was performed for the 2282del4 mutation. After PCR amplification with forward (5'-tccgccaccagctcca) and

TABLE II. Results of the TDT for the mutant *FLG* alleles in the European eczema families

Mutation	Allele frequency in founders	Eczema T:UT (<i>P</i> value)	Eczema and asthma T:UT (<i>P</i> value)	Eczema and allergic rhinitis T:UT (<i>P</i> value)	Eczema and elevated specific IgE T:UT (<i>P</i> value)
R501X	2.8%	64:37 (.016)	16:7 (.17)	21:8 (.059)	46:22 (.0090)
2282del4	6.6%	149:70 (6.5×10^{-7})	37:13 (.0017)	48:15 (5.0×10^{-5})	114:48 (1.5×10^{-6})
Combined allele	9.3%	209:98 (1.9×10^{-9})	53:19 (.00042)	69:22 (2.5×10^{-5})	157:63 (1.9×10^{-9})

T, Transmitted; UT, untransmitted.

reverse (5'-FAM-ctgatggtgaccagcctgt) primers, PCR products were size-fractionated by electrophoresis on an Applied Biosystems 3730 DNA Sequencer. Allele sizes were determined using the GeneMapper software (Applied Biosystems).

The genotyping success rate was 99.0% for R501X and 97.7% for 2282del4. Genotype frequencies of both markers were found to be consistent with Hardy-Weinberg equilibrium.

Genotypes were confirmed by sequencing of 80 randomly selected individuals and an additional 92 mutation carriers for each mutation. For R501X (2282del4), 72 (68) homozygous wild-type, 98 (91) heterozygous, and 2 (13) homozygous mutant individuals were included and demonstrated 100% agreement of genotyping and sequencing results.

Statistical analysis

In the GENUFAD cohort, we used a robust study design (family-based association) to avoid potential sources of error caused by population stratification in the association analysis.²² Furthermore, the investigation of complete families allowed us to check genotypes for Mendelian inconsistencies that were not detected. Because multiplex families were included in the analysis, we used the sib_TDT (transmission disequilibrium test) of the ASPEX software (version 2.4), which performs a permutation procedure to calculate empirical *P* values independent of linkage within families.²³ Analysis of the chromosomal transmissions in 490 families including 1596 individuals revealed not a single proband carrying both mutations on the same chromosome. This lack of linkage disequilibrium between the 2 mutations together with the equivalent effect of either mutation leading to a premature stop codon and a complete loss of filaggrin function⁵ allowed us to perform the association analysis for a combined allele. Here, individuals carrying any 1 mutated and 1 wild-type allele were coded heterozygous and individuals carrying any 1 of the loss-of-function mutations on both chromosomes were coded homozygous for the combined allele.

In the MAS cohort, case-control association analyses were performed using the χ^2 statistics. The population attributable risk fraction (PARF) was calculated according to Hennekens and Buring.²⁴ The disease prevalence in noncarriers was calculated by extrapolating the carrier frequencies observed in the MAS cohort for affected and healthy subjects to a population with 10% of patients with eczema¹ or with 2.5% of patients with both eczema and asthma.

RESULTS

To evaluate the role of the R501X and 2282del4 mutations in *FLG* in the susceptibility to atopic disease, 2 independent sample groups were analyzed (Table I). The first group was composed of 490 pan-European eczema families (366 multiplex and 124 simplex families). A total of 903 children with eczema were included, of whom 24.4% had asthma, 27.5% had allergic rhinitis,

15% had both, and 64.5% had elevated specific IgE. Genotyping of the *FLG* mutations revealed that, among the founders, the allele frequencies for R501X and 2282del4 were 2.8% and 6.6%, respectively, resulting in a combined allele frequency of 9.3% and in a combined carrier frequency of 17.9%. The TDT analysis yielded a strong association of either mutant allele with eczema (R501X, *P* = .016; and 2282del4, *P* = 6.5×10^{-7}), which increased for the combined allele (*P* = 1.9×10^{-9} ; Table II). Similar results for the combined allele were obtained in the subpopulations having eczema in combination with asthma (*P* = .00042), allergic rhinitis (*P* = 2.5×10^{-5}), or elevated specific IgE (*P* = 1.9×10^{-9}).

The MAS cohort was used to confirm the results and to evaluate the relevance of the *FLG* null mutations in allergic disorders at a population level. We performed a case-control association analysis including 871 children of this cohort, of whom 13.5% had atopic and 8.2% nonatopic eczema, 13.5% had asthma, 14.1% had allergic rhinitis, and 43.9% had elevated specific IgE (Table I). The extensive, prospective clinical characterization of this cohort enabled us to select a control group composed of 321 individuals matched for ethnic origin and age, and for whom the absence of signs and symptoms of allergic disease had repeatedly and consistently been documented.

The frequencies of the mutations R501X and 2282del4 were 0.8% and 1.7%, respectively, in the nonallergic control group, yielding a combined allele frequency of 2.5% and a combined carrier frequency of 5.1%. Interestingly, the combined carrier frequency was more than 3 times higher (16.7%) in the eczema group, resulting in an odds ratio (OR) of 3.73 (95% CI, 1.98-7.02; *P* = 3.5×10^{-5} ; Table III). Furthermore, this analysis revealed that filaggrin loss-of-function mutations predispose to both the atopic (OR, 3.84; 95% CI, 1.9-7.7; *P* = .00006) and the nonatopic (OR, 3.94; 95% CI, 1.7-8.9; *P* = .00065) forms of eczema, conferring the same risk of expressing the disease (Table IV). Among 74 filaggrin null allele carriers, 31 had eczema, resulting in an estimated penetrance of 42% in the MAS cohort.

Consistent with the family-based results, the analysis for eczema with concomitant allergic phenotypes revealed a strong association in the asthma plus eczema subpopulation with a combined carrier frequency of 25.0% and an OR of 6.21 (95% CI, 2.6-14.8; *P* = 5.4×10^{-8}). Likewise, we observed a highly significant association of filaggrin null mutations with allergic rhinitis plus eczema (carrier frequency, 20%; OR, 4.79; 95% CI,

TABLE III. Number of carriers of mutant *FLG* alleles in patients with eczema and controls of the German MAS birth cohort

Genotype	R501X		2282del4		Combined allele	
	Controls	Eczema	Controls	Eczema	Controls	Eczema
AA	314	170	305	172	298	155
Aa	5	17	11	14	16	27
aa	0	1	0	1	0	4
Total	319	188	316	187	314	186
<i>P</i> value	.00014		.057		3.5×10^{-5}	
OR	6.65		2.42		3.73	
(95% CI)	(2.43-18.22)		(1.09-5.38)		(1.98-7.02)	

TABLE IV. Number of carriers of the combined *FLG* allele in allergic disorders of the German birth cohort

Genotype	Controls	Atopic eczema	Nonatopic eczema	Asthma and eczema	Asthma, no eczema	Rhinitis and eczema	Rhinitis, no eczema	Specific IgE, no eczema
AA	298	97	52	30	68	35	72	237
Aa	16	17	10	7	9	7	4	18
aa	0	3	1	3	0	2	0	0
Total	314	117	63	40	77	44	76	255
<i>P</i> value		.00006	.00065	5.4×10^{-8}	.11	1.5×10^{-5}	1.00	.62
OR		3.84	3.94	6.21	2.47	4.79	1.03	1.41
(95% CI)		(1.9-7.7)	(1.7-8.9)	(2.6-14.8)	(1.1-5.8)	(2.0-11.6)	(0.3-3.2)	(0.7-2.8)

TABLE V. Evaluation of *FLG* mutations as independent risk factors for asthma in children with eczema of the GENUFAD and MAS cohorts

Genotype	GENUFAD		MAS	
	Eczema, no asthma	Eczema and asthma	Eczema, no asthma	Eczema and asthma
AA	515	171	125	30
Aa	148	36	20	7
aa	10	10	1	3
Total	673	217	146	40
<i>P</i> value	.0070		.0085	
(recessive)				
OR (recessive)	3.20		11.76	
(95% CI)	(1.2-7.8)		(1.2-116.3)	

2.0-11.6; $P = .000015$) and allergic sensitization plus eczema (OR, 3.84; 95% CI, 1.9-7.7; $P = 6.4 \times 10^{-5}$). In contrast, there was no association for asthma ($P = .11$), allergic rhinitis ($P = .99$), or elevated specific IgE ($P = .62$) in the absence of eczema (Table IV).

Furthermore, to investigate whether the association of filaggrin null mutations with asthma in the MAS cohort was independent of the association with eczema, we investigated the subset of children with eczema and evaluated the filaggrin mutations as a risk factor for asthma. This test revealed a significant association of *FLG* null mutations with asthma under a recessive mode of inheritance. Patients with eczema with 2 mutant alleles had a significantly increased risk (OR, 11.76; 95% CI, 1.2-116.3; $P = .0085$) of developing asthma compared with those carrying 1 or no *FLG*

mutation. This association and the recessive mode of inheritance were confirmed in the GENUFAD cohort (OR, 3.2; 95% CI, 1.3-7.8; $P = .0070$; Table V). Similar results were obtained for allergic rhinitis (data not shown) as a result of the high correlation of this phenotype with asthma.

Finally, the MAS cohort enabled us to estimate the PARF, which indicates the proportion of cases in the population attributable to the mutant *FLG* alleles. Assuming a prevalence of 10% for eczema in the German population¹ and considering the observed carrier frequencies of *FLG* mutations in the MAS cohort (Table IV), the PARF was 11.1% for eczema and 20.6% for eczema with asthma.

DISCUSSION

The impairment of the epidermal barrier function is a clinical hallmark of eczema.⁶ Only recently, the R501X and 2282del4 mutations in the filaggrin gene, encoding a structural protein essential for skin barrier formation, were found to be associated with eczema and concomitant asthma.⁵ To investigate the role of filaggrin loss-of-function mutations in the susceptibility to allergic disease, we have performed a comprehensive study using 2 different study designs (family-based and case-control association analysis) in 2 large and clinically well characterized sample groups including 1092 patients with eczema. All individuals were characterized for the phenotypes eczema, asthma, allergic rhinitis, and elevated specific IgE, allowing us to evaluate the influence of the *FLG* mutations on a wide range of atopic disorders.

In the family cohort, we found highly significant association of the 2 *FLG* nonsense mutations with eczema and with asthma in the context of eczema, confirming previous results.^{5,25} The strength of the association and the high risk conferred by the filaggrin loss-of-function mutations are remarkable, particularly in view of the large number of associations of genetic variants with allergic disease that have not been replicated in independent studies.⁴ In addition, our data provide strong evidence for an association between *FLG* mutations and other allergic phenotypes that occur in the context of the atopic march such as allergic rhinitis and elevated specific IgE levels. It is important to note that eczema, respiratory airways disease, and allergic sensitization are highly correlated traits and that the GENUFAD families were selected on the basis of children with eczema. Therefore, the finding of an association of asthma, allergic rhinitis, and elevated specific IgE with filaggrin null variants in this cohort may reflect the underlying association with eczema.

We have therefore performed a case-control association analysis in the well characterized German MAS cohort.^{18,19} The population-based recruitment enabled us to evaluate the association of *FLG* mutations and the different allergic phenotypes of the atopic march in an unbiased population, and to select affected and control individuals on the basis of longitudinal, prospectively collected data.

Three novel and important results emerged. In the analysis of the related phenotypes specific sensitization, asthma, and allergic rhinitis, we were able to distinguish between patients exhibiting these traits per se or in the context of eczema. It was striking that neither allergic airways disease nor specific sensitization was associated with the *FLG* mutations in the absence of eczema. These results suggest that the defect in filaggrin predisposes to the sequential acquisition of these allergic disorders instead of acting as an independent risk factor for each disease. The previous onset of eczema appears to be required for the subsequent development of specific sensitization and allergic airways disease. This finding supports the notion that the inflammatory reaction and further breakdown of the epidermal barrier in eczema facilitate transepidermal penetration of allergens and allergic sensitization. It also underlines the importance of epicutaneous allergen exposure in the development of bronchial hyperresponsiveness, which has previously been demonstrated in the mouse model.²⁶ In addition, we show in both cohorts that the presence of 2 null alleles of *FLG* is an independent risk factor for asthma in children with eczema, providing further evidence that the degree of epidermal barrier breakdown plays an important role in the subsequent development of allergic airways disease. The importance of *FLG* null mutations for the phenotype asthma after eczema is also underlined by the remarkably high PARF, which indicates that 20.6% of cases with eczema and asthma are caused by the investigated *FLG* mutations. Furthermore, this investigation revealed an equally strong effect of the filaggrin null mutations in patients with atopic and nonatopic eczema, demonstrating that the

development of eczema in filaggrin carriers is not dependent on allergic sensitization.

Finally, we assessed the role of filaggrin null mutations in allergic disease at the general population level. In the MAS cohort, we estimated a reduced penetrance of 42% in eczema. Functional redundancy among genes of the epidermal differentiation complex may compensate for filaggrin deficiency, as previously reported in a loricrin knockout mouse model.²⁷ Future studies may reveal which additional genetic or environmental factors modify the disease penetrance.

We have estimated that a sizable proportion of 11.1% of eczema cases in the German population are attributable to null mutations in the filaggrin gene. Palmer et al⁵ have shown that these variants are found only in white subjects and are absent in people of Asian or African descent, in whom other variants of filaggrin may exist. At a global level, this indicates that about 90% of eczema cases are caused by other, yet unidentified factors. One might speculate that genetic variants affecting the structural or functional integrity of the epidermis may emerge as possible culprit genes.

In conclusion, we provide highly significant replication of the previously reported association of the *FLG* mutations R501X and 2282del4 with atopic eczema and eczema-associated asthma. Furthermore, we show that these mutations predispose equally to the atopic and non-atopic forms of eczema, suggesting that the onset of eczema may occur independently of allergic sensitization. Finally, we demonstrate in the population-based cohort that the defect in a single gene may predispose to different allergic phenotypes of the atopic march and that the expression of the subsequent disease stages requires the presence of eczema. These findings highlight the role of the epidermal barrier in the pathogenesis of allergic sensitization and allergic disease and also stress the importance of maintaining or repairing the epidermal barrier in infantile eczema as a possible means of preventing allergic airways disease.

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