Filaggrin loss-of-function mutations are associated with persistence of egg and milk allergy

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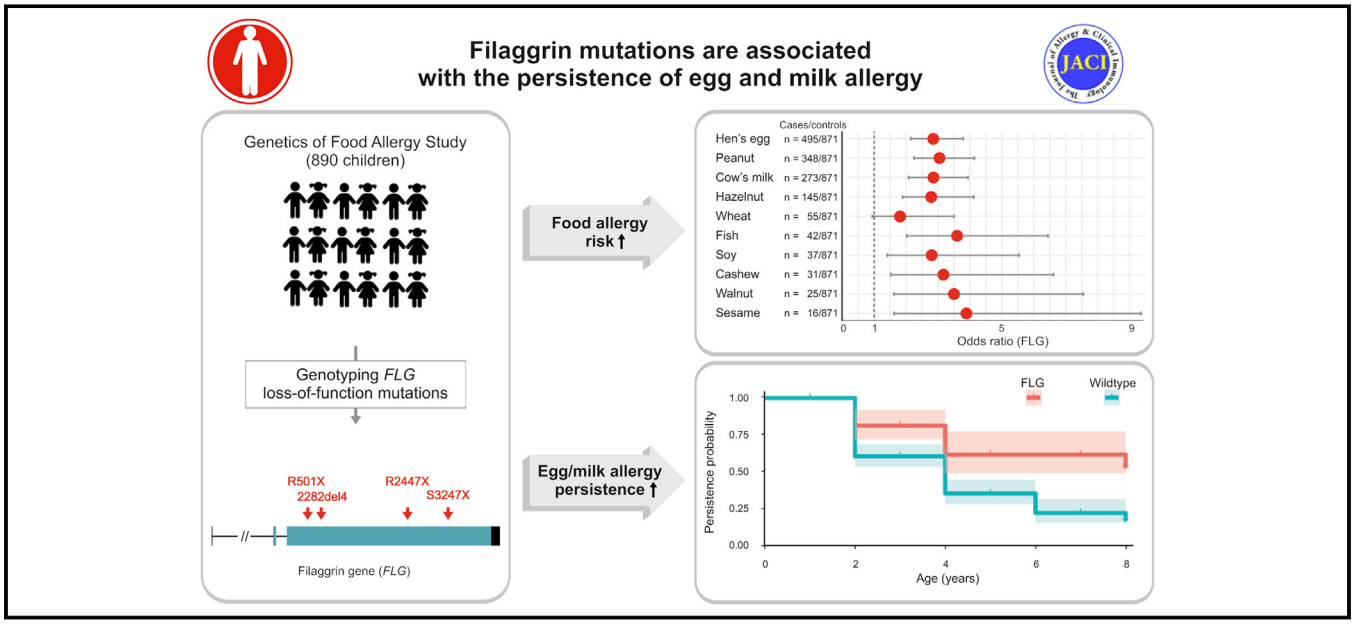
GRAPHICAL ABSTRACT



Background: A genetic defect in the epidermal barrier protein filaggrin (FLG) plays a major role in the etiology of eczema and associated allergic airways diseases. However, it is still controversial to what extend loss-of-function (LOF) mutations in *FLG* contribute to the development and persistence of food allergies.

Objectives: This study tested association of *FLG* LOF mutations with allergic reactions to diverse foods and investigated their potential effect on the persistence of early food allergies.

Methods: This study recruited 890 children with challenge-proven food allergy for the German Genetics of Food Allergy Study (GOFA). Longitudinal data were available for 684 children. All children were clinically characterized, including their allergic responses to specific foods, and genotyped for the 4 most common LOF mutations in *FLG*; R501X, 2282del4, R2447X, and S3247X. Associations between *FLG* mutations and food allergies were analyzed by logistic regression using the

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German Multicenter Allergy Study cohort as the control population.
Results: *FLG* mutations were associated with allergies to diverse foods including hen's egg (HE), cow's milk (CM), peanut, hazelnut, fish, soy, cashew, walnut, and sesame with similar risk estimates. Effects remained significant after adjusting for the eczema status. Interestingly, *FLG* mutations increased the risk of a persistent course of HE and CM allergy.
Conclusions: Using the gold standard for food allergy diagnosis, this study demonstrates that *FLG* LOF mutations confer a risk of any food allergy independent of eczema. These mutations predispose to the persistence of HE and CM allergy and should be considered in the assessment of tolerance development. (J Allergy Clin Immunol 2022;150:1125-34.)

Key words: *Persistence, food allergy, hen's egg, cow's milk, FLG, loss-of-function mutations, Genetics of Food Allergy Study, GOFA, double-blind placebo-controlled food challenge, eczema*

Abbreviations used
CM: Cow's milk
DBPCFC: Double-blind, placebo-controlled food challenge
FA: Food allergy
HE: Hen's egg
IoW: Isle of Wight
LOF: Loss-of-function
mut: Mutation
OFC: Oral food challenge
OIT: Oral immunotherapy
OR: Odds ratio
wt: Wild type

Food allergy (FA) is a global health problem with increasing prevalence over the past decades; in developed countries up to 10% of children are affected.[1-3] FA can trigger severe allergic responses and is the leading cause of life-threatening anaphylaxis in childhood.[4-6] The main elicitors in infancy are hen's egg (HE) and cow's milk (CM), whereas allergic responses to peanut and tree nuts usually occur later in childhood.[2] Therapeutic options for FA are limited. Avoidance of the allergenic food is usually the method of choice. While a large proportion of HE and CM allergy resolves spontaneously in early childhood,[7,8] some patients develop persistent FA with severe impact on their quality of life. The identification of patients at risk of persistent FA as well as understanding the underlying molecular mechanisms are therefore of major importance for the development of novel therapeutic strategies.

FLG is an epidermal protein that is essential for the integrity of the skin barrier. FLG monomers, which are cleaved from the large precursor protein profilaggrin, form tight bundles with keratin intermediate filaments and are linked to the protein-lipid cornified cell envelope.[9] This complex structure provides physical strength and the main barrier function of the stratum corneum. In addition, FLG degradation products serve as natural moisturizing factor, which is essential for elasticity of the stratum corneum and for normal desquamation.[10] Accordingly, defective FLG was linked to an impaired skin barrier and to a loss of natural moisturizing factor.[11] Loss-of-function (LOF) mutations in *FLG* were identified as the main genetic risk factors for eczema.[12] Interestingly, *FLG* mutations are also involved in the development of eczema-associated allergic airway diseases such as asthma and allergic rhinitis,[13,14] pointing to a key function in the pathogenesis of allergies. FLG is specifically expressed in stratified epithelia, mostly in the upper layers of the epidermis, but also in oral epithelia and esophageal mucosa.[15,16] In a mouse model, enhanced penetration of allergens through an impaired skin barrier led to systemic sensitization and subsequent allergic reactions on allergen contact in distant organs, demonstrating a link between epidermal barrier defect and airway diseases.[17]

FLG LOF mutations were detected in all ethnic groups with similar cumulative allele frequencies of about 5%, but mutation patterns were population-specific.[18] In populations of European ancestry, only 4 LOF mutations with allele frequencies >0.1% were identified: p.Arg501Ter, p.Ser761CysfsTer36, p.Arg2447Ter, and p.Ser3247Ter.[19] Each of the *FLG* mutations have an equivalent molecular biological effect, leading to truncated profilaggrin and complete absence of functional FLG monomers.[19,20] Because the mutations are located on different haplotypes, they are usually analyzed in a combined manner yielding the *FLG* genotypes wild type (wt/wt), heterozygous (wt/mut), and homozygous mutation (mut/mut) including homozygous and compound heterozygous mutation carriers.[19]

While the associations of *FLG* LOF mutations with eczema, asthma, and allergic rhinitis are well established, the role of *FLG* in FA is less clear. Genetic studies on FA often suffer from small sample sizes and weak phenotype definitions due to a lower prevalence and a higher diagnostic effort. To date, only a few studies on *FLG* in FA were conducted with different study designs and outcomes. In the Australian HealthNuts study, *FLG* LOF mutations increased the risk of food sensitization at age 1 year, but without an additional effect on FA.[21] In a Swedish birth cohort, *FLG* LOF mutations were associated with peanut sensitization only at 4 years of age.[22] In adults of a Danish cross-sectional study, a *FLG* effect on food sensitization was demonstrated only in the presence of eczema.[23] In addition, the English Isle of Wight (IoW) birth cohort reported no direct effect of *FLG* LOF mutations on FA, but did report an indirect effect through eczema and food sensitization in early childhood.[24] Finally, associations of *FLG* LOF mutations with peanut allergy and with clinical reactivity to food after adjusting for eczema were identified in 2 case control studies from England and the Netherlands, respectively.[25,26] However, these results were difficult to assess due to the different age groups under study (from infants to adults), diverse phenotype definitions ranging from questionnaire-based to the recommended gold standard, double-blind, placebo-controlled food challenges (DBPCFCs),[3,27] and the inclusion of varying allergenic foods across studies.

In this study, we investigate the role of *FLG* LOF mutations in the large Genetics Of Food Allergy Study (GOFA) including 890 children with FA who are of European ancestry from Germany. The majority of children was diagnosed by DBPCFC, the current gold standard. We demonstrate associations of *FLG* mutations with allergies to a wide range of allergenic foods, and we show that the *FLG* effect remains significant after adjusting for the eczema status. In addition, *FLG* mutations increase the risk of persistent HE and CM allergy, the 2 most common FAs in childhood.

METHODS

Study population

Children of the GOFA were recruited at pediatric clinics in Berlin, Wangen, Oldenburg, and Hannover. In line with the current guidelines,[3,27] FA was diagnosed based on an oral food challenge (OFC) (n = 766), most of which (n = 658; 85.9%) were conducted in a double-blind, placebo-controlled setting. All children with a suspected FA based on current symptoms or a clinical history plus sensitization to the corresponding food (elevated specific IgE and/or positive skin prick test) underwent an OFC. Children with a convincing history of an immediate, allergic reaction plus specific sensitization to the same food (IgE > 0.35 kU/L) were included as cases without further challenge (n = 124), as OFC was contraindicated due to the risk of a severe allergic reaction. OFCs were performed in an inpatient hospital setting under physicians' supervision. Children and their parents were instructed to quit any systemic anti-inflammatory medication 1 week before OFC. Only 1 food or placebo was investigated per 24-hour period and was administered in 7 escalating doses at 30-minute intervals. The cumulative allergen dose was given the next day if the 7 doses were tolerated without reaction. Consistent with the Practical Allergy (PRACTALL) guidelines, food challenges were scored as positive if objective cutaneous, gastrointestinal, respiratory, or cardiovascular reactions attributable to the allergen, but not to placebo were observed.[28] Organ-specific allergic responses to OFC and FA-associated allergic diseases were recorded. Allergen-specific IgE levels were determined using the ImmunoCAP test (Thermo Fisher Scientific/Phadia, Uppsala, Sweden). A physician's diagnosis of eczema was made according to standard criteria in the presence of a chronic or chronically relapsing pruritic dermatitis with the typical morphology and distribution.[29,30] In total, 890 children with FA for whom DNA samples were available for genotyping were included in this study. All individuals were of European ancestry as previously confirmed by principal component analysis.[31]

Children of the German Multicenter Allergy Study (MAS) were used as the control population. This cohort has previously been described in detail.[32,33] MAS consists of 1314 children born in 1990. Children were followed at the age of 1, 3, 6, 12, 18, and 24 months, and at yearly intervals thereafter until the age of 13 years. They were extensively characterized regarding their eczema and asthma status. However, food challenges were not performed and robust data on FA were not available. Accordingly, children from MAS were used as population-based controls. For regression analyses, eczema was defined by the presence of (1) a reported physician's diagnosis, (2) a parental report of eczema symptoms, or (3) visible eczema at the time of follow-up. DNA samples were available from 871 children of German descent. All samples were genotyped for the 4 *FLG* LOF mutations indicated below. The institutional review boards of all centers approved the study, and written informed consent was obtained from all participants or their legal guardians.

Genotyping

Genomic DNA was isolated from whole blood by standard methods. In all individuals, the most prevalent *FLG* LOF mutations in Europeans (R501X, 2282del4, R2447X, and S3247X) were genotyped by using TaqMan allelic discrimination, fluorescence-based semi-automated allele-sizing technology, or restriction enzyme digestion, as described previously.[13,19]

Statistical analyses

Because all investigated *FLG* mutations are LOF mutations located on different haplotypes, we combined them for the analysis as *FLG* status wt/wt, wt/mut, or mut/mut where wt/mut indicates a carrier of any 1 LOF mutation and mut/mut indicates a carrier of 2 mutated alleles (ie, homozygous or compound heterozygous). The association of the combined *FLG* mutations with FA was tested under an additive model with PLINK[34] using a logistic regression model adjusted by sex. Afterward, models were rerun including also eczema status as covariate to assess whether significant associations were dependent or independent of an eczema diagnosis. Association of the combined *FLG* mutations with age at FA diagnosis was analyzed by linear regression models including sex and eczema as covariates.[34] To assess the effect of *FLG* LOF mutations on the persistence of FA, we performed a Kaplan-Meier survival analysis.[35] Data were preprocessed so that for each individual with allergy, the data contained information about a new "event" happening as either "1" if participant had achieved tolerance, or "0" if participant was censored (or study termination), at each time point assessed. Kaplan-Meier curves enable analysis of incomplete sets of data (ie, after participants are lost to follow-up from visit to visit). Participants were stratified by *FLG* mutations carrier status (carriers vs noncarriers). Survival objects and Kaplan-Meier curves were fitted using the package "survival" in R (R Foundation, Vienna, Austria). Kaplan-Meier curves were visualized using the packages "survminer" and "ggpubr." Log-rank *P* values were calculated from the "survival" package and visualized using "survminer." Specific IgE levels were categorized into CAP classes 1 to 6. Correlation between allergen-specific IgE determined at the first visit below 2 years of age and FA persistence was assessed using the Kruskal-Wallace rank sum test. Finally, risk factors for persistence were analyzed using a 2×2 contingency table and Pearson chi-square test; for the *FLG* LOF mutations an allelic test was performed.

RESULTS

Characteristics of the study population: allergies to specific foods, comorbidities, *FLG* LOF mutations

A detailed description of the study population is provided in Table I. The most common FAs were those to HE (55.6%), peanut (39.1%), and CM (30.7%). Allergic symptoms on food challenge mainly involved the skin (93.6%), the gastrointestinal tract (32.2%), and the respiratory tract (23.2%). In 53.1% of children, the allergic response affected 2 or more organ systems (Table I). For 684 cases, at least 1 follow-up visit was available. The mean follow-up period was 39 months.

Overall, allergic comorbidities were present in 91% of children, with eczema being the most common (82.9%) among all children with FAs (Table I). The pattern of comorbidities varied according to the age of the children. In early childhood, the majority of children with FAs had concomitant eczema; below 4 years of age, asthma and hay fever were diagnosed only in 6.7% and 6.9% of the patients, respectively (see Fig E1 in this article's Online Repository at www.jacionline.org). Higher rates of asthma (37.4%) and hay fever (42.3%) were found in children who were followed until school age (6-8 years). Finally, of the children with FAs who had their last examination at ≥8 years of age, 63.7% had developed asthma and 54.8% hay fever.

All children were genotyped for the 4 most common *FLG* LOF mutations present in European populations: p.Arg501Ter, p.Ser761CysfsTer36, p.Arg2447Ter, and p.Ser3247Ter. In children with FAs, the allele frequency of the 4 *FLG* mutations combined was 13.3%, yielding a carrier frequency of 23.7% (Table I).

FLG mutations increase the risk of FA independent of the allergenic food

All 4 mutations under study were more frequent among food allergic cases with similar risk estimates (see Table E1 in this article's Online Repository at www.jacionline.org). Because they are located on different haplotypes, we performed all analyses using the combined genotype of the 4 mutations. Associations of the *FLG* mutations with allergies to different allergenic foods was analyzed by logistic regression using children of the German MAS study as population-based controls (n = 871). We found a strong association between the *FLG* status (odds ratio [OR] = 2.80; $P = 4.4 \times 10^{-15}$) and FA (Table II). This effect was not

TABLE I. Characteristics of the GOFA study

	GOFA (N = 890)
Male sex	573 (64.4)
FLG mutation carrier	211 (23.7)
Age at first diagnosis, mean ± SD	2.36 ± 2.69
Allergy to	n = 890
Hen's egg	495 (55.6)
Cow's milk	273 (30.7)
Peanut	348 (39.1)
Hazelnut	145 (16.3)
Other foods*	225 (25.3)
Polyvalent FA	405 (45.5)
Reacting organ system (at first challenge)	n = 715
Skin—eczema	50 (7.0)
Skin—other symptoms†	619 (86.6)
Gastrointestinal tract	230 (32.2)
Lower respiratory tract	166 (23.2)
Nasal mucosa/eye conjunctiva	122 (17.1)
Nervous system	58 (8.1)
Cardiovascular system	16 (2.2)
≥2 Organ systems affected	380 (53.1)
Allergic comorbidities	n = 890
Eczema	738 (82.9)
Asthma	201 (22.6)
Hay fever	211 (23.7)

Values are n (%) unless otherwise indicated.
*Other foods include wheat, fish, soy, cashew, walnut, pea, sesame, lens, and 26 additional foods with <1% of children in GOFA affected.
†Other skin symptoms are urticaria, angioedema, erythema, itch, flush, and wheals.

restricted to specific foods as similar effect sizes and significant *P* values (significance threshold corrected for the number of tests, $P < .005$) were observed for almost all foods tested. Only the association with wheat allergy comprising 55 cases did not reach significance. Because defective FLG is a major cause of eczema and because FA is often accompanied by eczema, we tested whether the observed association was due to eczema rather than FA. After adjusting for the eczema status, the effect of the *FLG* mutations on FA decreased slightly ($OR_{adj} = 2.10$; $P = 1.4 \times 10^{-7}$) but remained significant indicating that *FLG* mutations confer a risk of FA independent of eczema. We additionally conducted an eczema-stratified analysis, to test whether the *FLG* effect was different in children with eczema and in children without eczema. In both subgroups, significant effects of similar size were observed demonstrating that the *FLG* effect on FA was independent of the presence of eczema (see Table E2 in this article's Online Repository at www.jacionline.org). GOFA also enabled us also to investigate the effect of *FLG* mutations on polyvalent FA as 45% of the children had developed allergic responses to 2 or more foods. *FLG* mutations did not increase the risk of polyvalent versus monovalent FA ($P = .18$).

FLG LOF mutations are not associated with organ-specific allergic responses

To test the *FLG* status for association with organ-specific responses, we used data from the first OFC of each study participant (Fig 1). In carriers of *FLG* LOF mutations, there was a trend that skin and nasal mucosa/eye conjunctiva were less frequently involved in allergic reactions. However after correcting for the number of organ systems tested (n = 7), none of the association *P* values reached the significance threshold of $P < .0071$.

Association between *FLG* LOF mutations and the age at FA diagnosis

An age-of-onset analysis was performed for the 4 most common allergenic foods in GOFA: HE, peanut, CM, and hazelnut. According to the introduction of a specific food to the children's diet, allergies to CM and HE were usually diagnosed within the first 2 years of life (mean age of 1.4 years and 1.8 years, respectively), whereas hazelnut or peanut challenges were usually performed later (mean age of 3.3 years and 3.6 years, respectively). Comparing the age at FA diagnosis in *FLG* LOF mutation carriers versus noncarriers in a linear regression model revealed no difference for any of the foods (Table III). Because patients with FAs were asked to return for yearly follow-up visits to evaluate them for disease persistence or resolution, we also analyzed the age at the last positive OFC. Here we identified a significant difference in patients with allergies to CM ($P = 1.1 \times 10^{-4}$) and a nominally significant effect for HE ($P = .039$) (Table III). Carriers of *FLG* LOF mutations presented at the clinics for a longer period than those who did not carry a mutation, suggesting an effect of the combined *FLG* mutations on the persistence of CM and HE allergy.

Effect of *FLG* LOF mutations on the persistence of HE and CM allergy

In contrast to peanut and tree nut allergy, FA against HE or CM tends to resolve spontaneously in early childhood.[7,8,36] To investigate the effect of *FLG* on the persistence of HE and CM allergy in more detail, we performed a survival analysis in carriers of the *FLG* mutations versus noncarriers (Fig 2). Resolution of the respective allergy was used as the end point, which was either determined by a negative food challenge or by consumption of the formerly allergenic food without symptoms. We found significant differences in the curves for both HE and CM allergy ($P = .032$ and $P < .0001$, respectively). Carriers of the *FLG* LOF mutations were more likely to experience a persistent disease course than noncarriers were. The main separation of the curves occurred between 2 and 4 years, with minor increase of the deviation thereafter.

According to the results of the survival analysis, we defined 2 groups of children; those whose allergy resolved early (negative result for HE or CM at the first rechallenge between 2 and 4 years) and those whose allergy persisted (positive OFC for HE and CM, respectively, >2 years). Of 495 and 273 children with an early diagnosis of HE and CM allergy, respectively, 341 and 177 children fulfilled these criteria and were included in the analysis (Table IV). HE allergy resolved in 61 children (17.9%) at the first rechallenge (at a mean age of 35 months), CM allergy resolved in 70 children (39.5%) at a mean age of 31 months. Among children with persistent allergy, the mean age at the last follow-up visit with a positive OFC was 63 months for HE allergy (n = 280) and 62 months for CM allergy (n = 107).

Comparing the frequencies of carriers of at least 1 *FLG* LOF mutation between children with transient and persistent HE and CM allergy, respectively, yielded a significant increase of *FLG* mutation carriers among children whose allergy did not resolve early (Table IV). Among children who were HE-allergic with a positive rechallenge, the allele frequency of the combined *FLG* mutations was 16.8% compared with 6.6% in children whose HE allergy resolved. The presence of a *FLG* LOF mutation significantly increased the risk to develop persistent HE allergy (OR =

TABLE II. Association of *FLG* mutations with food allergic phenotypes

Allergic to	n_{cases}	$n_{controls}$*	OR_{unadj}	95% CI_{unadj}	*P* $value_{unadj}$†	OR_{adj}	95% CI_{adj}	*P* $value_{adj}$†
Any food	890	871	2.80	(2.16-3.62)	4.4E−15	2.10	(1.59-2.77)	1.4E−07
HE	495	871	2.85	(2.14-3.79)	6.0E−13	1.81	(1.33-2.46)	1.8E−04
Peanut	348	871	3.05	(2.24-4.14)	1.0E−12	2.43	(1.76-3.36)	8.0E−08
CM	273	871	2.86	(2.07-3.94)	1.7E−10	1.86	(1.32-2.62)	3.7E−04
Hazelnut	145	871	2.79	(1.89-4.12)	2.4E−07	1.85	(1.23-2.78)	3.3E−03
Wheat	55	871	1.82	(0.94-3.50)	.075	1.11	(0.57-2.19)	.75
Fish	42	871	3.60	(2.02-6.44)	1.5E−05	2.25	(1.24-4.07)	7.4E−03
Soy	37	871	2.80	(1.41-5.54)	3.2E−03	1.96	(0.97-3.94)	.06
Cashew	31	871	3.17	(1.52-6.61)	2.1E−03	2.21	(1.04-4.69)	.039
Walnut	25	871	3.50	(1.62-7.55)	1.4E−03	2.27	(1.04-4.95)	.04
Sesame	16	871	3.89	(1.62-9.36)	2.4E−03	2.71	(1.11-6.58)	.028
Polyvalent FA	405	485	1.20	(0.92-1.56)	.18	1.09	(0.84-1.43)	.52

unadj/adj, Values unadjusted/adjusted for eczema.
*Controls are from the German Multicenter Allergy Study (MAS) except for "Polyvalent FA," which compares only cases with polyvalent FA versus monovalent FA.
†The significance threshold was set at $P < .005$ according to the number of food allergens tested (n = 10).

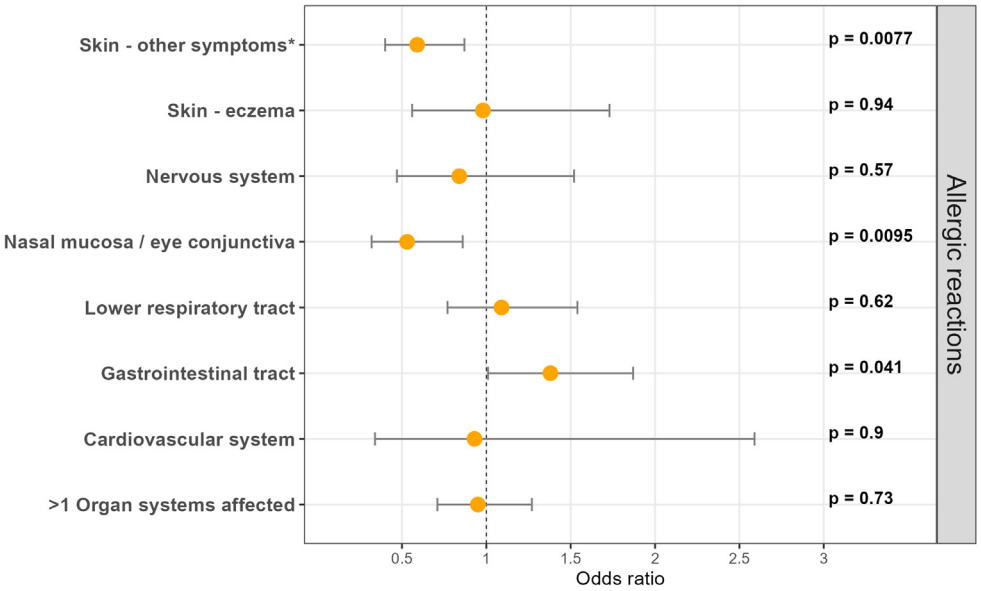

FIG 1. Association of *FLG* LOF mutations with organ-specific allergic responses. Allergic responses at the first OFC were used for the analysis. ORs (*dots*) and 95% confidence intervals (*whiskers*) are indicated. The significance threshold was set at $P < .0071$ according to the number of organ systems tested (n = 7).
*Other skin symptoms are urticaria, angioedema, erythema, itch, flush, wheals.

2.87; $P = .004$). A similar effect was observed for CM allergy. Here the allele frequency increased from 8.6% in children with a negative rechallenge to 23.8% in children with a positive rechallenge (OR = 3.34, $P = .0002$). Overall, 29.6% and 39.3% of the children with persistent HE and CM allergy, respectively, were carriers of an *FLG* LOF mutation. Notably, the presence of eczema or additional FAs had no impact on the persistence of HE or CM allergy.

The role of allergen-specific IgE levels in the persistence of HE or CM allergy

We additionally investigated whether the levels of specific IgE to HE or CM at the first visit correlated with the persistence of the respective FA. Specific IgE levels were categorized into 6 CAP classes. For HE, we detected a significant association of higher HE-specific IgE with persistence (median $CAP_{persistence} = 3$ vs median $CAP_{nonpersistence} = 2$; $P_{HE} = 1.5 \times 10^{-4}$) (Fig 3, *A*). For CM, a similar effect was observed (median $CAP_{persistence} = 3$ vs median $CAP_{nonpersistence} = 2$; $P_{CM} = 1.3 \times 10^{-4}$) (Fig 3, *B*). On the other hand, there was no difference in the IgE levels between carriers and noncarriers of *FLG* mutations, indicating that elevated specific IgE and *FLG* LOF mutations are independent risk factors for the persistence of HE and CM allergy (see Fig E2 in this article's Online Repository at www.jacionline.org).

Association of *FLG* LOF mutations with the severity of the allergic response

To assess whether *FLG* LOF mutations had an effect on the severity of the allergic response to HE or CM, we used data from the first OFC with the respective food. We applied the World Allergy Organization grading system[37] to classify the allergic responses into 5 categories from mild symptoms involving only 1 organ (grade 1) to severe lower/upper airway or cardiovascular

TABLE III. Effect of the *FLG* mutations on the age at diagnosis of FA

Allergic to	All	FLG_{mut} carriers	FLG_{wt}	Beta	SE	*P* value*
Any food, n	888	211	677			
Age at first diagnosis	2.36 ± 2.69	2.37 ± 2.64	2.35 ± 2.71	0.1249	0.1723	.47
Age at last diagnosis	4.40 ± 3.61	4.68 ± 3.49	4.32 ± 3.64	0.3914	0.2364	.098
HE, n	495	122	373			
Age at first diagnosis	1.78 ± 1.91	1.84 ± 2.05	1.77 ± 1.87	0.1605	0.1667	.34
Age at last diagnosis	3.18 ± 2.92	3.61 ± 3.18	3.04 ± 2.83	0.5326	0.2575	.039
CM, n	272	65	207			
Age at first diagnosis	1.38 ± 1.75	1.41 ± 1.39	1.38 ± 1.85	0.196	0.1962	.32
Age at last diagnosis	2.42 ± 2.67	3.43 ± 3.09	2.11 ± 2.45	1.156	0.2947	.00011
Peanut, n	347	89	258			
Age at first diagnosis	3.64 ± 2.91	3.82 ± 3.05	3.59 ± 2.87	0.083	0.297	.78
Age at last diagnosis	5.30 ± 3.68	5.28 ± 3.70	5.32 ± 3.68	−0.2223	0.3717	.55
Hazelnut, n	145	34	111			
Age at first diagnosis	3.27 ± 2.43	3.40 ± 2.07	3.23 ± 2.53	0.397	0.382	.3
Age at last diagnosis	4.40 ± 2.90	4.22 ± 2.45	4.45 ± 3.02	0.1118	0.457	.81

Values are mean ± SD unless otherwise indicated.
*The significance threshold was set at $P < .0063$ according to the number of independent tests performed for the 4 allergens (n = 8).

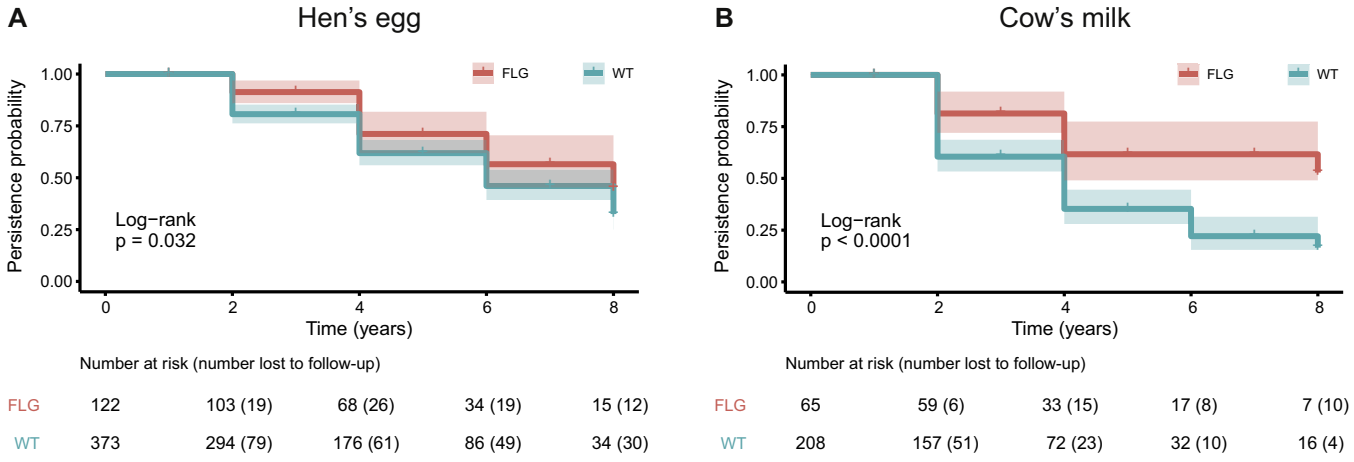

FIG 2. Persistence rates in **(A)** HE allergy and **(B)** CM allergy are dependent on the *FLG* mutation carrier status. Using a Kaplan-Meier survival curve, the persistence of FA from 0 to >8 years was analyzed in carriers of the *FLG* LOF mutations (*FLG*) versus in noncarriers (*WT*). Resolution of the respective allergy was used as the end point. The number of individuals who were lost to follow-up before reaching the end point is indicated.

symptoms (grades 4-5). There was no association of *FLG* mutations with the severity of the reaction neither for HE nor for CM allergy ($P_{HE} = .60$ and $P_{CM} = .69$) (see Table E3 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Markers for progression of early FA are urgently needed. Here we demonstrate that LOF mutations in *FLG* predispose to allergy against a wide range of foods, an effect independent of the known eczema effect. We additionally show that *FLG* LOF mutations increase the risk of a persistent course of HE or CM allergy suggesting their use as potential prognostic markers.

Previous studies investigated the association of *FLG* mutations with peanut allergy[25] and others considered FAs against diverse foods jointly to get sufficient numbers of cases.[21,24,26] In GOFA, we recruited a large number of children with FAs, almost doubling the number of cases compared with those of previous reports. This allowed us, together with a well-defined phenotype mainly based on DBPCFCs, to perform allergen-specific analyses. We identified significant associations between *FLG* mutations and almost all FAs tested including HE, CM, peanut, hazelnut, fish, soy, cashew, walnut, and sesame. Only the association with wheat allergy did not reach significance, which could be due to the lower numbers of cases. Considering the known association of *FLG* mutations with eczema and its high prevalence among children from GOFA (>80%), we investigated a potential role of eczema as a confounding factor. Eczema-adjusted effect sizes were consistent for all FAs (from 1.8 for HE to 2.7 for sesame), resembling the strong *FLG* effect on eczema. Thus, the association with *FLG* was independent of the allergenic food tested, suggesting a common barrier-related mechanism underlying all FAs. Abnormalities of the skin barrier have recently been reported in patients with peanut allergy

TABLE IV. Risk factors for a positive follow-up challenge in children with early allergy to HE or CM

	HE allergy (n = 341)				CM allergy (n = 177)			
Risk factors	Negative challenge/ consumption >2 years and <4 years (n = 61)	Positive challenge/ reaction >2 years (n = 280)	OR (95% CI)	*P* value*	Negative challenge/ consumption >2 years and <4 years (n = 70)	Positive challenge/ reaction >2 years (n = 107)	OR (95% CI)	*P* value*
Other FAs	34 (55.7)	183 (65.4)	1.50 (0.85-2.63)	.16	52 (74.3)	87 (81.3)	1.51 (0.73-3.10)	.27
Eczema	56 (91.8)	254 (90.7)	0.87 (0.32-2.37)	.79	63 (90.0)	95 (88.8)	0.88 (0.33-2.36)	.79
FLG mutations (het/hom)	8/0 (AF, 6.6)	72/11 (AF, 16.8)	2.87 (1.36-6.09)	.004	10/1 (AF, 8.6)	33/9 (AF, 23.8)	3.34 (1.71-6.52)	.0002

Values are n (%) unless otherwise indicated.
AF, Allele frequency; *het*, heterozygous carriers; *hom*, homozygous carriers.
*The significance threshold was set at $P < .0083$ according to the number of independent tests performed (n = 6).

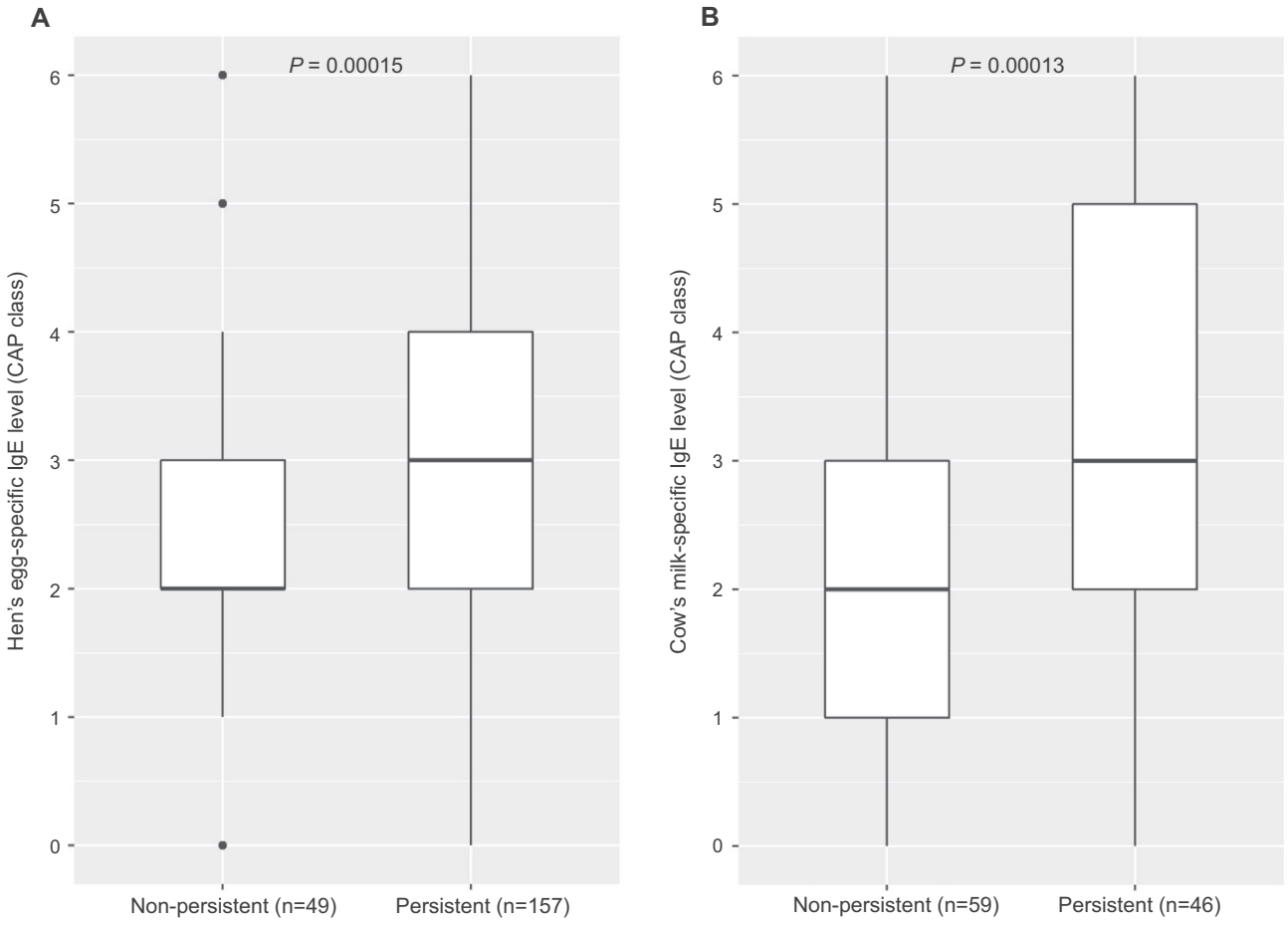

FIG 3. Allergen-specific IgE levels in **(A)** nonpersistent versus persistent HE allergy and **(B)** nonpersistent versus persistent CM allergy. Median (*horizontal line*), interquartile range (*box*), minimum and maximum corresponding to 1.5 times the interquartile range (*whiskers*), and outliers (*dots*) are indicated.

without eczema, supporting an eczema-independent effect of the epidermal barrier on FA.[38]

The role of *FLG* mutations in FA has been controversially discussed and seems to be dependent on the age group analyzed. In the Australian HealthNuts study, eczema-adjusted results revealed an association between *FLG* mutations and increased food IgE levels, mainly to HE and CM among 1-year-old infants but an additional effect on FA was not observed.[21] This outcome may be due to a lack of power to distinguish sensitization from clinically relevant FA, as the vast majority of children who were food sensitized were OFC positive (88%).

The IoW birth cohort study reported an association of *FLG* mutations with FA only in children from 10 years onward following food sensitization and eczema in early childhood.[24] Though the number of food allergic cases was small with a maximum of 67 children with FAs at age 1 year, this study pointed to an effect

of the *FLG* LOF mutations on FA mainly in older children. Our study found an age-dependent effect of the *FLG* LOF mutations in children with early allergies to HE and CM. We found a strong association between *FLG* mutations and persistent allergy to HE and CM (OR_{HE} = 2.9 and OR_{CM} = 3.3). Survival curves indicated that in HE and CM allergy the main effect of *FLG* occurs much earlier than in the IoW study, between 2 and 4 years, at a time point when natural tolerance usually develops.[7,8] Additionally, the IoW results may have been influenced by a less strict phenotype definition using questionnaire-reported FA symptoms, the inclusion of children with a negative skin prick test as well as by the lack of stratification for specific food allergens.

Therapeutic options for FA are still very limited. Apart from avoidance of the allergenic food, tolerance induction through oral immunotherapy (OIT), the repeated application of small allergen doses below the allergenic threshold level, is becoming more and more important. Recently, the first OIT product for the treatment of peanut allergy was approved.[39] However, OIT is largely restricted to children with a severe and more likely persistent disease course; trials have therefore focused mainly on peanut allergy, which is most common among school children and adults. In contrast, the most prevalent FAs in infancy are those against HE or CM.[7,8] Allergic responses to HE and CM typically develop during the first year of life and often show a transient disease course with most affected children acquiring clinical tolerance within a few years. However, some individuals develop a persistent FA. The factors influencing these divergent disease trajectories are largely unknown. It is therefore essential to gain a better understanding of the underlying mechanisms and to identify clinical features and biomarkers for tolerance development or persistence of FA. *FLG* LOF mutations are the first genetic marker associated with persistent HE or CM allergy. They might be used as prognostic markers to identify children who would most likely benefit from an OIT to HE and CM in the future. In addition, in carriers of a *FLG* mutation, the interval between oral rechallenges required to test for persistence of reactivity should be delayed, which would avoid undue stress in the patient and reduce the risk of severe reactions.

Other biomarkers associated with persistent HE and CM allergy were egg- and milk-specific IgE levels,[7,8] respectively, as well as the diversity of epitope-specific IgEs.[40,41] Confirming previous data, we found a highly significant association of allergen-specific IgE levels with the persistence of HE and CM allergy. HE and CM epitope-specific IgE levels were not available in GOFA. It would be interesting to test whether these phenotypes are also correlated with *FLG* mutations. In addition, a specific innate immune signature characterized by an increased number of monocytes and dendritic cells was associated with persistent HE allergy in the HealthNuts Study.[42] Finally, the gut microbiome seems to play a role in FA resolution because its composition in early infancy differed significantly between transient and persistent CM allergy.[43] A risk score combining genetic and immunological data could be a promising approach to identify children at risk of persistent HE and CM allergy.

A few limitations of our study have to be mentioned. While the definition of cases was based on the gold standard DBPCFC, controls were unselected for the absence of FA because food challenges were not available in the MAS cohort. According to the estimated prevalence of FAs in the German population, a small proportion of controls may actually be affected by FAs. This would reduce the power in the case-control design applied for the association analysis of *FLG* mutations with different allergenic foods. GOFA followed children at their routine clinical visits, which typically occur once per year. The end point of follow-up was therefore variable. In addition, some patients were lost to follow-up. Using the age at last positive challenge may therefore underestimate the duration of FA persistence because subsequent data were not available. On the other hand, loss to follow-up was more likely to occur in children with spontaneous tolerance development, which would lead to an underestimation of FA resolution. To alleviate this potential bias, we recontacted 96 children with HE allergy and 61 children with CM allergy who did not have a follow-up visit. Of 83 children who were HE-allergic and 57 children who were CM-allergic and who provided the requested information, 63% and 77%, respectively, reported spontaneous tolerance development, confirming that early loss to follow-up is more common among children who became tolerant.

We furthermore assessed how well the mutations under study represented the whole spectrum of LOF mutation in *FLG*. A total of 280 LOF mutations were reported in 129,196 individuals of European, non-Finnish ancestry in the gnomAD database.[18] Most of them are personal mutations identified in a single individual. The 4 most common mutations analyzed in our study represent 82.0% of all haplotypes with an LOF mutation in Europeans (see Table E4 in this article's Online Repository at www.jacionline.org). The fifth and sixth most common LOF mutations have a minor allele frequency of 0.05% and 0.04%, respectively. They are 5.5-fold less frequent compared with p.Ser3247Ter for which only 5 alleles were identified among our cases. Genotyping those is unlikely to significantly change our results. Hence, analyzing the 4 most common *FLG* LOF mutations is most efficient for association studies in populations of European ancestry.

Due to the ethnic homogeneity of the German study population, we were not able to investigate the role of *FLG* mutations in FA in other ethnic groups. Interestingly, the cumulative frequency of *FLG* LOF mutations is similar in Europeans (5.5% in non-Finnish Europeans), Africans (5.0% in African Americans), and Asians (5.5%, average between South and East Asians) (see Table E5 in this article's Online Repository at www.jacionline.org). In addition, a comparable effect on eczema susceptibility was reported for Asian-specific and African American–specific LOF mutations in *FLG*.[44-46] Moreover, equivalent biological effects were reported for LOF mutations identified in different populations,[19,20,44,47] suggesting that they may also increase FA risk in other ethnic groups. Indeed, a Japanese study confirmed the association of 6 population-specific *FLG* LOF mutations with FA in Asians.[48] A Turkish study investigating the 4 most common European mutations reported a combined allele frequency of 0.54% in patients with FAs,[49] an over 20-fold lower frequency than in our study, which may point to different, population-specific *FLG* mutations in the Turkish. Confirmation studies particularly in non-European populations would be of great value.

Using the gold standard DBPCFC for FA diagnosis, we show an association of *FLG* LOF mutations with FA independent of the allergenic food tested and not confounded by eczema. Moreover, we demonstrate an *FLG* effect on the persistence of HE and CM allergy. This study does not only highlight the role of the impaired barrier in the development of any FA, it also demonstrates its impact on the long-term disease course. Hence, *FLG* LOF mutations should be considered when planning oral food rechallenges.

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Clinical implications: In infants with HE or CM allergy, genotyping of *FLG* LOF mutations identifies children at high risk of a persistent disease course.

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