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Lower prevalence of common filaggrin mutations in a community sample of atopic eczema: is disease severity important?

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Geringere Prävalenz häufiger Filaggrin-Mutationen in einer unselektionierten Neurodermitis-Kohorte: Ist der Schweregrad der Erkrankung bedeutend?

Zusammenfassung. *Hintergrund:* In kürzlich publizierten Studien konnte ein Zusammenhang zwischen Funktionsverlust-Mutationen im Filaggringen (*FLG*) mit Ichthyosis vulgaris und Neurodermitis (AE) gezeigt werden. Die bisher berichteten Prävalenzen von *FLG* Mutationen und deren Relation zu atopischen Erkrankungen könnte jedoch durch Fall Selektion verzerrt werden. Daher war es Ziel dieser Studie, die wahre Populations-Prävalenz der *FLG* Mutationen in unselektionierten Kindern mit und ohne ärztliche Diagnose von Asthma bronchiale, allergischer Rhinitis und Neurodermitis sowie den familiären atopischen Hintergrund zu bestimmen.

Methoden: Verwendet wurde ein verschachteltes Case-control Design, bei dem Kinder mit ärztlicher Diagnose von Asthma bronchiale, allergischer Rhinitis und Neurodermitis sowie wahllos selektionierte Kontrollen aus einer größeren Querschnittsstudie ($n = 1263$) inkludiert wurden. Es wurde auf die häufigsten in Europa vorkommenden *FLG* Mutationen R501X, 2282del4 und R2447X gescreent, die DNA hierfür wurde aus aufgetauten Urinproben extrahiert. Das Verhältnis der kombinierten *FLG* Varianten mit atopischen Erkrankungen und mit berichteter Familienanamnese von Asthma bronchiale, allergischer Rhinitis und Neurodermitis wurde bestimmt.

Ergebnisse: In der Patientengruppe fanden sich ein homozygoter (R501X/R501X), 4 compound heterozygote (3 R501X/2282del4, ein 2282del4/R2447X) und 17 heterozygote (10 R501X/wt, 5 2282del4/wt, 2 R2447X/wt), in der Kontrollgruppe 9 heterozygote (5 R501X/wt, 4 2282del4/wt) Individuen. Die kombinierte Prävalenz von *FLG* Funktionsverlust-Mutationen betrug 5 % in der Kontroll- und 9 % in der Atopie-Gruppe. In einer Subtypenanalyse zeigte die Kombination von allergischer Rhinitis und Neurodermitis eine signifikante Assoziation mit *FLG* Mutationen, OR = 3,7 (1,01–12,67; $p = 0{,}024$). Ebenso wurden signifikante Zusammenhänge mit berichteter Familienanamnese von Asthma bronchiale, OR = 4,35 (1,78–10,62; $p = 0{,}0012$), allergischer Rhinitis, OR = 2,33 (1,49–3,63; $p = 0{,}0002$) und Neurodermitis, OR = 5,08 (2,78–9,30; $p \leq 0{,}0001$) gefunden. Im Gegensatz zu klinischen Studien mit prozentuell mehr schwer betroffenen Personen, zeigten *FLG* Mutationen in der vorliegenden Arbeit eine lediglich moderate Assoziation mit atopischen Erkrankungen.

Schlussfolgerung: Fall Selektion könnte zu einer Überschätzung der Prävalenz von *FLG* Mutationen in atopischen Erkrankungen führen.

Summary. *Background:* Recent studies have shown an association of loss-of-function mutations in the filaggrin gene (*FLG*) with ichthyosis vulgaris and atopic eczema (AE). Case selection may have distorted the hitherto reported prevalence of *FLG* mutations and their relation to atopic disease. The aim of the study was to determine the true population prevalence of *FLG* mutations in unselected children with and without reported physician diagnoses of asthma, allergic rhinitis and AE and their relationship with family history of atopic disease.

Methods: We used a nested case-control design by sampling children with reported doctor's diagnoses of AE, asthma and allergic rhinitis and randomly selected controls from a larger cross-sectional study ($n = 1263$). Most common *FLG* mutations R501X, 2282del4, and R2447X were screened in DNA extracted from defrosted urine samples. The relationship of the combined *FLG* variants with atopic diseases and with reported family history of AE, asthma, and rhinitis was assessed.

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Results: In the patient group one homozygote (R501X/R501X), 4 compound heterozygotes (3 R501X/2282del4, one 2282del4/R2447X), and 17 heterozygotes (10 R501X/wt, 5 2282del4/wt, and 2 R2447X/wt), in the control group 9 heterozygotes (5 R501X/wt, 4 2282del4/wt) were detected. The combined prevalence of *FLG* loss-of-function alleles was 5% in the control group and 9% in the atopic sample. In a subgroup analysis, the combination of allergic rhinitis and AE showed a significant relationship with *FLG* mutations, OR = 3.7 (1.01–12.67, $p = 0.024$). Likewise, significant relations with reported family history of asthma, OR = 4.35 (1.78–10.62, $p = 0.0012$), allergic rhinitis, OR = 2.33 (1.49–3.63, $p = 0.0002$), and AE, OR = 5.08 (2.78–9.30, $p \leq 0.0001$) were observed. In contrast to clinical studies with higher percentages of severely affected persons, *FLG* mutations here showed a moderate association with atopic disease.

Conclusions: Case selection may be responsible for overestimating the prevalence of *FLG* mutations in atopic disease.

Key words: Allergic rhinitis, asthma, atopic eczema, filaggrin, public health, Allergische Rhinitis, Asthma bronchiale, Neurodermitis, Filaggrin, Gesundheitswesen.

Abbreviations

AE	Atopic eczema
CAMP	Childhood Asthma Management Program
CI	Confidence interval
DNA	Desoxyribonucleic acid
FeNO	Fractional exhaled nitric oxide
FEV_1	Forced expiratory volume in one second
FLG	Filaggrin
FVC	Forced vital capacity
ISAAC	International Study of Asthma and Allergies in Childhood
MAS	Multicenter Allergy Study
MEF_{50}	Forced expiratory flow at 50% of FVC
OR	Odds ratio
PCR	Polymerase chain reaction
SCORAD	Scoring Atopic Dermatitis system

Introduction

Mutations in the filaggrin gene (*FLG*) proven to impair epithelial barrier function are strongly associated with ichthyosis vulgaris and atopic eczema (AE) [1–8]. In several European studies mainly in children and young adults strong associations of *FLG* loss-of-function alleles with AE, allergic rhinitis, early wheeze, and asthma occurring in the context of AE have been replicated [9–18]. A meta-analysis (nine studies that had been conducted until March 2007) of *FLG* associations with AE revealed an overall odds ratio (OR) for case-control studies of 4.09 (95% confidence interval (CI): 2.64–6.33) and 2.06 (95% CI: 1.76–2.42) for family studies [8]. Some analyses suggested that individuals with AE who carry *FLG* loss-of-function alleles are more likely to show earlier onset [14, 16] and persistent disease [13]. Eventually, *FLG* mutations were associated with greater asthma severity [19] and risk of asthma exacerbations in asthmatic children and young adults [20]. From this overall evidence it has been concluded that *FLG* mutations represent the single most important risk factors for the development of AE and associated illnesses such as asthma and allergic rhinitis [8, 21]. The observed ORs did, however, show a broader range from 3 to 7, with lower risk estimates from apparently less selected, population-based studies. Recently, a clinically well-characterized case-only cohort of white children (CAMP) with mild to moderate asthma confirmed the association of *FLG* mutations and AE using both population-based and family-based tests of association [21]. However, no association of *FLG* loss-of-function alleles with asthma or asthma severity was observed in the absence of AE. This result reminds of a cautious interpretation of earlier studies where subject ascertainment was strongly driven by known family history or by disease severity. It is well known that such studies often overestimate genetic associations due to strong case selection [22, 23]. Moreover, other potential biases can induce false-positive associations [24, 25]. A recent meta-analysis of 24 studies including both case control and family studies provided convincing evidence for a strong association of common *FLG* mutations with AE (OR = 3.12; 95% CI: 2.57–3.79), in particular more severe and dermatologist-diagnosed disease, and asthma in the presence of AE (OR = 3.29; 95% CI: 2.84–3.82) [26].

Since a genuine interest in both, clinical and public health medicine is to better predict the occurrence and the course of these prevalent and costly diseases, broader evidence is needed for *FLG* loss-of-function allele associations with asthma, allergic rhinitis, and AE from true population-based studies. For further clarification of hitherto reported mutant *FLG* associations we used urine samples for genetic analyses from a strict community-based study of schoolchildren with a participation proportion of 85.5%.

Patients, materials, and methods

Study population

As a method of choice [27], a (nested) case-control design was established by sampling all children, aged between 8 and 11 years, from a rural area in the center of Tyrol with International Study of Asthma and Allergies in Childhood (ISAAC) reported diagnoses of AE or asthma or allergic rhinitis as cases ($n = 382$ = "atopic illness") and stratified (after region) randomly selected controls without atopic illness ($n = 200$) from the original cross-sectional study ($n = 1263$). Due to missing information the final sample was reduced to $n = 576$. Complete genotyping information was available for 513 children. Sample size was based on power analyses using reviewed prevalence information provided by Irvine [8]. The study was approved by the ethics committee of the Innsbruck Medical University, the regional school board and all local schools.

Health outcome measures

Since the interest of the main study arm was the interplay between environment, predispositions and atopic disease symptoms, doctor's diagnoses, medications, family history of asthma, allergic rhinitis, and AE were collected from parent reports with a

translated German version of the ISAAC questionnaire [28]. The selection criteria to recruiting a case or a control were positive or negative responses to the following questions. AE: "Has your child ever had atopic eczema?" Asthma: "Has your child ever had asthma?". Further items used in the analyses to determine more recent and severe asthma were "In the last 12 months, has your child's chest sounded wheezy during or after exercise?", "Has your child taken any medication (or inhalation) against wheeze/asthma in the previous 12 months?" and "Has your child ever taken asthma medication?" Allergic rhinitis: "Has your child ever had hayfever?" Health status: "Considering the past 12 months, how would you rate the current health status of your child (poor-less satisfactory-satisfactory-good-very good)?" Further medical information was available through anthropometric measurements (height and weight), standardized spirometry, and fractional exhaled nitric oxide (FeNO) measurements.

Genetic analysis

Stored urine samples (at –80°C) of a volume between 5 and 7 ml were slowly defrosted in a two-step process and delivered to the genetics lab with an anonymized code. Thus, genetic analysis was blindly conducted. Genomic desoxyribonucleic acid (DNA) was extracted by using the GenoM48 automated extractor (Qiagen, Vienna, Austria) according to the protocols of the manufacturer. *FLG* variants R501X, 2282del4, and R2447X were initially screened by restriction enzyme digestion of polymerase chain reaction (PCR) products as described previously [6, 7]. However, most samples were screened for 2282del4 by a novel allel-specific PCR, amplifying a short fragment of 158 base-pairs and therefore leading to higher genotype completion rates. Genomic DNA was amplified by using Promega PCR buffer, Promega Taq polymerase mix, allel-specific forward primer AGAAGACTCAGACACACATTG, and reverse primer GGGAGGACTCAGACTGTTT. Amplification conditions were as follows: 95°C for 3 min; 35 cycles of 95°C for 20 s, 60°C for 30 s, 72°C for 40 s; final extension step at 72°C for 5 min. A fragment of 207 bp was amplified from an unrelated chromosomal region in each reaction to control for PCR success, using forward primer ATTCTTTGTCCCTGGCAGT and reverse primer GACAGTTCCCAAATGACAAGT. In patients showing the 2282del4 mutation a PCR for differentiation between homozygous and heterozygous followed using the same conditions, reverse primer and control fragment primers as described above and the forward primer AGAAGACTCAGACACACAGTTAG. Quality controls were performed for each mutation and in all screening steps with positive control DNA out of blood. All PCRs and digests were resolved on 2–2.5% agarose gels.

Statistical analyses

All analyses were conducted using R version 2.5.1. [29] Specialized R libraries (HMISC, design, epicalc, epi) were applied for the respective analyses. Binary and categorical variables were analyzed using Fisher exact test or Kendall's rank correlation tau. Binary logistic regression analysis was applied to determine the relative contribution of family history versus *FLG* mutations in the prediction of asthma, allergic rhinitis, and eczema in the presence of meaningful covariates. Continuous variables were analyzed by linear ordinary least square regression with adjustments for important variables as described in the respective result section.

Results

A comparison of the baseline characteristics of children with atopic illness (AE, asthma, and rhinitis) and those without (Table 1) shows significantly more male subjects and hours of school absence in the group with atopic illness. Lung function parameters do not indicate any significant difference and the average size also demonstrates that the study base consists of unselected children in good health.

In the patient group we detected one homozygote (R501X/R501X), 4 compound heterozygotes (3 R501X/2282del4, one 2282del4/R2447X), and 17 heterozygotes (10 R501X/wt, 5 2282del4/wt, 2 R2447X/wt), in the control group 9 heterozygotes (5 R501X/wt, 4 2282del4/wt). Genotype completion rates were high for R501X (98.6%) and lower for 2282del4 (89.1%). Only 3 R2447X alleles were detected (100% completion). For this reason we report the following results based on the samples for which a complete genotyping record and other diagnostic information

Table 1. Characteristics of the study population by atopic illness and Filaggrin gene (*FLG*)-status in % or mean (SD)

Variable	Patients with atopic illness (*n* = 382)	Controls without atopic illness (*n* = 192)	Combined *FLG* (*n* = 36)
Age (yrs)	9.4 (0.66)	9.3 (0.68)	9.3 (0.71)
Sex (male)	54.9%*	44.8%	72.2%**
Education (highest)	21.2%	17.1%	22.9%
BMI (height/weight^2)	16.9 (2.6)	16.9 (2.6)	16.8 (1.9)
Birthweight (gramm)	3309 (510)	3243 (503)	3351 (532)
Absence school (hrs)	22.1 (27.0)*	17.1 (17.1)	20.4 (15.3)
FEV_1 (ml)	1916 (31.6)	1867 (29.8)	1893 (31.5)
FEV_1 % FVC	91.2 (6.8)	92.1 (6.5)	88.7 (8.4)
MEF_{50} (ml/sec)	2555 (63.2)	2546 (66.8)	2313 (68.5)

*Significant difference (atopic *vs.* non-atopic); **Significant difference (*FLG vs.* non-atopic).

Table 2. Prevalence of *FLG* mutations in children with atopic illness and controls

Status	R501X	2282del4	R2447X	Combined genotype
	Percent (*n*)	Percent (*n*)	Percent (*n*)	Percent (*n*)
Atopic illness	5 (15)	3 (9)	1 (3)	9 (27)
Controls*	3 (5)	2 (4)	0 (0)	5 (9)

*No asthma, allergic rhinitis, or atopic eczema.

Table 3. Prevalence of family history in children with atopic and non-atopic illness

Status	Family history of asthma	Family history of rhinitis	Family history of atopic eczema
	Percent (*n*)	Percent (*n*)	Percent (*n*)
Atopic illness	13 (44)	41 (139)	22 (74)
Controls*	3 (6)	19 (35)	7 (12)

*No asthma, allergic rhinitis, or atopic eczema.

Table 4. Distribution of *FLG* mutations in children with combinations of illnesses with and without atopic sharing

Genotype	Controls	Asthma only	Rhinitis only	Atopic eczema only	Asthma and rhinitis	Asthma and atopic eczema	Rhinitis and atopic eczema	Asthma, rhinitis and atopic eczema
No mutation	168	15	97	116	9	8	30	10
Combined*	9	1	12	5	0	1	6	2
Total	177	16	109	121	9	9	36	12
Odds ratio		OR = 1.23	OR = 2.3	OR = 0.81	–	OR = 2.32	OR = 3.7	OR = 3.69
95% CI		(0.03–10.12)	(0.86–6.43)	(0.21–2.76)	–	(0.05–20.97)	(1.01–12.67)	(0.34–21.73)
Fisher exact		$p = 0.588$	$p = 0.1$	$p = 0.787$	–	$p = 0.399$	$p = 0.024$	$p = 0.148$

*R501X, 2282del4, R2447X, no differentiation between heterozygotes and homozygotes/compound heterozygotes; *OR* odds ratio.

Table 5. Multiple logistic regression models for asthma, allergic rhinitis, and atopic eczema

Factor	Asthma model		Rhinitis model		Atopic eczema model	
	Odds ratio (95% CI)	*p*-value	Odds ratio (95% CI)	*p*-value	Odds ratio (95% CI)	*p*-value
Sex (female)	0.71 (0.34–1.47)	0.3540	0.75 (0.49–1.14)	0.1770	0.72 (0.47–1.11)	0.1391
Education (high-low)	0.65 (0.21–2.08)	0.6346	1.25 (0.69–2.27)	0.6111	1.11 (0.61–2.02)	0.8251
Family history asthma	4.35 (1.78–10.62)	0.0012	3.30 (1.63–6.66)	0.0009	1.56 (0.77–3.17)	0.2183
Family history rhinitis	1.62 (0.77–3.43)	0.2038	2.33 (1.49–3.63)	0.0002	1.48 (0.94–2.33)	0.0904
Family history atopic eczema	0.89 (0.34–2.35)	0.8213	0.64 (0.34–1.18)	0.1499	5.08 (2.78–9.30)	<0.0001
Proneness to infections	2.15 (1.57–2.96)	<0.0001	1.26 (1.02–1.55)	0.0335	1.07 (0.86–1.32)	0.5575
FLG combined*	0.92 (0.25–3.35)	0.9049	1.22 (0.54–2.75)	0.6333	0.79 (0.34–1.86)	0.5972
Early risk**	1.63 (1.08–2.47)	0.0211	0.92 (0.70–1.21)	0.5317	1.06 (0.81–1.39)	0.6550
TOTAL	$R^2 = 23.9\%$	<0.0001	$R^2 = 13.6\%$	<0.0001	$R^2 = 14.6\%$	<0.0001

*R501X, 2282del4, R2447X; **perinatal risk, low birthweight, smoking during pregnancy/1st year, bronchiolitis/pneumonia 1st year; *CI* confidence interval.

(diagnosis and family history) was available (combined genotype: $n = 312$ with atopic illness, $n = 177$ without). No meaningful changes resulted when alleles were analyzed separately for the 3 distinct mutations. Boys carried significantly more often *FLG* loss-of-function alleles than girls (OR = 3.06; 1.45–6.49; Exact *p*-value: 0.0029). Children taking regularly asthma medication during the last 12 months had greater chances to show up positive too (OR = 2.50; 1.08–5.78; Exact *p*-value: 0.0474). Furthermore, children wheezing while exercising during the previous 12 months also had higher *FLG* rates (OR = 3.89; 1.76–8.62; Exact *p*-value: 0.0018). However, there was no correlation with asthma (OR = 1.10; 0.37–3.24; Exact *p*-value: 0.7782) or AE (OR = 1.11; 0.55–2.22; Exact *p*-value: 0.8578). On the other hand a significant link was established with allergic rhinitis (OR = 2.48; 1.25–4.91; Exact *p*-value: 0.0106).

Table 2 presents the frequencies of mutations in relation to atopic status and Table 3 in relation to reported specific family history stratified by atopic illness. The prevalence of *FLG* null alleles in the atopic sample nearly doubles (5 *vs.* 9%). However, we observed much stronger relations of atopic illness with reported positive family history. In children with reported family history of asthma, AE, and rhinitis the prevalence was 4.3 (respective 3.1 and 2.2) times higher in those with than in those without atopic background (Table 3). In subgroup analyses (Table 4) a significant association with *FLG* mutants is only established with the allergic rhinitis-AE combination. No significant relation is found for AE alone, without asthma or rhinitis. Unfortunately, the asthma subgroups suffer from low statistical power.

In the regression models (Table 5) the importance of family history is evident in all diseases. There is a strong overlap of family histories in allergic rhinitis. Adding a single or combined *FLG* mutation to the model does not make any significant contribution. However, mainly in asthma there are significant associations with exposure to pre-/peri- and postnatal risks and proneness to infections.

Discussion

In this true unselected population from Tyrol we found a *FLG* loss-of-function allele prevalence of approximately 5% in school children without asthma, allergic rhinitis, or AE, which is in conformity with our previous study and one larger population-based study from Germany [7, 10]. However, we could not replicate the much higher prevalences (16.7–56%) reported for the mutant *FLG* alleles in children and adults with asthma, rhinitis, or AE in previous studies as we observed 9% *FLG* mutation alleles in our at risk population (Table 2). On the other hand, we found the prevalence of family histories within a similar range that other studies reported for *FLG* loss-of-function alleles (AE: 22%; asthma: 13%; allergic rhinitis: 41%). The

Table 6. Comparison of available lung function results in *FLG* loss-of-function allele studies

Variable	Present study	Palmer et al. (2007)	Rogers et al. (2007)
	Atopic illness ($n=382$)	Asthma ($n=684$)	Atopic disease ($n=185$)
Mean age in yrs (SD)	9.4 (0.66)	10.4 (4.0)	8.5 (2.2)
Mean percent predicted FVC (SD)	99.0 (11.8)	95.8 (13.8)	–
Mean percent predicted FEV_1 (SD)	105.5 (13.4)	97.3 (15.2)	–
mean FEV_1/FVC (SD)	0.91 (6.8)	0.88 (12)	0.81 (12)

SD Standard deviation.

correlation between family histories and mutant *FLG* alleles was not significant (AE: $p=0.1255$; asthma: $p=0.974$; allergic rhinitis: $p=0.9766$). There may be several reasons for these findings.

To start, we assume that disease severity is important. In the more clinically oriented (so-called population based) studies exhibiting higher frequencies of *FLG* loss-of-function alleles, inclusion criteria for study entry were typically "moderate to severe" disease [10, 21]. In our study every third and fourth grader in a nearly complete cohort of children from 31 communities within a predefined area was included. The expected left shift in the distribution of general disease severity in such an unselected population certainly results in a larger proportion of children with diseases of low to moderate severity. We can provide three types of suggesting evidence for this hypothesis. First, comparing the results of lung function indicators from our study with other studies reporting lung function data, we observed better lung function values in our study sample (Table 6), which supports the argument of a more healthy atopic sample recruited from an unselected community background. In this context it is important to mention that we observed a similar prevalence of AE in our sample in comparison with the "population based" MAS-cohort. Our sample differed, however, significantly with respect to asthma (13.5 *vs.* 21.5%) and allergic rhinitis (35.7 *vs.* 14.1%). This suggests that the more severe inclusion criteria of the MAS-cohort (by an objective Scoring Atopic Dermatitis system (SCORAD) of >15 or involvement of >20% of the body surface) lead to higher inclusion of children with asthma and a lower inclusion of children with allergic rhinitis of less severe course of illness. Second, the finding of a significant relation of *FLG*-mutations in the subgroup of our children with "past 12 months" asthma medication (Exact *p*-value: 0.0474) but not with "ever" asthma medication (Exact *p*-value: 0.7073) supports the severity argument. Those experiencing wheezing during exercise in the past 12 month also show a strong relation with the combined *FLG* loss-of-function alleles (Exact *p*-value: 0.0018). Third, an analysis of the child's health status reported by parents reveals a higher proportion of children rated only in "good" health status who are on "current" asthma medication (31%) or experienced wheezing during exercise in the past 12 month (27%), compared with the overall sample of atopic children (10%). The corresponding proportions for a rating of "less than good" health status were very low and did not really differ (1.5, 1.7, 1.1). Thus, it seems that even those children showing current signs of active illness are only moderately impaired in view of their parents. Eventually, indirect support for the severity argument lends the finding of a very similar prevalence of *FLG* loss-of-function alleles (5%) found in our control population when compared with other true population controls [7, 10].

As children have not been investigated by a dermatologist but by general practitioners and were included with the question "Has your child ever had atopic eczema" a selection bias cannot entirely be ruled out, i.e., the number of cases with AE could be overestimated by accidentally including children with differential diagnoses of AE. However, diagnosis of atopic eczema in childhood is not very difficult and differential diagnoses such as scabies, Netherton syndrome, Wiskott-Aldrich-syndrome, selective IgA-deficiency, or Langerhans cell histiocytosis are rare.

While we found a strong association of atopic diseases with related family history, a much weaker or non-existent association with *FLG* loss-of-function alleles was obtained. Possible reasons for this observation are inadequate statistical power, population stratification and phenotypic misclassification. Although population stratification has been blamed mainly for producing false-positive results in genetic case-control association studies it can also be responsible for masking associations [25, 30]. However, when cases and controls stem from the same population, as in this study population, bias through population stratification is controlled by study design [25].

Low statistical power to detect significant associations is a possible reason in our study since we based the power calculations on the *FLG* allele prevalences provided by Irvine [8] in Table 1, observed in the population-based German MAS cohort [10]. Due to the low frequency of *FLG* mutants in the present study we were only able to show one significant allele association for the allergic rhinitis-AE combination but not for the asthma-AE and the triple combination (asthma+allergic rhinitis+AE). A statistical trend of similar size was also observed in "allergic rhinitis only" where power was reasonably good. Although statistical power was of equal size for AE not combined with asthma or allergic rhinitis, no association was observed with *FLG* loss-of-function alleles. The low combined allele frequency (4%; 5/121) is quite different from the one in the German birth cohort (17.1%; 20/117) or the CAMP cohort in the US-study (14.4%; 25/174). We believe that disease severity plays the decisive role here.

Another possible cause of false-negative results in genetic association studies is phenotypic misclassification. Here, insufficient stringency in the definition of disease may reduce the association in question. The ISAAC questionnaire approach [28] is clearly not able to establish clinically stringent diagnoses; however, the study of the CAMP cohort used a quite similar question for AE and could establish a clear relationship with the *FLG* loss-of-function

alleles [21]. We also evaluated an alternative question ("ever eczema"), which did not change the results. The same argument (misclassification) would likewise apply to the dichotomous questionnaire assessment of the family histories, where strong associations could be observed. Furthermore, multiple logistic regression analyses including family history, combined genotype, other co-determinants and possible confounders did not exhibit any correlation of *FLG* loss-of-function alleles with one of the diseases of the allergic triad.

Eventually, sufficient power was available to evaluate whether decrements in lung function were associated with mutant *FLG* alleles in asthma as shown in some studies. Multiple regression models could not demonstrate any association with several lung function parameters available in this study (basic data shown in Table 1, regression results not shown). Further multiple regression analyses in half of the sample with FeNO data available did not reveal an association with the *FLG* genotypes measured (not shown). Thus, the idea of using *FLG* mutations as clinical indicator of a higher risk with regard to secondary complications could not be supported in this study. It should be stressed that such broad secondary health information on study subjects with *FLG* genotyping data has not yet been provided by other studies.

This study shows that *FLG* mutations are less frequent at the community level where general practitioners deal with the large burden of the allergic triad of less severe disease. Much larger studies would be needed to firmly establish the actual prevalences of *FLG* mutations in unselected populations of different genetic descent. Our study confirmed a higher frequency of *FLG* mutations in children with atopic illness (9%) and a baseline prevalence of approximately 5% in controls without asthma, allergic rhinitis, or AE. On the other hand, subgroup analyses did not reveal a relationship of *FLG* mutants with simple AE, but with allergic rhinitis and AE. The strongly diverging results from family history analyses and *FLG* mutations need further thought about additional mechanisms of action.

Recently it was shown that *FLG* variants are not responsible for the significant genetic linkage signal to the epidermal differentiation complex on chromosome 1q21 alone [3]. An obvious requirement for biological plausibility of the *FLG* associations is the concordance of results from family history and genotyping analyses.

Since this study provides an indication of *FLG* mutations showing stronger predictive power in more severe atopic illness than in less severe illness, further genetic links and epigenetic pathways need to be explored. With the current state of knowledge it seems premature to consider *FLG* genotyping as possible screening method to control atopic disease at the population level.

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Conflict of interest

The authors declare no conflict of interest.

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