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Article (Unspecified)

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Original article

Filaggrin null mutations are associated with increased asthma exacerbations in children and young adults

Background: Filaggrin (*FLG*) null mutations are important genetic predisposing factors for atopic asthma and have recently been shown to influence controller and reliever medication needs in asthmatic children. Our objective was to study the role of *FLG* null alleles in asthma exacerbations.

Methods: *FLG* mutations R501X and 2282del4 were assayed in 1135 individuals ranging from 3 to 22 years old with asthma from Tayside and Dumfries, Scotland. Asthma exacerbations over the previous 6 months were also studied.

Results: The *FLG* mutations were significantly associated with greater risk of exacerbations in children with asthma. Exacerbations were significant for the R501X but not the 2282del4 mutation and the combined genotype compared to the wild-type with odds ratios of 1.97 (95% CI, 1.19–3.22; $P = 0.009$) and 1.61 (95% CI, 1.08–2.40; $P = 0.021$), respectively. Individuals with *FLG* null alleles were more likely to require oral steroids (31.4% vs 19.5%; OR = 1.89; $P = 0.021$) for their exacerbations. There was also a 1.71-fold increased risk (42.6% vs 30%; $P = 0.041$) of school absence owing to asthma exacerbations in asthmatic individuals with *FLG* null mutation. On sub-group analysis, the effect of *FLG* mutations on asthma exacerbations is significant ($P = 0.045$) only for participants with relatively mild asthma controlled on inhaled steroids, with inhaled albuterol according to need.

Conclusion: In addition to their effect on asthma medication requirements reported previously, there is an association between the presence of *FLG* null mutations and the risk of asthma exacerbations in asthmatic children and young adults.

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Key words: asthma exacerbation; child; filaggrin null mutation; oral steroid; school absence.

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Filaggrin is a highly abundant epidermal structural protein facilitating epidermal differentiation and skin barrier formation (1). The filaggrin gene (*FLG*) gene, located on human chromosome 1q21.3, encodes the giant (> 400 kDa) polypeptide profilaggrin, which consists of 10–12 tandemly repeated filaggrin subunits (2). Profilaggrin accumulates in dense granules within the keratinocytes of the stratum granulosum, the last living cell layers of the epidermis. Upon terminal differentiation of these cells to form the stratum corneum, the chemically modified, dead layers of the outermost epidermis, within which the skin barrier function resides, the inert profilaggrin molecule is proteolytically processed into multiple copies of active filaggrin. The liberated filaggrin aggregates the keratin cytoskeleton leading to cell compaction and squame formation. Enzymatic cross-linking of the protein and lipid components of the newly formed squames leads to formation of a chemically impermeable barrier whose

function is to retain water and resist entry of antigens, allergens and irritants from the environment (1). Disruption of barrier formation due to a reduction or complete absence of epidermal filaggrin expression has been postulated to lead to chronic transcutaneous antigen/allergen/irritant transfer, which via a Th2-mediated immune response, leads to atopic eczema and secondary allergic reactions, importantly including atopic asthma (3, 4).

Two independent mutations in the gene encoding filaggrin (*FLG*; R501X and 2282del4), carried by about 9% of people of European origin, result in the loss of processed functional filaggrin in the epidermis (3, 5). These genetic mutations, previously proven to impair the formation of stratum corneum (5), strongly predispose to childhood eczema in several white European populations, where these mutations are prevalent (3, 6–11), including Scottish, English, Irish, Danish and German populations. Analogous mutations leading to loss of function have

been recently reported to be significantly associated with atopic dermatitis and ichthyosis vulgaris in the Japanese population (12) and may even predict more severe and persistent form of atopy (6). Thus, this gene may contribute to atopic disease burden to varying degrees worldwide. Recently, the genetic architecture of filaggrin-related atopy has been shown to consist of a combination of a small number of prevalent null mutations as well as several rare or family-specific mutations (2), as recently reviewed (13).

The combined genotype of the two most prevalent filaggrin variants in Europeans, R501X and 2282del4, was the focus in our original study (3). However, further work suggested that the R501X mutation may have greater penetrance in determining higher serum IgE levels in patients with atopic eczema in comparison to 2282del4 (11). As similar penetrance differences may occur in asthma, we compared the relative effects of the null mutations as well as the combined genotype on the asthma severity outcomes and symptomatic control measures of the BREATHE study. The previous data demonstrated that individuals with *FLG* null alleles have a significantly increased disease burden, both in terms of lung function, the null mutation carriers having greater airway obstruction, and in the intensity of medication required for disease control (14). The individual contribution to the overall signal of the 2282del4 allele was lower than that observed for the R501X mutation (14). However, the association of these mutations with the risk of asthma exacerbations has never been assessed.

In children with asthma, school absences (15), use of short courses of oral steroids (16) and asthma-related hospital admissions (17) represent well-validated measures of asthma exacerbations. We have previously developed a combined score, involving yes/no responses for any of the above three measures of exacerbations over a 6-month period of reporting, to explore asthma exacerbation risk from *PPAR γ* genotype variation (18). Here, we have used this score to compare the relative effects of the two filaggrin mutations and the combined genotype on the risk of asthma exacerbations. We also explored the relative penetrance of the two mutations and the combined genotype on a larger asthmatic population for our study.

Methods

We have continued the recruitment of children with physician-diagnosed asthma for the BREATHE study beyond the publication of our initial results (3). The current dataset includes information about demographic, anthropometric and clinical details from 1135 individuals attending primary and secondary clinics in 29 primary care practices and 2 secondary care asthma clinics in Tayside and Dumfries, Scotland, from 2004 to 2007 (age 3–22 years).

The study was approved by the Tayside Committee on Medical Research and Ethics. Informed consent was provided by the patient and parent/guardian as relevant. The methods have been described in

detail (3, 19). The patients were seen in the asthma clinic setting, where a detailed history was obtained including information on school absences, usage of oral steroids and hospital admissions over the previous 6 months. Eczema status was determined using the question, 'Does the child have eczema?' The asthma prescribing level was determined in accordance with the British Thoracic Society (BTS) (20) guidelines for physician-led management of asthma, as follows: step 0 – no use of inhaled albuterol on demand within the past month; step 1: inhaled albuterol on demand; step 2: regular inhaled steroids plus inhaled albuterol on demand; step 3: regular inhaled salmeterol plus inhaled steroids with inhaled albuterol on demand; step 4: regular inhaled salmeterol plus inhaled steroids plus oral montelukast with inhaled albuterol on demand. From this data, a global index of asthma severity was derived through construction of a composite variable. Pulmonary function was measured by spirometry as per standard procedure described previously (18).

Genotyping for *FLG* R501X and 2282del4 was performed as described in our earlier publication (3). Mutation R501X creates a new *Nla*III restriction enzyme site, and 2282del4 creates a new *Dra*III site, which were used to screen short, highly specific polymerase chain reaction (PCR) fragments for these variants, as described previously (5). Genotyping for R501X was also performed using a TaqMan-based allelic discrimination assay (Applied Biosystems Europe, Warrington, UK). Standard procedures were used based on Applied Biosystems reagents and 10 μ L reaction volumes. Allelic discrimination was assessed using an Applied Biosystems 7700 sequence detection system. Mutation 2282del4 was also genotyped by sizing a fluorescently labeled PCR fragment on an Applied Biosystems 3100 or 3730 DNA sequencer. Ten-micro liter PCR reactions were carried out using primers DEL4.F2 and DEL4.R1 in AmpliTaq Gold buffer containing 1.5 mM MgCl₂ (Applied Biosystems), 10 nmol of each dNTP and 1 unit AmpliTaq Gold DNA polymerase. Reactions were amplified as follows: 94°C (12 min), 1 cycle; 94°C (15 s), 58°C (30 s) and 72°C (45 s), 30 cycles; and 72°C (5 min), 1 cycle. Fragments were diluted 1:60 and sized against ROX-500 size markers according to the manufacturer's recommended protocol (Applied Biosystems). The wild-type allele was 199 bp, and the 2282del4 allele was 195 bp.

AA refers to the wild-type *FLG* genotype for R501X and 2282del4 mutations, Aa refers to heterozygous genotype for either R501X or 2282del4 and aa refers to homozygous R501X or 2282del4 genotype or compound heterozygous genotype. The homozygous, heterozygous and compound heterozygous genotypes were considered together as Aa/aa.

All statistical analyses were performed by using SPSS for Windows version 14 (SPSS Inc., Chicago, IL, USA). To calculate the odds ratios (ORs) for comparison of risk, measures for asthma exacerbations were grouped according to severity. Thus, school absences, intake of oral steroids and admission to the hospital due to severity of asthma were grouped as present (minimum once over the previous 6 months) or absent. The total asthma exacerbation response was calculated as any of these measures during the same period of time. This was again grouped as present or absent. Chi-square test was used to compare the effects of the mutations on total asthma exacerbations as well as its constituent measures. Significance was assessed at $P < 0.05$. Both one-tailed and two tailed P -values are shown due to the predictable nature of the direction of effect of the variants of the traits under test.

Results

The population characteristics are fairly typical of young individuals with well-controlled asthma derived from

both primary and secondary care (Table 1) (21). Figure 1A shows the proportion of population on various stages of management as per BTS guidelines with asthma exacerbations over the previous 6 months. Figure 1B demonstrates frequencies of individuals with *FLG* null mutations with and without asthma exacerbations over the previous 6 months. The allele frequencies of the *FLG* mutations R501X and 2282del4 in children with asthma were increased relative to the Tayside population and it was limited to asthmatic children with a self-reported history of eczema.

Asthma exacerbations were found to be significantly increased in children with *FLG* mutation R501X and the combined genotype. The contingency analysis (Table 2)

Table 1. Characteristics of BREATHE study participants with asthma ($n = 1135$)

With eczema : without eczema ($n = 1125$)	580 : 544
Age	Range: 3–22 (mean, 10.3; SD, 5.1)
Sex (males : females)	671 (59.1%) : 464 (40.9%)
R501X AA : Aa/aa (%)	895 (92.6%) : 72 (7.4%)
2282del4 AA : Aa/aa (%)	856 (94.3%) : 52 (5.7%)
Combined genotype AA : Aa/aa (%)	774 (86.7%) : 119 (13.3%)
School absences (yes/no) over previous 6 months	339/753 (31%)
Courses of oral steroids (yes/no) over previous 6 months	228/895 (20.3%)
Hospital admissions (yes/no) over previous 6 months due to exacerbations	125/998 (11.1%)
Overall asthma exacerbations* (yes/no) over previous 6 months	394/694 (36.2%)
Family history of asthma and eczema	
Paternal asthma (yes/no)	219/901 (19.6%)
Paternal eczema (yes/no)	82/1037 (7.3%)
Maternal asthma (yes/no)	269/850 (24.0%)
Maternal eczema (yes/no)	162/958 (14.5%)
Mean percent predicted FEV1 (SD) ($n = 863$)	95.8 (15.6)
Mean percent predicted FVC (SD) ($n = 862$)	92.2 (14.5)
Mean FEV1/FVC (SD) ($n = 880$)	89.3 (14.8)
BTS asthma treatment steps† ($n = 1116$)	
Step 0	69
Step 1	189
Step 2	610
Step 3	149
Step 4	99
Inhaled bronchodilator use‡ ($n = 1111$)	
0	144
1	763
2	178
3	26

*Defined as any one of the following in previous 6 months: school absences, courses of oral steroids or hospital admissions.

†Step 0 = no use of inhaled albuterol within the past month; step 1 = inhaled β_2 agonists alone; step 2 = step 1 + inhaled steroids; step 3 = step 2 + inhaled long-acting β_2 agonists; step 4 = step 3 + montelukast.

‡Inhaled bronchodilator use: 0 = none, 1 = occasional, 2 = daily and 3 = excessive use.

shows that the heterozygous and homozygous genotypes for the R501X mutation and the combined genotype, were associated with higher risk for exacerbations of asthma. This is significant for the R501X mutation ($P = 0.009$) and the combined genotype ($P = 0.021$; Table 2). Thus, while 35% (301/859) of *FLG* wild-type participants were prone to exacerbations, a significantly

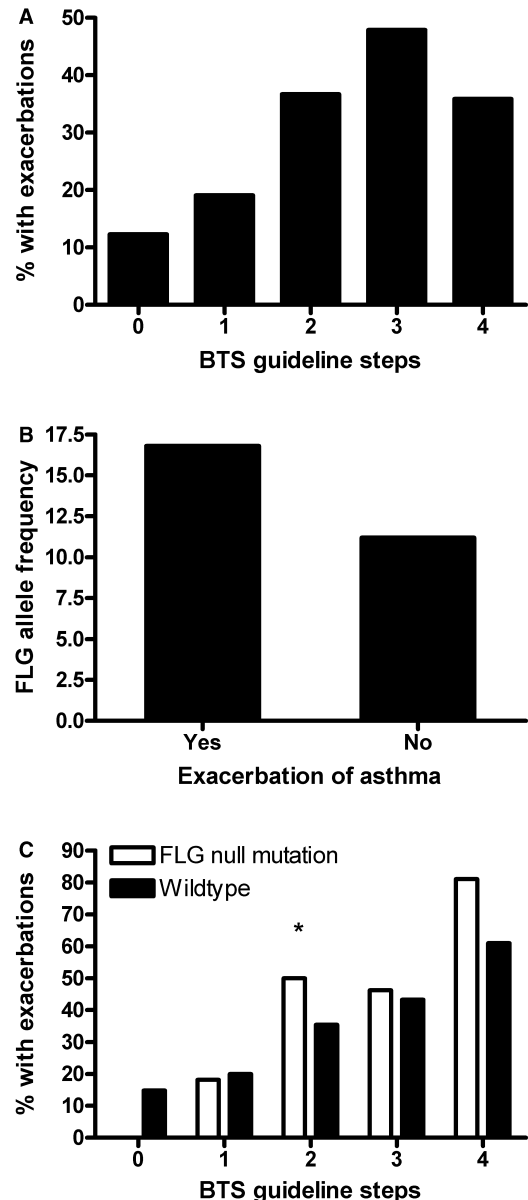


Figure 1. (A) Proportion of children suffering from asthma exacerbations across the stages of management as per British Thoracic Society (BTS) treatment guidelines. (B) Proportion of children with filaggrin allele mutations experiencing asthma exacerbations in comparison to those who did not experience asthma exacerbations over previous 6 months. (C) Proportion of subjects with asthma exacerbations over previous 6 months classified according to genotype and stages of treatment of asthma as per BTS guidelines. * $P = 0.045$.

Table 2. Contingency table for filaggrin genotype (co-dominant and mutant variants) vs exacerbation of asthma. Exacerbation was measured as school absence and/or asthma related hospital admission and/or use of short courses of oral steroids

		Exacerbation			<i>P</i> -value		OR
		Yes	No	Total	(one tailed)	(two tailed)	
R501X	AA	301	558	859	0.006	0.009	1.966 (1.198–3.228)
	Aa/aa	35	33	68			
	Total	336	591	927			
2282del4	AA	295	526	821	0.438	0.764	1.093 (0.607–1.969)
	Aa/aa	19	31	50			
	Total	314	557	871			
Combined	AA	257	486	743	0.013	0.021	1.612 (1.081–2.404)
	Aa/aa	52	61	113			
	Total	309	547	856			

aa, homozygous R501X or 2282del4 genotype or compound heterozygous genotype; Aa, heterozygous genotype for either R501X or 2282del4; AA, wild-type/wild-type *FLG* genotype for R501X and 2282del4 mutation; OR, odds ratio.

greater proportion 51% (35/68) of *FLG* null allele carriers with asthma suffered from exacerbation of their asthma. Hence, there was a 1.97-fold greater risk (95% CI, 1.19–3.22) of suffering from exacerbation of asthma in *FLG* null allele carriers in comparison to *FLG* wild-type participants with asthma.

Individual measures of asthma exacerbations were also found to be significantly increased in children with *FLG* mutation R501X and the combined type. On similar contingency table analysis (Table 3), we found that the heterozygous and homozygous genotypes for the R501X mutation and the combined genotype, were significantly associated with increased intake of oral steroids due to exacerbation of asthma. For the co-dominant model, this is significant for the R501X mutations ($P = 0.021$) and the combined genotype ($P = 0.025$). Significantly, increased absence from school was also noted in the children carrying R501X mutation ($P = 0.041$). Thus, 30.0% (261/862) and 19.5% (173/ 887) of *FLG* wild-type participants were absent from school or required oral steroids due to worsening of their asthma. This compares with 42.6% (29/68) and 31.4% (22/70) of *FLG* null allele carriers experiencing school absences or requiring a course of oral steroids over the previous 6 months. There was a 1.71-fold risk (95% CI, 1.04–2.83) of school absences due to asthma and a 1.89-fold risk (95% CI, 1.11–3.21) of requiring oral steroids to treat exacerbations in this population.

On further analysis, exacerbations of asthma were found to be significantly increased (OR 1.83, 95% CI, 1.013–3.29; $P = 0.045$) in individuals with the *FLG* null alleles compared to *FLG* wild-type only for BTS treatment step 2 (regular inhaled steroids plus inhaled short-acting beta agonists according to need) although a similar trend with greater risk with *FLG* null alleles compared to *FLG* wild-type was observed for BTS steps 3 and 4 (participants on regular inhaled long-acting beta agonists

Table 3. Contingency table for filaggrin genotype (co-dominant and mutant variants) vs individual parameters for exacerbation of asthma

	School absences			Hospital admissions			Oral steroid intake		
			<i>P</i> -value (one tailed)			<i>P</i> -value (one tailed)			<i>P</i> -value (two tailed)
	Yes	No		Yes	No		Yes	No	
R501X	AA	261	601	862	0.026	0.041	1.712 (1.036–2.829)	1.243 (0.598–2.584)	0.021
	Aa/aa	29	39	68	0.026	0.041	1.712 (1.036–2.829)	1.243 (0.598–2.584)	0.021
	Total	290	640	930	0.026	0.041	1.712 (1.036–2.829)	1.243 (0.598–2.584)	0.021
2282del4	AA	259	565	824	0.173	0.345	0.689 (0.354–1.340)	0.724 (0.255–2.057)	0.366
	Aa/aa	12	38	50	0.173	0.345	0.689 (0.354–1.340)	0.724 (0.255–2.057)	0.366
	Total	27	603	874	0.173	0.345	0.689 (0.354–1.340)	0.724 (0.255–2.057)	0.366
Combined genotype	AA	228	518	746	0.229	0.445	1.197 (0.788–1.819)	1.082 (0.581–2.015)	0.025
	Aa/aa	39	74	113	0.229	0.445	1.197 (0.788–1.819)	1.082 (0.581–2.015)	0.025
	Total	267	592	859	0.229	0.445	1.197 (0.788–1.819)	1.082 (0.581–2.015)	0.025

For abbreviations see footnote of Table 2.

with or without montelukast, in addition to regular inhaled steroids and inhaled short-acting beta agonists according to need) (Fig. 1C).

Discussion

Since the completion of our data collection for our initial study (3), we have continued recruiting patients with asthma for the Scottish cohort primarily ascertained with asthma to generate statistical power to investigate further the possible roles of filaggrin gene defects on asthma medication use (14) and, subsequently, risk of asthma exacerbations. Our data demonstrate that individuals with *FLG* null alleles have a significantly increased risk of exacerbations requiring hospital admissions, courses of oral steroids, or experiencing school absences.

On sub-group analysis, the effect of *FLG* mutations on asthma exacerbations is significant only for participants with relatively mild asthma controlled on inhaled steroids, with inhaled albuterol according to need. There is, however, a trend in the direction of greater morbidity in the presence of *FLG* mutations in participants on higher steps of asthma treatment (i.e. additional inhaled long-acting beta agonists with or without oral montelukast; Fig. 1C). This occurs against a background of an overall increasing prevalence of asthma exacerbations with greater asthma medication use (Fig. 1A). The overall prevalence of the *FLG* null alleles was higher in participants reporting asthma exacerbations in the previous 6 months in comparison to those that did not (46.0% vs 34.5%), and this overall difference was significant ($P = 0.01$; Fig. 1B).

The individual contribution to the overall signal (exacerbations) of the 2282del4 allele was lower than that observed for the R501X mutation. In our previous study, we observed a differential penetrance of these two mutations on the requirements for asthma medication, and other studies have seen a lower penetrance of the 2282del4 allele in asthma-related phenotypes, but not eczema-related phenotypes (22). The mechanism of this is not known, but may be related to an as yet uncharacterized functional difference in individuals with the 2282del4 allele which has the potential to encode filaggrin repeats, which is definitely not the case for the R501X allele, which truncates the protein at the beginning of the first repeat. Interestingly, a milder eczema phenotype has been reported for mutations that are much further towards the 3' end of the gene, even although it has proven difficult to detect any functional filaggrin in these individuals (2, 13). Further work is required to delineate possible mechanistic differences in these alleles that may lead to different disease susceptibility. A significantly greater proportion of *FLG* null allele carriers with asthma were on higher BTS treatment steps 3 and 4 (14). Together, the two papers, thus, reinforce the position that epithelial barrier defects resulting from *FLG* mutations have a major

influence on day-to-day aspects of asthma management and control, including overall risk of asthma exacerbations, use of oral steroids, together with 'as required' doses of inhaled bronchodilators (14) and regular asthma medication needs (14). Thus, *FLG* gene status appears to influence the overall burden of disease in asthmatic children and young adults. An understanding of the possible relationship between *FLG* gene defects and asthma thus might unfold newer hypotheses that focussed primary prevention strategies for asthma, may be particularly cost effective and beneficial in specific genotype-stratified populations (23).

Exacerbations cause the greatest concern to individuals with asthma and can be life-threatening. They also account for the largest proportions of health costs of asthma (24). Exacerbations of asthma symptoms diminish the quality of life of the patients and their families (25–27). Asthma exacerbations are triggered by several environmental factors including allergens, air pollutants (28) and respiratory viral infections, rhinoviruses being the most frequent (29–31). The mechanisms of viral-induced asthma exacerbations are different from those with allergen exposure, possibly explaining the degree of refractoriness to inhaled or oral corticosteroids (32, 33). We have discussed the possible up-regulation of epithelial T_H2 -type immunity with preferential activation of the immunological cascade as a likely mechanism for the role of epidermal permeability on asthma medication needs (14). Similar mechanisms could explain the associations between *FLG* gene defects and the increased risk of asthma exacerbations in children and young adults reported in this paper. However, asthma medication requirements do not necessarily reflect the risk of asthma exacerbations (Fig. 1A), while other forms of genetic variation that affect asthma exacerbation risk do not influence asthma medication requirements (18). Other mechanisms could contribute to the overall picture. Thus, keratinocytes differentiated in the presence of IL-4 and IL-13 exhibit significantly reduced filaggrin gene expression, suggesting a regulatory role for the atopic immune response on the skin barrier defect (34). It is thus possible that multiple mechanisms, including possible interactions between IL-13 (35) and filaggrin gene polymorphic variations, could be involved in mediating the observed associations between filaggrin gene defects and the overall burden of asthma [i.e. susceptibility (3), medication requirements (14) and risk of exacerbations].

Using this and other measurement tools for quantifying risk, other forms of genetic variation, such as in the β_2 adrenergic receptor gene, have been shown to influence the likelihood of asthma exacerbations, possibly through an interaction with pharmacological treatments (36–38). We predict that the study of genetic variation in relation to clinical outcomes in asthma will further explain underlying mechanisms for this disease, identify at-risk populations for susceptibility, severity and major life-events, define drug choice, and contribute overall to

significantly improved management strategies for asthma within 5–10 years' time.

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