

Filaggrin Loss-of-Function Variants are Associated with Atopic Comorbidity in Pediatric Inflammatory Bowel Disease

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Background: Pediatric inflammatory bowel disease (IBD) has a high prevalence of coexistent atopy. Filaggrin (*FLG*) loss-of-function variants (null-alleles) are associated with eczema and asthma in association with eczema. The aim was to assess the contribution of *FLG* null-alleles to pediatric IBD susceptibility and to co-existent atopy (eczema, asthma, allergic rhinitis, or food allergy).

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Conflict of Interest: Irwin McLean has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

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Methods: *FLG* variants (R501X and 2282del4) were genotyped in 403 children with IBD, 683 parents, and 996 population controls.

Results: In all, 11% of IBD patients carried at least 1 *FLG* null-allele compared to 11% of population controls ($P > 0.4$). Carriage of 1 or more null-alleles in patients with atopy (present in 52% of IBD patients) differed from IBD patients without atopy (14% versus 6%, $P = 0.01$; odds ratio [OR] 2.4, 95% confidence interval [CI] 1.2–5.1). The effect of *FLG* null-alleles was strongest for eczema (19% versus 7%, $P = 0.0003$; OR 3.3, 95% CI 1.7–6.6) and food allergy (28% versus 8%, $P = 0.0001$; OR 4.5, 95% CI 2.0–10.0). The presence of more than 1 atopic disease tended to increase the associated OR: eczema + asthma (23% versus 7%, $P = 0.001$; OR 3.9, 95% CI 1.6–9.1), eczema + asthma + allergic rhinitis (29% versus 7%, $P = 0.0006$; OR 5.4, 95% CI 1.9–15.4) and eczema + asthma + allergic rhinitis + food allergy (45% versus 6%, $P < 10^{-4}$; OR 12.2, 95% CI 3.2–46.3). Logistic regression analysis of IBD cases confirmed the association of carriage of an *FLG* null-allele with atopy ($P = 0.01$; OR 2.4, 95% CI 1.2–5.1) and co-occurrence of different forms of atopy ($P = 0.003$; OR 3.5, 95% CI 1.5–8.1).

Conclusions: Filaggrin null-alleles have no effect on IBD susceptibility but contribute to coexistent eczema and food allergy.

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Key Words: filaggrin, pediatrics, inflammatory bowel disease, asthma, eczema

The chronic inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are increasingly common causes of morbidity in the Western world.¹ Up to 1 in 250 of the general population is affected by IBD, with about 20% of these presenting during childhood and adolescence.^{2,3} Scotland has one of the highest incidence rates of pediatric IBD in the world.^{4,5} IBD pathogenesis is complex and polygenic, with evidence for the intestinal flora and other environmental factors triggering an inappropriate immune response in a genetically susceptible individual.⁶

Great progress in our understanding of the genetic susceptibility to IBD has been made through the study of common germline variants in genome-wide association

studies (GWAS).⁷ Barrett et al⁸ have recently reported on the meta-analysis of 3 GWAS from North America, the United Kingdom, and Belgium/France: more than 30 loci have now been identified and replicated satisfying stringent genome-wide significance criteria. Novel pathogenic mechanisms are being proposed and genetic evidence is accumulating for the empirical overlap with other immune-mediated diseases such as psoriasis, ankylosing spondylitis, and type 1 diabetes. However, the odds ratios (ORs) associated with these common variants are small (1.1–1.5) and the penetrance of these variants is likely to be very small.⁹

Compared with common variants, rare variants are more likely to have a greater contribution to the individual's genetic susceptibility to common diseases.⁹ Fine mapping of regions identified through previous linkage analysis has elucidated causal variants with a low allelic frequency in 2 genetic success stories which are noteworthy in this context.

In CD, variants of the intracellular pathogen-associated molecular pattern receptor NOD2/CARD15 (especially the 1007 frameshift mutation, allelic frequency in healthy controls <2%, with an OR of 3.99 in the recent meta-analysis and 7.71 in our own childhood-onset CD population) dominate in explaining the variance in genetic risk to CD.^{8,10,11}

Null-alleles of the epithelial barrier protein filaggrin (FLG) have been identified as the strongest genetic factors to date for the development of atopic eczema.^{12–14} These mutations also cause the semidominant skin-scaling disorder ichthyosis vulgaris.^{13,15} While each of the identified *FLG* null-alleles are relatively rare (minor allele frequency <3% in population controls of European ancestry), collectively up to 10% of the population carry 1 or more null-alleles.

FLG is located within the epidermal differentiation complex on chromosome 1q21.3, a gene cluster expressed late in epidermal differentiation.¹⁶ Previous linkage analysis studies have implicated this locus in susceptibility to atopic dermatitis as well as psoriasis and UC.^{17,18}

Filaggrin is a crucial component of the cornified envelope in the outer layer of the epidermis.¹⁹ The cornified envelope is a specialized membrane structure that forms during terminal differentiation of keratinocytes and consists of epidermal proteins that are cross-linked extensively by transglutaminases and enmeshed with keratinocyte-derived lipids, and as such is essential for the barrier function and hydration of the skin.²⁰

Epidemiological as well as genetic evidence is mounting for an important overlap between diseases associated with epithelial barrier dysfunction. Even though atopic disease affects up to 25% of the general population, it is more prevalent still among patients with IBD (e.g., 52% in the Scottish pediatric IBD cohort).^{21–25}

In the present study we aimed to assess the contribution of the *FLG* null-alleles to the genetic susceptibility and phenotype of IBD in the Scottish childhood-onset IBD population. As we have previously shown, this cohort of children with IBD is characterized by a high prevalence of atopic disease.²² Therefore, we also wanted to assess what role *FLG* null-alleles play in the genetic susceptibility to atopic disease in childhood-onset IBD.

MATERIALS AND METHODS

Subjects

A total of 2082 individuals consisting of 403 children with IBD (263 CD, 97 UC, 44 IBD type unspecified [IBDU]), diagnosed before their 17th birthday (median age 11.3 years [8.8–13.1]), their parents ($n = 683$), and 996 unselected pediatric population controls (both >95% Caucasian) were genotyped for *FLG* null-alleles R501X and 2282del4.

IBD was diagnosed by standard criteria.²⁶ Detailed subphenotypic data based on the Montreal Classification have been reported previously.^{27,28} During face-to-face interviews with each family of which a child was diagnosed with IBD, a detailed history was taken by a member of the research team (Linda Smith, R.K.R., or J.V.L.) for the presence of any family-reported atopic disease: asthma, eczema, allergic rhinitis (AR)/hayfever, and food allergy. For asthma, eczema, and allergic rhinitis, parental report of these conditions at any time during the patient's past medical history was noted. Specifically, before defining any cases of food allergy, the food antigen and the allergic reaction after exposure to it were discussed with the family. As all pediatric IBD patients were recruited for an ongoing IBD genetics project, additional food challenges or specific IgE assays were not performed, as they were not part of the original study protocol, which was approved by the local ethics committees at the participating centers (Edinburgh, Glasgow, Aberdeen, and Dundee — LREC 2002/6/18).

DNA Extraction and Genotyping

DNA was extracted from peripheral venous blood by a modified salting out technique or using the Nucleon-kit (Tepnel Life Sciences, Manchester, UK) and resuspended in 1× Tris-EDTA buffer (10 mM Tris, pH 7.5, and 1 mM EDTA) at a final concentration of 100 ng/μL.²⁹

All individuals were genotyped for the *FLG* null-alleles R501X and 2282del4 using the TaqMan system (7900HT sequence detection system; Applied Biosystems, Foster City, CA) at the Epithelial Genetics Group, Human Genetics Unit, University of Dundee. The *FLG* R501X and 2282del4 genotype frequencies in population

controls conformed to the Hardy–Weinberg equilibrium ($P > 0.15$).

The 3 most common NOD2/CARD15 polymorphisms (R702W, G908R, and Leu1007fsinsC) were previously genotyped.¹⁰

Power Calculations

Power calculations were performed using QUANTO v. 1.2 (<http://hydra.usc.edu/gxe>).^{30,31} Based on the most recent prevalence data of Scottish pediatric IBD (23/100,000 of the population <17 years), our case–control analysis in early-onset IBD had 80% power to detect an effect with an OR 1.41 or greater.³²

Statistical Analysis

Allelic, genotype, and carriage of any null-alleles frequencies were compared between cases and controls using Pearson's χ^2 or Fisher's Exact test (when $n < 6$) (Minitab software, Release 13.20, State College, PA, and GraphPad Instat software v. 3.06, San Diego, CA). A Transmission Disequilibrium Test (TDT) analysis was performed using PLINK software (<http://www.journals.uchicago.edu/doi/abs/10.1086/519795>).³³

Uncorrected P -values are given. In IBD and CD, multifactorial analysis (correcting for NOD2/CARD15 variant carriage) was performed using Minitab. The Mann–Whitney U -test was used to assess the relationship between carriage of an *FLG* null-allele with z-score of height, weight, and body mass index (BMI) at diagnosis. Z-scores were calculated using nomograms from the British 1990 growth reference (Child Growth Foundation, London, UK).

RESULTS

IBD Susceptibility and Genotype–Phenotype Analysis

Association analysis based on allelic, genotype, or carriage of at least 1 *FLG* null-alleles frequencies showed no effect of *FLG* null-alleles on IBD, CD, UC, or IBDU susceptibility (Table 1). Family-based association analysis showed no distortion of transmission of either the R501X or 2282del4 null-alleles in IBD, CD, or UC (R501X: all $P > 0.50$; 2282del4: all $P > 0.40$).

Detailed genotype–phenotype analysis based on the Montreal Classification, with stratification of the CD cases for the presence of atopic disease, showed no effect of *FLG* null-alleles on CD location or behavior (data not shown). In IBD and CD cases, carriage of an *FLG* null allele had no effect on z-scores of height, weight, and BMI at diagnosis (data not shown).

TABLE 1. Results of Case-Control Analysis of *FLG* R501X, *FLG* 2282del4 and Carriage of Either *FLG* Null Variant in Childhood-Onset IBD, CD, UC, and IBDU

Filaggrin R501X		Controls		IBD	P	CD	P	UC	P	IBDU	P
Wildtype=1 Null (X)=2											
Genotype frequencies	11	941/996 (94.5%)	354/381 (92.9%)	0.27	224/245 (91.4%)	0.07	87/92 (94.6%)	0.97	32/33 (97.0%)	0.53	
	12	53/996 (5.3%)	26/381 (6.8%)	0.28	21/245 (8.6%)	0.05	4/92 (4.3%)	0.68	1/33 (3.0%)	0.56	
	22	2/996 (0.2%)	1/381 (0.3%)	0.82	0/245	0.48	1/92 (1.1%)	0.12	0/33	0.79	
	2	57/1992 (2.9%)	28/762 (3.7%)	0.26	21/490 (4.3%)	0.10	6/184 (3.3%)	0.75	1/66 (1.5%)	0.51	
Allele											
Filaggrin 2282del4		Controls		IBD	P	CD	P	UC	P	IBDU	P
Wildtype=1 delACTG=2											
Genotype frequencies	11	899/951 (94.5%)	366/381 (96.1%)	0.24	233/244 (95.5%)	0.54	88/92 (95.7%)	0.64	34/34	0.16	
	12	52/951 (5.5%)	15/381 (3.9%)	0.24	11/244 (4.5%)	0.54	4/92 (4.3%)	0.64	0/34	0.16	
	22	0/951	0/381		0/244		0/92		0/34		
	2	52/1902 (2.7%)	15/762 (2.0%)	0.25	11/488 (2.3%)	0.55	4/184 (2.2%)	0.65	0/68	0.16	
Allele											
Filaggrin Null allele carriage		Controls		IBD	P	CD	P	UC	P	IBDU	P
At least one null allele*											
At least one null allele*		103/944 (10.9%)	40/378 (10.6%)	0.86	31/243 (12.8%)	0.41	8/91 (8.8%)	0.53	1/33 (3.0%)	0.14	

*Out of 378 IBD patients, there were only 2 compound heterozygotes (1 CD/1 UC).

TABLE 2. Association Analysis of *FLG* Null Variant Carriage in Childhood IBD, Stratified for the Presence of Atopic Disease

Filaggrin Null Carriage in Childhood IBD	Absent	Present	<i>P</i>
Atopy	11/179 (6.1%)	27/195 (13.8%)	0.01 OR 2.45 (1.18–5.11)
Eczema	17/254 (6.7%)	21/109 (19.3%)	0.0003 OR 3.33 (1.68–6.60)
Asthma	25/271 (9.2%)	13/92 (14.1%)	0.18
Hayfever	28/289 (9.7%)	10/74 (13.5%)	0.33
Food allergy	26/322 (8.1%)	11/39 (28.2%)	0.0001 OR 4.47 (2.00–10.00)
Eczema + asthma	15/210 (7.1%)	11/48 (22.9%)	0.001 OR 3.86 (1.65–9.08)
Eczema + asthma + hayfever	13/184 (7.1%)	7/24 (29.2%)	0.0006 OR 5.42 (1.90–15.41)
Eczema + asthma + hayfever + food allergy	11/172 (6.4%)	5/11 (45.5%)	<0.0001 OR 12.20 (3.21–46.34)

Atopy in Childhood-Onset IBD

Asthma was present in 25% of IBD patients, eczema in 29%, allergic rhinitis in 21%, and food allergy in 12% of IBD cases. In total, 52% of IBD patients had at least 1 manifestation of atopic disease.

In IBD and CD cases, carriage of an *FLG* null-allele differed significantly between patients with and those without coexistent atopic disease (13.8% [27/195] versus 6.1% [11/179], $P = 0.01$; OR 2.45, 95% CI 1.18–5.11, and 16.3% [22/135] versus 7.6% [8/105], $P = 0.04$; OR 2.36, 95% CI 1.01–5.54, respectively).

The atopic manifestations driving this association in both IBD and CD cases were eczema and food allergy (see Table 2 and Suppl. Table). In IBD cases, stratified for the presence of eczema, carriage of an *FLG* null-allele occurred in 19.3% (21/109) of IBD + eczema cases compared with 6.7% (17/254) of IBD without coexistent eczema ($P = 0.0003$; OR 3.33, 95% CI 1.68–6.60). We also observed a significant difference of carriage of *FLG* null-allele between the subgroup of IBD + eczema patients and population controls (19.3% versus 10.9%, $P = 0.01$; OR 1.95, 95% CI 1.16–3.27).

Stratifying IBD cases for the presence of food allergy demonstrated the association of coexistent food allergy in IBD with carriage of an *FLG* null-allele (28.2% [11/39] versus 8.1% [26/322], $P = 0.0001$; OR 4.47, 95% CI 2.00–10.00). The frequency of carriage of an *FLG* null-allele differed significantly between IBD + food allergy cases and population controls (28.2% versus 10.9%, $P = 0.0009$; OR 3.21, 95% CI 1.55–6.64).

Stratification for the presence of asthma and allergic rhinitis did not show a different carriage frequency of *FLG* null-alleles between IBD cases affected by asthma or allergic rhinitis and IBD cases without coexistent asthma or allergic rhinitis.

We demonstrated that atopic comorbidity additional to the presence of eczema in IBD cases tended to increase

the effect size (OR) of the association (Table 2). Carriage frequency of an *FLG* null-allele was significantly different between:

- 1) IBD patients with co-existent asthma and eczema (22.9% (11/48) vs IBD without asthma and eczema 7.1% (15/210), $P = 0.001$; OR 3.86, 95% CI 1.65–9.08, and vs population controls 10.9%, $P = 0.01$; OR 2.43, 95% CI 1.20–4.91).
- 2) IBD + asthma + eczema + allergic rhinitis (29.2% (7/24) vs IBD without asthma, eczema and allergic rhinitis (7.1% (13/184), $P = 0.0006$; OR 5.42, 95% CI 1.90–15.41, and vs population controls 10.9%, $P = 0.005$; OR 3.16, 95% CI 1.36–8.30).
- 3) IBD + asthma + eczema + allergic rhinitis + food allergy (45.5% (5/11) vs IBD without asthma, eczema, allergic rhinitis, and food allergy 6.4% (11/172), $P = 0.0009$; OR 12.19, 95% CI 3.21–46.35, and vs population controls 10.9%, $P = 0.004$; OR 6.80, 95% CI 2.04–22.69).

In the subgroup of childhood-onset CD, carriage of a *FLG* null-allele differed between cases also affected by eczema or food allergy and CD cases without (20.3% (16/79) vs 8.7% (14/161), $P = 0.01$ OR 2.67, 95% CI 1.23–5.79 and 29.0% (9/31) vs 9.6% (20/208), $P = 0.002$ OR 3.85, 95% CI 1.56–9.48, respectively). (See Supplementary Table for *FLG* association analysis of subgroup of childhood-onset CD).

In childhood-onset CD, carriage of an *FLG* null-allele differed between cases also affected by eczema or food allergy and CD cases without (20.3% [16/79] versus 8.7% [14/161], $P = 0.01$; OR 2.67, 95% CI 1.23–5.79 and 29.0% [9/31] versus 9.6% [20/208], $P = 0.002$; OR 3.85, 95% CI 1.56–9.48, respectively) (see Suppl. Table). Like in IBD overall, the coexistence of different manifestations of atopy in addition to eczema and food allergy tended to increase the effect size associated with the association: asthma + eczema ($P = 0.007$; OR 3.54, 95% CI 1.35–

9.30), asthma + eczema + allergic rhinitis ($P = 0.0005$; OR 7.00, 95% CI 2.07–23.71), and asthma + eczema + allergic rhinitis + food allergy ($P = 0.004$; OR 12.12, 95% CI 2.54–57.85). Like in IBD, these same associations were also seen when comparing pediatric CD cases with increasing atopic comorbidity in addition to eczema with population controls (data not shown).

Multifactorial analysis (also correcting for carriage of any of the 3 CD-associated variants of NOD2/CARD15) in both IBD and CD cases showed an association of carriage of an *FLG* null-allele with coexistent atopic disease ($P = 0.01$; OR 2.4, 95% CI 1.2–5.1) and co-occurrence of different forms of atopy ($P = 0.003$; OR 3.5, 95% CI 1.5–8.1). These associations did not alter when NOD2/CARD15 variant carriage and atopic disease were entered as interacting variables in the logistic regression model.

DISCUSSION

We report the first study into the contribution of *FLG* null-alleles to childhood-onset IBD susceptibility and to the high prevalence of coexistent atopic disease in childhood-onset IBD. We have demonstrated that the 2 most common *FLG* null-alleles and carriage of at least 1 *FLG* null allele are not associated with increased inherited risk of developing IBD per se. Our study was adequately powered to detect effect sizes greater than OR 1.40.

Our study is consistent with a previous report in adult-onset IBD by Ruether et al³⁴ in a population from Northern Germany, also showing no effect of these variants on disease susceptibility. These null-alleles have also been studied in psoriasis and rheumatoid arthritis (RA).^{35–37} Autoantibody formation to citrullinated (pro)filaggrin has been proven to be a highly specific serological marker for RA and the *FLG* R501X and 2282del4 null-alleles may contribute to the development of humoral autoimmunity against citrullinated peptides.³⁸ However, as in psoriasis, the *FLG* null-alleles do not contribute to the genetic susceptibility of RA.^{35–37,39}

FLG null-alleles have consistently been shown to be a major genetic risk factor for eczema as well as asthma occurring in the context of atopic eczema.^{13,40–55} A recent meta-analysis has added further support to the association with eczema.¹⁴

We have shown that *FLG* null-alleles influence coexistent atopic disease in IBD, particularly eczema and food allergy, with the novel finding of an increased effect size (OR) with increasing atopic disease-load. Although decreasing numbers in the subgroups of IBD patients with more atopic comorbidity lead to a widening of the confidence intervals associated with the increments in the corresponding ORs, this trend is noteworthy.

The question whether *FLG* null mutations are associated with IBD, independent of their effect on atopy (or more specifically atopic eczema), could not be addressed in

this study due to detailed atopic data not being available for our control cohort. It is noteworthy that the estimated incidence of atopic eczema in our birth cohort (based on epidemiological data for the region) is around 25%, compared with a reported incidence of 29% in our IBD cohort.^{48,56} An alternative assessment could be made by comparing our data of *FLG* null mutation carriage in IBD patients with eczema and associated asthma with other cohorts recruited in the Scottish association studies for atopic eczema and associated asthma. The contribution of the 2 *FLG* null-alleles studied in the present report to atopic comorbidity in childhood-onset IBD is comparable to that in the non-IBD population. In the study by Palmer,⁴⁰ 22.9% (64/279) of patients affected by asthma and eczema carried at least 1 null-allele. This is identical to the percentage of IBD patients affected by asthma + eczema carrying at least 1 *FLG* null allele in our study (11/48). Therefore, other determinants (genetic/environmental) likely underlie the higher prevalence of atopic disease in IBD.

It is also pertinent to address the issue of multiple testing in our association study. Although some of the *P*-values would not withstand stringent Bonferroni-correction for multiple testing, the clear interdependence of the phenotypes studied would make this type of correction too conservative. We have therefore opted to present the data uncorrected and expanded our analysis with a logistic regression analysis, also controlling for NOD2/CARD15 variant carriage.

Of note in our study is the strong independent association of food allergy with carriage of a null-allele in the context of IBD. The expression of (pro)filaggrin in the gastrointestinal tract could potentially shed further light on this association. De Benedetto et al⁵⁷ recently demonstrated that *FLG* is not expressed in the esophagus, whereas our own data suggest *FLG* is expressed in the human colon (W.H.I. McLean and A.T. Evans, in prep.). The parallels with the absence of filaggrin expression in the bronchial mucosa and the strongest effect on asthma in the context of atopic dermatitis are striking.⁵⁸ Allergic sensitization through an altered epithelial barrier (either via the skin or the gastrointestinal tract [colon]) could conceivably result in local symptoms (in the case of eczema and colitis, e.g., secondary to cow's milk protein allergy [CMPA]) and only secondarily in organs where there is no demonstrable *FLG*-related epithelial barrier defect via an upregulated adaptive immune response (e.g., atopic asthma and CMPA-driven gastroesophageal reflux disease).²¹

In summary, we have demonstrated that carriage of *FLG* null-alleles influences the high prevalence of atopic comorbidity in childhood-onset IBD without a significant contribution to the inherited susceptibility of IBD itself. As the contribution of *FLG* null-alleles to atopic comorbidity in pediatric IBD appears to be similar to that in the non-

IBD population, other determinants (genetic/environmental) likely underlie the higher prevalence of atopic disease in IBD. The identification of relatively rare variants as major determinants of inherited susceptibility in both eczema (*FLG*) and CD (*NOD2/CARD15*) illustrate the need for detailed resequencing and fine mapping of the loci identified in the recent series of GWAS. The epidemiological evidence for clustering of diseases within families and in individual patients will undoubtedly continue to prompt interest in molecular pathways, identified in coexistent immune-mediated diseases.

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