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The persistence of atopic dermatitis and Filaggrin mutations in a US longitudinal cohort

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Abstract

Background—Atopic dermatitis (AD) is a common skin disease that is characterized by recurrent episodes of itching. Filaggrin loss-of-function mutations (*FLG null*) have been associated with an increased risk of developing AD.

Objective—To evaluate the effect of individual *FLG null* mutations on the persistence of AD over time.

Methods—We evaluated a multiyear prospective cohort study of children with AD with respect to *FLG null* mutations (R501X, 2282del4, R2447X, and S3247X). We evaluated the association of these mutations with the persistence of AD symptoms over time, with respect to reports of no symptoms of AD and whether topical medication was needed for symptom resolution.

Results—857 subjects were followed for 3,684 person-years. One or more *FLG null* mutations were noted in 16.3% of subjects and specifically in 27.5% of whites and 5.8% of African-Americans. Individuals with a *FLG null* mutation were less likely [OR: 0.54 (95% CI: 0.41, 0.71)] to report that their skin was symptom-free at any time as compared to those without a *FLG null* mutation. The effect of these mutations was similar in whites [0.42 (0.31, 0.57) and African-Americans 0.53 (0.25, 1.12) (p=0.62)]. Children with the R501X mutation [0.44 (0.22, 0.88)] were the least responsive to therapy.

Conclusions—In a US cohort with AD, *FLG null* mutations were common. Children with *FLG null* mutations were more likely to have persistent AD. Although, these mutations were more common in those of European ancestry, their effect on persistence was similar in those of African ancestry. Response to therapy was not uniform among children with *FLG null* mutations.

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Keywords

Atopic Dermatitis; Filaggrin; Epidemiology; Genetic Epidemiology; Atopy

Introduction

Atopic dermatitis (AD) is a common, chronic, relapsing skin disease that often begins in early childhood and is characterized by recurrent episodes of itching. Many with AD also suffer from asthma and allergic rhinitis⁽¹⁻⁴⁾. The prevalence of AD has risen dramatically in the past 60 years and this increase has been well documented by an international work group called ISAAC (International Study of Asthma and Allergies in Childhood). In the US, AD affects 10–20% of all children^(1;2). However, reliably identifying affected patients with AD and prognosticating on the natural history of their illness can be difficult. Clues on the severity and persistence of AD have centered on a better understanding of the pathogenesis of this disorder and not on patient centered outcomes.

In the past, most investigations on the etiology and the pathogenesis of AD had centered on immunologic mechanisms and immunologic evaluations^(1;2). Starting in 2006 several seminal studies were published that revolutionized our understanding of the pathophysiology of AD. These studies described a defect in the skin barrier due to loss-of-function mutations in the gene responsible for the production of a protein called filaggrin⁽⁵⁾. Filaggrin, or filament-aggregating protein, is encoded by the *FLG* gene as the precursor profilaggrin, which is later intracellularly processed to filaggrin⁽⁵⁻⁷⁾. The gene is located within the epidermal differentiation complex (EDC) with 30 other genes on chromosome 1q21. In 2006, it was first demonstrated that the *FLG* gene was associated with ichthyosis vulgaris and then with AD⁽⁷⁾. Elucidating the association between *FLG* and AD was complicated because *FLG* was technically difficult to sequence and study using linkage-disequilibrium based techniques due to its location in the EDC (i.e., the 30 other genes of close proximity are also associated with keratinocyte development thus creating the potential for linkage disequilibrium to exist between many genes and AD)^(2;8-10).

Carrying a *FLG* null mutation has since been estimated to increase the odds of having AD by more than 3-fold⁽⁵⁾. The type of *FLG* loss-of-function mutations (*FLG null*) varies by population and country studied with the majority of those currently studied being of European origin^(6;7;11-14). Few large studies have been conducted on the heterogeneous US population and none have specifically evaluated the prognosis of those who have AD and a *FLG null* mutation with respect to patient centered outcomes concerning skin symptoms and the need for topical treatment.

The Pediatric Eczema Elective Registry (PEER), www.thepeerprogram.com, is an ongoing prospective 10-year observational registry that is part of a long-term safety post-marketing commitment by Valeant Pharmaceuticals International (formally the responsibility of Novartis) to the FDA and the European Drug Agency^(13;15;16). The objective of the PEER study is to evaluate long-term consequences of the use of pimecrolimus cream 1% in real-world settings^(5;17-19). All children enrolled in this nationwide registry have physician-confirmed diagnosis of AD that prior to enrollment was severe enough to require at least 6 weeks of treatment with topical medication. Because of this FDA mandate, this unique community-based dataset contains longitudinal information on AD as diagnosed by local care providers, information on the natural history of AD and the persistence of the clinical signs and symptoms of AD, information on other atopic illnesses associated with AD and information on the therapy used to treat AD.

To that end we identified *FLG null* mutations in a subcohort of our ethnically diverse PEER population and evaluated the persistence (or severity) of AD over time^(12;18).

Methods

Population

All children enrolled in our study were participants in the PEER study. Children were recruited nationwide by more than 1000 practicing Dermatologists, Allergists, Primary Care Providers, and Pediatricians. To be eligible for the PEER study, a child must have been diagnosed with AD, been more than two years of age at the time of enrollment, and used at least 6 weeks of topical therapy including pimecrolimus in the six months prior to enrollment. All subjects were cared for by their healthcare provider and the care was not based on a study protocol. Specifically, once a child was enrolled in PEER there was no requirement or expectation that they receive pimecrolimus. Data were collected every six months and, in most cases, the parent of the enrolled participant did this by completing a questionnaire. All participating children completed an additional informed consent approved by the Institutional Review Board of the University of Pennsylvania and provided a saliva sample from which DNA was extracted. Information on the enrollment process and the subcohort that provided a biospecimen has been previously published^(20;21).

Outcome

Two clinically important outcomes were measured from survey responses: 1) symptom free (i.e., if they reported their skin to be “clear” of findings of AD for the previous 6 month period); and 2) symptom free without using medications (i.e., if they reported that their skin was “clear” of findings of AD and that they had not used any medications to treat AD or maintain this response in the previous 6 month period).

Data Collection

The survey instruments used in PEER have been reported elsewhere^(20;22). Briefly, items pertinent to our study included questions on AD status and severity, medication use, related illnesses such as asthma and allergic rhinitis, and socio-demographic variables. Questions were modeled after the UK Working party’s Diagnostic Criteria for AD^(3;23). Questions on asthma, allergic rhinitis, and other allergic illnesses, were adopted from ISAAC^(4;20–22). Questions on AD status, AD severity, and medications used to treat AD were repeated every 6 months. For our current study, we had access to all survey materials collected by PEER.

Genotyping

The four most prevalent *FLG null* mutations, R501X (rs61816761), 2282del4, R2447X, and S3247X, were genotyped using custom-made TaqMan allelic discrimination assays (Applied Biosystems, Foster City CA) using previously published protocols^(20–22;24;25).

Because of concerns about the variability of *FLG* mutations with respect to race/ethnicity, ancestral race was inferred for all study subjects using a panel of ancestry informative markers (AIMS) similar to the panels implemented by Reiner *et al* and Stefflova *et al*^(5;19;26). AIMS genotyping was conducted using Illumina’s GoldenGate technology. Genetic ancestry was inferred using clustering techniques as implemented in STRUCTURE^(27–30).

Analysis

AD is a persistent waxing and waning disease. Our primary question was to investigate the association of *FLG null* mutations with the persistence of symptoms of AD using data

collected every six-months. This distinguishes us from previous studies because we accounted for longitudinal evaluations at multiple time points (starting with time of entry into the study) and defined improvement based on reports of absence of clinical disease or absence of disease in the setting of no treatment used^(31–33). In the past, persistence was usually based on a single report of absence of cutaneous symptoms thereby not allowing for subsequent exacerbation of skin symptoms⁽³³⁾. In addition, subjects in our study reported the presence or absence of skin symptoms on more than one occasion thereby mimicking the natural clinical course of AD.

In our primary analysis of *FLG* null mutations, we assumed an unadjusted additive genetic model. The *FLG* variants were evaluated independently and, as is common in the literature, jointly as “any *FLG* null mutation”^(5;34;35). For this outcome, subjects were categorized as: not having any *FLG* null mutations, having one mutant allele, or having more than one mutant allele^(5;34;35).

In order to investigate the association between *FLG* null mutations and repeated measures of persistence of disease, we used generalized linear latent and mixed models (GLLAMM) with a binary, logistic link function and a binomial family. GLLAMMs have both random effects and fixed effects terms, allowing for subject-specific regression coefficients or subject-specific estimates of the association of a risk factor with an outcome^(17;18). Secondary analyses were conducted using proportional hazards regression (to investigate time to symptom-free status), Poisson regression (to investigate rate of symptom-free status), logistic regression (to investigate any period of symptom-free status), and multilevel mixed-effects logistic regression (to compare to GLLAMM). We also investigated an outcome more similar to previous publications (e.g., based on no reported disease for one year)⁽¹⁸⁾. A p-value of < 0.05 was considered statistically significant and all effect estimates are presented with 95% CIs. All analyses were conducted using STATA 12.1 or PLINK⁽³⁶⁾.

Results

DNA was available from 857 PEER participants who were followed on average for 4.32 ± 1.79 person-years or, in total, for 3,684 person-years. A full description of the PEER participants has been previously published and shows no clinically important differences between those who provided DNA and those who did not⁽²¹⁾. In our study, the average age of disease onset was 2.1 ± 2.7 years. Females represented 51.9% of the cohort and 43.2% were self-described as African-American. Table 1 contains a more complete report of demographic and descriptive information. There was very tight concordance between self-reported ethnicity and genetically-inferred race based on AIMS (area under the receiver operator curve was 0.97 for genetically determined European ancestry versus those reporting they were white and 0.98 for genetically determined African ancestry versus those reporting that they were African-American). Only nineteen children were self-reported Asian which was too small a sample to separately analyze. At the time of enrollment in PEER, all participants had active disease that required topical treatment. However, 81.4% of subjects reported that their skin was free of symptoms associated with AD and 25.6% reported that their skin was symptom free without AD medication use for at least a one 6-month period (i.e. one survey) during their full follow-up period.

Study participants were genotyped for the four *FLG* null mutations (R501X, 2282del4, R2447X, and S3247X) and the allelic frequencies are presented in Table 1 and 2. Overall, any *FLG* null mutation was noted in 16.2% of subjects (Table 1). For the two most prevalent mutations (R501X and 2282del4), at least one null allele was noted in 8.3% and 6.8% of the children, respectively (Table 1). For all variants of *FLG*, the prevalence of these mutations was higher in whites, with 27.5% of Whites and 5.8% of African-Americans having at least

one *FLG null* mutation (Table 1). Allelic variation as percent of the minor allele frequency (MAF) per *FLG null* mutations is presented in Table 2. Variation was less common in African-Americans.

Tables 3 and 4 display the associations between *FLG null* mutations and the persistence of AD skin symptoms. Individuals with *any FLG null* mutation were less likely [OR: 0.54 (95% CI: 0.41, 0.71), $p < 0.0001$] to report that their skin was symptom-free at any time during the study observation period as compared to those without a *FLG null* mutation (Table 3 and Figure 1). The effect estimates for the likelihood that a subject with *any FLG null* mutation reported that their skin was symptom-free were similar: (0.42 (0.31, 0.57) versus 0.53 (0.25, 1.12) ($p = 0.62$) for whites and African-Americans, respectively (Table 3) as compared to those who did not have a *FLG null* mutation. With respect to individual mutations, the effect estimates were similar with odds ratios that varied from 0.47 to 0.77 and, in general, were not confounded by age, gender and ancestral race and the findings did not interact with race ($p > 0.20$).

There was, however, variation in the relationship between *FLG* mutations and those that reported that their skin was symptom-free and they were not using medications to treat AD (Table 4). Overall, the presence of *any FLG null* mutation was not associated with this more specific outcome [1.26 (0.90, 1.77)] as compared to those who did not have a *FLG* mutation. In other words, among a group of individuals with AD, those with *any FLG null* mutation were as likely as those without these mutations to be clear of symptoms and not require the use of AD therapy. However, this finding did vary by the location of the *FLG* mutation. For example, those with the R501X mutation [0.44 (0.22, 0.87)] were unlikely to achieve symptom free skin without topical treatment. Those with the 2282del4 and S3247X [(1.69 (1.01, 2.84); 3.56 (1.69, 7.52), respectively] mutations were more likely to report that their skin was symptom-free and that they did not use AD treatment as compared to those without these mutations. Furthermore, those with the R501X variant were almost two times more likely to use topical steroids during any 6-month interval (2.14 (1.5, 3.02)) as compared to all others in our cohort. No other variant was associated with increased use of topical steroids and none was associated with increased use of topical calcineurin inhibitor (a treatment that has become much less prevalent over time) while enrolled in the PEER study.

These findings with respect to the genetic variation of *FLG* and likelihood that a subject noted skin free of symptoms and skin free of symptoms not requiring the use of medication were tested by several secondary analyses to assess the sensitivity of our findings. These secondary analyses included the use of Poisson models, proportional hazards models, linear regression models, and multilevel mixed-effects logistic regression models. Furthermore, we also evaluated other assessments of persistence of AD, like the percentage of times a subject noted that their skin was not clear at a study visit, the total time an individual had AD, and whether the subject noted that they were symptom free at any visit or for two consecutive visits (i.e. clear one year). None of these evaluations changed our basic conclusions and are therefore not presented.

Discussion

The association between *FLG null* mutations and the risk of atopic dermatitis is well studied and is one of the most replicated with respect to genotype and phenotype for a complex trait like AD^(5;13;37;38). However, we had a different perspective. We evaluated the expected prognosis due to *FLG null* mutations among a community based, physician-diagnosed AD cohort who were receiving continuous care. Our US based cohort is longitudinal, multicultural, and contains more than 3500 person-years of follow up and our evaluations are based on continuous reports from our subjects. *Any FLG null* mutation was noted in

about 16% of our cohort of children with AD. The mutation panel that we studied was almost six times more common in whites than in African-Americans, but overall the influence of these combined mutations was similar in these two groups. Children in our study with *FLG* mutations were about 50% less likely than those without mutations to have 6-month time-periods when their skin was free of symptoms. This finding was not influenced by race. In addition, we explored whether achieving this outcome was dependent on the need to use medications. The effectiveness of medication did seem to vary by mutation. In whites, those with the 2282del4 and S3247X mutations were much more likely to clear their rash and no longer require the use of medication as compared to those with the R501X (rs61816761). Due to the rarity of *FLG null* mutations in our African-American cohort, we were not able to accurately evaluate children who were symptom-free and were not using medications to treat their AD. It is important to realize that our definition of improvement was different from that of previous studies^(5;18). In contrast to previous studies we allowed for the waxing and waning nature of AD over nearly 5 years of follow up, thereby more closely duplicating the clinical course and patient experience of a disease that changes over time. This is an important and novel difference between our study and others. Our study more closely represents the patient-centered outcomes currently under discussion by many groups like the Patient-Centered Outcomes Research Institute. We also evaluated the influence of treatment on the clinical course. Our findings reconfirm the ubiquitous nature of the *FLG* genotype-phenotype relationship in those undergoing treatment for AD with respect to the persistence of disease. We also reconfirmed that these mutations were much less common in those of with predominately African ancestry than in those with predominately European ancestry.

While many studies have shown that *FLG null* mutations are associated with the risk for developing AD and for having AD and other atopic illnesses very few have studied longitudinal cohorts with respect to persistence⁽⁵⁾. The Avon Longitudinal Study of Parents and Children (ALSPAC) study by Henderson *et al* is a good example of how our methods differed with respect to previous studies. The ALSPAC study measured eczema severity or persistence based on two consecutive reports of no flexural rash (i.e., no rash over about one year period)⁽¹⁸⁾. Using this scheme, medication use did not influence “persistence” and children could not become persistent in the future. In that study about 9% of the mostly white population had one or more allele for the mutations 2282del4 or R501X. The hazards ratio was 0.67 (0.58, 0.77) revealing that those with *FLG null* mutations were more likely to have persistent eczema than those without these mutations. While this finding was based on a somewhat different outcome than we report, the effect estimate was very similar to our odds ratio of 0.54 (0.41, 0.69), which could account for the natural variability over time. We were also able to replicate this finding in our study when we used the ALSPAC outcome measure and analysis. However, the ALSPAC study did not differentiate between those requiring treatments from those who did not. Our study is unique in that we allowed eczema symptoms to longitudinally vary and considered medication used to treat and control AD. We demonstrate for the first time that these mutations are associated with a protracted waxing and waning course and that each *FLG null* mutation does not result in the same prognosis.

It is not fully evident why in our study all *FLG null* mutations did not result in the same clinical response to topical therapy. The *FLG null* gene variants cause the production of a truncated profilaggrin molecule that results in the almost complete absence of filaggrin in the stratum corneum⁽⁵⁾. Further, it has been shown that those with *FLG null* mutations have lower quantities of natural moisturizing factor (NMF) in their skin⁽¹⁸⁾. However, supportive of the view that genetic variation in the *FLG* gene may not result in the same phenotype is a study of copy number variation of exon 3⁽¹⁷⁾. Copy number variation of the *FLG* gene is common with the number of filaggrin repeats occurring 10, 11 or 12 times⁽¹⁴⁾. Brown *et al*

demonstrated that in those without any *FLG null* mutations lower numbers of repeats are associated with the presence of AD^(14;39;40). In addition, a study using Raman spectroscopy showed that increasing numbers of *FLG null* alleles was inversely associated with NMF content in the skin⁽⁴⁰⁾. Others have also shown that the risk for developing more severe AD increases with the number of *FLG* mutations and that all *FLG null* genes penetrance is not the same^(17;41). Finally, most of the larger studies evaluating the association between *FLG null* mutations and the persistence of AD did not analyze individual *FLG* variants but a composite genotype like our *any FLG null* genotype⁽⁴⁰⁾. There is evidence that the risk of developing AD is dependent on a wide range of variation of the *FLG* gene. It is, therefore, possible that the persistence of AD symptoms also depends on the type of *FLG* variation and that others would have found similar effects if they had evaluated individual *FLG* variants and had recorded information on medication use.

As with all studies, ours has limitations. It is possible that those with specific *FLG* mutations were preferentially enrolled in our study creating a form of selection bias. However, when enrollment commenced in 2005 for the PEER study little was known about *FLG* variation and even today it is highly unlikely that any of the PEER children would have been genotyped for *FLG null* mutations prior to enrollment⁽⁷⁾. This is unlikely especially since phenotypic changes have been noted in those with *FLG null* mutations and we noted a differential effect of therapy by mutation⁽⁴²⁾. It is however possible that our finding of differential treatment effect is specific to our study. Treatment use is only known through patient reporting and we do not know how treatment was used. Also, our finding needs to be validated in other cohorts. It is also important to realize that the diagnosis of AD may be variable. Some investigators and investigations differentiate between atopic and non-atopic AD based on the presence of serum markers. In our study, we sought to determine the effect of *FLG null* mutations in a generalizable population of individuals receiving treatment for AD. AD was diagnosed by physicians and the diagnosis was confirmed by a commonly use epidemiologic tool (i.e. UK Working Party). It is possible that some of our subjects may not have AD as determined by more rigorous standards. However, these standards are infrequently used in clinic practice, which is the source of our population. Finally, we were only able to test for 4 mutations. Other mutations do exist but none have been found to be of high enough prevalence for an epidemiologic study of subjects from a US source population.

In conclusion, in a US population of AD children diagnosed by and seeking care with local physicians, *FLG null* mutations were common. Children with *FLG null* mutations were more likely to have persistent AD. These mutations were more common in those of European ancestry but the effect of these mutations on persistence was similar in those of African ancestry. In our cohort, not all individuals with *FLG null* mutations responded to therapy in a similar fashion. Specifically, those with the most common variant, R501X, were the least responsive to therapy and the most likely to be using topical steroids. This finding needs to be confirmed. Based on our findings, it may be possible to use a panel of *FLG null* mutations to predict the persistence of a child's AD over time.

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Abbreviations

AD	Atopic Dermatitis
AIMS	Ancestral informative markers

EDC	Epidermal Differentiation Complex
FLG	Filaggrin
FLG null	Filaggrin loss of function
GLLAMM	Generalized linear latent and mixed models
ISAAC	The International Study of Asthma and Allergies in Childhood
MAF	Minor allele frequency
NMF	Natural moisturizing factor
OR	Odds ratio
PEER	Pediatric Eczema Elective Registry

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Clinical Implications

FLG null mutations are associated with more persistent AD in children. Mutations are less likely to be found in African-Americans but when present affect African-Americans in a similar fashion to whites. However, among mutation carriers there is mutation-specific variability in the clinical response to treatment.

Figure 1.

The percentage of individuals reporting that they are symptom-free for those with no *FLG* mutations, i.e. wild type and for those with any *FLG* mutation per five years of 6-monthly study surveys.

Table 1

Basic demographic and descriptive information of the study population. For **any *FLG* null** subjects were categorized as: not having any *FLG* null mutations or having at least one mutant allele.

Variable	Full cohort	Any <i>FLG</i> null	R501X	2282del4
N (%) [‡]	857	139(16.2)	68(8.3)	56(6.8)
Female (%) [#]	445	64(14.4)	32(7.2)	29(6.5)
Male (%)	410	75(18.3)	36(8.8)	27(6.6)
Age at onset in years (sd)	2.1 (2.7)	1.8 (2.5)	1.7 (2.5)	1.8(2.5)
Age at enrollment in years (sd)	7.2 (3.8)	6.9(3.4)	6.7 (3.4)	6.5(3.3)
White (%)	433	119(27.5)	60 (13.8)	52(12.0)
African American (%)	370	21(5.8)	12(3.2)	2(0.5) [^]
Asthma (%)	447	85(19.0)	44(10.2)	30((7.0)
Seasonal allergy (%)	573	96(17.3)	51(9.4)	33(6.1)
Number of completed surveys (median 25%, 75%)	10 (7,12)	11(9,13)	11(9,13)	11(9,113)
Time in study in months (sd)	58.9 (22.1)	62.9 (19.7)	63.7 (21.2)	63.1 (19.5)
Symptom free at any time (%)	698	115 (16.5)	54(7.9)	47(6.8)
Symptom free without medication use at any time (%)	220	40(18.2)	15(6.9)	20 (9.2)
Probability of reporting no symptoms (sd) [*]	0.60 (0.34)	0.54 (0.35)	0.49(0.36)	0.58 (0.34)
Probability of symptoms-free and not using medication (sd) [*]	0.07 (0.16)	0.08(0.17)	0.05(0.13)	0.11 (0.19)

[‡] All percentages are based on the number of individuals who had that column characteristic divided by the number of individual in that row (e.g. percentage of individuals with any *FLG* null mutation (139/857) * 100)

[#] Gender not reported for two subjects.

^{*} The probability of reporting no symptoms or no symptoms and not using medication were based on the number of times these items were reported by an individual divided the number of complete surveys.

[^] Due to the small sample size, effect estimates for those who are African-American who have a 2282del4 mutation are not presented.

Table 2

Minor allele frequency (MAF) for each *FLG null* mutation by the full cohort and with respect to whites and African-Americans.

Allele type	R501X	2282del4	R2447X	S3247X
Full Cohort: MAF%	7.5	3.7	0.01	2.2
Whites: MAF%	7.7	6.8	1.4	2.8
African Americans: MAF%	3.1	0.3	0.7	1.5

Table 3

SNP	Full cohort	White	African-American
R501X	0.55(0.37,0.82) *	0.42(0.28,0.63) *	0.47(0.20,1.09)
2282del4	0.58(0.40,0.82) *	0.49(0.32,0.74) *	Unable to estimate
R2447X	0.47(0.24,0.91) *	0.48(0.24,0.94) #	0.37(0.08,1.59)
S3247X	0.77(0.41,1.45)	1.03(0.46,2.27)	0.30(0.13,0.64) +
Any FLG null	0.54(0.41,0.71) *	0.42(0.31,0.57) *	0.53(0.25,1.12)

Unadjusted odds ratios and 95% confidence intervals for individuals reporting that their skin was symptom free, * $p < 0.0001$, # $p < 0.04$, + $p < 0.01$.

Adjusted odds ratios were minimally different than those reported.

Table 4

SNP	Full cohort	White	African-American
R501X	0.44(0.22,0.87) *	0.44(0.22,0.88) *	Unable to estimate
2282del4	1.69(1.01,2.84)	1.72(0.99,3.00)	Unable to estimate
R2447X	0.97(0.36,2.58)	0.94(0.34,2.62)	0.66(0.04,11.20)
S3247X	3.56(1.69,7.52)	4.32(2.16,8.62)	0.10(0.01,1.44)
Any FLG null	1.26(0.90,1.77)	1.24(0.84,1.83)	0.15(0.02,1.43)

Unadjusted odds ratios and 95% confidence intervals for individuals reporting that their skin was symptom free and they were not using topical medication, *p<0.0001.

Adjusted odds ratios were minimally different than those reported.