



Uncommon Filaggrin Variants Are Associated with Persistent Atopic Dermatitis in African Americans

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Atopic dermatitis (AD) is a common illness that has been associated with filaggrin gene (*FLG*) loss of function (LoF) variation. In African Americans, a group that commonly has AD and has not been well studied, *FLG* LoF variation is rarely found. Our objective was to use massively parallel sequencing to evaluate *FLG* LoF variation in children of African ancestry to evaluate the association between *FLG* LoF variation and AD and AD persistence. We studied 262 African American children with AD. Nine unique *FLG* exon 3 LoF variants were identified for an overall minor variant frequency of 6.30% (95% confidence interval [CI] = 4.37–8.73). The most common variants were p.R501X (1.72%, 95% CI = 0.79–3.24), p.S3316X (1.34%, 95% CI = 0.54–2.73), and p.R826X (0.95%, 95% CI = 0.31–2.2). Over an average follow-up period of 96.4 (95% CI = 92.0–100.8) months, African American children with *FLG* LoF were less likely to be symptom free (odds ratio = 0.36, 95% CI = 0.14–0.89, $P = 0.027$) compared with a *FLG* wild-type child. In contrast to previous reports, uncommon *FLG* LoF variants in African American children exist and are associated with AD and more persistent AD. In contrast to Europeans, no *FLG* LoF variants predominate in African American children. Properly determining *FLG* LoF status requires advanced sequencing techniques.

Journal of Investigative Dermatology (2018) **138**, 1501–1506; doi:10.1016/j.jid.2018.01.029

INTRODUCTION

Atopic dermatitis (AD), also known as eczema or atopic eczema, is a common, chronic inflammatory skin condition characterized by periods of acute flares. It is often a lifelong ailment, and it frequently manifests as itchy, red patches on the flexural areas of the elbows and knees (Abuabara et al., 2017; Margolis et al., 2014b). The prevalence of AD is increasing worldwide, and it is a major public health burden. In the United States, AD affects roughly 5–20% of all children and adults of all races and ethnicities, with a total cost of more than 4.2 billion dollars per year (Abuabara et al., 2017; Lim et al., 2017; Shaw et al., 2011). Complex genetic,

immunologic, and environmental factors are thought to be responsible for the development of AD, including the disruption of the normally protective skin barrier. The most common gene associated with susceptibility to AD is filaggrin (*FLG*), which is located within the epidermal differentiation complex on chromosome arm 1q21. *FLG* encodes the protein filaggrin, an important epidermal barrier protein involved in maintaining an intact skin barrier (Brown and McLean, 2012; Margolis et al., 2012; Palmer et al., 2006; Sandilands et al., 2007).

FLG is transcribed as a large precursor protein, profilaggrin. In the granular cell layer of the epidermis, dephosphorylation and proteolysis of profilaggrin yields filaggrin, which binds to keratin intermediate filaments and subsequently assembles a keratin matrix (Brown and McLean, 2012). This matrix acts as a protein scaffold and, together with bound proteins and lipids, forms the topmost skin layer, the stratum corneum (Brown and McLean, 2012). Loss-of-function (LoF) mutations in exon 3 of the *FLG* gene are associated with markedly diminished or absent *FLG* protein production, presumably due to nonsense mediated decay and AD of earlier onset, increased severity, and/or increased persistence (Brown and McLean, 2012; Margolis et al., 2012).

More than 20 *FLG* LoF variants in exon 3 have been associated with susceptibility to AD. More than 300 *FLG* LoF variants have been identified in sequencing data available in the gnomAD browser, an international aggregate database of exome and genome sequencing data (Lek et al., 2016; Wong et al., 2018). As evidenced by several studies, *FLG* LoF

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Abbreviations: AD, atopic dermatitis; CI, confidence interval; LoF, loss of function; MPS, massively parallel sequencing; MVF, minor variant frequency; PEER, Pediatric Eczema Elective Registry

Received 27 December 2017; revised 29 January 2018; accepted 31 January 2018; accepted manuscript published online 8 February 2018; corrected proof published online 20 March 2018

mutations vary by race (Brown and McLean, 2012; Margolis et al., 2012, 2014a; Palmer et al., 2006; Sandilands et al., 2007; Wong et al., 2017). Four *FLG* LoF mutations have been consistently associated with AD in patients of European ancestry (p.R501X, c.2282del4, p.S3247X, p.R2447X), and many studies focus primarily on these variants (Brown and McLean, 2012; Margolis et al., 2012; Palmer et al., 2006; Sandilands et al., 2007). However, studies of patients of East Asian ancestry show larger numbers of variants, with c.3321delA the only variant associated with AD and found in Chinese, Japanese, Korean, and Taiwanese populations (Akiyama et al., 2010; Wong et al., 2018). Several recent studies, using a variety of genotyping and sequencing techniques, were not able to detect *FLG* LoF mutations in individuals of African ancestry with AD at frequencies noted in European and Asian populations (Margolis et al., 2012, 2014a; Policari et al., 2014; Taylan et al., 2015; Thawer-Esmail et al., 2014; Winge et al., 2011). The goal of this study was to use massively parallel sequencing (MPS) to evaluate *FLG* LoF variation in children of African ancestry to comprehensively evaluate the association between *FLG* LoF variation and AD and AD persistence.

RESULTS

Of the 370 African American Pediatric Eczema Elective Registry (PEER) subjects who provided DNA, 262 had sufficient DNA for this study. The mean age of onset of AD was 2.09 (standard deviation = 2.88) years of age, and 58.8% were female. Seasonal allergies were noted in 66.7%, and 55.7% had a history of asthma. The PEER study is an ongoing study. At the time of this analysis, 133 (50.7%) of the African American children had completed 10 years of follow-up. The African American cohort was followed on average for 96.4 (95% confidence interval [CI] = 92.0–100.8) months (see Table 1).

The interrater agreement for the four European variants (p.R501X, c.2282del4 [p.S761fs], p.R2447X, and p.S3247X) was assessed between the MPS approach and previous data that used traditional sequencing approaches (Table 2). The kappa score was in the *almost perfect* range (≥ 0.81) for the p.R501X, c.2282del4 (p.S761fs), and p.R2447X variants (Table 2) (Landis and Koch, 1977). The reliability for p.S3247X was in the *substantial* range (0.61–0.80) (Landis and Koch, 1977). The MPS approach did not identify three individuals heterozygous for p.S3247X noted by the TaqMan assay (Applied Biosystems, Waltham, MA).

In the African American children, the overall prevalence of any *FLG* LoF variant was 12.21%. Nine different *FLG* LoF variants were identified in 32 of the 262 children. The overall MVF of the *FLG* LoF composite was 6.30% (95% CI = 4.37–8.73). Each individual variant had a minor variant frequency (MVF) of less than 2.0%. The three most common variants were p.R501X (MVF = 1.72%, 95% CI = 0.79–3.24), p.S3316X (MVF = 1.34%, 95% CI = 0.54–2.73), and p.R826X (MVF = 0.95%, 95% CI = 0.31–2.21). The MVFs for *FLG* LoF were compared between our sample population and the African population described in gnomAD (release date October 3, 2017 r2.0.12). All but one variant (p.H440fs) was reported in gnomAD. All variants among those of African ancestry with AD were more frequent

in our cohort than in the reference gnomAD cohort (Table 3). Three of the variants (p.R501X, p.R2447X, and p.S3247X) were significantly more frequent than the gnomAD reference (Table 3). As expected, because of the reliability between the assays used, the MVFs for p.R501X, p.R2447X, c.S761fs, and p.S3247 for white children were very similar to those in our previous report; therefore, they were not reported (Margolis et al., 2012). Four variants, all uncommon, that were not previously reported by us in the white cohort and were not found in the African American children were noted: c.G1787fs, c.P685fs, c.T2496fs, and p.R1474X (Margolis et al., 2012).

African American children with *FLG* LoF variant composite, based on the nine variants, have more persistent AD than children with the wild type for *FLG* LoF (Figure 1). At nearly every time point (Figure 1), African American children with at least one *FLG* LoF were less likely to report that they were symptom free. The odds of an African American child reporting a 6-month period of disease-free skin at any given time point was less in those with a *FLG* LoF variant than in those who did not have a variant (odds ratio = 0.36, 95% CI = 0.14–0.89, $P = 0.027$). The overall effect of *FLG* LoF on the persistence of AD was similar in those of African ancestry to our white cohort (OR = 0.45, 95% CI = 0.22–0.93, $P = 0.037$). For the full cohort, the effect of the *FLG* LoF variant was 0.47 (95% CI = 0.27–0.8, $P = 0.006$), and when adjusted for age of onset, race based on ancestral informative markers (Margolis et al., 2012), and sex, the effect estimate was 0.42 (95% CI = 0.20–0.74, $P = 0.003$).

DISCUSSION

In contrast to previous reports, we present findings from a large longitudinal study showing that there exist uncommon *FLG* exon 3 LoF variants in children of African ancestry that are associated with AD and with more persistent AD. The overall prevalence of *FLG* LoF variants are less than was noted in a comparable cohort of white children, and all of the African American variants are uncommon (MVF < 5%), but the effect of these variants on persistence is similar to our previous observations in other races (Margolis et al., 2012). Although the prevalence of AD in the United States is highest among African Americans, patients of African ancestry have been largely understudied (Margolis et al., 2014a; Shaw et al., 2011). Studies have been unable to consistently identify *FLG* mutations in individuals with AD and African ancestry (Margolis et al., 2014a; Policari et al., 2014; Thawer-Esmail et al., 2014; Winge et al., 2011). Using our current sequencing methods and informatics pipeline, we were also able to identify additional *FLG* LoF variants in patients of European ancestry beyond the commonly reported variants (Margolis et al., 2012). In our previous study, only 5.8% of African Americans with AD had a *FLG* LoF variant (Margolis et al., 2012). We now are able to show that 12.2% of this cohort will have a *FLG* LoF variant. We have also shown the reliability of the MPS technique by comparing previous results with those of our current study (Margolis et al., 2012, 2013, 2014a). As expected, the MVF for the *FLG* LoF variants (p.R501X, c.2282del4 [p.S761fs], p.R2447X, and p.S3247X) in this study was similar to those noted in our previous study (Margolis et al., 2012).

Table 1. Participant demographics

Characteristics	African American	White
Number	262	133
Age at enrollment in years, mean (SD)	7.66 (0.24)	6.94 (0.30)
Age of AD onset, mean (SD)	2.09 (2.88)	1.46 (1.86)
Sex: female, n (%)	160 (61.0)	70 (52.6)
Asthma, n (%)	146 (55.8)	74 (55.8)
Seasonal allergies, n (%)	175 (66.8)	96 (72.6)
Observation time in months, mean (95% CI)	96.4 (92.0–100.8)	113.5 (109.1–117.8)
Abbreviations: AD, atopic dermatitis; CI, confidence interval; SD, standard deviation.		

Although previous studies have shown *FLG* LoF variants in those of African ancestry, because of their rarity they have not been consistently detected, and their importance with respect to AD may have been overlooked (Margolis et al., 2012, 2014a; Policari et al., 2014; Taylan et al., 2015; Thawer-Esmail et al., 2014; Winge et al., 2011). The results of this study are in direct contrast to these previous reports, including a report by some of the authors of this study (Margolis et al., 2012, 2014a; Taylan et al., 2015; Thawer-Esmail et al., 2014; Winge et al., 2011). A total of 53 subjects included in this report had been previously evaluated in a published whole exome study (Margolis et al., 2014a). The only meaningful technical difference between that study and our current analysis was the use of a local versus global alignment tool, which has been developed since those data were published (Margolis et al., 2014a; McKenna et al., 2010). Using the local alignment tool, we found that the number of *FLG* LoF variants noted in these subjects increased from only two to nine (all rare) (Margolis et al., 2014a). One previous study did show decreased FLG breakdown products on the skin of subjects of African ancestry with AD, which in retrospect could have been caused by the undetected *FLG* LoF mutations (Thawer-Esmail et al., 2014).

The difficulty of sequencing *FLG* is well known (Palmer et al., 2006). The gene *FLG* is over 23 kilo base pairs, and most of the initially coded profilaggrin polypeptide, which is later processed to form the filaggrin protein, is encoded from exon 3 (Brown and McLean, 2012; Palmer et al., 2006; Sandilands et al., 2007). Exon 3 is not only large (>12 kilo

base pairs) but also repetitive, with 10–12 nearly identical tandem repeats of about 972 base pairs, making the area difficult to sequence (Brown and McLean 2012; Lek et al., 2016; Margolis et al., 2014a; Palmer et al., 2006). Because of the intragenic homology of *FLG*, a recent report identified the *FLG* gene and several other genes as part of a so-called “NGS Dead Zone” (Mandelker et al., 2016). It will be important for future studies to use improved bioinformatics tools and fully targeted approaches when evaluating this gene (McKenna et al., 2010; Wong et al., 2018).

Our study does have some limitations. Prior convention suggests that *FLG* LoF mutations of exon 3 result in diminished production of filaggrin (Palmer et al., 2006). However, at this time we do not have any direct evidence that most of the variants described in this study directly resulted in diminished production of filaggrin. This limitation, however, is true of many studies of *FLG* LoF variants. Most of our samples were obtained in the mail, so we do not have easy access to study subjects and are not able to obtain tissue samples for FLG protein analysis. Even if possible, obtaining tissue to assess FLG for uncommon variants would require the identification and consent of a large number of participants. As has been recently noted, inflamed skin has diminished FLG production not specifically associated with *FLG* variation (Pellerin et al., 2013; Seykora et al., 2015). In addition, as expected, children with *FLG* LoF mutations had more persistent disease. It is probable that all of the *FLG* LoF variants do have a profound effect on the production of FLG and skin barrier function. We focused only on *FLG* exon 3 LoF mutations and did not assess copy number variation, which others also have found to be associated with AD (Brown and McLean, 2012). Our study focuses on African Americans and may not represent everyone with African ancestry. We included children with mild to moderate AD as per inclusion in the PEER cohort, and this cohort may not be generalizable to everyone with AD. Finally, the group who agreed to provide DNA was not a random sample, so it is possible that our findings may not be generalizable to the full PEER cohort.

In summary, we have shown that results from MPS and previous assays used for *FLG* LoF genotyping are highly concordant. Previous studies that found that *FLG* LoF variants were not associated with the prevalence and persistence of AD in those of African ancestry may have missed associations because of technical limitations. We show that children of African ancestry with *FLG* LoF variants are more likely to

Table 2. Interrater agreement between the previous approach¹ that used TaqMan assays and the current approach that used targeted next-generation sequencing

Allele	African American n = 262		White n = 133		All Subjects n = 353	
	% Agreement	Kappa (SD)	% Agreement	Kappa (SD)	% Agreement	Kappa (SD)
p.R501X	99.10	0.86 (0.06)	98.48	0.95 (0.08)	98.87	0.93 (0.05)
p.2447X	99.55	0.85 (0.07)	100.00	1.00 (0.09)	99.72	0.95 (0.05)
p.S3247X	98.67	0.72 (0.06)	100.00	1.00 (0.09)	99.16	0.80 (0.05)
p.S761fs (c.2282del4)	NR	NR	97.74	0.90 (0.08)	99.15	0.91 (0.05)
Composite based on variants listed above	98.64	0.88 (0.06)	96.21	0.92 (0.07)	97.73	0.91 (0.04)

Abbreviations: NR, not reported (no outcomes for African Americans); SD, standard deviation.

¹Margolis et al., 2012.

Table 3. Descriptive information for *FLG* loss-of-function stop gain variants noted in African American subjects in our study and comparisons with the African ancestry cohort from gnomAD¹

SNP	Chromosome Start	Our Study (Genomes = 524)		gnomAD		P-value	RSID	Change
		MVF, % (95% CI)	Variants Noted	MVF, % (95% CI)	Variants/Genomes			
p.R501X	152285861	1.72 (0.79–3.24)	9	0.43 (0.35–0.52)	103/23,984	0.001	61816761	c.1501C>T
p.R826X	152284886	0.95 (0.31–2.21)	5	0.77 (0.66–0.89)	184/23,970	0.607	115746363	c.C2476C>T
p.R2447X	152280023	0.76 (0.21–1.94)	4	0.05 (0.23–0.09)	13/23,962	0.0001	138726443	c.7339C>T
p.Q3818X	152275910	0.19 (0.00–1.06)	1	0.04 (0.02–0.08)	11/24,028	0.228	148606936	c.11452C>T
p.Q570X	152285654	0.19 (0.00–1.06)	1	0.02 (0.00–0.05)	3/15,304	0.126	192402912	c.1708C>T
p.R3409X	152277137	0.19 (0.00–1.06)	1	0.01 (0.00–0.04)	2/15,300	0.096	201356558	c.10225C>T
p.S3247X	152277622	0.76 (0.21–1.94)	4	0.05 (0.02–0.09)	12/24,008	<0.0001	150597413	c.9740C>A
p.S3316X ²	152277415	1.34 (0.54–2.73)	7	0.78 (0.67–0.90)	187/24,000	0.132	149484917	c.9947C>A
p.H440fs	152286044	0.19 (0.00–1.06)	1	NR	NR	—	NR	c.1318delC

Abbreviations: CI, confidence interval; MVF, minor variant frequency; NR, not reported in gnomAD; RSID, refSNP cluster identification; SNP, single nucleotide polymorphism.

¹All MVFs are presented as percentage with 95% CI and prevalence per hundred. All P-values were estimated using two-sided Fisher exact test.

²p.S3316X maps to p.S3640X using a 12 repeat *FLG* gene reference, which is not gnomAD standard.

have AD than those who do not have the LoF variants. The distribution of the frequency and the type of *FLG* LoF variants varies based on ancestry. The prevalence of these variants in children of African ancestry is less than those of European ancestry and Asian ancestry. Nonetheless, children with *FLG* LoF have more persistent AD, and the overall effect of LoF mutations in African American children is similar to that seen in white children. In contrast to Europeans, no *FLG* LoF variants predominate in African American children. The variation in the prevalence and diversity of variants is consistent with the genetic bottleneck hypothesized to have occurred with human migration out of Africa (Amos and Hoffman, 2010). Finally, based on our results, properly determining *FLG* LoF status requires next-generation sequencing techniques using proper informatics tools.

MATERIALS AND METHODS

Population and design

The PEER (www.thepeerprogram.com) is a US nationwide cohort of more than 8,000 subjects with pediatric-onset AD. This study represents a subcohort of the 370 African Americans who previously participated in a study of the genetics of AD (Chang et al., 2017;

Margolis et al., 2012). Self-described race was previously confirmed in this group using ancestral informative markers (Margolis et al., 2012). At the time of enrollment, children were 2–17 years old, had a physician-confirmed diagnosis of AD, and had used pimecrolimus cream for at least 6 months (Margolis et al., 2012). Subjects were followed for up to 10 years, and during that time they were not required to (and most did not) continue therapy with pimecrolimus (Margolis et al., 2015). All subjects, or if appropriate a guardian, provided written informed consent approved by the University of Pennsylvania Institutional Review Board. Full details of the PEER cohort have been previously reported (Margolis et al., 2012, 2015; Margolis et al., 2014b). To confirm the reliability of the MPS, we also evaluated 133 previously assessed white subjects (Margolis et al., 2012, 2013).

Genetic analysis

Overall, 262 of the 370 eligible African American children had DNA sufficient to conduct MPS targeted capture. To confirm the reliability of the MPS technology, a sample of 133 of 433 white subjects from the PEER cohort was sequenced, and their results were compared with the results that used a long-range PCR and TaqMan-based approach (Margolis et al., 2012). The goal of sequencing was the identification of exon 3 LoF *FLG* variants in African American children with AD. Exon 3 LoF variants have been previously reported to diminish the production of FLG (Brown and McLean, 2012; Palmer et al., 2006; Sandilands et al., 2007).

DNA was collected using Oragene DNA collection kits (DNA Genotek, Ottawa, Canada). MPS was performed by creating a library preparation using the NEBNext Ultra DNA Library Prep kit (New England BioLabs, Ipswich, MA). Briefly, 500 ng of input DNA was sheared on a Covaris (Woburn, MA) Sonicator to a size of 150 base pairs. End repair was performed to remove overhangs, and sequencing adapters were subsequently ligated to the blunt-ended fragments. Adapter-ligated fragments were then PCR-amplified, incorporating barcode sequences to enable downstream sample pooling. PCR-amplified products were then size-selected by Ampure XP beads (Beckman Coulter, Indianapolis, IN) to ensure optimal insert sizes. To verify insert sizes and library concentration, size-selected genomic libraries were analyzed on an Agilent (Santa Clara, CA) 2100 Bioanalyzer instrument. Library concentrations were determined by Bioanalyzer analysis so that equimolar sample

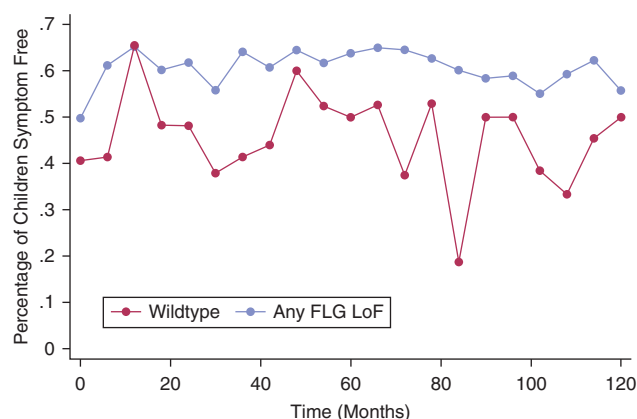


Figure 1. Children symptom free. Percentage of African American children reporting symptom-free skin during each 6-month period since time of enrollment in the Pediatric Eczema Elective Registry. LoF, loss of function.

pooling before targeted capture-based enrichment of *FLG* occurred. Targeted capture was designed using Agilent SureDesign and included the entire *FLG* gene (exons and introns). Overall, 88% of the *FLG* gene was covered, and the read depth was 185 times. Samples were dual-indexed using appropriate SureSelect indexing primers (A01–H12; New England BioLabs), so that 96 samples were sequenced on a single lane of Illumina (San Diego, CA) HiSeq 4000 instrument. Raw sequencing data were aligned and mapped to the reference genome hg19 using the Burrows-Wheeler aligner (Li and Durbin, 2010). Single nucleotide variant and insertion/deletion calling was accomplished by using the Genome Analysis Toolkit HaplotypeCaller, after following Genome Analysis Toolkit Best Practices realignment and recalibration (DePristo et al., 2011; McKenna et al., 2010; Van der Auwera et al., 2013). All calls were confirmed using VarDict and visualize using the integrative genomics viewer (Lai et al., 2016; Robinson et al., 2011).

Statistical analysis

For the genetic prevalence study embedded in the longitudinal cohort study, variant frequency was reported as prevalence per individual (percentage) of the minor variant and as genome MAF (percentage with 95% CI). Also, as is the convention, a composite was created for all of the *FLG* LoF variants (no variant, a single variant, two or more variants including homozygote variant) (Brown and McLean, 2012; Margolis et al., 2012; Palmer et al., 2006; Sandilands et al., 2007). For this study, we created a composite consistent with previous publications, which used the most common European (white) alleles and made a composite based on the findings from this study (Margolis et al., 2012). The observed frequencies were compared with frequencies available from the gnomAD browser (<http://gnomad.broadinstitute.org/>) (Wong et al., 2018).

To assess the reliability of the MPS technology, genotyping results from this study were compared with previously reported long-range PCR and TaqMan data using the kappa statistic (Margolis et al., 2012, 2013). Comparisons between *FLG* LoF variant frequencies and the gnomAD browser were made using Fisher exact test. No correction was made for multiplicity because each comparison represented a predetermined hypothesis.

Persistence was evaluated based on the self-reported outcome of whether or not a child's skin was AD symptom free during the previous 6 months (Margolis et al., 2012). Persistence was determined by survey responses to a series of questions, including *During the last six months would you say that your child's skin disease (AD) has shown: complete disease control, good disease control, limited disease control, or uncontrolled disease. Symptom free* was defined as an affirmative response of *complete disease control*. This finding has been shown to correlate with other tools used to evaluate symptom control (Chang, et al., 2017). The association between these outcomes (multiple outcomes recorded over time per participant) and the *FLG* variant composite were evaluated using generalized estimating equations for binary outcomes, assuming an independence working correlation structure with empirical standard errors. All analyses were conducted with Stata, version 15.1 (StataCorp, College Station, TX).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was funded by support from the National Institutes of Health National Institute of Arthritis and Musculoskeletal and Skin Diseases (R01-AR069062). The sponsor did not have a role in the design of the study; the collection, analysis, or interpretation of the data; the preparation of this report; or the decision to submit this report for publication.

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