

Abbreviations used

AD: Atopic dermatitis
 FLG: Filaggrin
 HRNR: Hornerin
 OR: Odds ratio
 qPCR: Quantitative PCR
 TEWL: Transepidermal water loss

stratum corneum pH, and dryness^{15,16}; and abnormal bacterial colonization.¹⁷

However, *FLG* loss-of-function mutations explain the AD-associated stratum corneum abnormalities in a maximum of one in 3 northern European patients,¹⁸ and a very low frequency of *FLG* null alleles has been observed in patients with AD from the southern European¹⁹ and Ethiopian²⁰ populations. In addition, some patients with AD have increased TEWL and percutaneous penetration irrespective of their *FLG* genotype.²¹ These observations imply that in addition to *FLG* loss-of-function mutations, other factors that modulate epidermal barrier functions are involved in the pathogenesis of AD. Several proinflammatory cytokines, including IL-4, IL-13, IL-22, and IL-25, can reduce the expression of FLG through transcriptional regulation.²²⁻²⁵ Also, mutations in a tight junction protein and in some protease/protease inhibitors involved in desquamation have been tentatively associated with AD susceptibility.²⁶⁻²⁸

In this study we focused on 2 other S100-fused type proteins: hornerin (HRNR) and FLG2. These proteins share many properties with FLG: a closely related structural organization, including a large central repetitive domain; a similar amino acid composition; an identical pattern of expression in the epidermis; and an analogous proteolytic processing.²⁹⁻³³ In addition, HRNR is a component of cornified cell envelopes.²⁹ Altogether, this suggests that HRNR and FLG2 abnormalities might well be involved in AD-associated epidermal barrier defects. Reinforcing this idea, an AD predisposition factor distinct from *FLG* was suggested to be present in the 1q21 chromosomal region in which the *HRNR* and *FLG2* genes are located.³⁴ We compared the expression of FLG, HRNR, and FLG2 in the skin of healthy volunteers and in lesional and nonlesional skin of a large cohort of patients with AD. In addition, we investigated the effect of proinflammatory cytokines on expression of the 3 proteins.

METHODS**Study subjects**

Seventy-three unrelated adults with a history of mild-to-severe AD were recruited and clinically characterized by an experienced dermatologist. Disease severity was determined by using the SCORAD and Nottingham Eczema Severity Score evaluations (see Table E1 in this article's Online Repository at www.jacionline.org). Seventy-three unrelated sex- and age-matched healthy subjects with no personal or familial history of AD, ichthyosis, asthma, or allergic rhinitis were recruited. All patients underwent four 3-mm punch biopsies, 2 on lesional and 2 on nonlesional skin sites. Two biopsy specimens were obtained from the control subjects at the corresponding sites. Blood was taken from all subjects for genotyping. Genomic DNA was prepared from whole blood by using standard methods. *FLG* genotyping was performed in patients and control subjects for the 4 *FLG* mutations most prevalent in the European population^{3,4,11}: R501X, 2282del4, S3247X, and R2447X (see Table E2 in this article's Online Repository at www.jacionline.org). For further details, see the Methods section in this article's Online Repository at www.jacionline.org.

All experiments were performed according to the principles of the Declaration of Helsinki. Participants provided written informed consent before inclusion. The study was authorized by the General Board of the French Ministry of Health (Direction Générale de la Santé, DGS2008-0259, August 2008) and approved by the Comité de protection des Personnes Sud-Ouest et Outre Mer I (Ethics Committee).

Histologic, immunohistologic, and Western blot analyses of skin samples

Five-micrometer cryosections of skin biopsy specimens were used for either hematoxylin and eosin staining or indirect immunofluorescence analysis with the AHF₃ anti-FLG mAb³⁵ and with the AHP₂ anti-HRNR²⁹ and AIP₂ anti-FLG³¹ affinity-purified rabbit antibodies. Total epidermal proteins were separated on 10% acrylamide gels and immunodetected with either AHF₃, AHP₂, or a polyclonal goat antibody directed against the FLG2 spacer³² by using an ECL kit (Pierce/Thermo Scientific, Rockford, Ill). The National Institutes of Health ImageJ software (Bethesda, Md) was used to quantify immunoreactive bands on Western blot films after scanning. Signals were normalized for total protein concentration (for details, see the Methods section in this article's Online Repository).

Effects of inflammatory cytokines on primary normal keratinocyte

Primary human keratinocytes were obtained from abdominal dermolipectomy of healthy subjects who had provided informed consent. They were cultured in DermaLife medium (CellSystems, Troisdorf, Germany), supplemented as recommended by the manufacturer, until they reached confluence. Keratinocytes were then differentiated for 4 days in DermaLife medium supplemented with 1.3 mmol/L CaCl₂. Recombinant IL-4, IL-25, IL-22 (Cell-Systems), and IL-13 (R&D Systems, Minneapolis, Minn) were added at 100 ng/mL to the keratinocyte culture medium for the entire differentiation period to investigate the effect of cytokines. For immunofluorescence analysis, cells were fixed in 4% formaldehyde buffered solution, permeabilized, and incubated with the primary antibodies. Total proteins were extracted for Western blot analysis, as described above. The previously described primary antibodies and the anti-HRNR antibody HPA031469 (1:500; Sigma-Aldrich, St Louis, Mo) were used. Immunodetection intensities were normalized against actin immunoreactivity. For RT-quantitative PCR (qPCR), total RNA was extracted with the RNeasy Plus Minikit (Qiagen France, Courtaboeuf, France), according to the manufacturer's instructions. Reverse transcription was performed by using Improm-II Reverse Transcriptase (Promega, Madison, Wis) with a combination of oligo(dT) and random hexamers. qPCR amplification was performed with the 7300 Real Time PCR System (Applied Biosystems, Foster City, Calif) by using the Sybr qPCR SuperMix W/ROX (Invitrogen Life Technologies, Carlsbad, Calif). Relative levels of gene expression among samples were determined by using the $\Delta\Delta$ cycle threshold method. Hypoxanthine-guanine phosphoribosyltransferase gene expression was used for normalization. For details, see the Methods section in this article's Online Repository.

Statistical analyses

MedCalc software 12.2.1 (MedCalc Software, Mariakerke, Belgium) was used for statistical calculations. Statistical differences between groups were determined with the Mann-Whitney test, Bonferroni correction was used to account for multiple testing, and adjusted *P* values are indicated. We analyzed more precisely paired nonlesional and lesional skin samples using the Wilcoxon nonparametric test. The Kruskal-Wallis test was used to compare the proFLG/FLG ratio between control, nonlesional, and lesional skin samples. For the correlation analyses, the Spearman rank correlation test was used. Differences were considered significant when the *P* (or *P*_{corrected}) value was less than .05. A 3-dimensional graph was made with Statistica software (Statsoft, Tulsa, Okla) by using a distance-weighted least-squares smoothing procedure. Full logistic regression models were constructed to quantify the effect of protein expression on AD risk and to assess the potential effect of FLG mutational

status on AD risk. For this analysis, FLG and FLG-like protein levels were dichotomized based on the median values in control samples.

RESULTS

Decreased FLG expression in AD skin

Expression and localization of proFLG, processing intermediates, and FLG monomers (collectively called [pro]FLG) in nonlesional and lesional skin of 73 patients with AD and in healthy skin from 73 paired healthy subjects were first analyzed by using indirect immunofluorescence. Differences in immunostaining intensities were observed, as shown in Fig 1, A. Most control samples presented a strong (pro)FLG expression in the granular and lower cornified layers. Low immunostaining intensity was observed in the lesional skin of several patients. A complete absence of staining was noted in the lesional skin of 8 patients, with 6 of them showing agranulosis when sections of the same biopsy specimens were stained with hematoxylin and eosin (data not shown) and 4 of them carrying a heterozygous *FLG* mutation. Immunostaining of the nonlesional skin was usually normal or presented a moderate decrease in intensity. Absence of (pro)FLG staining was noted in the nonlesional skin of 1 patient, a carrier of an *FLG* heterozygous mutation.

Western blotting of total epidermal protein extracts was then performed, as shown in Fig 1, B. Signals were quantified and normalized to the total amount of extracted protein. This allowed the FLG monomer expression to be precisely quantified (Fig 1, C). A large variation in protein detection was observed in the control subjects, with a factor of 75.1 between the lowest and highest values. A similar variation was observed among patients with AD. FLG was hardly detected in the extracts of the 6 lesional skin biopsy specimens that showed agranulosis and absence of immunohistochemical staining. We then compared FLG expression between patients and control subjects. A statistically significant ($P_{\text{corrected}} \leq 10^{-4}$, Bonferroni correction, Mann-Whitney test) reduction in FLG monomer levels was noted in both lesional and nonlesional skin samples, with a decrease in the median of 88% and 61%, respectively. However, in some patients a large amount of FLG was immunodetected. For most patients (63/73 [86.3%]), the detected amounts of FLG were lower in the lesional compared with nonlesional skin extracts (Fig 1, D) but did not seem to be clearly correlated ($\rho = 0.223$, $P = 7.32 \times 10^{-2}$, Spearman rank correlation).

Three control subjects were carriers of an *FLG* loss-of-function mutation. Two of them carried either an R501X or an R2447X heterozygous mutation. These control subjects showed an FLG amount (0.930 and 1.767 AU/ μ g, respectively) of less than the median of the control subject cohort (2.461 AU/ μ g). Interestingly, 1 control subject carried a R2447X/R2447X homozygous mutation and presented one of the lowest FLG amounts (0.491 AU/ μ g). Nevertheless, a truncated form of proFLG, as well as FLG monomers, was clearly immunodetected. In accordance, the epidermis of this control subject showed very low but detectable (pro)FLG immunofluorescence staining (see Fig E1 in this article's Online Repository at www.jacionline.org). Among the 73 patients, 9 carried a heterozygous mutation of *FLG*. They exhibited significantly lower FLG expression in both lesional ($P_{\text{corrected}} = 4 \times 10^{-4}$, Bonferroni correction, Mann-Whitney test) and nonlesional ($P_{\text{corrected}} = 3.1 \times 10^{-3}$, Bonferroni correction) skin compared with that seen in the other patients. Interestingly, the reduced expression of FLG in lesional and nonlesional

skin was also evident in the subgroup of patients who did not carry an *FLG* mutation compared with control subjects (see Fig E2 in this article's Online Repository at www.jacionline.org).

Abnormal proFLG/FLG monomer ratio in AD skin

We then tried to understand why the amount of FLG monomers was reduced in patients independently of *FLG* gene mutations. We hypothesized that proFLG processing might be altered. We therefore evaluated the proFLG/FLG monomer ratio, as determined by means of Western blotting. We selected patients and control subjects without any detected *FLG* mutations. Because the amounts of some epidermal extracts were insufficient, this was performed for 16 patients with AD and 16 matched control subjects (Fig 2). Whereas the proFLG/FLG ratio was always less than 3 (mean, 1.70 ± 0.64) in the epidermal extracts of control subjects, it was frequently greater than 3 in the extracts of lesional (8/16 samples; mean, 4.44 ± 3.44) and nonlesional (6/16 samples; mean, 3.19 ± 2.80) AD skin (eg, compare lanes Co with lane AD-4 in Fig 2, A). These differences were statistically significant ($P < .05$, Kruskal-Wallis test).

Decreased HRNR expression in AD skin

HRNR expression in the epidermis for all 73 patients and 73 control subjects was analyzed by means of indirect immunofluorescence. In healthy skin HRNR has previously been detected in the upper stratum granulosum and entire stratum corneum.²⁹ Representative examples of the immunodetection patterns are shown in Fig 3, A.^{29,35} HRNR was detected in the stratum granulosum of all but 1 of the control subjects and was undetectable in the stratum corneum of 6 (8%) control subjects. The staining intensity was strongly reduced or the protein was undetectable in the stratum corneum of the lesional skin of 57 (78%) patients and of the nonlesional skin of 37 (51%) patients.

HRNR expression was also analyzed by means of Western blotting of total epidermal extracts and quantified as described for FLG expression (Fig 3, B and C). Huge interindividual differences in the amounts of HRNR were observed within the control population, with a factor of 500.0 between the lowest and highest values. Large variations in HRNR detection were also observed among patients with AD. A very statistically significant reduction in HRNR levels was noted in lesional ($P_{\text{corrected}} \leq 10^{-4}$, Bonferroni correction, Mann-Whitney test) and nonlesional ($P_{\text{corrected}} = 2 \times 10^{-4}$, Bonferroni correction) skin, with decreases in the medians of 75% and 56%, respectively. This reduction was independent of the presence of an *FLG* mutation. For a majority of patients (54/73 [74%]; $P_{\text{corrected}} = 10^{-4}$, Bonferroni correction), the detected amount of HRNR was lower in the lesional compared with nonlesional skin extracts (Fig 3, D). We observed a strong correlation in HRNR expression between nonlesional and lesional skin ($\rho = 0.657$, $P < 10^{-4}$, Spearman rank correlation).

Decreased FLG2 expression in AD skin

Expression of FLG2 was similarly analyzed by using indirect immunofluorescence and Western blotting (Fig 4). In skin samples from all control subjects, FLG2 was detected in the stratum granulosum and lower stratum corneum, as previously published.^{31,32} We did not observe any obvious staining abnormalities in the nonlesional skin of patients with AD compared with

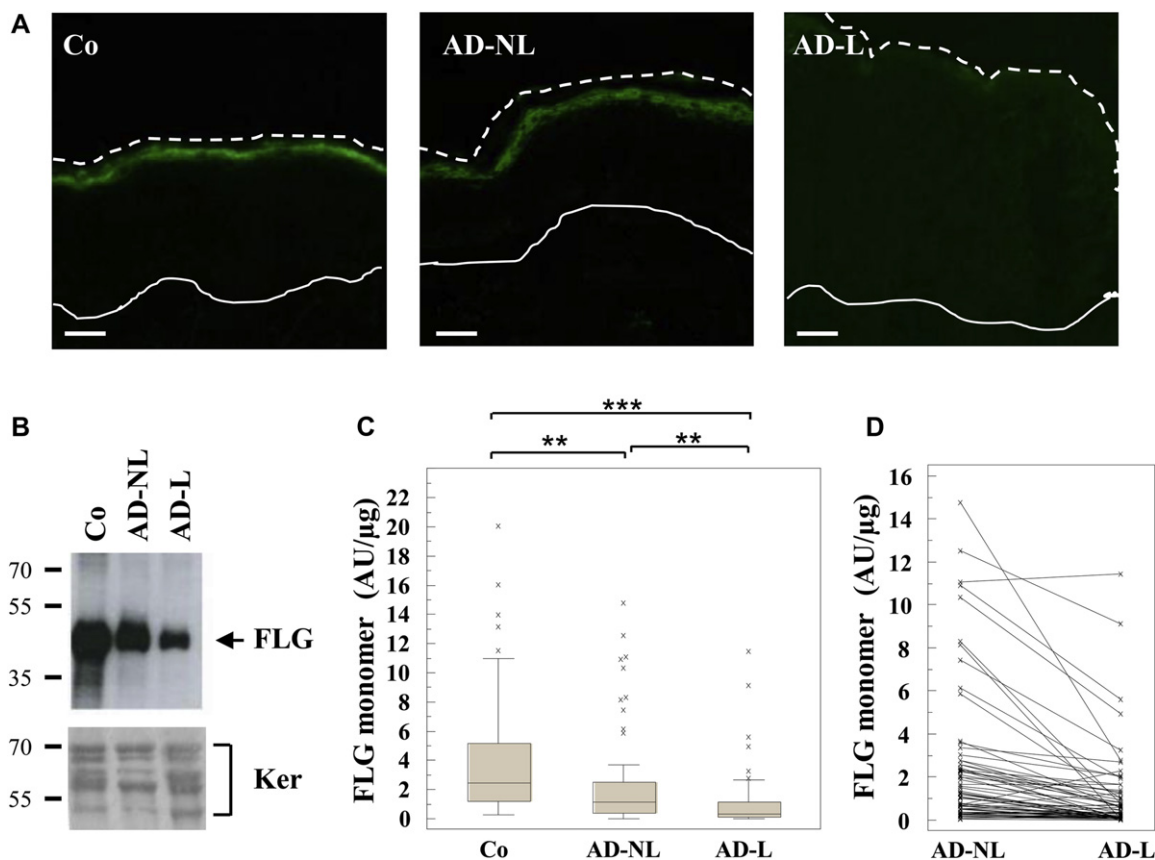


FIG 1. Reduced expression of FLG in AD skin. **A**, Detection of (pro)FLG on skin cryosections of control subjects (Co) and patients with AD. Bars = 20 μm. **B–D**, equal amounts of epidermal proteins were analyzed by using Western blotting. Representative results are shown in Fig 1, **B**. Molecular masses are on the left in kilodaltons. Immunodetected FLG monomer was quantified and indicated by using a box plot (Fig 1, **C**) and a dot-and-line diagram (Fig 1, **D**) for patients. ** $P = 10^{-4}$ and *** $P < 10^{-4}$. Ker, Stained keratins; L, lesional skin; NL, nonlesional skin.

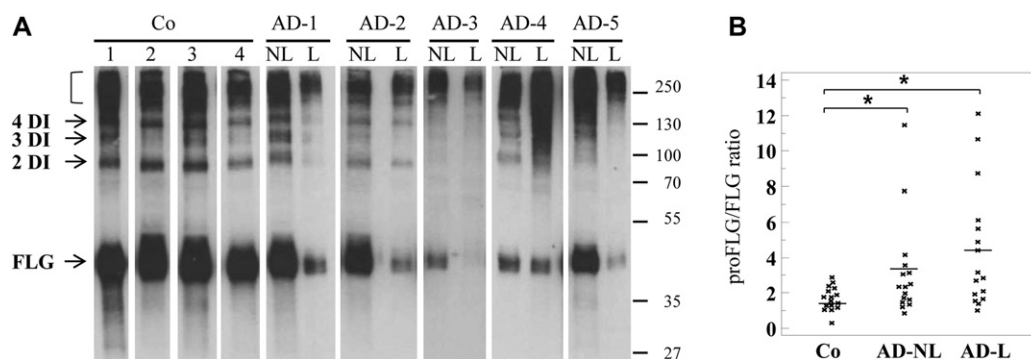


FIG 2. Alteration of the pro-FLG/FLG monomer ratio in AD skin. Epidermal extracts of 16 control subjects (Co) and 16 patients with AD were immunodetected for the expression of (pro)FLG. **A**, Representative Western blots. Pro-FLG is indicated by brackets, and domain intermediates (4DI, 3DI, and 2DI) and FLG are indicated by arrows. **B**, Immunodetected proFLG plus intermediates (referred to as proFLG amount) and FLG monomers were quantified. The proFLG amount/FLG monomer and mean values are indicated. * $P < .05$. L, Lesional skin; NL, nonlesional skin.

control subjects. However, the immunostaining intensity was reduced, discontinuous, or both in the lesional skin of 39 (53%) patients with AD, as shown in Fig 4, **A**. Quantification of FLG2 expression by using Western blotting showed a large variation in the amounts of FLG2 in the control population, with a factor

of 34 between the lowest and highest values. Variations were also noted between patients. Very significant ($P_{\text{corrected}} \leq 10^{-4}$, Bonferroni correction, Mann-Whitney test) but less marked reduction in FLG2 amounts in the lesional and nonlesional skin of patients with AD compared with those seen in control subjects

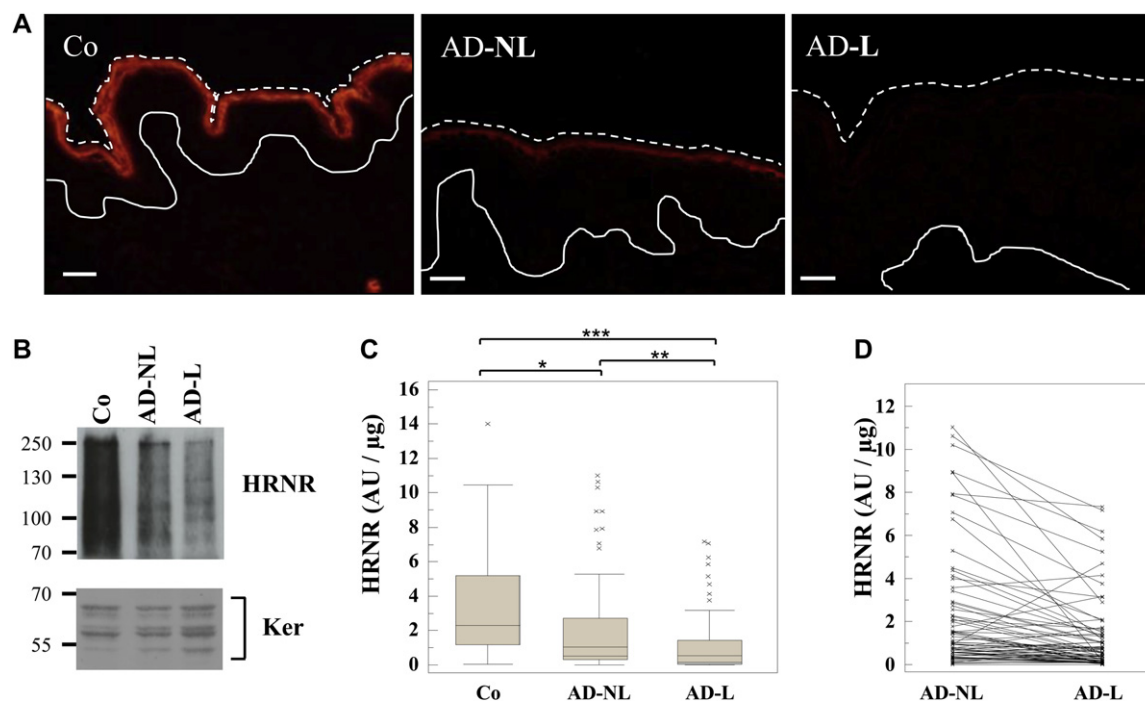


FIG 3. Reduced expression of HRNR in AD skin. **A**, Representative immunodetection of HRNR with AHP2 antibody in skin of control subjects (Co) and patients with AD. Bars = 20 μ m. **B–D**, Equal amounts of epidermal proteins were analyzed by means of Western blotting. Representative data are shown in Fig 3, B. Note the numerous bands corresponding to the HRNR precursor and processed forms, as previously described.^{29,35} Immunodetected HRNR was quantified and indicated by using a box plot (Fig 3, C) and dot-and-line diagram for each patient (Fig 3, D). * $P = 2.10^{-4}$, ** $P = 10^{-4}$, and *** $P < 10^{-4}$ ($P_{\text{corrected}} \leq 10^{-4}$, Bonferroni correction). Ker, Stained keratins; L, lesional skin; NL, nonlesional skin.

were also observed (Fig 4, B and C). This reduction was independent of the presence of an *FLG* mutation. As observed for *FLG* and HRNR, *FLG2* expression was lower in lesional than in nonlesional skin of many patients with AD ($n = 59/73$ [81%]; $P_{\text{corrected}} \leq 10^{-4}$, Bonferroni correction; Fig 4, D). We observed a medium correlation in *FLG2* expression between nonlesional and lesional skin ($\rho = 0.447$, $P = 10^{-4}$, Spearman rank correlation).

Correlation between *FLG*, HRNR, and *FLG2* amounts

We investigated a possible correlation between the amounts of immunodetected *FLG*, HRNR, and *FLG2* (see Table E3 and Fig E3 in this article's Online Repository at www.jacionline.org). Very significant positive correlations in amounts of the 3 proteins were observed in control and AD skin ($P \leq 3 \times 10^{-3}$, Spearman rank correlation). The highest correlation coefficient value was 0.72 (between HRNR and *FLG2* in the nonlesional skin samples). When considering both lesional skin in patients and skin of healthy control subjects, the correlation coefficient between HRNR and *FLG* expression was 0.630 ($P < 10^{-4}$), the correlation between *FLG* and *FLG2* was 0.647 ($P < 10^{-4}$), and the correlation between HRNR and *FLG2* was 0.679 ($P < 10^{-4}$).

Predictive value of *FLG*-related protein expression on AD status

We did not find any correlations among the 3 protein levels and disease duration, disease severity assessed by using the Nottingham

or SCORAD scores, and the age and sex of the volunteers, except a surprising lower level of *FLG* in male compared with female control subjects ($P = .0371$, Mann-Whitney test). A logistic regression analysis showed that low *FLG* (odds ratio [OR], 5.51; 95% CI, 1.91–15.94; $P = 2 \times 10^{-3}$) and *FLG2* (OR, 16.21; 95% CI, 4.05–64.86; $P < 10^{-4}$) expression in lesional skin was strongly associated with AD. HRNR expression displayed no association with AD (OR, 1.11; 95% CI, 0.37–3.37; $P = .85$). The effect of *FLG* mutations on AD susceptibility was evident but not statistically significant, probably because of a lack of power (OR, 4.7; 95% CI, 0.78–28.34; $P = .091$).

Search for HRNR and *FLG2* loss-of-function mutations

Loss-of-function mutations in the *HRNR* and *FLG2* genes might explain the reduced expression of HRNR and *FLG2* in patients with AD. We hypothesized that, just as for *FLG*, such mutations would most likely be at the beginning of the repetitive domain-encoding parts of the genes. PCR fragments corresponding to the intron 2/exon 3 junction and to the regions encoding the beginning of the repetitive domain were amplified from the genomic DNA of each patient with AD and healthy control subject and fully sequenced (see Fig E4 in this article's Online Repository at www.jacionline.org). No nonsense mutations were detected. Numerous single nucleotide polymorphisms were observed, including 4 in *HRNR* not already described in published libraries. However, there was no statistical difference in their frequency between control subjects and patients with AD (see Figs E5 and E6 in this article's Online Repository at www.jacionline.org).

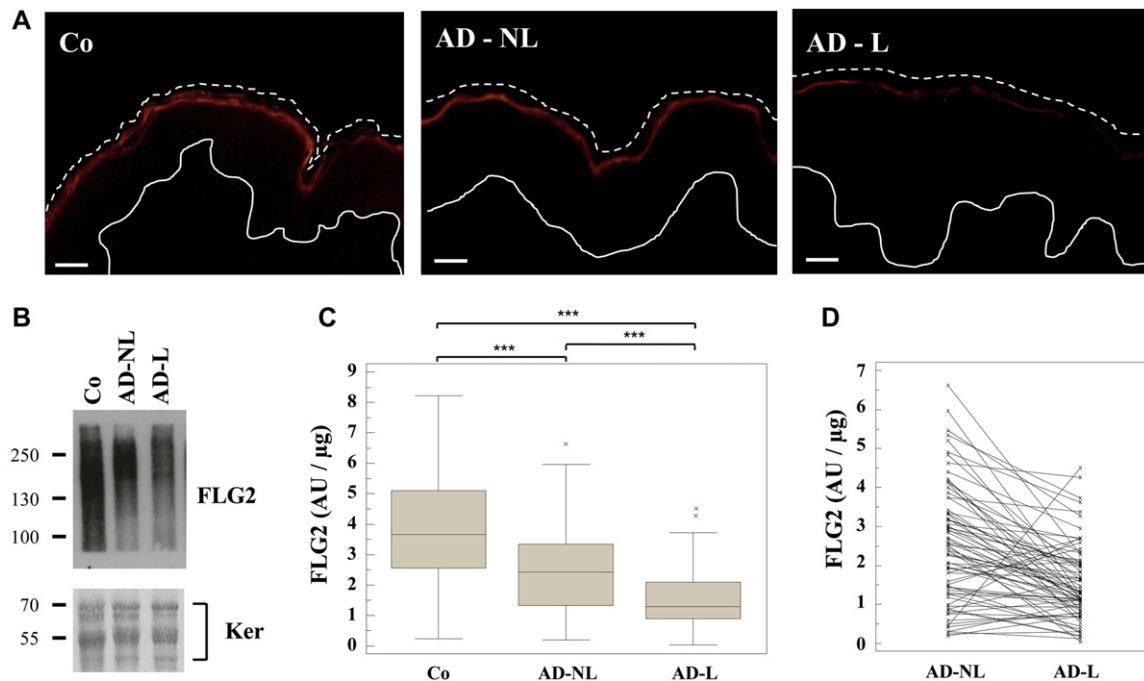


FIG 4. Reduced expression of FLG2 in AD skin. **A**, Representative immunodetection of FLG2 with AIP₂ in skin from control subjects (Co) and patients with AD. Bars = 20 μ m. **B-D**, Equal amounts of epidermal proteins were analyzed by using Western blotting. Representative data are shown in Fig 4, B. Immunodetected FLG2 was quantified and represented by using a box plot (Fig 4, C) and a dot-and-line diagram for each patient (Fig 4, D). *** $P < 10^{-4}$. Ker, Stained keratins; L, lesional skin; NL, nonlesional skin.

Reduced expression of FLG, HRNR, and FLG2 in keratinocytes exposed to IL-4, IL-13, and IL-25

Because FLG expression is known to be downregulated by proinflammatory cytokines involved in AD, we tested the effects of recombinant IL-4, IL-13, and IL-25 on the expression of HRNR and FLG2 in cultured keratinocytes at both the mRNA and protein levels using RT-qPCR and Western blotting, respectively (Fig 5). Keratinocytes at confluence were induced to differentiate for 4 days in 1.3 mmol/L CaCl₂ in the absence and presence of these cytokines. FLG and FLG2 mRNA levels were strongly reduced after exposure to 100 ng/mL cytokines (Fig 5, A). We were not able to accurately determine the effect of cytokine treatments on HRNR mRNA levels because they were already low in untreated cells. Indirect immunofluorescence analysis indicated that (pro)FLG, HRNR, and FLG2 levels were reduced after treatment with IL-4 (Fig 5, B), IL-13, and IL-25 (data not shown). Western blotting clearly demonstrated that the 3 proteins were downregulated by each cytokine at the protein level (Fig 5, C). When we treated the keratinocytes with IL-22 in the same conditions, no clear effect was observed on the expression of the 3 proteins (data not shown).

DISCUSSION

The discovery that nonsense mutations in the gene encoding FLG are a strong genetic risk factor for AD has been a significant breakthrough in the understanding of the disease. Because some patients with AD without *FLG* gene mutations have nevertheless a defective epidermal barrier,²¹ there must be additional mechanisms impairing barrier integrity. In addition, many of the

available data concern the relationship between *FLG* mutations and clinical characteristics of the disease, but few studies have evaluated the expression and processing of proFLG in the nonlesional skin of patients. Here, we precisely analyzed the levels of FLG and of 2 FLG-like proteins, HRNR and FLG2, in the nonlesional and lesional skin of a large cohort of healthy control subjects and adults with AD. We also investigated the processing of proFLG in a subset of control subjects and patients without *FLG* null mutations.

In control subjects we observed large interindividual variations in the amounts of detected FLG, HRNR, and FLG2. Considering the importance of these proteins for the biophysical properties of the stratum corneum, this result strongly suggests unsuspected differences in the epidermal barrier efficacy in the healthy population. In agreement, interindividual variations in TEWL values of healthy volunteers have been reported. Percutaneous absorption of corticoids topically applied on the forearm can vary by up to 30-fold between subjects (discussed by Cork et al⁷ and references cited therein). Similarly, marked interindividual variations have been reported in the constitutive skin expression of antimicrobial peptides³⁶ and drug-metabolizing enzymes.³⁷ These differences could be part of an adaptive response to various environmental insults.

Our data reveal significantly decreased FLG, HRNR, and FLG2 levels in the lesional skin of patients with AD compared with levels seen in nonatopic control subjects. This result confirms that genes involved in epidermal barrier function are downregulated in AD skin lesions.^{38,39} In particular, decreased amounts of HRNR and other components of the cornified cell envelope, including involucrin and loricrin, might be responsible for the previously described defects in this resistant protein

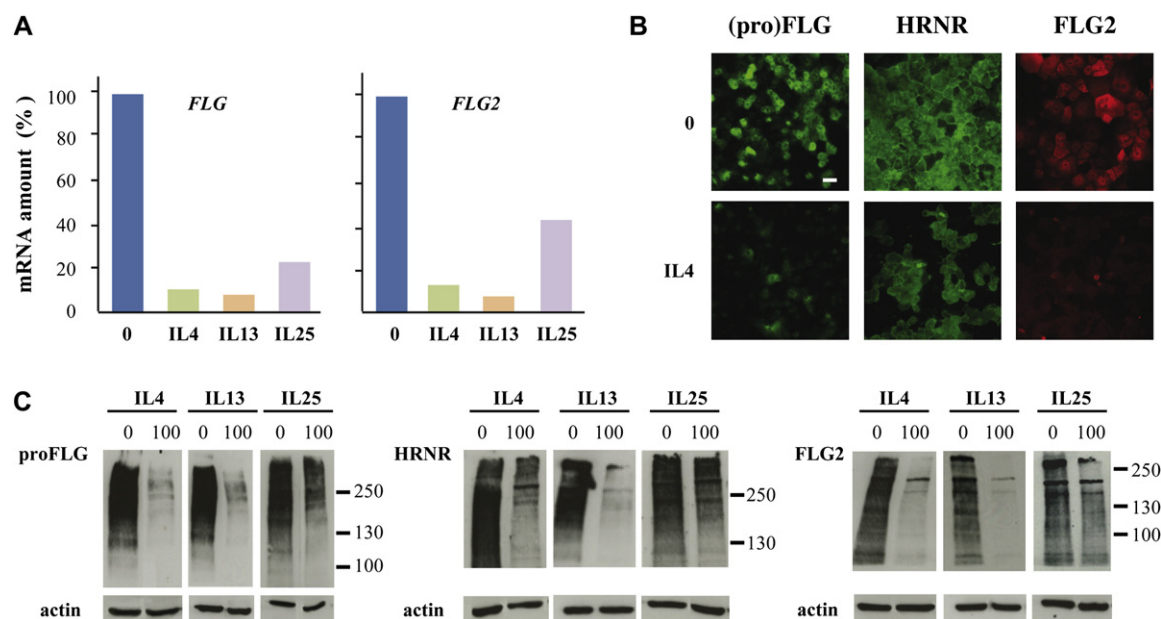


FIG 5. Downregulation of FLG, HRNR, and FLG2 expression by IL-4, IL-13, and IL-25. Human keratinocytes were cultured in the absence (0) and presence (100 ng/mL) of the indicated cytokines. Total RNA was extracted, and *FLG* and *FLG2* mRNA levels were evaluated by using real-time RT-PCR (A; $n = 2$). The expression of the 3 proteins was analyzed by means of indirect immunofluorescence (for the effect of IL-4, B; $n = 2$; bar = 50 μ m) and Western blotting (C; $n = 3$).

shell at the corneocyte periphery.³⁹ Decreased levels of the 3 proteins were also observed in nonlesional atopic skin, although to a lesser extent. Similarly, a proteomic analysis of superficial stratum corneum samples obtained by means of skin taping showed reduced amounts of FLG2 in nonlesional atopic versus nonatopic skin and a greater reduction in lesional skin.³⁸ The low FLG and FLG2 levels might explain the skin dryness in the absence of *FLG* mutations.⁴⁰ These data indicate that the stratum corneum in clinically unaffected skin of adults with AD is abnormal. They are consistent with the previously reported increase in the percutaneous penetration of sodium lauryl sulfate and polyethylene glycols into the uninvolved skin of patients with AD.^{41,42} This defect probably allows intraepidermal penetration of environmental antigens, triggering the inflammatory response that precedes the formation of lesions. These data also emphasize the importance of treatments with “barrier repair” cream to prevent eczema flares.

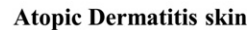
We explored whether nonsense mutations existed in *HRNR* and *FLG2*. Only silent and missense mutations were detected, and their frequencies did not differ between control subjects and patients. Likewise, the allelic frequency of a recently identified nonsense mutation in *FLG2* (Ser2377X; rs12568784) is similar in patients with AD (13.4%) and nonatopic control subjects (15.1%).⁴³

The parallel reduction in levels of FLG, HRNR, and FLG2 in adults with AD, apparently in the absence of any mutations in their encoding genes, implies a systemic downregulation of their expression. We tested the effect of proinflammatory cytokines known to be overexpressed in AD skin.⁶ We confirmed that IL-4, IL-13, and IL-25 downregulate proFLG expression in cultured keratinocytes. We demonstrated that these cytokines also downregulated HRNR and FLG2 at the mRNA level, protein level, or both. Taken together, these results strongly suggest that the expression

of FLG and FLG-like proteins is modulated by the inflammatory response in the skin of patients with AD. Consistently, mRNA levels of involucrin and loricrin are also downregulated by IL-4 and IL-13.⁴⁴ The same cytokines were recently shown to increase kallikrein 7 expression and activity.⁴⁵ Because this protease plays a major role in corneodesmosome degradation, its overexpression might reduce stratum corneum cohesion and therefore participate in the epidermal barrier deficiency of AD skin. T_H2 and T_H22 cytokines might have a more general effect on keratinocytes, inhibiting terminal differentiation.^{22,24,25}

The large effect of IL-4, IL-13, and IL-25 on FLG, HRNR, and FLG2 mRNAs, proteins, or both together with the positive correlations we observed between the amounts of the 3 proteins suggests a coordinated regulation of their expression. Consistently, exposure of keratinocytes to dioxin upregulates the 3 genes.⁴⁶ In favor of this hypothesis, noncoding sequences conserved during the evolution and shown to display keratinocyte- and differentiation-specific enhancer activities at long range have been identified throughout the epidermal differentiation complex genomic locus.⁴⁷ These long-range enhancers might regulate FLG, HRNR, and FLG2 expression at the transcriptional level.

We carefully examined the proFLG immunodetection patterns in epidermal extracts of patients and control subjects who do not carry an R501X, 2282del4, S3247X, or R2447X *FLG* mutation. The extracts of lesional and nonlesional skin obtained from the patients with AD had a higher proFLG/FLG ratio than those obtained from control subjects, confirming that the clinically uninvolved skin is abnormal. Tan et al⁴⁸ studied the proFLG to FLG processing in the epidermis obtained by using suction blistering performed on the uninvolved skin of patients with AD. Their data support our own results: patients with AD without any *FLG* mutations present a higher proFLG/FLG ratio



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SUPPLEMENTARY METHODS

Study patients

Seventy-three unrelated adult subjects (33 men and 40 women) aged between 18 and 49 years (mean age 29.8 ± 9.03) with a history of mild-to-severe AD were recruited and clinically characterized by an experienced dermatologist. Disease severity was determined using the SCORing Atopic Dermatitis (SCORAD) and Nottingham Eczema Severity Score evaluations (see Table E1 in this online repository). A medical interview, including questions about familial antecedent of atopy and history of the disease, was carried out. The presence of palmar hyperlinearity, keratosis pilaris and atopic manifestations (food allergy, asthma and allergic rhinitis) was recorded. Treatments with topical corticosteroids, antihistamines, immunosuppressants or antibiotics were not allowed during the eight days preceding the inclusion.

Seventy-three unrelated sex- and age-matched healthy individuals with no personal or familial history of AD, ichthyosis, asthma or allergic rhinitis were recruited. Maximum time between the AD patient and the matching control being recruited was one month. All patients underwent four 3-mm punch biopsies, two on L and two on NL skin sites, usually on their arms (forearm, antecubital fossa or wrist). Two biopsies were obtained from controls at the corresponding skin site. As soon as they had been taken, the biopsies were either embedded in OCT compound (Tissue-Tek®) for immunohistochemical analyses, or frozen in liquid nitrogen for Western blotting experiments. Blood was taken from all individuals for genotyping.

Genotyping

Genomic DNA was prepared from whole blood using standard methods. *FLG* genotyping was performed in patients and controls for the four *FLG* mutations most prevalent in the European population: R501X, 2282del4, S3247X and R2447X (see Table E2 in this online repository). The presence of R501X and 2282del4 were determined by direct sequencing (BigDye Terminator v3.1 cycle sequencing kit, Applied Biosystems, Foster City, CA) of fragments PCR-amplified from genomic DNA. Mutations R2447X and S3247X were detected using matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry (Sequenom, San Diego, CA) as already described.^{E1} *HRNR* and *FLG2* gene fragments were PCR-amplified and fully sequenced in both directions. Primer sequences and annealing temperature for PCR amplifications are indicated in Table E4 (see this online repository).

Histological and immunohistological analyses of skin samples

Five-micron cryosections were used for either hematoxylin-eosin staining or indirect immunofluorescence analyses. After incubation in a blocking solution (2% fish skin gelatin and 0.05% Tween-20 in PBS), sections were incubated with the AHF₃ anti-FLG monoclonal antibody^{E2} diluted to 0.5 µg/ml, and with the AHP₂ anti-hornerin^{E3} and AIP₂ anti-FLG2^{E4} affinity-purified rabbit antibodies, both diluted to 1:50. AHF3 recognizes the proFLG, processing intermediates and FLG monomers. Alexa-fluor® conjugated secondary antibodies (Invitrogen Life Technologies, Grand Island, NY) were used for the detection of primary antibodies. Images were taken using a Nikon Eclipse 80i microscope equipped with a Nikon DXM 1200C digital camera and NIS image analysis software (Nikon, Tokyo, Japan).

Epidermal protein extraction – Western blotting analyses

After heat-induced dermo-epidermal cleavage, total epidermal proteins were extracted by homogenization in 35 mM Tris-HCl pH 6.8, 1.5% SDS, 5% glycerol, and 2.5% β-mercaptoethanol. The homogenates were cleared by centrifugation for 15 min at 15,000 rpm and the supernatants were kept at -80°C until used. Proteins were separated on 10% acrylamide-bisacrylamide gels and transferred onto nitrocellulose membranes. The membranes were stained with Ponceau red, incubated in 5% skimmed milk, and then incubated with either AHF₃ (50 ng/ml), AHP₂ (1:200) or a polyclonal goat antibody directed against the FLG2 spacer^{E5} diluted to 1:1000. Secondary horseradish peroxidase-conjugated IgGs (1:10,000; Zymed Laboratories, San Francisco, CA) were used. The detection was performed using an ECL kit (Pierce/Thermo Scientific, Rockford, IL). The NIH ImageJ free software (Bethesda, MD) was used to quantify immunoreactive bands on Western blot films after scanning.

Signals were normalized for total protein concentration determined against bovine serum albumin using Coomassie blue stained dots on PVDF membranes.

Effects of inflammatory cytokines on normal keratinocytes

To investigate the effect of cytokines, recombinant IL4, IL25, IL22 (CellSystems®) and IL13 (R&D Systems, Minneapolis, MN) were added at 100 ng/ml to the keratinocyte culture medium for the entire differentiation period.

For immunofluorescence analysis, cells were washed twice with PBS, fixed for 10 min in 4% formaldehyde buffered solution, washed twice again and permeabilized for 10 min with 0.2% Triton X-100 in PBS. After blocking in 2% bovine serum albumin in PBS for 20 min, cells were incubated with the primary antibodies diluted in the same buffer. For nucleus counterstaining, 4',6-diamidino-2-phenylindole (100 ng/ml; Invitrogen) was added to the secondary antibody solution. Slides were then mounted in fluorescent mounting medium (Dako France, Trappes, France) and analyzed using an inverted Zeiss LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Total proteins were extracted and analyzed by Western blotting as described above, except that 7.5% acrylamide gels were used. The previously described primary antibodies and the anti-HRNR antibody HPA031469 (1:500; Sigma-Aldrich, St-Louis, CO) were used. Immunodetection intensities were normalized against actin detected using MAB1501 (Merck Millipore, Billerica, MA).

For RT-qPCR, keratinocytes were rinsed twice in PBS then dissociated in trypsin (Invitrogen Life Technologies), washed twice in PBS and centrifuged. Pellets were quickly frozen in liquid nitrogen and stored at -80°C. Lysis was performed by thawing in RLT-1% β -mercaptoethanol (Sigma-Aldrich) buffer, and total RNA was extracted using the RNeasy Plus Minikit after homogenization on QIAshredder columns (Quiagen France, Courtaboeuf, France) according to the manufacturer's instructions. Reverse transcription was performed by means of Improm-II Reverse Transcriptase (Promega, Madison, WI) using a combination of oligo(dT) and random hexamers. Quantity and quality of the RNA were checked on agarose gels and using the 260 nm/280 nm and 260 nm/230 nm absorbance ratios recorded with a Nanodrop 1000 (Pierce/Thermo Scientific). qPCR amplification was performed with the 7300 Real Time PCR System (Applied Biosystems) using the Sybr qPCR

SuperMix W/ROX (Invitrogen Life Technologies). Sequences of the primers used are indicated in Supplementary Table S1. Their efficiency was checked to be $\geq 99.5\%$. Relative levels of gene expression among samples were determined using the $\Delta\Delta C_t$ method. Hypoxanthine-guanine phosphoribosyl transferase gene expression was used for normalization.

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- E3-Henry J, Hsu CY, Haftek M, Nachat R, de Koning HD, Gardinal-Galera I, et al. Hornerin is a component of the epidermal cornified cell envelopes. *Faseb J* 2011;25:1567-76.
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SUPPLEMENTARY FIGURE LEGENDS

FIGURE E1. ProFLG and FLG are detected in the epidermis of a control subject with an R2447X/R2447X homozygous mutation. **A**, equal amounts of epidermal proteins from the *FLG*^{R2447X/R2447X} control, the matched AD wild-type (wt) patient and an *FLG* wt control skin biopsies were immunodetected with AHF3 monoclonal antibody directed against proFLG and FLG. The wt (arrow) and truncated mutated (arrowhead) forms of proFLG, and FLG monomers are indicated. Molecular mass standards are shown on the left (kDa). **B**, a biopsy of the skin obtained from the *FLG*^{R2447X/R2447X} control subject was analyzed by indirect immunofluorescence with AHF3. Scale bar = 20 µm. The external surface of the *stratum corneum* and the dermo-epidermal junction are indicated by continuous and dashed lines, respectively.

FIGURE E2. Expression of FLG is decreased in lesional and non-lesional skin of AD patients who did not carried a FLG mutation. Immunodetected amounts of FLG in total epidermal extracts of controls (n = 70) and AD patients (n = 64) who did not carry a FLG mutation were quantified and are indicated (in arbitrary units (AU) per µg of proteins) using box plots showing the median (midline), superior and inferior deciles and quantiles, and outlier values (x); * p = 4x10⁻³ *** p < 10⁻⁴; Mann-Whitney non-parametric rank sum test.

FIGURE E3. Correlations between the detected amounts of FLG, HRNR and FLG2. Three-dimensional graphic representations of HRNR (x-axis), FLG (y-axis) and FLG2 (z-axis) Western blotting detection values (in arbitrary units (AU) per µg of proteins) corresponding to the normal skin of control individuals (**A**), and to the non-lesional (**B**) and lesional (**C**) skin of the group of AD patients. Colors represent the intensity of FLG2 immunodetection, ranging from green (weak) to brown (high). Note the differences in scales, depending on the graph.

FIGURE E4. Schematic representation of FLG2 and HRNR genes and protein structures. *FLG2* and *HRNR* genes have the same intron/exon structure, and consist of three exons. Exon 1 corresponds to 5' UTR sequences, and exon 2 contains the initiation codon. Exon 3 is unusually large (lengths are indicated in bp). Open boxes represent the 5' and 3' noncoding sequences. Coding sequences (black and colored boxes) are split into the exons 2 and 3. They correspond to the S100A homologous region, the spacer, and the multiple tandem repeat domain (amino acid numbers are indicated). The repeat domain of *FLG2* is formed by two types of repeats, nine A-type and fourteen B-type repeats. The repeat domain of *HRNR* is formed by seventeen repeats. Positions of the sequenced genomic regions (named IFPS-1, IFPS-2, IFPS-3, H-1 and H-2) are shown below the genes, with the corresponding length in bp. For gene mutations analysis, the sequences of both sense and antisense strands were read and further compared to the genomic reference according to the NT004487.19 contig. After Blast alignments, sequence differences were confirmed manually. The drawings are not to scale. UniProtKB/SwissProt accession numbers of *HRNR* and *FLG2* are Q86YZ3 and Q5D862, respectively.

FIGURE E5. Location and frequency of FLG2 mutations observed. **A**, *FLG2* is schematically represented with the position of S100 homologous domain, and A1, A2, B1 and B2 repeats (amino acid numbers are indicated below). Arrows show the position of the mutations we identified, with National Center for Biotechnology Information single-nucleotide polymorphism identification numbers in brackets (rs#). **B**, observed frequency of the *FLG2* mutations in the controls (n = 73) and AD patients (n = 73). The % of individuals carrying at least one mutated allele is shown.

FIGURE E6. Location and frequency of HRNR mutations observed. **A**, *HRNR* is schematically represented with the position of S100 homologous domain, and A1, A2, B1, B2 and C1 repeats (amino acid numbers are indicated below). Arrows show the position of the mutations we identified, with identification numbers in brackets (rs#). **B**, observed frequency of the *HRNR* mutations in the controls (n = 73) and AD patients (n = 73).

SUPPLEMENTARY TABLES

Table E1. Characteristics of atopic dermatitis patients (n = 73).

	n (%)
Nottingham score	
Mild	12 (16.5%)
Moderate	29 (39.5%)
Severe	32 (44.0%)
SCORAD	
Mild (< 20)	5 (6.8%)
Moderate (20-40)	26 (35.7%)
Severe (> 40)	42 (57.5%)

Table E2. Frequency of the *FLG* gene mutations.

Genotypes	R501X		2282del4		S3247X		R2447X		Combined	
	Co	AD	Co	AD	Co	AD	Co	AD	Co	AD
AA*	72	67	73	70	73	73	72	73	70	64
Aa	1	6	0	3	0	0	1	0	2	9
aa	0	0	0	0	0	0	1	0	1	0
Total	73	73	73	73	73	73	73	73	73	73

* AA, wild-type for the mutation; Aa, heterozygote for the mutation; aa, homozygote or compound heterozygote for the mutation

Table E3. Correlations between the detected amounts of FLG, HRNR and FLG2.

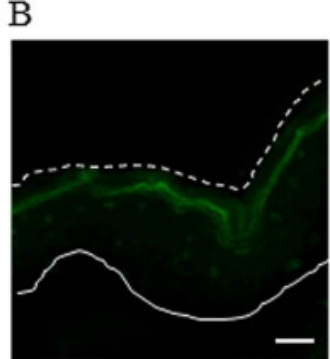
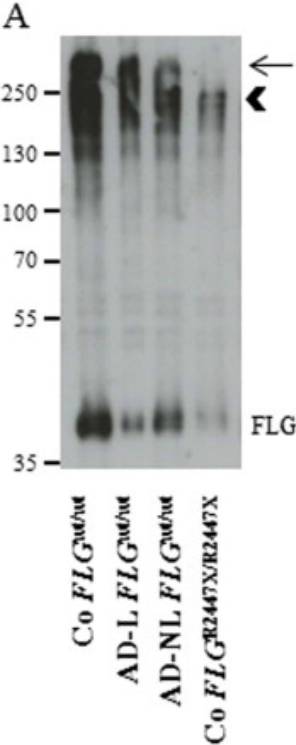
	Co	AD-NL	AD-L
FLG vs HRNR	0.5* $p < 10^{-4}$	0.57 $p < 10^{-4}$	0.45 $p = 10^{-4}$
FLG vs FLG2	0.34 $p = 0.0029$	0.49 $p < 10^{-4}$	0.5 $p < 10^{-4}$
HRNR vs FLG2	0.59 $p < 10^{-4}$	0.72 $p < 10^{-4}$	0.54 $p < 10^{-4}$

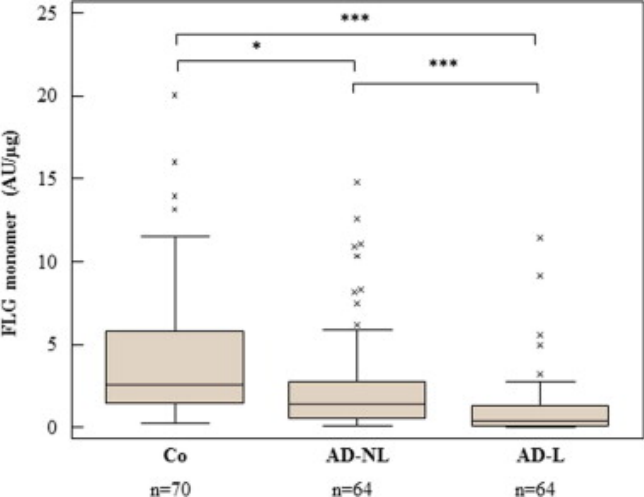
* Results are presented as Spearman correlation coefficient and p value for each group of data: controls subjects (Co), and non-lesional (AD-NL) and lesional (AD-L) skin of atopic dermatitis patients.

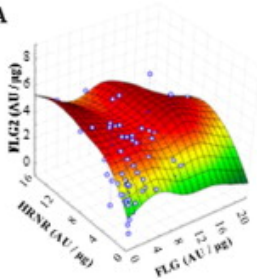
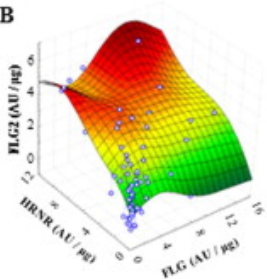
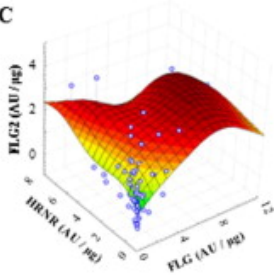
Table E4. Sequences (5'-3') of primers used for genotyping and q-PCR.

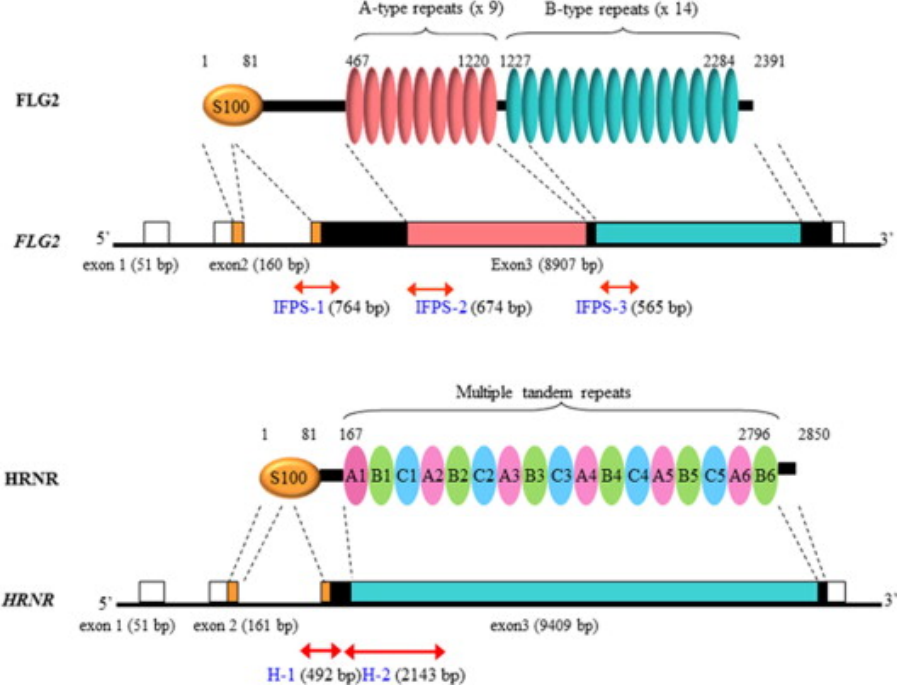
Gene name (°C)*	Forward primer	Reverse primer	Extension primer
<i>FLG-R501X</i> (57°C)	CACGGAAAGGCTGGGCTGA	ACCTGAGTGTCAGACCTATT	
<i>FLG-2282del4</i> (57°C)	AATAGGTCTGGACACTCAGGT	GGGAGGACTCAGACTGTTT	
<i>FLG-R2447X</i> (72°C)	ACGTTGGATGAGACAAGGATCCCACCACA	ACGTTGGATGTGTCCATGAATGGTGTCTG	GCCTGGAGCTGTCTC
<i>FLG-S3247X</i> (72°C)	ACGTTGGATGAAACCATCGTGGATCTGT	ACGTTGGATGTGTCTTCGTGATGGGACCTG	GTCTGGAGCCGTGCCTT
<i>FLG2-IFPS1</i> (58°C)	CTGCAACAAGGTCCTCAGC	CACATCCAGAGGGCTGACC	
<i>FLG2-IFPS2</i> (58°C)	TCCTGTGGCCGCTTTTCAAAC	CCAGAACTGTGTTGGCCATAAC	
<i>FLG2-IFPS3</i> (58°C)	GGCTCAGGTCAATCAACTGG	TGGCCTCTCCTGTCTGTC	
<i>HRNR-H1</i> (59°C)	CCTTCAGTTCGTTAGCCAGA	TGGGAGTCGGAGTTTTGC	
<i>HRNR-ABC</i> (72°C)	AGGAGAACAAACGGCAAGAA	CCATGCTGACTATAGCCCTGT	
<i>FLG-qPCR**</i> (72°C)	GCAAGGTCAAGTCCAGGAGAA	CCCTCGGTTTCCACTGTCTC	
<i>HPRT-qPCR</i> (72°C)	ACCCACGAAGTGTGGATA	AAGCAGATGGCCACAGAACT	
<i>FLG2-qPCR**</i> (72°C)	ACCAGGTTCACTTAAACTTGCA	ATGACATCCACTGTGTCTGGAT	

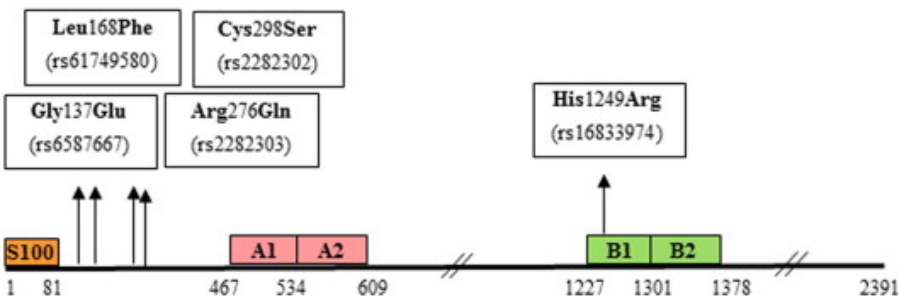
*Annealing temperature. ***FLG* and *FLG2* primers have been already described (Peyret-Lacombe et al. Cell Tissue Res 2007;328:85-95 and de Koning H, et al. Br J Dermatol 2012;166:1245-54, respectively). HPRT, hypoxanthine-guanine-phosphoribosyl transferase.





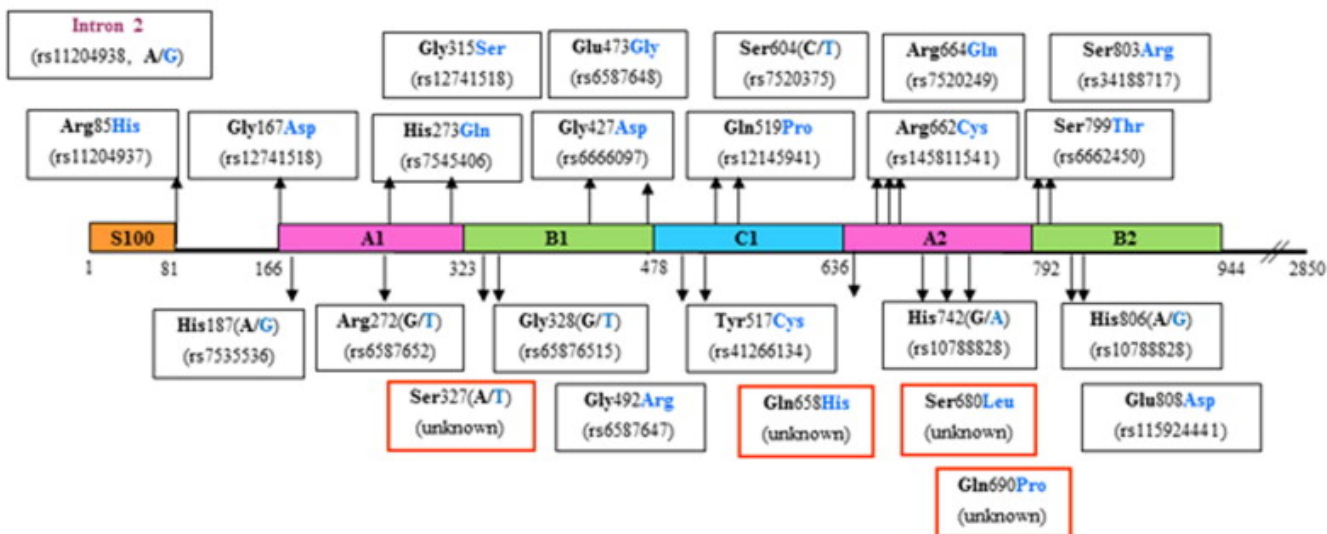
A**B****C**



A**B**

	AD	Control
Gly137Glu	5.5 %	2.7 %
Leu168Phe	1.4 %	2.7 %
Arg276Gln	30.1 %	26.0 %
Cys298Ser	28.8 %	37.0 %
His1249Arg	1.4 %	2.7 %

A



B

	AD	Control
Intron	39.7 %	27.4 %
Arg85His	95.9 %	95.9 %
Gly167Asp	6.4 %	3.8 %
His187	91.8 %	91.8 %
Arg272	93.2 %	91.8 %
His273Gln	78.1 %	74.0 %
Gly315Ser	5.5 %	4.1 %
Ser327	1.4 %	0 %
Gly328	90.4 %	91.8 %

	AD	Control
Gly427Asp	78.1 %	74.0 %
Glu473Gly	100 %	100 %
Gly492Arg	93.2 %	100 %
Tyr517Cys	26.0 %	21.9 %
Gln519Pro	8.2 %	13.7 %
Ser604	93.2 %	91.8 %
Gln658His	1.4 %	0 %
Arg662Cys	0 %	2.7 %
Arg664Gln	93.2 %	100 %

	AD	Control
Ser680Leu	1.4 %	0 %
Gln690Pro	0 %	1.3 %
His742	93.2 %	93.2 %
Ser799Thr	100 %	100 %
Ser803Arg	5.5 %	4.1 %
His806	5.5 %	4.1 %
Glu808Asp	1.4 %	0 %