

Letter to the Editor

Bleomycin hydrolase downregulation in lesional skin of adult atopic dermatitis patients is independent of *FLG* gene mutations

To the Editor:

Atopic dermatitis (AD) pathogenesis is still incompletely understood. The major genetic risk factor is nonsense mutations of the *FLG* gene encoding filaggrin.¹ Filaggrin is a key protein for the stratum corneum (SC) barrier functions.^{2,3} It is synthesized as a 400 kDa precursor named profilaggrin. During the late keratinocyte differentiation, profilaggrin is cleaved by proteases, including calpain-1. The produced filaggrin monomers associate with keratin filaments and promote their aggregation. Then, the monomers are proteolyzed into hygroscopic free amino acids by caspase-14, bleomycin hydrolase (BLMH), and calpain-1. The consequences of *FLG* nonsense mutations are increased photosensitivity of the lower keratinocytes, enhanced outside-in permeability of the SC, and exacerbated percutaneous immune

response.^{4,5} Other mechanisms beyond *FLG* mutations certainly are important in the development of AD. Filaggrin expression is downregulated in the epidermis of adult AD patients independently of *FLG* mutations through the action of proinflammatory cytokines.⁶ Also an enhancement of the profilaggrin/filaggrin ratio has been observed in some patients,^{6,7} suggesting decreased profilaggrin processing or increased filaggrin degradation. To test this hypothesis, we comparatively analyzed the expression of calpain-1, caspase-14, and BLMH in a cohort of 28 adult AD patients and 28 age- and sex-matched healthy control subjects. The individuals were genotyped for the most common *FLG* mutations (R501X, 2282del4, S3247X, and R2447X). Total epidermal proteins were prepared and analyzed by Western blotting.

All experiments were performed according to the principles of the Declaration of Helsinki. The detailed methodology and characteristics of the patients are described in this article's [Methods](#) section and [Table E1](#) in this article's [Online](#)

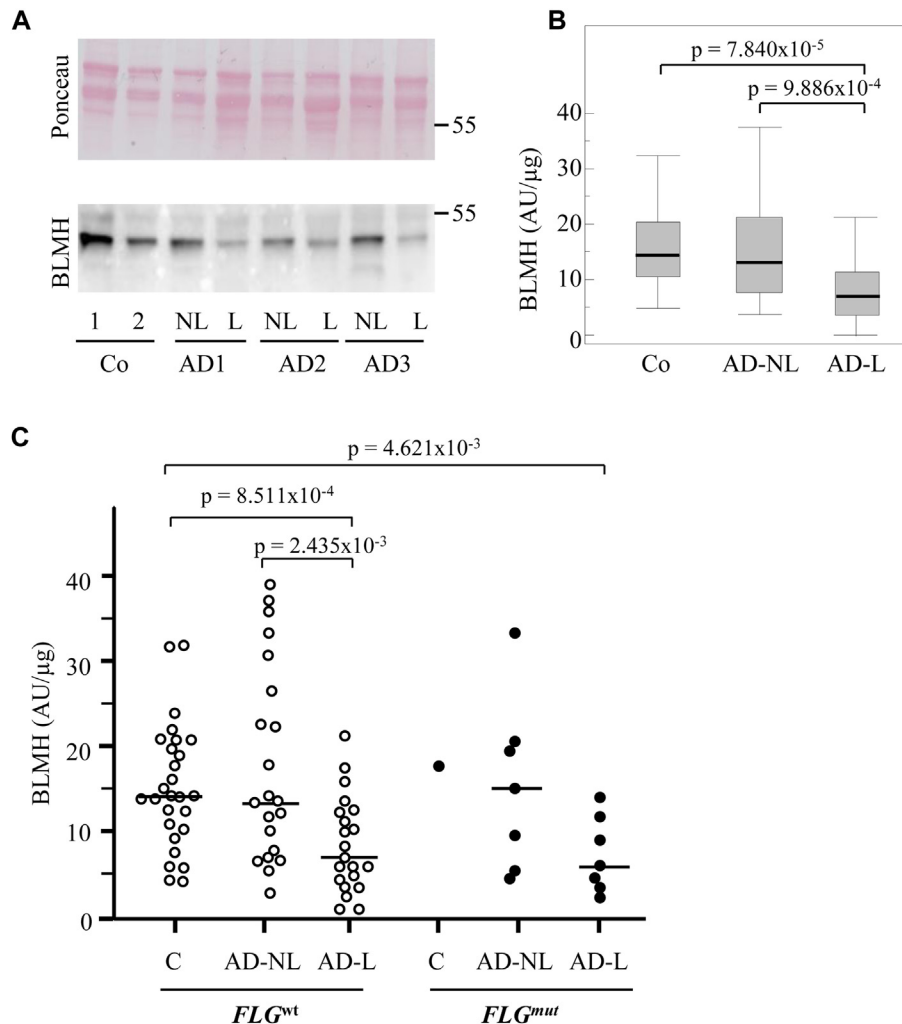


FIG 1. Reduced detection of BLMH in AD skin. Total epidermal proteins of 28 controls (Co) and 28 patients (AD) were immunodetected. Representative Ponceau red staining and blots (A). Immunodetected BLMH amounts were quantified and are indicated (AU/μg total proteins) using box plots (B) and individual values (C). Only statistically significant *P* values are shown.

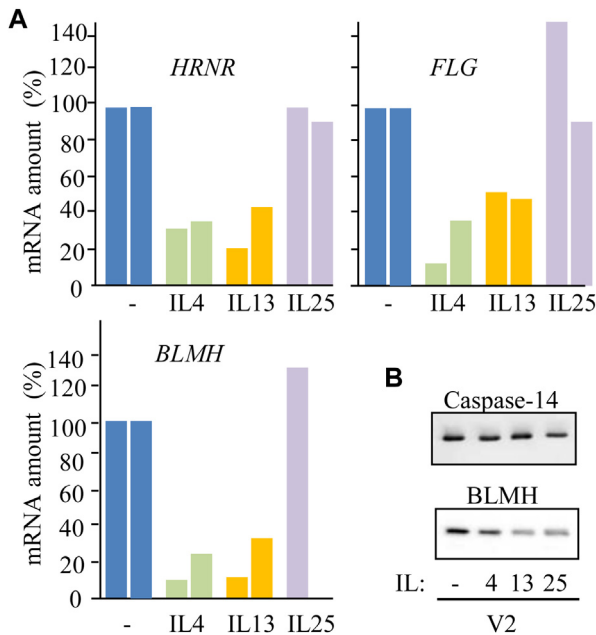


FIG 2. In normal keratinocytes, BLMH is downregulated by IL4, IL13, and IL25. Keratinocytes from 2 healthy volunteers (V1 and V2) were cultured without (-) and with (100 ng/mL) the indicated cytokines. Levels of *HRNR*, *FLG*, and *BLMH* mRNA were evaluated by real-time RT-PCR (V1, left bars; V2, right bars) (A). Expression of BLMH and caspase-14 was analyzed by Western blotting (B).

Repository at www.jacionline.org. One control subject and 3 patients carried a 2282del4 *FLG* mutation and 4 patients an R501X mutation (see Table E2 in this article's Online Repository at www.jacionline.org).

We first evaluated the profilaggrin/filaggrin ratio (Fig E1, in this article's Online Repository at www.jacionline.org). Considering all patients and controls, a statistically significant ($P < .008$; Kruskal-Wallis test) increased ratio was observed in atopic (median value = 7.21 and 10.10 for nonlesional [NL] and lesional [L] skin, respectively) as compared to normal (median = 2.76) skin. In addition, the ratio was higher in L than in NL skin extracts for 18 (64%) of the 28 patients. When the values were stratified according to the *FLG* mutation status, a similar increased ratio was observed in patients without *FLG* mutations (*FLG*^{wt}; $n = 21$) and patients with a *FLG* mutation (*FLG*^{mut}; $n = 7$). In the latter group, statistical significance was only achieved when considering the NL skin. The difference in ratios between *FLG*^{mut} and *FLG*^{wt} was not statistically significant. Moreover, the individual ratio was higher in L than in NL skin extracts for 16 *FLG*^{wt} patients (76%) and 1 *FLG*^{mut} patient (14%).

The extracts were then analyzed for the expression of BLMH (Fig 1, A and B). The protein was immunodetected as a band of 50 kDa. Variations were observed in the amounts of protein detected, with a factor of 6.8, for example, between the lowest and the highest values in the control group. The amounts of BLMH were lower in the extracts of AD-L as compared to AD-NL and to control skin (median = 6.56, 13.23, and 14.37 arbitrary units [AU]/μg of proteins, respectively). When considering the *FLG*^{wt} groups (Fig 1, C), very similar results were observed (median = 6.93, 13.41, and 14.30 AU/μg of proteins, respectively). Considering the *FLG*^{mut} patients, the amounts of BLMH in the extracts of L skin were also lower

(median = 5.75 AU/μg) than the controls. A similar tendency was observed as compared with the NL skin extracts (median = 15.29 AU/μg); however, the difference was not statistically significant. In addition, the amount of BLMH was lower in L than in NL skin extracts for 24 (86%) patients, including 18 (86%) *FLG*^{wt} and 6 (86%) *FLG*^{mut}.

The proteins were then immunodetected with an antibody recognizing both the proform and the large subunit of the active form of caspase-14. The amounts of each and the procaspase-14/caspase-14 ratio were then computed (Fig E2 in this article's Online Repository at www.jacionline.org). Procaspase-14 was increased in the L skin samples of both *FLG*^{wt} and *FLG*^{mut} patients as compared with the controls ($P < 2 \times 10^{-2}$). The procaspase-14/caspase-14 ratio was increased in the patients (either as a whole or stratified), suggesting that the procaspase-14 was less efficiently activated. This is in agreement with the work of Yamamoto et al.⁸

When the amounts of immunodetected calpain-1 were computed, no relevant differences were observed (Fig E3 in this article's Online Repository at www.jacionline.org).

The expression of several late differentiation proteins, including filaggrin and hornerin, is known to be downregulated by proinflammatory cytokines.⁶ Therefore, we tested the effects of recombinant IL4, IL13, and IL25 on the expression of BLMH in cultured keratinocytes from 2 healthy volunteers, at both the mRNA and protein levels (Fig 2). Used as a control, the downregulation of *FLG* and *HRNR*, the gene encoding hornerin, was confirmed after exposure to 100 ng/mL of IL4 and IL13. The amount of *BLMH* mRNAs was also reduced by IL4 and IL13, whereas IL25 had no effect in our conditions. Western blotting performed with extracts from 1 of the volunteers evidenced that BLMH was downregulated by IL4, IL13, and IL25 (23.1%, 58.9%, and 52.9% reduction, respectively) at the protein level. The cytokines had a moderate effect on caspase-14.

We showed that the expression of BLMH is diminished in the epidermis of the L skin of atopic patients. This has already been observed in a small series of patients and controls of Asian origin.⁹ Inflammatory cytokines may be responsible for this reduction. Less BLMH could lead to a reduced production of amino acids that compose the natural moisturizing factor and, therefore, SC dryness.

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METHODS

Patients

All experiments were performed according to the principles of the Declaration of Helsinki. Participants gave their written informed consent before being included. 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).

Twenty-eight unrelated adult subjects (12 men and 16 women) aged between 18 and 49 years (mean age 28.21 ± 8.09) 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). Treatments with topical corticosteroids, antihistamines, immunosuppressants, or antibiotics were not allowed during the 8 days preceding the inclusion. Twenty-eight 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 1 month. These volunteers were part of a previously studied cohort.^{E1} All patients underwent 4 3-mm punch biopsies, 2 on L and 2 on NL skin sites, on their arms (arm, forearm, antecubital fossa, or wrist) except for 2 (dorsal face of the finger and thigh). Two biopsies were obtained from controls at the corresponding skin sites. As soon as they had been taken, the biopsies were either embedded in Tissue-Tek OCT compound (Fisher Scientific, Illkirch, France) for immunohistochemical analyses or frozen in liquid nitrogen for Western blotting experiments. Starting from blood, *FLG* genotyping was performed, as described previously,^{E1} for the 4 *FLG* mutations most prevalent in the European population: R501X, 2282del4, S3247X, and R2447X (see Table E2).

Epidermal protein extraction and analysis using Western blotting

After 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, separated on acrylamide-SDS gels, and transferred onto nitrocellulose membranes. The membranes were stained with Ponceau red, and Western blotting was performed as described previously,^{E1} with AHF₃ anti-(pro)filaggrin^{E2} (diluted to 50 ng/mL) and D-10 anti-caspase-14 (Santa Cruz Biotechnology, Heidelberg, Germany; 1:100) monoclonal antibodies, and with rabbit polyclonal antibodies directed against bleomycin hydrolase (HPA039548; Sigma Aldrich, Saint-Louis, Mo; 1:1000) and calpain-1 (HPA005992; Sigma Aldrich; 1:250). Quantity One software (Bio-Rad Laboratories, Hercules, Calif) was used to quantify immunoreactive bands on Western blot films after scanning. Signals were normalized for total protein concentration.

Immunohistologic analyses of skin samples

Five-micron cryosections were used for indirect immunofluorescence analyses, as described previously,^{E1} with the HPA039548 anti-bleomycin hydrolase rabbit antibody (Sigma Aldrich), diluted to 1:1000. Alexa-fluor conjugated secondary antibodies (Invitrogen Life Technologies, Grand Island, NY) were used for the detection of the primary antibody. 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).

Effects of inflammatory cytokines on normal human primary keratinocytes

Keratinocytes were obtained from the abdominal dermolipectomy of 2 healthy individuals who had given their 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 mM CaCl_2 . To investigate the effect of cytokines, recombinant IL4, IL25 (CellSystems), and IL13 (R&D Systems, Minneapolis, Minn) were added at 100 ng/mL to the keratinocyte culture medium for the entire differentiation period. Total proteins were extracted and analyzed by Western blotting, as described previously^{E1}; immunodetection intensities were normalized against actin detected using MAB1501 (Merck Millipore, Billerica, Mass) diluted to 1:10,000. For RT-qPCR, keratinocytes were rinsed in PBS and dissociated in trypsin (Invitrogen Life Technologies); then, total RNA was extracted using the RNeasy Plus Minikit (Qiagen, France; Courtaboeuf, France) according to the manufacturer's instructions. Reverse transcription using a combination of oligo(dT) and random hexamers and qPCR amplification performed with the 7300 Real Time PCR System (Applied Biosystems) have been described previously.^{E1} Sequences of the primers used are indicated in Table E3. Relative levels of gene expression among samples were determined using the $\Delta\Delta\text{Ct}$ method. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene expression was used for normalization.

Statistical analyses

Statistical differences between groups were determined with the Kruskal-Wallis test. For the correlation analyses, the Spearman rank correlation test was used. Differences were considered significant when $P < .05$.

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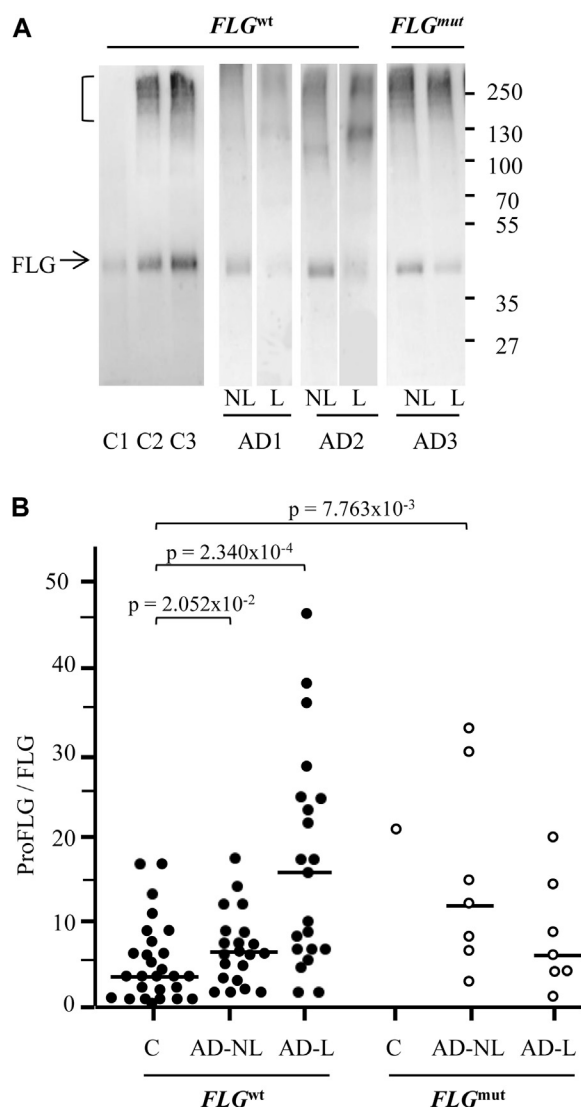


FIG E1. Profilaggrin/filaggrin ratio is increased in AD skin. Total epidermal proteins from NL and L skin of 28 healthy controls (C) and 28 atopic dermatitis patients (AD) were immunodetected with AHF3 antibody for the expression of filaggrin. One control and 7 patients carried an *FLG* mutation (black shaded circle; *FLG^{mut}*); the others did not (non-shaded circle; *FLG^{wt}*). Representative Western blots (**A**). Profilaggrin and processing intermediates (referred to as proFLG) are indicated by a bracket and filaggrin monomers (FLG) by an arrow. Molecular masses are indicated on the right in kDa. Immunodetected amounts of profilaggrin and filaggrin were quantified (**B**). Profilaggrin/filaggrin ratio and the median values are indicated. Calculated *P* values are shown only when statistically significant.

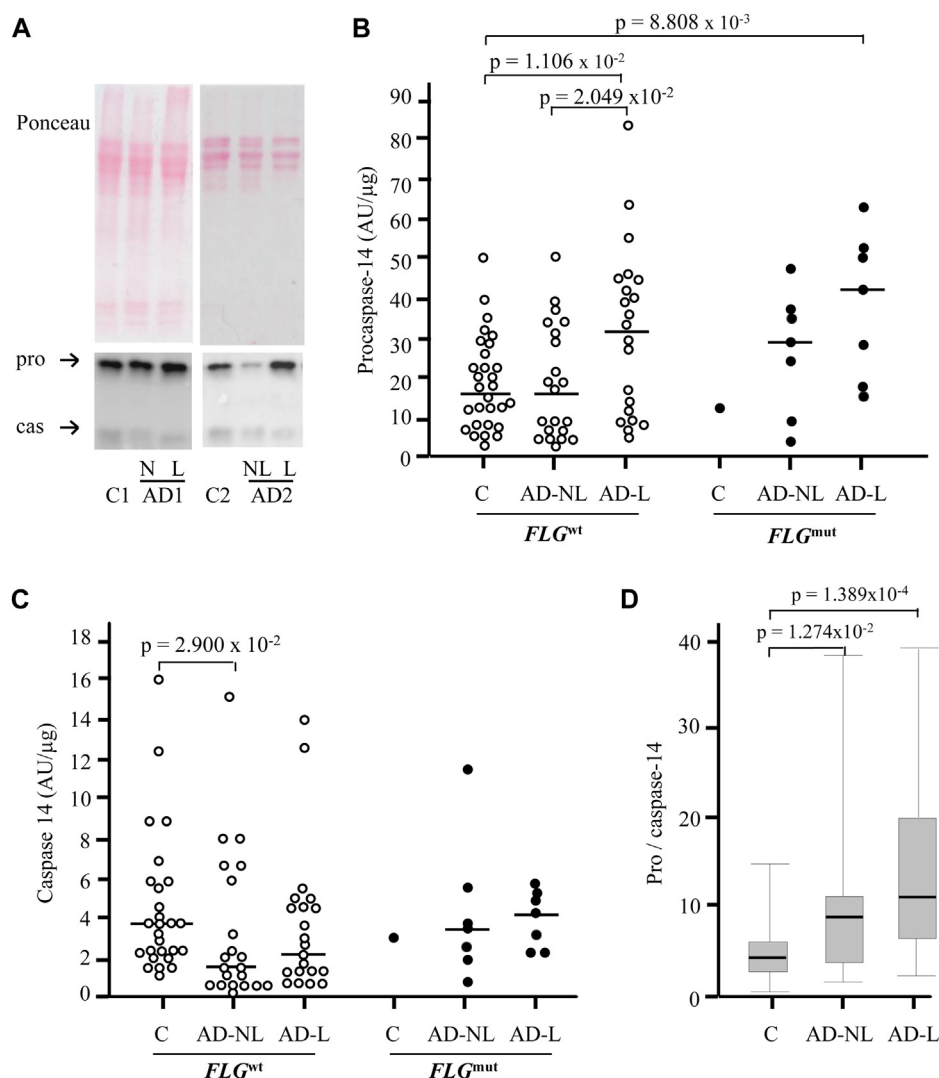


FIG E2. Procaspase-14 is increased, and its activation is reduced in atopic skin. Total epidermal proteins of 28 controls (C) and 28 atopic dermatitis patients (AD) were separated by SDS-PAGE, transferred to membranes, stained with Ponceau red, and immunodetected with an antibody recognizing both the procaspase-14 (pro) and the large subunit of the active caspase-14 (cas). Representative results for 2 controls (C1 and C2) and 2 patients (AD1 and AD2) are shown (A). Both the NL and L skin were analyzed. The immunodetected procaspase-14 (B) and active caspase-14 (C) were quantified, normalized to the total amounts of proteins in the samples, and plotted as arbitrary units (AU) per μg of proteins for each of the extracts. Bars represent the median values. The subjects were stratified according to the absence (FLG^{wt}) or the presence (FLG^{mut}) of an *FLG* gene mutation. The proform/active form ratios were calculated and are indicated using box plots showing the median (midline), first and third quartiles, and minimal and maximal values (D). Calculated *P* values are shown when significant.

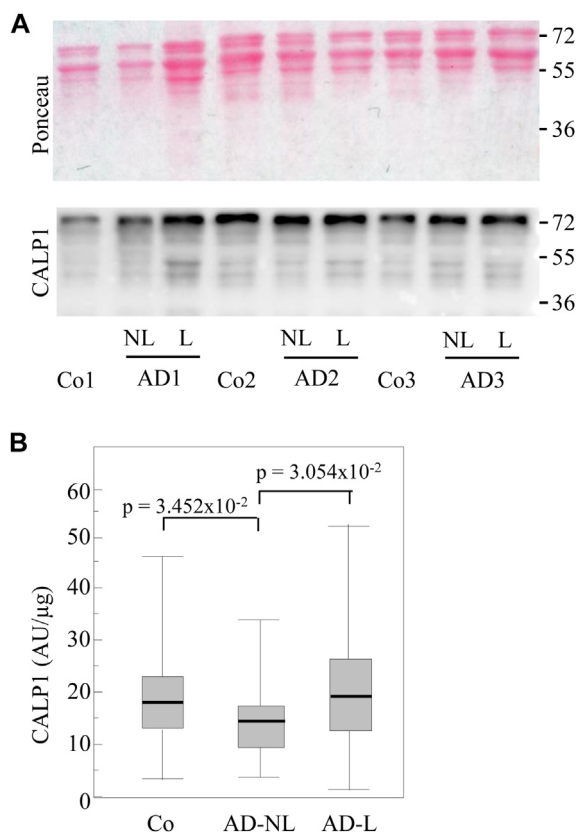


FIG E3. Detection of calpain-1. Total epidermal proteins of 28 controls (C; one was a carrier of an *FLG* mutation) and 28 atopic dermatitis patients (AD, 7 were carriers of an *FLG* mutation) were separated by SDS-PAGE, transferred to membranes, stained with Ponceau red, and immunodetected with an antibody recognizing calpain-1 (CALP1). Representative results corresponding to the extracts of 3 controls (C1-C3) and 3 patients (AD1-AD3) are shown (A). Both the NL and L skin were analyzed. The amounts of immunodetected calpain were quantified (AU per μg) and are indicated using *box plots* showing the median (midline), first and third quartiles, and minimal and maximal values (B). Calculated *P* values are shown when significant.

TABLE E1. Characteristics of atopic dermatitis patients (n = 28)

	n (%)
Nottingham score	
Mild	3 (10.7%)
Moderate	13 (46.4%)
Severe	12 (42.9%)
SCORAD	
Mild (<20)	3 (10.7%)
Moderate (20-40)	8 (28.6%)
Severe (>40)	17 (60.7%)

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	28	24	27	25	28	28	28	28	27	21
Aa	0	4	1	3	0	0	0	0	1	7
aa	0	0	0	0	0	0	0	0	0	0
Total	28	28	28	28	28	28	28	28	28	28

AA, Wild-type for the mutation; Aa, heterozygote for the mutation; aa, homozygote or compound heterozygote for the mutation.

TABLE E3. Sequences of primers used for q-PCR

Gene name	Forward primer	Reverse primer
<i>FLG</i>	5'-GCAAGGTCAAGTCCAGGAGAA-3'	5'-CCCTCGGTTTCCACTGTCTC-3'
<i>HPRT</i>	5'-ACCCACGAAGTGTGGATA-3'	5'-AAGCAGATGGCCACAGAACT-3'
<i>HRNR</i>	5'-AGGACAGGGCTATAGTCAGCA-3'	5'-CCGAAGCGTGATGGGAGG-3'
<i>CASP14</i>	5'-TGCACGTTTATTCCACGGTA-3'	5'-TGCTTTGGATTTCAGGGTTC-3'
<i>BLMH</i>	5'-GTGGTGGACAGGAAGCATGT-3'	5'-TCCTTTGCAGCTACGTCAGG-3'

HPRT, Hypoxanthine-guanine-phosphoribosyl transferase.