Severe skin inflammation and filaggrin mutation similarly alter skin barrier in atopic dermatitis patients

Running head: Filaggrin alterations in atopic dermatitis

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What's already known about this topic?

There is a strong genotype–phenotype link in atopic dermatitis (AD) patients suffering from filaggrin (*FLG*) haploinsufficiency, but acquired FLG deficiency can also occur in AD patients. It is not known whether the clinical and laboratory characteristics of AD are influenced only by genetic or also by acquired FLG alterations.

What does this study add?

1. Actual skin barrier impairment in AD patients with severe skin inflammation is similar in *FLG* wild-type patients and *FLG* mutants and correlate with the severity of skin inflammation (SCORAD).

2. On the other hand the constant barrier deficiency in filaggrin mutants results in an increased risk of allergic sensitisation compared to wild-type patients.

Summary

Background: Filaggrin (FLG) deficiency is a well-known predisposing factor for the development of atopic dermatitis (AD). Decreased FLG expression can be the result of haploinsufficiency or severe inflammation, which can cause acquired FLG alterations. *FLG* mutations are related to several clinical and laboratory parameters of AD; however, some recent data seem to contradict these associations.

Objectives: Our aim was to determine which clinical and biochemical parameters are connected to *FLG* haploinsufficiency and which ones are also associated with acquired FLG alterations due to severe skin symptoms in AD patients.

Methods: We introduced a novel classification of AD patients based on *FLG* mutations and SCORAD. Based on these parameters, we created three groups of AD patients: mild-to-moderate wild-type (A), severe wild-type (B) and severe mutant (C) patients. In all groups, we assessed laboratory and clinical parameters and performed immunohistochemical analyses.

Results: Groups B and C contained patients with equally severe symptoms based on the SCORAD. The two severe groups did not differ significantly with respect to barrier-specific parameters, whereas group A had significantly better results for the barrier function

measurements. However, significant differences were detected between groups B and C with respect to the allergic sensitisation-specific parameters.

Conclusions: These findings suggest that skin barrier function correlates with the severity of skin inflammation and can be equally impaired in FLG mutant and wild-type AD patients with severe symptoms. Nevertheless, our results also suggest that *FLG* mutant patients may have a higher risk of allergic sensitisation compared to wild-type patients.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects up to 20% of the white European paediatric population[1]. AD is often accompanied by other allergic diseases (e.g., allergic rhinitis and bronchial asthma) and leads to an impaired quality of life[2,3]. Previously, the development of AD was primarily explained by the dysregulation of immune responses (the inside-out theory); however, in the last few years, the role of skin barrier alterations has been emphasised (the outside-in theory)[4-6].

The filaggrin (FLG) protein, which is produced from the profilaggrin precursor and is located in the granular and corneal layers of the skin, plays a pivotal role in the formation of the skin barrier[6,7]. Active FLG plays a major role in crosslinking keratin filaments and participates in the development of the cornified envelope, and its degradation products are important components of natural moisturising factors (NMFs)[8,9]. NMFs buffer the pH of the skin and play a role in UV protection (e.g. urocanic acid), as well as in immunomodulation[10-12]. Intragenic variation with respect to the copy number of FLG monomers is correlated with the occurrence of AD, and the presence of fewer FLG repeats in the profilaggrin gene contributes significantly to the development of AD[13].

Previous investigations have demonstrated that major (R501X and 2282del4) as well as minor (S3247X, R2447X and 3702delG) *FLG* null mutations are responsible for the development of ichthyosis vulgaris (IV), and these mutations are also major predisposing factors for AD[14,15]. Recently, several research groups have reported associations between *FLG* mutations and the severity of AD, early disease onset, allergic sensitisation, the frequency of eczema herpeticum outbreaks and the degree of skin barrier defects, which are characterised by high transepidermal water loss (TEWL)[16-18]. However, others have not detected any correlations between *FLG* mutations and TEWL, skin diffusion or permeability,

and suggest that *FLG* haploinsufficiency may play a minor role in the barrier abnormalities characteristic of AD[19], or attribute this contradiction to low patient numbers[10]. We suggest that this discrepancy may result from the fact that until now, most studies investigating the effects of *FLG* haploinsufficiency have not taken into consideration the SCORAD (SCORe Atopic Dermatitis) value as an independent parameter to differentiate the compared groups. Patients without *FLG* mutations (wild-type) can also suffer from severe AD, and the actual severe skin inflammation (high SCORAD) can lead to FLG reduction and alter barrier functions.

In the current investigation our aim was to study whether acquired FLG deficiency as a consequence of actual skin inflammation can result in the same barrier disruption as *FLG* mutations. Therefore, we established three patient groups (mild-to-moderate and severe wild-type and severe mutant) and systematically analysed and compared the most frequent AD-related clinical (SCORAD, TEWL, patients' atopic history) and laboratory parameters (serum thymic stromal lymphopoietin [TSLP] levels, total and specific IgE levels) among these groups, as well as FLG content and epidermal thickness, which were measured after immunohistochemical staining. Of great importance, our results clearly identified the parameters that are mainly related to *FLG* genotype and those that are also associated with the severity of skin inflammation.

Materials and methods

Patients

Peripheral blood was obtained from 49 Caucasian AD patients, 22 males and 27 females (mean age: 19 years, range: 5–36 years) with mild-to-moderate or severe clinical symptoms. Skin biopsy specimens were also collected from six patients. All patients suffered from extrinsic type of AD. Their mean total IgE serum level was 3370 kU/L, mean objective SCORAD (OSCORAD) was 31.51, mean LDH was 436.9 U/L, and mean eosinophil count was 0.49 G/L. Patients with AD did not suffer from any concomitant skin diseases at the time of the examination and had not been treated with any moisturisers for 1 day, topical corticosteroids for 3 days or with systemic immunosuppressants for 28 days prior to examination. The following laboratory parameters were examined: serum TSLP level, total IgE and specific IgE levels (house dust mites, ragweed and cat dander). Data on the patients'

history of other allergic diseases and sensitisation were recorded. The severity of AD was determined using OSCORAD (Objective SCORe Atopic Dermatitis) and was also checked by measuring epidermal thickness in biopsy specimens. Three groups were formed according to their *FLG* status and clinical severity: Group A, patients with mild-to-moderate AD symptoms (OSCORAD ≤ 25) without *FLG* mutations (n=10); Group B, patients with severe AD symptoms (OSCORAD > 25) without *FLG* mutations (n=22); and Group C, patients with severe AD symptoms (OSCORAD > 25) who carried *FLG* mutations (n=17, of which 15 were heterozygotes for one of the two alleles [11 patients for 2282del4 and 4 patients for R501X], and two were compound heterozygotes). The compound heterozygous patients belonged to the severe *FLG* mutant group, and they had no concomitant IV. Healthy controls (n=10) were included as the basis for the comparison of barrier function and serum TSLP levels. All participants provided written informed consent according to the principles of the Declaration of Helsinki. The study was approved by the local ethics committee.

Filaggrin genotyping

Analysis of the *FLG* mutations R501X and 2282del4 was performed for all patients. DNA isolated from peripheral blood mononuclear cells using the GenElute Blood Genomic DNA Kit (Sigma, Chemical Co., St. Louis, MO, USA) was subjected to polymerase chain reaction (PCR) amplification. Primers for genotyping were ACG TTC AGG GTC TTC CCT CT and ATG GGA ACC TGA GTG TCC AG for R501X; CAG TCA GCA GAC AGC TCC AG and AAA GAC CCT GAA CGT CGA GA for 2282del4. PCR amplification conditions were as follows: 1 cycle of 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 64°C for 30 seconds, and 72°C for 30 seconds; and 1 cycle of 72°C for 10 minutes. All PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Inc) and bidirectionally sequenced on an ABI Prism 3100 automated sequencer with Big-Dye terminator cycle sequencing reagents (Applied Biosystems, Foster City, CA) as previously described[21].

Measurement of TEWL

Measurements were performed under standardised laboratory conditions at a temperature of 22–25 ˚C and a humidity level of 40–60%. Before the measurements were taken, patients were allowed to adapt to the room conditions for 5 minutes. TEWL measurements (g/hm^2) were carried out with Tewameter TM300 (Courage and Khazaka, Cologne, Germany) on

nonlesional and lesional skin on both forearms, from the cubital fossa down to the wrist. The duration of the measurements, performed in triplicate, was 30 seconds.

Immunohistochemical staining and whole-slide imaging

For immunohistochemical analyses, paraffin-embedded sections from lesional AD skin (two patients from each group were selected randomly, giving six samples altogether), and healthy controls (n=2) were deparaffinised using xylene and ethanol. Heat-induced antigen retrieval was performed using citrate/TRIS buffer, and sections were preprocessed with H_2O_2 for 5 minutes, followed by the blocking of endogenous peroxidase activity and nonspecific binding sites for 15 minutes. Sections were stained with an antibody against human filaggrin (mouse IgG: Abcam, Cambridge, UK). Subsequently, anti-mouse polyclonal antibodies from the Dako Real EnVision Detection System kit (Dako, Glostrup, Denmark) were employed. Staining was detected using the Vector VIP Kit (VECTOR Laboratories, Burlingame, CA). Sections were counterstained with methylene green. The slides were digitalised using a *Pannoramic SCAN* digital slide scanner with a Zeiss plan-apochromat objective (magnification: 20X, Numerical aperture: 0.8) and Hitachi (HV-F22CL) 3CCD progressive scan colour camera (resolution: 0.2325 μm/pixel). Epidermal thickness as a well-accepted method for the measurement of the severity of skin inflammation in AD[20] and immunostainings were analysed with Pannoramic Viewer 1.15.2 (3DHistech Ltd., Budapest, Hungary), using the HistoQuant application. Regions of interest (ROIs) (n=20/slide) were selected in the corneal layer, and then the Field area [FA (mm^2)] and the Mask area [MA (mm^2)] were calculated by the software. The FA shows the whole area of the ROI, and the MA represents the filaggrin-positive area. The MA/FA values were calculated for all ROIs.

TSLP ELISA

Serum was isolated from patients and aliquoted, and the TSLP levels were determined using the ELISA Human TSLP Quantikine Immunoassay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

To determine the statistical significance of the results, the Kruskal-Wallis test and the Mann-Whitney test were used to analyse nonparametric distributions, and Fisher's exact test was applied to compare specific IgE values and the history of sensitisation. P-values <0.05 were considered statistically significant (*p<0.05; **p<0.01; ***p<0.005).

Results

Skin barrier dysfunction and serum TSLP levels are elevated in severe AD patients irrespective of *FLG* genotype

When comparing the OSCORAD of each patient group, significant differences were observed between the mild-to-moderate (Group A) and severe groups (Groups B and C) (P<0.0001). Comparison of the severe *FLG* wild-type (Group B) with the severe *FLG* mutant group (Group C) revealed no significant difference in their OSCORAD levels (Fig. 1a).

Then, TEWL was measured on nonlesional (Fig. 1b) and lesional AD skin (Fig. 1c). Significantly increased TEWL was observed in the severe groups (Group B, and C) relative to the mild-to-moderate group (Group A) for both nonlesional skin (P=0.0100 and P=0.0262, respectively) and lesional skin (P=0.0234 and P=0.0464, respectively). No difference was detected between the two severe groups (Groups B and C) with respect to TEWL for either the lesional or the nonlesional skin. When determining serum TSLP levels in the three AD groups, no significant difference in serum TSLP levels was detected between the mild-to-moderate group and the severe groups. When combining all severe patients into one group, significant elevation of serum TSLP levels was observed in severe patients compared to healthy controls (P=0.0236) (Fig. 1d).

Importantly, TEWL measured for nonlesional skin (Fig. 1e) and lesional skin (Fig. 1f) showed a strong correlation with the OSCORAD score (P=0.0063, Spearman's rho=0.48 for nonlesional skin and P=0.0029, Spearman's rho=0.61 for lesional skin).

Allergic sensitisation is associated with *FLG* haploinsufficiency

In contrast to the barrier measurements, for which the two severe groups had similar results, our data indicate that the level of allergic sensitisation differed between the two severe groups. The occurrence of allergic asthma and rhinitis in the personal medical history of the patients was detected significantly more frequently in the *FLG* mutant group (P=0.0166 and P=0.0154) than in the wild-type groups (groups A and B, respectively) (Fig. 2a). With respect to the levels of serum total IgE (Fig. 2b), a three-level tendency was observed. Prominent differences were found between the severe groups (groups B and C) and between the wild-type groups (P=0.0181). In addition, a significant difference was found between groups A and C (P=0.0229) (Fig. 2B). These distinctions appeared even stronger when measuring specific IgE levels for ragweed (Fig. 2c) and cat dander (Fig. 2d) in AD patients; indeed,

significant differences were observed between the mutant and the wild-type groups (P=0.0090 and P=0.0472 for ragweed and P=0.0338 and P=0.0021 for cat dander in groups A and B and groups B and C, respectively). No significant differences were found between groups B and C with respect to the specific IgE levels for house dust mites (Fig. 2e).

Epidermal FLG content changes equally in severe AD patients with and without *FLG* mutations

Beside the SCORAD, skin inflammation was also detected by measuring epidermal thickness, and the two severe groups (B and C) showed significantly increased acanthosis compared to group A (P<0.0001) (Fig. 3f). Reduced or no FLG staining was observed in the skin of severe AD patients, in both FLG mutants (Fig. 3c) and wild-type patients (Fig. 3b). Normal FLG immunostaining was found in the upper granular layer and the lower corneal layers of the epidermis of the normal controls (Fig. 3d) and AD patients with mild-to-moderate symptoms (Fig. 3a). When the FLG content was measured using the HistoQuant analysis software, a significantly lower FLG level was observed in AD skin biopsies relative to samples from normal controls (P=0.0001 for mild-to-moderate, and P<0.0001 for severe groups). In addition, there were significant differences between group A and groups B and C (P=0.0010 and P=0.0036, respectively) (Fig. 3e). No differences were detected between the severe groups.

Discussion

AD is a multifactorial disease that is driven by different genetic and environmental factors. Crucial events that have been identified in the development of the disease[22,23] are overactive adaptive and dysregulated innate immune responses and also impaired skin barrier function. One basic component of the physicochemical barrier is FLG, which may show genetic alterations (e.g. *FLG* null mutations and copy number variations in Caucasian AD patients), or acquired damage due to the effects of cytokines produced by T helper (Th) cell subtypes (Th2 and Th22) in AD[24-26]. Other barrier gene mutations (KLK7, SPINK5, Claudin-1) may also predispose to AD, although the occurrence of these alterations against the background of disease development is still not clearly known[27-29]. Acquired barrier disruption can also be caused by the frequent usage of detergents, as well as allergens and toxic mediators; however,

up to now only Th2 and Th22 cytokines have been confirmed to modify *FLG* expression[30]. *FLG* haploinsufficiency exhibits one of the strongest genotype–phenotype associations with the clinical and laboratory characteristics of AD, but recent studies are not consistent concerning the link between *FLG* mutations and skin barrier parameters.

In this study, our aim was to determine whether actual severe skin inflammation can cause as severe barrier defects as genetic *FLG* alterations, which clinical and laboratory parameters are associated with *FLG* haploinsufficiency, and which ones are also associated with acquired FLG alterations.

In order to answer this specific question, a novel subdivision of AD patients was introduced in this study. Based on actual disease severity, defined by the OSCORAD, and on the *FLG* genotype, we created three patient groups (wild-type patients with mild-to-moderate symptoms or severe symptoms – Groups A and B; and mutant patients with severe symptoms – Group C). Using this system, we were able to investigate two groups suffering from severe symptoms (B, C) but differing in their *FLG* genotype, and two groups with a wild-type *FLG* genotype (A, B) but with different SCORAD values. Therefore, we were able to determine which investigated parameters are related to the actual severe skin inflammation (which is responsible for the acquired FLG alterations) and which are related to the *FLG* genotype. Previous studies suggest that minor FLG mutations (S3247X, R2247X and 3702delG) are less prevalent in continental Europe than in the United Kingdom and Irish populations[6], and in a larger German cohort were present in <1%[7]. Therefore our patients were genotyped for the two most common loss-of-function mutations (R501X and 2282del4), for the first time in the Hungarian population. We do not believe that the exclusion of the minor variants would have altered our findings significantly.

In our study, significant differences in TEWL were found between the mild-to-moderate and severe groups for both nonlesional and lesional skin areas whereas no differences were observed between the two severe AD groups irrespective of their *FLG* genotype. This observation emphasises that beside genetic *FLG* haploinsufficiency, actual disease severity can also greatly influence barrier functions, as also supported by the strong correlation between the OSCORAD and TEWL.

In the last few years, measuring TEWL has become the most acceptable noninvasive way to examine skin barrier alterations in AD patients[31,32]. Certain groups have found significant differences in TEWL between AD patients with and without *FLG* haploinsufficiency[33,34];

however, others could not confirm these results[10,20]. The reason for this contradiction could be that in those studies in which an association between the *FLG* genotype and TEWL was detected, the SCORAD values also differed between patient groups; whereas when no differences were found between the above-mentioned groups, the SCORAD values were almost the same, so the effect of SCORAD on TEWL was not calculated.

The importance of measuring TSLP levels in patients suffering from AD has also increased remarkably in the last few years. Elevated TSLP levels in the lesional skin are highly characteristic of AD, although the direct association between AD severity and TSLP expression in the epidermis has not yet been clarified[35]. Significant elevation of serum and stratum corneum TSLP levels was also detected in AD patients, and the expression in the stratum corneum correlated with disease severity[36-38]. On the other hand one workgroup failed to detect elevated serum TSLP levels in AD patients[39]. In our study, serum TSLP levels did not differ significantly among the three AD groups, although TSLP levels in the sera of the mild-to-moderate group were lower than those of the severe wild-type group. In a comparison of the serum TSLP levels of the combined severe and mild-to-moderate AD group with the TSLP levels of healthy controls, only the severe group showed significant elevation, providing further data regarding the previously mentioned unsolved contradiction in the literature. In summary, the skin barrier functions (measured by TEWL on both nonlesional and lesional skin) were influenced equally by a hereditary lack of *FLG* and by acquired FLG insufficiency, driven by severe skin inflammation.

To demonstrate that actual skin inflammation (measured by OSCORAD and epidermal thickness) is strongly linked with FLG alterations in AD patients, immunohistochemical staining of skin biopsies was also performed. Similar to the TEWL and TSLP results, FLG expression was not detected or was significantly decreased in both severe groups, irrespective of the origin of the FLG deficiency[40].

In contrast to barrier functions, allergic sensitisation was mainly associated with *FLG* haploinsufficiency. The medical history data, which indicated the occurrence of other allergic diseases, the serum total IgE levels, as well as the presence of specific IgE against ragweed and cat dander, differed prominently between the mutant and wild-type patient groups. These indicators of allergic sensitisation were remarkably more frequent in the mutant group. The reason for this difference could be that hereditary *FLG* deficiency results in continuous disruption of the skin barrier over the whole lifespan, whereas acquired FLG deficiency,

which is the result of actual skin inflammation, fluctuates and is not continuously present. Regarding levels of specific IgE against house dust mites no differences were found between the distinct genotype groups. The reason for this difference could be that a shorter duration of skin inflammation and barrier impairment is sufficient to sensitise individuals against this aggressive allergen, which is not just extremely frequent, but has prominent proteolytic activity that induces inflammatory responses.

Our results are consistent with those of recent studies that showed that allergic rhinitis, eosinophilic esophagitis and traceable specific IgE against cat dander were more frequently present in AD patients with *FLG* mutations than in wild-type patients[30,41].

In summary, our results show that in AD patients with severe skin inflammation, the skin barrier can be disrupted as much in *FLG* wild-type patients as in *FLG* mutants and correlates with the severity of skin inflammation (SCORAD). In contrast, our results also suggest that *FLG* mutant patients may have a higher risk of allergic sensitisation compared to wild-type patients.

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Figure legends

Figure 1. Comparison of skin barrier functions and serum TSLP levels in subgroups of AD patients. No difference was found between the severe groups with respect to the OSCORAD, although the mild-moderate group had significantly lower values (P<0.0001) (1a). Significant TEWL elevation (for both nonlesional skin (1b) – P=0.0100, P=0.0262, and lesional skin (1c) P=0.0234, P=0.0464) was observed in the severe groups relative to the mild-moderate group. In the three-group-subdivision, no significant difference in serum TSLP levels was detected between the mild-to-moderate group and the severe groups. When combining all severe patients into one group, a significant increase in serum TSLP levels was observed in the severe groups compared to the healthy control group (P=0.0236) (1d). A strong correlation was found between TEWL and the OSCORAD score for nonlesional (1e) and lesional (1f) skin.

Figure 2. Occurrence of allergic sensitisation-specific parameters in subgroups of AD patients. Significant differences were found between the severe mutant and wild-type patients (P=0.0166 between group A and group C, and P=0.0154 between group B and group C) when analysing the data on patient history (2a). A remarkable difference was detected in the level of total IgE between the severe groups, and significant differences were found between the severe and mild-moderate groups (P=0.0181 between group A and group B, and P=0.0229 between group A and group C) (2b). With respect to the ragweed-specific (2c) and cat dander-specific (2d) IgE levels, significant differences were observed between the severe groups and between the severe mutant and mild-moderate groups (P=0.0090 and P=0.0472

for ragweed; P=0.0338 and P=0.0021 for cat dander in groups A and B and groups B and C, respectively). No differences were found between the severe groups in the occurrence of house dust mite-specific IgE (2e).

Figure 3. Immunohistochemistry and whole-slide imaging of FLG in skin biopsies from healthy controls and AD patients. FLG immunostaining was performed for mild-to-moderate patients (3a), severe wild-type patients (3b), severe mutant patients (3c) and healthy controls (3d). A significantly lower FLG content was observed in the AD skin biopsies relative to the biopsies of the normal controls (P=0.0001 for mild-to-moderate, and P<0.0001 for severe groups). There were also differences between group A and groups B and C (P=0.0010 and P=0.0036, respectively) (3e). Scale bar = 100 µm. The epidermis was significantly thicker in groups B and C compared to group A (P<0.0001) (3f).