Stratum corneum biomarkers for inflammatory skin diseases
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Citation for published version (APA):

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CHAPTER 4

Biomarkers for atopic dermatitis,
clinical studies
Filaggrin breakdown products determine corneocyte conformation in patients with atopic dermatitis

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(J Allergy Clin Immunol. 2015 Dec;136(6):1573-80)
ABSTRACT

Background: Loss-of-function (LOF) mutations in the filaggrin gene (FLG) are a well-replicated risk factor for atopic dermatitis (AD) and are known to cause an epidermal barrier defect. The nature of this barrier defect is not fully understood. Patients with AD with FLG LOF mutations are known to have more persistent disease, more severe disease, and greater risk of food allergies and eczema herpeticum. Abnormalities in corneocyte morphology have been observed in patients with AD, including prominent villus-like projections (VP); however, these ultrastructural features have not been systematically studied in patients with AD in relation to FLG genotype and acute and convalescent status.

Objective: We sought to quantitatively explore the relationship between FLG genotype, filaggrin breakdown products (natural moisturizing factor (NMF)), and corneocyte morphology in patients with AD.

Methods: We studied 15 children at first presentation of AD and after 6 weeks of standard therapy. We applied atomic force microscopy to study corneocyte conformation in patients with AD stratified by FLG status and NMF level. By using a new quantitative methodology, the number of VPs per investigated corneocyte area was assessed and expressed as the Dermal Texture Index score. Corneocytes were also labeled with an anti-corneodesmosin antibody and visualized with scanning electron microscopy.

Results: We found a strong correlation between NMF levels and Dermal Texture Index scores in both acute and convalescent states (respective $r = -0.80$ and $-0.75$, $P < .001$ and $P = .002$). Most, but not all, VPs showed the presence of corneodesmosin abundantly all over the cell surface in homozygous/compound heterozygous FLG patients and, to a lesser extent, in heterozygous and wild-type patients.

Conclusions: NMF levels are highly correlated with corneocyte morphology in patients with AD. These corneocyte conformational changes shed further insight into the filaggrin-deficient phenotype and help explain the barrier defect in patients with AD with FLG LOF mutations.
INTRODUCTION

A recent study using scanning electron microscopy (SEM) showed abnormal surface structures of corneocytes from patients with atopic dermatitis (AD) that the authors named as villus-like projections (VPs)\(^1\). Similar structures were described as protrusions \(^2\) and rough corneocytes \(^3\). Bead- or nipple-like elevations have also been observed in abdominal \(^4\), cheek, and plantar corneocytes \(^5\), as well as in 2,4,6-trinitro-1-chlorobenzene–sensitized hairless mice \(^6\). They seem to be absent in forearm healthy skin \(^2\) or exclusively present in the periphery of corneocytes from the inner upper arm \(^5\). A villous appearance with an irregular fine nodular surface pattern has also been shown in patients with ichthyosis vulgaris and in squamous cells from patients with psoriasis \(^7\). In most of these studies, VPs were observed qualitatively, and only in one study were the VPs determined semiquantitatively \(^5\), suggesting a correlation between VP numbers and skin barrier function, as assessed based on transepidermal water loss (TEWL). The nature and cause of VPs on the stratum corneum (SC) surface is not well understood. Several mechanistic suggestions have been proposed for the occurrence of VPs, including disturbed organization of the cytoskeleton on desmosome disruption, immature and fragile cornified envelopes (CEs), and attachment sites of desmosomes \(^1,2,6,8\). Rankl et al. \(^4\) showed that staining for corneodesmosin protein mostly matched the beadlike topographic features, although not all of these structures showed corneodesmosin immunoreactivity.

Because filaggrin (gene name FLG) is a component of the CE \(^9\) and filaggrin-deficient corneocytes display gene dose-dependent alterations in CE structure,\(^{10}\) we aimed to investigate the relationship between VPs on the SC with levels of filaggrin degradation products in children with AD. The filaggrin degradation products histidine, pyrrolidone-5-carboxylic acid, and urocanic acid can be used as an indirect measure of filaggrin expression that is dependent not only on FLG loss-of-function (LOF) mutations but also on other factors, including genetic factors, filaggrin degradation pathway factors \(^{11}\), and both local and systemic inflammation \(^{12, 13}\). Furthermore, because these products are the main source of the constituents of natural moisturizing factor (NMF) and contribute to SC hydration, their levels might influence structural conformation of the CE.
In this study we used high-resolution atomic force microscopy (AFM) to investigate the topography of corneocytes in patients with AD in relation to FLG genotype and levels of filaggrin degradation products. AFM provided nanoscale 3-dimensional resolution of native corneocytes collected by means of adhesive tape stripping. AFM involves a sharp tip at the end of a soft silicon cantilever touching and scanning the surface of a sample. Because of the change in topography, the deflection of the cantilever is transformed into a 3-dimensional image. Recently, we have developed and evaluated a software method through which VP surfaces on the corneocyte can be quantitatively determined (the Dermal Texture Index (DTI); technical manuscript in preparation, full details available on request from the authors (CR)). We measured DTI scores in corneocytes of children with active AD at first presentation and after 6 weeks of standard topical therapy with skin care regimens and appropriate topical steroids. In addition to DTI scores, we measured NMF levels; skin barrier function, as assessed based on TEWL; and severity of AD based on the SCORAD score (14). Next, we investigated the distribution of corneodesmosome remnants by using SEM and corneodesmosin immunocytochemical labeling.

METHODS

Study population
Patients with AD were recruited from a dedicated AD clinic in a tertiary referral center. An experienced pediatric dermatologist (ADI, MAM, or both) made the diagnosis and recorded the disease phenotype. All patients met the United Kingdom diagnostic criteria (15) and had moderate or severe disease. Exclusion criteria from the study included patients who had pyrexial illness in the preceding 2 weeks; those who had received immunosuppressive systemic therapy, such as oral corticosteroids, in the preceding 3 months; and those whose ancestry was not exclusively Irish (4/4 grandparents). The study was conducted in accordance with the Helsinki Declarations and was approved by the Research Ethics Committee of Our Lady’s Children’s Hospital, Dublin, Ireland. Full written informed consent was obtained from all patients’ parents. The children were treatment naive at presentation and were assessed at first presentation and after 6 weeks of standard treatment with skin care regimens and appropriate topical steroids.
Severity assessment
The severity of a patient’s AD was assessed by using the SCORAD index. A single dermatologist performed all SCORAD measurements. SCORAD is one of the most valid and reliable instruments to assess the clinical severity of AD. SCORAD is a composite score on a scale of 0 to 103 that incorporates both objective physicians’ estimates of extent and severity and subjective patient or parental assessments of itch and sleep loss. SCORAD is internally consistent, responsive, and interpretable and has adequate interobserver reliability (Cohen $\kappa = 0.82$, $P < .001$).

Biophysical measurements of the SC
All topical therapies, including emollients, were withheld from the patients’ upper limbs for 48 hours before skin biophysical measurements were performed. All measurements were done in standardized environmental conditions (room temperature, 22°C to 25°C; humidity levels, 30% to 35%). Before testing, the patient’s forearm was acclimatized to this controlled environment for a minimum of 10 minutes. All measurements were done by the same investigator and on a clinically unaffected area of skin on the volar forearm. TEWL was determined by using a Tewameter 300 (Courage and Khazaka Electronic GmbH, Cologne, Germany).

Sampling of the SC by using tape stripping
The SC was sampled by using the previously described method. A clinically unaffected site on the patient’s volar forearm, where the TEWL measurement was also taken, was used for SC sampling. Circular adhesive tape strips (3.8 cm², D-Squame; Monaderm, Monaco, France) were attached to volar forearm skin and pressed for 10 seconds with a constant pressure (225 g/cm²) by using a D-Squame Pressure Instrument D500 (CuDerm, Dallas, Tex). The tape strip was then gently removed and placed in a closed vial. Eight consecutive tape strips were sampled, all from the same site. The tape strips were immediately stored at −80°C until analysis.

FLG genotyping
All patients were screened for the 9 most common FLG mutations found in the Irish population (R501X, Y2092X, 2282del4, R2447X, S3247X, R3419X, 3702X, S1040X, and G1139X) from DNA extracted from a blood sample. The methods used have been previously described.
NMF determination

NMF analysis was performed on the fifth consecutive strip, according to methods described in detail elsewhere (19). Briefly, each tape strip was extracted with 25% (wt/wt) ammonia solution. After evaporation of the ammonia extract, the residue was dissolved in 250 µL of pure water and analyzed by using HPLC. The NMF concentration was normalized for the protein amount determined with a Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, Ill; referred to as the Pierce assay) to compensate for a variable amount of the SC on the tape.

Skin nanotexture analysis (DTI)

Corneocytes from patients were analyzed with AFM, as previously described.21 Briefly, in each case the seventh tape strip was subjected to AFM measurements carried out with a Multimode AFM equipped with the Nanoscope III controller and software version 5.30sr3 (Digital Instruments, Santa Barbara, Calif). Silicon-nitride tips on V-shaped gold-coated cantilevers were used (0.01 N/m, MLCT; VEECO, Mannheim, Germany). Imaging was performed at ambient temperature with forces of less than 1 nN and 1 to 3 scan lines per second (1-3 Hz) with 512 × 512 pixel resolution. For texture analysis, subcellular scan areas of 20 µm² were recorded. Ten random images were analyzed from each sample. Topographic cell-surface data were analyzed with the nAnostic method, applying custom-built proprietary algorithms (Serend-ip GmbH, Munster, Germany). The principle of this method has been described elsewhere (22). Briefly, each nanostructure protruding from the mean surface level was morphometrically evaluated. These objects were then filtered by size and shape through computer vision. At this stage, only structures of positive local deviational volume smaller than 500 nm in height and with an area of less than 1 µm² were considered. The DTI score is the count of identified objects per image (a mean value from 10 randomly recorded images).

Corneodesmosin immunolabeling

Corneocytes from 3 patients with different FLG mutation genotypes collected on D-squame discs were labeled with an anti-corneodesmosin antibody and visualized with SEM, as described elsewhere (23). Briefly, the native cells exposed to the mouse mAb to corneodesmosin (diluted 1:100; Abnova, Jhongli City, Taiwan) were immunogold labeled with the goat anti-mouse Ultra Small probe (0.8 nm, diluted
1:10; Aurion, Wageningen, The Netherlands). The labeling was silver enhanced with the BBI kit (BBI Solutions, Cardiff, United Kingdom), and the samples, after dehydration in ethanol, were observed in a partial vacuum by using secondary and backscattered electron detection modes.

**Statistics**

Data were checked for normality by using the Shapiro-Wilk test. The relationship between DTI scores and clinical parameters was tested either by using the Pearson correlation test or Spearman rank correlation if the variables were not normally distributed or the relationship between the variables was not linear. Because of skewed distribution, DTI scores and NMF levels were log-transformed before linear regression analysis. Differences in the investigated parameters (DTI score, TEWL, SCORAD score, and NMF level) between 2 measurement points (0 and 6 weeks) were tested by using the paired 2-sided t test (NMF and DTI score) or by using the Wilcoxon matched signed-rank test in the case of deviation from normal distribution (TEWL and SCORAD score). Differences in DTI scores among 3 FLG genotypes were tested by using the Kruskal-Wallis test, followed by Dunn multiple comparison. The relationship between the DTI score as a dependent variable versus the SCORAD score and NMF level as dependent variables was tested by using a linear regression model with SPSS software (version 22; IBM, Somers, NY). For other statistical analyses, GraphPad Prism version 5.00 software for Windows (GraphPad Software, San Diego, Calif) was used. A P value of less than .05 was considered statistically significant.

**RESULTS**

Demographic characteristics of the investigated populations and values of measured parameters are presented in Table 1. Fig 1 shows representative AFM images of the surfaces of corneocytes sampled from patients with AD with 3 different FLG mutation genotypes. On simple inspection, VP numbers were clearly increased in carriers of FLG mutations. The DTI score, which quantifies the number of VPs per investigated surface area, showed a trend toward higher mean values in the carriers of FLG mutations compared with FLG wild-type subjects at week 0 (427.0
and 336.2, respectively) and after 6 weeks of therapy (296.6 and 224.3, respectively), although the differences did not reach statistical significance (data not shown). At week 6, however, the DTI in the FLG−/− group was significantly higher than in the FLG+/+ group (respective median values were 496.8 and 208.2, respectively; P < .05, as assessed by using the 2-tailed Mann-Whitney test), whereas there was no significant difference in SCORAD scores between these 2 FLG genotype groups. When DTI scores were plotted against the NMF levels, a significant correlation was observed at both weeks 0 and 6 (respective correlation coefficients amounted to −0.80 and −0.75, P < .001 and P = .002, respectively; Fig 2, A). VP numbers reach a plateau at normal NMF levels (approximately 0.5 mmol/g protein).

Table 1. Demographic data of patients, DTI and NMF assessed at baseline (treatment naïve) and after 6 weeks of treatment

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<th>Sex</th>
<th>Age (months)</th>
<th>Number of FLG mutations</th>
<th>SCORAD Week 0</th>
<th>SCORAD Week 6</th>
<th>TEWL (g/m² hr) Week 0</th>
<th>TEWL (g/m² hr) Week 6</th>
<th>DTI (AU) Week 0</th>
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* under the lower limit of quantification
NMF AND CORNEOCYTE CONFORMATION IN AD PATIENTS

Fig. 1. Representative AFM images of the surfaces of corneocytes sampled from patients with AD with 3 different FLG mutation genotypes: +/+, wild-type homozygote; +/-, heterozygote for FLG LOF mutation; −/−, compound heterozygote or homozygote for FLG LOF mutation. On simple inspection, numbers of VPs were clearly increased in carriers of FLG mutations.

Fig 2. A. Relationship between DTI scores and NMF levels at first presentation of disease (squares) and after 6 weeks of topical therapy with skin care regimens and appropriate topical steroids (circles), with corresponding correlation coefficients (r). B, Linear regression analysis of log-transformed DTI scores and NMF levels at first presentation of disease (squares) and after 6 weeks of topical therapy with skin care regimens and appropriate topical steroids (circles). □ ○, Patients with AD wild-type with respect to FLG LOF mutations; ◇ ◆, patients with AD heterozygous for FLG LOF mutations; ⊗⊠, patients with AD homozygous or compound heterozygous for FLG LOF mutations.
Regression analysis of log-transformed values of DTI scores and NMF levels showed almost identical regression coefficients for 0 and 6 weeks (−0.726 and −0.730, respectively; Fig 2, B). The relationship of DTI scores with TEWL and SCORAD scores (see Fig E1 in this article’s Online Repository at www.jacionline.org) was weaker than that of DTI scores and NMF levels. Furthermore, in a linear regression model with the DTI score as a dependent variable versus the NMF level and SCORAD score, only NMF levels showed a significant effect on DTI scores (P = .005 and .015, respectively, for weeks 0 and 6; see Table E1 in this article’s Online Repository at www.jacionline.org).

Fig. 3. TEWL, SCORAD score, DTI score, and NMF level at first presentation of disease and after 6 weeks of topical therapy with skin care regimens and appropriate topical steroids.

Changes in DTI scores, NMF levels, TEWL, and SCORAD scores measured at the first presentation of disease and after 6 weeks of standard topical therapy with skin care regimens and appropriate topical steroids are shown in Table I and Fig 3. Although the skin barrier, as measured based on TEWL and SCORAD scores, significantly improved after 6 weeks of therapy, NMF levels and DTI scores did not mirror these improvements in all patients.
Representative SEM images of D-Squames after immunogold labeling are shown in Fig 4 for 3 patients with AD of different FLG genotype status. The high abundance of VPs on the corneocytes obtained from an FLG−/− subject (Fig. 4, C) was confirmed by means of SEM. The VPs were decorated at their tips with corneodesmosin labeling, indicating the presence of disrupted corneodesmosome structures (Fig. 4, D). The corneocytes of a homozygous subject (FLG−/−) demonstrated labeling over the entire surface (Fig. 4, C). In contrast, in a patient who is wild-type with respect to FLG mutations (FLG+/+; Fig 4, A), the labeling was almost exclusively distributed on the lateral rims of the cell. In the heterozygous patient (FLG+/−; Fig 4, B) the central area of corneocytes remained largely free of the label, even though it was partially occupied by the VPs (Fig 4, B, arrows).

Fig. 4. SEM immunolabeling of corneodesmosin. A, Corneodesmosomes at the cell surface of a patient wild-type with respect to FLG LOF mutations (FLG+/+). B, A patient heterozygous for FLG LOF mutations (FLG+/−). C, A patient homozygous for FLG LOF mutations (FLG−/−). Insert in Fig 4, C, Corneodesmosin-expressing junctions present at the tops of the VPs in the patient homozygous for FLG LOF mutations. Arrows in Fig 4, B, show the presence of VPs (not labeled for corneodesmosin).
DISCUSSION

Filaggrin deficiency results in a definite skin barrier defect, but the pathomechanisms underlying this defect are poorly understood (11). Within the corneocytes, filaggrin aggregates intermediate keratin filaments that are linked to the corneodesmosomes, which interconnect the corneocytes, providing a physical barrier structure at the top of the skin (24, 25). Together with keratin filaments, filaggrin has been proposed to provide a scaffold for the assembly of structural proteins, such as involucrin, loricrin, and small proline-rich proteins, which are cross-linked by several transglutaminases to form the CE (24, 25). Some CE proteins serve as an anchor for attachment of ceramides, and thus lack of filaggrin might also affect the structural organization of the intercellular SC lipid lamellae responsible for barrier function.

In the present study we demonstrate that deficiency of filaggrin is associated with altered topography of the corneocyte surface, likely caused by defects in the CE. In a recent study (2) similar villous structures were observed on the palmar skin of healthy subjects, although not on forearm skin, which is in contrast to the present study. We found that levels of filaggrin degradation products (NMF) used as a marker of filaggrin expression (12, 26) were strongly associated with corneocyte VP numbers. These corneocytes were sourced from the upper middle part of the SC (seventh strip); however, the same pattern concerning distribution of VPs was also seen in the more superficial strips (eg, strip number 4; data not shown). VP numbers were more closely related to NMF levels than to SCORAD scores, suggesting that the absence of filaggrin is important for the formation of VPs rather than inflammation per se. This is supported by similar regression coefficients of the DTI score versus NMF level relationship at weeks 0 and 6, despite the sharp decrease in SCORAD scores. Furthermore, in a linear regression model with the DTI score as a dependent variable versus the NMF level and SCORAD score as independent variables, only NMF levels showed a significant effect on DTI scores at both weeks 0 and 6. Local inflammation might have affected the presence of DTI scores indirectly by influencing NMF levels, an effect that previously has been shown in vitro and in vivo (12, 13). This might explain the lack of a significant difference in DTI scores between patients with AD with FLG LOF mutations and patients with AD without FLG LOF mutations, although the former group tended
to have higher DTI scores, and the lack of statistical significance seen here might simply be due to a lack of power in this study. Furthermore, at week 6, the FLG−/− patients, in whom inflammation is controlled and NMF levels are mainly influenced by FLG LOF mutations, had significantly higher DTI scores compared with FLG+/+ patients, despite clinical improvement, as measured based on SCORAD scores. Also of note is our observation that the relationship between TEWL and DTI scores was significant at 6 weeks (after anti-inflammatory therapy) but not at week 0 (see Fig E1). This suggests a relationship between corneocyte conformation as measured by DTI scores and barrier function (TEWL).

During the transition from the stratum compactum to the stratum disjunctum, corneocyte morphology and mechanical properties change from a “fragile” and soft to a more robust, smooth, and “rigid” phenotype (27-29). This transition process is accompanied by loss of nonperipheral corneodesmosomes because only peripheral corneodesmosome attachments connecting consecutive layers of corneocytes remain (28, 29). Interestingly, we observed corneodesmosin on the tips of VPs, all over the cell surface in FLG−/− patients, and, to lesser extent, in heterozygous patients, which suggests changes in their maturation because of a disturbed terminal differentiation program. As discussed by Rawlings (28) and shown by Watkinson and Rawlings (30), the loss of nonperipheral corneodesmosomes and CE maturation changes seem to parallel filaggrin degradation. Lack of filaggrin in the CE and between the keratin filaments might lead to conformational changes, and the adhesive portions of the peripheral corneodesmosomes might become less accessible for degradation enzymes. In addition to the direct effect of filaggrin, the existence of VPs might also be caused by a reduction in filaggrin degradation products and reduced hydration of the SC. Matsumoto et al. (6) observed the emergence of villi on the rear surfaces of corneocytes after topical exposure to the contact allergen 2,4,6-trinitrochlorobenzene, which caused dry and inflamed skin. However, the villi disappeared after topical treatment with a moisturizer at a higher rate than after topical corticosteroid therapy. The surfactant-induced xerosis led to a considerable increase of the immature and fragile CE phenotype (29). The perturbation of CE maturation coincided with reduced hydrolysis of corneodesmosomes, which was paralleled by altered activity of transglutaminase. Recently, we have shown that exposure to sodium lauryl sulfate caused a dramatic decrease in NMF levels in the
SC,31 and therefore the changes in corneocyte maturation might have also been caused by the lack of NMF. Interestingly, also in the study of Harding et al. (29), the balance between the 2 CE phenotypes was recovered after treatment with a moisturizer, emphasizing the importance of SC hydration for the maturation process.

The size of the VPs (ie, several hundreds of nanometers: average height, 350 nm; width at half-maximum, 250-400 nm) and their high abundance is intriguing. The CE is approximately 20 nm thick, implying that considerable mechanical force lies behind its protrusion. The present results do not allow firm conclusions to be drawn regarding the relationship between VPs and retention of the nonperipheral corneodesmosomes because the presence of VPs was not always accompanied by central distribution of corneodesmosin. The persistence of VPs in both the acute and convalescent phases of AD with FLG loss-of-function mutations offers an intriguing insight into the persistent abnormalities in “normal” or “unaffected” AD skin, an area of great interest (32). The persistence of an underlying physical and structural abnormality, even in light of apparent clinical improvement, might explain why patients with AD with FLG loss-of-function mutations have more severe and persistent disease (33), why they are more likely to have eczema herpeticum,34 and why they have more food allergies (35).

In conclusion, we have shown for the first time a significant structural difference in corneocytes in patients with AD with FLG loss-of-function mutations that can be quantitatively measured. These structural changes correlate well with NMF levels and persist despite apparent clinical improvement and might explain some of the observed phenotypic differences in patients with AD with FLG loss-of-function mutations.

Acknowledgements
The SEM images were obtained at the Centre Technologique des Microstructures, CTµ, an LBI platform of University Lyon 1.
REFERENCES


4.2

Efficacy of a cream containing ceramides and magnesium in the treatment of mild to moderate atopic dermatitis: a randomized, double-blind, emollient- and hydrocortisone-controlled trial

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(Acta Derm Venereol. 2016 Nov 2;96(7):948-953)
ABSTRACT

The aim of this randomized controlled trial was to assess the efficacy of a cream containing ceramides and magnesium (Cer-Mg) in the treatment of mild to moderate atopic dermatitis and to compare it with hydrocortisone and a commonly used emollient (unguentum leniens; cold cream). A total of 100 patients, randomized into 2 groups, were treated for 6 weeks simultaneously (left vs. right side of the body) with either Cer-Mg and hydrocortisone (group I) or Cer-Mg and emollient (group II). The primary outcome was a reduction in severity of lesions as assessed by (local) SCORAD (SCORing Atopic Dermatitis). Levels of trans-epidermal water loss (TEWL), skin hydration, and natural moisturizing factors (NMF) were then measured. After 6 weeks, group I showed comparable significant improvement in SCORAD and TEWL, while in group II, the decrease in SCORAD and TEWL was significantly greater after Cer-Mg compared with emollient. Finally, Cer-Mg cream was more effective in improving skin hydration and maintenance of levels of NMF than hydrocortisone and emollient.
INTRODUCTION

Atopic dermatitis (AD), a chronic, inflammatory skin disease characterized by dry, pruritic and erythematous skin, affects up to 10% of adults and up to 20% of children in the Western world (1–3). Patients with mild to moderate AD are constrained for long periods to over-the-counter (OTC) emollients or, in some countries, such as the UK and the USA, to low-potency corticosteroids. However, long-term use of corticosteroids is associated with adverse side-effects, such as skin atrophy (4). Such side-effects are well known among the general public and (not always justifiable) anxiety about corticosteroids is a major factor in poor adherence to therapy (5–8). Therefore, emollient therapy is often preferred by patients and is shown to reduce corticosteroid use significantly (9). In general, emollients aim to prevent water loss from the skin, e.g. by occlusion (petrolatum) or by addition of hygroscopic compounds (e.g. glycerol and urea) and lipids (e.g. ceramides). Identification of an inherited deficiency of the epidermal protein filaggrin as a major risk factor for AD, points to the importance of the skin barrier in the aetiology of AD (10–12). The barrier is located mainly in the stratum corneum (SC), which is composed of corneocytes surrounded by lipid lamellae composed of ceramides, cholesterol and free fatty acids (13–15). Although emollients are regarded as basic therapy by the European Task Force on Atopic Dermatitis/European Academy of Dermatology and Venereology (EADV) Eczema Task Force, their efficacy in randomized controlled trials (RCT) has been insufficiently investigated (16–20). Therefore, the aim of the present double-blinded RCT was to assess the efficacy of an emollient containing ceramides and magnesium (Cer-Mg), compounds involved in the maintenance of the skin barrier (21). SC ceramide composition is altered in AD, and reduced levels of ceramides and changes in their relative composition have been shown to correlate with trans-epidermal water loss (TEWL) (12). The role of magnesium in AD is relatively unknown; however, bathing in magnesium-rich water has been shown to have a beneficial effect on the skin barrier in dry atopic skin (22). Furthermore, magnesium is known to be involved in synthesis of ceramides, regulation of epidermal proliferation and differentiation. In addition, children with AD showed a reduced level of serum magnesium (23, 24). Although there is some evidence that both ceramides and magnesium might improve barrier function in AD, their efficacy remains to be elucidated, preferably in RCTs. In the present study the efficacy of the Cer-Mg cream was compared side-by-side with 2 other creams,
which are frequently used in treatment of mild and moderate AD: a low-potency topical corticosteroid (hydrocortisone acetate 1% in petrolatum-cetomacrogol) and a commonly used OTC emollient, unguentum leniens; cold cream).

**Trial population**
A total of 100 patients were recruited from the outpatient clinic at VU University Medical Center Amsterdam (VUmc). Inclusion criteria were: (i) clinically diagnosed AD conforming to the Hanifin & Rajka criteria (25), (ii) mild to moderate AD, (iii) age 18–70 years, (iv) at least 2 symmetrical (i.e. left and right side of the body) skin sites with comparable AD severity. The exclusion criteria were: (i) extensive ultraviolet (UV) exposure in the last 14 days and/or expected exposure during the study, (ii) skin disease other than AD, (iii) use of antibiotics prior (at least 4 weeks) to the study and/or expected use during the study, (iv) use of systemic immuno-suppressing drugs prior (at least 4 weeks) to the study and/or expected use during the study, (v) severe disorders within the last 6 months, (vi) investigator’s uncertainty about the willingness or ability of the patient to comply with the protocol requirements (e.g. mental disability). In the case of adverse health effects, such as allergic reaction or severe deterioration of the symptoms, patients were prevented from further participation. Patients could not use any AD medication for at least 2 weeks prior to participation (wash-out period). The study was approved by the medical ethics committee of the Academic Medical Centre and VUmc. All patients gave their written informed consent prior to participation.

**Patients’ experience**
After participation patients were asked, in a short questionnaire, what their personal preferred treatment was.

**Registration and medical ethics approval**
The trial was registered under the number NTR 4541. Medical ethics approval was obtained on the basis of the study protocol (AMC registration number: METC 2014_090).
Randomization and blinding
The randomization list was produced prior to treatment by a random number sequence generated in Microsoft Excel™. Treatment combinations (Cer-Mg and HC or Cer-Mg and EM) were linked to a unique inclusion number. The allocation list was prepared by an investigator (SK) with no executive tasks in the trial and handed over to the VUmc pharmacy. After the enrolment of a second investigator (SAK) who had access only to the inclusion numbers, each patient was given the inclusion number and collected the creams at the pharmacy. Creams were packed in identical tubes labelled only with the 3 possible treatments (hydrocortisone, EM or Cer-Mg cream) and the body side on which to apply the cream (left or right). For safety reasons 2 investigators (TR, MFD) were given the allocation list so that in case of an adverse event medical care could be given without delay.

Intervention
Patients were randomly allocated into 2 groups. Group I was treated with Cer-Mg cream on a lesion on one side of the body and simultaneously with HC on a lesion on the contralateral side. Group II was treated simultaneously with Cer-Mg and EM (unguentum leniens, also called cold cream) contralaterally. Patients were instructed to apply one fingertip unit (approximately 1 g) of both creams twice daily for 6 weeks. Patients were instructed not to apply cream on the morning of measurements. Furthermore, patients were asked not to apply any other product on other lesions, except the study creams. Measurements were performed under the same climate conditions (21°C, controlled humidity) between September and January, by one investigator (SAK). In weeks 0, 3 and 6 the parameters were measured and samples of the SC were collected for analysis. A flow diagram is shown in Fig. 1.
Study material

The Cer-Mg cream (Dermalex™ Eczema, Omega Pharma, Nazareth, Belgium) contained: water, ceramide 1 (0.001%), ceramide 3 (1%), ceramide 6 II (0.5%), phytosphingosine, cholesterol, magnesium chloride hexahydrate, zeolite (the combination of magnesium and zeolites are trademarked as MagneoLite™), glycerol, cocoglycerides, cetyl alcohol, isopropyl myristate, emulsifiers and preservatives. The control products; hydrocortisone acetate 1% in petrolatum-cetomacrogol (HC) and unguentum leniens (EM, also called cold cream, consists of arachis oil (peanut oil), purified water, white beeswax and glyceryl monooleate) both produced by Fagron, NL, BF (Capelle aan den IJssel, the Netherlands) were, together with the Cer-Mg, packed in blinded tubes by Thiopharma (Maassluis, the Netherlands) according to the good manufacturing practice guidelines. The total lipid content of the Cer-Mg cream was 30%, of the EM 75%, and of the HC 49%.

Clinical parameters (primary outcome)

The primary outcome of the study was the comparison of the treatments based on the change in symptom severity as assessed by the difference in the SCORAD (SCORing Atopic Dermatitis) at 3 and 6 weeks from baseline. SCORAD is based on the total body surface area affected by a disease and visually apparent symptoms (erythema, oedema, excoriation, oozing/crusts, lichenification, dryness) and on 2
subjective parameters (pruritus and sleep deprivation, both measured on a visual analogue scale) (16). Due to the split-body study design a modified SCORAD (local SCORAD) was used (26). By local SCORAD, the scoring parameters were performed on the investigated skin sites and the body surface area was set to 1%.

**Biophysical parameters and natural moisturizing factors (secondary outcomes)**
The biophysical parameters included TEWL, skin surface pH and erythema. The measurements were conducted within a time-period of 60 min at each visit under controlled environmental conditions. TEWL was measured using a Tewameter 300 (Courage and Khazaka Electronic GmbH, Cologne, Germany) (27). Hydration was measured using a Moisture Meter SC Compact (Delfin, Inc, Kuopio, Finland). Skin pH was measured by a skin pH meter (pH900, Courage and Khazaka Electronic GmbH, Cologne, Germany) and erythema by an erythema meter (DermaSpectrometer; Cortex Technology, Hadsund, Denmark).

**Natural moisturizing factors in the stratum corneum**
The SC samples were collected with an adhesive tape (3.8 cm2, D-Squame, CuDerm, Dallas, Texas, USA) as described previously (12) and analysed for natural moisturizing factors (NMF) by HPLC-UV (22, 28).

**Statistical analysis**
Sample size was calculated using power analysis (nQuery advisor). Based on data from our pilot study (unpublished, results available on request) a difference of 5 arbitrary units (AU) (standard deviation (SD) 4.0) on the SCORAD index could be detected in a population of 39 patients (power 80%). Anticipating a drop-out percentage of 20%, we included 50 patients per group. Data analysis was performed using IBM SPSS Statistics® version 20.0. The Shapiro-Wilk test was used to check for data normality. The differences within the investigated parameters or between the 2 treatments were tested by a paired Student’s t-test (normally distributed data, data are shown as the mean value and standard error of the mean (SEM)) or a Wilcoxon signed-rank test (non-normally distributed data, shown as median value with interquartile ranges). A per-protocol analysis was performed as described in the study protocol.
RESULTS

Of 100 patients recruited between October and December 2014, 95 completed the study according to the protocol (group I: 48 patients; 16 males/32 females, median age 28.5 years (range 23.0–51.0 years) and group II: 47 patients 19 males/28 females, median age 25.0 years (range 21.0–35.0 years). Five patients were excluded during the study because of an allergic reaction to EM (n = 2), severe worsening of eczema symptoms (n = 1) or non-compliance with the study protocol (n = 2) (see Fig. 1). Due to technical failure, no reliable measurements of erythema by DermaSpectrometer could be performed; however, visual erythema was measured as a part of the SCORAD index. Furthermore, the measurement of proteins on the tapes from 3 subjects in group II could not be performed, and thus the levels of NMF in those individuals could not be determined. As the main outcome is the difference in parameter change between 2 treatments (e.g. Cer-Mg vs. HC in group I and Cer-Mg vs. EM in group II), the results will be presented separately for each group.

SCORing Atopic Dermatitis

At baseline, there was no significant difference in the (local) SCORAD between the 2 treated skin sites in either arm of the study.

Group I: HC vs. Cer-Mg. Both treatments led to clinical improvement in the test areas, as evidenced by a significant decrease in local SCORAD after week 3 and week 6 (Fig. 2A). The reduction in SCORAD from baseline (ΔSCORAD) was significantly greater for HC compared with Mg-Cer at 3 weeks; however, after 6 weeks there was no significant difference in ΔSCORAD between HC and Cer-Mg (Table I). At week 6, the ΔSCORAD amounted to −11.5 (IQR: −17.4; −5.6) for HC and −9.0 (IQR: −15.9; −5.6) for Cer-Mg.
Group II: EM vs. Cer-Mg. Cer-Mg treatment led to a significantly greater decrease in SCORAD from baseline (ΔSCORAD) compared with EM at both week 3 and week 6 (Table I). At week 6, the ΔSCORAD was −3.5 (IQR: −10.5; 3.0) for EM and −6.7 (IQR: −14.5; −2.0) for Cer-Mg.

Local pruritus (itch) intensity
Results for pruritus show a similar pattern as the SCORAD results; an extensive description can be found in online Appendix SI1.

TEWL as a marker of skin barrier
Group I: HC vs. Cer-Mg. The TEWL levels after both Cer-Mg and HC decreased significantly compared with their corresponding baseline values (Fig. 2B) reflecting an improvement of the skin barrier. The decrease in TEWL from baseline (ΔTEWL) after HC and Cer-Mg was comparable and did not significantly differ at both measurement points (Table I).

Group II: EM vs. Cer-Mg. Cer-Mg treatment did not lead to a significant change in the TEWL from baseline (Fig. 2B), while the EM treatment showed a significant increase in TEWL at 3 weeks. The change in TEWL from baseline (ΔTEWL) was significantly greater after EM compared with Cer-Mg at both time-points (Table II).

Table 1. Change from baseline of clinical and biophysical parameters in the treatment Group I (Cer-Mg vs. HC).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I: Cer-Mg versus HC</th>
<th>HC</th>
<th>IQR</th>
<th>p-value1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔSCORAD (AU)</td>
<td>Week 3 -6.25 (-8.40; -1)</td>
<td>-7.75 (-15.38; -3.63)</td>
<td>0.0078</td>
<td></td>
</tr>
<tr>
<td>ΔPruritus (AU)</td>
<td>Week 3 -1.00 (-2; 0)</td>
<td>-1.00 (-4; 0)</td>
<td>0.0104</td>
<td></td>
</tr>
<tr>
<td>ΔTEWL (g/m²/h)</td>
<td>Week 3 -4.75 (-13.66; 1.473)</td>
<td>-7.24 (-15.70; 2.21)</td>
<td>0.104</td>
<td></td>
</tr>
<tr>
<td>ΔHydration (AU)</td>
<td>Week 6 -6.28 (-12.20; 5.15)</td>
<td>-5.19 (-14.36; 2.21)</td>
<td>0.083</td>
<td></td>
</tr>
<tr>
<td>ΔNMF (nmol/ug protein)</td>
<td>Week 6 0.08 (-0.12; 0.25)</td>
<td>-0.10 (-0.23; 0.06)</td>
<td>0.0015</td>
<td></td>
</tr>
<tr>
<td>ΔpH</td>
<td>Week 3 0.00 (-0.20; 0.28)</td>
<td>0.00 (-0.28; 0.40)</td>
<td>0.2475</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Week 6 0.00 (-0.40; 0.20)</td>
<td>0.10 (-0.30; 0.40)</td>
<td>0.024</td>
<td></td>
</tr>
</tbody>
</table>

P-significance level of the difference in changes from baseline between two treatments (Wilcoxon signed-rank test); AU: arbitrary units; IQR: interquartile range; SCORAD: SCORing Atopic Dermatitis; TEWL: trans-epidermal water loss; NMF: natural moisturizing factors.
**Hydration**

Group I: HC vs. Cer-Mg. Treatment with HC and Cer-Mg significantly improved skin hydration (Fig. 2C). The increase in hydration from baseline (ΔHydration) after Cer-Mg was significantly greater after Cer-Mg compared with HC at weeks 3 and 6 (Table I).

Group II: EM vs. Cer-Mg. Hydration after Cer-Mg was significantly higher than the baseline values at weeks 3 and 6 (Fig. 2C), while hydration after EM treatment improved significantly only after 6 weeks. The changes in hydration from baseline (ΔHydration) were significantly larger after Cer-Mg compared with EM at week 3 (Table II).

**Natural moisturizing factors**

Group I: HC vs. Cer-Mg. Treatment with Cer-Mg showed a tendency of NMF increase (p = 0.09) (Fig. 2D). In contrast to Cer-Mg, treatment with HC resulted in a significant decrease (by 22%) of NMF levels after 6 weeks. The difference in NMF change from the baseline (ΔNMF) between HC and Cer-Mg emollient was significant at week 6 (p < 0.05), (Table I).

Group II: EM vs. Cer-Mg. EM treatment showed a significant decrease in NMF at week 3 (Fig. 2D). Treatment with Cer-Mg did not influence NMF levels. No significant difference in ΔNMF could be detected between the 2 treatments (Table II).
Fig. 2. (A) Local SCORAD (SCORing Atopic Dermatitis). (B) Trans-epidermal water loss (TEWL). (C) Hydration and (D) natural moisturizing factors (NMF) at baseline, after 3 and 6 weeks of treatment in group I (hydrocortisone (HC) vs. ceramides and magnesium (Cer-Mg); n = 48) and group II (emollients (EM) vs. Cer-Mg; n = 47). Results are shown as medians and interquartile ranges. Significance levels as tested by Wilcoxon signed-rank test: *p < 0.05; **p < 0.01; ***p < 0.001.
Table 2. Change from baseline of clinical and biophysical parameters in the treatment Group II (Cer-Mg vs. EM).

<table>
<thead>
<tr>
<th>Group II: Cer-Mg versus Emollients</th>
<th>Cer-Mg</th>
<th>IQR</th>
<th>EM</th>
<th>IQR</th>
<th>p-value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔSCORAD (AU) Week 3</td>
<td>-8.50</td>
<td>(-11.5; -1.5)</td>
<td>-3.50</td>
<td>(-8; 1)</td>
<td>0.0058</td>
</tr>
<tr>
<td>ΔPruritus (AU) Week 3</td>
<td>-1.00</td>
<td>(-2; 0)</td>
<td>0.00</td>
<td>(-1; 1)</td>
<td>0.0173</td>
</tr>
<tr>
<td>ΔTEWL (g/m²/h) Week 3</td>
<td>-3.48</td>
<td>(-8.24; 3.66)</td>
<td>2.75</td>
<td>(-3.68; 10.07)</td>
<td>0.005</td>
</tr>
<tr>
<td>ΔHydration (AU) Week 3</td>
<td>3.10</td>
<td>(-3.1; 9.6)</td>
<td>1.20</td>
<td>(-3.2; 6.5)</td>
<td>0.0401</td>
</tr>
<tr>
<td>ΔNMF (nmol/ug protein) Week 6</td>
<td>-0.02</td>
<td>(-0.19; 0.10)</td>
<td>-0.07</td>
<td>(-0.20; 0.09)</td>
<td>0.9767</td>
</tr>
<tr>
<td>ΔpH Week 3</td>
<td>0.30</td>
<td>(-0.1; 0.5)</td>
<td>0.10</td>
<td>(-0.1; 0.3)</td>
<td>0.5189</td>
</tr>
<tr>
<td></td>
<td>0.00</td>
<td>(-0.2; 0.3)</td>
<td>0.00</td>
<td>(-0.3; 0.3)</td>
<td>0.4739</td>
</tr>
</tbody>
</table>

¹P-significance level of the difference in changes from baseline between two treatments (Wilcoxon signed-rank test) ; AU: arbitrary units; IQR: interquartile range; SCORAD: SCORing Atopic Dermatitis; TEWL: trans-epidermal water loss; NMF: natural moisturizing factors.

An extensive description of pH results can be found in the online supplementary file (S1, Results).

Online supplement contains additional information on:
- Patient characteristics (S1, results)
- Local Pruritus (itch) intensity (S1, results)
- skin surface pH (S1, results)
- Tolerability and subjective preference (S1, results)

DISCUSSION

The results of the present study show that the Cer-Mg cream is an effective approach in improving the clinical symptoms and skin barrier. Although all 3 treatments led to significant improvement in clinical symptoms after 6 weeks, only the HC and Cer-Mg cream reduced SCORAD by more than 8.7 units, which is considered clinically relevant (26). After 3 weeks of treatment HC showed slightly, but significantly, greater reduction in SCORAD than Cer-Mg (–7.8 vs. –6.3), while Cer-Mg showed significantly greater reduction than EM (–8.5 vs. –3.5). The subjective VAS-pruritus
scale and the skin barrier function parameter TEWL showed similar results: Cer-Mg and HC showed a significantly beneficial effect, which was, however, not observed after EM treatment. Overall subjective preference slightly favoured the Cer-Mg, which might be of importance in patients’ adherence to therapy. Topical corticosteroids (TCS) are the first-line treatment for AD; however, their long-term use can lead to the deterioration of the skin barrier, which is an important aetiological factor in AD. Moreover, a recent study has shown that therapy with a potent TCS leads to a reduction in NMF levels, which play an important role in skin hydration, antimicrobial defence and skin inflammatory status (29, 30). Our study shows, for the first time, that a low-potency corticosteroid such as HC can lead to a significant reduction of NMF. A decrease in NMF has also been observed after EM treatment at 3 weeks, while Cer-Mg showed a tendency to increase NMF. This emphasizes the importance of this adverse side-effect of HC, as reduced NMF levels may contribute to the recurrent flares. The greatest improvement in SC hydration was observed after Cer-Mg cream that, similarly to HC, showed a decrease in TEWL, but in contrast to HC had no negative effect on NMF levels.

The Cer-Mg cream contains 2 components that might beneficially influence the skin barrier: ceramides (1, 3 and 6 II) and a complex of magnesium and zeolites (31). Huang & Chang (32) have shown that topical application of ceramide 1 and 3 reduces TEWL and increases hydration in sodium lauryl sulfate-irritated, thus beneficial effect of these ceramides, which are also present in Cer-Mg cream, might also have occurred in patients with AD in the present study. As the molecular size of the skin ceramides is > 500 Da, which is proposed as a molecular size cut-off for percutaneous penetration (33), the question arises whether and to which extent each of individual ceramides can penetrate across the SC, realizing that not only the amount, but also their balance is crucial for the skin barrier. Recently, Zhang et al. (34) demonstrated that topically applied ceramides are located mainly in the SC glyph and that the penetration into the lipid layers is minimal. It is likely that penetration of ceramides through the impaired skin barrier is enhanced in AD; however, RCT studies on the penetration of various ceramides, and their efficacy in improvement of the skin barrier in AD, are lacking.
Another rationale candidate to explain the effectiveness of Cer-Mg cream is magnesium, which is known to be involved in synthesis of ceramides (23). Topical treatments with magnesium-rich Dead Sea salts showed a beneficial effect in dry and pruritic dermatoses (27). Whether the effect of the Cer-Mg cream could be assigned to the presence of ceramides or magnesium remains to be elucidated in a vehicle-controlled trial as some constituents of the vehicle in the Cer-Mg cream, such as glycerol, are also known to lead to improvement in the skin barrier (35, 36).

**Strengths and limitations**

In this RCT the efficacy of Cer-Mg cream was compared with that of 2 currently used therapeutic options for mild to moderate AD. In most RCTs the efficacy is compared only with either corticosteroid or OTC emollient. The double-blind, split-body design offers a well-paired comparison between 2 treatments, compensating partly for the heterogeneity of the disease severity among patients with AD. The inclusion of biophysical and biochemical parameters provides more insight into the target of the treatment (37). This study did not account for spontaneous resolution of the disease over the study period. However, as the primary aim was to compare the efficacy of Cer-Mg to the upper (hydrocortisone) and lower spectrum of recommended OTC therapy for mild to moderate AD, we did not include an untreated site. Finally, the study does not provide insight into the working mechanism of Cer-Mg, which needs to be confirmed in the separate vehicle-controlled clinical trial.

**CONCLUSION**

The present study shows that, after 6 weeks of treatment, Cer-Mg cream offers benefits over high lipid-OTC emollients and comparable clinical efficacy to hydrocortisone. In addition, in contrast to hydrocortisone, it does not influence negatively the concentration of NMF. Cer-Mg may therefore offer a non-steroid alternative for the treatment of mild to moderate AD. Furthermore, the fact that Cer-Mg might be used as a stand-alone treatment for mild and moderate AD as well as a maintenance therapy might improve adherence to AD therapy.
**Funding:** Omega Pharma provided study medication free of charge. However, it had no involvement in generating, analysing or processing data, nor in scientific input, and it had no input into the generation of this article.

**Statement of all funding sources that supported the work:** This study was supported by Omega Pharma, Nazareth, Belgium.

**Conflict of interest:** S. A. Koppes was reimbursed by Omega Pharma for international conference attendance.
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4.3

Stratum corneum tape stripping: monitoring of inflammatory mediators in atopic dermatitis patients using topical therapy

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\textit{(International Archives of Allergy Immunology 2016;170(3):187-93)}
ABSTRACT

Objective: The aim of this study was to explore the tape strip sampling technique in the assessment of stratum corneum levels of inflammatory mediators in a clinical trial setting.

Methods: Thirty-eight inflammatory mediators were analyzed by a multiplex-assay in the stratum corneum, collected by adhesive tapes before and after 6 weeks of therapy, in mild and moderate atopic dermatitis (AD) patients (n = 90). Treatment was a ceramide- and magnesium-containing emollient.

Results: Twenty-four mediators could quantitatively be determined. The Th2 mediators interleukin (IL)-4, IL-13, CCL2 (monocyte chemotactic protein-1), CCL22 (macrophage-derived chemokine), and CCL17 (thymus and activation-regulated chemokine (TARC)) were significantly decreased after therapy as well as IL-1β, IL-2, IL-8 (CXCL8), IL-10, acute-phase protein serum amyloid A, C-reactive protein, and vascular adhesion molecule-1. The decrease of CCL17 and IL-8 was correlated with the decrease in disease severity in a subgroup of moderate AD individuals.

Conclusion: Stratum corneum tape stripping offers a minimally invasive approach for studying local levels of immunomodulatory molecules in the skin. CCL17 (TARC) and IL-8 were found to be the most promising biomarkers of AD and might be useful for investigating the course of skin diseases and the effect of local therapy.
INFLAMMATORY MEDIATORS IN AD PATIENTS

INTRODUCTION

Atopic dermatitis (AD) is a chronic remitting inflammatory skin disorder. It has a complex etiology in which immunological dysregulation and skin barrier alterations play an important role (1-3). The fact that mutations in the gene encoding for the epidermal protein filaggrin predispose for AD points toward the skin barrier as an important factor in the pathophysiology of the disease (4). In addition to genetic factors, filaggrin expression is down-regulated by Th2 cytokines (5). This further emphasizes the interaction between the immune system and skin barrier in AD, particularly since flares of AD are characterized by activation of Th2 pathways (6, 7) (See Fig 1). The profile of inflammatory mediators might provide more insight into specific immunopathological pathways, offering potential targets for more personalized therapy, the sub-classification of AD and for the monitoring of therapy (8). Clinical assessment of AD lesions is often hampered by symptoms such as erythema and lichenification. Furthermore, as addressed by Mansouri and Guttman-Yasky, AD has a high placebo response rate, which might influence assessment of the efficacy of therapeutics in clinical trials (8). The profiles of inflammatory mediators in AD skin lesions during exacerbation or after treatment have been insufficiently investigated because most studies are based on blood samples, and thus only provide information on systemic profiles and not on the cutaneous microenvironment (9, 10). The lack of data on the local milieu of inflammatory mediators is partly due to the invasive and laborious nature of many procedures used to obtain skin samples, such as biopsies, harvesting of skin-derived interstitial fluid or cutaneous microdialysis (11). In contrast, the collection of the stratum corneum (SC), the uppermost layer of the epidermis, by adhesive tape offers the advantage of obtaining skin samples in a simple and non-invasive manner. This technique has been used to determine different skin biomarkers, such as IL-1 cytokines, enzymes, lipids and filaggrin degradation products (12-16). However, so far the poor sensitivity of the analytical methods used in this approach has hampered the analysis of inflammatory mediators relevant to AD. Recently, several multiplex assays have been introduced which are more sensitive and which also permit the determination of a broad range of inflammatory mediators in a single (tape strip) sample (17).
In the present study, we used the SC tape stripping technique to determine various inflammatory mediators in lesional skin of AD patients before and after treatment with an emollient containing ceramides and magnesium (EM). Both ingredients are involved in the maintenance of the skin barrier. Furthermore, as a control, inflammatory mediators of 20 healthy, non-AD individuals were measured. We analysed 38 different cytokines, chemokines, and vascular growth factors, most of which, to our knowledge, have not previously been determined in the SC.

METHODS

Patients
Ninety adult patients from the VU Medical Center Amsterdam outpatient clinic (average age 33 years, 57 female, 33 male) with AD as defined by the Hanifin and Rajka criteria entered the study after their written informed consent (18) was obtained. Only patients with mild to moderate AD based on the OSCORAD (Objective SCORing Atopic Dermatitis) score (range 12.5-50) were included (19, 20). Apart from the emollient used in the study, patients were not allowed to apply local steroids or other products such as calcineurin inhibitors and OTC emollients on the investigated lesions. Patients undergoing systemic immunosuppressive or antibiotic therapy were excluded. SC samples were collected before and after six weeks of topical treatment with the study emollient. A healthy population of twenty individuals without a history of AD was included as a control group. The research was conducted according to the principles of the Declaration of Helsinki and was approved by the ethics committee of the Academic Medical Center (study number: METC 2014_090).

Emollient
The AD-patients were asked to apply an emollient containing ceramides and magnesium chloride hexahydrate (Dermalex® Eczema, Omega Pharma, Nazareth, Belgium) twice daily. Other components of the emollient included water, cholesterol, zeolite, glycerol, cocoglycerides, cetyl alcohol, isopropyl myristate, emulsifiers, and preservatives. The total lipid content was 30%. The control population was asked not to use any cream for at least 3 days preceding the study.
**Tape stripping of the stratum corneum**

Tape stripping was performed as previously described (12, 13). Briefly, adhesive tapes (3.8 cm², D-Squame; CuDerm, Dallas, TX, USA) were placed on a skin site affected by AD, e.g. the volar forearm or popliteal fossa, and briefly pressed with a standardized pressure pen of 225 g/cm² (D-Squame pressure instrument D500, CuDerm, Dallas, TX, USA). Eight consecutive tapes were collected from the same skin site. Six weeks later, another eight consecutive tapes were taken from an adjacent AD-affected and emollient-treated area as close as possible to the skin site sampled during the first visit. For the analysis, the 6th tape was used (stored at -80˚C). The tape strips of the control group were taken from the upper back of the participant and the 6th and 7th strip (1.5 cm² D-squames) were used for analysis. These strips represent the middle part of the SC where the cytokine concentrations reach stable levels (21).

**Sample preparation and analysis**

The SC samples were extracted from the tapes by ultrasonification (15 min) with 0.5 ml of phosphate buffered saline (PBS) containing 0.05% Tween 20. After vortexing, the extract aliquots were distributed in vials and stored at -80˚C. Analysis was performed using the MESO QuickPlex SQ 120 assay (MSD, Rockville, Maryland, USA). If more than 50% of the samples were under the level of detection, no further analysis was performed for that specific mediator (22). An overview of the inflammatory mediators can be found in Table 1 and supplement 1. All panels used human antibodies. For the analysis 50 µl of the SC extract and a calibrator (provided by MSD) were incubated overnight on the sealed plate at 2-8˚C. Reading was done after washing with PBS with Tween 20 and after adding the provided reading buffer. The extracts of tape 6 and 7 of the control group were pooled, as smaller D-squame tapes were used. Concentrations of all cytokines in both groups were corrected by the total protein content of the extract, which has been determined by Pierce assay (23). Samples under the limit of detection were substituted for by a value of half the level of the detection limit.

**Clinical and biophysical parameters**

The OSCORAD (Objective SCORing Atopic Dermatitis) was used to determine the clinical severity of AD lesions (19). Since only isolated skin sites were treated
and clinically assessed, the body surface area was set to 1% when calculating the OSCORAD. To assess the skin barrier function, transepidermal water loss (TEWL) was measured using a Tewameter 300 (Courage and Khazaka Electronic GmbH, Cologne, Germany).

**Statistics**

Statistical calculations were performed using Graphpad Prism 6.0 (Graphpad software, La Jolla, CA, USA). The Shapiro-Wilk test was used to check for data distribution. In the case of deviation from normal distribution, a non-parametric two-sided Wilcoxon signed-rank test was used. For normally distributed data, a two-sided t-test was applied. A p value of <0.05 was considered statistically significant. Because of the explorative character of the study, no correction for multiple testing was applied. Spearman’s rank correlation coefficient was used to perform correlation analysis of individual immunomodulators, clinical and biophysical parameters.

**RESULTS**

In total, 90 AD patients and 20 healthy controls completed the study and their samples were analyzed. The concentrations of 24 out of 38 measured inflammatory mediators could be quantitatively determined (i.e. the concentrations were above the detection limit of the method) in the majority of the samples (Table 1). Immunomodulators of which the concentrations in majority of samples (>50%) were below the detection limit were not further analysed, a summary of these mediators can be found in Supplement 1. The levels of eleven inflammatory mediators were significantly decreased after the six week topical emollient treatment. Among them were Th2 inflammatory mediators interleukin (IL)-4 (P=0.0033), IL-13 (P=0.0016), CC chemokine ligand (CCL)2/monocyte chemotactic protein (MCP)-1 (P=0.0025), CCL22/macrophage-derived chemokine (MDC) (P=0.0153) and CCL17/thymus and activation-regulated chemokine (TARC) (P=0.0047). A significant decrease was also found for the levels of the regulatory cytokines IL-1β (P=0.0008), IL-2 (P=0.0359), IL-4 (P=0.0033), IL-8 (P=0.0011) and IL-10 (P=0.0069) as well as the acute phase proteins serum amyloid A (SAA) (P=0.0276) and C-reactive protein (CRP) (P=0.0006) and vascular adhesion molecule (VCAM-1) (P=0.0087). The
other mediators did not show a significant change over time. When compared with healthy controls most of inflammatory mediators were significantly higher in the treatment group, both before and after therapy (Table 1).

Both the clinical severity as assessed by OSCORAD and the skin barrier function as measured by TEWL improved significantly after six weeks of therapy (Table 2). The OSCORAD decreased from 14.20 to 10.70 (p<0.0001) and the TEWL from 29.91 to 28.2 g/m2/h (P=0.008). The decrease in OSCORAD was 14.0 units (p<0.0001) in the patients with moderate AD and 3.5 (p<0.0001) in patients with mild AD (Table 2). A representative picture of lesions in patients with a mild and moderate disease severity is shown in Fig. 1.

![Representative pictures of lesions.](image_url)

Fig. 1. Representative pictures of the studied lesions. Tape strip samples were taken in the center of the marked region. Left: mild AD, right: moderate AD.
Table 1. Concentrations of inflammatory mediators in the SC before and after topical therapy

<table>
<thead>
<tr>
<th></th>
<th>BEFORE THERAPY</th>
<th>AFTER THERAPY</th>
<th>HEALTHY CONTROL</th>
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<tbody>
<tr>
<td></td>
<td>M (IQR)</td>
<td>M (IQR)</td>
<td>n</td>
</tr>
<tr>
<td>CRP *</td>
<td>3.799 (0.9;12.7)</td>
<td>3.516 (1.0;8.3)</td>
<td>75 ND</td>
</tr>
<tr>
<td>CCL11/ Eotaxin-1</td>
<td>270.2 (146.70;439.9)</td>
<td>315.5 (179.5;443.7)</td>
<td>75 ND</td>
</tr>
<tr>
<td>CSF2/GM-CSF</td>
<td>9.20 (3.60;21.58)</td>
<td>7.0 (2.91;19.24)</td>
<td>88 4.6 (2.5;11.5)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>49.9 (38.0;80.20)</td>
<td>44.3 (31.5;79.5)</td>
<td>57 33.4 (17.5;42.2)</td>
</tr>
<tr>
<td>IL-10</td>
<td>8.7 (5.6;13.9)</td>
<td>6.6 (4.6;12.3)</td>
<td>57 0.8 (0.01;1.16)</td>
</tr>
<tr>
<td>IL-13</td>
<td>80.9 (53.7;114.8)</td>
<td>67.1 (45.2;94.8)</td>
<td>90 2.0 (1.6;8.2)</td>
</tr>
<tr>
<td>IL-16</td>
<td>171.2 (83.4;363.3)</td>
<td>186.5 (71.3;334.9)</td>
<td>90 18.3 (1.3;42.0)</td>
</tr>
<tr>
<td>IL-17a</td>
<td>74.3 (40.9;120.4)</td>
<td>84.5 (42.2;118.5)</td>
<td>88 13.7 (9.6;24.3)</td>
</tr>
<tr>
<td>IL-1α *</td>
<td>2.3 (0.08;5.6)</td>
<td>2.4 (0.6;6.3)</td>
<td>88 42.1 (35.1;59.9)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>95.5 (37.7;243.6)</td>
<td>59.7 (31.8;137.6)</td>
<td>88 137.4 (48.3;523.5)</td>
</tr>
<tr>
<td>IL-2</td>
<td>20.14 (12.2;56.8)</td>
<td>19.4 (11;39.3)</td>
<td>90 17 (0.04;4.0)</td>
</tr>
<tr>
<td>IL-4</td>
<td>11.4 (8.4;16.5)</td>
<td>9.7 (6.1;16.0)</td>
<td>41 0.3(0.3;0.3)</td>
</tr>
<tr>
<td>IL-5</td>
<td>25.0 (13.0;43.3)</td>
<td>26.0 (15.0;46.8)</td>
<td>88 10 (7.4;14.7)</td>
</tr>
<tr>
<td>IL-8</td>
<td>97.8 (33.5;320.3)</td>
<td>46.1 (16.3;152.8)</td>
<td>90 14.2 (2.7;33.6)</td>
</tr>
<tr>
<td>IL-12/23p40</td>
<td>54.8 (28.7;124.1)</td>
<td>58.0 (18.9;141.7)</td>
<td>49 34.8 (13.2;49.8)</td>
</tr>
<tr>
<td>CCL2/MCP1</td>
<td>16.5 (8.4;32.8)</td>
<td>11.4 (6.2;21.7)</td>
<td>90 7.4 (3.2;13.8)</td>
</tr>
<tr>
<td>CCL13/MCP4</td>
<td>247.4 (166.5;472.4)</td>
<td>295.8 (160.2;534.1)</td>
<td>90 67.3 (25.0;95.6)</td>
</tr>
<tr>
<td>CCL22/MDC</td>
<td>1152 (656.2;1941)</td>
<td>955.4 (522.1;1465)</td>
<td>90 71.7 (465.2;1325)</td>
</tr>
<tr>
<td>CCL3/MIP1a</td>
<td>210.8 (137.7;325.7)</td>
<td>245.5 (167.7;335.7)</td>
<td>88 5.82 (1.7;9.139)</td>
</tr>
<tr>
<td>CCL4/MIP1b</td>
<td>164.6 (102.6;335.6)</td>
<td>179 (94.6;318.8)</td>
<td>90 22.6 (7.3;29.2)</td>
</tr>
<tr>
<td>SAA *</td>
<td>9.8 (102.6;335.6)</td>
<td>8490 (94.6;2318.8)</td>
<td>75 ND</td>
</tr>
<tr>
<td>CCL17/TARC</td>
<td>85.0 (35.7;187.2)</td>
<td>56.3 (34.1;105.1)</td>
<td>90 26.1 (35.6;52.7)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>8.0 (4.6;20.5)</td>
<td>8.2 (4.3;18.4)</td>
<td>51 1.16 (3.4;15.3)</td>
</tr>
<tr>
<td>VCAM-1 *</td>
<td>3.8 (1.2;10.7)</td>
<td>2.4 (0.7;5.7)</td>
<td>57 ND</td>
</tr>
</tbody>
</table>

Values are expressed as medians (interquartile ranges), unless otherwise indicated. *Before vs. after* was tested by a two-sided Wilcoxon signed rank test an ‘before vs. healthy’ and ‘after vs. healthy’ by a Mann-Whitney test. n = Number of samples in which the inflammatory mediators' concentration could quantitatively be determined; p = level of significance (significant differences are highlighted in bold). CSF2 = Colony-stimulating factor 2; GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN = interferon; MCP = monocyte chemotactic protein; MDC = macrophage-derived chemokine; MIP = macrophage inflammatory protein; TNF = tumor necrosis factor; NM not determined. * All concentrations are expressed as ng/µg protein, except CRP, IL-1, SAA and VCAM-1 that are expressed in µg/µg protein.
Table 2. Clinical and biophysical parameters measured at baseline and after 6 weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BEFORE THERAPY</th>
<th>AFTER THERAPY</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total OSCORAD</td>
<td>14.2 (10.7;21.2)</td>
<td>10.7 (7.2;14.2)</td>
<td>90</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mild OSCORAD</td>
<td>14.2 (10.7;17.70)</td>
<td>10.7 (7.2;14.20)</td>
<td>69</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Moderate OSCORAD</td>
<td>28.2 (26.45;31.70)</td>
<td>14.2(10.7;22.95)</td>
<td>21</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>TEWL (g/m²/h)</td>
<td>29.91 (23.29;39.37)</td>
<td>28.2 (19.73;36.18)</td>
<td>90</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Values are expressed as medians (interquartile ranges), unless otherwise indicated. P = Level of significance for the difference between the baseline and after therapy (two sided Wilcoxon signed-rank test).

**Correlation analysis**

In order to investigate relationships between the various inflammatory mediators, TEWL and OSCORAD, a correlation analysis was performed (online suppl. tables 2.1 and 2.2). A strong and significant correlation was found between the change in Th1 cytokines CCL11/Eotaxin-1 and IL-5 (r = 0.61, p < 0.001), the Th2 cytokines IL-4 and IL-13 (r = 0.72, p < 0.001), and tumor necrosis factor-β and IL-12 (r = 0.75, p < 0.001). The acute-phase proteins SAA and CRP both correlated well with VCAM-1 (r = 0.90; p < 0.001 and r = 0.74; p < 0.001, respectively). There was no correlation in changes of any of the investigated mediators with changes in OSCORAD and TEWL. However, in a subgroup analysis of moderate AD cases (OSCORAD >25), CCL17 and IL-8 showed to be significantly correlated with the decrease of OSCORAD (r = 0.56; p = 0.01 and r = 0.45; p = 0.05). Furthermore, significant correlations between the values of various inflammatory mediators and OSCORAD and TEWL at baseline as well as after the therapy (online suppl. tables 3.1 and 3.2) were found.

**DISCUSSION**

In the present study, we explored the potential of a minimally invasive sampling technique for the determination of inflammatory mediators in the SC of the lesional skin of AD patients with mild-to-moderate AD before and after treatment with an emollient. In total, 24 out of 38 investigated mediators could quantitatively be determined. The absolute levels of various inflammatory mediators correlated with the skin severity (OSCORAD) and skin barrier function (TEWL) at baseline and/or after therapy (online suppl. table 3).
After 6 weeks of topical therapy with an emollient containing magnesium and ceramides, both the severity of skin lesions and the skin barrier function improved. This was consistent with a significant decrease in the levels of various inflammatory mediators, mainly Th2-related cytokines and chemokines characteristic of acute AD lesions (23,24,25,26), including IL-4, IL-13, CCL2, CCL22, and CCL17. As expected, the levels of most inflammatory mediators were significantly higher in AD patients (before and after therapy) than in the healthy control group (7). Furthermore the levels of IL-4 and IL-13 were strongly correlated ($r = 0.70$). IL-4 and IL-13 are known as drivers of key pathogenic mechanisms of AD such as the survival of Th2 cells, differentiation and activation of myeloid and dendritic cells, activation of B cells, stimulation of the IgE class switch, and recruitment of eosinophils (27,28). Furthermore, they suppress lipid production, filaggrin expression, and keratinocyte differentiation, impairing the skin’s barrier function (5,29). The importance of IL-4 and IL-13 in AD has been previously confirmed in clinical studies. Thus, systemic therapy of AD patients with dupilumab, a monoclonal IL-4 and IL-13 receptor antagonist, resulted in a rapid improvement of disease activity (5,30,31,32,33,34,35).

In the present study, there was no significant change in the levels of the Th1 mediator interferon-$\gamma$. However, the levels of IL-1$\beta$ and IL-2 decreased significantly during the 6 weeks of therapy. The latter is associated with pruritus, a key symptom of AD (36,37,38). Apart from the Th2- and Th1-mediated molecules, significant changes were observed for the more general inflammatory mediators SAA, CRP, and VCAM-1 during therapy. Similarly, Caproni et al. (39) found a decrease of VCAM-1 in lesional skin biopsies of AD patients after local therapy with tacrolimus. Elevated CRP serum levels have previously been reported in AD (40). However, to the best of our knowledge, this is the first time that CRP was measured directly in human AD skin lesions.

Data on the local cytokine and chemokine milieu in AD are scarce (30). Morita et al. (41) showed a significant correlation between the SC levels of CCL17 and disease severity, Amarbayasgalan et al. (42) showed that IL-8 was correlated with AD-severity. Szegedi et al. (7) determined a wide range of Th1 and Th2 cytokines in dermal interstitial fluid collected from healthy subjects, and in the lesional and nonlesional skin of AD patients. IL-8 and the levels of Th2 cytokines/chemokines
IL-13, CCL2, and CCL17 were lower in healthy skin compared to lesional skin. Data on inflammatory mediators obtained from skin samples (i.e. SC tapes and interstitial fluid) are consistent with those from blood samples. Utilizing a multiplex analysis, Thijs et al. (43) found a significant decrease in the levels of 7 out of 31 investigated biomarkers, including CCL17 and CCL22, in blood samples from patients with moderate-to-severe AD who were treated with a potent corticosteroid (44). This is in line with the results of the present study in which a significant decrease in CCL17 and IL-8 was found after the therapy with the emollient containing magnesium and ceramides. CCL17 and CCL22 act via the Th2 cell chemokine receptor CCR4 (44,45,46,47). In a recent meta-analysis, serum levels of CCL17 were the most reliable available biomarker of AD disease severity, showing correlation coefficients of 0.60 (95% CI 0.48-0.70) and 0.64 (95% CI 0.57-0.70) in longitudinal and cross-sectional studies, respectively (41,43,44,48,49).

Although the levels of several immunomodulators in the present study were significantly decreased after therapy and were associated with the OSCORAD and TEWL at baseline and/or after therapy, there was no correlation between the changes in their levels and an improvement of clinical symptoms (OSCORAD) or skin barrier function (TEWL). One of the reasons for this might be the inclusion of patients with mild-to-moderate AD who showed a slight but significant reduction of OSCORAD from 14.2 to 10.7. For instance, patients in the study conducted by Thijs et al. (43) had more severe AD, and the study showed a large decrease of the severity index SASSAD (‘six area, six sign AD’) from 36.9 at baseline to 8.0 at the endpoint. Indeed, in the present study a subgroup analysis of moderate-AD individuals did show that declining concentrations of CCL17 and IL-8 were significantly correlated with the decrease in symptoms as asssed by OSCORAD. The performance of these inflammatory mediators as biomarkers of disease activity should therefore be further investigated in patients with more severe AD. One of the limitations of the present study is that the SC samples of the control group were collected from the upper back. However, Amarbayasgalan et al. (42) have shown no regional differences for various inflammatory mediators in healthy skin, including the back, neck, and arm. Furthermore, most of the investigated cytokines are produced upon inflammation due to AD and are not constitutively present in healthy skin.
In conclusion, tape stripping of the SC is a simple, minimally invasive approach to the study of local levels of various immunomodulatory molecules relevant to AD. CCL17 (TARC) and IL-8 were found to be the most promising biomarkers for AD. These biomarkers provide not only a better understanding of the pathogenesis of AD but also information on treatment efficacy, further enabling more targeted clinical care.

Acknowledgments
This work was supported by COST Action TD1206 StanDerm.
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Determination of natural moisturizing factors in the skin: Raman microspectroscopy versus HPLC

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(Biomarkers. 2017 Jan 19:1-6)
ABSTRACT

**Background:** Natural moisturizing factor (NMF) is used as genotypic and phenotypic biomarker in diagnostics. This study is a side-to-side comparison of two different methods to determine NMF in atopic dermatitis patients: Raman microspectroscopy and stratum corneum tape stripping followed by HPLC.

**Results:** Measured NMF values were significantly correlated ($R^2 = 0.61; p < 0.0001$), both methods demonstrated a concentration-depth dependence of NMF and reduced NMF levels in the carriers of filaggrin null mutations. Good agreement between measurements of left and right arms indicated robustness and good reproducibility of both methods.

**Conclusions:** Both methods showed comparable performance, choice of method will rather be influenced by practical consideration.
INTRODUCTION

The function of the skin barrier has gained renewed interest in dermatological research after identification of loss-of-function mutations in the filaggrin gene as a main risk factor for Atopic Dermatitis (AD), a common inflammatory skin disease (1). Filaggrin is a histidine-rich protein that is degraded into free amino acids in the uppermost layer of the skin; the stratum corneum (SC). The amino acids may be further metabolized into hygroscopic derivatives such as pyrrolidone carboxylic acid (PCA) from glutamine and urocanic acid (UCA) from histidine (His). This makes filaggrin a major source of hygroscopic compounds, which are collectively named Natural Moisturizing Factor (NMF), known to play an important role in skin hydration and barrier function (2, 3). Up to 55% of AD patients carry a filaggrin mutation; furthermore the carriers have an early onset and a more persistent and severe course of disease (1, 4) (5). We have demonstrated that filaggrin degradation products can be used as a biomarker for filaggrin genotype, which might enable sub-classification of AD (6-8). Next to genetic factors, filaggrin levels are influenced by exposure to detergents, organic solvents or topical therapy by corticosteroids (9, 10). Thus, they might also be used as a biomarker of the damaging effect of environmental exposures (10). The potential of filaggrin degradation products as a biomarker demands a feasible and reliable analytical method, which might be used for diagnostics and research purposes. Currently, for their determination the most used methods are Raman microspectroscopy (RMS) and stratum corneum tape stripping technique followed by high performance liquid chromatography (TS/HPLC). RMS is an optical method determining filaggrin degradation products via spectroscopic measurements within the SC. It is a non-invasive method, which determines, in real-time, levels of several individual filaggrin degradation products simultaneously at different SC depths (11, 12). TS/HPLC determination is based on the tape stripping technique by which the SC layers are collected in a minimally invasive manner by adhesive tapes. After extraction by acid or base, the filaggrin degradation products are determined by HPLC (6). In most published studies, RMS measurements include several free amino acids (serine, glycine, ornithine, alanine, histidine and proline) and PCA, UCA, and urea, while main TS/HPLC studies measured PCA and UCA, and in some studies also His is included (13, 14). The measured filaggrin degradation products are referred to as NMF, although taken
strictly NMF contains next to filaggrin degradation products also lactate, urea and sugars and inorganic salts (15).

So far, there is no side-to-side comparison between the two methods concerning agreement of the measurements and practical considerations. Therefore, in the present study we compared the results of both methods applied to the same AD-patient population.

METHODS

Subjects
Twenty patients with mild to moderate AD were recruited from the outpatient dermatological clinic (Waterland Hospital, the Netherlands). Diagnosis was done according to the Hanifin and Rajka criteria(16) for AD by a trained dermatologist. From all patients a buccal swab sample was collected for genotyping of the four most prevalent Filaggrin mutations in the European Caucasian population (R501X, 2282del4, R2447X, and S3247X)(17, 18). The experimental protocol followed the Declaration of Helsinki Principles and was approved by the Medical Ethics Committee of the Academic Medical Centre. Written informed consent was obtained from each participant.

Raman microspectroscopy (RMS)
NMF were determined on visibly non-affected volar forearm skin using confocal Raman microspectroscopy (Model 3510 Skin Composition Analyzer; RiverD International BV, Rotterdam, The Netherlands). In one subject it was difficult to find a non-affected skin site which was accessible for the RMS measurement, therefore the measurement was done on the volar aspect of the wrist. Raman spectra were recorded in the 400 - 1800 cm⁻¹ spectral region using a 785-nm laser with 25 mW of laser power on the skin. Levels of NMF relative to keratin (NMF/keratin ratio in arbitrary units) are displayed in real-time by the data acquisition software included with the RMS instrument. For more detailed analysis and comparison with the TS/HPLC results, the data were further processed offline. NMF/keratin ratios were determined from the Raman spectra by classical least-squares fitting using the
SkinTools 2.0 software (RiverD International BV, Rotterdam, The Netherlands). In this analysis NMF is defined as the sum of 7 dominant constituents of NMF: serine, glycine, PCA, ornithine, alanine, histidine (His), and proline (19). A reference spectrum of NMF has been constructed from the weighted sum of the Raman spectra of these dominant constituents and is used by the least-squares-fitting algorithm to determine the NMF/keratin ratio. The details of this method have been described elsewhere (11, 12). Possible negative values from the unconstrained classical least-squares-fit, due to low Raman signals from NMF, were replaced by value 0. NMF values from individual Raman spectra with insufficient spectral quality based on signal-to-noise ratio or insufficient quality of the fit based on too large residual between the original spectrum and the calculated fit were excluded from further analysis. The measurements were performed without any pretreatment of the skin. The subject positioned the arm on the instrument with the skin resting on the 2x4 cm² fused silica measurement window. After the operator had set a starting point at the skin surface, the instrument recorded a profile consisting of 8 Raman spectra from the skin surface to 28 μm below the skin surface at 4 μm depth increments. The total time to record one profile of 8 Raman spectra was 48 s. A maximum of 8 profiles from different areas of the volar aspect of the left and the right forearm were measured for each patient. The NMF values measured between 2 and 8 μm from the skin surface were pooled and averaged to obtain the NMF value of each patient per location (left or right arm). This depth range overlaps with the central portion of the SC. To enable a comparison of depth-dependent NMF concentrations with the results from HPLC-TS, the NMF profiles measured with Raman spectroscopy were interpolated on 1 μm depth intervals and then pooled for all patients, resulting in the average NMF value and standard deviation in the depth range 0 to 12 micrometer. All results are presented as NMF/keratin ratio in arbitrary units.

Tape stripping/ HPLC
To enable comparison of NMF-concentrations determined with HPLC-TS and RMS, which determines NMF levels at different depths, the average concentration of three tapes (4, 6 and 8) was calculated and compared to an average value of the RMS results at corresponding position in the SC. The depth of each tape has been estimated from the cumulative amount of protein obtained by measuring optical density of the tape as described previously(20, 21).
The SC samples for TS/HPLC were taken in the vicinity (unaffected by AD lesions) of the skin sites used by RMS on the left and right volar forearm. Eight D-squame tapes (22-mm diameter, Cuderm, Dallas, TX, USA) were consecutively placed on the skin and gently pressed with a standardized pressure pen (D500 - D-Squame Pressure Instrument Cu-derm, Dallas, TX, USA). Tapes were then removed, placed in vials and stored at -20 °C. NMF components His, PCA and UCA (trans-cis isomer) in the SC on the tape were extracted with 500 µl of 25% (w/w) ammonia solution, evaporated to dryness, and reconstituted in 500 µl of pure water. The HPLC separation was achieved by using a 250 * 3 mm reversed-phase Prevail column (Grace /Alltech, Breda, the Netherlands), with a flow rate of 0.4 mL min. The effluent was monitored by a UV / Vis detector (UV-975; Jasco, The Netherlands) set at 210 nm for PCA and His and at 270 nm for both UCA isomers. The total amount of UCA, PCA and His referred here collectively to as NMF, has been calculated by summing up their individual molar amounts. The amount of SC collected by the tapes was assessed by measurement of the optical density (OD) of each D-Squame® disc with the D-Squame Scan 850A (Monaderm, Monaco, France). The amount of SC on the tape was calculated from the OD, and expressed as the mass of protein/cm² according to a standardized procedure described elsewhere (20). The concentrations of NMF were corrected for the amount of protein, and expressed as nmol/µg protein.

Statistics
All calculations were performed by GraphPad version 6.0 (GraphPad Software, La Jolla California USA). Distribution of data was tested by Shapiro-Wilk normality test. The difference in NMF values between the tapes was tested by repeated measures ANOVA using Bonferroni correction for multiple testing. The comparison of the NMF values between RMS and TS/HPLC method was tested by linear regression model. The comparison of the NMF values between left and right arm was performed by linear regression analysis and Bland-Altman analysis. The Bland-Altman plots evaluate a bias between the mean differences, and to estimate an interval, within which 95% of the differences of the second measurement, compared to the first one, fall (Giavarina, 2015).
RESULTS

Genotyping for the four most prevalent filaggrin mutations in the Caucasian population revealed that four patients were heterozygous and one patient was compound heterozygous carrier of a filaggrin loss-of-function mutation.

Depth dependence of the NMF levels in the SC

As shown in Fig. 1, the NMF concentrations on the 4th tape strip were significantly lower (paired 1-way ANOVA test with Bonferroni correction for multiple testing) than the NMF values on the 6th and 8th tapes which originate from deeper SC layers. The NMF concentrations on all tapes were very strongly correlated (Table inserted in Fig. 1). RMS shows a similar pattern of concentration-depth dependency, with maximum NMF concentrations around 2-4 μm (Fig. 2).
Spearman's correlation coefficients (R)

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Fig. 1. The NMF concentrations on the 4th, 6th and 8th tape and the correlations between NMF concentrations determined on the three tapes (table). The respective estimated depths of the 4th, 6th and 8th tapes were 1.9 (SD 0.32); 2.8 (SD 0.41) and 3.6 (SD 0.51) µm. P<0.0001 (ANOVA paired test with Bonferroni correction). The results are shown as mean ±SD (n=20)

Fig. 2. The concentrations of NMF (serine, glycine, PCA, ornithine, alanine, histidine, and proline) measured by RMS, interpolated on 1 µm depth intervals and averaged for all patients. The results are shown as mean ±SD (n=20)

Comparison of NMF measured on the left and right arm

As shown in Fig. 3 (left panel), both methods showed strong correlations of NMF values between the left and right arm. Furthermore, as assessed by a Bland-Altman plot, a preference method when analyzing agreement between two measurements
of the same parameter (right panel, Fig. 3), there was a good agreement in the measured values between left and right arm with the average bias (average of the difference between left and right arm) of 0.02 for TS/HPLC and 0.005 for RMS. The fact that the average bias is close to zero indicates that the measurements on the left and right arm do not produce systematically different results.

\[ R^2 = 0.82; P < 0.0001 \]

\[ \text{Equation: } Y=0.9140+0.06757 \]

\[ R^2 = 0.92; P < 0.0001 \]

\[ \text{Equation: } 0.8441x+0.131 \]

Fig. 3. Comparison of the NMF levels determined on the left and right arm by TS/HPLC (upper panel) and RMS (lower panel). In the Bland-Altman plots the horizontal lines represent the mean difference, and at the limits of agreement (mean difference plus and minus 1.96 times the standard deviation of the differences)

**Correlation RMS vs TS/HPLC**

As there was no systematic difference in the NMF values between left and right arm, for the comparison of the results obtained by two methods, the corresponding NMF values measured on the left and right arm were averaged. To compare the two methods linear regression analysis was performed including residual analysis. As shown in Fig. 4, both methods showed a strong correlation between the NMF values measured in the same AD patients \(r^2=0.61; P<0.0001\), and furthermore the
residuals showed a random distribution around the horizontal axis (Fig. 4, right panel). The average NMF value measured by the TS/HPLC method for the carriers of a filaggrin mutation (indicated with X in Fig.) was significantly lower than the average NMF value for the patients who were wild-type for filaggrin mutations (respective NMF values of 0.24 and 0.62 nmol/µg protein; P=0.005). A similar trend was found by RMS, however, the difference in NMF values between filaggrin mutation carriers and wild-type individuals did not reach significance (respective average NMF values were 0.53 and 0.98 AU; P=0.079).

Fig. 4. The correlation between NMF concentrations determined in AD patients by TS/HPLC and RMS. The X symbol marks the patients with a filaggrin loss-of-function mutation. $R^2$= coefficient of determination.

**DISCUSSION**

NMF concentrations in the SC of AD patients as measured by of Raman microspectroscopy (RMS) and stratum corneum tape stripping method followed by HPLC (TS/HPLC) show a strong correlation despite the fact that the investigated methods did not measure the same NMF components. RMS includes in the measurement serine, glycine, PCA, ornithine, alanine, histidine and proline, while TS/HPLC includes PCA, histidine and both isomers of urocanic acid. However, all mentioned NMF components are degradation products of the same protein: filaggrin. So the choice and the number of measured NMF components seem not to be critical. This is consistent with previous data, showing that different filaggrin degradation products are highly correlated (21) (22).
Both RMS and TS/HPLC show a similar pattern in depth dependency of the NMF concentration. Although NMF concentrations on all three tapes were strongly correlated, the NMF concentrations on the 4th tape was lower than the levels on the 6th and 8th tapes (approximate SC depth of 1.9; 2.8, and 3.6 µm, respectively). Also by RMS, the NMF levels show lower concentrations in the more superficial SC layers (up to 2 µm). (14) (23)

A strong correlation and agreement in the NMF values measured on the left and right arm indicate good reproducibility and robustness of both methods.

The average NMF value in the carriers of filaggrin mutations measured by TS/HPLC was significantly lower than the average NMF value in the patients who were wild type for filaggrin mutations, which is in accordance with literature data. NMF results obtained by RMS showed a similar trend although the difference in NMF values between carriers and non-carriers did not reach the level of significance (P=0.08). It has to be noted that the present study was not specifically designed to differentiate carriers of a filaggrin mutation from non-carriers. Especially for RMS, measurements for prediction of filaggrin genotype can be more accurately and rapidly performed on the palm of the hand where the SC is much thicker than on the forearm enabling more measurement points at SC depths where the NMF values level off (7). Previous studies have shown that NMF measured by RMS on the palm of the hand is a strong predictor of filaggrin genotype (8).

On the practical level, both methods have advantages and limitations. RMS can provide a quick and real-time outcome on NMF concentration in the SC in a non-invasive manner. Due to the thorough research protocol in this study, with multiple measuring points per skin site the total period of measurement, including preparations, per patient was 15 minutes. TS/HPLC enables rapid collection of tape strip samples from the subjects (roughly half the time of RMS, including preparation), but samples require further processing and analysis. If (relatively) a quick assessment of NMF levels is required, e.g. in the clinics, RMS would therefore be the method of choice. On the other side, TS/HPLC can be implemented fast in research and clinical environments; tape stripping can be performed on-site with minimal facilities which could be advantageous in multicenter or field studies. The
used RMS hardware as a bench-top device limits the practical use to hand palm (preferably) and arms, whereas tape stripping for HPLC analysis is not limited to a specific body site which might be advantageous by monitoring of local therapy. Costs of RMS equipment are currently relatively high, but unlike HPLC RMS it does not require laboratory facilities. The current allround research RMS instrument for in vivo skin analysis, used in this study, is commercially available in various versions ranging from €195.000 to €290.000. However devices dedicated to a single specific task will have a very significantly reduced price and dedicated software for RMS and streamlining of the workflow can reduce training needed to work with RMS to a minimum. While RMS is still a specialized field, expertise in HPLC is present in most analytical laboratories and the costs of the instrument are relatively low (approximately €25.000).

An important advantage of RMS to TS/HPLC is that NMF can be measured at specified depths, although at the cost of increased measurement time. Thus the optimal SC depth (i.e. the depth where the NMF levels level off or reach maximum) can be derived from the recorded spectra. As shown in the present study, the NMF levels are depth-dependent, which is in accordance with our previous study(7). Although there was a strong correlation between all three strips, using the tapes collected from the most superficial layers would create an underestimation of the actual NMF concentration. In the present study, there was no difference between the 6th and 8th strip number which corresponds to the SC depth around 3-4 µm. Due to intra-individual variability in the SC thickness and cohesiveness the SC in other diseases or other body locations the optimal strip number might be different from AD-forearm SC and should therefore be investigated before implementation of tape stripping in a different disease or body location. Both methods normalize the NMF values with the protein levels. The RMS normalizes the NMF values with the simultaneously measured keratin levels whereas TS/HPLC uses proteins derived from the optical density (OD) of the TS which needs to be done separately. The prerequisite for using OD is that the SC cells on the tape are homogeneously distributed over the tape, which especially in diseased skin is not always the case. When SC is non-homogeneously distributed over the tape, protein concentration have to be determined by another method e.g. by weighing or by a spectrophotometric assays (21). In that case the determination of NMFs by TS/HPLC is more time consuming.
CONCLUSION

The concentrations of NMF determined by Raman microspectroscopy and tape stripping followed by HPLC show a good correlation. Good agreement between measurements obtained on the left and right arm indicate robustness and good reproducibility of both methods. The choice between these two methods therefore will likely be influenced by practical considerations such as price, accessibility, available expertise and time-constrains. RMS can provide fast and detailed results on the profile of NMF components across the SC. However the current cost and accessibility of state-of-the-art Raman equipment may be an obstacle for using this technique more widely. Development of a small portable device with user-friendly software and quick read-off would be of a great value for clinical practice. Tape stripping enables simple and quick collection of a large number of samples which can be stored for a longer period of time which is convenient for large clinical studies. However, in contrast to RMS, further processing of the samples for HPLC analysis is required, and the sampling process (i.e. the tape number) should be optimized before application in a specific population.

Acknowledgments

We acknowledge the supported by H2020 COST Action TD1206 ‘StanDerm’. We also like to would like to thank F. Charles and M. Bos for their assistance with the measurements.
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