Skin tape proteomics identifies pathways associated with transepidermal water loss and allergen polysensitization in atopic dermatitis

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Background: Atopic dermatitis (AD) and food allergy (FA) are associated with skin barrier dysfunction. Objective: Skin biomarkers are needed for skin barrier interventions studies.

Methods: In this study, skin tape strip (STS) samples were collected from nonlesional skin of 62 children in AD FA+, AD FA−, and nonatopic groups for mass spectrometry proteomic analysis. Transepidermal water loss and allergic sensitization were assessed. STS proteomic analysis results were validated in an independent cohort of 41 adults with AD with and without FA versus nonatopic controls.

Results: A group of 45 proteins was identified as a principal component 1 (PC1) with the highest expression in AD FA+ STSs. This novel set of STS proteins was highly correlative to skin transepidermal water loss and allergic sensitization. PC1 proteins included keratin intermediate filaments; proteins associated with inflammatory responses (S100 proteins, alarmins, protease inhibitors); and glycolysis and antioxidant defense enzymes. Analysis of PC1 proteins expression in an independent adult AD cohort validated differential expression of STS PC1 proteins in the skin of adult patients with AD with the history of clinical reactions to peanut.

Conclusions: STS analysis of nonlesional skin of AD children identified a cluster of proteins with the highest expression in AD FA+ children. The differential expression of STS PC1 proteins was confirmed in a replicate cohort of adult AD patients with FA to peanut, suggesting a unique STS proteomic endotype for AD FA+ that persists into adulthood. Collectively, PC1 proteins are associated with abnormalities in skin barrier integrity and may increase the risk of epicutaneous sensitization to food allergens.

Key words: Atopic dermatitis, food allergy, skin barrier, proteomics, transepidermal water loss, IgE

The stratum corneum (SC) serves as a skin barrier, providing protection from potentially damaging external factors and controlling transcutaneous water loss. Transepidermal water loss (TEWL) measurement quantifies water diffusion across the SC and is commonly used for the physiologic assessment of skin barrier function.1 In addition to basal TEWL to assess the undisturbed permeability of the skin barrier, TEWL measurements have also been conducted together with skin barrier perturbation using skin tape stripping (STS) to measure skin barrier integrity.2 Healthy skin is not very sensitive to STS and can withstand mild perturbation, while disrupted skin, and skin with low structural integrity, exhibit greater changes in TEWL. The area under the curve (AUC) for TEWL measurements done over a defined number of STSs is used to assess the overall integrity of the SC. Skin barrier dysfunction and increased TEWL are major pathologic features of atopic dermatitis (AD).3-5 TEWL has been shown to correlate with AD severity.6-8

Epidermal proteins including filaggrin (FLG) are essential for the maintenance of the epidermal permeability barrier that prevents allergen penetration and loss of fluid.5 Both FLG gene mutations and FLG inhibition by type 2 cytokines contribute to reduced FLG expression in AD skin.5,10 Several research groups have reported reduced FLG breakdown products and long-chain skin barrier lipid products in the skin of patients with AD versus nonatopic (NA) subjects using STS.11,12 Even in subjects with AD who do not carry FLG gene mutations, significant changes in the skin expression of enzymes involved in lipid metabolism have been demonstrated.13 These changes in the SC that are detectable by STS support the concept that skin barrier abnormalities in AD could allow penetration of environmental allergens and microbes and are accompanied by the release of epithelial-derived cytokines (eg, thymic stromal lymphopoietin [TSLP], IL-25, IL-33), which drive type 2 immune responses.5

The utility of noninvasive approaches, such as TEWL measurement and STS protein and lipid profiling, to identify endotypes and skin biomarkers of clinical AD phenotypes is an area of active investigation.14-17 However, the mechanisms underlying increased TEWL and changes in skin barrier function are not well understood. Identification of skin barrier components that control TEWL is critical for designing skin barrier interventions and establishing biomarkers and could facilitate the identification of AD subtypes.

In a recent prospective clinical study, funded by the National Institutes of Health/National Institute of Allergy and Infectious Diseases Atopic Dermatitis Research Network, we utilized a
multimomics approach with STS of nonlesional skin to show that children with AD and food allergy (FA) (AD FA+) represent a unique endotype that can be distinguished from children with AD but without FA (AD FA−) and subjects who are NA using a constellation of epithelial biomarkers including increased TEWL, low FLG breakdown products, changes in SC lamellar bilayer structure, and lipid composition.17 In this study, we report detailed information on STS proteomic analysis of this study cohort and examine associations of the proteomic data with TEWL and clinical allergy. The utility of STS proteomic analysis for the endotyping of patients who are AD FA+ and AD FA− is reported in this article.

METHODS
Study subjects
In this study, we used data of 62 children from a cohort of patients from a prospective, clinical mechanistic study registered at ClinicalTrials.gov (Atopic Dermatitis and Food Allergy; NCT03168113). The details of the study cohort were reported earlier by our research group. All participants with AD had active skin disease diagnosed using published criteria18 and were stratified into 2 groups based on their FA status: (1) AD FA+ (n = 21); (2) AD FA− (n = 19). The children in the AD FA+ group were allergic to peanut with a peanut skin prick test wheal ≥ 8 mm, which has been reported to significantly correlate with immediate clinical reactions to oral peanut challenge,19 as well as documentation of a previous positive oral food challenge to peanut or convincing history of an immediate allergic reaction to peanut. In the AD FA− group, the participants had no personal history of FA, as well as a negative skin prick test (wheal < 3 mm) to peanut, milk, egg, wheat, soy, shellfish mix (clam, crab, oyster, scallops, and shrimp), almond, English walnut, hazelnut, cashew, brazil nut, and sesame seed. A third group included NA controls (n = 22) who were defined as those without a personal history of ADs and negative skin prick tests to the same foods as well as common aeroallergens (cat, dog, dust mite, cockroach, mold mix, tree mix, grass mix, weeds mix). Children who were AD FA+ and AD FA− were also skin tested for the aeroallergens and the information was recorded. The full inclusion and exclusion criteria for this study and study subject characteristics are detailed in our prior publication. All participants avoided any treatments, baths, and skin creams or emollients that may potentially affect skin barrier function before skin sample collection.

Skin disease severity assessments
All patients who are AD FA+ and AD FA− in the study had active AD. None of the study subjects had an overt skin infection. AD severity was evaluated using the Scoring Atopic Dermatitis,20 and the Eczema Area and Severity Index.21 Persistence of AD was evaluated using the Nottingham Eczema Severity Score,22 a validated index incorporating clinical course over the past 12 months, disease intensity over the past 12 months, and extent of body surface area involvement. Enrollment of participants with AD FA+ and AD FA− was actively matched based on AD severity (mild or moderate) as determined by the Nottingham Eczema Severity Score.

Skin barrier assessment
Comparisons between groups for the TEWL AUC and TEWL at baseline before STS and after 5, 10, 15, and 20 STSs were done on nonlesional skin of the forearm. TEWL was evaluated using the AquaFlux AF200 (Biox, London, UK) as previously described.17

STS collection
Thirty D-Squame tape strips (22-mm diameter, CuDerm; Synergy Biologics, West Midlands, UK) were collected from nonlesional skin of the upper arms (forearm) using a D-Squame D500 pressure instrument to apply all STSs with equivalent pressure (e.g., 225 g/cm²). Nonlesional sites selected for STS collection were at least 2 inches away from the lesion. Collected STSs were stored individually in 12-well plates and kept frozen at −80°C prior to processing.

Sample preparation for global proteomic analysis
For each sample collection, protein content was extracted from each of 15 STSs (every other STS to tape 30) with 50 mmol/L ammonium bicarbonate, 1% SDS buffer supplemented with HALT protease inhibitors and 10 mmol/L dithiothreitol (all reagents from Thermo Fisher Scientific, Waltham, Mass). SDS was removed using a detergent removal spin column, and then protein concentrations were determined. For proteomic analysis, 50 μg of protein from each sample were reduced in 5 mmol/L dithiothreitol and alkylated in 10 mmol/L iodoacetamide. The protein content was precipitated with cold (−20°C) 10% wt/vol trichloroacetic acid in acetone. The pellet was rinsed with cold acetone and dried. The dried protein pellet was dissolved in 5% trifluoroethanol 50 mmol/L trethylammonium bicarbonate and digested with 2 μg of Trypsin Lys-C mix overnight at 37°C. A 10-μg sample of the protein was labeled with TANDEM mass tag (TMT) 10-plex reagents (Thermo Fisher Scientific) for 2 hours at room temperature. The reaction was quenched with 1% ethanolamine. The TMT-labeled samples were combined into sets of 9 samples containing samples from each study group. Each TMT set was step-fractionated on an Oasis plate (Waters, Milford, Mass) using 7 mmol/L tetraethylammonium bromide (pH = 8) in 5%, 10%, 25%, and 70% acetonitrile. Each fraction was lyophilized, stored at −20°C, and reconstituted in 0.1% formic acid, 2% acetonitrile prior to tandem mass spectrometry (MS/MS) analysis.

LC-MS/MS
Using liquid chromatography (LC)-MS/MS, each fraction was separated over a 90-minute reversed phase gradient. Precursor ion scans spanning 400 to 1600 m/z (ie, mass/charge number) were acquired at 120,000 (m/z = 200) resolution every 3 seconds. Detected ions in the +2 to +6 charge states were individually isolated by the quadrupole in 0.4 m/z bins and fragmented in order of highest intensity by high-energy collisional dissociation (38 normalized collision energy). Fragment ion scans were acquired at 50,000 resolution (m/z = 200) with automatic gain control set to 50,000 and a maximum ion accumulation time of 86 milliseconds. Fragmented ions were excluded from redundant fragmentation for 15 seconds.

Data analysis
The mass spectrometry (MS) data were searched against the SwissProt Homo Sapien database (May 2019, 20,431 entries, https://www.uniprot.org) with MASCOT (version 2.6.2; Matrix Science, Boston, Mass) and Percolator 2,21

Abbreviations used
AD: Atopic dermatitis
AUC: Area under the curve
FA: Food allergy
kUA: Kilo allergy unit
LASSO: Least absolute shrinkage and selection operator
LC: Liquid chromatography
MS/MS: Tandem mass spectrometry
m/z: Mass/charge number
NA: Nonatopic
PC1: First principal component
PCA: Pyroglutamic acid
SC: Stratum corneum
STS: Skin tape strip
TEWL: Transepidermal water loss
TMT: Tandem mass tag
UCA: Urocanic acid

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rescoring followed by quantitative analysis in Proteome Discoverer 2.2 (Thermo Fisher Scientific). This ensured that only human-derived proteins were analyzed. Spectra were matched to theoretical tryptic peptides using 5 parts-per-million precursor and 0.01 Da fragment mass error tolerances and filtered to 1% false discovery rate. For quantitation, the identified spectra were filtered to exclude chimeric spectra with >30% coisolation interference and peptides with deamidation or oxidation. Peptides were quantified based on the reporter ion signal-to-noise values. Each channel was adjusted for loading by leveling the summed peptide abundance to the channel with the highest summed peptide intensity. Proteins were quantified using only unique peptides, and the average protein abundance across all samples was scaled to 100. Fold changes and ANOVA significance values were calculated based on the scaled protein abundances. Protein grouping was performed according to molecular function and protein class using the Gene Ontology database (STRING version 11.0; https://string-db.org/).

**FLG breakdown products and skin microbiome analysis**

In this article, the data on the FLG breakdown products and skin microbiome analysis were collected for the study cohort in our previous work. For details of FLG breakdown products and microbiome analysis, refer to our previously published work.

**Statistical data analysis**

All statistical analyses were performed in the R programming environment (R Foundation, Vienna, Austria) and a P value <0.05 was considered significant. Figures were created using the Latte package. The protein expression data set acquired in this study is high dimensional (n = 149) and contains variables that correlate with each other and this introduces multicollinearity in regression models. Multicollinearity, therefore, may interfere in determining the precise effect of each predictor and also tends to result in larger standard errors (and, hence, wider confidence intervals and greater P values). Given these considerations, we have used a principal component analysis in our study to reduce the set of 149 proteins into a few dimensions that reduce a large amount of the variability of the original variables. These dimensions, called components, have the properties of collecting highly correlated variables within each component and being uncorrelated with each other. The first principal component (PC1) explains the highest amount of the variability of the original data. Principal component analysis and correlation analysis was executed using the Stats package in R. The PC1 loadings were compared across the 3 patient groups with an ANOVA model including pairwise comparisons using the Emmeans package in R.

To identify differentially expressed proteins between the patient groups, we conducted an analysis using the Bioconductor Limma package. This approach uses linear models to analyze the entire experiment as an integrated whole, rather than as individual pairwise comparisons. The model borrows information across proteins to smooth out variances and uses posterior variances in a classical t-test setting. The method is completely data-dependent and uses an empirical Bayes approach to estimate hyperparameters. Contrasts were set up for pairwise comparisons between the patient groups. P values were adjusted using the false discovery rate correction method. A Venn diagram was constructed using the Eulerr package in R.

**RESULTS**

**STS proteomic data principal component analysis**

Protein extracts were prepared from STS samples of 62 study children. Using LC-MS/MS analysis, 371 individual proteins were detected in STS extracts. A total of 149 of these proteins were quantified in STS extracts from all study subjects. Due to stochastic sampling of data-dependent MS, 222 proteins were only quantified in some TMT sets. No statistically significant differences were found in the expression of these 222 proteins between the NA subjects and subjects with AD FA− versus those with AD FA+ (P = .64).

The 149 proteins that were detected in all of the STS extracts were further evaluated in an unsupervised principal component analysis. The leading principal component (PC1) explained 30% of the variance in the proteomic data and consisted of 45 proteins (see Table I for the list of these proteins). Importantly, as a group, these PC1 proteins were differentially expressed across the 3 patient groups in our cohort (Fig 1), with the highest PC1 composite score for subjects with AD FA+, intermediate for those with AD FA−, and lowest for NA controls (Fig 1).

**Relationship between STS PC1 proteins and skin TEWL**

We have previously reported increased TEWL in the skin of patients with AD FA+. Given that PC1 proteins had the highest expression in subjects with AD FA+, we evaluated the relationship between skin PC1 protein expression and skin TEWL. Importantly, we observed a significant positive correlation between PC1 protein expression and the cumulative TEWL estimate over 20 STSs (TEWL AUC) (Fig 2, A). In our recent study, we reported dissociation in TEWL measurements between subjects with AD FA+ versus those with AD FA− and NA controls with tape stripping, with significant enhancement in skin TEWL at STSs 15 and 20 in subjects with AD FA+ as compared to subjects with AD FA− and NA controls. Using TEWL data measurement collected at STS 15, we observed a significant positive correlation from the prediction model, chi-square tests were used for categorical variables, and the Wilcoxon test was used for the 2-group comparisons.

**Validation of proteomic findings in the independent patient cohort**

In this study, we have utilized an independent proteomic data set that examined nonlesional skin STS protein expression in adults with AD and NA controls. The patient cohort was a separate cohort gathered by the Atopic Dermatitis Research Network. As part of the patients’ registry, the study gathered data on patients’ history of clinical reactions to peanuts, which included development of itchy skin rashes, swelling of the lips, tongue or eyes, breathing difficulties, gastrointestinal symptoms, and anaphylactic reactions to peanut consumption. Total serum IgE to peanut levels were analyzed.

Twenty STS samples were collected from nonlesional skin of the volar surface of forearm at least 2 inches away from the lesion. Protein extracts were prepared from 10 STSS as described above, and protein extracts were analyzed as the extracts from the pediatric cohort. STS PC1 protein expression was extracted from the data set. Protein IDs of PC1 proteins were used for data extraction and cumulative PC1 expression was compared between adult patients with AD with and without history of allergic reactions to peanut. In addition, cumulative PC1 protein expression was compared between patients with AD stratified by serum levels of total IgE to peanut.
A total of 371 individual proteins were detected in STS extracts by LC-MS/MS analysis. Of those, 149 proteins were quantified in STS extracts from all study subjects and are summarized in this table. The 45 proteins identified as PC1 in the principal component analysis of the STS proteomic data are boldfaced.

### TABLE I. Proteins identified in STS samples from all study participants

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<th>A2ML1</th>
<th>CAPNS2</th>
<th>DSG1</th>
<th>GSDMA</th>
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<th>KRT5</th>
<th>NPC2</th>
<th>PSMA1</th>
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<th>SPRR1A</th>
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<td>FLG</td>
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<td>PSMA7</td>
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<td>PSMB1</td>
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<td>PSMB2</td>
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<td>HSPB1</td>
<td>KRT17</td>
<td>ME1</td>
<td>PRDX2</td>
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<td>IgG heavy chain</td>
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<td>FSAF</td>
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<td>IGKC</td>
<td>KRT23</td>
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<td>PSAPL1</td>
<td>S100A7</td>
<td>SPINK5</td>
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by skin PC1 protein expression and skin TEWL at STS 15 (Fig 2, B).

### Relationship between STS PC1 proteins and parameters of allergic sensitization in subjects with AD FA+ and AD FA−

We have evaluated a relationship between cumulative STS PC1 protein expression and total serum IgE in patients with AD as a marker of allergic sensitization. For this analysis, 2 of AD groups were combined. A significant positive correlation between the cumulative expression level of STS PC1 proteins and total serum IgE was found (Table II). We further examined relationships between STS PC1 protein expression and IgE to foods (peanut, milk, egg) and aeroallergens (Phadiatop IgE) in AD groups. A significant correlation between serum levels of IgE to peanuts and PC1 protein skin expression was observed, while a trend for a positive correlation between serum IgE to milk and egg and PC1 was found. A significant correlation between the aeroallergen Phadiatop IgE panel and PC1 skin expression was also identified (Table II). No association between STS PC1 expression and aforementioned serum IgE parameters was found if the data were analyzed separately for the AD FA+ and AD FA− groups (data not shown).

Skin prick test assessment confirmed a significant correlation between the number of positive allergen skin prick tests and STS PC1 protein expression (Table III). A positive correlation between the number of skin tests to foods and PC1 was found. Similarly, a positive correlation between the number of positive skin prick tests to aeroallergens and PC1 was observed (Table III). No association between number of positive skin tests and PC1 expression was observed if the data were analyzed separately for the AD FA+ and AD FA− groups (data not shown).

### Differential expression of PC1 proteins among subject in AD FA+, AD FA−, and NA groups

The expression of each of the 45 PC1 proteins was compared across the 3 study groups and a summary of results is included in Fig 3.

All 45 PC1 proteins identified in STS analysis were differentially expressed in subjects with AD FA+ when compared with the NA controls; specifically, the expression of 36 proteins was upregulated and 9 proteins were downregulated in AD FA+ STS protein extracts. Of these 36 upregulated proteins in AD FA+ STSs, 27 were also significantly increased, when comparing the STS protein extracts from AD FA+ to those from AD FA− STSs (see Fig E1 in the Online Repository at www.jacionline.org). Of the 9 downregulated proteins in the comparison of the AD FA+ versus the NA STSs (see Fig E2 in the Online Repository at www.jacionline.org), the expression of 4 proteins (N-acylsphingosine aminohydrolase 1, acid ceramidase [ASAH1],...
The expression of several keratins was found to be selectively altered only in STS samples from subjects with AD FA+ An increase in the levels of KRT16, a keratin associated with epidermal proliferation, was observed in AD FA+ STSs as compared to AD FA− and NA STSs (Fig E1). KRT17, another keratin reported to be induced by inflammatory cytokines or in response to wound healing and oxidative or UV stress, was found increased in AD FA+ only (Fig E2). At the same time, the cornified envelop of AD FA+ skin was enriched in keratins that are normally found in the basal layer of epidermis, that is, KRT5 and KRT14 (Fig E1), suggesting that the keratinocyte layers are not fully differentiated in AD FA+ skin. The expression of keratins that are part of epidermal maturation and differentiation was decreased in AD FA+ skin (KRT10, KRT78) (Fig E2).

In contrast, the expression of KRT77 was equally decreased in AD FA+ and AD FA− STS as compared to NA STS samples (Fig E3). KRT77 has been reported to be weakly expressed by supra-basal cells in human epidermis and is highly expressed by the epithelial cells of sweat gland ducts. Rearrangement of keratins in AD FA+ skin SC and to some extent in AD FA− skin SC may result in tissue fragility and reduced mechanical stress resilience of the skin.

Concomitantly, a significant increase in expression of S100 calcium binding protein A family proteins (S100A7, S100A8, S100A9) in AD FA+ STSs was observed (Fig E1). An increase in alarmins, such as heat shock protein family B (small) member 1

Functional analysis of proteins in PC1 cluster

Functional analysis of the PC1 proteins identified in STS proteomic analysis revealed 3 major functional groups of proteins: (1) a group of keratin-intermediate filaments; (2) proteins associated with inflammatory response (S100 proteins, alarmins, protease inhibitors); (3) glycolysis and oxidative stress response proteins (glycolytic enzymes, oxidative stress response enzymes) (Fig 4). The expression of these proteins was altered in non-lesional skin STS samples from patients with AD FA+.

In the stratified epithelia, keratins are expressed in a specific pattern tightly regulated by the differentiation program of the tissue. The expression of several keratins was found to be selectively altered only in STS samples from subjects with AD FA+ An increase in the levels of KRT16, a keratin associated with epidermal proliferation, was observed in AD FA+ STSs as compared to AD FA− and NA STSs (Fig E1). KRT17, another keratin reported to be induced by inflammatory cytokines or in response to wound healing and oxidative or UV stress, was found increased in AD FA+ only (Fig E2). At the same time, the cornified envelop of AD FA+ skin was enriched in keratins that are normally found in the basal layer of epidermis, that is, KRT5 and KRT14 (Fig E1), suggesting that the keratinocyte layers are not fully differentiated in AD FA+ skin. The expression of keratins that are part of epidermal maturation and differentiation was decreased in AD FA+ skin (KRT10, KRT78) (Fig E2).

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**TABLE II.** Total serum IgE, food-specific IgE, and aeroallergen IgE correlations with PC1 proteins among children with AD FA+ and children with AD FA− (n = 40)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Correlation (95% CI) with PC1</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgE log10 (kU/L)</td>
<td>0.35 (0.04 to 0.59)</td>
<td>.028</td>
</tr>
<tr>
<td>IgE a-peanut log10 (kUA/L)</td>
<td>0.33 (0.02 to 0.58)</td>
<td>.037</td>
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<tr>
<td>IgE a-milk log10 (kUA/L)</td>
<td>0.29 (−0.02 to 0.55)</td>
<td>.068</td>
</tr>
<tr>
<td>IgE a-egg log10 (kUA/L)</td>
<td>0.23 (−0.09 to 0.50)</td>
<td>.20</td>
</tr>
<tr>
<td>Phadiatop (PAU/L)*</td>
<td>0.32 (0.01 to 0.57)</td>
<td>.047</td>
</tr>
</tbody>
</table>

*Phadiatop IgE analysis was restricted to aeroallergens.

**TABLE III.** Skin test associations with PC1 proteins among children with AD FA+, children with AD FA−, and NA children (n = 62)

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.</th>
<th>Correlation (95% CI) with PC1</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive skin tests to allergens</td>
<td>20</td>
<td>0.54 (0.33−0.69)</td>
<td>.001</td>
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<tr>
<td>Positive skin tests to foods</td>
<td>12</td>
<td>0.48 (0.26−0.65)</td>
<td>.001</td>
</tr>
<tr>
<td>Positive skin tests to aeroallergens</td>
<td>8</td>
<td>0.52 (0.31−0.68)</td>
<td>.001</td>
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</tbody>
</table>

**FIG 2.** The associations of skin TEWL with STS proteomic PC1. The associations for the TEWL AUC (A) and TEWL 15 (TEWL measurement in the skin after 15 STSs) (B) are shown. In this and all other figures with correlation analysis, correlation coefficients with 95% CIs and associated P values are shown in the upper part of the graphs.

**TABLE II.** Total serum IgE, food-specific IgE, and aeroallergen IgE correlations with PC1 proteins among children with AD FA+ and children with AD FA− (n = 40)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Correlation (95% CI) with PC1</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgE log10 (kU/L)</td>
<td>0.35 (0.04 to 0.59)</td>
<td>.028</td>
</tr>
<tr>
<td>IgE a-peanut log10 (kUA/L)</td>
<td>0.33 (0.02 to 0.58)</td>
<td>.037</td>
</tr>
<tr>
<td>IgE a-milk log10 (kUA/L)</td>
<td>0.29 (−0.02 to 0.55)</td>
<td>.068</td>
</tr>
<tr>
<td>IgE a-egg log10 (kUA/L)</td>
<td>0.23 (−0.09 to 0.50)</td>
<td>.20</td>
</tr>
<tr>
<td>Phadiatop (PAU/L)*</td>
<td>0.32 (0.01 to 0.57)</td>
<td>.047</td>
</tr>
</tbody>
</table>

*Phadiatop IgE analysis was restricted to aeroallergens.
intracellular protease inhibitor.34,35
surfaces and, unlike other protease inhibitors, functions as an proteinases (CSTA). SERPINB12 is abundant at epithelial
ases (SERPINB3), trypsin, plasmin (SERPINB12), and thiol

taining the activity of skin proteases, such as papain-like prote-
B member 3 (SERPINB3) in AD FA
(FIG 5,E1) and a decrease in catalase (CAT) (as compared to NA STSs only) were found in AD FA+ STSs, support-
ing a demand on antioxidant defense in the AD FA+ skin with perturbed barrier (FIG E1 and E2).

In summary, among the STS PC1 proteins, the expression of keratins, proteases, inflammatory mediators, alarmins, glycolytic enzymes, and antioxidant defense proteins was found to be selectively altered in AD FA+ STS samples in comparison to AD FA− and NA STS samples. The expression of 4 proteins (KRT77, protease inhibitors SERPINB12, CSTA, and acid ceramidase ASAH1) was significantly decreased in nonlesional skin STS samples from all patients with AD, irrespective of FA.

**Contribution of individual PC1 proteins to skin TEWL**

Importantly, all of the 45 proteins that make up PC1 had a significant correlation with TEWL AUC (see Table E1 in the Online Repository at www.jacionline.org). SERPINB3 expression in the skin had the highest positive correlation with TEWL AUC, while KRT10 expression in skin samples had the highest negative correlation with TEWL AUC in our data set (Table E1).

Using a statistical method for the determination of the relative importance of individual PC1 proteins to skin TEWL, we were able to quantify the independent effect of each of the 45 PC1 proteins on skin TEWL AUC. The LASSO regularized regression model selected 9 of the 45 proteins that were highly predictive for TEWL AUC (in decreasing order of the absolute penalized regression coefficient): SERPINB3, gelsolin (GSN), KRT77, tubulin alpha 1b (TUBA1B), ALDOA, histone H2B type 1-K (HIST1H2BK), KRT16, S100A8, premature ovarian failure protein 1B (POF1B) (FIG 5, A). Selected proteins had the largest contribution to \( R^2 \) in independently explaining TEWL AUC as an outcome variable, that is, selected proteins were weighted equally (unrelated to the order or position of the predictor in the model statement) and were found to be independent from other proteins selected by the model, suggesting that these proteins out of the 45 proteins analyzed are the top representatives of several independent pathways/processes that contribute to skin TEWL. SERPINB3 (FIG 5, B), GSN (FIG 5, C), and KRT77 (FIG 5, D) were also identified as top 2 positive and 1 negative correlates of skin TEWL AUC.

We have previously determined a significant inverse correlation between the levels of FLG breakdown products pyroglutamic acid (PCA) and urocanic acid (UCA) in nonlesional skin STSs of this study cohort and skin TEWL.17 SERPINB3 and GSN had a significant inverse correlation with the levels of PCA (see FIG E4, A and B, in the Online Repository at www.jacionline.org) and UCA (FIG E4, D and E) in nonlesional skin of the study subjects, while KRT77 had a significant positive correlation with PCA and UCA levels in the nonlesional skin (FIG E4, C and F).

Microbial dysbiosis and *Staphylococcus aureus* colonization have been implicated in regulation of skin barrier function regulation in AD skin.18 Skin microbiome analysis of this study cohort has been recently reported.17 We, therefore, examined the relationship between *S. hominis* and *S. aureus* expression in the skin of the study subjects and PC1 protein expression in STS

**FIG 3.** Differential expression of the 45 PC1 proteins in STS samples from children with AD FA+, children with AD FA−, and NA children. The large circle illustrates the number of proteins that were differentially expressed between AD FA+ and NA subjects. Circles of smaller diameter reflect the number of proteins that were differentially expressed between subjects with AD FA+ and those with AD FA− (medium dark gray circle) and between subjects with AD FA− and NA subjects (small light gray circle). Four proteins were decreased in all subjects with AD in comparison to NA subjects.

<table>
<thead>
<tr>
<th></th>
<th>AD FA+ vs NA</th>
<th>AD FA+ vs AD FA−</th>
<th>AD FA− vs NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ Up-Regulated</td>
<td>36 (9+27+0)</td>
<td>27 (27+0+0)</td>
<td>0 (0+0+0)</td>
</tr>
<tr>
<td>↓ Down-Regulated</td>
<td>9 (5+0+4)</td>
<td>0 (0+0+0)</td>
<td>4 (4+0+0)</td>
</tr>
<tr>
<td>↔ Not Significant</td>
<td>0</td>
<td>18</td>
<td>41</td>
</tr>
<tr>
<td>↑ Total</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
</tbody>
</table>

(HSPB1), galectins (eg, LGALS3) was found in AD FA+ STSs
(Fig E1). The increase in these proteins, in general, reflects
ongoing inflammatory response in the skin. Importantly, the
expression of protease inhibitors was altered in AD FA+ and AD FA− STSs, with increased serine protease inhibitor glade B member 3 (SERPINB3) in AD FA+ STSs only and decreased SERPINB12 and CSTA in both AD FA+ and AD FA− STSs (Figs E1 and E3). These protease inhibitors are critical in main-
taining the activity of skin proteases, such as papain-like prote-
ases (SERPINB3), trypsin, plasmin (SERPINB12), and thiol
proteinases (CSTA). SERPINB12 is abundant at epithelial
surfaces and, unlike other protease inhibitors, functions as an
intracellular protease inhibitor.34,35

Increased levels of glycolytic enzymes were found in STSs of patients with AD FA+, including enolase 1 (ENO1), lactate dehy-
drogenase (LDHA) (as compared to both AD FA− and NA STSs
(Fig E1)), aldolase, fructose-bisphosphate A (ALDOA), and
pyruvate kinase M1/2 (PKM) (as compared to NA STSs only
(Fig E2)), suggesting an increased energy requirement in AD
FA+ STSs. Lastly, a significant increase in glutathione S-trans-
ferase Pi 1 (GSTP1) (as compared to AD FA− and NA STSs), per-
oxiredoxin 1 (PRDX1), and a decrease in catalase (CAT) (as
defined as nonlesional skin only) were found in AD FA+ STSs, sup-
porting a demand on antioxidant defense in the AD FA+ skin with perturbed barrier (Figs E1 and E2).
A significant positive correlation between *S. aureus* skin levels and PC1 protein expression was found (Fig E5, B).

**Validation of nonlesional STS PC1 in an independent patient cohort**

Our data suggest that despite the normal appearance of AD nonlesional skin in the pediatric cohort, structural and proinflammatory changes were already present in the skin of these patients. In particular, the most significant changes in STS protein composition were observed in AD FA*1* STS samples.

In the next set of experiments, we examined whether the identified differences in PC1 protein expression in nonlesional skin STS proteomic analysis could be confirmed in an independent cohort of patients with AD. We studied the nonlesional STS proteome from a separate cohort of adult patients with AD enrolled in the Atopic Dermatitis Research Network. STS samples were collected from nonlesional skin of 28 adults with AD and 13 NA controls (see Table IV for study subjects’ characteristics). STS proteomic analysis was done and the expression of 45 PC1 proteins previously identified in the pediatric cohort STS analysis was analyzed from these samples. In this cohort, the clinical history of allergic reactions to peanuts was collected, and we were able to subgroup AD patients into AD FA*+* or AD FA*−* based on the clinical history of allergic reactions to peanuts. Using these definitions, we have found that the PC1 proteins previously identified in the pediatric cohort were also differentially expressed in nonlesional skin STS samples from adults with AD with a history of clinical reactions to peanuts versus adults with AD and no history of peanut allergy versus NA controls. Importantly, the cumulative expression of the previously identified PC1 proteins was the highest in adults with AD and peanut allergy and significantly different from adults with AD without peanut allergy and NA controls (Fig 6, A). As in the pediatric cohort, the expression of STS PC1 proteins was intermediate in adults with AD without peanut allergy and was the lowest in NA adults (Fig 6, A).

Separately, serum analysis for total IgE to peanuts was done and adult study participants were subgrouped into AD patients with peanut allergy or AD patients with no peanut allergy using 2 kUA/L (ie, kilo allergy units/L) of total serum IgE to peanut as a cutoff. Again, the expression of STS PC1 proteins in adult patients with AD with serum IgE levels to peanut above 2 kUA/L was significantly higher than in adult patients with AD with serum IgE to peanut below this cutoff (Fig 6, B). Lastly, a positive correlation between total serum IgE levels to peanut and STS PC1 expression in this adult independent cohort was found (Fig 6, C).

**DISCUSSION**

In this study, STS proteomic analysis identified abnormalities in skin barrier function of children with AD and adults with AD with and without FA that can be noninvasively assessed using
STSs. We report several novel findings. First, in this study we took a novel bioinformatics approach to STS proteomic data analysis, and instead of using a predetermined list of skin biomarkers, we applied an unbiased principal component analysis to the list of 150 proteins that was found to be expressed by all of the nonlesional skin samples. Through this approach we determined a group of 45 proteins, PC1, that depicts major differences among the 3 study groups — NA controls, subjects with AD FA−, and subjects with AD FA+ (30% of the variance explained). Importantly, the expression of this group of proteins in the skin samples

![Image](image.png)

**FIG 6.** Summary of the top PC1 proteins with the greatest contribution to skin TEWL. (A) Top 9 PC1 proteins identified by the classification model (LASSO generalized linear model, with 5-fold cross-validation) based on their contribution to skin TEWL. A univariate correlation analysis between SERPINB3 (B), GSN (C), and KRT77 (D) expression in STS samples and skin TEWL in the study cohort.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AD FA+</th>
<th>AD FA−</th>
<th>NA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y.)</td>
<td>33.0 ± 9.5</td>
<td>36.9 ± 11.9</td>
<td>41.0 ± 8.4</td>
<td>.16</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9 (75.0)</td>
<td>11 (61.1)</td>
<td>10 (76.9)</td>
<td>.57</td>
</tr>
<tr>
<td>Male</td>
<td>3 (25.0)</td>
<td>7 (39.9)</td>
<td>3 (24.1)</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>10 (83.3)</td>
<td>16 (88.9)</td>
<td>10 (76.9)</td>
<td>.67</td>
</tr>
<tr>
<td>Black or African American</td>
<td>2 (16.6)</td>
<td>2 (11.1)</td>
<td>3 (24.1)</td>
<td></td>
</tr>
<tr>
<td>EASI score</td>
<td>17.3 ± 10.8</td>
<td>16.4 ± 13.9</td>
<td>16.4 ± 13.9</td>
<td>.85</td>
</tr>
<tr>
<td>Total serum IgE (kU/L)</td>
<td>1368 ± 1332</td>
<td>2475 ± 3733</td>
<td>35.6 ± 40.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Total IgE to peanut (kUA/L)</td>
<td>9.4 ± 14.9</td>
<td>4.4 ± 16.4</td>
<td>0.11 ± 0.03</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

EASI, Eczema Area and Severity Index.

Values are mean ± SD or n (%).

*Patients were defined as AD FA+ based on the history of clinical reactions to peanut.
was found to be a significant positive correlate of skin TEWL in these patients. Second, the proteins that make up PC1 provide novel insights into biology of AD and FA. All of the proteins that make up PC1 were found to significantly correlate with skin TEWL. Through a multivariant analysis, we determined the top 9 contributors to TEWL, with SERPINB3, GSN, and KRT77 identified to have the greatest impact on skin TEWL. Third, according to the data presented by our group recently, TEWL alone was not a sufficient parameter to differentiate non-lesional skin of patients with AD FA from NA controls. In this article, we observed that skin expression of PC1 proteins can distinguish nonlesional skin samples among all 3 study groups. Lastly and worth noting, the findings of the study were confirmed and validated in an independent cohort of adults with AD with the history of clinical reactions to peanut. The expression of PC1 proteins was the highest in adults with AD with the positive total IgE to peanut defined by the cutoff above 2 kUA/L. A significant positive correlation between total serum IgE levels to peanut and STS PC1 expression was observed.

Collected data suggest that TEWL abnormalities in the skin stem from disorganization in expression of skin KRT intermediate filaments with increased expression of KRTs associated with epidermal proliferation, wound healing, and oxidative stress responses (KRT16, KRT17), increased detection of immature keratins that normally are associated with basal skin layer (KRT4, KRT15), with concomitant decrease in keratins that are part of epidermal maturation and differentiation (KRT10, KRT78). Keratin intermediate filaments protect the epidermis against mechanical force, support strong adhesion, help barrier formation, and regulate growth. Mice lacking all type I or type II keratins display severe barrier defects and fragile skin, leading to perinatal mortality, if fully penetrant. Comparative proteomics of cornified envelopes from these mice collected prenatally demonstrates that absence of keratin intermediate filaments causes dysregulation of many cornified envelop constituents, with some of these features also observed in our proteomic analysis, including the increase in desmosomal proteins desmoplakin (DSP) and junction plakoglobin (JUP), deregulated expression of protease inhibitors (SERPINs), increased expression of epidermal alarmins (annexin A2 [ANXA2], HSPB1, S100 proteins, galec
tins), and activation of targets that are involved in antioxidant defense in the skin, such as small proline rich protein 2D (SPRR2D). Of note, the absence of keratin intermediate filaments puts excessive stress on mitochondria to supply energy for increased mechanical stress resistance and cell proliferation. Notably, in our study among PC1 proteins, an increased expression of glycolysis enzymes (ALDOA, ENO1, PKM, LDHA) and deregulated expression of antioxidant defense proteins (GSTP1, CAT, PRDX1) was found. We propose that observed changes in expression of glycolytic enzymes, antioxidant defense proteins, proteases, and inflammatory mediators in AD STS samples is likely a compensatory mechanism to counterbalance initial changes and disorganization of keratin intermediate filaments under the influence of type 2 cytokine environment in AD skin.
We report here that all of the 45 proteins that make up PC1 had a significant correlation with TEWL. SERPINB3 expression in the skin had the highest positive correlation with TEWL, while KRT10 expression in skin samples had the highest negative correlation with TEWL in our data set. The expression of both proteins has been previously shown to be regulated by IL-4/IL-13, as SERPINB3 has been found to be directly induced by IL-4/IL-13 in bronchial epithelial cells, while KRT10 was reported to be inhibited by IL-4 in keratinocytes, suggesting the involvement of type 2 inflammation in AD skin in regulation of skin barrier function. In addition, a multivariable analysis identified 9 proteins expressed in PC1 as TEWL predictors, with SERPINB3, GSN, and KRT77 identified as top independent predictors of TEWL. The data acquired suggests that type 2 cytokines are only one of several potential pathways involved in regulation of skin TEWL. We propose that STS protein analysis can provide broad knowledge about biochemical pathways involved in TEWL and skin barrier, including (but not limited to) an alternative and independent, minimally invasive skin targeted approach for the readout of type 2 inflammation in AD skin.

Serine proteases are critical for epidermal barrier homeostasis, and their aberrant expression and/or activity is associated with chronic skin diseases. Elevated levels of the serine protease inhibitors SERPINB3 and SERPINB4 have been found in AD skin. In an experimental mouse model, allergen exposure induced SerpinB3 expression in the skin, along with increased TEWL, epidermal thickness, and skin inflammation, all of which were attenuated in the absence of SerpinB3. Attenuated TEWL correlated with decreased expression of the proinflammatory marker S100A8. RNA-sequencing analysis following allergen exposure identified a network of proinflammatory genes induced in wild-type mice that was absent in SerpinB3-null mice, suggesting that SerpinB3 contributes to early inflammatory responses in the skin following allergen exposure, disrupting the skin barrier. The involvement of other factors, including microbes, environmental pollutants, detergents, and such, in regulation of SERPINB3 expression in the skin is unexplored.

KRT10 deficiency was previously shown to result in profound changes in permeability barrier function. Baseline TEWL in skin with normal appearance of newborn homozygous Krt10-deficient mice was increased 8-fold compared with that of wild-type controls. Adult heterozygotes exhibited delayed barrier repair after experimental barrier disruption. SC hydration was reduced in homozygous and heterozygous mice. Skin fragility in Krt10 knockout mice is suggested to be a consequence of 2 complementing mechanisms, namely, a decrease of normal Krt1/Krt10 filaments and an increase in Krt6/Krt16 with a poor filament-forming capacity. Krt10 knockout mice displayed hyperproliferation of basal keratinocytes, with the induction of c-Myc, cyclin D1, and 14-3-3σ (stratifin [Sfn]). The study suggested a direct involvement of KRT10 in cell cycle control. Of note, in our study KRT16 and SFN were also identified as PC1 proteins, were significantly increased in AD skin, and had a significant positive correlation to TEWL.

Of interest, KRT77 is highly expressed in luminal duct cells of eccrine sweat glands in the skin, including intraepidermal duct region, but is only weakly expressed by suprabasal epithelia. An impaired sweating ability is observed in patients with AD. The mechanisms of decreased sweat production in AD include obstruction of sweat pores by keratin plugs, sweat production and secretion abnormalities from sweat glands, and sweat leakage into surrounding tissues. Decreased sweating exacerbates the symptoms of dermatitis, as it prolongs heat retention, skin dryness, and increased susceptibility to infection. The decreased levels of KRT77 in STS of patients with AD identified in our study and significant negative correlation with TEWL may suggest previously unrecognized decreased eccrine sweat glands density in AD skin. Of interest, reduced sweat production, sweat glands obstruction, and abnormalities in sweat gland morphology have been found in the footpads of Flg mutant mice as compared to wild-type mice, also suggesting a potential regulation of sweat glands by Flg. Our data demonstrate a significant positive correlation between KRT77 levels in STS and levels of FLG in the same skin site as determined by FLG breakdown products PCA and UCA.

The role of GSN in TEWL regulation has not been previously reported. GSN acts as a key regulator of actin filament assembly and disassembly, which binds to the ends of actin filaments, and prevents monomer exchange (end-blocking or capping). Moreover, GSN has also been suggested to exert multifunctional roles inside the cell, functioning as a transcription or apoptosis regulator. GSN and other actin-associated proteins are reported to be less abundant in suprabasal cells than in basal cells in the epidermis. On the contrary, increased GSN expression in AD STS was found in our study, suggesting abnormal epidermal differentiation in AD skin. Loss of integrins occurs during keratinocyte differentiation and changes in actin cytoskeleton through the action of cytoplasmic proteins that control actin assembly have been proposed to be involved in signaling between extracellular matrix–bound integrins and cell nuclei. Further studies are required to examine the role of altered GSN-regulated actin assembly/integrin signal transduction in AD skin in epidermal differentiation and skin barrier homeostasis.

We also determined a positive correlation between S. aureus expression in the skin of the study subjects and PC1 protein expression in STS. The data suggests that S. aureus can be a contributing factor involved in the regulation of the PC1 proteins expression in the skin. It is possible that observed association is a consequence of type 2 inflammation in AD skin, which supports microbial dysbiosis and S. aureus colonization. The direct role of S. aureus in AD development remains controversial. TEWL as a research tool enables noninvasive measurement of skin barrier function. TEWL elevation may precede clinical manifestation of eczema, suggesting that TEWL measurement may be useful in guiding AD prevention strategies. Recent studies have demonstrated that TEWL measured during the first days of life can predict the development of AD in infancy, independent of Flg status. These findings suggest that TEWL could potentially be used to identify neonates at increased risk of AD and help guide prevention strategies. However, the variability and insensitivity of TEWL measurements contributes to the lack of precision for individual subjects. Our data propose that STS proteomic analysis can also provide a minimally invasive assessment of the skin in critical disease groups and can provide insights into the structural and inflammatory changes that are ongoing in atopic skin.

Importantly, we were able to validate the findings of a unique proteomic profile of pediatric patients with AD with FA in an independent cohort of adults with AD with the history of clinical reactions to peanut and positive total serum IgE levels to peanut. As part of patients’ registry, the data on clinical history of allergic reactions to foods, including peanuts was gathered and serum IgE
levels to peanut, egg, and milk were collected. STS PC1 protein expression in this cohort was examined in 2 ways, first, by subgrouping patients based on prior history of clinical reactions to peanuts and, second, based on total serum IgE levels to peanut. Both strategies for stratification of patients with AD determined that skin expression of PC1 proteins previously identified in children with AD and FA to peanut was also significantly increased in adults with AD with allergic sensitization to peanuts. This is an important finding, as it suggests that patients with AD with FA are a unique endotype as a unique proteomic profile of this group can be seen in STS proteomic analysis and it is similar in children and adults with AD and FA. We have previously shown that alterations in lipids, gene expression, and skin protein levels in nonlesional skin, detected by STS, individually distinguish patients with AD FA+ from those with AD FA−. The comprehensive STS proteomic analysis conducted in this study provides further evidence that nonlesional skin distinguishes AD FA+ as a unique phenotype.

Identification of patients who are prone to development of AD, FA, and environmental allergy prior to clinical manifestation of these diseases in early childhood is of tremendous value as this is the group that is at high risk to undergo the atopic march. Early prognosis for patients at risk may direct these patients for specific treatment strategies and early intervention. In the current study, all nonlesional proteomic skin analyses were done in patients with existing AD, and, therefore, the validation of this established set of proteins for TEWL assessment and AD and FA prediction in subjects prior to the establishment of clinical features of AD is warranted in future birth cohort studies.

In conclusion, in this study we identified STS PC1 proteins that demonstrated a significant positive correlation with TEWL and allergic sensitization, suggesting that skin expression of these proteins is involved in the determination of skin structures that may be associated with skin barrier function. We propose that altered expression of these skin proteins may support penetration of allergens through a damaged barrier and also may support consequent type 2 inflammatory response that ensures after penetration, suggesting potential new targets for future early interventions to prevent AD and FA.

The authors would like to thank the nursing staff (Susan Leung, Caroline Bronchick, Shirley Palombi) and coordinators (Marco Ramirez-Gama and Shannon Garcia) of the Clinical Translational Research Center at National Jewish Health for their work on patient recruitment, patient clinical characterization, and STS sample collection. The authors also thank Ms Brittany Richers for her assistance with the preparation of STS protein extracts for LC-MS analysis.

Clinical implications: Skin tape proteomic analysis identified abnormalities in barrier function of AD in children and adults with and without FA that can be non invasively assessed using STSs.

REFERENCES