An Integrated Model of Atopic Dermatitis Biomarkers Highlights the Systemic Nature of the Disease

Benjamin Ungar¹,², Sandra Garcer², Juana Gonzalez², Nikhil Dhingra¹,², Joel Correa da Rosa², Avner Shemer³, James G. Krueger², Mayte Suarez-Farinas¹,²,⁴,⁵,⁶ and Emma Guttman-Yassky¹,²,⁷

Current atopic dermatitis (AD) models link epidermal abnormalities in lesional skin to cytokine activation. However, there is evolving evidence of systemic immune activation and detectable abnormalities in nonlesional skin. Because some of the best single correlations with severity (Scoring of AD, or SCORAD) are detected not only in lesional but also nonlesional skin and blood, more complex biomarker models of AD are needed. We thus performed extensive biomarker measures in these compartments using univariate and multivariate approaches to correlate disease biomarkers with SCORAD and with a combined hyperplasia score (thickness and keratin 16 (K16) mRNA) at baseline and after cyclosporine A treatment in 25 moderate to severe AD patients. Increases in serum cytokines and chemokines (IL-13, IL-22, CCL17) were found in AD versus healthy individuals and were reduced with treatment. SCORAD correlated with immune (IL-13, IL-22) and epidermal (thickness, K16) measures in lesional and, even more strongly, in nonlesional AD. Serum cytokines also had higher correlations with nonlesional markers at baseline and with treatment. Multivariate U statistics improved baseline and treatment-response SCORAD correlations. Nonlesional models showed the strongest correlations, with further improvement upon integration of serum markers. Even better correlations were obtained between biomarkers and the hyperplasia score. Larger cohorts are needed to confirm these preliminary data.

Journal of Investigative Dermatology (2017) 137, 603–613; doi:10.1016/j.jid.2016.09.037

INTRODUCTION

Atopic dermatitis (AD) is the most common inflammatory skin disease (Leung and Guttman-Yassky, 2014), with a prevalence of 3–7% (Eichenfield et al., 2014; Hanifin et al., 2007; Margolis et al., 2014; Silverberg and Hanifin, 2013). Since emerging as immune-driven (Jensen et al., 2009, 2012; Khattri et al., 2014; Tintle et al., 2011), AD has experienced a translational revolution, with testing of targeted therapeutics (Noda et al., 2015a). Evidence suggests that several AD variants (intrinsic vs. extrinsic, Asian vs. European American, adult vs. pediatric) might have differences in polar cytokine activation (Gittler et al., 2012; Noda et al., 2015b; Suarez-Farinas et al., 2013). Consequently, specific therapeutics

¹Department of Dermatology, Icahn School of Medicine at Mount Sinai, New York, New York, USA; ²Laboratory for Investigative Dermatology, The Rockefeller University, New York, New York, USA; ³Department of Dermatology, Tel-Hashomer, Tel-Aviv, Israel; ⁴Department of Population Health Science and Policy, Icahn School of Medicine at Mount Sinai, New York, New York, USA; ⁵Department of Genetics and Genomics Science, Icahn School of Medicine at Mount Sinai, New York, New York, USA; ⁶Icahn Institute for Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, New York, New York, USA; and ⁷Department of Immunology, Icahn School of Medicine at Mount Sinai, New York, New York, USA

Correspondence: Emma Guttman-Yassky, Department of Dermatology, Icahn School of Medicine at Mount Sinai Medical Center, 5 East 98th Street, New York, New York 10029, USA. E-mail: Emma.Guttman@mountsinai.org

Abbreviations: AD, atopic dermatitis; CsA, cyclosporine A; K16, keratin 16; SCORAD, Scoring Atopic Dermatitis; Th, T helper

Received 11 May 2016; revised 25 September 2016; accepted 30 September 2016; accepted manuscript published online 4 November 2016; corrected proof published online 4 November 2016

© 2016 The Authors. Published by Elsevier, Inc. on behalf of the Society for Investigative Dermatology.
Identifying AD biomarkers is important for the following reasons. (i) Unlike in psoriasis (Langley et al., 2014; McInnes et al., 2015), it is difficult to quantify improvements in erythema and lichenification because of lack of demarcation between lesional and nonlesional areas, contributing to very high placebo responses in AD (up to 25%) (Hamilton et al., 2014) compared with only 3–5% in psoriasis (Lowes et al., 2008; Zaba et al., 2007). Another factor that may contribute to high placebo responses in AD is insufficient patient education and continuum of care during clinical trials. (ii) Tissue analysis has shown increased sensitivity at earlier time points compared with clinical responses (Hamilton et al., 2014). (iii) Identification of compartment-specific biomarkers in responders can ultimately lead to a precision medicine approach. (iv) Finally, through biomarker studies, we can uncover pathogenic pathways, as in psoriasis (Zaba et al., 2009). Additionally, maximizing disease models using serum biomarkers may be especially important for pediatric studies, where obtaining skin biopsy samples may be impossible.

We studied blood biomarkers before and after CsA treatment and integrated lesional, nonlesional, and serum AD biomarkers, using nonparametric multivariate modeling. We conducted an exhaustive approach that estimates multivariate associations between any combinations of biomarkers with disease outcomes. This allowed us to identify unbiased sets of biomarkers that reflect disease activity and therapeutic response with no distributional assumptions. We identified blood biomarkers associated with severity and treatment response, and our integrated model improved correlations with disease activity. Even better correlations than with clinical scores were obtained between skin biomarkers and a clinical score with disease activity. Even better correlations than with blood biomarkers associated with severity and treatment response, and our integrated model improved correlations with disease activity.

RESULTS

Study patients

Twenty-five moderate to severe AD patients were included (16 male, 9 female; age = 18–73 years; median age = 43.5 years; Scoring Atopic Dermatitis [SCORAD] score range = 44–97, mean score = 62.3; standard deviation = 13.0) (see Supplementary Table S1 online), some from a previously published study of CsA treatment for 12 weeks (Khattri et al., 2014). We studied pre- and posttreatment serum analytes versus published lesional and nonlesional skin biomarker data. We performed analyses of serum cytokines and chemokines including T helper (Th) type 1, Th2, and Th17/Th22 products (see Supplementary Table S2 online). Serum markers were measured using sensitive ELISA-based techniques (see Methods section). Extensive single and multivariate correlations were performed at baseline and after treatment.

Baseline AD serum biomarkers

Compared with controls, AD serum samples showed significantly increased levels of Th2-related (IL-13, CCL13, CCL17, CCL22, CCL2; all P < 0.05) and Th22 markers (IL-22, P < 0.001), with no significantly increased IL-17A (see Supplementary Figure S1 online) or Th1 (IFN-γ, CXCL10) markers (Figure 1a, and see Supplementary Table S2).

Although not significantly elevated overall, there was high variability, and many individuals had increased IFN-γ levels compared with control subjects (see Supplementary Figure S1). We also compared baseline extrinsic and intrinsic patient subsets and found that although one of the main Th2 chemokines, CCL26/eotaxin-3, was overall decreased in AD serum, it was higher in extrinsic versus intrinsic patients but did not reach significance (see Supplementary Figure S2 online).

Several markers that were increased at baseline in AD patients also showed significant or approaching significant correlations with disease severity (measured by SCORAD). These included Th2 (IL-13 [r = 0.53], CCL22 [r = 0.59], CCL13 [r = 0.40], CCL2 [r = 0.41]) and Th22 (IL-22 [r = 0.54]) markers (see Supplementary Table S3 online). Additionally, SCORAD significantly correlated with serum IgE and eosinophil levels (see Supplementary Table S3).

Serum therapeutic responses

Treatment with CsA, a broad immune modulator, led to significant decreases in multiple Th pathways including Th2 (IL-13, CCL13, CCL22, CCL26, CCL17, CCL2), Th22 (IL-22), and Th1 (IFN-γ, CXCL10) (see Supplementary Table S4 online). Among these, CCL13 (r = 0.48, P = 0.036) and CCL10 (r = 0.47, P = 0.049) also significantly correlated with SCORAD improvement with treatment, with additional markers approaching significance (CCL2 [r = 0.42], IL-13 [r = 0.41], CCL17 [r = 0.43]) (see Supplementary Table S5 online). Neither serum eosinophil nor IgE levels were correlated with SCORAD improvement.

Lesional and nonlesional AD biomarkers at baseline

Although predominantly considered a Th2-skewed disease, the highest univariate correlations with SCORAD at baseline were seen with Th17- (IL17A, P13, IL-22, CXCL1) and Th17/Th22-related (IL22, S100A7, S100A9) markers (see Supplementary Table S6 online). Terminal differentiation markers (PPL, FLG) (see Supplementary Table S6) showed inverse correlations with SCORAD.

Nonlesional markers generally showed higher and more significant correlations with SCORAD at baseline than lesional markers did including Th17 (CXCL1), Th22 (IL22), Th17/Th22 (S100A7, S100A9), inflammatory (MMP12), and proliferation (K16) markers (see Supplementary Table S7 online). Terminal differentiation markers (PPL, LOR, FLG) showed stronger inverse correlations with SCORAD than lesional skin did (see Supplementary Table S7).

Overall, serum and lesional data were largely not well correlated with each other, and only IL-22 showed significant serum/skin correlation (r = 0.66, P < 0.01) (Figure 2a, and see Supplementary Table S8a online). However, serum and nonlesional skin showed significant correlations for several markers, including IL-22 (r = 0.69, P < 0.01), IL-13 (r = 0.69, P < 0.01), CCL17 (r = 0.56, P = 0.016), and CXCL10 (r = 0.48, P = 0.04) (Figure 2b, and see Supplementary Table S8b). Of note, IL-22 expression was below detection in baseline nonlesional skin of 10 patients, but levels of serum IL-22 among patients with detectable nonlesional IL-22 at baseline were significantly higher than those with undetectable IL-22 (see Supplementary Figure S3 online).
Many nonlesional skin response biomarkers show greater correlations with clinical improvement than lesional measures

Both lesional and nonlesional biomarkers correlated with SCORAD improvement. Lesional response biomarkers included Th2 (IL13 [r = 0.65, P < 0.01], CCL18 [r = 0.42, P = 0.077]), Th22 (IL22 [r = 0.51, P = 0.027]), and cellular measures (CD206+ [marking inflammatory dendritic epidermal cells/IDECs]; r = 0.44, P = 0.063) (see Supplementary Table S9 online). Higher and more numerous correlations were seen between nonlesional markers (CCL18 [r = 0.73, P < 0.01], CCL13 [r = 0.68, P < 0.01]) and SCORAD improvement compared with lesional skin (see Supplementary Table S10 online).

Similar to baseline disease correlations between serum and skin, changes in serum markers correlated much better with changes in nonlesional markers than those with lesional skin, with no significant correlations detected between serum and lesional response biomarkers. In contrast, changes in CCL17 (r = 0.80, P < 0.001), CXCL10 (r = 0.63, P < 0.01), CCL22 (r = 0.55, P = 0.018), CCL2 (r = 0.55, P = 0.012), and IL-13 (r = 0.49, P = 0.037) all significantly correlated between serum and nonlesional skin with treatment (Figure 2c, and see Supplementary Table S8c).

Multivariate correlation models for AD at baseline

Some of the best single correlations with disease severity were identified not only with lesional but also with nonlesional expressions, possibly suggesting that an integrated model of disease severity is needed to reflect cytokine activation and corresponding epidermal responses. To evaluate which sets of biomarkers in lesional and nonlesional compartments correlate best with disease, we used multivariate mScores, performing a computationally intensive search that estimates the association of any combination of n biomarkers with disease severity and treatment response as measured by SCORAD. The best multivariate associations with disease outcomes (baseline disease activity and therapeutic response) are shown.

At baseline, when we considered lesional markers, most sets showing the highest correlations with SCORAD included Th17, Th22, and epidermal barrier markers...
Representative models are shown in Figure 3f. A few selected models are shown as scatterplots (Figure 3b). The multivariate approach improved correlations with disease activity compared with univariate analyses (i.e., \( r = 0.63, P < 0.001 \) vs. \( r = 0.48, P = 0.034 \) for PI3).

Compared with lesional markers, integrating nonlesional markers resulted in substantially greater correlations with baseline SCORAD, with a top correlation of \( r = 0.73 (P < 0.001) \) (Figure 3c, and see Supplementary Table S12 online; scatterplots are shown in Figure 3d). Top models included nonlesional CD11c+ and CD3+ cell counts, MMP12, IL13, IL22, additional Th2 (CCL18) and Th17/Th22 markers (S100A9, CXCL11, PI3), and epidermal thickness, with inverse correlation with PPL (see Supplementary Table S12).

Analyses that integrated both nonlesional and lesional biomarkers showed improved correlations versus lesional skin, with only a small improvement compared with those of nonlesional skin alone (i.e., \( r = 0.73, P < 0.001 \) in nonlesional vs. \( r = 0.76, P < 0.001 \) for combined lesional and nonlesional) (see Supplementary Tables S12 and S13 online). The top integrated lesional and nonlesional models included epidermal thickness, CD11c+ and CD3+ cell counts, IL13 and IL22 expressions in nonlesional skin, lesional expressions of CXCL11, and inverse expression of MX1 and PPL in lesional and nonlesional skin (see Supplementary Figure S4a and Supplementary Table S13 online). A few models are represented in Figure S4b.

Integrating serum with lesional and nonlesional skin markers provided the highest correlations with baseline SCORAD baseline \( (r = 0.81, P < 0.001 \) vs. \( r = 0.76, P < 0.001 \) for combined lesional and nonlesional) (see Supplementary Table S13 and S14 online). Top models included serum eosinophil, CCL11, and IgE levels; lesional PI3 and S100A12; and nonlesional PI3, IL13, CCL18, and IL22 (Figure 3e, and see Supplementary Table S14 online). Representative models are shown in Figure 3f.

**Multivariate models for AD therapeutic response biomarkers**

As seen in univariate correlations, markers associated with baseline disease severity are not necessarily those that reflect treatment response. We thus also examined multivariate models of response biomarkers after 12 weeks of treatment with CsA. For lesional skin, top sets of biomarkers with the best correlations included IL22, IL9, MMP12, CCL17, IL31 and markers of epidermal proliferation (thickness and S100A8/2) (see Supplementary Table S15 online), with selected models highlighted (Figure 4a and b). The set of biomarkers with the highest correlation showed \( r = 0.73 \) \((P = 0.001)\).

Similar to what was seen at baseline, correlations of SCORAD therapeutic response with multivariate nonlesional markers were substantially greater than for lesional markers \( (r = 0.91, P < 0.001 \) vs. \( r = 0.73, P = 0.001 \) for lesional) (see Supplementary Tables S15 and S16 online). Markers in top models included Th2 (CCL18, CCL17), Th9/IL9, Th17 (IL12B, CXCL1, S100S12), and innate immunity (IL8, IL1B) (see Supplementary Table S16; examples are given in Figure 4c and d).

When integrating lesional and nonlesional markers, we again obtained a correlation of \( r = 0.91 \) \((P < 0.001)\), highlighting the impact of nonlesional contributions to therapeutic response measured by SCORAD. Furthermore, most top models included predominantly nonlesional markers, with lesional IL22 seen in some (see Supplementary Figure S5a and b and Supplementary Table S17 online). Additionally, integrating serum with skin markers improved correlations to \( r > 0.94 \) \((P < 0.001)\) (see Supplementary Table S18 online). Lesional markers were absent from these models, emphasizing nonlesional and serum (IL-13, CCL17, CCL13, CCL22) markers (Figure 4e and f, and see Supplementary Table S18).

**Even higher correlations were found between biomarkers and epidermal hyperplasia**

There are multiple limitations in using clinical scoring for assessing disease severity and therapeutic responses. These limitations include blending of involved and uninvolved AD
Figure 3. μScore models of baseline biomarker correlations with SCORAD. Representative top multivariate correlation models of SCORAD with baseline biomarkers in (a) lesional (LS) skin with (b) associated scatterplots, (c) nonlesional (NL) skin (d) with associated scatterplots, and (e) LS + NL + serum (f) with associated scatterplots. Regression lines in blue, with gray smoothed confidence interval. SCORAD, Scoring of AD; val, value.
Figure 4. μScore models of therapeutic response biomarker correlations with SCORAD. Representative top multivariate correlation models of SCORAD with therapeutic response biomarkers in (a) lesional (LS) skin (b) with associated scatterplots, (c) nonlesional (NL) skin (d) with associated scatterplots, and (e) LS + NL + serum (f) with associated scatterplots. Regression lines in blue, with gray smoothed confidence intervals. SCORAD, Scoring of AD; val, value.
skin and lack of a single robust scoring system, ultimately leading to very high placebo responses in AD (Beck et al., 2014; Hamilton et al., 2014). Because epidermal hyperplasia (measured by thickness and K16) is a measure of disease activity, similar to psoriasis (Krueger et al., 2012), we also correlated baseline and response biomarkers (excluding the hyperplasia biomarker thickness and K16) with a combined score of K16 and thickness that we termed as “HistScore.”

Overall, higher correlations were obtained between the HistScore and both baseline and response biomarkers than with corresponding clinical scores. For lesional skin, correlations reached $r = 0.92$ ($p < 0.001$), and the best sets included IL17A and related markers (IL19, S100A8/9/12, P13) and inverse expression of FLG (see Supplementary Figure S6a and b and Supplementary Table S19 online). In contrast to SCORAD results, nonlesional models had lower correlations ($r = 0.71$, $p < 0.001$) than lesional models ($r = 0.92$, $p < 0.001$) (see Supplementary Figure S6c and d and Supplementary Tables S19 and S20 online). However, integrating lesional and nonlesional markers further improved correlations to $r = 0.94$ ($p < 0.001$) (see Supplementary Figure S6e and f and Supplementary Table S21 online). Integrating serum with skin provided incrementally increased correlations reaching $r = 0.95$ ($p < 0.001$) (Figure 5a and b, and see Supplementary Table S22).

For the same reasons that the histology score perhaps provides a better metric to reflect disease activity in tissues, we used the same approach to assess therapeutic response. Similar to baseline evaluations, much stronger correlations were obtained between histological response biomarkers and skin response biomarkers than those observed for SCORAD. Correlations between HistScore therapeutic response and lesional skin markers approached $r = 0.93$ ($p < 0.001$). These models included IL17-related markers (IL19, S100A8/12), proliferation (Ki67), dendritic cell (CD11c), TH2 markers (IL13, CCL17, CCL18) and inverse FLG (Figure 5c and d, and see Supplementary Table S23 online).

Similar to baseline observations, nonlesional markers provided lower correlations than lesional markers (see Supplementary Table S24 and Supplementary Figure S7a and b), but integrating nonlesional with lesional skin improved response correlations with HistScore to $r = 0.96$ ($p < 0.001$) (see Supplementary Figure S7c and d and Supplementary Table S25 online). However, unlike at baseline, further integrating serum markers did not improve correlations (Figure 5e and f, and see Supplementary Table S26 online). Overall, many markers that correlate with HistScore at baseline and with treatment response (i.e. IL22, IL19) are members of the IL-20 cytokine family (Sa et al., 2007) and related markers (S100A8s), along with terminal differentiation markers (FLG, LOR).

**DISCUSSION**

AD is complex, with increased activation of multiple, polar T-cell subsets in the skin (Leung and Guttman-Yassky, 2014) and alterations in circulating T-cell (Czarnowicki et al., 2015b, 2015c) and B-cell subsets (Czarnowicki et al., 2016). AD is characterized not only by cutaneous inflammation (Esaki et al., 2015; Gittler et al., 2012; Suarez-Farinas et al., 2011, 2015) but also by systemic immune activation with significant increases in circulating T-cell subsets and T-cell activation in both effector, skin-homing (CLA+) and central memory, noncutaneous (CLA-) T-cell populations. However, although circulating T cells bear activation markers, these cells are not actively producing polar cytokines, as determined by intracellular cytokine staining (Czarnowicki et al., 2016). The marked increase in polar cytokines measured AD patients’ serum in this study adds an additional dimension to assessing pathogenic immune activation, because it provides an integrated measure of immune activation that occurs in the skin and other tissues, such as lymph nodes, which would be the presumptive site for activation of central memory T cells. High levels of circulating inflammatory cytokines might in turn drive tissue inflammation outside of skin lesions, for example, having effects on tissues such as lungs (asthma), mucosal surfaces (allergy), and even on background nonlesional AD skin.

Although variable results have been obtained using skin, and particularly blood, biomarkers (Guttman-Yassky et al., 2008, 2009; Krause et al., 2016; Nomura et al., 2003; Quaranta et al., 2014; Suarez-Farinas et al., 2011; Wenzel et al., 2008), the independent correlations between the serum cytokines and disease activity as measured by SCORAD, and of skin biomarkers with disease activity, argue that both may be important in generating clinically relevant disease activity. Nevertheless, the lack of good correlation between serum biomarkers and lesional skin biomarkers also argues that the circulating cytokine profile reflects activation of T cells and other cells reported to produce cytokines, including eosinophils, basophils, macrophages, epithelial cells (Lacy and Stow, 2011), and cellular responses beyond the skin. Understanding immune pathways that are elevated outside of skin lesions is key to deriving a deeper understanding of the atopic march, as well as increasingly recognized comorbidities of AD such as an elevated cardiovascular risk (Silverberg, 2015; Silverberg and Hanifin, 2013; Zhang and Silverberg, 2015).

When pathogenic disease models are focused only on the formation of acute or chronic skin lesions (Gittler et al., 2012), activation responses of cutaneous dendritic cells and T cells, including increased local production of TH2, Th22, TH1, and Th17 cytokines, provides a reasonable explanation for resulting cytokine-induced changes in keratinocytes and other skin-resident cell types. This pathogenic model, where locally produced cytokines have “short-range” effects on keratinocytes, fits well with the extremely high correlations between the epidermal hyperplasia HistScore, representing tissue alterations, and underlying changes in immune-related biomarkers. The fact that correlations are higher for multiple versus single biomarkers also fits well with the current concept that different polar immune cytokines are required to induce the range of cutaneous alternations seen in chronic AD lesions (Leung and Guttman-Yassky, 2014; Malajian and Guttman-Yassky, 2015; Noda et al., 2015a).

Although considered primarily a Th2-driven disease, the large representation of TH17 markers in both univariate and multivariate models suggest that the Th17/IL-23 axis may be involved in AD pathogenesis, which is also suggested by some other data. Elevated IL-17 levels have been found in some AD subsets, including intrinsic (Suarez-Farinas et al.,...
**Biomarker correlations with HistScore**

**Baseline Disease Severity (LS + NL + serum biomarkers)**

<table>
<thead>
<tr>
<th>Set of markers</th>
<th>r</th>
<th>p-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL19[LS], IL22[NL], CCL26[serum], IL22[LS], -FLG[LS]</td>
<td>0.950</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL19[LS], IL22[NL], CCL26[serum], -FLG[LS], PI3[LS]</td>
<td>0.941</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL19[LS], IL22[NL], CCL26[serum], IL22[LS], PI3[LS]</td>
<td>0.940</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Therapeutic Response (LS biomarkers)**

<table>
<thead>
<tr>
<th>Set of markers</th>
<th>r</th>
<th>p-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>-FLG, S100A12, Ki67, CCL18, CCL17</td>
<td>0.927</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>-FLG, Ki67, S100A8, IL9, CD11c</td>
<td>0.924</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>-FLG, IL13, IL19</td>
<td>0.922</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Therapeutic Response (LS + NL + serum biomarkers)**

<table>
<thead>
<tr>
<th>Set of markers</th>
<th>r</th>
<th>p-val</th>
</tr>
</thead>
<tbody>
<tr>
<td>-FLG[LS], IL13[LS], -FLG[NL], IL17A[NL], IL19[LS]</td>
<td>0.959</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>-FLG[LS], IL13[LS], -FLG[NL], IL17A[NL]</td>
<td>0.954</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>-FLG[LS], IL13[LS], -FLG[NL]</td>
<td>0.947</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Figure 5.** \( \mu \text{Score} \) models of biomarker correlations with \( \text{HistScore} \). Representative top multivariate correlation models of \( \text{HistScore} \) with biomarkers in (a) lesional (LS) + nonlesional (NL) + serum at baseline (b) with associated scatterplots, (c) therapeutic response biomarkers in LS skin (d) with associated scatterplots, and (e) therapeutic response biomarkers in LS + NL + serum (f) with associated scatterplots. Regression lines in blue, with gray smoothed confidence intervals. val, value.
2013) and Asian AD (Noda et al., 2015b) patients. Increased IL-17 expression was also shown in acute AD skin lesions (Koga et al., 2008; Toda et al., 2003). Furthermore, an AD mouse model showed that IL-17 can induce Th2 responses (Nakajima et al., 2014). Additionally, Th2 antagonism in AD shows unexpectedly large suppression of IL-17/IL-23 pathway genes (Hamilton et al., 2014). Ongoing studies with IL-17 antagonists (clinicaltrials.gov:NCT02594098) will be able to shed light on contributions of IL-17 to disease pathogenesis.

The lesional skin disease model provides a tissue source for high-level production of cytokines and chemokines that could diffuse from skin lesions into blood and thus contribute to systemic cytokine abnormalities as measured in this study. However, past studies have also identified increases in mRNAs encoding polar cytokines in nonlesional AD skin compared with healthy control skin (Hamid et al., 1994; Leung et al., 2004; Suarez-Farinas et al., 2011). In the current study, expression levels of disease-associated biomarkers in nonlesional skin also correlated strongly with clinical disease activity (SCORAD), generally showing better correlations than lesional skin biomarkers (e.g., IL22). This surprising outcome potentially suggests that the cutaneous disease burden in AD patients may involve physiological alterations in both lesional and nonlesional skin. Indeed, multivariate models of nonlesional markers tended to have higher correlations than lesional markers. Integrating lesional with nonlesional, and even more so with serum, markers yielded the highest correlations, strongly suggesting a systemic component in AD that requires nonlesional and serum markers to measure more accurately.

The disease-related nonlesional biomarkers could arise either from increased local production of polar cytokines from skin-resident T cells, which are increased in nonlesional skin versus skin from healthy control subjects, or from exposure to high levels of immune cytokines in the blood, as has also been suggested for psoriasis vulgaris nonlesional skin (Chiricozzi et al., 2016; Davidovici et al., 2010). Because activation of systemic T cells and B cells has been recently shown to be much higher in AD than in psoriasis patients (Czarnowicki, 2015c, 2016) with similar disease severity, circulating cytokines may contribute to nonlesional AD skin being much more abnormal (and closer to lesional skin) than its psoriasis counterpart (Chiricozzi et al, 2016; Gudjonsson et al., 2009; Noda, 2015a; Suarez-Farinas, 2011).

Another important focus of this work has been to derive biomarkers that are strongly associated with therapy-induced disease improvements. These biomarkers potentially provide a means of assessing disease changes that are more objective and independent of SCORAD measures, particularly because this tool mainly quantifies features of skin lesions, but boundaries of AD lesions are often difficult to pinpoint, because they often blend with the surrounding skin (Leung, 2004, 2014; Noda, 2015a).

Our study also presents several limitations. The systemic treatment used in this report was CsA, a broad immune antagonist that might not be a mainstay of moderate to severe AD treatment in the near future due to emerging targeted therapeutics (Noda, 2015a). Thus, these findings need to be further validated in future studies with targeted treatments. Additionally, we did not include serum biomarkers in our integrated models because unlike AD skin data, in which immune biomarkers have been well established and a wide array of markers representative of all immune axes were included, the serum data are still preliminary and do not include a large representation of all possible pathways. We thus chose to avoid examining multivariate models of serum markers alone, only integrating them with lesional and nonlesional skin markers; future studies with larger panels of serum markers with skin are needed. Because of sample size, we did not have the power to delve more deeply into differences between serum profiles of extrinsic versus intrinsic AD patients. Furthermore, we could not develop any predictive modeling to address confounding clinical and demographic factors. A limitation of the nonparametric technique used to estimate the multivariate associations is that it does not provide a formula that can be directly used in other populations. Future studies should evaluate the utility of our biomarkers in various AD endotypes (Czarnowicki et al., 2015a; Malajian and Guttman-Yassky, 2015; Mansouri and Guttman-Yassky, 2015; Noda et al., 2015b; Suarez-Farinas et al., 2013), with different treatments, and in larger cohorts.

Integrated skin and blood biomarkers may prove helpful to stratify patients in a precision medicine approach to treatment. As one moves to more targeted therapeutic approaches, the ability to sequentially follow disease-associated immune pathways may help to better define the contribution of specific immune axes to different disease features across the spectrum of age ranges and disease phenotypes. Blood response biomarkers may be particularly important in pediatric AD (Czarnowicki et al., 2015a), where obtaining skin biopsy samples is challenging. Hence, the set of response biomarkers identified in this study would need to be tested in future studies in pediatric patients in the context of disease treatment with available agents. In sum, our study highlights the systemic abnormalities of AD, as seen in the nonlesional skin models, reflecting a high state of immune activation in the disease. Future studies with larger cohorts are needed to further explore these preliminary results.

MATERIALS AND METHODS

Patients and samples

A cohort of 25 patients with moderate to severe AD was included in this study, with lesional and nonlesional biopsies performed at baseline. Of the 25 patients, 19 completed 12 weeks of CsA treatment, with baseline and week 12 lesional and nonlesional biopsies. Lesional results from these 19 patients were previously published (Khattri et al., 2014). All biopsies were 6-mm punch biopsies, and patients gave written informed consent under an institutional review board-approved protocol. Lesional biopsy samples were taken from chronic lesions, largely from the extremities. Nonlesional samples were taken from previously uninvolved skin in proximity but at least 1 cm away from lesional samples. Serum samples were available at baseline for 20 of 25 patients and all 19 patients at week 12. Additionally, 25 healthy control subjects were included for evaluation of chemokines, eight of whom also had IFN-γ, IL-13, and IL-22
cytokine assessments. Baseline serum IL-17A levels were available for six AD patients and four healthy control subjects. Intrinsic AD was defined as IgE level less than 200 kU/L.

**Immunohistochemistry**

Immunohistochemistry was performed on cryostat sections using purified mouse anti-human monoclonal antibodies, as reported (Esaki et al., 2015; Khattri et al., 2014). See Supplementary Materials and Methods online for details.

**Real time-PCR**

Real time-PCR was performed on 32 preselected key AD-related genes as described (Khattri et al., 2014; Suarez-Farinas et al., 2015). Real time-PCR data were normalized to the housekeeping gene hARP and log2-transformed for analysis. The primers used are listed in Supplementary Table S27 online.

**Serum immunoassays**

Electrochemiluminescence immunoassay was used in a multiplex format to quantify concentrations of nine chemokines in each sample: CCL11/eotaxin, CCL26/eotaxin3, CXCL10/IP-10, CCL2/MCP-1, CCL13/MCP-4, CCL22/MDC, CCL3/MIP-1α, CCL4/MIP-1β, and CCL17/TARC. Because serum IFN-γ, IL-17A, and IL-22 cytokine levels are very low, we used Erenna (EMD Millipore, MO) immunoassays, which have a lower limit of quantification that is 10–100 times smaller than electrochemiluminescence. See Supplementary Materials and Methods for details.

**Statistical analyses**

For each model/data set (baseline lesional, baseline lesional + nonlesional, lesional therapeutic response, lesional + nonlesional therapeutic response), we used a multivariate regression analysis (Wittkowski et al., 2004). See Supplementary Materials and Methods for details.

For serum data, we used t test (paired t test for pre-/posttreatment comparison) on log2-transformed data. Univariate correlations were calculated using the Pearson correlation coefficient.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**ACKNOWLEDGMENTS**

BU, JGK, and MSF were supported by grant number SUL1RR024143-02 from the National Center for Research Resources, a component of the National Institutes of Health, and National Institutes of Health Roadmap for Medical Research.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.09.037.

**REFERENCES**


Silverberg JI. Association between adult atopic dermatitis, cardiovascular disease, and increased heart attacks in three population-based studies. Allergy 2015;70:1300–8.


