Received: 16 December 2020 | Revised: 14 April 2021 | Accepted: 29 April 2021

DOI: 10.1111/exd.14389

ORIGINAL ARTICLE

Experimental Dermatology WILEY

Insights into adult atopic dermatitis heterogeneity derived from circulating biomarker profiling in patients with moderate-to-severe disease

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Funding information
This study was funded by Eli Lilly and Company, under license from Incyte Corporation. The funder of the study had a role in study design, data collection, data analysis, data interpretation, writing of the report and the decision to submit the article for publication.

Abstract

Atopic dermatitis (AD) is a heterogeneous systemic inflammatory skin disease associated with dysregulated immune responses, barrier dysfunction and activated sensory nerves. To characterize circulating inflammatory profiles and underlying systemic disease heterogeneity within AD patients, blood samples from adult patients (N = 123) with moderate-to-severe AD in a phase 2 study of baricitinib (JAHG) were analysed. Baseline levels of 131 markers were evaluated using high-throughput and ultrasensitive proteomic platforms, patient clusters were generated based on these peripheral markers. We implemented a novel cluster reproducibility method to validate cluster outcomes within our study and used publicly available AD biomarker data set (73 markers, N = 58 patients) to validate our findings. Cluster reproducibility analysis demonstrated best consistency for 2 clusters by k-means, reproducibility of this clustering outcome was validated in an independent patient cohort. These unique JAHG patient subgroups either possessed elevated pro-inflammatory mediators, notably TNFβ, MCP-3 and IL-13, among a variety of immune responses (high inflammatory) or lower levels of inflammatory biomarkers (low inflammatory). The high inflammatory subgroup was associated with greater baseline disease severity, demonstrated by greater EASI, SCORAD Index, Itch NRS and DLQI scores, compared with low inflammatory subgroup. African-American patients were predominantly associated with the high inflammatory subgroup and increased baseline disease severity. In patients with moderate-to-severe AD, heterogeneity was identified by the detection of 2 disease subgroups, differential clustering amongst ethnic groups and elevated pro-inflammatory mediators extending beyond traditional polarized immune responses. Therapeutic strategies targeting multiple pro-inflammatory cytokines may be needed to address this heterogeneity.

Jonathan T. Sims, Ching-Yun Chang and Richard E. Higgs contributed equally to this work.

KEYWORDS
atopic, biomarkers, computational biology, dermatitis, population heterogeneity, Th1-Th2 balance

1 | INTRODUCTION

Atopic dermatitis (AD) is a heterogeneous, complex, inflammatory skin disease.[1]

Lesional AD skin is postulated to result from dysregulated immune responses, a defective barrier, microbial dysbiosis, activated sensory nerves and various environmental factors.[2] Clinically, the disease course includes exacerbations and remissions, and it is unclear whether cellular immune reactions that define these clinical episodes are the result of multiple pathogenic pathways operating in parallel or discrete sequential events.[3] Lesional AD skin is characterized by activated epidermal keratinocytes accompanied by a significant dermal immune infiltration involving resident and recruited inflammatory cells producing a subacute spongiotic dermatitis.[2] Interestingly, T cells comprising the AD dermal infiltrate and circulating T-cell subsets vary depending on age of disease onset, ethnicity, disease status (acute vs. chronic AD), serum Immunoglobulin (Ig) E levels (extrinsic vs. intrinsic AD) and mutations in various epidermal proteins contributing to barrier function such as filaggrin.[1,4-8]

Primarily T_H2/T_H22, but also T_H1 and T_H17 immune pathways have been implicated in AD pathogenesis, and with recent reports suggesting systemic immune involvement, it is unclear whether epidermal barrier dysfunction is a primary or secondary driver of AD.[4,6,9-11] Moreover, once the disease shifts into a chronic status, it is unclear whether a core pathological pathway is distinguishable, nor is it clear how topical corticosteroids (TCS) modulate adaptive versus innate immune responses. One study indicated progression from acute to chronic AD lesions was associated with an intensification along a progression of inflammatory mediators rather than distinct immunological mechanisms.[12] The admixture and continuum among various T-cell immune pathways are contrary to the original basic immunological principle in which major T-cell responses such as T_H1 versus T_H2 were portrayed as mutually exclusive and self-reinforcing.[13,14] Such a perspective led early clinical investigators to administer the T_H2 cytokine, IL-4, to psoriasis patients and the T_H1 cytokine, IFNγ, to AD patients.[15,16]

While the inflammatory milieu in AD has been characterized extensively in skin, only a few studies reported alterations in blood.[17-19] These studies included limited numbers of patients, which did not allow for the evaluation of disease heterogeneity. To gain insight into this basic immunological conundrum, beyond the multiple T-cell responses occurring in the skin, we sought to define the breadth and scope of immunomodulatory mediators in the blood of AD patients. Hence, we focused on defining disease heterogeneity and various immune responses using quantitative analysis of circulating pro-inflammatory mediators.[20]

The aforementioned translational gaps in knowledge have therapeutic implications, as multiple reports have emerged from the blood biomarker field suggesting as many as 4 different endotypes may be present amongst AD patients.[21] Should these different endotypes be reflected in responders or non-responders when exposed to targeted therapies, it would be important to avoid using therapies unlikely to be effective in subgroups not enriched for the targeted pathway. Hence, as the practice of precision medicine in dermatology advances, it is important to gain a deeper understanding into the immunopathogenesis of AD. Additionally, the use of blood-based biomarkers was a line of inquiry representing the subject of this study.

To fill this translational gap, we comprehensively examined blood samples derived from a large population of adult patients with moderate-to-severe AD enrolled in a phase 2 randomized, multi-centre controlled study treated with baricitinib an oral selective Janus kinase (JAK) 1 and JAK2 inhibitor.[22] We examined the baseline serum levels of 123 moderate-to-severe AD patients using 131 different inflammatory markers derived from both innate and adaptive immunity for the analyses across different high-throughput proteomic platforms. We also utilized a publicly available biomarker data set derived from the serum Olink proteomic analysis of 58 moderate-to-severe AD patients who were not treated with TCS (2 week washout period for TCS) or systemic therapy.[23]

2 | METHODS

2.1 | Patients and samples

This study included a cohort of 123 patients with moderate-to-severe AD from a phase 2 study of baricitinib (JAHG) who underwent a 4-week TCS standardization period (ClinicalTrials.gov: NCT02576938). The trial was conducted in Japan and the United States.[22] Patients were at least 18 years of age and had moderate-to-severe AD with a baseline Eczema Area and Severity Index (EASI) score ≥12, body surface area (BSA) involvement >10% and a disease diagnosis occurring at least 2 years prior to baseline evaluation. The SCORing Atopic Dermatitis (SCORAD) index, Itch Numeric Rating Scale (NRS) and Dermatology Life Quality Index (DLQI) were also assessed. Baseline blood samples from patients enrolled in JAHG were collected, and serology specimen was separated by centrifugation. The samples were stored at −80°C until analysis. The study was conducted in accordance with ethical principles of the Declaration of Helsinki and Good Clinical Practice guidelines. All investigation sites received approval from the appropriate authorized institutional review board or ethics committee. All patients provided written informed consent before the study-related procedures were undertaken.

A public cohort (TCS washout) of 58 patients with moderate-to-severe AD who underwent a 2-week TCS washout period served for comparison, and 20 age/sex-matched healthy control subjects with

no history of autoimmune disorders was included for evaluation of markers. Samples were collected as previously described.[23] The demographic descriptions for both AD cohorts are shown in Table 1.

2.2 | ELISA and multiplex immunoassays

2.2.1 | Olink multiplex assay

Serum samples were analysed with the Olink Inflammation I Proseek multiplex assay, a proximity extension assay (PEA) technology that utilizes oligonucleotide-labelled antibody probes, according to manufacturer's specifications. The levels of analyte-specific deoxyribonucleic acid (DNA) amplicon for each patient were quantified on the Fluidigm Biomark HD.

2.2.2 | Luminex bead-based multiplex immunoassay

Luminex bead-based sandwich immunoassay platform was used to assess serum protein levels, in which captured analytes are detected using a biotinylated detection antibody and streptavidin-phycoerythrin (S-PE).

Circulating cytokine and chemokine profiles for each patient were assessed by MILLIPLEX MAP Human Cytokine/Chemokine 41-plex Magnetic Bead Panel (HCYTOMAG-60K-PX41, Millipore) using the Curiox Biosystems (San Carlos, CA) DA-Bead plates and DropArray LT210 washing station. Five microlitre of sample was added to beads and incubated overnight at 4°C on the DA-Bead plates. The beads were washed in the washing station between incubations with detection antibody cocktail and S-PE. After the final wash, the beads were resuspended and transferred to a polymerase chain reaction (PCR) plate for analysis with the Millipore Luminex 200 Bead Reader System.

Levels of the following analytes were assessed by Luminex bead-based multiplex assays characterized by AssayGate, Inc.: IL-4, IL-5, IL-10, IL-12p40, IL-12p70, IL-13 and IFNγ according to manufacturer's protocol and detected with the Bio-Plex 200 Bead Reader System.

ELISA

For additional quantitative assessment of important analytes, or where multiplex-based immunoassays were not available, conventional sandwich enzyme-linked immunoassays (ELISA) were utilized. Samples were added to wells coated with monoclonal antibody specific to an analyte and allowed to incubate. After a wash step, an anti-target antibody horseradish peroxidase (HRP) conjugate was added to the wells. Any unbound target and HRP conjugate were washed off, and substrate was added. Intensity of colour was measured with an ELISA plate reader and was proportional to the concentration of analyte in the serum samples.

TABLE 1 Patient demographics and clinical characteristics

	JAHG (*N* = 123)	TCS Washout (*N* = 59)	High inflamm (*N* = 54)	Low inflamm (*N* = 69)
Age (years), mean (SD)	37.9 (14.1)	40.5 (15.2)	41.3 (15.2)	35.2 (12.5)
Gender, *n* (%)				
Male	68 (55.3)	31 (52.5)	26 (48.2)	42 (60.9)
Female	55 (44.7)	28 (47.5)	28 (51.8)	27 (39.1)
Ethnicity, n (%)				
Asian	33 (26.8)	15 (25.4)	7 (21)	26 (79)
African American	25 (20.3)	24 (40.7)	16 (64)	9 (36)
Caucasian	60 (48.8)	20 (33.9)	27 (45)	33 (55)
Other[a]	5 (4.1%)	NA	4 (80%)	1 (20%)
SCORAD, mean (SD)	57.0 (13.2)	54.1 (13.2)	61.3 (13.5)	53.7 (12.1)
EASI score, mean (SD)	25.6 (14.2)	NA	29.9 (16.6)	22.2 (10.9)
Itch NRS, mean (SD)	6.4 (2.2)	NA	6.9 (2.0)	6 (2.3)
DLQI, mean (SD)	13.3 (7.6)	NA	15 (7.8)	12 (7.1)
Eosinophils (10^9/L)	0.4 (0.3)	NA	0.4 (0.3)	0.3 (0.3)
IgE (pg/ml)	8141.0 (834.0)	NA	7464 (1.1)	8713 (1.1)

Note: Data are *n* (%) or mean (SD).

Abbreviations: DLQI, Dermatology Life Quality Index; EASI, Eczema Area and Severity Index;N, number of subjects in the analysis population; n, number of subjects in the specified category; NA, not available; NRS, Numerical Rating Scale; SCORAD, SCORing Atopic Dermatitis; TCS, topical corticosteroid.

[a]Other: native Hawaiian or other Pacific Islander, or multiple.

Levels of IL-22 were measured using a Mesoscale Delivery (MSD)-based ELISA, consisting of Lilly proprietary anti-IL-22 antibodies and a recombinant IL-22 protein. This assay utilized a commercially available Small Spot SA–MSD plate (MSD, Rockville, MD; Cat # L45SA-1), along with various in-house diluents. Following each incubation step, wells were thoroughly washed using a Bio-TEK ELx405. MSD's electrochemiluminescence (ECL) detection technology used SULFO-TAG labels that emit light upon electrochemical stimulation, initiated at the electrode surfaces of MULTI-ARRAY microplates, when submerged in conductive Read Buffer. ECL measurement was captured by the Meso Quick Plex SQ 120 plate reader, and analysis was performed using the Discovery Workbench Software.

Levels of IL-13 and IL-17a were assessed by Quanterix single molecule array (Simoa) bead-based 2.0 assays on the Simoa HD-1 analyser. Conjugated paramagnetic beads, biotinylated detection antibodies and associated buffers were resuspended and loaded onto the Simoa HD-1 instrument per manufacturer protocol. Patient samples were diluted in 96-well plates and loaded onto the instrument for automated analysis. The Simoa HD-1 mixes each sample with beads, incubates and adds detection antibodies, building an immunocomplex on the bead itself. These bead complexes (containing up to 1 target-specific immunocomplex) are then pushed over a specialized disk to separate out individual beads into thousands of femtoliter sized wells on the disk and finally read by the Simoa HD-1 giving results down to the fg/mL level in assayed fluids.

Levels of the following analytes were assessed by ELISA platforms characterized by AssayGate, Inc.: IL-31, thymus and activation-regulated chemokine (TARC), IgE, lactate dehydrogenase (LDH), Periostin, S100A9 and S100A12.

2.3 | Statistical analyses

2.3.1 | Cluster reproducibility analysis

To characterize cytokine heterogeneity, cluster algorithms can be used to group samples. However, it is challenging to validate defined clusters. In order to quantify the robustness of clusters defined in AD patients, we proposed a novel cluster reproducibility method based on multiple splits of the data into training and testing portion. Unlike current practice, where cluster analysis was implemented using all the data, our proposed method could systematically evaluate 2 clustering algorithms (*k*-means and hierarchical cluster) and different numbers of clusters (2, 3 or 4 clusters) so we could select the optimal clustering method and the number of clusters to characterize the heterogeneity of AD based on reproducibility performance. Based on simulation, this machine learning method could generate reliable estimation of cluster number and patient cluster heterogeneity. The method is described as follows: first, samples were split into 2 groups (training or testing data sets). Second, the same training and testing sets were applied with the 2 cluster algorithms and different number of clusters. Third, the cluster prediction rule was established using random forest (R package: randomForest function)[24] from the training set and the per cent accuracy of the training set was calculated. The same cluster prediction rule from the training set was used to predict membership for each sample in the testing set, and the per cent accuracy of the testing set was calculated. Fourth, the above procedure was repeated 100 times for each scenario and the per cent accuracy values for 100 iterations of both the training and testing sets for a given clustering algorithm and number of clusters were calculated, respectively. In order to assess the reproducibility of cluster methods, the median per cent accuracy across these 100 iterations was compared. The optimal cluster methods (*k*-means and 2 clusters) for AD heterogeneity was determined based on the reproducibility with the highest median per cent accuracy in both the training and testing sets and would be used for subsequent cytokine cluster comparison (Figure 1A).

2.3.2 | Heatmap analysis

To visualize cytokine patterns, heatmap on the scaled and centralized cytokine data of AD patients and healthy controls was applied (R package heatmap.2 function).[25]

2.3.3 | *t*-SNE analysis

t-distributed Stochastic Neighbour Embedding (*t*-SNE) is an automatic algorithm for representing data in lower dimensions while preserving potential clusters.[26] Here, we use *t*-SNE to project cytokine data in 2 dimensions so it can be displayed graphically to evaluate strength of clustering.

2.3.4 | Cluster comparison analysis (volcano plot)

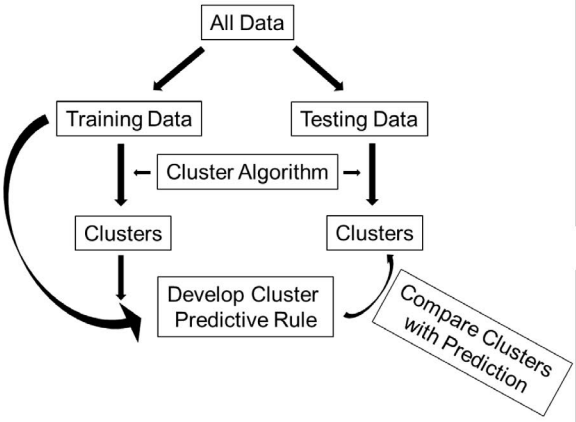
Once the optimal cluster was defined, for each cytokine, a one-way ANOVA (R package: lm function) with cluster as fixed effect on the log transformation of cytokine concentration was applied to generate the fold change and raw *p*-values. The Benjamini & Hochberg method[27] was further applied to calculate the adjusted *p*-values to control false discovery rate across markers (R package: *p*.adjust function). Volcano plot was applied to visualize markers with log2 fold change and negative of log transformed adjusted *p*-values. Significant markers were defined based on adjusted *p*-value<0.05 and fold change (FC)>1.5 (R package: ggplot2 function).

3 | RESULTS

3.1 | Patient demographics and disease activity of JAHG and TCS washout cohorts

Serum samples were collected from a total of 123 AD patients in the JAHG cohort and 58 AD patients in the TCS washout cohort, with patient demographics and disease activity data shown from

(A)



(B)

JAHG Cohort

Median % Accuracy Rate	K-means		Hierarchical Cluster	
	Training set	Testing set	Training set	Testing set
2 Cluster Scenario	**92%**	**83%**	79%	64%
3 Cluster Scenario	80%	63%	61%	54%
4 Cluster Scenario	73%	58%	50%	47%

TCS Washout Cohort

Median % Accuracy Rate	K-means		Hierarchical Cluster	
	Training set	Testing set	Training set	Testing set
2 Cluster Scenario	**90%**	**88%**	90%	78%
3 Cluster Scenario	76%	64%	63%	60%
4 Cluster Scenario	56%	54%	49%	50%

FIGURE 1 Cluster reproducibility methodology for atopic dermatitis patients with corresponding age/sex-matched healthy controls. (A) Samples were split into either training or testing datasets in order to assess optimal cluster method and reproducibility of the clusters. (B) Summary table of median percent accuracy rate for 2 methods: *k*-means and hierarchical cluster with 2–4 cluster scenarios. Abbreviations: AD, atopic dermatitis; HC, healthy controls; TCS, topical corticosteroid

all 59 patients included in the original study[23] (Table 1). Age was similar between the 2 studies with the mean age in JAHG and TCS washout cohorts being 37.9 years and 40.5 years, respectively. Gender was also comparable in the JAHG cohort and the TCS washout cohort. Ethnicity was different between the 2 datasets with the JAHG cohort having a higher percentage of Caucasians compared with the TCS washout cohort (48.8% vs. 33.9%, respectively) and the TCS washout cohort having a higher percentage of African Americans compared with the JAHG cohort (40.7% vs. 20.3%, respectively). The percentage of Asians was similar between the 2 data sets. Disease severity was measured in both cohorts with the SCORAD index being utilized in the TCS washout cohort and the SCORAD index, EASI score, Itch NRS and DLQI being utilized in the JAHG cohort. The mean SCORAD index score for the JAHG and TCS washout cohorts were 57.0 (SD 13.2) and 54.1 (SD 13.2), respectively. The JAHG cohort had a mean EASI score of 25.6 (SD 14.2), mean Itch NRS of 6.4 (SD 2.2) and mean DLQI of 13.3 (SD 7.6).

3.2 | Cluster analyses suggests 2 clusters of patients with AD

To determine the heterogeneity of AD patient pro-inflammatory profiles, we performed multiple cluster analyses on the JAHG and TCS washout cohort biomarker datasets with healthy controls. Based on proposed cluster reproducibility analysis (Figure 1A), the median per cent accuracy over 100 iterations for the 2 cluster methods (*k*-means and hierarchical clusters) with 2–4 number of clusters for both training and testing sets were calculated (Figure 1B). In both JAHG and TCS washout cohorts, the *k*-means algorithm with 2 clusters method had the highest median per cent accuracy in both the training and testing sets (≥80% accuracy) and was therefore used for subsequent cytokine cluster comparison. The per cent accuracy from *k*-means and hierarchical clustering decreased with the addition of more clusters. Likewise, we also observed the *k*-means algorithm with 2 clusters method to be the most reproducible population categorization method after excluding healthy controls (HC) for both JAHG and TCS washout cohorts, and increasing the number of analytes measured in AD samples for JAHG (Figure S1).

In Figure 2, heatmap and t-SNE analyses were utilized to visualize the heterogeneity of the JAHG and TCS washout data sets. Healthy controls were utilized as a reference point for each data set (JAHG: 20 HC, TCS Washout: 17 HC) and are represented in the figure by a striped bar. The suggested *k*-means method was used to classify samples into 2 clusters and highlighted in black as low inflammatory subgroup and in red as high inflammatory subgroup in both heatmap and *t*-SNE plots. The colour scheme of data matrix is based on scaled and centralized cytokine data per marker across donors: red, higher expression and blue, lower expression (Figure 2A-B). The JAHG data set (123 AD and 20 HC, 71 Olink markers after %CV >20 filtering) was clustered using *k*-means into 2 clusters: 72 AD patients (59%) in the low inflammatory subgroup marked with a black bar and 51 AD patients (41%) in the high inflammatory subgroup marked with a red bar in the

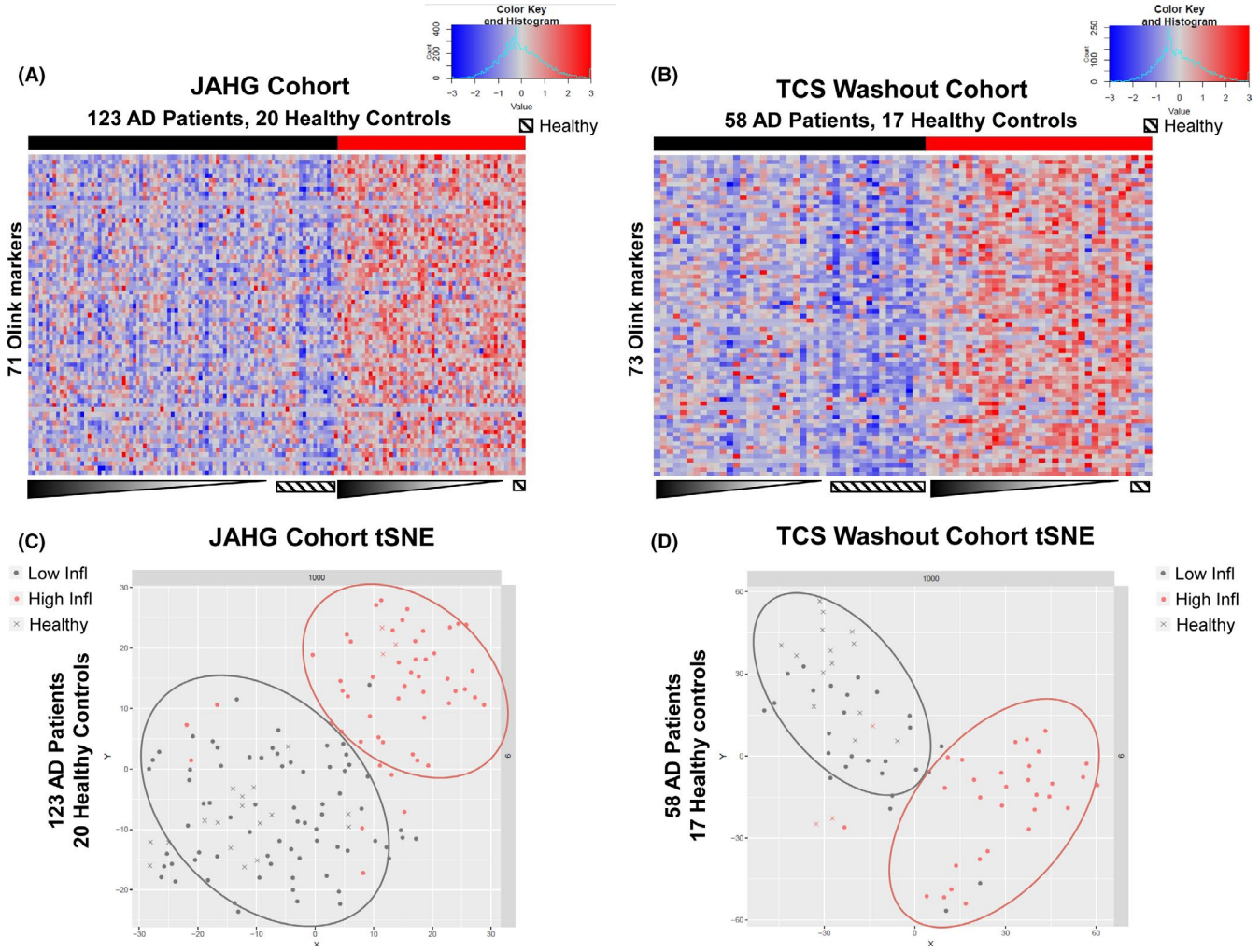


FIGURE 2 Heatmap and *t*-SNE analyses of atopic dermatitis patients with corresponding age/sex-matched healthy controls. Samples in the heatmap are sorted on the *x*-axis by disease severity from most to least severe (displayed in the gradients) within the 2 clusters: low inflammatory subgroup (black) and high inflammatory subgroup (red). Healthy controls are represented by a striped bar. The colour scheme of data matrix is based on scaled and centralized cytokine data per marker across samples: red, higher expression; blue, lower expression. For *t*-SNE, the colour scheme refers to 2 clusters: low inflammatory subgroup (grey) and high inflammatory subgroup (orange). Healthy controls are signified by "X." (A) Heatmap analysis of JAHG cohort (123 AD, 20 HC, 71 Olink markers). (B) Heatmap analysis of TCS washout cohort (58 AD, 17 HC, 73 Olink markers). (C) *t*-SNE analysis of JAHG cohort (123 AD, 20 HC, 71 Olink markers). (D) *t*-SNE analysis of TCS washout cohort (58 AD, 17 HC, 73 Olink markers). Abbreviations: AD, atopic dermatitis; EASI, Eczema Area and Severity Index; hc, healthy control; infl, inflammatory; TCS, topical corticosteroid; *t*-SNE, *t*-distributed Stochastic Neighbour Embedding

heatmap (Figure 2A). The TCS washout data set (58 AD and 17 HC, 73 Olink markers after %CV >20 filtering) was clustered using *k*-means into 2 clusters: 27 AD patients (47%) in the low inflammatory subgroup marked with a black bar and 31 AD patients (53%) in the high inflammatory subgroup marked with a red bar in the heatmap (Figure 2B). The lower inflammatory subgroup predominately associated with the HC in both data sets (Figure 2A-D). The per cent of HC which overlapped with the low inflammatory AD patient profile were as follows: JAHG: 17 of 20 (85%), TCS washout: 14 of 17 (82%).

In Figure 2C-D, the inflammatory markers were spatially mapped and reduced to low dimensional graphs using the *t*-SNE analysis. The same 2 clusters from *k*-means in both cohorts were annotated with the lower inflammatory subgroup represented by grey dots, higher inflammatory subgroup represented by orange dots, and HC represented by "X."

Heatmaps and t-SNE analyses without healthy controls and with all 131 markers (after %CV >20 filtering) spanning multiple proteomic platforms are displayed in Figure S2A-B for JAHG.

3.3 | Biomarkers differentially regulated in clusters 1 and 2

To investigate whether certain biomarkers showed directional change between clusters, the expression of protein markers from the high inflammatory subgroup was compared with the low inflammatory subgroup and visualized via volcano plot (Figure 3). The

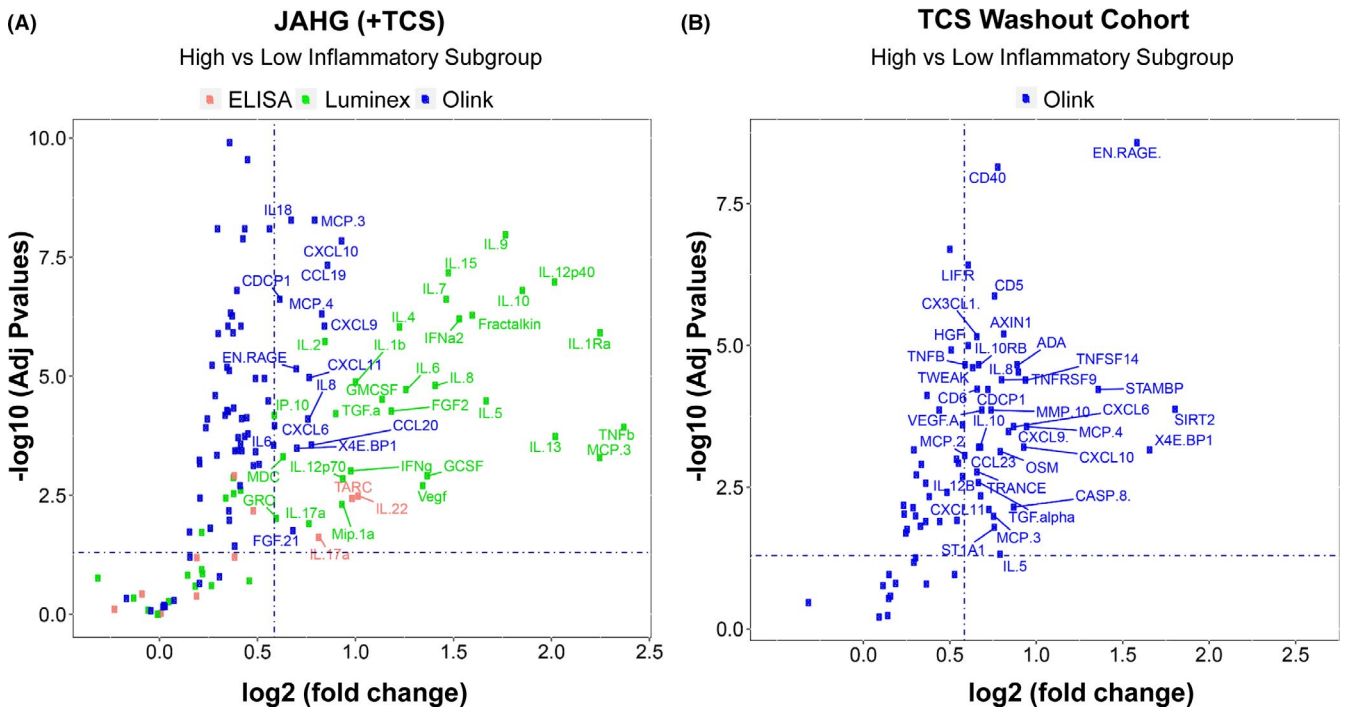


FIGURE 3 Volcano plot shows significantly upregulated markers of high inflammatory subgroup to low inflammatory subgroup (adjusted *p*-value <0.05, fold change >1.5). The analyte log2 fold change is plotted on the *x*-axis, and the negative log10 adjusted *p*-value is plotted on the *y*-axis. Detection platform is denoted by color (ELISA, pink; Luminex, green; Olink, blue). (A) Volcano plot of JAHG cohort. (B) Volcano plot of TCS washout cohort. Abbreviations: Adj, adjusted; TCS, topical corticosteroid

analyte log2 fold change is plotted on the *x*-axis, and the negative log10 adjusted *p*-value is plotted on the *y*-axis. In the JAHG data set, 47 out of 131 markers were found to be significantly upregulated in the high inflammatory subgroup as compared with the low inflammatory subgroup (using criteria of fold change (FC)>1.5, adjusted *p*-value <0.05,) (Figure 3A). Markers are coloured by the proteomic platform utilized. In the TCS washout cohort, 37 out of 73 markers were significantly upregulated in the high inflammatory subgroup as compared to the low inflammatory subgroup (Figure 3B). Biomarkers of note in the high inflammatory subgroup of the JAHG cohort included T_H2: IL-4, IL-5, IL-6, IL-10, G-CSF; T_H9: IL-9; T_H22: IL-22; T_H17: IL-17a; and T_H1: IFNγ, IL-12p70, MIP1a, IL-2, IL-12p40. Across the multiple detection platforms used (Olink, Quanterix and 2 Luminex panels), IL-17a, IL-6, IL-5, IL-13 and MCP-3/CCL7 were among the most consistently upregulated pro-inflammatory mediators in the high inflammatory subgroup, providing enhanced confidence in the observations reported (Figures S3 and S4A).

3.4 | High inflammatory subgroup of the JAHG cohort associates with higher disease severity

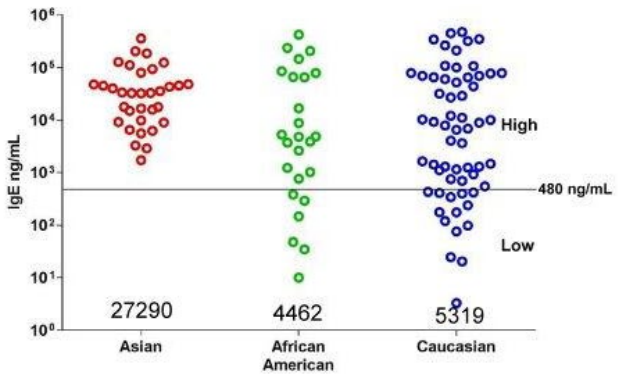
To determine whether patients in the 2 JAHG cohort clusters associated with disease severity, we compared EASI Score, SCORAD Index, Itch NRS and DLQI scores between the low inflammatory subgroup and the high inflammatory subgroup (Figure S5). We found the higher inflammatory JAHG subgroup was associated with higher disease severity and displayed significantly greater EASI (29.9 [high] vs. 22.2 [low]; $p = 0.002$), SCORAD Index (61.3 [high] vs. 53.7 [low]; $p = 0.001$), Itch NRS (6.9 (high) vs. 6.0 (low); $p = 0.03$) and DLQI (15 [high] vs. 12 [low]; $p = 0.03$) scores when compared with the lower inflammatory subgroup (Figure S5A-D). In the TCS washout data set, no association to SCORAD ($p = 0.63$) was observed between the 2 subgroups (data not shown).

To further determine the objective value for individual baseline cytokine/chemokine measurements, we assessed correlations of the log-transformed concentrations with JAHG's primary disease severity measure, EASI. Pearson correlation coefficients for circulating levels of key T_H2/T_H22 cytokine indices (IL-13, IL-22, TARC/CCL17, MDC/CCL22, MCP-4/CCL13, IgE) and MCP-3/CCL7 were compared with baseline EASI measurements, showing moderately strong correlations with EASI severity scores (Figures S6A-F and S4B).

3.5 | JAHG patient subtype characteristics by ethnicity

Given the presence of 2 AD patient clusters in the JAHG analysis, we evaluated ethnicity-related characteristics. All Asian patients had elevated IgE levels (>480 ng/ml), while among Caucasian and African-American patients, the relative distribution for elevated IgE levels was ~80% (Figure 4A). Also, Asian AD patients were predominately

(A)



(B)

Ethnicity and Cluster Demographics			
	Low Infl	High Infl	Total
Asian (N)	26	7	33
% Individual Population	79	21	
African American (N)	9	16	25
% Individual Population	36	64	
Caucasian (N)	33	27	60
% Individual Population	55	45	
Other (N)	1	4	5
% Individual Population	20	80	
All Patients (N)	69	54	123
% Total Population	56	44	

(C) (D) (E)

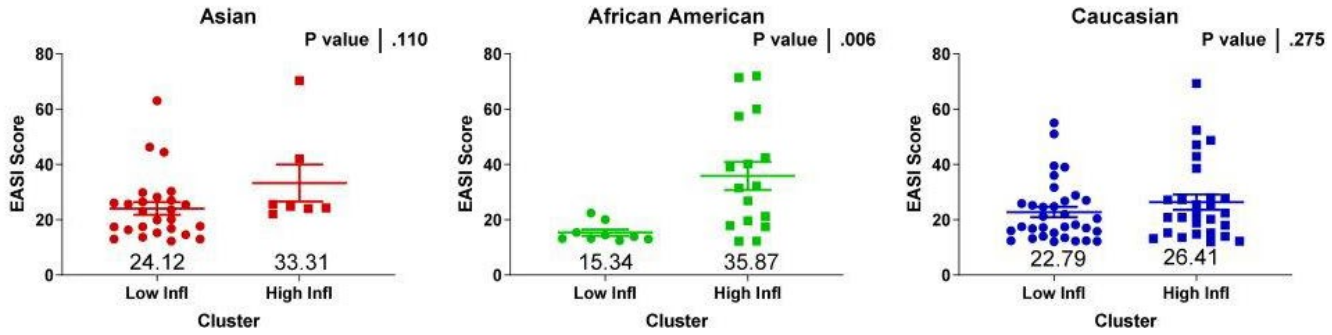


FIGURE 4 IgE levels and disease severity vary between ethnicities. (A) IgE levels (log ng/mL) were plotted by ethnicity (Asian, red; African American, green; and Caucasian, blue). Mean values are provided along the *x*-axis. The cut-off of 480 ng/ml (black line) was set for high versus low IgE. (B) For each race and cluster, the relative distribution of patients enrolled in the study is shown as patient number, percentage of patients within each cluster, and the percentage of each race between clusters. Patients separated by ethnicity (C-E) were plotted for relative disease severity (EASI score) and respective patient cluster. Mean values are provided along the *x*-axis. Abbreviations: EASI, Eczema Area and Severity Index; Infl, Inflammatory; Ig, immunoglobulin

associated with the low inflammatory subgroup, whereas African-American patients were predominantly associated with the high inflammatory subgroup, and Caucasian patients were more evenly distributed among the 2 clusters (Figure 4B-E). Furthermore, the interaction between cluster and ethnicity indicated that African-American patients with severe disease possess a high inflammatory profile ($p = 0.006$, FC = 2.34; Figure 4D).

4 | DISCUSSION

A primary goal of this study was to propose patient endotypes that could be readily reproduced by objectively and robustly assessing the clustering output. Using 2 independent clinical patient data sets, remarkably similar blood biomarker profiles were characterized in adults with moderate-to-severe AD either in the presence or absence of TCS. These blood-based analyses of numerous and wide-ranging pro-inflammatory mediators revealed a highly inflammatory molecular profile spanning broad T_H2 and T_H1-type immune reactions in both AD patient cohorts. The diversification of immune responses likely reflects the complexity of immune signalling networks in the skin lesions which are influenced by several internal and external factors, with contributions from multiple pathways activated in parallel, involving both innate and adaptive arms of the immune system.[2,28,29] Age of disease onset, ethnicity, disease status, serum IgE levels and mutations in genes involved in skin barrier function all appear to contribute to the diversity of the disease and the overall complexity of the inflammatory loops in the skin and blood of adult moderate-to-severe AD patients.[2,30]

In addition to deciphering the array of pro-inflammatory mediators in the blood derived from a coalition of cell types emanating from lesional AD skin,[31] we next determined whether patient subgroups could be identified along the continuum of immune responses observed in both patient cohorts. Indeed, 2 patient clusters or sub-populations of approximately equal frequency were found in both cohorts. It was remarkable that diverse pro-inflammatory

profiles spanning the T_H-cell landscape were similarly elevated in the high inflammatory subgroup compared with the low inflammatory subgroup in both cohorts (Figure 3). It appears the primary distinction between the subgroups is a matter of intensity rather than large qualitative differences. Thus, these results are in agreement with an earlier report indicating that transition from non-lesional skin to chronic lesions is more closely related to a continuum of similar disease mechanisms with increasing intensity rather than to distinct immune response characteristics.[12,32]

This study of 2 independent AD moderate-to-severe patient cohorts is distinguished from both a moderate-to-severe adult AD study conducted by Thijs et al (2017), and a paediatric AD analysis by Seo et al. (2019), in which serum biomarker profiling led to the identification of 4 clusters within their AD data set.[21] In our large data set of the 2 moderate-to-severe AD cohorts, we only found evidence of 2 clusters based on our rigorous clustering methodology and stringent standards of accuracy. While it may be possible in independent studies to subjectively discern patient endotypes with a greater degree of granularity, the prior studies did not associate the findings with various characteristics that may impact the various clusters, such as disease severity or ethnicity. Further, we were able to recapitulate our findings in a large data set with data from a second independent cohort.

Interestingly, in our 2 cohorts, the presence of TCS did not influence patient inflammatory profiles. Our study differs from recent studies due to the inclusion of stringent analytical methods and multiple commercially available assays, including several ultrasensitive serum biomarker assays, to identify the existence of biomarker clusters within a large AD patient population. We pursued an agnostic approach to determining whether we could reproducibly identify patient subpopulations within these 2 data sets. The results of this rigorous assessment of patient clustering are shown in Figure 1 and indicate the *k*-means clustering method was superior to hierarchical clustering in terms of accuracy rate and provided reasonable evidence we could reliably reproduce clustering indicative of 2 patient clusters. Consistent with the cluster reproducibility analysis, heatmap and t-SNE analyses also suggested 2 clusters further supporting this notion of only 2 subgroups of patients.

Within the JAHG cohort, we found the 2 clusters show unbiased association to baseline EASI Score, SCORAD Index, Itch NRS and DLQI. Specifically, the high inflammatory subgroup was associated with higher disease scores as compared to the low inflammatory subgroup (Figure S5). The patients in the low inflammatory subgroup did not significantly separate from the HC samples included in the clustering analysis. This is consistent with the current literature as high inflammatory subgroups in both cohorts had an upregulation of pro-inflammatory mediators spanning all polar cytokine pathways, which contribute to AD.[6,9,33,34] The largest fold changes between clusters were found in TNFβ (FC = 5.19), MCP-3/CCL7 (FC = 4.76) and IL-13 (FC = 4.07) levels (Figure S7). Due to the key role of the T_H2 cytokine IL-13 in driving AD,[35] we pursued quantitative assessment of this cytokine which corroborated the demonstration by Luminex that IL-13 is significantly upregulated in the high inflammatory subgroup compared with the low inflammatory subgroup (Figure S4A) correlating IL-13 with disease severity in AD patients (Figure S4B). Currently, there is no reliable quantitative assay for the key T_H2 cytokine IL-4, and future studies will be needed to determine its association with AD clusters. The diversity of elevated cytokines and chemokines supports the notion that targeting 1 or 2 inflammatory mediators may not be adequate to control disease activity in many AD patients and raises the possibility that clinicians could require a number of therapeutic interventions in order to control disease activity across the broad spectrum of AD patients. This further supports the potential therapeutic utility of AD clusters in the search for personalized treatments for AD patients and the utility of agents blocking more than one inflammatory pathway.

The rise of serum biomarker profiling has led to the quest of finding baseline cytokines or chemokines that offer objective and consistent outcome measures of disease severity. We demonstrated that Th2/Th22-related biomarkers, including IL-13, IL-22, TARC/CCL17, IgE, MDC/CCL22, MCP-4/CCL13 and MCP-3/CCL7, correlate with disease severity (EASI score –Figures S6 and S4B). Several studies have shown these biomarkers not only correlate with disease severity,[23,36-43] but some are also targets of active clinical programmes.[44,45] Further studies are needed to determine whether assessment of blood alone is adequate for significant correlation with disease scoring metrics or if combination with biomarkers in AD patient serum or skin is required for greater accuracy than a single circulating biomarker can provide.

Finally, we characterized the JAHG AD population by ethnicity and determined that all Asian patients in our cohort had high IgE levels. African-American and Caucasian patients were distributed at an 80:20 ratio between high and low IgE levels suggesting a greater incidence of so called “extrinsic” AD among Asian patients. Interestingly, high inflammatory subgroup patients who were predominately of African American descent had significantly higher EASI scores than Asians and Caucasians. Overall, these baseline demographic characteristics support earlier reports indicating that African-American patients tend to have higher disease activity[20,46,47] but differ from another report indicating African-American patients having the highest IgE levels when compared with Asians and Caucasians.[48] One explanation for this difference may be the relatively small population of Asians and African Americans in the JAHG cohort (*N* = 33 and *N* = 25, respectively) compared with other studies.

Limitations of this study include the limited clinical annotation beyond overall clinical severity parameters, and future studies will require genotyping, assessment of the microbiome, greater numbers of ethnic and racial diversity, phenotyping lesions as regards acute and chronic with presence or absence (and extent) of lichenification, as well as anatomical locations of lesions (eg facial). Another limitation of the study is that our investigation was focused on peripheral blood only. While it is likely that systemic TCS use will alter the inflammatory profile of patients in peripheral blood, we attempted to define a common systemic inflammatory profile of AD,

with real-world implications, since the majority of patients will have utilized TCS at various stages during their treatment course. In addition, we feel that a definite need exists for a less invasive approach than biopsies, and robust examination of peripheral blood is the first step in addressing that need.

In summary, we have undertaken a wide-ranged exploration of protein mediators of inflammation and used the most stringent analysis of AD patient endotyping presented thus far in the literature to our knowledge. Overall, these data support a systemic inflammatory state accompanying the visible lesional inflammation typically seen in adult AD. These data will be useful for researchers with interest in broadly applying their findings in highly heterogenous disease, including moderate-to-severe AD.

ACKNOWLEDGEMENTS

The authors would like to thank Brian J. Nickoloff and Fabio P. Nunes, formerly with Eli Lilly and Company, for support throughout this project, many thought-provoking scientific discussions, and for assistance with both the interpretation of our data and manuscript preparation. The authors would also like to thank all patients, study investigators, healthy volunteers and the following colleagues at Eli Lilly and Company: David McIlwain and Nicole L. Byers for manuscript preparation and process support, Robert J. Benschop and Jochen Schmitz for scientific input, Guilherme Rocha for technical assistance, and Nicoletta Bivi and Robert J. Konrad for assay development.

CONFLICT OF INTEREST

JTS, CC, REH, SME, YL, SES, GHR, JJ and SCC are employees and may be shareholders of Eli Lilly and Company. ELS has been a consultant with honorarium from AbbVie, Eli Lilly and Company, Forte Bio, Galderma, Incyte, LEO Pharma, Menlo Therapeutics, Novartis, Pfizer, Regeneron, Sanfoi Genzyme and Valeant; and an investigator for AbbVie, Eli Lilly and Company, Galderma, Kyowa Hakko Kirin, LEO Pharma, Merck, Pfizer and Regeneron. JIS has received consultancies from Abbvie, AnaptysBio, Arena, Asana, Boehringer-Ingelheim, Celgene, Dermavant, Dermira, Eli Lilly and Company, Galderma, GlaxoSmithKline, Glenmark, Incyte, Kiniksa, Leo, Menlo, Novartis, Pfizer, Regeneron, Sanofi-Genzyme; speaker honoraria from Regeneron and Sanofi-Genzyme; and research grants from Galderma and GlaxoSmithKline. SBF has received research grants from AbbVie, AstraZeneca, Celgene Corporation, Cutanea Life Sciences, Eli Lilly and Company, Incyte, Innovaderm Research Inc, Novan, Novartis, Pfizer, Promius Pharma LLC, Regeneron, UCB, Valeant Pharmaceuticals North America LLC and Xbiotech; consultancies and honoraria from Abb-Vie, Eli Lilly and Company, Pfizer and Xbiotech; speaker honoraria from Eli Lilly and Company and Novartis; and principal investigator fees from Pfizer Inc. EG-Y is an employee of Mount Sinai and has received research funds (grants paid to the institution) from Abbvie, Almirall, Amgen, AnaptysBio, Asana Biosciences, AstraZeneca, Boerhinger-Ingelhiem, Celgene, Dermavant, DS Biopharma, Eli Lilly and Company, Galderma, Glenmark/Ichnos Sciences, Innovaderm, Janssen, Kiniksa, Kyowa Kirin, Leo Pharma, Novan, Novartis, Pfizer, Ralexar, Regeneron Pharmaceuticals, Inc., Sienna Bio pharma, UCB and Union Therapeutics/Antibiotx; and is a consultant for Abbvie, Aditum Bio, Almirall, Alpine, Amgen, Arena, Asana Biosciences, AstraZeneca, Bluefin Biomedicine, Boerhinger-Ingelhiem, Boston Pharmaceuticals, Botanix, Bristol-Meyers Squibb, Cara Therapeutics, Celgene, Clinical Outcome Solutions, DBV, Dermavant, Dermira, Douglas Pharmaceutical, DS Biopharma, Eli Lilly and Company, EMD Serono, Evelo Bioscience, Evidera, FIDE, Galderma, GSK, Haus Bioceuticals, Ichnos Sciences, Incyte, Kyowa Kirin, Larrk Bio, Leo Pharma, Medicxi, Medscape, Neuralstem, Noble Insights, Novan, Novartis, Okava Pharmaceuticals, Pandion Therapeutics, Pfizer, Principia Biopharma, RAPT Therapeutics, Realm, Regeneron Pharmaceuticals, Inc., Sanofi, SATO Pharmaceutical, Sienna Biopharma, Seanegy Dermatology, Seelos Therapeutics, Serpin Pharma, Siolta Therapeutics, Sonoma Biotherapeutics, Sun Pharma, Target PharmaSolutions and Union Therapeutics/AntibioTx, Vanda Pharmaceuticals, Ventyx Biosciences, and Vimalan.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. JTS, JMJ and EG contributed to study conception and design. JTS, SME and SES contributed to acquisition of data. JTS, CC, REH, SME, YL, SES, GHR, ELS, JIS, SBF, JMJ, SCC and EG contributed to analysis and interpretation of data.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1: Cluster reproducibility methodology for atopic dermatitis patients alone.

Figure S2: Heatmap and t-SNE analyses of atopic dermatitis patients alone.

Figure S3: a) MCP-3, b) IL-6, c) IL-5, and d) IL-17a were consistently identified across multiple testing platforms as distinguishable signature proteins for the high inflammatory subgroup in JAHG cohort.

Figure S4: Ultrasensitive measurement of IL-13 by Quanterix Simoa assay confirms our Luminex-based observation of: a) IL-13 upregulation in patients comprising JAHG's high inflammatory subgroup and b) correlation of baseline IL-13 protein levels with disease severity (cor=0.47).

Figure S5: JAHG cohort cluster associations with disease skin severity.

Figure S6: Representative scatterplots of top analyte correlations with skin disease severity (EASI score).

Figure S7: Ranking of fold changes between Cluster 2 (High Inflammation) and Cluster 1 (Low Inflammation) for all examined biomarkers.

How to cite this article: Sims JT, Chang C-Y, Higgs RE, et al. Insights into adult atopic dermatitis heterogeneity derived from circulating biomarker profiling in patients with moderate-to-severe disease. *Exp Dermatol*. 2021;30:1650–1661. https://doi.org/10.1111/exd.14389