Predictive biomarker modeling of pediatric atopic dermatitis severity based on longitudinal serum collection

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Abstract

Background: The pathogenesis of atopic dermatitis (AD) results from complex interactions between environmental factors, barrier defects, and immune dysregulation resulting in systemic inflammation. Therefore, we sought to characterize circulating inflammatory profiles in pediatric AD patients and identify potential signaling nodes which drive disease heterogeneity and progression. Methods: We analyzed a population of 87 infants that were at high risk for atopic disease based on dermatitis diagnoses. Clinical parameters, serum, and peripheral blood mononuclear cells (PBMCs) were collected upon entry, and at one and four years later. Within patient serum, 126 unique analytes were measured using a combination of multiplex platforms and ultrasensitive immunoassays. Results: We assessed the correlation of inflammatory analytes with AD severity (SCORAD). Key biomarkers, such as IL-13 (corr=0.47) and TARC (corr=0.37), among other inflammatory signals, significantly correlated with SCORAD across all timepoints in the study. Flow cytometry and pathway analysis of these analytes implies that CD4 T cell involvement in type 2 immune responses were enhanced at the earliest time point (year 1) relative to the end of study collection (year 5). Importantly, forward selection modeling identified 18 analytes in infant serum at study entry which could be used to predict change in SCORAD four years later. Conclusions: We have identified a pediatric AD biomarker signature linked to disease severity which will have predictive value in determining AD persistence in youth and provide utility in defining core systemic inflammatory signals linked to pathogenesis of atopic disease.

INTRODUCTION

Atopic dermatitis (AD) is a highly complex, heterogeneous, chronic inflammatory skin condition characterized by a pruritic, erythematous rash whose immune pathophysiology remains unclear. While AD has been shown to affect up to 25% of children and 3% of adults worldwide, incidence rates has been increasing 2- to 3-fold over the last several decades [1]. Although many pediatric cases are transient in nature, AD is often the first step of the atopic march and active disease throughout life with up to 75% of adults with AD reporting onset during childhood [2]. An early diagnosis of AD has been linked with the development of additional atopic comorbidities, such as allergic rhinitis, food allergy, and asthma [3]. This link may be the result of similar underlying mechanisms of disease development involving the circulating inflammatory immune milieu in AD patients since type 2 cytokines, barrier function proteins, and allergen-specific IgE responses have been implicated in both disease onset and persistence [4, 5].

The comorbid nature of AD, as well as its development in early childhood, makes it an attractive target for predictive studies of disease development highlighting the importance of characterizing these early stages of pathogenesis. The systemic nature of AD has only recently been explored within younger patients demonstrating the presence and persistence of cell populations that produce biomarkers like IL-22, IL-17a, and

IFNγ, and strong correlations to cells capable of producing IL-13 were shown to increase from childhood to adulthood [6, 7]. Because AD is a complex disease involving multiple cell types, chemokines, and cytokines, further longitudinal studies identifying additional biomarkers and correlating them to disease manifestations are needed to truly characterize the complex underlying immune signals of AD pathogenesis that drive barrier disruption and atopy development. Therefore, using high-throughput and ultrasensitive protein quantitation methods, we evaluated 126 secreted proteins from the serum of 87 children diagnosed with AD in their first year of life and correlated levels of inflammatory proteins to disease progression over the course of this 5-year study.

Our findings provide further insight into the systemic inflammatory serum protein and cell population profile present in the circulation of pediatric AD patients. Previous studies utilizing this cohort of patients revealed correlations with AD to atopic and nonatopic eczema, food allergy, and asthma [8, 9]. Several biomarkers indicative of active type 2 immune responses were correlated with both progression of disease and cell phenotypes which have been linked to comorbidities associated with non-AD-like conditions such as childhood wheezing [10]. These correlations in early life demonstrate the potential for development of targeted therapeutics for younger patients with mild to moderate disease. In this manuscript, we highlight key insights into the pathology of AD, outlining the correlations with analytes previously identified in adult moderate to severe disease and their role in disease development even in this mild to moderate cohort of pediatric subjects.

METHODS

Patients and samples This study included a cohort of 87 American pediatric patients recruited in infancy (Figure 1). Serum samples were collected from children diagnosed with chronic dermatitis as previously described [9]. All subjects or their parents/guardians provided written consent for participation in the study. Sample collection and subsequent use of data was approved by the IU Institutional Review Board. Flow cytometry analysis of PBMCs collected throughout the course of the study was previously described [11].

ELISA and Multiplex Immunoassays

*Olink multiplex assay*Serum samples were analyzed with the Olink Inflammation I Proseek multiplex assay (95302) according to manufacturer's specifications. The levels of analyte-specific DNA amplicons for each patient were quantified by Fluidigm Biomark HD.

*Luminex bead-based multiplex immunoassay*Luminex bead-based sandwich immunoassay was used to assess serum protein levels quantitatively and simultaneously on the Millipore Luminex 200 Bead Reader System. Two MILLIPLEX MAP Human Cytokine/Chemokine 41-plex Magnetic Bead Panels (HCYTOMAG-60K-PX41 and HCP2MAG-62K-PX23) were assayed on Curiox Biosystems DA-Bead plates and plates were washed on a DropArray LT210 washing station following the Curiox low-volume protocol.

*ELISA*For additional quantitative assessment of important analytes conventional sandwich enzyme-linked immunoassays (ELISA) were utilized including Periostin (DY3548B) and sST2 (DST200) from R&D Systems. Levels of IL-22 and TARC were measured using MesoScale Discovery (MSD)-based sandwich ELISA. The IL-22 assay, consisting of proprietary antibodies and recombinant protein, utilizes a Small Spot SA–MSD plate (L45SA-1) and in-house diluents. Following incubations, wells were washed with 1x TBST using a Bio-TEK ELx405 and analyzed on the Meso Quick Plex SQ 120 plate reader. TARC (K151NTD) was measured according to manufacturer's specifications. Levels of IL-13 (102732) and IL-17A (101599) were assessed by Quanterix single molecule array (Simoa) bead-based 2.0 assays on the Simoa HD-1 analyzer per manufacturer protocols.

Statistical Analyses

Correlation analysis of AD severity

Repeated measures correlation (R package: rmcorr) was applied to SCORAD and log2-transformed serum

protein concentration across all years to account the dependent structure of the same patient over time for each marker and Benjamini-Hochberg multiple comparison adjustment across markers to report the correlation values and adjusted p -values [12]. Volcano plots were used to identify significant correlations (i.e., adjusted p -values <0.05 and repeated measures correlation >0.3 or <-0.3).

Serum protein changes over time

For each analyte, a mixed-effect model with year, gender, and race as fixed covariates was applied to the log2-transformed analyte concentration. Comparisons of each analyte at each time point (Y1 to Y2, Y1 to Y5, and Y2 to Y5) were made and significance assessed using the Tukey Multiple Comparison Test. Volcano plots were generated and used to identify changes from Y5 to Y1 and from Y2 to Y1 (p -values <0.05 and fold change >1.5X or <-1.5X).

Prediction model of SCORAD change

The prediction model of the change in SCORAD at Y5 to Y1 with all the baseline (Y1) analytes concentrations was built using JMP [13]. With forward selection, starting with null model and add the analyte most predicted the most on dependent measure (e.g. smallest p -values) one by one until including analyte predictors provided no additional predictive power. The stopping criteria is based on minimum corrected Akaike Information Criterion (AIC) [14]. To evaluate the performance of prediction model, we calculate the R squares and RMSE (Root Mean Square Error) between predicted values and observed values for all patients and RMSE. Furthermore, the cross validation on the proposed prediction model with selected markers was applied with 100 iterations [15]. At each iteration, the entire baseline patient set was split into training set (75%) and testing test (25%). The training set data was used to build the model and estimate model parameters. The model was then used to predict the testing set data. The R squares and RMSE between predicted values and observed values were calculated for each iteration to assess the robustness of prediction model.

Pathway analysis

Pathway analysis was conducted with MetaCore using Fisher's exact test to define relevant pathways based on differentially expressed serum protein signatures between Y5 and Y1 samples [16].

RESULTS

This study sought to understand the interplay among analytes, relationships to disease severity, and to delineate patterns of dysregulation through the first five years of life. Serum samples were collected from a total of 87 pediatric AD patients upon entry, one, and four years later [9]. The mean age at study entry was 10.4 months and the population was evenly distributed both by gender and between Caucasian and African American ethnicities (Figure 1). Incidence of asthma at Y5 was observed in 65% of patients. AD severity, measured by SCORAD, was mild to moderate at time of enrollment (range 1-28) and maintained an average between 10.1-14.7 across the study years (Figure 1). A total of 126 serum proteins, including IgE, were analyzed across different platforms to identify relevant connections between various immunological markers and disease progression, using SCORAD, across each time point of data collections. An important measure of atopy, circulating IgE, was measured on average to be 29.6 IU/mL at year 1 (Y1; baseline), 32.7 IU/mL at year 2 (Y2; one year after baseline), and 29.9 IU/mL at year 5 (Y5; four years after baseline). In this patient population, IgE was only weakly correlated to SCORAD (corr=-0.22) based on combined analysis across all timepoints of the study (data not shown). Considering this weak correlation, we then shifted focus to other key inflammatory serum markers possessing positive correlation with clinical severity across all 3 visits. The strongest positive correlation to SCORAD across all visits were CASP-8 (corr=0.48) and IL-13 (corr=0.47) (Figure 2). Additional proteins positively correlated with SCORAD in these pediatric AD patients that have been previously linked to severity in adult AD included TARC (corr=0.37) and MCP-4 (corr=0.3) (Figure 2) [17]. Interestingly, we observed proteins shown to be elevated in the circulation of adult AD patients to be negatively correlated with SCORAD across all 3 timepoints taken in the first 5 years of life from these pediatric AD patients, such as sCD40L (corr=-0.3), Eotaxin-1 (corr=-0.3), and IL-7 (corr=-0.4) (Figure 2,

Supplemental Table 1) [17].

Understanding the dramatic developmental changes that occur in early childhood, we utilized volcano plots to visualize differentially expressed proteins between Y1 and both Y2 and Y5 (Figure 3a-b). Proteins which had the highest positive correlations with disease severity, such as IL-13, CASP-8, and TARC (Figure 2a) were observed to have the highest concentrations early at Y1 relative to Y5 (Figure 3a). We also observed decreasing serum concentrations of Th2 markers (TARC, IL-13, and MCP-4) from Y1 to Y5, perhaps indicative of the dramatic elevation at early stages of life for these patients (Figure 3c, Supplemental Figure 1). The Th22 marker, IL-22, a highly expressed cytokine in adult AD was not observed to change over the course of the study, suggesting the absence of a significant role for IL-22 in the first 5 years of pediatric disease within this cohort (Supplemental Figure 1) [18]. Similarly, many Th1/Th17 markers, including CXCL10, IL-17A, and IFNγ shown to be upregulated in adult AD were unchanged in our study [6]; however, elevated serum concentration of the related marker, CXCL11, was observed as early as Y2 (Supplemental Figure 1).

Given the correlation of inflammatory markers with SCORAD, we wanted to determine how these serum proteins associated with each other. Thus, we examined correlations among SCORAD-associated analytes across all time points of the study. We observed strong interconnectivity within two groups of analytes: 1) CD5, CASP8, IL-12b, TRANCE, and TNFRSF9 and 2) CXCL12, CCL19, TRAIL, and IL-7 (Figure 4). Surprisingly, IL-13, which emerged as one of the most significant correlates with SCORAD showed only moderate associations with other protein concentrations across all timepoints (Figure 4, Supplemental Table 1). This observation, coupled with our finding that many inflammatory markers, such as IL-13, decrease in concentration over the course of the study as patients age and utilize standard of care therapies initiated further assessment of pathway nodes connecting these markers to disease severity.

Molecular processes linked to the analytes with higher concentrations observed at Y1 relative to Y5 were significantly associated with pathways related to T cell activation and cytokine secretion profiles including Immune response pathways in T cell differentiation and cytokine secretion (Figure 3a, Figure 5a). Juxtaposed with the Y1 pathway analysis of T cell driven response mechanisms in early AD development, Y5 pathway analysis highlights the shift from T cell driven disease to include innate immunity, links to Langerhans and Dendritic cells' presence in allergic dermatitis, and the rise of asthma-related mast cell mechanisms over time (Figure 5a-b). Proteins increased in concentration at subsequent visits (Y2 and Y5) relative to Y1 were sCD40L, ST1A1, 4E-BP1, CXCL12, CXCL11, CCL19 and pathway analysis connected these to innate mechanisms (Figure 3b, Figure 5b). Most notably, a 34-fold magnitude increase was observed in the circulating levels of sCD40L at Y5 as compared to Y1 (Figure 3a). Molecular processes observed to be upregulated at Y2 from Y1 were also affiliated with innate immunity (Figure 3b) and tracked with similar innate cell-influenced mechanisms observed at Y5 from Y1 (Figure 5b). Several of these markers overlapped between Y2 and Y5 and all markers significantly higher concentrations in Y5 were also seen in Y2 highlighting the early onset of these mechanisms (Figure 3a-b). These differences reiterate the idea that blood samples from pediatric AD patients contain strong Th2-driven signals early, though levels decrease slightly with age as Th1 and innate-linked inflammatory markers develop.

Utilizing heatmap visualizations, we next assessed the correlations of serum protein analytes to T cell phenotypes using previously described flow cytometry analysis of PBMCs from the same individuals demonstrating sweeping responses for analytes shown to track with changes in T cell development from Y1 to Y5 (Figure 5c) [11]. We observed distinct profiles at Y1 between circulating Th2 and Treg cells and epithelial-derived chemokines, including two proteins most significantly upregulated at Y1 to Y5; MIP-1a, sCD40L, CCL20, and IL-8 all showed positive correlations with Th2 populations and were unchanged or negatively correlated with Treg populations indicating these signaling mediators promote T-cell mediated immune responses very early in pediatric development (Figure 5c). sCD40L showed strong correlations at both Y1 and Y5 with Th2 cells and did not correlate with any other cell types (Figure 5c). Th2 cytokines, IL-4 and IL-5, strongly correlated with NKT cell populations at Y1 and Y5, respectively, and IL-9 and PDFGa2 positively correlated with Treg levels in Y1, but not at Y5 (Figure 5c).

Notwithstanding the complexity of immune responses observed in these samples, the longitudinal nature of this study suggested that we may be able to identify predictive biomarkers related to the course of disease. We utilized the forward selection modeling approach with an Akaike information criterion (AIC) stopping rule to predict the change of SCORAD from Y1 to Y5 using the Y1 inflammatory protein concentrations. A biomarker panel of 18 analytes was selected in the prediction model to predict progression of severity from infancy to Y5 (Figure 6a, R^2=0.64, p=0.0058). To further illustrate the robustness of prediction, a cross validation with 100 iterations on the proposed 18 analytes predicted model resulted in the mean of the R squares and root of mean square error (RMSE) of 0.77 and 3.5 for the 100 iteration, respectively (Figure 6b). The highlighted serum proteins, which could be involved in persistence or resolution of AD, include IL-18, MMP-10, and IL-13 (Figure 6).These analyses highlight the objective and predictive nature of correlated analytes present in the serum of infants for subsequent disease severity.

DISCUSSION

Minimally invasive biomarkers, such as serum proteins, are widely sought after for their value in defining disease states and outcomes. In adolescent and adult AD patients, a high total serum IgE level was a predictive factor for poor disease prognosis [19]. Interestingly, in these pediatric patients, many inflammatory serum biomarkers demonstrated stronger correlation to disease severity better than IgE alone [20]. Thus, the focus of our study was to highlight key biomarkers in early AD that correlate with disease and potentially predict the persistence or resolution of disease.

Multiple analytes correlated strongly with pediatric AD severity and, most significantly, known disease markers, IL-13, MCP-4, and TARC, correlated with SCORAD across all time points from infancy to 5 years of age, recapitulating the importance of type 2 immune response early in AD pathogenesis. Correlations with chemokines and monocyte chemoattractant proteins underscores the role of eosinophils and monocytes in early AD, while TARC and IL-13 are both mediators of Th2 inflammation and implicated in pediatric and adult AD, emphasizing that these analytes could represent critical nodes for better understanding AD heterogeneity and disease progression [7, 21, 22]. Although standard of care treatments lead to a decreased concentration of these biomarkers, the observations and changes observed in our study demonstrate the strong relationship these markers have with the disease pathology in pediatric patients. Importantly, this study included ultrasensitive measurements of numerous cytokines for which quantitative assessment has only recently been possible, including IL-13, which is implicated as a driver of AD based on clinical responses to lebrikizumab and dupilumab, further implicating IL-13 in disease severity even in infancy [5, 23, 24].

Analytes described as drivers of adult AD, such as IL-22 and IL-17A [7, 18], did not possess strong correlations to disease severity within these pediatric AD samples. This is likely due to the slow increase in serum concentration over time, limiting the role of IL-22 in pediatric AD as seen in previous reports [7]. Though levels of IL-17A were not correlated to SCORAD and present in very low levels, as seen in previous pediatric studies [25], Th17-related fibroblast markers, CCL19 and CCL20, were observed at various timepoints throughout our study and CCL20 positively correlated with Th2 circulating cell populations at Y1 pointing toward an intriguing immunological overlap of inflammatory fibroblasts and T cells. Pathway analysis of proteins upregulated in Y5 samples reveals the emergence of innate pathways over time and multiple gene ontology results indicate that dendritic cells (DCs) may increase with age. Increased conventional dendritic cells were previously described as being protective for wheezing in these pediatric eczema patients [10], and stimulation of these conventional DCs isolated from atopic infants produced more interferon and IL-10 than those derived from non-atopic infants at initial visit and the one year follow-up visit [26]. Mounting evidence demonstrates this appearance of symptoms known as the atopic march tracks with early disruption of immune development and can have lasting effect into adulthood [27]. This link between increased CCL19 and DCs further indicates a role in atopic march from AD to asthma for CCR7, the receptor for CCL19, which can coordinate T cell, B cell, and DC responses across pathologies [28].

Matrix remodeling proteins (MMP-10) and chemoattractant molecules (MCP-3, MCP-2, CXCL5) provide further insights into the complexity of disease mechanisms significantly associated with the progression of the pediatric form of AD. The connections of these markers to disease severity contextualize a shift

toward Th1 immunity and myeloid involvement developing throughout the course of the pediatric AD patient journey concomitant with dominant Th2 responses throughout. The identification of CXCL5 and IL-5 as important disease severity predictive biomarkers for pediatric AD highlights the innate allergic response early in disease progression. The high levels of sCD40L that we observed in pediatric patients aligned with prior published observations that this protein is elevated in the blood of pediatric patients [6]. Additionally, sCD40L concentrations correlated throughout the study with CD4+Th2 cells which might link to the role of CD4+CD40L+ cells in the priming of other immune cell types including CD14+ monocytes and CD8+ T cells [29]. This work aids our contextualization of a shift toward Th1 immunity and myeloid involvement developing throughout the course of the pediatric AD patient journey concomitant with Th2 responses presenting initially and persisting throughout the course of disease.

The previously reported circulating protein profiles of pediatric AD have provided robust assessment of correlation analysis at a single timepoint [6]. Our study builds upon these important observations via the monitoring of circulating biomarkers over a development period of four years adding value to the concept that objective biomarkers could be used to objectively assess disease severity, as has been demonstrated in adult AD [30]. We observed subsets of inflammatory proteins that correlated with each other in a manner consistent with early disease skewing toward type 2 immune responses. Importantly, forward selection modeling identified 18 analytes, such as IL-18, IL-13, and MMP-10, in infant serum that could be used to predict AD severity four years later; therefore, these analytes might represent critical nodes to better understand AD heterogeneity and disease progression. The measurement of markers identified in Figure 6 should provide guidance for therapeutic intervention strategies at early stages of life for pediatric patients suffering from AD. This model will likely have predictive value for AD persistence past infancy and will be useful in further defining the pathogenesis of atopic disease allowing us to begin to see ever branching nodes delineating the complex underlying immune regulation. This, in turn, may guide therapeutic intervention and the prognostic value of such serum protein measurement, outlining the need for early treatment options in pediatric patients.

Our data highlights additional questions for the field regarding the progression of AD as children age through adolescence into adulthood. We did not observe elevation of IL-4 in the serum of these patients, which could be attributed to the technical limitations of assays available at time of testing, considering that IL-13 was measured on an ultra-sensitive platform. We also note that though MCP-4 and TARC were moderately correlated with disease severity, they did not factor into the predictive modeling of disease progression possibly due to these proteins being more broadly involved in atopic mechanisms beyond AD [31]. Future studies with focused outcomes on other atopy measures can provide predictive insight into mechanisms of wheezing or lung function. Correlations to SCORAD allow us to minimize potential error that is introduced in a longitudinal study, especially in pediatrics as maturation and growth are heavily involved in immune system development. This cohort and data included in this manuscript enhance our knowledge regarding the systemic presentation of AD in children and potential biomarker profiles which aid in predication of disease persistence or resolution.

Abbreviations:

AD: atopic dermatitis

AIC: Akaike information criterion

Corr: correlation

DC: dendritic cell

IL: interleukin

MCP: monocyte chemoattractant protein

MSD: mesoscale discovery

PBMC: peripheral blood mononuclear cell

TARC: thymus and activation regulated chemokine

Y1: year 1

Y2: year 2

Y5: year 5

FIGURE LEGENDS

Figure 1: Sample Population Characteristics. This study included a cohort of 87 patients with serum sample collection at infancy (Y1), 1-year follow-up (Y2), and 4 years later (Y5). Samples collected from children diagnosed with dermatitis for this study have been previously described and assessed for clinical features including asthma [9].

Figure 2: Correlations to SCORAD. (a) Repeated measurement correlation of SCORAD to the concentration of all analytes across all study years. (b) Log2 protein expression of serum protein concentrations correlated to SCORAD for key analytes of interest. 1= year 1; 2= year 2; 5= year 5.

Figure 3: Analyte Patterns in the Population. (a, b) Volcano plot showing serum protein change from Y1. Proteins expressed at Y1 that decrease in Y5 or Y2 are colored blue. Proteins expressed at Y1 that increase at Y5 or Y2 are colored red. (c) Log2 protein expression of serum protein concentrations for key analytes of interest. *significant change from Y1, p-value <0.05; Y1= year 1; Y2= year 2; Y5= year 5.

Figure 4: Correlations Among Cytokines. (a) Heatmap representation of cytokine correlations to one another shown with additional proteins of interest across the course of the study. (b) Visual representation of cytokine correlations to each other (thickness of connecting line) and to SCORAD (size of circle) across all time points of the study. Red color denotes correlations [?]0.35. Blue color denotes correlations [?]0.35.

Figure 5: Pathway analysis. (a) Top 10 pathway maps for highly expressed proteins at year 1 that decrease expression by year 5. The 28 specific proteins used for this analysis are outlined in Figure 3a. (b) Top 10 pathways of most highly expressed proteins at year 5 that were low or absent at year 1. The 6 specific proteins used for this analysis are outlined in figure 3a. (c) Heat map correlations of cell phenotypes previously assessed by flow cytometry [11] to serum protein analysis from same time point.

Figure 6: Predictive Model. (a) Using forward modeling with AIC, we use the listed 18 serum analytes to predict change in SCORAD over time. The fit of these 18 analytes within this model are defined by root mean square error (RMSE) and R square adjusted (R^2). (b) Samples were split into either test or training sets to assess the reproducibility of the model.

Supplemental Figure 1: Analyte Patterns in the Population. Log2 protein expression of serum protein concentrations for analytes of interest. Y1= year 1; Y2= year 2; Y5= year 5.

Supplemental Table 1: Correlations to SCORAD and Among Cytokines . Repeated measurement correlations of SCORAD to the concentration of selected analytes and correlations of cytokine correlations to one another across all study years.

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