The blood proteomic signature of early-onset pediatric atopic dermatitis shows systemic inflammation and is distinct from adult long-standing disease

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Background: Despite increasing evidence that adults with long-standing atopic dermatitis (AD) have systemic inflammation, little is known about systemic inflammation in recent-onset early pediatric AD.

Objective: To analyze blood inflammatory proteins of early pediatric AD.

Methods: Using high-throughput proteomics (proximity extension assay), we assessed 257 inflammatory and cardiovascular risk proteins in the blood of 30 children with moderate to severe AD younger than 5 years of age (within 6 months of onset) compared with age-matched pediatric control individuals and adult patients with AD.

Results: In pediatric AD blood, T helper (Th) type 2 (CCL13, CCL22) and Th17 (peptidase inhibitor-3/elafin) markers were increased, together with markers of tissue remodeling (matrix metalloproteinases 3/9/10, urokinase receptor), endothelial activation (E-selectin), T-cell activation (IL2RA), neutrophil activation (myeloperoxidase), lipid metabolism (FABP4), and growth factors (FGF21, transforming growth factor-α). Total numbers of dysregulated proteins were smaller in pediatric AD (n = 22) than in adult AD (n = 61).

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Clinical severity scores were positively correlated with receptors for interleukins 33 and 36 and inversely correlated with some Th1 markers (interferon gamma, CXCL11).

Limitations: Different baseline expression levels in healthy pediatric vs adult samples.

Conclusions: Within months of pediatric AD onset, systemic immune activation is present, with Th2/Th17 skewing but otherwise different proteomic patterns from adult AD. Future correlation of proteomic patterns with disease course, comorbidity development, and drug response may yield predictive biomarkers. (J Am Acad Dermatol 2019;81:510-9.)

Key words: Atopic dermatitis; infant; multiplex assay; Olink; pediatric; peripheral blood; proximity extension assay.

CAPSULE SUMMARY

- Pediatric patients with early-onset moderate to severe atopic dermatitis already show systemic signs of inflammation within months of onset and before chronic disease develops.
- These data suggest the need for early intervention in pediatric atopic dermatitis to prevent disease chronicity and perhaps development of atopic comorbidities ("atopic march") and other associated disorders.

Atopic dermatitis (AD) is often associated with allergic comorbidities such as asthma, rhinoconjunctivitis, and food allergies.[1] Increasing evidence in adults suggests that AD is also associated with cardiovascular disease,[2-6] potentially rooted in decades of chronic systemic inflammation, because the majority of AD cases begin before 5 years of age. Consistently, adults with moderate to severe AD show increases in levels of multiple circulating inflammatory mediators,[7,8] including cardiovascular risk proteins, and increased frequencies of activated blood T cells.[9] While most data on systemic inflammation are based on studies in adults with decades of chronic disease, blood profiling from early pediatric AD (within the first months of disease onset) is limited. Increased odds of obesity, hypertension, and hyperlipidemia have been noted in children ages 0 to 17 years.[10] Pediatric AD is also associated with increases in autism spectrum and attention deficit hyperactivity disorders (ADHD), particularly among those with earlier-onset or more-severe AD.[11] The link of ADHD with pediatric AD is hypothesized to relate to early exposure of the brain to inflammatory cytokines.[12] Given the challenge of obtaining skin biopsy samples, finding biomarkers for comorbidity risk in blood would be ideal.

Recent profiling studies in infants, young children,[13,14] and adolescents[15] show that early AD is T helper (Th) type 2, but also Th17, polarized in skin.[13,14] Blood studies have investigated smaller numbers of preselected markers[15-40] and have identified biomarkers correlating with disease activity, especially Th2-associated mediators (ie, interleukin [IL]-31, CCL17 (thymus and activation regulated chemokine [TARC]), CCL22, and CCL27).[32-36] Increases in memory T cells have been observed throughout life,[41-44] and we recently described expansion of Th2 T cells, but not other polar T-cell subsets, in early pediatric AD blood.[29] Overall, newborns show lower percentages of $CD4^+$ and $CD8^+$ interferon gamma (IFN-γ)—producing peripheral blood mononuclear cells,[43] and further reduction in IFN-γ secretion predisposes to atopy.[18] Consistently, increases in Th2 activation and lower frequencies of IFN-γ responses early in life are suggested to increase the risk of developing AD.[16,17] However, unlike in adults with chronic AD,[7,8,45] a broad, array-based proteomic approach to characterize the peripheral blood phenotype of early AD is lacking.

METHODS

Patients

Thirty children (≤5 years) with moderate to severe AD within 6 months of disease onset were included (Table I). Exclusion criteria were active skin infection and use of topical glucocorticosteroids/immunomodulators within 1 week and systemic immunosuppressants within 4 weeks. Before study inclusion, parents signed institutional review board—approved written consent, according to the Declaration of Helsinki. Clinical severity scores included the SCORAD (SCORing of Atopic Dermatitis), EASI (Eczema Area and Severity Index), BSA (body surface area; as a percentage), and Atopic Dermatitis Quickscore pruritus score. Transepidermal water loss (TEWL) of lesional and nonlesional skin was performed by using a Tewameter (Courage and Khazaka GmbH,

Abbreviations used:

AD:	atopic dermatitis
BSA:	body surface area
EASI:	Eczema Area and Severity Index
FDR:	false discovery rate
IFN:	interferon
MMP:	matrix metalloproteinase
PI3:	peptidase inhibitor-3
SCORAD:	SCORing Atopic Dermatitis
SELE:	E-selectin
TARC:	thymus and activation regulated chemokine
TEWL:	transepidermal water loss
TGF:	transforming growth factor

Cologne, Germany). Symptoms of current food allergy, allergic rhinoconjunctivitis, and asthma were present in 4, 2, and no children, respectively. Nineteen age-matched control samples were obtained from healthy children without personal or family history of atopy. Proteomic results were compared with blood data from 58 adult AD patients with comparable severity, as well as 18 adult control individuals, both from a published cohort (Table I).[46] Transcriptomic skin biopsy data (Affymetrix Human U133Plus 2.0 arrays, Affymetrix, Santa Clara, CA) from a published cohort of 19 lesional pediatric AD (≤5 years of age and within 6 months of disease onset) and 17 pediatric healthy control skin samples were used for blood-skin comparisons.[13] Transcriptomic whole-blood data (Affymetrix Human U133Plus 2.0 arrays) from 15 of our pediatric AD patients (same blood draw) were available for blood cell-plasma correlations from a previously published data set.[47]

Blood protein quantification

Blood was collected and centrifuged, and plasma samples were stored at −80°C until further processing. Aliquots were analyzed by a proteomic Olink (Uppsala, Sweden) Proseek multiplex assay (a proximity extension assay with oligonucleotide-labeled antibody probe pairs).[48-50] This method quantifies hundreds of protein levels from 10 μL serum by using an antibody-mediated detection system linked to synthetic DNA for quantification by a real-time polymerase chain reaction platform.[49] Samples were analyzed with the Olink Inflammation I, and the cardiovascular disease II and III panels, which contain 257 established and exploratory markers.[49]

Statistical analyses

Analyses were performed using R language (R-project.org) and packages available through the Bioconductor Project (www.bioconductor.org).[46] Quality control of Olink data was performed with Olink's standard quality control pipeline.[48,49] Protein expression profiles were modeled by using linear models for high-throughput data on R's *limma* framework. The model used disease as a factor and age, sex, and body mass index (BMI) as covariates. Fold changes for comparisons were estimated with an empirical Bayesian method,[51] and hypothesis testing was conducted using contrasts under the general framework for linear models in the *limma* package. *P* values from the moderated (paired) *t* test were adjusted for multiple hypotheses by using the Benjamini-Hochberg procedure. Genes with a fold change greater than 1.3 and a false discovery rate (FDR) of less than 0.1 were considered differentially expressed. Protein annotations and gene-protein relationships were obtained by using the UniProt ID and R's *AnnotationDbi* package.

RESULTS

Proteomic differences in peripheral blood between early pediatric and adult AD

Differentially expressed proteins were plotted in a heat map (Fig 1). Consistent with the Th2- and Th17-skewed inflammatory milieu described in pediatric skin,[14] Th2 (CCL13, CCL22) and Th17 (PI3/elafin) markers were most strongly increased in pediatric compared with healthy control blood. We also found increases in E-selectin (SELE), a cell adhesion molecule of endothelial cells. Other inflammatory markers upregulated in pediatric AD included proteins involved in tissue remodeling (matrix metalloproteinase [MMP3], MMP9, MMP10, myeloblastin), T-cell activation (IL2RA or CD25, a receptor component of the T-cell growth factor IL-2), lipid metabolism (fatty acid binding protein-4 [FABP4], a treatment target in cardiovascular disease and diabetes[52]), and angiogenesis/coagulation (urokinase receptor). We also found increases in growth factors FGF21 (fibroblast growth factor-21), a liver-derived molecule that is increased in insulin-resistant morbidities such as type 2 diabetes mellitus or obesity,[53] and transforming growth factor (TGF)-α, an analogue of epidermal growth factor mediating epithelial development, epidermal hyperproliferation, and tumorigenesis. Other upregulated molecules included the potent leukocyte chemoattractants CCL2 and CCL15, neutrophil protein myeloperoxidase (MPO), lymphotoxin-β receptor LTBR (a member of the tumor necrosis factor receptor superfamily involved in lymphoid tissue development), and the IL-1 decoy receptor IL1R2, which limits IL-1-α/β pathway activation by preventing receptor binding of IL-1α and IL-1β.

Table I. Baseline characteristics of study cohorts for proteomic blood analyses

Characteristics	AD pediatric	Healthy pediatric	*P* value, pediatric	AD adult	Healthy adult	*P* value, adult
Sample size, n	30	19		58	18	
Age, y, mean (SD)	1.8 (1.6)	2.1 (1.3)	.526	40.3 (15.2)	41.3 (10.3)	.801
BMI, kg/m^2, mean (SD)	17.1 (1.9)	15.9 (1.7)	.891	27.8 (6.1)	27.6 (4.4)	.891
SCORAD, mean (SD)	52.4 (16.7)	NA		54.3 (13.1)	NA	
Sex, n (%)			.733			.474
Female	13 (43.3)	10 (52.6)		27 (46.6)	6 (33.3)	
Male	17 (56.7)	9 (47.4)		31 (53.4)	12 (66.7)	
Race, n (%)			.087			.656
Asian	6 (20.0)	0 (0.0)		15 (25.9)	3 (16.7)	
Black	6 (20.0)	3 (15.8)		23 (39.7)	9 (50.0)	
White	18 (60.0)	16 (84.2)		20 (34.5)	6 (33.3)	

AD, Atopic dermatitis; *BMI*, body mass index; *NA*, not applicable; *SCORAD*, SCORing Atopic Dermatitis; *SD*, standard deviation.

Overall, children with early-onset AD had a smaller set of upregulated proteins (n = 22) than adults with long-standing AD (n = 61) (Fig 1). Only 3 proteins (CCL13, MMP10, TGF-α) were upregulated versus control samples in both pediatric and adult blood. Upregulated proteins in adult AD included a broader array of Th2- (CCL17, CCL13, IL-13) and Th17- (CCL20) associated markers but also of Th1 (CXCL9, CXCL10, CXCL11, interferon gamma [IFN-γ]) markers, which were not upregulated in pediatric AD blood, consistent with Th1 activation as a marker of AD chronicity.[54]

Among healthy control individuals, levels of Th1- (CXCL9, CXCL10, CXCL11), Th2- (CCL13, CCL17), Th1/Th17- (IL12B-IL12/IL23p40) and Th17- (CCL20) associated proteins, the regulatory cytokine IL-10, and markers of general inflammation (MMP12), endothelial activation (SELE), the neutrophil chemoattractant CXCL6, and the β-oxidation enzyme 2,4-dienoyl-CoA reductase (DECR1) were all greater in young children than in adult healthy control individuals (Fig 1). In contrast, healthy adult control individuals had increased levels of chemokines CCL28 and CCL8 (which have antimicrobial activity),[55,56] the hematopoietic growth factor IL-7, and matrix metalloproteinases 3 and 9 compared with healthy pediatric control individuals (Fig 1).

We also performed Spearman correlation of absolute blood (plasma) protein levels with matching whole-blood cell messenger (mRNA) expression in the 15 AD children for whom the same sample was available for both assays. Although a few markers were significantly correlated ($P < .05$ in Table II), none of these were among the 22 markers upregulated in pediatric AD plasma, suggesting the independent regulation of soluble blood protein and blood cell gene-expression patterns in early-onset pediatric AD.

Skin and blood comparisons

We next compared upregulated blood proteins (fold change >1.3 vs healthy control samples) with corresponding upregulated skin mRNA levels from a recently described similar pediatric AD population.[13] In our panels, there were only 9 shared upregulated inflammatory and cardiovascular markers: PI3/elafin (Th17), CCL22 (Th2), markers of endothelial activation (SELE) and tissue remodeling (MMP 10), T-cell activation (IL2RA), and the growth factor TGF-α, chemokine CCL2, lymphotoxin-β receptor LTBR, and kallikrein 6 (KLK6) (Fig 2).

Correlations between proteomic markers and skin disease scores

Given the limited number of circulating markers increased in both blood and skin, we focused on blood protein biomarkers of AD severity, regardless of skin expression. We plotted blood proteomic levels and clinical severity measures (SCORAD, EASI, BSA) in a correlation heat map (Fig 3). We also added lesional and nonlesional TEWL, a functional measure of the skin barrier,[57] and BMI, which is correlated with cardiovascular risk.[58] Positive correlations with SCORAD, EASI, and BSA (Fig 3, blue box) were found for the soluble cytokine receptors ST2 (IL1RL1) and IL1RL2. ST2 is the receptor for the Th2-inducing cytokine IL-33,[59] which is critically involved in IL-33–mediated food allergy via skin sensitization.[60] IL1RL2 is a receptor component for IL-36 (IL-36A, IL-36B, IL-36G), which is increased in skin lesions of early-onset pediatric, but not adult, AD.[13] BSA and SCORAD were also positively correlated with the endothelial activation marker SELE. BSA was positively correlated with MMP12, KLK6, and CCL22. Other positive correlations between SCORAD, EASI, and/or BSA were found for heat shock protein HSP27, protease-activated receptor PAR-1, the IL-1 decoy receptor IL-1RT2, and galectin-3

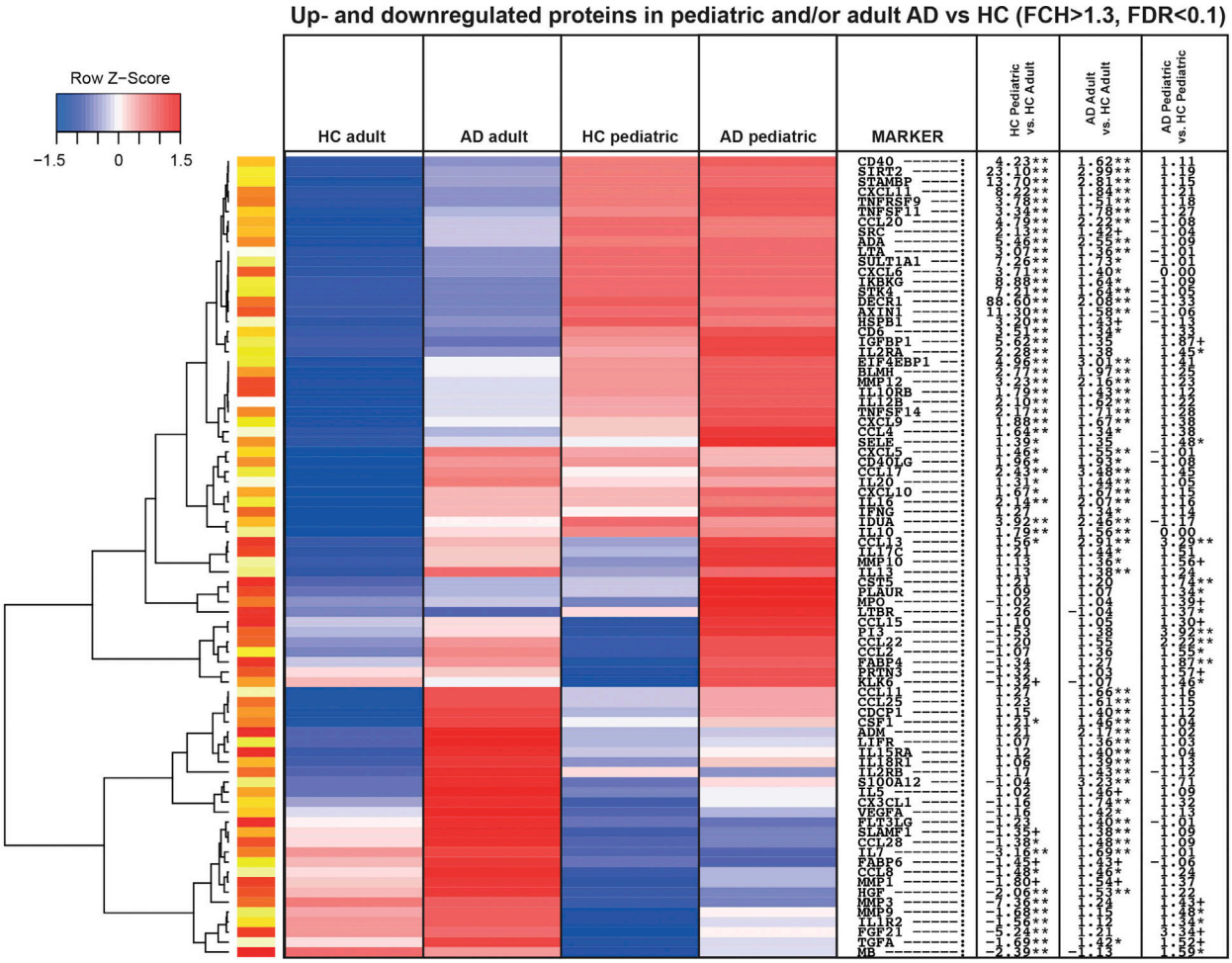

Fig 1. Atopic dermatitis. Heatmap of differentially regulated proteins (fold change > 1.3, FDR < 0.1), with markers grouped by unsupervised hierarchic clustering (data-driven organization of patterns into useful groups) in either adult or pediatric AD compared with their age-matched control samples. $^{+}P < .1$, $^{*}P < .05$, and $^{**}P < .01$ are the *P* values for significance versus comparator, as shown in each column. The expression of many blood proteins is higher or lower in pediatric versus adult AD, but not when compared with age-matched pediatric healthy control blood. Red color denotes higher and blue color denotes lower mean expression levels. *AD*, Atopic dermatitis; *FCH*, fold change; *FDR*, false discovery rate; *HC*, healthy contol individuals.

(Gal-3) (Fig 3, blue box). Surprisingly, the level of CCL17 (TARC), considered the most reliable serum biomarker of AD skin disease activity in adults and children,[28] was not correlated with disease severity.

Negative correlations between skin activity scores (SCORAD, BSA, and/or EASI) and blood proteins (Fig 3, green box) were found with the Th1-associated markers IFN-γ, CXCL11, and CCL2 (MCP-1). Skin scores were also negatively associated with neutrophil chemoattractants CXCL5 and CXCL6, the hematopoietic growth factor macrophage colony-stimulating factor (CSF-1), leukemia inhibitory factor receptor (LIF-R), MMP1, the β-oxidation enzyme DECR1, and fractalkine (CX3CL1). Some were also negatively correlated with pruritus or lesional TEWL (Fig 3, black box). BMI showed only weak correlations with PI3/elafin, IL-16, and IL-2RA (Fig 3). We also found a larger correlation cluster (Fig 3, pink box) comprising the general inflammation marker MMP12 with PI3/elafin (Th17), SELE (endothelial activation), CCL22 (Th2), and others, indicating their common regulation.

DISCUSSION

Whole-skin profiling studies of infants and young children with recent-onset AD have shown considerable differences versus adults with long-standing disease.[13,14] This study extends these differences to

Table II. Correlation of plasma protein levels with corresponding whole-blood messenger RNA levels in pediatric blood samples*

Marker	Correlation coefficient (r)	P value
TFRC	0.689285714	.0058
CD274	0.620790837	.014
CXCL1	−0.6	.02
HSPG2	−0.591607978	.02
IL1R1	0.592857143	.022
THBD	−0.589285714	.023
PAPPA	0.565267106	.028
TNFSF11	0.553571429	.035
TNFRSF10A	0.542857143	.039
CA5A	−0.536774032	.039
TNFSF13B	0.528571429	.045
CD93	−0.521428571	.049

*Protein and messenger RNA data from the same individual were available for 15 pediatric AD patients. Spearman correlation was limited to markers that were detectable in both assays (n = 202 markers, data not shown). Correlations with $P \leq .05$ are depicted in this table.

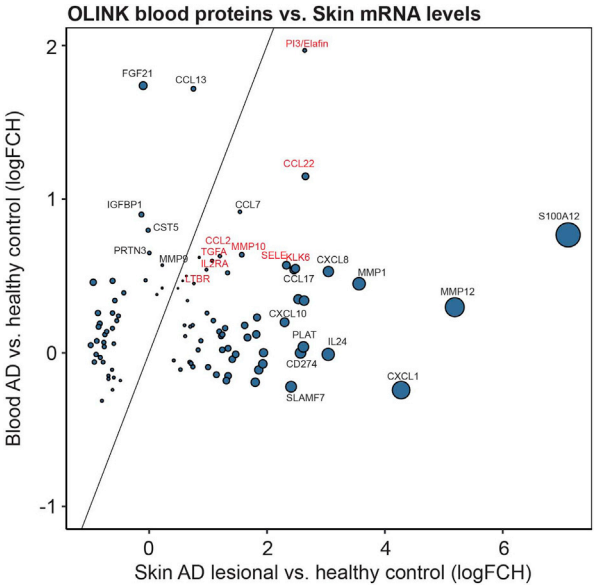

Fig 2. Atopic dermatitis. Scatterplots comparing upregulated differentially expressed genes in skin (mRNA levels) and proteins in blood (fold change > 1.3, FDR < 0.1), depicted as fold change over respective healthy control samples; the size of the circle represents the absolute difference between $\log_2$ fold change blood and skin. Markers significantly upregulated in both blood and skin are indicated in red letters. *AD*, Atopic dermatits; *FCH*, fold change; *FDR*, false discovery rate; *mRNA*, messenger RNA.

blood by using a large-scale proteomic platform. Overall, blood profiles of early pediatric AD show Th2/Th17 skewing of soluble proteomic markers, with absence of the Th1 upregulation seen in adults with AD, consistent with the skin immune phenotype in early AD,[13,14] and complementing our recent article[47] showing increased eosinophil and Th2 mRNA markers in early pediatric AD whole blood cells on a transcriptomic level, with decreases in IFN-γ/Th1 that were also negatively correlated with clinical severity. Together with increases in inflammatory markers of endothelial cell activation (SELE) and tissue remodeling (MMP3, MMP9, MMP10, urokinase receptor), our data emphasize the systemic alterations within months of disease onset, potentially suggesting the need for more aggressive intervention before the establishment and perpetuation of chronic disease.

Blood fractalkine (CX3CL1), which is involved in T-cell retention in inflamed tissue and leukocyte adhesion to endothelial cells,[61] is significantly increased in adult AD[7,62] and was previously reported to be increased in pediatric AD, correlating with clinical severity.[63] In our study, CX3CL1 negatively correlated with SCORAD, which may reflect the early disease, young age, and lack of disease chronicity of our AD patients; the age of previously published children was 5.4 ± 2.2 years (mean ± standard deviation)[63] versus our cohort age of 1.8 ± 1.6 years.

Surprisingly, the previously described positive correlation of SCORAD with CCL17 (TARC) was absent.[34,64] It is unlikely that the difference reflects our evaluation of plasma versus the more typical serum samples. However, most studies showing correlations of CCL17 with AD severity in children involved patients older than 4 years.[28] The few studies that evaluated blood in infants and/or toddlers investigated Asian populations,[28,34,36] whereas our patients were mostly white/European American. The only other exception is a study by Ahrens et al[65] in Germany, which showed a correlation between CCL17 levels and SCORAD in infants who were food and airborne-allergen sensitized but not those who showed no food sensitization. Our infants with AD largely had no allergic comorbidities (food allergies, asthma, or allergic rhinoconjunctivitis), although specific types of immunoglobulin E to detect food sensitization were not tested. Thus, the difference may, in part, relate to the age, recent AD onset, and limited atopic profile of our cohort.

We also compared blood protein profiles with both blood and skin transcriptomic profiles of early AD. We did not find correlations between soluble blood protein levels that were upregulated in pediatric AD and mRNA expression of corresponding whole-blood cell samples, suggesting that blood proteins might be derived from sources

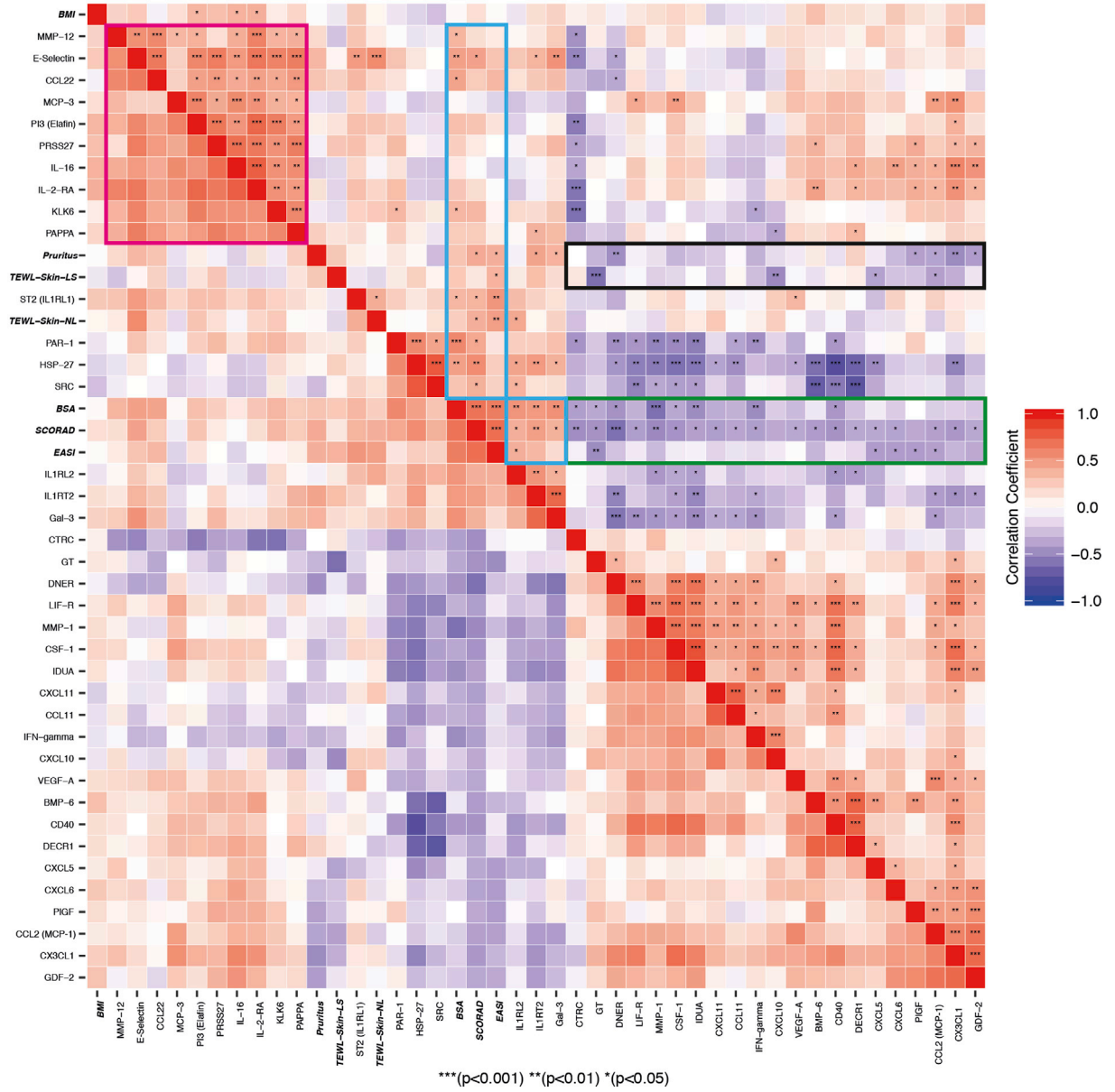

Fig 3. Atopic dermatitis. Heatmap of Spearman correlations between blood proteins and clinical scores such as SCORAD (SCORing Atopic Dermatitis, which captures itch and insomnia), EASI (Eczema Area and Severity Index), BSA (body surface area), pruritus intensity, BMI (body mass index), and TEWL (transepidermal water loss). Markers were selected for top significance levels for positive or negative correlations with SCORAD; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. Red denotes positive and blue denotes negative correlations. Colored boxes denote correlation clusters with inflammatory markers (pink box) and clinical disease characteristics (blue for positive and green for negative correlations with SCORAD, BSA, and EASI severity scores; black for negative correlations with pruritus and TEWL). Clinical scores are in bold font.

independent of circulating blood cells, such as the skin. When comparing blood proteins with skin mRNA levels, we found a limited set of mutually upregulated markers, including those associated with Th2 and Th17 responses. Differences between extracellular versus intracellular expression and posttranscriptional changes could also account for inconsistencies between protein and mRNA levels.

Food sensitization tends to appear in pediatric patients with more severe AD.[65,66] In this regard, we found strong positive correlations between soluble

blood ST2 levels and pediatric AD skin severity scores. ST2 (IL1RL1), the IL-33 receptor, is highly expressed on Th2 cells, mast cells, and type 2 innate lymphoid cells,[67] mediating IL-33–mediated food and dust-mite allergy via skin sensitization in mouse models.[60,68] Its role in humans, however, is less clear. Despite the fact that our pediatric patients did not (yet) suffer from allergic comorbidities, the observed correlation of ST2 expression with early disease severity (which has not been observed in older children and adults with AD[69]) suggests the need for investigation of the IL-33/ST2 axis in longitudinal studies beginning early after AD onset. Nevertheless, these data might suggest that IL-33–targeting strategies, which are being tested in adults (www.clinicaltrials.gov nos. NCT02920021, NCT03533751), could benefit some children with early AD.[70]

Blood IL1RL2, the receptor for IL-36 cytokines, was also positively correlated with skin disease. IL-36 cytokines are upregulated in infant AD skin but much less so in adult AD skin[13] and might thus be a biomarker specific for early disease. Furthermore, anti-IL-36 treatment approaches, which are being tested for pustular psoriasis (www.clinicaltrials.gov nos. NCT03135548, NCT02978690), may also be applicable for children with AD.

Low IFN-γ responses have been described as a risk factor for developing AD.[16-18] The negative correlation of skin severity with Th1 markers (IFN-γ, CXCL11, and CCL2) was also present in our blood samples, together with negative correlations of multiple additional markers, including mediators of neutrophil chemotaxis (CXCL5, CXCL6). One might speculate that these molecules could have a protective role, but future studies will be needed to confirm this hypothesis.

Overall, our study sheds a more comprehensive light on proteomic regulation in the peripheral blood of early AD. This initial stage might be an attractive window for novel therapeutic approaches to treat both the skin disease and early systemic inflammation. Although blood proteomic and previously published mRNA investigations[47] elicited only a limited number of regulated markers, proteomic blood profiling (potentially in conjunction with noninvasive skin investigations such as tape stripping), during trials or longitudinally, might identify predictive markers of disease response and the natural course of AD in children, including the development of multimorbidities.

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