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Biomarkers in asthma in the context of atopic dermatitis in young children

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Abstract

Background: Diverse pathways stemming from a history of atopic dermatitis (AD) might modulate different biomarkers associated with the development of asthma. Biomarkers associated with AD and asthma separately have been investigated, but none have characterized a combined AD+asthma phenotype. We investigated the clinical and molecular characteristics associated with an AD+asthma phenotype compared with AD, asthma and controls.

Methods: From a prospective birth cohort and the outpatient allergy clinic, we included four groups of 6–12-year-old children: (1) healthy controls (2) previous, current, or present AD without asthma, (3) previous, current, or present AD *and* current asthma and (4) current asthma without AD. We performed clinical examinations and interviews and measured serum IgE, natural moisturizing factors (NMF), and plasma cytokine levels.

Results: We found an increased number of IgE sensitizations in AD+asthma, prominent after stratifying for food allergens ($p < .05$). Pro-Th_2 cytokines CCL18, TSLP, and Eotaxin-3 were elevated in AD+asthma, though not significantly higher than asthma, and elevated in asthma compared with controls. NMF levels were decreased in AD compared with asthma and control groups ($p = .019$, $p < .001$, respectively). NMF levels correlated negatively to sensitization ($p = .026$), though nonsignificant with only the patient groups.

Conclusion: Our results indicate that Th_2 cytokines and increased number of sensitizations are associated with AD + asthma phenotypes compared with AD alone and

that skin barrier impairment as well as decreased airway epithelial integrity may play a role in sensitization and immune modulation. Our findings suggest candidate biomarkers that should be further explored for their functional roles and prognostic potential.

KEYWORDS
asthma, atopic dermatitis, biomarkers, CCL18, NMF, sensitization, TSLP

1 | INTRODUCTION

A recent theory in the atopic march touts a connection between skin barrier impairment and immune dysregulation and implicates diverse pathways initiated by atopic dermatitis (AD) that might modulate specific biomarkers associated with the development of asthma.[1] One of the leading hypotheses in this AD-asthma association is the "outside-inside-outside" theory in which skin barrier impairment triggers immune dysregulation that leads to asthma.[1,2] While potential biomarkers have been studied in the context of AD and asthma separately, information on the clinical phenotype of combined AD and asthma is lacking. Our objective was to explore this leg of the atopic march, from AD to asthma, by describing the clinical characteristics and investigating candidate biomarkers that may be associated with different AD and asthma phenotypes.

We aimed to explore components of this epidermal-immunologic connection by investigating natural moisturizing factors (NMF), a biomarker for skin barrier impairment. NMF is the breakdown product of filaggrin protein and is strongly associated with filaggrin (*FLG*) gene variants, one of the strongest genetic determinants of AD. FLG gene is also associated with an increased risk of asthma; this risk seems increased in the context of AD.[3] We also investigated plasma cytokines including thymic stromal lymphopoietin (TSLP), which belongs to the group of alarmins, skin-associated cytokines triggered by epithelial and endothelial tissue damage to induce Th_2 cytokine production.[4] C–C Motif Chemokine Ligand 18 (CCL18) is also of interest in the atopic dermatitis-asthma association as it has been associated with both an AD and asthma phenotype in several studies albeit investigated separately in these disease entities.[5–8] Cutaneous T cell-attracting chemokine (CTACK/CCL27) and Eotaxin-3 are pro-Th_2 cytokines. CTACK and Eotaxin-3 have been shown to correlate with disease activity in AD.[9,10]

We also explored serum IgE levels that have been demonstrated to be upregulated in asthma and AD as well as the associations between these biomarkers.

Key Message

Biomarkers have been studied in relation to atopic dermatitis (AD) and asthma separately, but studies investigating a combined AD-asthma phenotype, which could elucidate key biomarkers in the development of disease, are lacking. Our results demonstrate that AD-asthma and asthma phenotypes are characterized by elevated levels of pro-Th_2 cytokines, including CCL18, TLSP, and Eotaxin-3, and increased number of allergen sensitization. This suggests that both a defective skin barrier and a leaky bronchial epithelial barrier could lead to increased allergen presentation and upregulation of Th_2 cytokines and are potential biomarkers of disease and disease progression to asthma that could be useful in the clinical setting for diagnosis, prognosis, and therapy.

2 | METHODS

2.1 | Study subjects

Participants aged 6–12 years were recruited consecutively from the Odense Child Cohort (OCC), a population-based prospective birth cohort (described elsewhere[11,12]) and the outpatient allergy clinic at the Hans Christian Andersen Children's Hospital, Odense University Hospital (see Appendix S1). The data collected for this study are from two ancillary sub-studies and the data overlap but are not identical to these studies. The participants were invited for a clinical interview and focused examination where biological samples were collected. After confirmation of diagnosis based on previous questionnaires, medical records, and clinical interviews, the participants were allocated into the following groups: (1) healthy controls without a history of allergic or other chronic disease [controls], (2) previous, current, or present AD [AD], (3) previous, current, or present AD and current asthma [AD + asthma] and (4) current asthma without present or prior history of AD [asthma]. The UK Working Party criteria were used to confirm an AD diagnosis.[13] *Previous* AD was defined as the absence of lesions in the last year, *current* AD was lesions in the last year but not at the time of examination and *present* AD if lesions were present at the time of examination. Current asthma diagnosis was based on the fulfillment of at least one of the following three criteria in the last 12 months: (1) at least 2 out of 3 indicative symptoms (cough, wheeze, and shortness of breath) not only associated with concurrent infection, (2) physician-diagnosed asthma with ongoing treatment or (3) symptoms suggestive of asthma plus a positive subjective effect of beta-agonists and/or inhaled steroids.

2.2 | Data collection

2.2.1 | Clinical data

Clinical assessments were obtained from the participants' medical records and/or previous questionnaires from the OCC database. In addition, an allergy-specific questionnaire including personal and family history of allergy and current medications was administered and supplemented with a clinical examination and interview. An objective SCORing Atopic Dermatitis (oSCORAD) severity score was assessed for AD participants with lesions present at the time of clinical visit, and xerosis scoring using the SCORAD dryness criteria was evaluated at the skin sampling site for OCC participants.[14]

2.2.2 | *FLG* genotyping

Participants were genotyped for the most common *FLG* variants in Europe—R501X, 2282del4, R2447X, and S3247X (Pentabase).[15,16]

2.2.3 | Total and specific IgE

We analyzed for total IgE and specific IgE (s-IgE) to the (1) food allergens: cow's milk, egg white, egg yolk, wheat, hazelnut, and peanut and the (2) inhalation allergens including pollens: timothy grass, birch, and mugwort; danders: horse, dog, and cat; molds: *Cladosporium herbarum* and *Alternaria alternata*; and house dust mites: *Dermatophagoides pteronyssinus* and *farinae* (ImmunoCAP, Thermo Fisher Diagnostics ApS). Values of ≥0.35 kU/L were considered indicative of sensitization.

2.2.4 | NMF and cytokine levels

NMF levels were analyzed in the stratum corneum layer of the epidermis using a tape-strip technique.[17,18] We measured plasma CCL18, TSLP, Eotaxin-3/CCL26, and CTACK/CCL27 (Th_2 polarized cytokines), interferon-gamma (IFN-γ) (Th_1 polarized), and IL-10 (regulatory) cytokine levels (Mesoscale Discovery [MSD]).

2.3 | Statistical analysis

Pearson's chi-squared test was performed to evaluate differences in categorical characteristics in the four groups. Visual diagnostic plots were used to determine normal distribution, and the data were found to be non-normally distributed. Wilcoxon rank-sum test or Kruskal–Wallis with post hoc Dunn test for multiple testing was performed for comparisons between groups. Spearman rank correlation test was used to evaluate associations. *p*-values <.05 were considered significant using STATA 16.1.

2.4 | Ethics

The study was conducted in accordance with the Helsinki Declarations and approved by the local ethics committee (S-20160169) and the Danish Data Protection Agency (17/9138). Informed consent was obtained from participants' parents.

3 | RESULTS

3.1 | Study population

The study population consisted of 169 controls and 89 AD from the OCC and 26 AD + asthma and 24 asthma participants from the OCC and outpatient allergy clinic. An overview of clinical and para-clinical data is outlined in Table 1.

3.2 | Biological specimens

Samples were collected from ancillary studies, and therefore, not all data points are complete for the biological specimens. Samples are missing because it was not part of the inclusion criteria for the substudy, participants declined venipuncture or laboratory error (<3%).

3.3 | Associations with NMF levels

Children with AD had lower levels of NMF than asthma and controls, also when stratified by present AD, current AD during the last year, and previous AD (Figure 1). NMF levels were negatively associated with increased sensitization, total number of sensitizations, plasma CCL18, and CTACK levels ($p < .05$, Table 2). These associations became nonsignificant when we excluded the controls from our analysis (data not shown).

3.4 | Associations with participant age and severity of disease

There was a significant positive correlation between participant age and plasma cytokine levels of CTACK, TSLP, and Eotaxin-3 (Table 3). These positive correlations remained significant with TSLP and Eotaxin-3 when we excluded our healthy controls from the analysis (data not shown).

There was a significant positive correlation between plasma IL-10 levels and AD disease severity as measured by oSCORAD score in our AD groups (rho = 0.26, $p = .043$) but no significant correlation with the other plasma cytokines (data not shown).

NMF levels were measured at 7 years of age. There was a significant negative correlation between NMF levels and AD disease severity when we investigated participants in the AD groups (rho = −.20, $p = .048$).

TABLE 1 Basic clinical and para-clinical characteristics of the study population, *n* (%)

	Controls	Atopic dermatitis	AD + Asthma	Asthma	Intergroup differences[†]
	n = 169	*n* = 89	*n* = 26	*n* = 24	*p*-value
Gender					
Female	76 (45)	39 (44)	10 (38)	5 (21)	.153
Male	93 (55)	50 (56)	16 (62)	19 (79)	
Age					
Mean value in years (min, max)	7.1 (7.0, 7.5)	7.1 (6.9, 7.4)	8.8 (6.6, 12.7)	8.8 (6.9, 12.3)	**<.001**[‡]
Family history of allergy[*]					**<.001**
Maternal	55 (33)	51 (57)	14 (54)	15 (63)	**<.001**
Paternal	57 (34)	51 (58)	10 (38)	12 (50)	**.003**
Personal history of other allergy					
Food Allergy	n/a	4 (4)	1 (4)	0	.575
Allergic rhinoconjunctivitis	n/a	15 (17)	14 (54)	13 (54)	**<.001**
oSCORAD score[**ab] mean (min, max)	n/a	8.3 (0, 39.3)	2.1 (0, 9)	n/a	**<.001**
AD disease severity					
None to mild	n/a	65 (73)	26 (100)	n/a	
Moderate	n/a	24 (27)	0 (0)	n/a	
Severe	n/a	0 (0)	0 (0)	n/a	
Xerosis score[abcd] (mean, *n* = 280)	0.15	0.42	0.20	0.42	**<.001**[d]
Inhaled steroids -					
Daily equivalent dosage (mcg)					
Mean value	n/a	n/a	246	283	.792
FLG gene variant[ab] (*n* = 277)					.339
Wild Type	161 (95)	77 (89)	8 (89)	11 (92)	
Heterozygous	8 (5)	8 (9)	1 (11)	1 (8)	
Homozygous/double heterozygous	0	2 (2)	0	0	
NMF[ab] (mean [SD], *n* = 280)	0.85 (0.25)	0.66 (0.23)	0.79 (0.26)	0.84 (0.32)	**<.001**
Total IgE[a] (mean, *n* = 230)	112	307	422	306	**<.001**
Serum IgE[a] (*n* = 230)					
Nr. of food allergy sensitizations (mean)	0.16	0.67	1.00	0.48	**<.001**
Nr. of aeroallergen sensitizations (mean)	0.27	1.70	2.81	2.14	**<.001**
Sensitized (No/Yes)	105/22	30/31	5/16	9/12	**<.001**

Note: [a]Missing data specified for >5% of missing values. [b]Missing data from outpatient clinic participants. [c]Based on dryness criteria from SCORAD severity index. [d]Not significant between patient groups.

[†]Assessed with chi-squared, rank-sum, or Kruskal–Wallis test.

[‡]No difference between asthma groups.

*Self-reported.; **oSCORAD severity index score and AD disease severity[14]:mild (<15), moderate (15–40), severe (>40).

3.5 | Group associations with total and specific sensitizations

Patient groups had significantly higher sensitization parameters of total IgE and total number of sensitizations (also after stratifying to food and inhalation allergens) compared with healthy controls, with the same trend for the AD+asthma group having the highest sensitization parameters among the patient groups (Figure 2).

3.6 | Associations with cytokines

Plasma cytokine level results are shown graphically in Figure 3. Specifically, CCL18, TSLP, and Eotaxin-3 plasma levels were higher in AD+asthma compared with the other groups ($p < .01$), though not reaching significance to the asthma group (Figure 3A–C). Plasma CTACK levels were higher in the patient groups compared with controls (Figure 3D). Plasma IFN-γ levels were significantly higher in controls

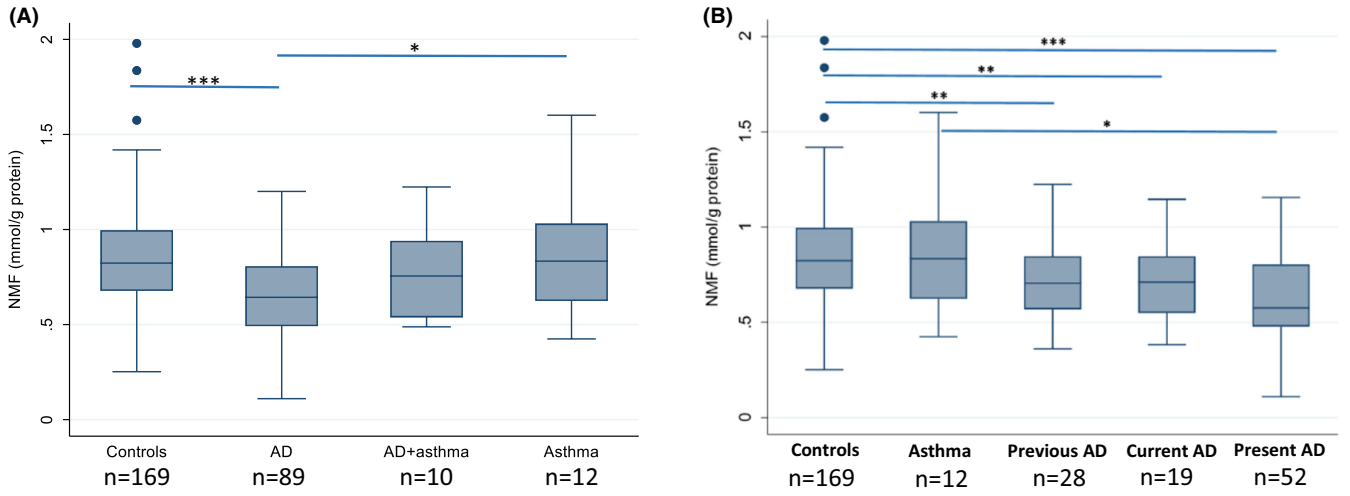

FIGURE 1 Distribution of NMF levels according to group. Box-and-whiskers plot with NMF levels by (A) groups and (B) group with stratification to AD subgroups showing the median (midline) and inter-quartile ranges. *$p < .05$ **$p < .01$ ***$p < .001$ using Kruskal–Wallis with post hoc Dunn test

TABLE 2 Correlations between NMF and IgE sensitization and cytokine levels

NMF	total IgE ($n = 202$)	sensitized (no/yes) ($n = 202$)	nr. of sensitizations ($n = 202$)
Spearman's rho		−0.16	−0.17
p-value	ns	**.026**	**.017**

NMF	CCL18 (plasma) ($n = 194$)	TSLP (plasma) ($n = 194$)	CTACK (plasma) ($n = 194$)	Eotaxin-3 (plasma) ($n = 194$)	IFN-γ (plasma) ($n = 194$)	IL-10 (plasma) ($n = 194$)
Spearman's rho	−0.15		−0.18			
p-value	**.037**	ns	**.011**	ns	ns	ns

Note: Associations analyzed with Spearman's rank correlation test.

compared with the asthma and AD + asthma groups, and there were no significant intergroup differences in IL-10 levels (not shown).

4 | DISCUSSION

To our knowledge, this is the first study to investigate the clinical characteristics and biomarkers associated with a combined AD + asthma phenotype compared with AD or asthma alone and healthy controls. We analyzed for differences in biomarkers between the four groups to elucidate whether these differences could suggest different regulatory pathways leading to these clinical phenotypes.

Decreased NMF levels were significantly associated with IgE sensitization and increased levels of CCL18 and CTACK, though the correlation coefficient demonstrated only a small association. When we investigated the associations excluding the control group from our analysis, we found no significant correlations, which may indicate the weight of influence our control group had on these significant results. We found a significantly higher total number of sensitizations in our AD+asthma group compared with the AD group and near significant compared with the asthma group. This supports different routes of sensitization from impermeable barriers in both skin and lung tissue.

NMF levels were lower in our AD group, which could be an indication that NMF is a marker of disease activity; however, this cannot be substantiated in our cross-sectional study design. NMF levels are known to be affected by FLG variant status. There was no difference in the proportion of variants between the four groups. This could be due to the fact that our AD groups had relatively mild disease while FLG variants are associated with moderate–severe disease.

CCL18, TSLP, and Eotaxin-3 levels were higher in the AD + asthma group compared with AD and controls. There was no significant difference between the AD + asthma and asthma groups. It may be that these levels are higher in asthma patients regardless of their AD status; however, the majority of our AD+asthma group had *previous* AD, as indicated by a low SCORAD score (2.1—see Table 1) in that group. One theory is that our group of AD + asthma participants, where a majority had mild, transient AD, could have a different immune regulatory response than those with severe, persistent AD + asthma. That there is a clear trend of higher levels in these pro-Th_2 cytokines in our AD + asthma group

TABLE 3 Correlations between participant age and cytokine levels

Age	CCL18 (plasma) ($n = 194$)	TSLP (plasma) ($n = 194$)	CTACK (plasma) ($n = 194$)	Eotaxin-3 (plasma) ($n = 194$)	IFN-γ (plasma) ($n = 194$)	IL-10 (plasma) ($n = 194$)
Spearman's rho		0.23	0.17	0.28		
p-value	ns	**<.001**	**.010**	**<.001**	ns	ns

Note: Associations analyzed with Spearman's rank correlation test.

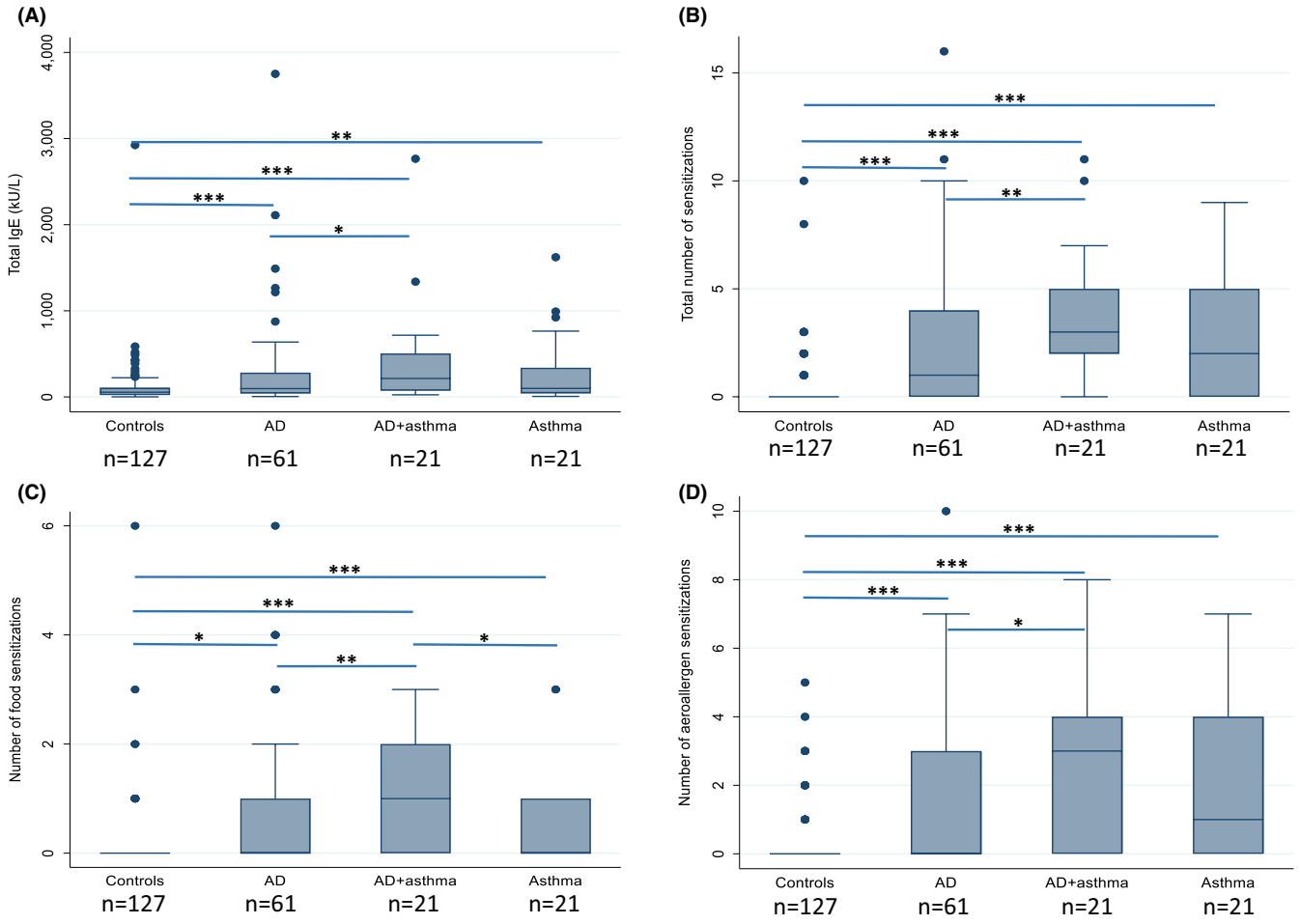

FIGURE 2 Distribution of IgE sensitizations according to group. Box-and-whiskers plot with (A) total IgE levels (B) total number of analyzed s-IgE sensitizations (food and aeroallergens, $n = 16$ total), (C) number of food allergens ($n = 6$), and (D) number of aeroallergens ($n = 10$) by groups showing the median (midline) and inter-quartile ranges. $^{*}p < .05$ $^{**}p < .01$ $^{***}p < .001$ using Kruskal–Wallis with post hoc Dunn test

compared with asthma alone raises the question of whether severe, persistent AD + asthma would result in a significant difference between these two groups. CCL18 is of particular interest as few studies have indicated its relevance to the pathogenesis of AD and asthma. CCL18 attracts diverse immunological cells, including skin-homing memory T cells, naive T cells, and immature dendritic cells.[19] While CCL18 is expressed by dermal APCs and epidermal Langerhans and inflammatory dendritic cells, it is also expressed in lung tissue.[6,19] A recent publication from the Manchester Asthma and Allergy Study (MAAS) demonstrated that elevated levels of CCL18 at one year of life were significantly associated with an increased risk of asthma from early school age to adolescence.[8] Our study is limited by power in the asthma subgroups, though it supports the results from the MAAS study, and raises a pertinent question of whether CCL18 is increased from an early age in children with AD by way of skin barrier dysfunction and plays a functional role in the pathogenesis of asthma development at a later age.

Both TSLP and Eotaxin-3 plasma levels were elevated in AD + asthma compared with the other groups, although not significant to the asthma group. TSLP is produced by epidermal keratinocytes and epithelial cells and is highly expressed in AD skin lesions

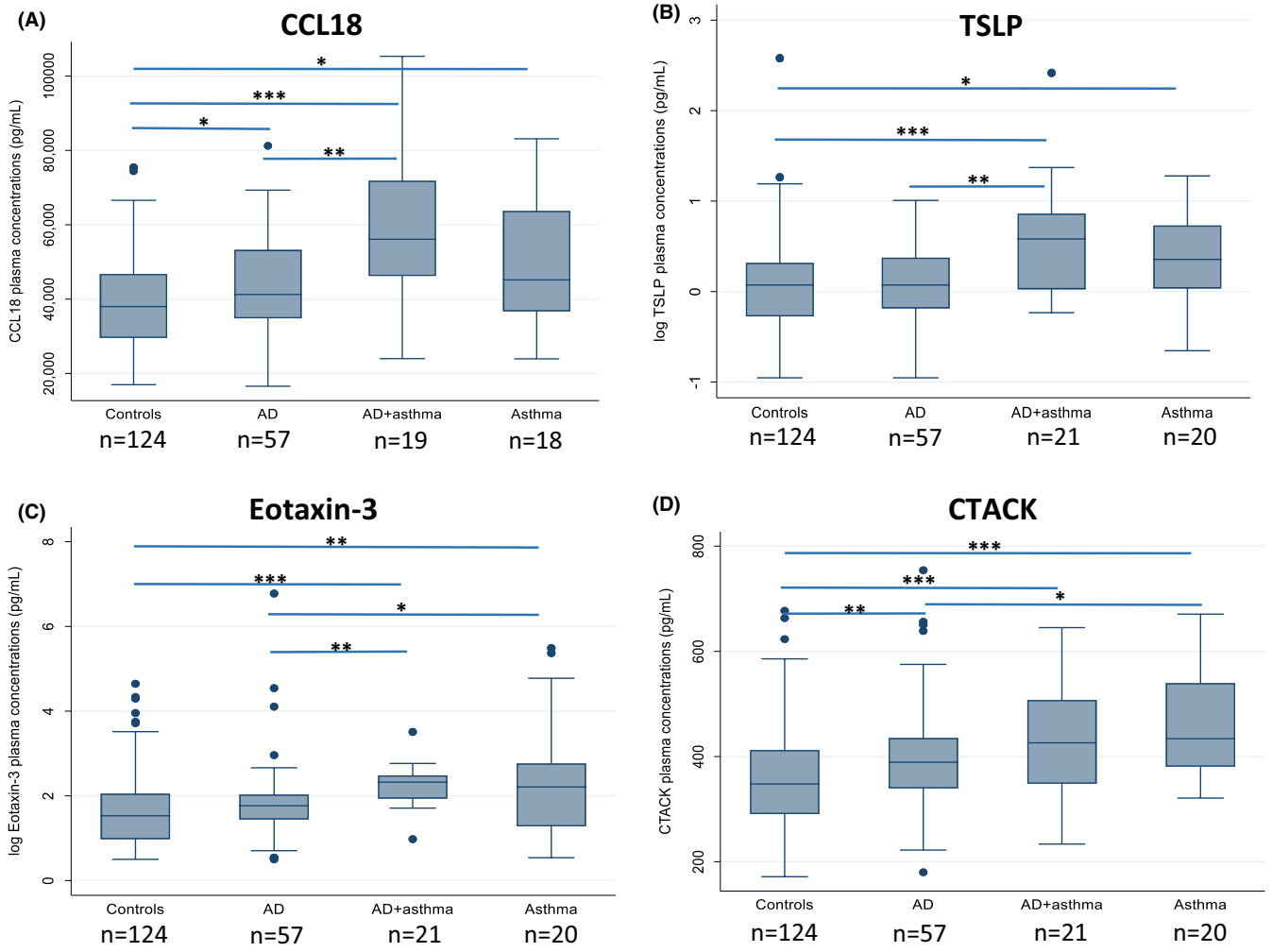

FIGURE 3 Distribution of cytokine levels according to group. Box-and-whiskers plot with (A) CCL18 plasma levels, (B) log-transformed TSLP plasma levels, (C) log-transformed Eotaxin-3 plasma levels, and (D) CTACK plasma levels, showing the median (midline) and interquartile ranges. $^{*}p<.05$ $^{**}p<.01$ $^{***}p<.001$ using Kruskal–Wallis with post hoc Dunn test

and bronchial biopsies of asthmatics compared with controls.[20–22] There was no significant difference in TSLP levels between our AD and control group. Previous studies have shown that TSLP levels correlate with disease severity in asthma but this correlation is equivocal in results from AD disease severity studies.[22–25] One might speculate whether the mild transient AD phenotype in our AD group might have affected these levels. Eotaxin-3 is produced by endothelial cells and dermal fibroblasts, and limited studies have demonstrated its upregulation in AD and asthma after allergen challenge.[10,26–28] Eotaxin-3 levels were higher in our patient groups compared with controls, though not reaching significance in our AD group.

CTACK plasma levels were significantly increased in all patient groups compared with controls and elevated in the asthma group compared with the AD group. This is surprising given that CTACK is expressed predominantly in keratinocytes and in contrast to findings from another report.[9,29] Studies on CTACK in asthma are limited; however, one recently demonstrated that CTACK levels were increased in bronchoalveolar lavage fluid in asthmatic mice models compared with controls.[30] Interestingly, IFN-γ levels were increased in controls compared with the patient groups, in contrast to the associations we found with the pro-Th_2 cytokines, which were generally higher in the patient groups. This supports the cross-regulation between Th_1 and Th_2 subsets.

Our study has both strengths and limitations. It is the first, to our knowledge, to characterize clinical and molecular features in a combined AD + asthma phenotype compared with AD and asthma alone. We had access to participant medical records and questionnaire responses from infancy and early childhood, which mitigated recall bias and improved diagnosis validity. Our asthma subgroups were small in sample size. Our participants were recruited consecutively from both a population-based cohort and outpatient clinic, and this heterogeneous group and sampling bias may have a confounding effect on our results. Missing samples may potentially bias our results. However, there was no significant difference in the number of missing samples in our AD + asthma and asthma group (data not shown). The control and AD groups were significantly younger with proportionally more females compared with the asthma groups,

which could affect the results given that sensitizations and allergic diseases are known to increase with age and the male sex. Age associations with cytokine biomarkers in AD and asthma are still largely unknown. There was no significant age difference between our asthma and AD + asthma groups and no difference in sex proportions in our three patient groups. The asthma participants were using inhaled steroids, which could have affected their cytokine levels. However, there were no significant differences between daily equivalent steroid doses, nor was there a significant difference in place of recruitment or missing samples between our AD + asthma and asthma group (not shown). As this is a cross-sectional study design, the results do not infer causation.

In conclusion, these results demonstrate that an AD+asthma phenotype is characterized by elevated levels of pro-Th_2 cytokines, including CCL18, TSLP, and Eotaxin-3, and increased number of allergen sensitization compared with controls and AD alone. Our findings support the theory of a defective barrier increasing antigen/allergen presentation and upregulation of Th_2 cytokines as proposed by the "outside-inside-outside" theory. However, they suggest that a leaky bronchial epithelial barrier may also play a role. These are preliminary findings in a cross-sectional study that need to be further explored in larger, longitudinal studies supplemented by mechanistic experiments to better clarify causality and pathomechanisms.

AUTHOR CONTRIBUTIONS

Millie Basu: Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (lead); project administration (lead); resources (lead); validation (lead); writing – original draft (lead); writing – review and editing (lead). **Charlotte Gotthard Mortz:** Conceptualization (supporting); formal analysis (supporting); funding acquisition (supporting); methodology (supporting); supervision (supporting); writing – review and editing (supporting). **Tina Kold Jensen:** Conceptualization (supporting); formal analysis (supporting); resources (supporting); supervision (supporting); writing – review and editing (supporting). **Torben Barington:** Formal analysis (supporting); methodology (supporting); resources (supporting); supervision (supporting); writing – review and editing (supporting). **Kate Lykke Lambertsen:** Formal analysis (supporting); investigation (supporting); writing – review and editing (supporting). **Susanne Halken:** Conceptualization (supporting); formal analysis (supporting); funding acquisition (supporting); methodology (supporting); project administration (supporting); resources (supporting); supervision (lead); writing – review and editing (supporting).

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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