Received: 17 May 2022 | Revised: 14 July 2022 | Accepted: 8 August 2022

DOI: 10.1111/all.15518

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI WILEY

ORIGINAL ARTICLE

Atopic Dermatitis, Urticaria and Skin Disease

Skin biomarkers predict development of atopic dermatitis in infancy

Maria Rasmussen Rinnov[1] | Anne-Sofie Halling[1,2] | Trine Gerner[1] | Nina Haarup Ravn[1] | Mette Hjorslev Knudgaard[1] | Simon Trautner[3] | Susan M. I. Goorden[4] | Karen J. M. Ghauharali-van der Vlugt[4] | Femke S. Stet[2] | Lone Skov[1] | Simon Francis Thomsen[2] | Alexander Egeberg[2] | Aske L. L. Rosted[5] | Troels Petersen[5] | Ivone Jakasa[6] | Christoph Riethmüller[7] | Sanja Kezic[8] | Jacob P. Thyssen[2]

[1]Department of Dermatology and Allergy, Herlev and Gentofte Hospital, University of Copenhagen, Hellerup, Denmark

[2]Department of Dermatology and Venereology, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark

[3]Department of Neonatology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

[4]Laboratory Genetic Metabolic Diseases, Department of Clinical Chemistry, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

[5]Niels Bohr Institute, University of Copenhagen, Copenhagen, Denmark

[6]Laboratory for Analytical Chemistry, Department of Chemistry and Biochemistry, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia

[7]Serend-ip GmbH, Centre for Nanotechnology, Munster, Germany

[8]Amsterdam Public Health research institute, Department of Public and Occupational Health Amsterdam UMC, Department of Public and Occupational Health, University of Amsterdam, Amsterdam Public Health Research Institute, Amsterdam, The Netherlands

Correspondence
Jacob P. Thyssen, Department of Dermatology and Venereology, Bispebjerg Hospital, University of Copenhagen, Bispebjerg Bakke 23, build. 4, DK-2400 Copenhagen N, Denmark.
Email: jacob.pontoppidan.thyssen@regionh.dk

Funding information
Herlev Hospital; Kongelig Hofbuntmager Aage Bangs Fond; LEO Fondet; Lundbeckfonden; Novo Nordisk Fonden; Savvaerksejer Jeppe Juhl og Hustru Ovita Juhls Mindelegat

Abstract

Background: There is currently no insight into biomarkers that can predict the onset of pediatric atopic dermatitis (AD).

Methods: Nested in a prospective birth cohort study that examined the occurrence of physician-diagnosed AD in 300 children, 44 random children with onset of AD in the first year of life were matched on sex and season of birth with 44 children who did not develop AD. Natural moisturizing factor (NMF), corneocyte surface protrusions, cytokines, free sphingoid bases (SBs) of different chain lengths and their ceramides were analyzed from tape strips collected at 2 months of age before onset of AD using liquid chromatography, atomic force microscopy, multiplex immunoassay, and liquid chromatography mass spectrometry, respectively.

Results: Significant alterations were observed for four lipid markers, with phytosphingosine ([P]) levels being significantly lower in children who developed AD compared with children who did not (median 240 pmol/mg vs. 540 pmol/mg, $p < 0.001$). The two

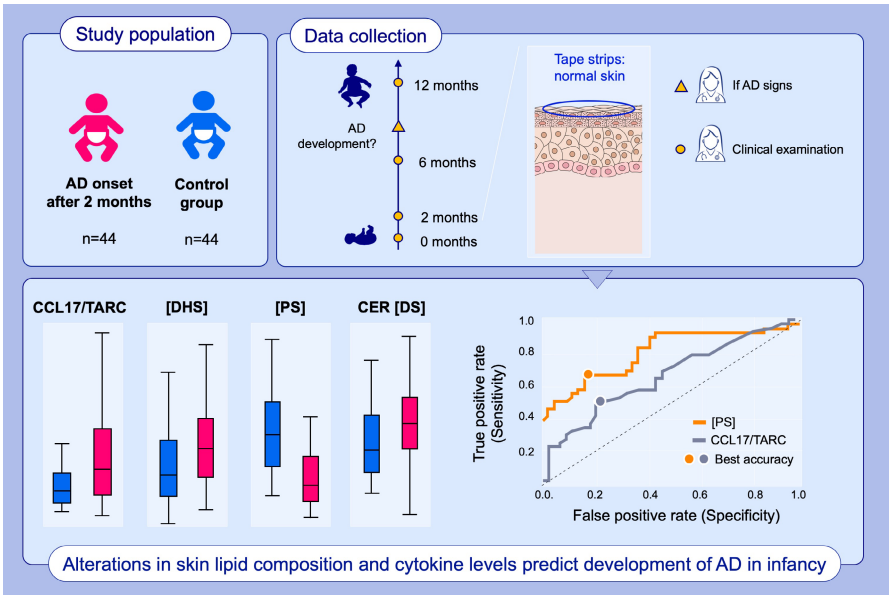
Abbreviations: AD, atopic dermatitis; AUC, area under the curve; C, carbon atoms; FA, fatty acid; *FLG*, filaggrin gene; FPR, false positive rate; NMF, natural moisturizing factor; PPAR, peroxisome proliferator-activated receptor; ROC, receiver operating characteristic; SC, stratum corneum; SPT, serine palmitoyl transferase; TARC/CCL17, thymus- and activation-regulated chemokine; TEWL, transepidermal water loss; TPR, true positive rate.

Sanja Kezic and Jacob P. Thyssen contributed equally to this article.

groups of children differed in the relative amounts of SB of different chain lengths (C17, C18 and C20). Thymus- and activation-regulated chemokine (TARC/CCL17) was slightly higher in children who developed AD, whereas NMF and corneocyte surface texture were similar. AD severity assessed by the eczema area and severity index (EASI) at disease onset was 4.2 (2.0;7.2). [P] had the highest prediction accuracy among the biomarkers (75.6%), whereas the combination of 5 lipid ratios gave an accuracy of 89.4%.

Conclusion: This study showed that levels and SB chain length were altered in infants who later developed AD, and that TARC/CCL17 levels were higher.

KEYWORDS
atopic dermatitis, biomarker, birth cohort, prediction, skin barrier



GRAPHICAL ABSTRACT

This study examines whether selected skin biomarkers in stratum corneum tape strips from infants with clinically normal skin at 2 months of age are different in children who develop AD in the first year of life. Skin CCL17/TARC levels as well as lipid markers, including [P], [DS] and CER [DS] show significant alteration between children who developed AD and those who did not. ROC curve demonstrate that phytosphingosine, and to a lesser extend CCL17/TARC separate children who developed AD from those who did not.
Abbreviations: AD, atopic dermatitis; CCL17/TARC, C-C motif chemokine ligand 17/ TARC, thymus- and activation-regulated chemokine; CER [DS], ceramide class with dihydrosphingosine as a sphingoid base; [DHS], dihydrosphingosine (sphinganine) [PS], phytosphingosine.

1 | INTRODUCTION

A parental history of atopic disease as well as occurrence of common filaggrin gene (*FLG*) mutations are strongly associated with pediatric atopic dermatitis (AD).[1,2] Identification of predictive biomarkers is important to guide future prevention efforts and ultimately halt the atopic march.[3,4]

The skin barrier resides in the uppermost cornified layer of the skin, stratum corneum (SC). Filaggrin proteins are degraded into amino acids contributing to the pool of cytosolic natural moisturizing factors (NMF). The main quantitative determinant of NMF is *FLG* loss-of-function mutations,[5,6] of topical corticosteroids, and skin inflammation.[7] Nano-size protrusions of unknown composition have been identified on the surface of corneocytes that are deficient in NMF, and on corneocytes collected from xerotic, lesional, and non-lesional AD skin.[8,9]

Extracellular covalently bound esterified ω-hydroxyl fatty acid ceramides provide a scaffold on which extracellular lipids organize in lipid lamellae, which are regarded as the principal barrier of the skin.[10] These lipid lamellae consist of a total lipid mass basis of 50% ceramides, 25% cholesterol, 15% free fatty acids (FA), and very little phospholipids.[11] Several hundreds of ceramide species can be identified, which differ in the type of sphingoid base (SB) and FA chain. There are four main SBs, dihydrosphingosine ([DS]), sphingosine ([S]), 6-hydroxy-sphingosine ([H]) and phytosphingosine ([P]), which in combination with varying FA chains give rise to 12 main ceramide

(CER) classes (Figures S1 and S2). Patients with AD have important alterations in extracellular lipid composition,[12] for example, shorter chain lengths of FA in ceramides and of free FA, further reducing the performance of the skin barrier.[13] Only the length of the acyl chain of the CER or the total number of carbon atoms (C) in CER have been reported, so the length of the SB is not specified. CER with SB whose length are C16–26 and C18 (28.6%) was the most abundant, followed by C20 (24.8%), and C22 (12.8%).[14] The role of the chain length variability of the SB in AD has not been investigated.

We examined whether selected skin biomarkers in SC tape strips collected in infants with clinically normal skin at 2 months of age are different in children who develop AD in the first year of life compared with children who do not.

2 | METHODS

This case–control study was nested in the prospective Copenhagen Baby Skin birth cohort,[15] approved by the local ethics committee (H-16042289 and H-16042294) and conducted according to the Declaration of Helsinki.

2.1 | Study population

300 children, singletons and born to term (gestational age GA: 37 + 0 to 41 + 6), were followed prospectively from birth and until 2 years of age. Recruitment was independent of their family history of atopic disease. The skin was examined 1–3 days after birth, and at 2 and 12 months of age. If children who showed signs of AD, they were seen immediately by a physician to confirm the diagnosis and assess severity of AD using the Eczema Area and Severity Index (EASI).[16] For the present study, we identified 44 random children who developed AD in the first year of life and 44 reference children who did not develop AD in the first year of life. All children had clinically normal skin at 2 months of age and no history of eczema. The two groups were matched on sex and season of birth. Parents completed questionnaires before the 2 months study visit about lifestyle factors, pregnancy, and frequency of emollient use. The use of emollients was grouped as "every day or every 2. Day," "1-2 times every week," "1-2 times a month/rarely, or never used." Parental history of atopy was defined as either one having past or current history of AD, asthma, rhinitis, or aeroallergen sensitization measured from skin prick testing or blood analysis. Due to missing data in parental atopy, imputations were made for 9 individuals (11%). Due to other missing entries for a few of the patients, analysis comparing prediction powers of all biomarkers was based on 43 children who developed AD in the first year of life, and 43 who did not.

2.2 | Sampling and tissue analysis

At age 2 months, all children had clinically normal skin and no history of eczema. Transepidermal water loss (TEWL) was measured three times on the same non-lesional skin area on the central part of the flexor forearm using a portable, closed condenser-chamber device (AquaFlux model AF200, Biox Systems Ltd, UK). Eight skin tape strips were collected from the dorsal side of the hand. No preference was given to the left or right side, but selection depended on the positioning of the child. Tape strip no. 2 underwent examination with atomic force microscopy and analysis for the number of corneocyte protrusions using the nAnostic™-method (Serend-ip GmbH, Münster, Germany).[17] Free SBs and their ceramides were determined in tape strip no. 3 and 4 collected at 2 months of age.[18] Tape strip no. 5 was examined for NMF.[19] Tape strip no. 6 was analyzed for interleukins (IL)-1α, IL-1RA, IL-8 (C-C motif chemokine ligand 8, CCL8), macrophage-derived chemokine (MDC, CCL22), cutaneous T-cell attracting chemokine (CTACK/CCL27), interferon gamma (IFN-γ), IL-1β, IL-18, IL-4, IL-5, IL-10, IL-13, IL-31, IL-33, macrophage inflammatory protein (MIP)-1β, thymic stromal lymphopoietin (TSLP), thymus and activation-regulated chemokine (TARC/CCL17), macrophage inflammatory protein (CCL20, MIP-3α), and IL-22. Cytokine concentrations in the extracts were measured on multiplex panels using MESO QuickPlex SQ 120 (MSD, Rockville, MA, U.S.A.). DNA was genotyped for three common *FLG* mutations (R501X, 2282del4, and R2447X).[20]

2.3 | Methods for ceramide and sphingoid base measurements

Free SBs and their CERs were determined in a 2-step procedure which is described in detail elsewhere.[21] Briefly, free SBs were separated from CERs by extraction with methanol:chloroform:ammonium formate buffer, yielding CERs in the lower phase and free SBs in the upper phase.[22] The CERs in the lower phase were subsequently deacylated to corresponding SBs by use of microwave-assisted hydrolysis in methanolic NaOH.[23] Free SBs and the SBs derived after deacylation of CERs were determined in separate runs by LC–MS/MS[24] (Figure S3). Details on the LC–MS/MS analysis are described in the Table S1. Concentrations of SBs, CER, and GlcCER were normalized by protein amount, determined from optical density of the tape measured by SquameScan (CuDerm, Dallas TX, USA).

2.4 | Statistical analysis

Statistical analysis was performed using Python (Python 3 Reference Manual. Scotts Valley, CA: CreateSpace) version 3.8 (Windows) with statistical packages from SciPy (Algorithms for Scientific Computing in Python. Nature Methods, 17,[3] 261–272). All distributions were presented as medians with 25th/75th percentiles. Data distribution was assessed using Shapiro–Wilks normality test. Differences between groups (AD vs non-AD) were tested by unpaired *t*-test for normally distributed data, and otherwise using Mann–Whitney U test. Checking observation categories for assumption of same shape was done by subtracting the mean of all values and comparing the distributions using Kolmogorov–Smirnov test with a *p*-value of

0.05. A two-tailed nonparametric Spearman's correlation was used to test the strength of the association between biomarkers. Levels of biomarkers were corrected for protein levels. If more than 50% of the samples of one immune biomarker had undetectable levels it was excluded.[25] The ability of parental atopy, *FLG* mutation status, TEWL, NMF, cytokines, and lipid ratios in discriminating AD children from *non-AD children* was evaluated by constructing receiver operating characteristic (ROC) curves. Logistic regression fits were used to construct prediction scores combining biomarkers, where all data was standardized for stability.[26] The sensitivity and specificity were calculated. Quantitative comparisons were made using Area Under the Curve (AUC), and by comparing sensitivity (True Positive Rate [TPR]) and 1 – specificity (False Positive Rate [FPR]), at working points found by maximizing accuracy:

$$\text{Accuracy} = \frac{TP + TN}{TP + TN + FP + FN} = \frac{\text{correct predictions}}{\text{number of samples}},$$

where TP = True Positive, TN = True Negative, FP = False positive, and FN = False Negative.

2.5 | RESULTS

2.6 | Clinical characteristics

Forty-four (50%) children were male. Children were born throughout the year; winter (27.3%), spring (18.2%), summer (25.0%), and autumn (29.5%) (Table 1). Among children who developed AD, 66% had parental history of atopy compared with 52% among children who did not develop AD ($p = 0.4$). At AD onset, the median age was 4.0 months (25th/75th percentiles 3.0;7.0), and the median EASI score 4.2 (25th/75th percentiles 2.0;7.2). There were 8 (18.2%) heterozygote *FLG* mutation carriers in the AD group, and 2 (4.5%) in the control group ($p < 0.05$). *FLG* mutation carriers had significantly lower NMF levels compared with the *FLG* wild type group ($p < 0.001$), independent of AD onset in the first year of life or not ($p < 0.001$ and $p = 0.03$). No difference in the number of corneocyte protrusions ($p = 0.1$), levels of phytosphingosine ([P]) ($p = 0.3$), TARC/CCL17 levels ($p = 0.2$), or TEWL values ($p = 0.2$) was observed between *FLG* mutation and wildtype carriers at 2 months of age (Table S2). Parents reported at 2 months that emollient was used significantly more frequent (every day or every other day) in the group of children that later developed AD compared with the control group (27% vs. 9%, $p = 0.04$, Table S3).

2.7 | Biomarkers as predictor of atopic dermatitis in the first 12 months of life

There were no significant differences in NMF levels, corneocyte protrusion numbers, or TEWL values at 2 months of age between children who developed AD in the first 12 months and children who did not (Figure 1 and Table 2). Skin TARC/CCL17 levels (median [pg/μg]) collected at 2 months showed a small but significant difference between children who developed AD in the first 12 months and those who did not (0.02 vs. 0.01, $p = 0.01$). Analysis of various skin lipids collected at 2 months of age showed significant alterations for 4 lipids; in particular phytosphingosine ([P]) levels (median [pmol/mg]) were much lower in children who developed AD compared with children who did not (238 vs. 535, $p < 0.001$) (Figure 1 and Table 2). We then calculated ratios between short compared with long SB chain lengths of free SBs and CERs and different SB types (Table 3). Ten lipid ratios in tape strips collected at 2 months of age were significantly different between children who developed AD in the first 12 months compared with children who did not. Most ratios

TABLE 1 Characteristics of 44 infants who developed atopic dermatitis and 44 controls who did not develop atopic dermatitis during the first year

	Atopic dermatitis developed within the first year ($n = 44$)	No atopic dermatitis developed within the first year ($n = 44$)
	n (%)	
Males	22 (50.0)	22 (50.0)
Birth season		
Winter (Dec-Feb)	12 (27.3)	12 (27.3)
Spring (March–May)	8 (18.2)	8 (18.2)
Summer (June-Aug)	11 (25.0)	11 (25.0)
Fall (Sept-Nov)	13 (29.5)	13 (29.5)
Filaggrin gene mutations	8 (18.2)	2 (4.5)
Parental history of atopic dermatitis, asthma or hay fever	29 (65.9)	23 (52.3)
Parental history of AD only	6 (13.6)	9 (20.5)
Age at AD onset (months), median (25th/75th percentile)	4.0 (3.0/7.0)	-
AD severity measured by EASI score at disease onset, median (25th/75th percentile)	4.2 (2.0/7.2)	-

reflected increased concentrations of shorter SBs as compared to longer SBs. In addition, the ratio of CER[DS]-(d18:1)/CER[S]-(d20:1) was lower in children who developed AD reflecting a variation in SB structure.

We examined biomarker levels in children born in different seasons (Figure S4). Across all birth seasons, the levels of [P] were consistently lower, and the levels of TARC/CCL17 consistently higher, in children who developed AD in the first 12 months compared with those who did not.

2.8 | Sub-analyses examining biomarkers as predictors of earlier onset atopic dermatitis and disease severity

We examined the levels of biomarkers in children who developed AD within the first 4 and 6 months of life, respectively, vs. those who did not develop AD in the first 12 months (Tables S4 and S5, Figure S5). The number of corneocyte protrusions was significantly higher in children who developed AD after 2 months of age, but before 6 months of age, compared with AD onset between 6 and 12 months of age (median [AU] 2.1 vs. 1.9, $p = 0.03$), and compared with children who did not develop AD (median [AU] 2.1 vs. 1.9, $p = 0.03$) (Figure S6). No differences were observed for NMF levels between these groups. TARC/CCL17 and [P] levels remained significantly different between AD and non-AD children when examining the difference for children who develop AD within 6 months, respectively. No significant difference in [P] and TARC/CCL17 levels was seen when comparing children with AD onset before 6 months of age and children with AD onset at 6–12 months of age ($p = 0.2$ and $p = 0.6$ for [P] and TARC/CCL17, respectively) (Figure 2). Biomarker levels did not differ according to AD severity (high vs. low EASI scores).

2.9 | Correlations between biomarkers

To study associations between various biomarkers, we performed Spearman's correlation analysis. NMF levels correlated weakly

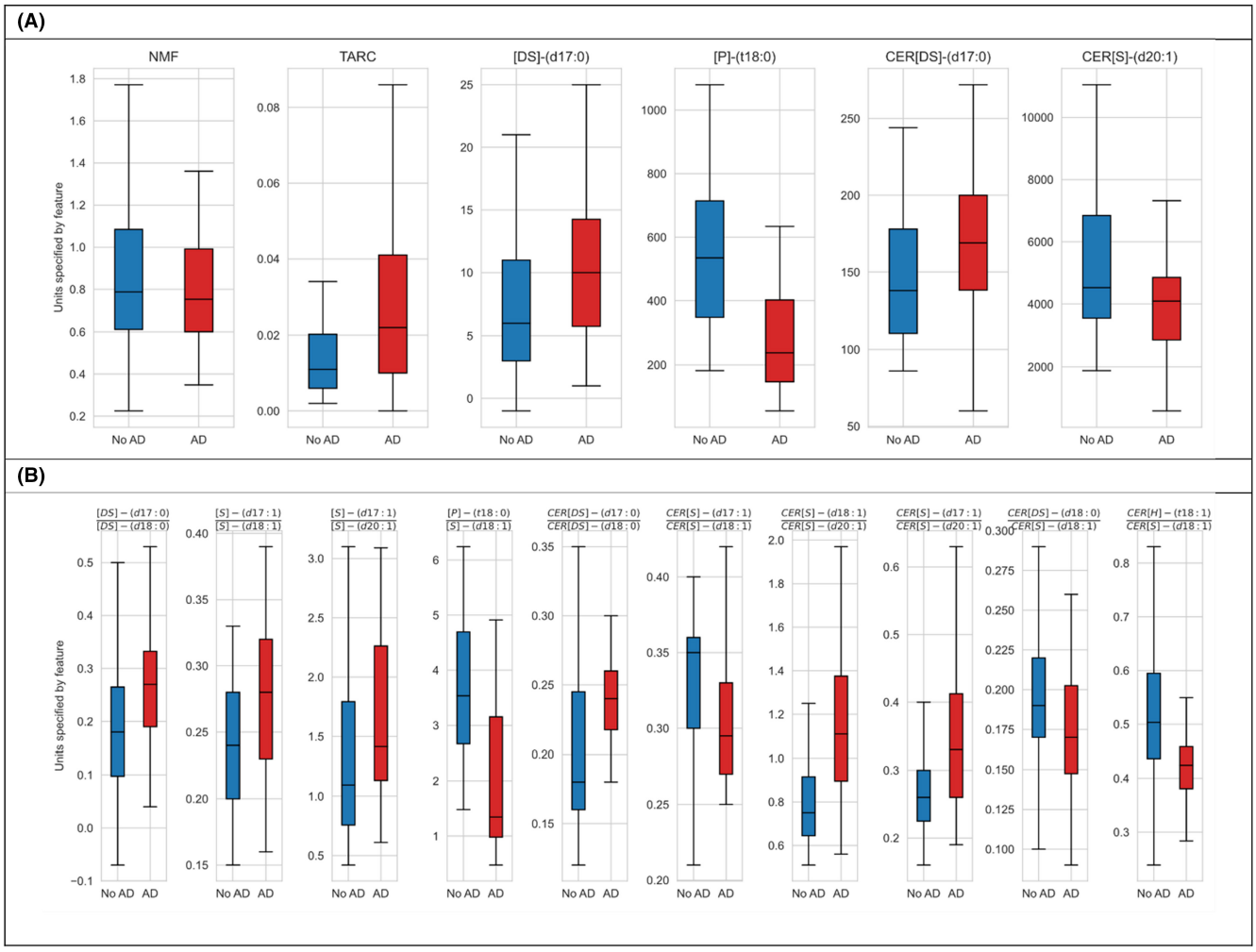


FIGURE 1 Biomarkers measured at 2 months of age in children with clinically normal skin and who were then followed prospectively for development of AD within the first year of life. All apart from NMF were significantly altered in children with subsequent AD. (A) single measurement of various biomarkers, (B) median ratios of sphingoid bases and ceramides (pmol/mg).

TABLE 2 Biomarkers measured at 2 months of age in healthy children with clinically normal skin at the time of tissue collection and who were then followed prospectively for development of atopic dermatitis within the first year of life

	Atopic dermatitis developed within the first year ($n = 44$)	No atopic dermatitis developed within the first year ($n = 44$)	*p*-value
	Median (25th/75th percentile)	Median (25th/75th percentile)	Mann–Whitney/T-test
TEWL ([g/m^2]/h)	14.0 (11.0;17.0)	13.3 (11.1;17.2)	0.9
Corneocyte protrusions[a]	2.0 (1.8; 2.1)	1.9 (1.8; 2.0)	0.1
NMF (mmol/g protein)	0.8 (0.6;1.0)	0.8 (0.6;1.1)	0.7
Cytokines (pg/μg)			
IL-1β	0.02 (0.01; 0.04)	0.02 (0.01; 0.03)	0.6
IL-8	0.1 (0.0; 0.2)	0.1 (0.04; 0.2)	0.4
TARC/CCL17	0.02 (0.01; 0.04)	0.01 (0.01; 0.02)	**0.01**
IL-18	0.1 (0.1; 0.3)	0.11 (0.1; 0.1)	0.2
IL-31	0.4 (0.3; 0.5)	0.4 (0.3; 0.6)	0.7
MIP3α	0.1 (0.1; 0.1)	0.1 (0.04; 0.2)	0.6
IL-1α	18.0 (10.0; 27.0)	18.0 (14.0; 22.0)	0.8
IL-1Ra	90.0 (50.0; 160.0)	70.0 (50.0; 140.0)	0.5
Sphingoid Bases (SB)			
[DS]-(d17:0)	10.0 (5.3; 14.8)	6.0 (3.0; 11.0)	**0.02**
[DS]-(d18:0)	32.5 (24.3; 47.3)	30.5 (21.3; 43.0)	0.3
[P]-(t18:0)	238.0 (142.35; 411.5)	535.0 (346.5; 719.5)	**1×10^{-6}**
[S]-(d17:1)	37.0 (25.3; 58.0)	35.0 (22.5; 43.0)	0.2
[S]-(d18:1)	134.5 (110.5; 194.8)	137.0 (101.5; 188.0)	0.9
[S]-(d20:1)	24.5 (19.3; 35.8)	24.5 (20.0; 38.0)	0.8
[H]-(t18:1)[b]	80.0 (59.0; 114.0)	91.0 (69.0; 108.0)	0.4
Ceramides			
CER[DS]-(d17:0)	169.0 (134.8; 208.0)	138.0 (110.0; 179.0)	**0.02**
CER[DS]-(d18:0)	680.0(556.5; 884.0)	687.0 (560.0; 994.0)	1.0
CER[P]-(t18:0)	9484.0 (5569.0; 20302.50)	9785.0 (4954.0; 21747.0)	0.9
CER[S]-(d17:1)	1242.0 (849.5; 1690.0)	1323.0 (865.0; 1529.0)	0.9
CER[S]-(d18:1)	4320.0(2692.3; 6057.8)	3645.0 (2730.0; 4884.0)	0.3
CER[S]-(d20:1)	4095.0 (2650.3; 4872.5)	4528.0 (3534.0; 7127.0)	**0.05**
CER[H]-(t18:1)[b]	1700 (1300; 2200)	1900 (1400; 2700)	0.3
GlcCER[S]-(d18:1)	173.5(108.3; 259.3)	148.0 (94.0; 212.0)	0.3
GlcCER[H]-(t18:1)[b]	14.0 (11.0; 23.0)	14.0 (9.0; 20.0)	0.8
GlcCER[S]-(d20:1)	126.5 (84.5.0; 187.3)	113.0 (79.0.0; 153.0)	0.3

Abbreviations: CER, ceramide; GlcCer, glucosylceramide; NMF, natural moisturizing factor; TEWL, transepidermal water loss; [DS], dihydrosphingosine; [H], 6-hydroxy-sphingosine; [P], phytosphingosine; [S] sphingosine. Bold values indicates $p < 0.05$ was considered significant.

[a]Arbitrary unit, (Log10).

[b]Approximate concentrations (since there is no standard available for this metabolite).

to moderately with most SBs and CERs, whereas correlations for corneocyte protrusions and TARC/CCL17 were less pronounced (Table S6).

2.10 | Prediction accuracy of skin biomarkers

We then constructed ROC curves to identify biomarkers that could potentially separate children who developed AD in the first 12 months from those who did not (Figures 2 and 3). Figure 2 shows that in particular [P], and to a lesser degree TARC/CCL17, were indicators of AD occurrence, while corneocyte protrusions and NMF levels were not. Parental atopy and *FLG* mutations showed weak ability to differentiate between AD and non-AD occurrence. The combination of these biomarkers only slightly increased the ability to separate AD children from non-AD children compared with [P] alone. For [P], the highest accuracy was 75.6%. At this working point, the TPR (true positive rate reflecting sensitivity) was found to be 0.67 and the FPR (false positive rate reflecting specificity) was found to be 0.16 (Specificity = 1–0.16 = 0.84). A separation

TABLE 3 Differences in median ratios of sphingoid bases (SBs) and ceramides (pmol/mg) measured at 2 months of age in children with clinically normal skin at the time of tissue collection and who developed atopic dermatitis in the first year of life vs. children who did not

Sphingolipid ratios	Atopic dermatitis developed within the first year ($n = 44$)	No atopic dermatitis developed within the first year ($n = 44$)	*p*-value
	Median (25th/75th percentile)	Median (25th/75th percentile)	Mann–Whitney/T-test
Sphingoid base (SB) ratios			
Ratios between SBs of different chain lengths			
[DS]-(d17:0)/[DS]-(d18:0)	0.3 (0.2; 0.34)	0.2 (0.1; 0.3)	**0.004**
[S]-(d17:1)/[S]-(d18:1)	0.3 (0.2; 0.3)	0.2 (0.2; 0.3)	**0.004**
[S]-(d18:1)/[S]-(d20:1)	5.8 (4.3; 7.7)	5.5 (3.9; 6.4)	0.3
[S]-(d17:1)/[S]-(d20:1)	1.4 (1.1; 2.3)	1.1 (0.8; 1.8)	**0.03**
Ratios between SBs of different structures			
[DS]-(d18:0)/[S]-(d18:1)	0.2 (0.2; 0.3)	0.2 (0.2; 0.3)	0.1
[P]-(t18:0)/[S]-(d18:1)	1.3 (1.0; 3.2)	3.5 (2.7; 4.7)	$\mathbf{1 \times 10^{-6}}$
[H]-(t18:1)/[S]-(d18:1)	0.6 (0.5; 0.7)	0.6 (0.6; 0.8)	0.2
Ceramide ratios[a]			
CER[DS]-(d17:0)/CER[DS]-(d18:0)	0.2 (0.2; 0.3)	0.2 (0.2; 0.2)	**0.002**
CER[S]-(d17:1)/CER[S]-(d18:1)	0.3 (0.3; 0.3)	0.4 (0.3; 0.4)	**0.002**
CER[S]-(d18:1)/CER[S]-(d20:1)	1.1 (0.9; 1.4)	0.8 (0.6; 0.9)	$\mathbf{3 \times 10^{-7}}$
CER[S]-(d17:1)/CER[S]-(d20:1)	0.3 (0.3; 0.4)	0.3 (0.2; 0.3)	**0.0002**
CER[DS]-(d18:0)/CER[S]-(d18:1)	0.2 (0.1; 0.2)	0.2 (0.2; 0.2)	**0.02**
CER[P]-(t18:0)/CER[S]-(d18:1)	2.7 (1.1; 5.6)	2.1 (1.5; 6.2)	0.9
CER[H]-(t18:1)/CER[S]-(d18:1)	0.4 (0.4; 0.5)	0.5 (0.4; 0.6)	**0.0004**

Abbreviations: CER, ceramide; [DS], dihydrosphingosine; [H], 6-hydroxy-sphingosine; [S], sphingosine; [P], phytosphingosine. Bold values indicates $p < 0.05$ was considered significant.

[a]Ceramides have AD positive $n = 44$/AD negative $n = 43$.

value of 433.5 was identified, that is, measurements below the value of 433.5 will lead to development of AD and anything above will not. In a similar analysis, but where lipid ratios listed in Table 3 were examined (Figure 3), the single best predicting lipid ratio was CER[S]-(d18:1)/CER[S]-(d20:1). When examining all lipid ratios, we found that [DS]-(d17:0)/[DS]-(d18:0), [DS]-(d18:0)/[S]-(d18:1), [P]-(t18:0)/[S]-(d18:1), CER[DS]-(d17:0)/CER[DS]-(d18:0), CER[S]-(d17:1)/CER[S]-(d18:1) together gave the best prediction of AD within the first 12 months. The accuracy was 89.4% (TPR = 0.95 and FPR = 0.17). Adding TARC/CCL17, NMF and corneocyte protrusions only slightly increased the ability to separate the two groups of children. We also constructed ROC curves for AD onset within the first 6 months, but they showed a similar pattern (Data not shown).

3 | DISCUSSION

This case–control study[15] is the first to identify significant alterations in skin barrier biomarkers in clinically normal skin of neonates that were associated with later onset of AD in the first year. Children who developed AD had an altered SC lipid composition with a higher proportion of shorter-chain SBs and their CERs. ROC curves showed that ratios between shorter and longer SBs and/or corresponding CERs, and furthermore SB [P], strongly distinguished children who subsequently developed AD from those who did not. These findings lend support to the outside–inside hypothesis and put lipid alterations in the center of the AD pathogenesis.

Our findings emphasize a hitherto unrecognized importance of lipids for skin homeostasis and AD pathogenesis. We observed strongly reduced levels of the free SB [P] in children who developed AD compared with those who did not. However, the corresponding CER[P] was not significantly changed in AD. While most studies have focused on the role of FA chain length in ceramides, it is less recognized that also SBs show structural diversity when it comes to chain length and composition.[27] Although SBs with 18 carbon atoms (C18) are the most common, shorter and longer SB have been identified in human SC.[28,29] Indeed, the impact of SB length may be more significant than FA chain length with respect to biophysical properties of sphingolipids.[27]

[P] has anti-inflammatory effects and stimulates epidermal differentiation through the activation of peroxisome proliferator-activated receptors (PPARs),[30] lending support to the use of [P] as a treatment modality[31] not just for the visible lesions of different inflammatory skin disorders, but also in a preventive context.[30,32] Furthermore, [P] increases both filaggrin synthesis and degradation, leading to increased NMF levels and skin hydration.[32] This effect may be caused by the anti-inflammatory properties of [P], as inflammation showed to negatively affect filaggrin synthesis and

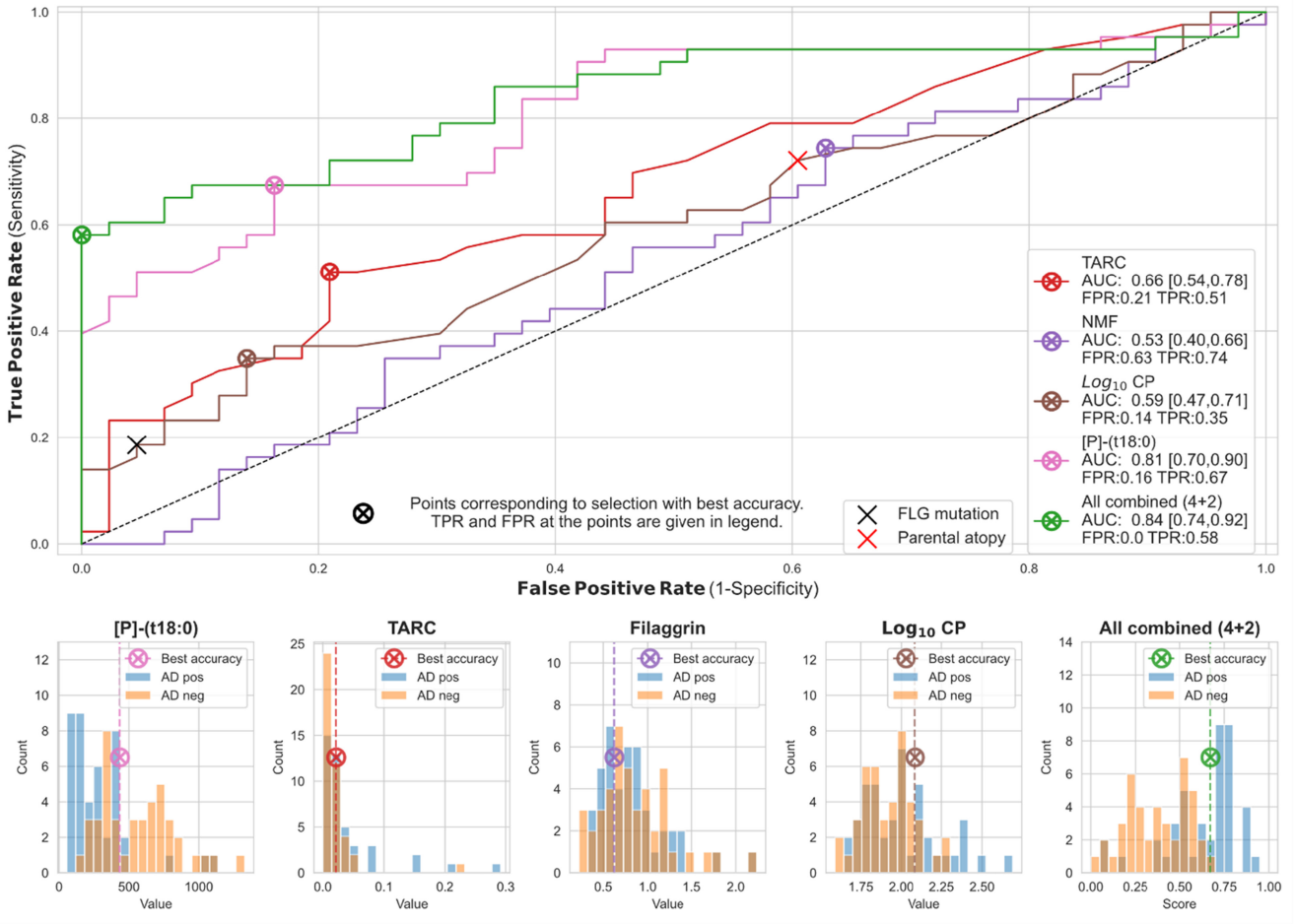


FIGURE 2 ROC curve showing the ability of individual skin biomarkers as well as filaggrin gene mutations and a history of parental atopy to distinguish between atopic dermatitis and non-atopic dermatitis children.

NMF levels, and in particular one of its components urocanic acid, which contributes to the regulation of pH in the SC.[10] Higher NMF levels might shift SC pH toward optimal values for the enzymes involved in metabolism of sphingolipids.[33] Clearly, [P] has multiple roles in formation and maintenance of the skin barrier function; however, the reason for lower levels of [P] in the SC of children who developed AD is not clear. Interestingly, [P] is metabolized to odd-numbered fatty acids[34] which might indicate degradation of [P] as a source of odd-numbered FA (and Coenzyme A). We found abundant levels of C17 SB and corresponding CER in children with AD, and the two groups of children significantly differed in the relative amounts of SB of different chain lengths (C17, C18, and C20). SBs with 18 C-atoms are normally the most abundant in the SC and are generated through the action of Serine Palmitoyl Transferase (SPT), using palmitoyl- Coenzyme A and serine as substrates, which is the rate limiting step in de novo synthesis of CERs (Figure S2). SPT has different isoenzymes and their expression can modulate the affinity toward different fatty acyl-coenzyme A substrates, and thereby affect relative composition of SBs with different chain lengths.[35] For instance, HEK293 cells that express SPTLC3, one of the SPT isoforms, have a higher affinity toward shorter acyl-CoAs, as compared to the predominant usage of palmitoyl-CoA by SPT in the absence of SPTLC3.[36] In human SC, SB chain length varies in size from C16 to C26,[37,38,14] with the most prominent odd chain SB being C17. In a recent comprehensive analysis of CER composition in human SC, the highest abundance of SB was C18 (28.6%) followed by C20 (24.8%) and C22 (12.8%).[14] We found higher ratios of C17/C18, C17/C20 and C18/C20 for [DS] and [S] SBs in children who developed AD. Consistently, these children showed significantly higher levels of [DS]-(d17:0) and CER[DS]-(d17:0). The mechanisms underlying the relatively higher proportion of SBs with shorter chain length, and consequences for skin barrier function, are not clear. Affinity of the SPT enzyme complex for certain FA-CoAs and expression of individual subunits may play a role, but influence of the altered activity and/or expression of certain elongases and ceramide synthases (Figure S2) should also be considered, as they may increase availability of FA-CoAs with shorter chain lengths.

TARC/CCL17 is the hitherto best biomarker in AD.[39] Cytokine and TARC/CCL17 levels were consistently higher in the skin at 2 months of age in those who later developed AD but were inferior to lipid alterations for differentiation between the children

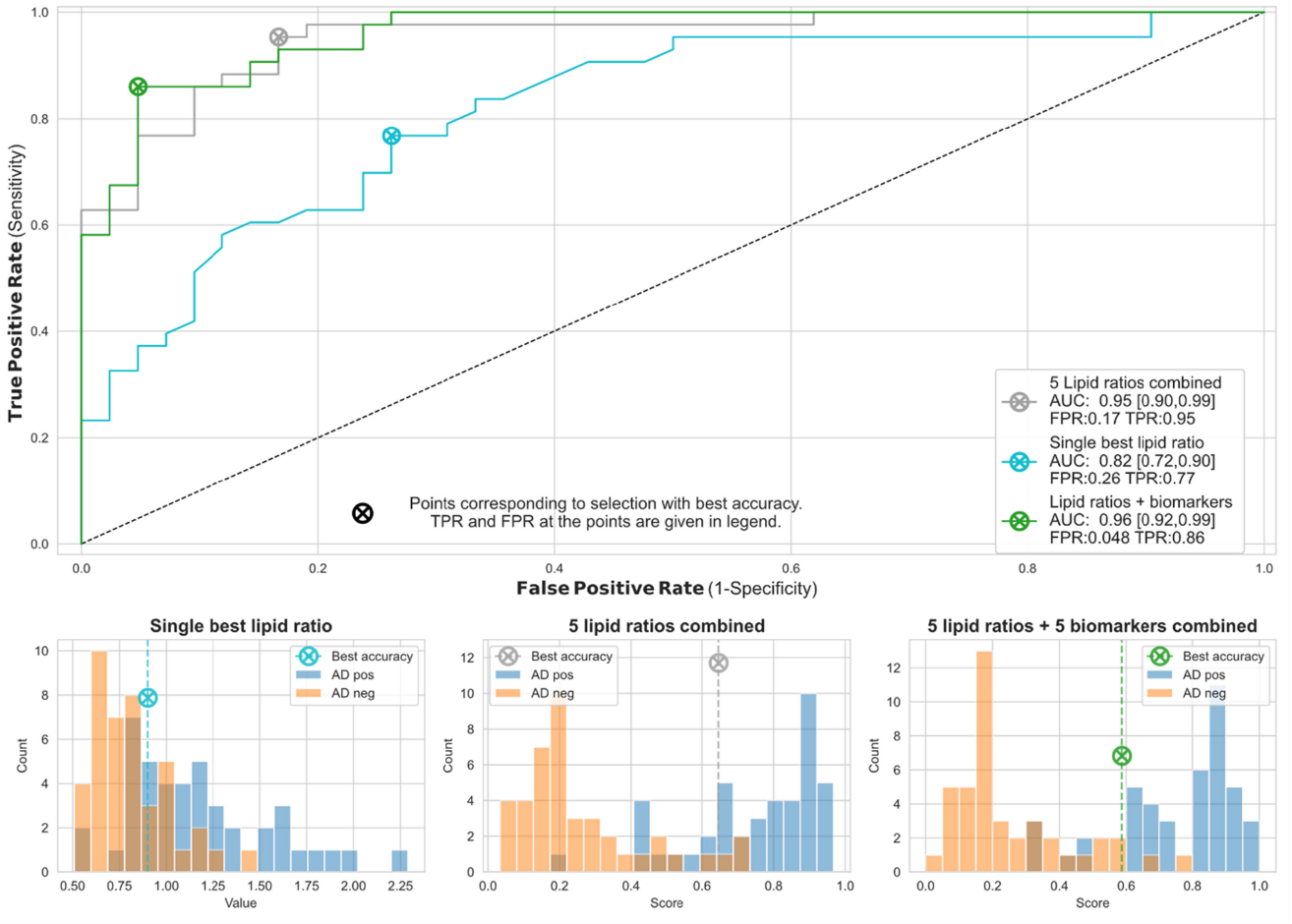


FIGURE 3 ROC curve showing the ability of single and multiple lipid ratios, as well as in combination with other biomarkers, to distinguish between atopic dermatitis and non-atopic dermatitis children.

in ROC curves. The question arises whether observed changes in lipids are intrinsically present and may be regarded as individual susceptibility factors, or if they are a downstream effect of already initiated immunological processes. Several studies showed that changes in lipid composition are more pronounced in lesional AD skin and that addition of Th2 cytokines suppresses lipid metabolism.[40] Our study findings lend support to the outside–inside hypothesis, which suggests that skin barrier impairment is the initial driver of AD, and that immunological activity is secondary.[41] The use of emollients was significantly more frequent at age 2 months in children that later developed AD suggesting in turn that the elevated lipid levels in the control group was not explained by increased use of emollients.

There was no predictive value of TEWL, which is consistent with other studies.[42,43] Since lipid levels were measured on the hands and TEWL measurements were done on the forearm skin, this could potentially have influenced the results. Low epidermal levels of NMF could not predict onset of AD in our study, whereas the prevalence of *FLG* mutations was higher in children who developed AD. The number of corneocyte protrusions was increased in children with AD onset before 6 months of age compared with those with an AD onset between 6 and 12 months and those who did not develop AD in the first 12 months. Their numbers are elevated in children and adults with AD, and also in individuals with dry skin and *FLG* mutations,[6,8] and have been associated with any deficiency in the filaggrin-NMF axis, suggesting that reduced skin hydration is a central cause.[9]

A strength is the prospective design with close follow-up and clinical examination for AD. Children with other types of eczema of childhood, for example, irritant, or nummular may incorrectly be classified as having AD, but this risk was minimized in our design. Since SC was only collected from the dorsal aspect of the hands, it is possible that these were not representative of other skin sites. Our study was limited in size with only 88 children being studied. We did multiple testing, which may increase the risk of random findings; however, we found very consistent results for the biomarkers that best predicted AD in sensitivity analyses, for example, age at AD onset, AD severity, and birth season. No serum biomarkers were analyzed, and consequently no information on, for example, the role of IgE sensitization was determined. Since no predictive studies have

been done for other skin diseases (e.g., psoriasis), our findings may not be specific to AD. Biomarker selection was based on previous studies and we may therefore have overlooked important biomarkers that were not examined.

We conclude that cutaneous biomarkers can be used to predict the onset of AD in the first year of life. Alterations in skin lipid composition, in particular relative composition of SB and CER with different chain length of the SB, seem to be of particular importance in the etiopathogenesis of AD.

AUTHOR CONTRIBUTIONS

MRR, SK, and JPT designed the study. MRR, AH, TG, NHR, MHK, ST, and JPT were responsible for data collection. MRR, ALLR, and TP contributed to the statistical analysis. MRR, SG, KGV, FS, LS, SFT, AE, IJ, CR, SK, and JPT contributed to the interpretation of the data. MRR, SK, and JPT wrote the manuscript. All authors critically revised the manuscript.

ACKNOWLEDGMENTS

We thank all families for their participation in the BABY cohort. The study received financial support from The LEO Foundation, The Lundbeck Foundation, The Novo Nordisk Foundation, Aage Bangs fond, Savværksejer Jeppe Juhl og hustru Ovita Juhls Mindelegat and Herlev and Gentofte Hospital Research Foundation. The study was conducted independent of these and they had no influence on study design or data analysis.

CONFLICTS OF INTERESTS

Dr. Thyssen is an advisor for AbbVie, Almirall, Arena Pharmaceuticals, OM Pharma, Coloplast, Aslan Pharmaceuticals, Union Therapeutics, Eli Lilly & Co, LEO Pharma, Pfizer, Regeneron, and Sanofi-Genzyme, a speaker for AbbVie, Almirall, Eli Lilly & Co, LEO Pharma, Pfizer, Regeneron, and Sanofi-Genzyme, and received research grants from Pfizer, Regeneron, and Sanofi-Genzyme. Dr. Thomsen has been a speaker or has served on advisory boards for Sanofi-Genzyme, AbbVie, LEO Pharma, Pfizer, Eli Lilly and Company, Novartis, UCB Pharma, Union Therapeutics, Almirall, and Janssen Pharmaceuticals; and has received research support from Sanofi-Genzyme, AbbVie, LEO Pharma, Novartis, UCB Pharma, and Janssen Pharmaceuticals, outside the submitted work. Dr. Skov has been an advisor, or speaker for Abbvie, Eli Lilly, Novartis, Sanofi, Celegen, Leo Pharma, Janssen-Cilag, UCB, BMS, Boehringer Ingelheim and Almirall, has received research support from Sanofi, LEO Pharma, UCB, BMS and Janssen, outside the submitted work. Dr. Halling has been a consultant for Coloplast A/S and speaker for LEO Pharma. The remaining authors declare no conflict of interest.

SPHINGOLIPIDS

Sphingoid bases (SBs)

[DS] Dihydrosphingosine (sphinganine)

[H] 6-Hydroxy-sphingosine

[S] Sphingosine

[P] Phytosphingosine

CERAMIDES (CERS)

CER[DS] A ceramide class with dihydrosphingosine as a sphingoid base

CER[H] A ceramide class with 6-hydroxy-sphingosine as a sphingoid base

CER[S] A ceramide class with sphingosine as a sphingoid base

CER[P] A ceramide class with phytosphingosine as a sphingoid base

GlcCER[H] Glucosylceramide with 6-hydroxy-sphingosine as a sphingoid base

GlcCER[S] Glucosylceramide with sphingosine as a sphingoid base

NOMENCLATURE OF SPHINGOID BASES

For example: d18:1 (most abundant sphingoid base, belonging to the class of sphingosine).

→ d = number of hydroxyl-groups. Most common is two (d), less common are one (m) and three (t).

→ 18 = number of C-atoms, with a minimum of 12 and a maximum of 24.

→:1 = number of double bonds.

ORCID

Maria Rasmussen Rinnov https://orcid.org/0000-0003-4624-6180

Trine Gerner https://orcid.org/0000-0002-5377-4013

Mette Hjorslev Knudgaard https://orcid.org/0000-0001-7072-0933

Jacob P. Thyssen https://orcid.org/0000-0003-3770-1743

REFERENCES

1. Ravn NH, Halling AS, Berkowitz AG, et al. How does parental history of atopic disease predict the risk of atopic dermatitis in a child? A systematic review and meta-analysis. *J Allergy Clin Immunol*. 2020;145(4):1182-1193. doi:10.1016/j.jaci.2019.12.899
2. Palmer CN, Irvine AD, Terron-Kwiatkowski A, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet*. 2006;38(4):441-446. doi:10.1038/ng1767
3. Chalmers JR, Haines RH, Bradshaw LE, et al. Daily emollient during infancy for prevention of eczema: the BEEP randomised controlled trial. *Lancet (London, England)*. 2020;395(10228):962-972. doi:10.1016/s0140-6736(19)32984-8
4. Foolad N, Brezinski EA, Chase EP, Armstrong AW. Effect of nutrient supplementation on atopic dermatitis in children: a systematic review of probiotics, prebiotics, formula, and fatty acids. *JAMA Dermatol*. 2013;149(3):350-355. doi:10.1001/jamadermatol.2013.1495
5. Kezic S, Kemperman PM, Koster ES, et al. Loss-of-function mutations in the filaggrin gene lead to reduced level of natural moisturizing factor in the stratum corneum. *J Invest Dermatol*. 2008;128(8):2117-2119. doi:10.1038/jid.2008.29
6. Engebretsen KA, Bandier J, Kezic S, et al. Concentration of filaggrin monomers, its metabolites and corneocyte surface texture in individuals with a history of atopic dermatitis and controls. *J Eur Acad Dermatol Venereol*. 2018;32(5):796-804. doi:10.1111/jdv.14801
7. Kezic S, O'Regan GM, Yau N, et al. Levels of filaggrin degradation products are influenced by both filaggrin

genotype and atopic dermatitis severity. *Allergy*. 2011;66(7):934-940. doi:10.1111/j.1398-9995.2010.02540.x

8. Riethmuller C, McAleer MA, Koppes SA, et al. Filaggrin breakdown products determine corneocyte conformation in patients with atopic dermatitis. *J Allergy Clin Immunol*. 2015;136(6):1573-1580.e2. doi:10.1016/j.jaci.2015.04.042
9. Thyssen JP, Jakasa I, Riethmüller C, et al. Filaggrin expression and processing deficiencies impair corneocyte surface texture and stiffness in mice. *J Invest Dermatol*. 2020;140(3):615-623.e5. doi:10.1016/j.jid.2019.07.716
10. Thyssen JP, Kezic S. Causes of epidermal filaggrin reduction and their role in the pathogenesis of atopic dermatitis. *J Allergy Clin Immunol*. 2014;134(4):792-799. doi:10.1016/j.jaci.2014.06.014
11. Feingold KR. Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. *J Lipid Res*. 2007;48(12):2531-2546. doi:10.1194/jlr.R700013-JLR200
12. Jungersted JM, Scheer H, Mempel M, et al. Stratum corneum lipids, skin barrier function and filaggrin mutations in patients with atopic eczema. *Allergy*. 2010;65(7):911-918. doi:10.1111/j.1398-9995.2010.02326.x
13. Janssens M, van Smeden J, Gooris GS, et al. Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *J Lipid Res*. 2012;53(12):2755-2766. doi:10.1194/jlr.P030338
14. Suzuki M, Ohno Y, Kihara A. Whole picture of human stratum corneum ceramides, including the chain-length diversity of long-chain bases. *J Lipid Res*. 2022;63(7):100235. doi:10.1016/j.jlr.2022.100235
15. Gerner T, Halling AS, Rasmussen Rinnov M, et al. 'Barrier dysfunction in atopic newBorns studY' (BABY): protocol of a Danish prospective birth cohort study. *BMJ Open*. 2020;10(7):e033801. doi:10.1136/bmjopen-2019-033801
16. Hanifin JM, Thurston M, Omoto M, Cherill R, Tofte SJ, Graeber M. The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. EASI Evaluator Group. *Exp Dermatol*. 2001;10(1):11-18. doi:10.1034/j.1600-0625.2001.100102.x
17. Franz J, Beutel M, Gevers K, et al. Nanoscale alterations of corneocytes indicate skin disease. *Skin Res Technol*. 2016;22(2):174-180. doi:10.1111/srt.12247
18. Toncic RJ, Jakasa I, Hadzavdic SL, et al. Altered levels of sphingosine, sphinganine and their ceramides in atopic dermatitis are related to skin barrier function, disease severity and local cytokine milieu. *Int J Mol Sci*. 2020;21(6):1-14. doi:10.3390/ijms21061958
19. Dapic I, Jakasa I, Yau NLH, Kezic S, Kammeyer A. Evaluation of an HPLC method for the determination of natural moisturizing factors in the human stratum corneum. *Anal Lett*. 2013;46(14):2133-2144. doi:10.1080/00032719.2013.789881
20. Meldgaard M, Szecsi PB, Carlsen BC, et al. A novel multiplex analysis of filaggrin polymorphisms: a universally applicable method for genotyping. *Clin Chim Acta*. 2012;413(19–20):1488-1492. doi:10.1016/j.cca.2012.06.014
21. Kezic S, McAleer MA, Jakasa I, et al. Children with atopic dermatitis show increased activity of β-glucocerebrosidase and stratum corneum levels of glucosylcholesterol that are strongly related to the local cytokine milieu. *Br J Dermatol*. 2022;186(6):988-996. doi:10.1111/bjd.20979
22. Mirzaian M, Wisse P, Ferraz MJ, et al. Simultaneous quantitation of sphingoid bases by UPLC-ESI-MS/MS with identical (13) C-encoded internal standards. *Clin Chim Acta*. 2017;466:178-184. doi:10.1016/j.cca.2017.01.014
23. Groener JE, Poorthuis BJ, Kuiper S, Helmond MT, Hollak CE, Aerts JM. HPLC for simultaneous quantification of total ceramide, glucosylceramide, and ceramide trihexoside concentrations in plasma. *Clin Chem*. 2007;53(4):742-747. doi:10.1373/clinchem.2006.079012
24. Gold H, Mirzaian M, Dekker N, et al. Quantification of globotriaosylsphingosine in plasma and urine of fabry patients by stable isotope ultraperformance liquid chromatography-tandem mass spectrometry. *Clin Chem*. 2013;59(3):547-556. doi:10.1373/clinchem.2012.192138
25. Jepsen AA, Chawes BL, Carson CG, et al. High breast milk IL-1β level is associated with reduced risk of childhood eczema. *Clin Exp Allergy*. 2016;46(10):1344-1354. doi:10.1111/cea.12770
26. Gelman A. Scaling regression inputs by dividing by two standard deviations. *Stat Med*. 2008;27(15):2865-2873. doi:10.1002/sim.3107
27. Lam BWS, Yam TYA, Chen CP, Lai MKP, Ong WY, Herr DR. The noncanonical chronicles: emerging roles of sphingolipid structural variants. *Cell Signal*. 2021;79:109890. doi:10.1016/j.cellsig.2020.109890
28. Maula T, Artetxe I, Grandell PM, Slotte JP. Importance of the sphingoid base length for the membrane properties of ceramides. *Biophys J*. 2012;103(9):1870-1879. doi:10.1016/j.bpj.2012.09.018
29. Zhao L, Spassieva S, Gable K, et al. Elevation of 20-carbon long chain bases due to a mutation in serine palmitoyltransferase small subunit b results in neurodegeneration. *Proc Natl Acad Sci U S A*. 2015;112(42):12962-12967. doi:10.1073/pnas.1516733112
30. Pavicic T, Wollenweber U, Farwick M, Korting HC. Anti-microbial and -inflammatory activity and efficacy of phytosphingosine: an in vitro and in vivo study addressing acne vulgaris. *Int J Cosmet Sci*. 2007;29(3):181-190. doi:10.1111/j.1467-2494.2007.00378.x
31. Kim S, Hong I, Hwang JS, et al. Phytosphingosine stimulates the differentiation of human keratinocytes and inhibits TPA-induced inflammatory epidermal hyperplasia in hairless mouse skin. *Mol Med*. 2006;12(1–3):17-24. doi:10.2119/2006-00001.Kim
32. Choi HK, Cho YH, Lee EO, Kim JW, Park CS. Phytosphingosine enhances moisture level in human skin barrier through stimulation of the filaggrin biosynthesis and degradation leading to NMF formation. *Arch Dermatol Res*. 2017;309(10):795-803. doi:10.1007/s00403-017-1782-8
33. Hachem JP, Roelandt T, Schürer N, et al. Acute acidification of stratum corneum membrane domains using polyhydroxyl acids improves lipid processing and inhibits degradation of corneodesmosomes. *J Invest Dermatol*. 2010;130(2):500-510. doi:10.1038/jid.2009.249
34. Kondo N, Ohno Y, Yamagata M, et al. Identification of the phytosphingosine metabolic pathway leading to odd-numbered fatty acids. *Nat Commun*. 2014;5:5338. doi:10.1038/ncomms6338
35. Han G, Gupta SD, Gable K, et al. Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities. *Proc Natl Acad Sci U S A*. 2009;106(20):8186-8191. doi:10.1073/pnas.0811269106
36. Hornemann T, Penno A, Rütti MF, et al. The SPTLC3 subunit of serine palmitoyltransferase generates short chain sphingoid bases. *J Biol Chem*. 2009;284(39):26322-26330. doi:10.1074/jbc.M109.023192
37. Laffet GP, Genette A, Gamboa B, Auroy V, Voegel JJ. Determination of fatty acid and sphingoid base composition of eleven ceramide subclasses in stratum corneum by UHPLC/scheduled-MRM. *Metabolomics*. 2018;14(5):69. doi:10.1007/s11306-018-1366-4
38. Wakita H, Nishimura K, Takigawa M. Composition of free long-chain (sphingoid) bases in stratum corneum of normal and pathologic human skin conditions. *J Invest Dermatol*. 1992;99(5):617-622. doi:10.1111/1523-1747.ep12668019
39. Thijs J, Krastev T, Weidinger S, et al. Biomarkers for atopic dermatitis: a systematic review and meta-analysis. *Curr Opin Allergy Clin Immunol*. 2015;15(5):453-460. doi:10.1097/aci.0000000000000198
40. Ewald DA, Malajian D, Krueger JG, et al. Meta-analysis derived atopic dermatitis (MADAD) transcriptome defines a robust AD signature highlighting the involvement of atherosclerosis and lipid

metabolism pathways. *BMC Med Genomics*. 2015;8:60. doi:10.1186/s12920-015-0133-x

41. Elias PM, Hatano Y, Williams ML. Basis for the barrier abnormality in atopic dermatitis: outside-inside-outside pathogenic mechanisms. *J Allergy Clin Immunol*. 2008;121(6):1337-1343. doi:10.1016/j.jaci.2008.01.022
42. Rehbinder EM, Advocaat Endre KM, Lødrup Carlsen KC, et al. Predicting skin barrier dysfunction and atopic dermatitis in early infancy. *J Allergy Clin Immunol Pract*. 2020;8(2):664-673.e5. doi:10.1016/j.jaip.2019.09.014
43. Berents TL, Lødrup Carlsen KC, Mowinckel P, et al. Transepidermal water loss in infancy associated with atopic eczema at 2 years of age: a population-based cohort study. *Br J Dermatol*. 2017;177(3):e35-e37. doi:10.1111/bjd.15157

SUPPORTING INFORMATION

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

How to cite this article: Rinnov MR, Halling A-S, Gerner T, et al. Skin biomarkers predict development of atopic dermatitis in infancy. *Allergy*. 2023;78:791-802. doi: 10.1111/all.15518