Natural moisturizing factors in children with and without eczema

Associations with lifestyle and genetic factors

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Conflicts of interest

The authors have no conflicts of interest to declare.

Data Availability Statement

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

Abstract

Background

Filaggrin-derived natural moisturizing factors (NMF) play an important role in skin barrier function and in atopic dermatitis (AD). Its deficiency is associated with dry skin and increased

surface pH. Studies on childhood environmental exposures and associations to NMF levels are scarce.

Objectives

We investigated previous exposures and genetic factors and their associations with NMF levels in young children.

Methods

In a case-control study nested in a prospective birth cohort (Odense Child Cohort), 169 healthy controls (HC) and 99 children with AD were included consecutively at the age of 7 years based on previous responses from questionnaires administered at 18 months, 3 years and 5 years, pertaining to past medical history including allergy-specific questions. NMF levels were measured via a stratum corneum tape-stripping technique, genotyping for filaggrin (*FLG)* gene variants was performed and data on external exposures including usage of moisturizer and topical steroids, antibiotics and early pet exposures were obtained from questionnaires.

Results

NMF levels were significantly lower in AD participants compared to HC ($p<0.001$). This significance persisted after stratifying for AD subgroups of *present* AD, *current* AD during the last year and *previous* AD ($p<0.001$, $p=0.039$, $p=0.009$ respectively). There was a significant association between NMF and *FLG* genotype ($p=0.016$, $p=0.002$ for HC, AD respectively). NMF levels were negatively correlated with early age moisturizer use (<18 months, $p=0.001$) in HC but not significant in AD.

Conclusions

We found decreased levels of NMF with early moisturizer use and a genetic influence of the *FLG* variant on these levels. NMF was decreased in the AD subgroup with *previous* AD compared with HC, which could suggest the persistence of a Th_2 cytokine milieu suppressing these levels.

INTRODUCTION

Atopic dermatitis (AD) affects numerous children worldwide, with a previous report of a six-year cumulative incidence of 22.8%[1]. Recent consensus papers highlight skin barrier dysfunction in the etiopathogenesis of AD[2, 3]. Limited studies have investigated the

association of natural moisturizing factors (NMF) in AD patients[4-8]. NMF is the breakdown product of filaggrin and plays an important role in skin barrier function by its humectant properties and contribution to the acid mantle[9]. NMF levels are strongly associated with filaggrin (*FLG)* gene loss-of-function variants, however, it is found to be decreased in AD patients with and without a *FLG* variant[5, 6, 8, 10]. Decreased NMF levels have mainly been associated with moderate to severe AD phenotypes until recently, where decreased levels were also demonstrated in patients with mild or previous history of AD[8]. Decreased NMF levels have been shown to be associated with environmental factors including humidity, steroid use, soaps and water exposure[9, 11, 12].

NMF levels can be decreased already from an early age - increased transepidermal water loss (TEWL) is associated with lower NMF concentrations present in the newborn period and has been associated with a higher risk of developing AD at one year of age[13, 14]. NMF levels can give insight into skin barrier function - however it is unknown whether external exposures in childhood have an effect on these levels. In this case-control study, we investigated selected exposures in infancy and early childhood as well as genetic factors that might be associated with NMF concentrations. We investigated potential exposures based on previous known or investigated associations with AD, including use of moisturizers in the first years, long term use of moisturizer, exposure to antibiotics, indoor cat and dog exposure in the first years, and association with the most common *FLG* variants found in the European population.

MATERIALS AND METHODS

Study design

Our case-control study is an ancillary project within the Odense Child Cohort (OCC), a prospective birth cohort of 2549 children born between 2010-2013 in the Odense municipality, as previously described elsewhere[15]. These children at the time of our study were invited to their 7-year-old visit according to their birthdates – the fifth out of nine planned standardized physical examinations in addition to questionnaires and biological specimens taken and stored in a biobank. Healthy controls were pre-selected based on a negative response to questions regarding any chronic illnesses and medications from the questionnaire administered at 5 years of age, in addition to negative responses to the

presence of eczema, flexural rash, diagnosis and treatment for AD, asthma, food allergy or hay fever from the 18-month and 3-year-old questionnaires. AD participants were pre-selected based on positive eczema-related responses. We invited consecutively these pre-selected participants in our recruitment period from January 2018 to March 2020 in extension of their standard OCC 7-year-old visit to participate in an additional focused clinical examination, allergy-specific questionnaire and interview. Participants were included or excluded in the study after confirmation of group allocation as healthy control or AD at the clinical visit, based on clinical interview and physical examination supplemented with allergy-related details obtained from the participant's electronic medical record. We collected biospecimens including skin tape strips, blood samples for those who agreed to venipuncture and/or buccal swabs for *FLG* analysis.

The STROBE checklist was used as a guidance in formulating the manuscript.

Diagnostic criteria

Healthy controls were defined as participants without atopic or chronic disease. Participants with AD were included based on the UK Working Party diagnostic criteria and excluded if they had other chronic inflammatory diseases. AD participants were further categorized into: 1) *present AD* – lesions at the time of clinical examination 2) *current AD* – lesions within the last year but none at the time of clinical examination or 3) *previous AD* – no lesions within the last year. In cases with *present* AD, disease severity was scored at the clinical examination using the SCORAD index [16, 17]. The degree of xerosis was assessed at the body site where the NMF samples were collected based on the same scoring evaluation as in the SCORAD index for all participants.

Data collection

Risk predictor and confounder variables

Data variables were obtained prospectively from the OCC questionnaires and pregnancy/birth records – these questionnaires were administered with the standard OCC visits during the in utero period and at the three and eighteen months, three, five, and seven year visits and supplemented with our questionnaire at the extended 7 year visit. The use of emollients, including age at first regular use and lifetime duration of regular use, was ascertained from our supplementary questionnaire at the 7 year visit - possible responses

were sub-categorized into time windows, which are detailed in **Tables 1-3** with the other variables.

Assessment of NMF levels in the stratum corneum

We measured NMF levels in the stratum corneum level of the epidermis using a noninvasive tape-stripping technique as earlier described[10]. Five disc samples (1.5 cm^2, D-Squame; CuDerm, USA) were sequentially taken from the same location on the volar surface of unaffected non-lesional skin (a minimum of 3 cm away from lesional skin) on the forearm at the midpoint between the wrist and the antecubital fossa, after equal pressure was applied with a disc pressure applicator (D500 D-Squame; CuDerm, USA) for 10 seconds. We discarded the first four samples to improve sample homogeneity and limit contamination error. We collected and stored the fifth sample at -20°C until further analysis. To normalize for the variable stratum corneum amount collected by a disc, total protein content was measured using the Pierce Micro BCA Protein Assay Kit (Thermo Fischer Scientific, USA). The major NMF components histidine, pyrrolidone-5-carboxylic acid (PCA), trans- and cis-urocanic acid (UCA) were measured with high-performance liquid chromatography (HPLC) analysis as described elsewhere[18]. The four components were combined to give a 'total NMF' value. Participants were instructed to refrain from using moisturizer on the forearms for at least one day prior to their visit.

Genotyping

The participants were genotyped for R501X, 2282del4, R2447X, S3247X - the most common *FLG* variants in Europe[19]. Buffy coats from blood samples or buccal swabs were analyzed for these four SNPs using nuclease resistant probes in a 2-step real-time PCR, followed by melt analysis (PentiPlex, PentaBase, Denmark)[20].

Statistics

We performed Pearson's chi-squared test to test for differences in categorical characteristics in AD and healthy controls. We used rank sum to test for differences in numerical non-normal distributed characteristics between groups. NMF was normally distributed per diagnostic plots. Student's t-test was used to test intergroup differences between two

samples if normally distributed. Kruskal Wallis test was performed for data not normally distributed followed by *post hoc* Dunn test for multiple testing.

Multivariate regression was performed using NMF concentration as the dependent variable and adjusting for confounders. We evaluated the effect of group allocation (AD/healthy controls) by performing a simple regression with NMF and the exposure variables. An overall trend for a larger effect of our main exposure variable was seen in the AD group compared to healthy controls in the unadjusted model. We therefore performed multivariate regression models separately for healthy controls and AD participants. Confounders were determined by literature review and background scientific knowledge with the assistance of direct acyclical graphing to visualize associations between potential confounders to exposures and the main outcome. We selected the following potential confounders - mode of delivery, maternal smoking in pregnancy, maternal education level, sibling rank order, breastfeeding, xerosis score and SCORAD severity score, *FLG* variant status, season of clinical visit and parental history of atopy. We selected the following exposures: time point of first moisturizer use (main exposure), lifetime duration of moisturizer use, early life indoor cat and dog exposure, early life use of antibiotics, bathing habits and potency of steroid use in the last 12 months. All analysis were performed with STATA 16.1 (Texas, USA). P-values < 0.05 were considered significant.

Missing data were evaluated by performing Pearson's chi-squared or Fisher's exact test and found that the distribution of missing data was similar in both AD and healthy control groups - evidence for the data being missing completely at random. We therefore treated missing values by performing complete case analysis.

Sample size

The optimal sample size was difficult to assess based on the novelty of our study – we attempted to estimate the needed number in each group using statistical considerations for a study of the effect of one variable on another, based on estimated standard deviations from a published pilot study[5, 21]. The sample size was estimated to n=200 in each group, power=80%, significance level=5%, drop-out rate of 15%. The dependent variable was NMF level and the independent variable chosen for the calculations was use of moisturizer within

the last 90 days, based on data accessibility and limited literature. Detectable differences were clinically relevant based on estimated standard deviations of the dependent variable. Due to the uncertainty of our estimations, the population size was re-evaluated around the halfway point of the study and found to delineate associations in the two groups.

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.

RESULTS

Study population

458 children were potentially eligible to be included in the study. Out of those invited, 211 healthy controls and 157 children with possible AD accepted and participated in the clinical visit. There was no difference in non-participation between the AD vs. healthy control group (Pearson's chi-squared test, p=0.30, not shown). The participant group and inclusion criteria were confirmed in 169 healthy controls and 99 children with AD (**Fig 1**). DNA genotyping was analyzed from 137 blood and 128 buccal swab samples; 3 participants had missing samples.

Baseline characteristics and frequencies are listed in **Table 1**. There was a significant group difference in parental history of allergy (p<0.001) and *FLG* variant status (p=0.041), and a significant difference in xerosis score (p<0.001). Frequencies of exposures are outlined in **Table 2**. There was a significant group difference in age at first use and duration of moisturizer use (p<0.001), antibiotic use in infancy (p<0.001), and potency of steroid use (p<0.001) with greater frequencies in the AD group.

Associations with NMF and covariates

NMF levels were significantly lower in AD participants compared to healthy controls (p<0.001), also when stratifying for heterozygous gene variant (p=0.014, not shown). This significance also persisted after stratifying for the AD subgroups – NMF levels remained lower in *present* AD (p<0.001), *current* AD with lesions in the last year (p=0.039) and *previous* (p=0.009) AD compared with healthy controls (**Fig 2**). There was an association between

NMF levels and *FLG* genotype for both healthy controls and AD (p=0.016, p=0.002, respectively, not shown).

Associations with NMF and covariates in the multivariate linear regression models

The final regression model included the following variables: age of first regular moisturizer use, lifetime duration of moisturizer use, cat and dog exposure and use of antibiotics in the first years, potency of steroids used in the last 12 months, *FLG* variant status, parental history of allergy, season of clinical visit, breastfeeding, xerosis and SCORAD score. Lifetime duration of moisturizer use was omitted in the healthy control group due to collinearity. We excluded bathing habits as an exposure nor did we adjust for sibling rank order, maternal smoking during pregnancy, maternal education group and mode of delivery because these variables were not significantly different between the two groups (**Tables 1-2**) and a limited sample size.

Table 3 shows the associations with NMF and the tested covariates in the multivariate regression models for both healthy controls and AD participants. There was a significant negative association between NMF levels with different ages of first regular moisturizer use for healthy controls and a negative non-significant trend in AD participants.

DISCUSSION

NMF levels are significantly lower in AD participants than healthy controls which is consistent with other studies in European Caucasian populations[5, 8, 22-25]. *FLG* variants are the strongest genetic determinant of filaggrin expression and NMF levels in the stratum corneum - NMF levels were negatively associated with *FLG* variants in our study, as demonstrated in previous studies [5, 6, 26]. Levels of NMF were reduced in AD compared to healthy controls, also after stratifying for *FLG* variants. While only approximately 10% of the European population have a *FLG* variant, up to 40% are asymptomatic carriers[27 28]. This further supports the hypothesis of multifactorial pathways leading to clinical disease that is independent of *FLG* variant.

We sampled non-lesional skin sites in all the participants, including AD participants with *present* AD where the samples were collected from the skin site at a minimum of 3 cm from

lesional skin. The reduced NMF levels in non-lesional skin in AD patients supports the theory of inflammatory changes that may be present in subclinical disease. This finding correlates with previous studies and is reiterated in a recent study investigating the effect of the distance from the lesion on the NMF levels[29].

An important finding in the present study is the persistence of decreased NMF levels in our group of *previous* AD participants i.e. those who have not had symptoms in at least one year. This is consistent with the study of Engebretsen et al., however in that study, the majority of the patient population had a *FLG* variant, which can alone contribute to decreased NMF levels[8]. There were no statistical difference in *FLG* variant status between our healthy control and *previous* AD groups (data not shown), which points to mechanisms other than *FLG* variant. One hypothesis is that although these children "superficially" have outgrown their AD, there may still be a persistent underlying skewed Th_2 cytokine milieu which downregulates the filaggrin expression and consequently NMF levels. Whether this might have future clinical implications for remitting-relapsing disease in adulthood or the development of other atopic comorbidities, is uncertain.

NMF levels were inversely associated with different early age periods of first regular moisturizer use in healthy controls – from birth to six months and twelve to eighteen months. The same inverse trend was seen at the other age periods in both healthy controls and AD participants, as well as for duration of moisturizer use in AD participants, though not reaching significance. In vitro studies have demonstrated the occluding effect of emollients with better retention of moisture and its subsequent inhibitory effect on filaggrin proteolysis and breakdown resulting in lower NMF levels[30, 31]. This effect is corroborated in our case-control study and could suggest that the general effect on NMF concentrations is not transient but may persist over time and be related to the duration of moisturizer use. While we found a significant inverse association between early age use of moisturizer and NMF levels in healthy controls, this does not entail causality nor elucidate temporality. One cannot assert that early use of moisturizer decreases NMF levels. Nor can one exclude that the healthy controls had dry skin or other skin conditions at an early age; this may have led to the increased use of moisturizer. We also lacked details regarding the type, content and

amount of moisturizer used during the early years, which may have had a differential effect on moisture retention and skin function and subsequently NMF levels[32].

Methodological considerations

This is the first case-control study to our knowledge that investigated infancy and young childhood exposures and NMF levels. Furthermore, NMF levels were evaluated in children with *present* AD, *current* AD in the last year and *previous* AD. Our investigation is a sub-study in a larger ongoing prospective, population-based, observational birth cohort. Our study population is relatively homogenous in the maternal education levels and ethnic demographics in both our healthy control and AD groups, which mitigates selection bias. The ethnic proportions of the cohort are generalizable to the Danish population. All the participants were examined by the same pediatrician who had completed a practical manual and test developed for the UK Working Party diagnostic criteria for AD[33]. It is possible that our control and AD participants were misclassified. However, we had access to questionnaire responses completed prospectively from the prenatal period and during infancy and early childhood and therefore less prone to recall bias. These responses were used for pre-selection of healthy control and AD groups, which decreases the risk of information bias and group misclassification. Questions regarding early life use of moisturizer were primarily ascertained from our supplementary questionnaire at the 7-year-old visit and could be strongly influenced by recall bias. There was a significantly higher frequency of parental allergy in our AD group and subsequently possible higher rates of pet allergy and avoidance, which could have a significant confounding effect on pet ownership and its association with NMF levels. Our study and results are limited by power, which was difficult to ascertain by power calculation due to the novelty of the study.

Conclusion

In our case-control study, we corroborated the findings of earlier studies and found decreased levels of NMF in AD participants compared with healthy controls, and a genetic influence of the *FLG* variant on these levels in a larger sample population. NMF levels remained decreased after stratifying our AD group into *present* and *current* AD *and* notably *previous* AD - participants who had not had active disease in at least one year. We found significant inverse associations for earlier use of moisturizer and NMF levels in healthy

controls, with a similar non-significant trend in early use and duration of use with NMF levels in our AD participants. Our study did not find any significant associations with NMF levels and exposures to cats, dogs and antibiotics in the first years, nor with steroids used in the last 12 months. Larger longitudinal studies examining NMF levels with other skin barrier parameters and exposures from birth might better elucidate an understanding of the influence of external exposures on skin barrier impairment, how it correlates with disease progression and its role in the pathogenesis of AD.

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REFERENCES

1. Eller E, Kjaer HF, Høst A, Andersen KE, Bindslev-Jensen C. Development of atopic dermatitis in the DARC birth cohort. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 2010;21(2 Pt 1):307-14.

2. Davidson WF, Leung DYM, Beck LA, Berin CM, Boguniewicz M, Busse WW, et al. Report from the National Institute of Allergy and Infectious Diseases workshop on "Atopic dermatitis and the atopic march: Mechanisms and interventions". The Journal of allergy and clinical immunology. 2019.

3. Werfel T, Allam JP, Biedermann T, Eyerich K, Gilles S, Guttman-Yassky E, et al. Cellular and molecular immunologic mechanisms in patients with atopic dermatitis. The Journal of allergy and clinical immunology. 2016;138(2):336-49.

4. Kezic S, O'Regan GM, Lutter R, Jakasa I, Koster ES, Saunders S, et al. Filaggrin loss-of-function mutations are associated with enhanced expression of IL-1 cytokines in the stratum corneum of patients with atopic dermatitis and in a murine model of filaggrin deficiency. The Journal of allergy and clinical immunology. 2012;129(4):1031-9.e1.

5. Kezic S, O'Regan GM, Yau N, Sandilands A, Chen H, Campbell LE, et al. Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. Allergy. 2011;66(7):934-40.

6. O'Regan GM, Kemperman PM, Sandilands A, Chen H, Campbell LE, Kroboth K, et al. Raman profiles of the stratum corneum define 3 filaggrin genotype-determined atopic dermatitis endophenotypes. The Journal of allergy and clinical immunology. 2010;126(3):574-80.e1.

7. Riethmuller C, McAleer MA, Koppes SA, Abdayem R, Franz J, Haftek M, et al. Filaggrin breakdown products determine corneocyte conformation in patients with atopic dermatitis. The Journal of allergy and clinical immunology. 2015;136(6):1573-80.e2.
8. Engebretsen KA, Bandier J, Kezic S, Riethmuller C, Heegaard NHH, Carlsen BC, et al. Concentration of filaggrin monomers, its metabolites and corneocyte surface texture in individuals with a history of atopic dermatitis and controls. Journal of the European Academy of Dermatology and Venereology : JEADV. 2018;32(5):796-804.
9. Rawlings AV, Harding CR. Moisturization and skin barrier function. Dermatol Ther. 2004;17 Suppl 1:43-8.
10. Kezic S, Kammeyer A, Calkoen F, Fluhr JW, Bos JD. Natural moisturizing factor components in the stratum corneum as biomarkers of filaggrin genotype: evaluation of minimally invasive methods. The British journal of dermatology. 2009;161(5):1098-104.
11. Engebretsen KA, Kezic S, Jakasa I, Hedengran A, Linneberg A, Skov L, et al. Effect of atopic skin stressors on natural moisturizing factors and cytokines in healthy adult epidermis. The British journal of dermatology. 2018;179(3):679-88.
12. Koppes SA, Charles F, Lammers L, Frings-Dresen M, Kezic S, Rustemeyer T. Efficacy of a Cream Containing Ceramides and Magnesium in the Treatment of Mild to Moderate Atopic Dermatitis: A Randomized, Double-blind, Emollient- and Hydrocortisone-controlled Trial. Acta Derm Venereol. 2016;96(7):948-53.
13. Chittock J, Cooke A, Lavender T, Brown K, Wigley A, Victor S, et al. Development of stratum corneum chymotrypsin-like protease activity and natural moisturizing factors from birth to 4 weeks of age compared with adults. The British journal of dermatology. 2016;175(4):713-20.
14. Kelleher M, Dunn-Galvin A, Hourihane JO, Murray D, Campbell LE, McLean WH, et al. Skin barrier dysfunction measured by transepidermal water loss at 2 days and 2 months predates and predicts atopic dermatitis at 1 year. The Journal of allergy and clinical immunology. 2015;135(4):930-5.e1.
15. Kyhl HB, Jensen TK, Barington T, Buhl S, Norberg LA, Jorgensen JS, et al. The Odense Child Cohort: aims, design, and cohort profile. Paediatric and perinatal epidemiology. 2015;29(3):250-8.
16. Severity scoring of atopic dermatitis: the SCORAD index. Consensus Report of the European Task Force on Atopic Dermatitis. Dermatology. 1993;186(1):23-31.
17. Schmitt J, Langan S, Deckert S, Svensson A, von Kobyletzki L, Thomas K, et al. Assessment of clinical signs of atopic dermatitis: a systematic review and recommendation. The Journal of allergy and clinical immunology. 2013;132(6):1337-47.
18. Dapic I JI, Yau NLH, Kezic S, Kammeyer A. Evaluation of an HPLC Method for the Determination of Natural Moisturizing Factors in the Human Stratum Corneum. Analytical Letters. 2013;46:2133-44.

19. Sandilands A, Terron-Kwiatkowski A, Hull PR, O'Regan GM, Clayton TH, Watson RM, et al. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. Nature genetics. 2007;39(5):650-4.
20. Christensen UB. EasyBeacons for the detection of methylation status of single CpG duplets. Methods Mol Biol. 2008;429:137-60.
21. Schoenfeld D. Statistical considerations for a study of the effect of one variable on another: Massachusetts General Hospital Biostatistics Center; 2010 [Available from: http://hedwig.mgh.harvard.edu/sample_size/js/js_associative_quant.html.
22. Hulshof L, Hack DP, Hasnoe QCJ, Dontje B, Jakasa I, Riethmüller C, et al. A minimally invasive tool to study immune response and skin barrier in children with atopic dermatitis. The British journal of dermatology. 2019;180(3):621-30.
23. Haftek M, McAleer MA, Jakasa I, McLean WI, Kezic S, Irvine AD. Changes in nano-mechanical properties of human epidermal cornified cells in children with atopic dermatitis. Wellcome Open Res. 2020;5:97.
24. Jurakic Toncic R, Kezic S, Jakasa I, Ljubojevic Hadzavdic S, Balic A, Petkovic M, et al. Filaggrin loss-of-function mutations and levels of filaggrin degradation products in adult patients with atopic dermatitis in Croatia. Journal of the European Academy of Dermatology and Venereology : JEADV. 2020;34(8):1789-94.
25. Mlitz V, Latreille J, Gardinier S, Jdid R, Drouault Y, Hufnagl P, et al. Impact of filaggrin mutations on Raman spectra and biophysical properties of the stratum corneum in mild to moderate atopic dermatitis. Journal of the European Academy of Dermatology and Venereology : JEADV. 2012;26(8):983-90.
26. Brown SJ, McLean WH. One remarkable molecule: filaggrin. The Journal of investigative dermatology. 2012;132(3 Pt 2):751-62.
27. Barker JN, Palmer CN, Zhao Y, Liao H, Hull PR, Lee SP, et al. Null mutations in the filaggrin gene (FLG) determine major susceptibility to early-onset atopic dermatitis that persists into adulthood. The Journal of investigative dermatology. 2007;127(3):564-7.
28. Weidinger S, O'Sullivan M, Illig T, Baurecht H, Depner M, Rodriguez E, et al. Filaggrin mutations, atopic eczema, hay fever, and asthma in children. The Journal of allergy and clinical immunology. 2008;121(5):1203-9.e1.
29. Toncic R, Kezic S, Ljubojevic S, Marinović B, Jakasa I. Stratum Corneum Biomarkers in Atopic Dermatitis: Biological and Spatial Variability. The Open Biomarkers Journal. 2020;10:47-54.
30. Scott IR, Harding CR. Filaggrin breakdown to water binding compounds during development of the rat stratum corneum is controlled by the water activity of the environment. Dev Biol. 1986;115(1):84-92.

429 31. Takahashi M, Tezuka T. The content of free amino acids in the stratum corneum is increased in
430 senile xerosis. Arch Dermatol Res. 2004;295(10):448-52.
431 32. Elias PM, Wakefield JS, Man MQ. Moisturizers versus Current and Next-Generation Barrier Repair
432 Therapy for the Management of Atopic Dermatitis. Skin Pharmacol Physiol. 2019;32(1):1-7.
433 33. Williams H. UK Diagnostic Criteria for Atopic Dermatitis [Available from:
434 https://www.nottingham.ac.uk/research/groups/cebd/resources/uk-diagnostic-criteria-for-atopic-
435 dermatitis.aspx.

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Figure legends

Box 1. Overview of abbreviations and definitions

Figure 1. Flow diagram of participant inclusion

Figure 2. Distribution of NMF values according to healthy controls and atopic dermatitis (AD) subgroups.

Table 1. Basic characteristics and frequencies of the study population, n(%)

	Healthy Controls n=169	Atopic Dermatitis n=99	Chi^2/ranksum p-value
Gender			0.571
Female	76 (45)	41 (41)	
Male	93 (55)	58 (59)	
Maternal ethnicity			0.743
Danish	157 (93)	93 (94)	
Other	12 (7)	6 (6)	
Maternal education			0.880
Upper secondary	20 (12)	11 (11)	
Bachelor or equivalent	88 (52)	50 (51)	
Master or equivalent	59 (35)	38 (38)	
Maternal smoking during pregnancy			0.196
No	153 (91)	95 (96)	
Yes	6 (4)	1 (1)	
Mode of delivery			0.791†
Spontaneous vaginal	128 (76)	74 (75)	
Instrumental vaginal	0 (0)	0 (0)	
Caesarean section	40 (24)	25 (25)	
Breastfeeding‡ (exclusive, n=236; *missing*=32§)			0.272
Mean number of weeks	14	15	
Sibling rank			0.890
1	72 (43)	44 (44)	
2	75 (44)	44 (44)	
≥3	22 (13)	11 (11)	
Season of clinical visit			0.270
Winter	39 (23)	18 (18)	
Spring	56 (33)	26 (26)	
Summer	38 (22)	25 (25)	
Autumn	36 (21)	30 (30)	
Family history of allergy¶			
Maternal	55 (33)	56 (57)	**<0.001**
Paternal	57 (34)	55 (56)	**<0.001**
Personal history of other allergy			
Food Allergy	0	4 (4)	

Asthma[¶]	0	10 (10)	
Allergic rhinoconjunctivitis	0	17 (17)	
FLG gene variant			**0.041**[a]
Wild Type	161 (95)	85 (86)	
Heterozygous	8 (5)	9 (9)	
Homozygous/double heterozygous	0 (0)	2 (2)	
SCORAD score[b] (mean)	n/a	7.7	
Xerosis score[c] (mean)	0.15	0.39	**<0.001**

Notes: † tested between spontaneous vaginal and caesarean section;

‡ assessed at the 3 and 18 month questionnaire; §missing data specified for >5% of missing values;

¶ self-reported; a- tested between wildtype and FLG variants;

b- SCORAD severity index score: clear(0-9.9), mild(10.0-28.9), moderate(29.0-48.9), severe(49.0-103);

c- based on dryness criteria from SCORAD severity index: 0 (absent) 1(mild) 2(moderate) 3 (severe)

Table 2. Frequency of exposures in the study population, n(%)

	Healthy controls	Atopic Dermatitis	Chi^2 test
	n=169	n=99	p-value
Pet exposure from infancy† (n=207; *missing*=61‡)			
Cats	27 (16)	8 (8)	0.071
Dogs	21 (12)	14 (14)	0.611
Antibiotic use in infancy†§ (n=210; missing=58‡)			**<0.001**
No	93 (55)	33 (33)	
Yes	40 (24)	44 (44)	
Bathing habits¶			0.488
< 3 per week	83 (49)	43 (43)	
3-6 per week	80 (47)	50 (51)	
≥ 1 daily	6 (4)	6 (6)	
Age at first regular moisturizer use¶			**<0.001**
Birth - 1 month	6 (4)	9 (9)	
1-6 months	4 (2)	29 (29)	
> 6 months	6 (4)	16 (16)	
>12 months	6 (4)	20 (20)	
never	143 (85)	25 (25)	
Lifetime duration of regular moisturizer use¶			**<0.001**
< 1 year	145 (86)	33 (33)	
1-3 years	5 (3)	18 (18)	
4-6 years	10 (6)	29 (29)	
7 years	5 (3)	17 (17)	
Steroid group¶[a] (use in last 12 months)			**<0.001**
None	160 (95)	44 (44)	
Mild potency - group 1	1 (1)	7 (7)	
Moderate potency - group 2	4 (2)	30 (30)	
High potency - group 3-4	4 (2)	17 (17)	

Notes: † assessed at the 18 month questionnaire; ‡ missing data specified for >5% of missing values; § assessed at the 3 month questionnaire; ¶ assessed with the supplemental 7 year questionnaire; a- based on medicin.dk professional classification

Table 3. Associations between NMF and covariates in the final multivariate regression models†

		Healthy controls		Atopic dermatitis	
Covariate‡	**Comparison**	**β-coef**	**p-value**	**β-coef**	**p-value**
FLG variant					
(Wild type vs.)	Heterozygous	-0.19	0.071	-0.29	**<0.001**
	Homo-/Double heterozygous	n/a	n/a	-0.39	0.080
Season of clinical visit					
(Winter vs.)	Spring	0.01	0.924	0.10	0.380
	Summer	0.00	0.952	0.08	0.579
	Autumn	0.01	0.880	0.15	0.167
Parental history of allergy§					
(None vs.)	Single disposition	-0.04	0.336	0.07	0.455
	Double disposition	0.08	0.425	-0.11	0.411
Breastfeeding (exclusive)¶	Per week increase	0.00	0.837	-0.01	0.077
SCORAD severity score[a]	Per unit increase	--	--	0.00	0.525
Xerosis score[b]	Per unit increase	0.09	0.115	-0.19	0.136
Age at first regular moisturizer use					
(Never vs.)	Birth to 1 month	-0.33	**0.003**	-0.24	0.342
	1-6 months	-0.45	**<0.001**	-0.12	0.556
	6-12 months	-0.07	0.245	-0.11	0.531
	12-18 months	-0.30	**<0.001**	-0.04	0.862
	18-24 months	--	**--**	-0.15	0.552
	After 24 months	-0.19	0.117	-0.15	0.474
Duration of moisturizer[c]	Per year increase	--	--	-0.02	0.243
Early cat exposure	No/Yes	0.04	0.518	-0.09	0.301
Early dog exposure	No/Yes	0.00	0.948	-0.11	0.464
Early antibiotic exposure	No/Yes	-0.03	0.534	-0.18	0.108
Steroid group use[d]					
(None vs.)	Mild potency	-0.03	0.779	0.18	0.266
	Moderate potency	0.02	0.807	0.09	0.614
	High potency	-0.13	0.211	0.06	0.634

Notes- † refer to statistics section for details on the model fitting; ‡ reference point in parentheses;

§ self-reported; ¶ mmol/g protein change in NMF for each additional week increase in breastfeeding;

a- SCORAD severity index score: clear(0-9.9), mild(10.0-28.9), moderate(29.0-48.9), severe(49.0-103);

b- based on dryness criteria from SCORAD severity index: 0 (absent) 1(mild) 2(moderate) 3 (severe);

c- mmol/g protein change in NMF for each additional year of moisturizer use;

d- based on medicin.dk professional classification; -- omitted due to collinearity