ASSOCIATION BETWEEN SKIN BARRIER DEVELOPMENT AND EARLY-ONSET ATOPIC DERMATITIS: A LONGITUDINAL BIRTH COHORT STUDY

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ABSTRACT

Background

A diagnosis of atopic dermatitis (AD) is common during infancy; however, it is unclear whether differential skin barrier development defines this period and signals disease onset in predisposed individuals.

Objective

A longitudinal observational cohort study (NCT03143504) assessed the feasibility of remote skin testing from birth to monitor skin barrier maturation and model association with an AD diagnosis by 12-months of age.

Methods

Biophysical testing and infrared spectroscopy were conducted at the maternity ward and family home. Tape stripping collected samples for desquamatory protease and Natural Moisturising factor (NMF) analysis. The four common European Filaggrin (*FLG*) risk alleles were screened.

Results

A total of 128 infants completed the study with 20% developing mild disease. Significant changes in permeability barrier function, desquamatory protease activity and molecular composition assessed spectroscopically were observed longitudinally, but only subtle evidence of differential skin barrier development was noted between infant subgroups. Common *FLG* risk alleles were strongly associated with early onset disease and conferred a significant reduction in NMF and water content by four weeks of age. Accounting for a family history of atopy, these parameters alongside a greater lipid/protein ratio and reduced chymotrypsin-like activity at birth were associated with AD. Measured in ambient conditions, transepidermal water loss did not signal disease risk at any stage.

Conclusion

Skin barrier dysfunction lacked an acquired modality but was considered proportional to cohort severity and suggests that a portfolio of tests used in a community setting, has the potential to improve current AD risk evaluations from birth.

KEY MESSAGES

- Biophysical skin testing at birth was well received by the participating families with subtle skin barrier dysfunction accompanying the mild disease observed in this study.
- An inherited loss of Filaggrin conferred a reduction in NMF at four weeks of age determined by real time IR spectroscopy at the 'bedside' that was associated with AD onset.
- Reduced chymotrypsin-like activity combined with inherited *FLG* loss provided the strongest indication of disease (AUC=0.78) highlighting a novel mechanism for further exploration.

CAPSULE SUMMARY

Remote skin testing from birth is a feasible approach to detect early signals of AD prior to the onset of clinical signs over the first 12 months of life.

KEY WORDS

Atopic dermatitis, remote skin testing, infant skin barrier, infrared spectroscopy.

ABBREVIATIONS

Attenuated total reflectance infrared spectroscopy (ATR-FTIR), Atopic Dermatitis (AD), Natural Moisturising Factor (NMF), Chymotrypsin-like (C-L), Filaggrin (FLG).

INTRODUCTION

Skin barrier breakdown is a significant component of atopic dermatitis (AD) pathogenesis. Although uninvolved skin appears healthy, underlying structural defects render it functionally inadequate and inflamed that corresponds to disease severity (1, 2). A less ordered lipid structure, protease hyperactivity and low levels of natural moisturising factor (NMF) within the stratum corneum (SC) are hallmarks of the barrier abnormality that associate with weakened permeability barrier function, signified by elevated transepidermal water loss (TEWL) (3-5).

With disease prevalence greatest in younger children (aged ≤4 years) worldwide, (6) infants predisposed to AD are not born with clinical signs but have an increased risk of diagnosis before their first birthday (7). Over this time period the developing skin barrier is structurally and functionally immature, suggesting a fragility as it adapts to a terrestrial environment (8, 9). Considering the pathological evidence from established adult AD, skin barrier breakdown may therefore define a differential trajectory from birth that predates active disease. Modifying factors here may include climate, the home environment and parental skin care practices, but their interaction with the maturing skin barrier is unclear.

In a medical era where AD prevention is a key objective, pinpointing susceptible babies for early intervention is an unmet clinical need. One of the best indicators is familial atopic disease, (10) but when screening using this metric, it is estimated that around 40% of cases may be missed altogether (11). Conversely, a risk of unnecessary treatment intervention exists, burdening new parents when time is scarce and mental health may be challenged (12). Additional tools are therefore required to

evaluate disease risk in a maternity or community setting. One possibility is TEWL measurement, but this may be unsuitable due to strict environmental requirements (13).

To address these research questions, a longitudinal study was designed to monitor skin barrier development from birth, and pilot non-invasive measures of lipid structure, protease activity and NMF alongside TEWL for disease risk evaluation. Improving the early detection of at-risk infants may empower parents in future to take measured actions from birth to prevent or delay the possible emergence of AD in their baby.

MATERIALS AND METHODS

Study design

A longitudinal observational cohort study (clinicaltrials.gov ref: NCT03143504) was performed to monitor infant skin development from birth to 12-months of age. By capturing the incidence of AD, the association between subclinical barrier breakdown and disease risk by 12-months could be preliminarily explored. The study was conducted by an experienced team of midwife and technical researchers trained in the instrumentation and clinical scoring, overseen by a senior dermatologist providing consultancy where required. Ethical permission was granted by the Preston North West NHS Research Ethics committee (16/NW/0848) and informed parental consent obtained. The reporting of this study conforms to STROBE (14).

Participants

Full-term, healthy, singleton neonates (≤72 hours old) and their mothers (≥18 years old) living within a 5-mile radius of the University of Sheffield were recruited at Jessop Wing Maternity Unit, Sheffield Teaching Hospital, UK, between April 2017 and December 2019. Table E1 in the Online Repository details the full study eligibility criteria.

Sample size

A recruitment target of 180 neonates was considered appropriate to explore the feasibility of skin testing from birth, with 15-30% anticipated to develop AD by 12-months of age (27-54 cases of disease). Based on a TEWL standard deviation of 2.31g/m^2/hr, (15) a difference of 2g/m^2/hr at birth could be adequately detected between babies that do and do not develop AD (22 babies required for 80% power).

137

138 Skin assessments

139 Remote measurements were performed on the maternity ward (≤72 hours old) and
140 the family home at 4±2weeks and 12±1months, with all follow up visits completed by
141 February 2020. The volar forearms, the right antecubital fossa and thigh were the
142 designated assessment sites. Measurements were performed at the test sites in the
143 presence of eczema. The Neonatal Skin Condition Score (16) was calculated at birth
144 and a visual inspection of dryness and erythema conducted at all time points. ***Skin***
145 ***Barrier Function*****:** A single TEWL reading was obtained in ambient conditions from
146 each skin site using an AquaFlux AF200 closed chamber condensing device (Biox
147 Systems Ltd, London, UK). A measurement was aborted if the infant was distressed.
148 ***Infrared Spectroscopy*****:** A portable 4300 Handheld Fourier Transform Infrared (FTIR)
149 spectrometer equipped with mercury cadmium telluride detector and one bounce/one
150 pass diamond Attenuated Total Reflectance (ATR) accessory (Agilent Technologies,
151 Santa Clara, USA) collected absorption spectra in the mid infrared region from 32
152 scans at 4cm^{-1} resolution. A single spectrum was obtained from each skin site and
153 visually checked for quality. Due to inconsistent ATR-FTIR signal encountered at birth,
154 a prototype three-bounce/two-pass (3B2P) diamond ATR accessory was implemented
155 by the recruitment of participant number 035. All subsequent babies were therefore
156 assessed using the 3B2P accessory, with all prior participants (001-034) excluded
157 from the spectroscopic endpoints at birth for consistency. For quantitative ATR-FTIR
158 parameters, peak intensities related to total lipid (2850cm^{-1} CH_2 stretching mode),
159 sebum (1740cm^{-1} C=O stretching mode) and water (1640cm^{-1} H_20 deformation) were
160 baseline corrected and normalised to Amide II at 1540cm^{-1} to account for contact
161 pressure (17). Lipid structure was assessed by the position of the 2850cm^{-1} CH_2

stretching vibration (4). Negative peak intensities were excluded from the analysis. A commercial baby wipe was piloted for sebum removal prior to an additional ATR-FTIR measurement being taken. ***Tape stripping*****:** Three serial 14mm D squame discs (CuDerm, Dallas, TX, USA) were pooled for *ex vivo* desquamatory protease analysis (left forearm) and Natural Moisturising Factor quantification (left forearm and right antecubital fossa).

Desquamatory protease activity

Caseinolytic and chymotrypsin-like activities were assayed using EnzCheck® (Invitrogen, Paisley, UK) and MeOSuc-Arg-Pro-Tyr-AMC (Peptide Protein Research Ltd, Southampton, UK) substrates. Specific activity (nU/µg) was calculated using SC mass estimated by densitometry.

Measuring NMF abundance *in vivo by* ATR-FTIR

As previously described, (18) a real time spectroscopic measure of NMF was developed by calibrating IR absorption across the fingerprint spectral region (1090-1653cm^{-1}) using a single composite quantitative measure of NMF obtained by *ex vivo* laboratory analysis. Three serial tape strips were extracted and pooled; with pyrrolidone carboxylic acid and urocanic acid analysed by a high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) combined with free amino acid quantification by o-phthalaldehyde derivatisation. Sampling sites in a proportion of infants at four weeks and 12-months old were randomly allocated to model calibration and validation sets. Spectra were normalised to Amide II (1540cm^{-1}) prior to modelling. The final chemometric model was validated using a within-cohort subset of infants, independent to the model build.

187

188 Filaggrin genotyping

189 Saliva was collected at 12-months and genomic DNA extracted using the Oragene
190 OG-250 sampling device and PrepIT L2P kit (DNA Genotek, Ottawa, Canada). The
191 four common European Filaggrin risk alleles were screened by Taqman (R501X and
192 2282del4) or Sanger sequencing (R2447X and S3247X) using established probe and
193 primer sets (19).

194

195 Outcome measures

196 Primary outcomes were the change in skin barrier function, molecular composition and
197 desquamatory protease activity from birth to 4-weeks and 12-months of age.
198 Secondary outcomes included (a) the incidence of AD diagnosed by (i) general
199 practitioner in primary care (remote diagnosis, parental-reported to study team by 12
200 months) or (ii) study investigator using the UK working party criteria at the 12 month
201 visit; (20) (b) parental reported skin rashes; (c) the frequency of Filaggrin risk alleles;
202 and (d) the association of skin barrier function, molecular composition and
203 desquamatory protease activity measured at birth and 4-weeks of age with an AD
204 diagnosis by 12-months. In the presence of clinical signs, the Eczema Area and
205 Severity Index (EASI) score was conducted by a trained researcher at the home visits
206 as a measure of disease severity. Completion of the 12-month visit was the study end
207 point. Additional outcomes on infant skin care practices and parental satisfaction
208 captured by diary, questionnaire and semi-structured interviews, will be reported by
209 subsequent manuscripts.

210

211 Statistical analysis

Study data was captured using FileMaker Pro (Claris, London, UK) and collated in Excel. Normality was checked by Q-Q plot and parameters log transformed where appropriate. For primary outcomes, a matched, mixed model, one-way analysis of variance (ANOVA) or nonparametric equivalent compared means over time. For secondary outcomes associated with AD diagnosis; (a) a two-way ANOVA compared parameter means over time between infants with and without disease; (b) a matched, mixed model, analysis of covariance (ANCOVA) investigated the relationship between TEWL, temperature and time; and (c) multiple logistic regression using the log likelihood ratio test (p=<0.05) for stepwise selection, modelled parameters at birth and four weeks (including *FLG* status) controlling for familial atopy, sex and gestation period. Withdrawn participants were excluded from AD risk analysis. Statistical tests were performed using GraphPad prism 9 (San Diego, California, USA) or IBM SPSS statistics (version 27; Armonk, NY, USA). Panorama PRO was used for spectroscopic chemometric analysis (LabCognition, Cologne, Germany).

RESULTS

A total of 689 eligible families were screened. Of the 180 neonates recruited, (mean age 33.61±17.66 hours at first assessment) 128 completed the 12-month home visit (Figure 1). Two babies were >72 hours old at the first assessment but included in the analysis. Following informed consent, 52 participants withdrew due to screening failure following enrolment (*n*=3); retraction of parental consent (*n*=9); or were loss to follow-up after three failed attempts to schedule a home visit (*n*=40). Mean age at the 4-week and 12-month assessment was 33.43±6.68 days and 12.02±0.75 months respectively. Twenty-five infants (20%) developed AD by 12-months of age (Table I) confirmed by general practitioner (*n*=19) or investigator-diagnosed (*n*=6). The proportion of infants carrying at least one common *FLG* variant allele was 13% (13/99) with a higher prevalence in the AD group (35%) compared to no disease (8%). Familial atopy was high (79%) suggesting infants highly predisposed to AD development. None of the children developed AD by 4 weeks of age. At 12-months the mean whole body EASI score was 1.5 (18/25 infants with clinical signs) indicative of mild disease well controlled by the study endpoint. The infant AD group reported a greater proportion of skin rashes (Table I) and general skin complaints (see Table E2 in the Online Repository). Overall, parents were satisfied with the skin tests performed when asked at the 12 month exit questionnaire (see Table E3 in the Online Repository).

Skin barrier development from birth

As a biomarker of permeability barrier function, TEWL proved highly variable at all developmental time points (Figure 2a) that likely reflected the wide range of ambient temperatures encountered at assessment (Figure 2b). For example at birth, a far greater cohort standard deviation (8.89g/m^2/hr) was noted compared to climate

controlled conditions (15). Accordingly, temperature (but not humidity) correlated with TEWL across all study timepoints (r=0.29, *p*=<0.001, data not shown). As expected, higher temperatures were encountered on the maternity ward at birth compared to the family home at 4-weeks and 12-months. Accounting for this covariate using a matched, mixed model ANCOVA confirmed a significant effect of time (*p*=<0.001) on mean TEWL (log transformed) from birth to 12-months of age, indicating a weakening infant permeability barrier over this period (Figure 2c).

When comparing averaged skin surface ATR-FTIR spectra collected over the course of the study, clear differences in absorption related to SC lipid abundance and confirmational ordering (2850cm^{-1}) sebum (1740cm^{-1}) water and NMF (1653-1090cm^{-1}) existed between age groups that warranted further investigation (see Figure E2 in the Online Repository). By developing and validating a real time *in vivo* measurement of NMF, a reduction was observed at the skin surface in infants carrying *FLG* risk alleles at four weeks of age (see Figure E3 in the Online Repository). After collecting baseline ATR-FTIR spectra, cleansing the skin by commercial baby wipe to remove sebum proved unsuccessful, therefore the post-wipe measurements were not pursued further in the cohort analysis (data not shown).

A summary of skin development over the first year of life is presented by Figure 3. Like TEWL, several biomarkers changed significantly from birth. Caseinolytic protease activity increased 5-fold by 4-weeks of age suggesting a rapid rise in desquamation and cell turnover (Figure 3a). Supportive of this was the 1.4-fold rise in chymotrypsin-like (C-L) protease activity (Figure 3b). An increase in ATR-FTIR modelled NMF abundance from birth was accompanied by a greater 1640/1540cm^{-1} peak ratio at 4-

weeks indicative of higher water content (Figure 3c and d). SC lipids and sebum (peak 2850/1540cm^{-1} and 1740/1540cm^{-1}) decreased from birth alongside an improvement in lipid structure denoted by the shift to a more orthorhombic phase (lower peak 2850cm^{-1} centre of gravity) (21). Although minimal vernix caseosa (VC) presence was noted across the cohort, these observations were presumably due to greater sebaceous lipid deposition at birth (Figure 3e-g). There was little change in investigator-observed skin dryness from birth, but an inverse relationship with spectroscopically determined water content was noted across all timepoints (Figure 3h). Cohort stratification by season of birth did not reveal any significant differences in skin barrier development throughout the first 4-weeks of life (data not shown). Between 4-weeks and 12-months of age were further reductions in NMF, water and lipid abundance in conjunction with increased TEWL.

Subtle differential skin barrier development between infants with and without disease

For TEWL and ATR-FTIR parameters, similar trends were observed across anatomical sites between infants with and without AD (see Figure E4 in the Online Repository). Consequently, the mean of these body sites was calculated (one measurement parameter per infant, per timepoint) allowing greater precision to address the secondary endpoints of AD risk. Overall, few differences in early skin barrier maturation were observed between infants with and without AD; however, subtle patterns existed prior to onset of clinical disease (Figure 4). For example, chymotrypsin-like protease activity at birth was on average 40% lower in infants that developed AD (Figure 4b). Although no difference in visual dryness was apparent by 4-weeks of age (Table I), spectroscopically modelled NMF and water content was on average 15% and 7% lower respectively in infants that developed disease, indicating

a dryer skin surface at this developmental point (Figure 4c and d). The strong inherited NMF defect evident at this timepoint likely influenced this finding (see Figure E3 in the Online Repository). Using a matched mixed model ANOVA with Fisher's LSD post-test, a significant effect of infant subgroup on ATR-FTIR lipid peak 2850/1540cm^{-1} (p=0.0371) was highlighted (Figure 4f). Here, surface lipids at birth were 27% greater (relative to protein) in infants that developed disease, a trend corroborated by lipid esters (Figure 4f-g). Although mean TEWL was elevated at birth in the infant AD group, no significant differential permeability barrier function was noted, representing comparable skin barrier health throughout the study (Figure 2d). Correlation analysis was performed on all parameters tested but yielded no significant associations.

Factors associated with disease risk by 12-months of age

To explore parameters associated with an AD diagnosis by 12-months, logistic regression modelling was employed controlling for sex and gestation period (Table II). Modelling 1st degree atopy alone was not associated with an AD diagnosis (AUC=0.61 ns, data not shown) nor was any form of atopy (AD, asthma and hay fever) when modelled in turn (1st degree relative, data not shown). By comparison, the forward selection of independent variables using a log likelihood ratio threshold (p=<0.05), revealed 5 parameters of additive value to family atopy for early disease risk evaluation (models 1-5) indicated by a significant area under the receiver operating characteristic curve (AUC). A greater ATR-FTIR peak 2850/1540cm^{-1} (birth), reduced C-L protease activity (birth), low NMF (4 weeks) and a drier skin surface (4 weeks) were early signals of disease development. Individuals carrying at least one common *FLG* variant allele (model 5) were 6 times more likely to develop AD by 12-months (AUC=0.73). A second round of forward selection combined sequentially each

significant parameter. Modelling *FLG* status with C-L protease activity (model 6, AUC=0.78) correctly classified 30% and 99% of cases with and without AD respectively at a 50% cut off. In contrast, TEWL was not associated with AD at any timepoint.

DISCUSSION

From birth, a significant period of skin barrier maturation (8, 9) coincides with an increased risk of developing AD (7). By performing a portfolio of tests remotely in a longitudinal birth cohort, this exploratory study identified subtle signals that predated AD onset. A primary *FLG* defect strongly conferred early disease with reduced NMF and a drier skin surface observed by four weeks of age spectroscopically; highlighting potential as a rapid, bedside genotyping tool compared to laboratory analysis. In conjunction with reduced chymotrypsin-like protease activity and increased surface lipids at birth, assessment of these parameters in a community setting proved feasible, and were of additive value for early AD risk evaluation when accounting for a family history of atopy; a metric used currently to identify high risk infants for prophylactic intervention (22).

This study reports that developmentally, skin barrier maturation continues to at least the conclusion of the first year. From birth the SC rapidly acidifies and hydrates (23, 24). Here, using ATR-FTIR peak 1640cm^{-1} attributed to the bending mode of water, (25) a significantly more hydrated skin surface was reported at four weeks of age. This is in agreement with Raman Spectroscopy measuring greater water content throughout the entire depth of the infant SC compared to adults (8). Underlying this evolution towards hydrophilicity may be changing NMF abundance; from low levels at birth increasing with postnatal age (26, 27). A similar trend was observed for C-L desquamatory activity – attributable to KLK-7 (28) - that can be modulated through changes in biochemical environment (29). As a biomarker of skin health, TEWL increased from birth over this early period, although there is uncertainty in this finding due to the extreme range of ambient conditions encountered by the study.

Nevertheless, the observed trend in TEWL is corroborated by a larger infant study, (30) with further weakening of permeability barrier function noted in our cohort beyond more steady state conditions at four weeks, supporting an extended period of optimisation from birth (8). Although minimal vernix caseosa (VC) was noted by this study, bulk surface lipids and lipid esters measured spectroscopically were elevated at birth. With VC comprising of triglycerides, wax esters and squalene of sebaceous origin, any remnants would increase lipid/protein ratio and disorder lipid organisation (increased proportion of branched chain fatty acids) beyond the background SC signal (31). The gradual decline in lipid esters reflects the change in sebum at the neonate skin surface from birth (32, 33).

A secondary outcome measure of this study was disease incidence; allowing the relationship between early skin barrier dysfunction and AD diagnosis to be retrospectively assessed. Although a small cohort, the rate of AD was consistent with two similar UK cohorts, (22, 34) albeit lacking more active disease cases that may reflect a bias towards the more common early-onset/resolving prognosis (35). However, a 2% and 14% rate of moderate-severe AD reported in infants suggests the early onset trajectory although prevalent, is predominantly mild (22, 34).

This study reinforces the association between common European *FLG* variants and early AD development (35, 36). Here, a functional consequence was reduced NMF abundance at 4-weeks representative of *FLG* genotype; an observation supported by Raman Microspectroscopy (AUC=0.79-0.83) performed around birth (37). Considering the constraint of ATR-FTIR measurements to the skin surface, where NMF levels are low following birth, it is plausible that around four weeks of age is the

optimum time to differentiate *FLG* endotypes using this technique. We saw little indication of an acquired NMF defect, representative of a cohort devoid of overt inflammation (5, 38).

There is evidence that weakened permeability barrier function predates early onset AD (39, 40). Although a similar trend here was observed at birth, no significant difference in TEWL was noted between infant subgroups throughout the study. With a clear TEWL abnormality observed in more severe infant disease cases by 12 months of age (5) this likely reflects an absence of inflammation. A further contributory factor could also be the omission of TEWL measurements from the cheek or face, a common site of symptomatic onset (39, 40). Nor was TEWL performed in environmentally controlled conditions where the passage of water through the SC is the sole, rate-limiting factor of vapour flux following an appropriate period of acclimatisation (41). The lack of precision we observed, suggests that 25 babies with AD was underpowered to obtain a reliable cohort estimate of TEWL when measured in unstandardised conditions.

The study produced some novel results. One skin parameter associated with disease that modelled independently to the pronounced inherited *FLG* defect, was reduced KLK7 activity at birth. This contrasts to the elevated expression and activity of desquamatory proteases as a hallmark of established AD (28, 42). Relevant to this finding is increased serine protease inhibitor (Serpin) A12 abundance within the VC of infants that develop AD by 24 months old (43). Expressed in the adult epidermis and an inhibitor of KLK7, Serpin A12 activity may explain the initial reduction in

chymotrypsin-like proteolytic activity observed in the AD infants while the VC is retained (44, 45).

A strength of this project was its pragmatic design, balancing scientific objectives alongside consideration of the families involved over the longer-term study duration. To maximise participant retention, portable equipment was used enabling remote data collection at the bedside and family home; skin tests were restricted to the surface layers; and follow up ceased at 12-months to capture the majority of AD cases over a minimum timeframe. Even under these circumstances, 23% of the families recruited either withdrew or were lost to follow up by four weeks; a comparable rate to a similar study conducted by our group, (15) suggesting more flexible assessment points are required in future work.

The study was limited by design to primarily focus on the feasibility of the remote methodology tested. A single instance of general practitioner confirmed AD was employed as a study endpoint - albeit diagnosis in early childhood challenging - when multiple clinical observations are required throughout infancy to ensure established criteria of disease are fulfilled (46). For example, at 12 months of age only 28% of infants with current eczematous lesions received a UK working party diagnosis due to the uncertainty of itch (46). Further study limitations include the employment of early, fixed assessment points at a time of significant adaptation to terrestrial life, and the decision to combine anatomical sites in the longitudinal analysis. Both may have contributed to clearer evidence of skin barrier breakdown being missed at a later developmental stage. Nor was a hypothesised SC lipid ordering defect assessed spectroscopically due to the contamination of sebum (4). Recent evidence has

associated early infant AD development with altered SC lipid composition, characterised by higher ratios of shorter chain sphingosine bases and lower phytosphingosine levels, but the subsequent effect on skin barrier function remains unclear (47). Finally, a limitation of the observational design is unintended sources of confounding. Here for instance, the majority of participating families were of white ethnicity with a history of atopic disease, limiting the applicability of our findings to lighter skin phototypes at a high-risk of AD.

In conclusion, it can be reasoned that the subtle skin barrier dysfunction noted, indicative of the inherited *FLG* defect, was proportional to the mild AD observed that lacked the anticipated acquired defect in TEWL, lipid structure and NMF (5, 38, 48). Larger, more definitive trials should therefore incorporate a greater proportion of more active disease cases to place the current dataset into context; employing a more robust AD diagnosis to explore further skin barrier risk signals independently of the established inherited *FLG* defect to improve the current disease modelling. Early detection and intervention of at-risk babies remains an unmet clinical need, with the more severe, persistent cases predisposing to further atopic comorbidities in childhood (34, 35, 49).

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REFERENCES

1. Jensen JM, Folster-Holst R, Baranowsky A, Schunck M, Winoto-Morbach S, Neumann C, et al. Impaired sphingomyelinase activity and epidermal differentiation in atopic dermatitis. J Invest Dermatol. 2004;122(6):1423-31.

2. Suarez-Farinas M, Tintle SJ, Shemer A, Chiricozzi A, Nograles K, Cardinale I, et al. Nonlesional atopic dermatitis skin is characterized by broad terminal differentiation defects and variable immune abnormalities. J Allergy Clin Immunol. 2011;127(4):954-64 e1-4.

3. Danby SG, Chittock J, Brown K, Albenali LH, Cork MJ. The effect of tacrolimus compared with betamethasone valerate on the skin barrier in volunteers with quiescent atopic dermatitis. Br J Dermatol. 2014;170(4):914-21.

4. Janssens M, van Smeden J, Gooris GS, Bras W, Portale G, Caspers PJ, 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):2755-66.

5. McAleer MA, Jakasa I, Hurault G, Sarvari P, McLean WHI, Tanaka RJ, et al. Systemic and stratum corneum biomarkers of severity in infant atopic dermatitis include markers of innate and T helper cell-related immunity and angiogenesis. Br J Dermatol. 2019;180(3):586-96.

6. Laughter MR, Maymone MBC, Mashayekhi S, Arents BWM, Karimkhani C, Langan SM, et al. The global burden of atopic dermatitis: lessons from the Global Burden of Disease Study 1990-2017. Br J Dermatol. 2021;184(2):304-9.

7. de Lusignan S, Alexander H, Broderick C, Dennis J, McGovern A, Feeney C, et al. The epidemiology of eczema in children and adults in England: A population-based study using primary care data. Clin Exp Allergy. 2021;51(3):471-82.

8. Nikolovski J, Stamatas GN, Kollias N, Wiegand BC. Barrier function and water-holding and transport properties of infant stratum corneum are different from adult and continue to develop through the first year of life. J Invest Dermatol. 2008;128(7):1728-36.

9. Stamatas GN, Nikolovski J, Luedtke MA, Kollias N, Wiegand BC. Infant skin microstructure assessed in vivo differs from adult skin in organization and at the cellular level. Pediatr Dermatol. 2010;27(2):125-31.

10. Ravn NH, Halling AS, Berkowitz AG, Rinnov MR, Silverberg JI, Egeberg A, 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):1182-93.

11. Williams HC, Chalmers JR, Simpson EL. Prevention of atopic dermatitis. F1000 Med Rep. 2012;4:24.

12. Gavin NI, Gaynes BN, Lohr KN, Meltzer-Brody S, Gartlehner G, Swinson T. Perinatal depression: a systematic review of prevalence and incidence. Obstet Gynecol. 2005;106(5 Pt 1):1071-83.

13. Pinnagoda J, Tupker RA, Agner T, Serup J. Guidelines for transepidermal water loss (TEWL) measurement. A report from the Standardization Group of the European Society of Contact Dermatitis. Contact Dermatitis. 1990;22(3):164-78.

14. von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche PC, Vandenbroucke JP, et al. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. J Clin Epidemiol. 2008;61(4):344-9.

15. Cooke A, Cork MJ, Victor S, Campbell M, Danby S, Chittock J, et al. Olive Oil, Sunflower Oil or no Oil for Baby Dry Skin or Massage: A Pilot, Assessor-blinded,

509 Randomized Controlled Trial (the Oil in Baby SkincaRE [OBSeRvE] Study). Acta Derm
510 Venereol. 2016;96(3):323-30.
511 16. Lund CH, Osborne JW. Validity and reliability of the neonatal skin condition
512 score. J Obstet Gynecol Neonatal Nurs. 2004;33(3):320-7.
513 17. Brancaleon L, Bamberg MP, Sakamaki T, Kollias N. Attenuated total reflection-
514 Fourier transform infrared spectroscopy as a possible method to investigate
515 biophysical parameters of stratum corneum in vivo. J Invest Dermatol.
516 2001;116(3):380-6.
517 18. Chittock J, Cork MJ, Danby SG. Real-Time Infrared Spectroscopic
518 Measurement of Natural Moisturizing Factor. J Invest Dermatol. 2023;143(4):676-9
519 e5.
520 19. Sandilands A, Terron-Kwiatkowski A, Hull PR, O'Regan GM, Clayton TH,
521 Watson RM, et al. Comprehensive analysis of the gene encoding filaggrin uncovers
522 prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. Nat Genet.
523 2007;39(5):650-4.
524 20. Williams HC, Burney PG, Pembroke AC, Hay RJ. The U.K. Working Party's
525 Diagnostic Criteria for Atopic Dermatitis. III. Independent hospital validation. Br J
526 Dermatol. 1994;131(3):406-16.
527 21. Boncheva M, Damien F, Normand V. Molecular organization of the lipid matrix
528 in intact Stratum corneum using ATR-FTIR spectroscopy. Biochim Biophys Acta.
529 2008;1778(5):1344-55.
530 22. Chalmers JR, Haines RH, Bradshaw LE, Montgomery AA, Thomas KS, Brown
531 SJ, et al. Daily emollient during infancy for prevention of eczema: the BEEP
532 randomised controlled trial. Lancet. 2020;395(10228):962-72.

23. Garcia Bartels N, Mleczko A, Schink T, Proquitte H, Wauer RR, Blume-Peytavi U. Influence of bathing or washing on skin barrier function in newborns during the first four weeks of life. Skin Pharmacol Physiol. 2009;22(5):248-57.

24. Hoeger PH, Enzmann CC. Skin physiology of the neonate and young infant: a prospective study of functional skin parameters during early infancy. Pediatr Dermatol. 2002;19(3):256-62.

25. Gloor M, Willebrandt U, Thomer G, Kupferschmid W. Water content of the horny layer and skin surface lipids. Arch Dermatol Res. 1980;268(2):221-3.

26. McAleer MA, Jakasa I, Raj N, O'Donnell CPF, Lane ME, Rawlings AV, et al. Early-life regional and temporal variation in filaggrin-derived natural moisturizing factor, filaggrin-processing enzyme activity, corneocyte phenotypes and plasmin activity: implications for atopic dermatitis. Br J Dermatol. 2018;179(2):431-41.

27. Visscher MO, Utturkar R, Pickens WL, LaRuffa AA, Robinson M, Wickett RR, et al. Neonatal skin maturation--vernix caseosa and free amino acids. Pediatric dermatology. 2011;28(2):122-32.

28. Voegeli R, Rawlings AV, Breternitz M, Doppler S, Schreier T, Fluhr JW. Increased stratum corneum serine protease activity in acute eczematous atopic skin. Br J Dermatol. 2009;161:70-7.

29. Watkinson A, Harding C, Moore A, Coan P. Water modulation of stratum corneum chymotryptic enzyme activity and desquamation. Arch Dermatol Res. 2001;293(9):470-6.

30. 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. J Allergy Clin Immunol. 2015;135(4):930-5 e1.

31. Rissmann R, Groenink HW, Weerheim AM, Hoath SB, Ponec M, Bouwstra JA. New insights into ultrastructure, lipid composition and organization of vernix caseosa. J Invest Dermatol. 2006;126(8):1823-33.

32. Agache P, Blanc D, Barrand C, Laurent R. Sebum levels during the first year of life. Br J Dermatol. 1980;103(6):643-9.

33. Henderson CA, Taylor J, Cunliffe WJ. Sebum excretion rates in mothers and neonates. Br J Dermatol. 2000;142(1):110-1.

34. Flohr C, Perkin M, Logan K, Marrs T, Radulovic S, Campbell LE, et al. Atopic dermatitis and disease severity are the main risk factors for food sensitization in exclusively breastfed infants. J Invest Dermatol. 2014;134(2):345-50.

35. Paternoster L, Savenije OEM, Heron J, Evans DM, Vonk JM, Brunekreef B, et al. Identification of atopic dermatitis subgroups in children from 2 longitudinal birth cohorts. J Allergy Clin Immunol. 2018;141(3):964-71.

36. Hoyer A, Rehbinder EM, Fardig M, Asad S, Lodrup Carlsen KC, Endre KMA, et al. Filaggrin mutations in relation to skin barrier and atopic dermatitis in early infancy. Br J Dermatol. 2022;186(3):544-52.

37. Ni Chaoimh C, Nico C, Puppels GJ, Caspers PJ, Wong X, Common JE, et al. In vivo Raman spectroscopy discriminates between FLG loss-of-function carriers vs wild-type in day 1-4 neonates. Ann Allergy Asthma Immunol. 2020;124(5):500-4.

38. 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.

39. Horimukai K, Morita K, Narita M, Kondo M, Kabashima S, Inoue E, et al. Transepidermal water loss measurement during infancy can predict the subsequent

development of atopic dermatitis regardless of filaggrin mutations. Allergol Int. 2016;65(1):103-8.

40. Ye Y, Zhao P, Dou L, Zhang Y, Ken K, Gu H, et al. Dynamic trends in skin barrier function from birth to age 6 months and infantile atopic dermatitis: A Chinese prospective cohort study. Clin Transl Allergy. 2021;11(5):e12043.

41. Imhof RE, De Jesus ME, Xiao P, Ciortea LI, Berg EP. Closed-chamber transepidermal water loss measurement: microclimate, calibration and performance. Int J Cosmet Sci. 2009;31(2):97-118.

42. Morizane S, Yamasaki K, Kajita A, Ikeda K, Zhan M, Aoyama Y, et al. TH2 cytokines increase kallikrein 7 expression and function in patients with atopic dermatitis. J Allergy Clin Immunol. 2012;130(1):259-61 e1.

43. Holm T, Rutishauser D, Kai-Larsen Y, Lyutvinskiy Y, Stenius F, Zubarev RA, et al. Protein biomarkers in vernix with potential to predict the development of atopic eczema in early childhood. Allergy. 2014;69(1):104-12.

44. Heiker JT, Kloting N, Kovacs P, Kuettner EB, Strater N, Schultz S, et al. Vaspin inhibits kallikrein 7 by serpin mechanism. Cell Mol Life Sci. 2013;70(14):2569-83.

45. Schultz S, Saalbach A, Heiker JT, Meier R, Zellmann T, Simon JC, et al. Proteolytic activation of prochemerin by kallikrein 7 breaks an ionic linkage and results in C-terminal rearrangement. Biochem J. 2013;452(2):271-80.

46. Endre KMA, Landro L, LeBlanc M, Gjersvik P, Lodrup Carlsen KC, Haugen G, et al. Diagnosing atopic dermatitis in infancy using established diagnostic criteria: a cohort study. Br J Dermatol. 2022;186(1):50-8.

47. Rinnov MR, Halling AS, Gerner T, Ravn NH, Knudgaard MH, Trautner S, et al. Skin biomarkers predict development of atopic dermatitis in infancy. Allergy. 2023;78(3):791-802.

48. Brunner PM, Israel A, Zhang N, Leonard A, Wen HC, Huynh T, et al. Early-onset pediatric atopic dermatitis is characterized by TH2/TH17/TH22-centered inflammation and lipid alterations. J Allergy Clin Immunol. 2018;141(6):2094-106.

49. Roduit C, Frei R, Depner M, Karvonen AM, Renz H, Braun-Fahrlander C, et al. Phenotypes of Atopic Dermatitis Depending on the Timing of Onset and Progression in Childhood. JAMA Pediatr. 2017;171(7):655-62.

TABLES

Table I: Cohort characteristics stratified by AD diagnosis.

	No AD	Confirmed AD	Unknown (W/LTFU)	Total
n	103	25	49	177
Sex, male (%)	51 (50)	10 (40)	17 (35)	78 (44)
Birth weight (g)	3480±408 [2330-4380]	3477±480 [2620-4540]	3487±517 [2560-4890]	3482±447 [2330-4890]
Gestation (days)	280±9 [260-299]	282±9 [262-290]	280±9 [263-295]	280±9 [260-299]
Mode of delivery (%Caesarean section)	42 (41)	9 (36)	23 (47)	74 (42)
Season of birth (%)				
Autumn	32 (31)	11 (44)	20 (41)	63 (36)
Winter	13 (13)	6 (24)	6 (12)	25 (14)
Spring	26 (25)	3 (12)	10 (20)	39 (22)
Summer	32 (31)	5 (20)	13 (27)	50 (28)
Age at assessment				
Birth (hours)	33.38±16.84 [9-88]	39.72±21.57 [11-95]	30.85±16.72 [9-71]	33.61±17.66 [9-95]
4-weeks (days)	33.16±7.11 [25-64]	34.52±4.54 [28-45]	-	33.43±6.68 [25-64]
12-months	12.01±0.76 [11-15]	12.04±0.73 [11-14]	-	12.02±0.75 [11-15]
NSCS	3.46±0.72 [3-7]	3.44±0.51 [3-4]	3.57±0.58 [3-5]	3.49±0.66 [3-7]
^Parental-reported rash (%)	34 (33)	16 (70)	-	50 (28)
Mean dryness score				
Birth	1.25±0.41 [1-3]	1.24±0.39 [1-2]	1.49±0.56 [1-3]	1.31±0.46 [1-3]
4-weeks	1.18±0.35 [1-3]	1.22±0.39 [1-2]	-	1.19±0.36 [1-3]
12-months	1.08±0.24 [1-2]	1.02±0.07 [1-2]	-	1.07±0.22 [1-2]
Baby's ethnicity (%)				
Asian	4 (4)	2 (8)	4 (8)	10 (6)
Black	5 (5)	-	1 (2)	6 (3)
Other	3 (3)	1 (4)	-	4 (2)
Mixed	10 (10)	2 (8)	3 (6)	15 (8)
White	81 (79)	20 (80)	41 (84)	142 (80)
[#]Family atopy (%)				
AD	40 (39)	14 (56)	21 (43)	75 (42)
Asthma	33 (32)	7 (28)	19 (39)	59 (33)
Allergic rhinitis	58 (56)	18 (72)	32 (65)	108 (61)
Any atopy	76 (74)	22 (88)	42 (86)	140 (79)
[+]*FLG* variants (%)	6/79 (8)	7/20 (35)	-	13/99 (13)
[§]EASI score	-	1.5±1.5 [0.2-5.8]	-	-

^One or more episodes of a parent-reported erythema throughout the study period. [#]At least one 1st degree relative with atopic disease. [+]Babies carrying at least one common risk allele. [§]Averaged whole-body assessment of active disease at 12 months (*n*=18). NSCS: neonatal skin condition score (range=3-9). Dryness (mean of forearm, antecubital fossa and thigh) scored visually by

three-point scale (1= no dryness; 2=dry skin; 3= very dry skin). Mean±SD and [range] presented. Percentages in brackets. W: withdrawn; LTFU: loss-to-follow-up; *FLG*: filaggrin.

Table II: Forward selection modelling of parameters associated with AD by 12-months.

	Model 1	**Model 2**	**Model 3**	**Model 4**	**Model 5**	**Model 6**
No AD/AD(n)	73/22	94/25	96/24	97/24	79/20	74/20
Parameter (OR,95%CI, *p*)	**˜2850/1540cm^{-1}** 1.7(1.1-2.9)0.04	**˜C-L activity** 0.6(0.3-0.9)0.05	**^NMF** 0.4(0.2-0.9)0.03	**^1640/1540cm^{-1}** 0.2(0.1-0.7)0.01	***FLG* variant** 6.0(1.6-23.2)0.008	***FLG* variant** 6.2(1.6-27.6)0.01 **˜C-L activity** 0.6(0.3-1.0) 0.07
Sex	0.6(0.2-1.6)0.30	0.7(0.3-1.7)0.39	0.7(0.2-1.7)0.39	0.8(0.3-2.2)0.70	1.(0.3-3.3)0.93	1.1(0.3-3.5)0.93
Gestation	1.0(1.0-1.1)0.41	1.0(1.0-1.1)0.34	1.0(1.0-1.1)0.72	1.0(1.0-1.1)0.52	1.0(1.0-1.1)0.22	1.0(1.0-1.1)0.17
Family atopy	2.0(0.6-9.5)0.32	2.9(0.9-13.2)0.12	1.9(0.6-9.0)0.33	2.1(0.6-9.8)0.28	5.3(0.90-104)0.13	8.0(1.3-166)0.07
AUC	0.68**	0.69**	0.67*	0.69**	0.73**	0.78**

Spectroscopic parameters (models 1-4) meaned from a minimum of two anatomical sites. Odds ratio (95% confidence interval) and *p* value shown. ˜birth; ^four weeks of age; family atopy: at least one 1st degree relative with atopic disease; AUC: area under the receiver operating characteristic curve; C-L (chymotrypsin-like) protease activity. *FLG* variant: Babies carrying at least one common risk allele

FIGURE LEGENDS

Figure 1: Participant pathway

Figure 2: Development of skin barrier function from birth measured in ambient conditions. (a) A wide range of raw TEWL was observed in accordance with (b) the highly variable ambient conditions encountered at assessment. (c) Controlling for temperature, a significant weakening of skin barrier function from birth was observed to 12-months of age, albeit (d) no significant difference in TEWL was noted between infants that did (darker shading) and did not develop AD. A matched, mixed model ANCOVA reported a significant effect of time (p=0.002) and temperature (p=<0.001) but not AD group on log-transformed TEWL, with asterisks denoting the result of a Bonferroni post-test to compare groups (*p=<0.05). The mean of a single TEWL measurement collected from the right forearm, right thigh and right antecubital fossa is presented as comparable trends were observed across anatomical sites over time (see Figure E1 in the Online Repository).

Figure 3: Skin barrier development from birth. Mean change in surface (a) Caseinolytic and (b) Chymotrypsin-like protease activity, (c) *in vivo* ATR-FTIR modelled NMF, (d) water content (e) lipid structure, (f) total lipids and (g) lipid esters from birth to 4-weeks and 12-months of age. Protease measurements were obtained from one repeat at the left forearm only. For spectroscopic parameters, a single measurement was meaned from both forearms, right antecubital fossa and thigh as comparable patterns were observed across anatomical sites over time (see Figure E1 in the Online Repository). Infants with a minimum of two successful ATR-FTIR spectra were included for analysis. A Friedman test with asterisks denoting a significant

Dunn's multiple comparison test was used for panels (a) and (b) only. For all other panels, a matched, mixed model ANOVA was performed with asterisks denoting a significant Tukey post-test to compare timepoints (*p=0.05). Log transformation was used where indicated. (h) A significant association was noted between investigator-observed dryness and spectroscopically determined water content across all timepoints using the Spearman rank test (r=-0.29, ***p=<0.0001).

Figure 4: Subtle differential skin barrier development between infants with and without disease. (a) Mean surface caseinolytic (b) chymotrypsin-like protease activity (c) *in vivo* ATR-FTIR modelled NMF (d) water content (e) lipid structure (f) total lipid and (g) lipid esters in infants that developed AD by 12-months (darker shading) compared to those who did not. Protease measurements were obtained from one repeat on the left forearm only. For all skin parameters determined spectroscopically, a single measurement was meaned from both forearms, the right antecubital fossa and thigh as comparable patterns were observed between anatomical sites across time (see Figure E4 in the Online Repository). Infants with a minimum of two successful ATR-FTIR spectra were included for analysis. A matched mixed model ANOVA reported an overall significant effect of infant subgroup (AD versus no AD) on ATR-FTIR lipid peak 2850/1540cm^{-1} only (f). p values denote the result of an exploratory uncorrected Fisher's LSD. Log transformations were used where indicated. Statistics were performed on log transformed data for panels a and b.