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Highly accurate, noninvasive early identification of infants with a filaggrin loss-of-function mutation by in vivo Raman spectroscopy, followed from birth to 12 months

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ABSTRACT

Background: Loss-of-function *FLG* mutation (*FLGmut*) carriers are at an increased risk of developing atopic dermatitis (AD), characterized by earlier onset and more severe disease. AD is driven by a complex interplay between skin barrier function, T_H2 and T_H2-dominant immune dysregulation, and dysbiosis. Results from the Short-Term Topical Application for Prevention of Atopic Dermatitis study suggest 2 early initiating AD pathogenetic pathways: an *FLGmut*-related skin barrier deficiency pathway and an immune function-related inflammatory pathway. The Short-Term Topical Application for Prevention of Atopic Dermatitis study suggested that early preventative intervention with specialized emollients for barrier function augmentation may benefit newborns with *FLGmut*. This requires early identification of *FLGmut* carriers, for which noninvasive Raman spectroscopic determination of natural moisturizing factor (NMF) levels in the stratum corneum of the thenar eminence provides a surrogate marker.

Objective: To identify strategies for early identification of infants with *FLGmut*.

Methods: *FLG* sequencing was performed on 253 infants, and NMF concentrations were measured in the stratum corneum of the palmar eminence (pSC-NMF) using noninvasive Raman spectroscopy at 6 time points after birth. Furthermore, the pSC-NMF concentrations were obtained from both parents of 150 infants.

Results: Babies are born with little to no NMF. In the first days after birth, NMF levels rapidly increase and 65% of newborns with *FLG* wild type already reach pSC-NMF concentrations, which excludes them as *FLGmut* carriers with high specificity. At 2 weeks of age, *FLGmut* carriers could be distinguished from newborns with *FLG* wild type with high sensitivity (97%) and specificity (97%). In addition, parent pSC-NMF concentrations offer the possibility to exclude their newborn as *FLGmut* carriers with high specificity.

Conclusion: Noninvasive Raman spectroscopy enables the accurate early identification of infants with *FLGmut*.

Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a common inflammatory skin condition, affecting up to one-fifth of children and 10% of adults in developed countries.[1] Onset is in infancy in 60% of the patients. AD is characterized by chronic skin inflammation, dryness, and itching and can have a severe impact on quality of life.[2] AD is a complex disease resulting from interactions between genetic and environmental factors.[3] Loss-of-function (LoF) mutations in the filaggrin gene (*FLG*), resulting in filaggrin deficiency and impaired skin barrier function, are the most well-known genetic factors in AD pathogenesis.[4] LoF-*FLG* mutations (*FLGmut*) are associated with a particular AD endotype, characterized by early onset, severe, and persistent disease.[5,6]

Filaggrin, which is a major structural skin protein, undergoes a cascade of proteolytic steps in the stratum corneum (SC) resulting in a mixture of hygroscopic amino acids and their derivatives in the corneocytes, which collectively account for most natural moisturizing factor (NMF).[7] NMF, making up approximately 20% to 30% of the dry

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weight of the SC, plays a central role in skin barrier function and SC hydration.[8,9]

NMF concentration in the SC is much reduced in subjects with *FLGmut*.[10,11] In vivo Raman confocal microspectroscopy allows for noninvasive measurement of NMF.[12]

Animal studies have revealed that activation of filaggrin proteolysis is dependent on the drop in external humidity during the transition from the intrauterine to the extrauterine environment.[13] NMF content increases in the first days of life, while the skin adjusts to its new extrauterine environment starting with posttranslational modification of profilaggrin in the stratum granulosum and ending with enzymatic breakdown of filaggrin present in the SC. Measurement of NMF content in the palmar SC of the thenar eminence (pSC) using noninvasive in vivo Raman spectroscopy has been found to be a robust surrogate marker for LoF-*FLGmut* carriers, independent of ethnicity and actual disease status.[10,14-16] Despite these advantages, the cost of high-end Raman instrumentation for in vivo skin research has been an impediment to its more widespread use.[17,18]

The recent results of the Short-Term Topical Application for Prevention of Atopic Dermatitis (STOP-AD) study revealed that the initiation of daily specialized emollient use, within 4 days after birth to 2 months of age, significantly reduced the incidence of AD in the first year of life in high-risk infants (defined as having at least 1 parent with a self-reported history of AD, asthma, or allergic rhinitis).[19] Although STOP-AD was not powered for this, the results of the study also invite the hypothesis that this effect may be particularly strong in newborns with LoF-*FLGmut*.

In addition, STOP-AD[20] found that in newborns with *FLG* wild type (*FLGwt*), elevated titers of the inflammatory marker cytokine S100A8/9 from skin swabs taken at the antecubital fossa at 8 weeks of age were strongly predictive of the development of AD in the first year of life, whereas no such association was found in newborns with *FLGmut*. On this basis, the existence of different initiating paths leading to AD has been proposed: one related to barrier impairment (linked to LoF-*FLGmut*) and another more dominantly linked to immune-related mechanisms. Although *FLGmut*-induced barrier impairment could be addressed with topical barrier-enhancing emollients, intervention with such an emollient in the STOP-AD study had no effect on S100A8/9 expression in newborns with *FLGwt*. Stratification of newborns according to their potential risk of AD pathogenesis is critical for the development of targeted prevention strategies. This creates a need for neonatal screening for *FLGmut* status, which may help identify a subgroup that could greatly benefit from early targeted interventions with barrier-enhancing specialized emollients.

To address the need for neonatal screening, a prototype-dedicated in vivo Raman instrument was developed. It can be used at the point of care and returns an immediate result in the form of a mass ratio: g_{NMF}:$g_{protein}$. It was recently used in an exploratory study to measure the NMF content increase in the first days after birth, while the skin adjusts to its new extrauterine environment.[21]

Here, we report the evolution of NMF content in pSC, as determined by Raman spectroscopy, from birth to 12 months, in newborns included in the STOP-AD study cohort[19] and determine the opportunities for early identification of *FLGmut* carriers. We also investigated how parental pSC-NMF content values could be used to preselect or exclude newborns from testing their pSC-NMF content.

Methods

The STOP-AD study was conducted in accordance with the Declaration of Helsinki and approved by the Clinical Research Ethics Committee of the Cork Teaching Hospital (ref ECM 5 [2] 18/12/18).

FLGmut analysis and Raman spectroscopic NMF analysis of infants were performed after the STOP-AD study (ClinicalTrials.gov registration: NCT03871998). The STOP-AD study was a single-center, 2-armed randomized controlled trial that recruited newborn-term (≥37 weeks of gestation) infants at high risk of AD within 4 days of birth, as described recently.[19]

Parents' atopic profiles were determined using a questionnaire, and their NMF values were determined at postdischarge study visits when attending with their children.

Natural Moisturizing Factor Measurements

NMF concentration in the SC of the thenar eminence was measured using Raman spectroscopy. The instrument that was used is a benchtop, dedicated prototype, dubbed "NMFscan," developed by RiverD International B.V. (Rotterdam, The Netherlands). It is a class 1M laser device and is both eye and skin safe, in accordance with the International Laser Safety Standard IEC 60825-1:2014. In vivo Raman spectra were recorded using a 785-nm laser illuminating the skin with a laser power between 25 and 30 mW. Before measurement, the thenar eminence surface was cleaned using a dry tissue wipe. To optimize the optical contact with the skin, a drop of sterile water was placed on the measurement window to optimize the optical contact between the skin and the window of the instrument, after which the thenar eminence was placed on the window and the measurement was started (Fig 1). After the device is switched on, it performs a self-test that takes a few minutes, after which it is ready for measurement. All measurements are performed at the thenar eminence (Fig 1A). Initially the area is dried using a tissue wipe (Fig 1B). A drop of water is added on the measurement window (Fig 1C). The thenar eminence is positioned on the measurement window (Fig 1D). A 1-minute measurement is taken (Fig 1E). A real-time measurement result is given (Fig 1F). Infant pSC-NMF measurement is shown (Fig 1G).

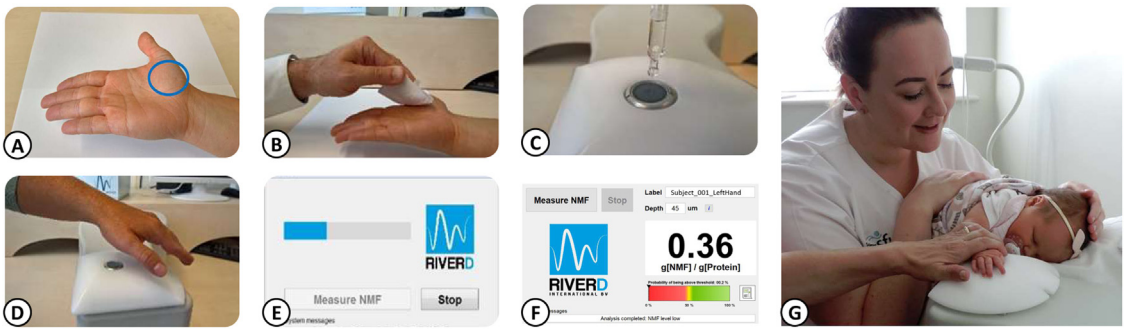

Figure 1. pSC-NMF measurement procedure. A, All measurements are performed at the thenar eminence. B, Dry using a tissue wipe. C, Drop of water on the measurement window. D, Positioning of the thenar eminence on the measurement window. E, A 1-minute measurement. F, Real-time measurement result. G, Infant pSC-NMF measurement (picture with parental publication consent). pSC-NMF, natural moisturizing factor at the stratum corneum of the palmar eminence.

For infants with a relatively thin pSC, measurements were performed at a depth of 35 m below the skin surface. A parent or a research nurse held the infant during measurement, keeping the thenar eminence in contact with the window. For parents, measurements were performed 70 m below the skin surface.

To account for local variance in NMF concentration, the NMFscan records 12 Raman spectra in sequence at a fixed distance to the skin surface, at locations spaced 100 μm apart. A signal collection time of 5 seconds per measurement location was used, for a total measurement time of approximately 60 seconds. Spectra of insufficient quality were discarded. The NMF concentrations were then calculated for each recorded spectrum following the Raman spectral analysis method of Nico et al,[22] which builds on the original method of Caspers et al.[12] The results were averaged to yield the final results expressed in grams of NMF per gram of protein (g_{NMF}:$g_{protein}$).

Infant pSC-NMF concentration analysis was performed at 6 different time points: before postnatal discharge of the mother and child, between 0 and 4 days after birth, then at approximately 2, 4, and 8 weeks and 6 and 12 months after birth. Parent pSC-NMF concentration analysis was performed once.

FLG Genotyping

Buccal swabs for DNA extraction were collected at a scheduled study visit using Isohelix SK-3S swabs and BFX/S1/05/50 prefilled buccal fixation tubes (Cell Projects, Ltd). DNA was used for *FLG* genotyping by microfluidic polymerase chain reaction for full coverage of *FLG* using the method previously described by Wong et al.[23] All identified mutations were validated using Sanger sequencing.

Analysis of Development of Palmar Stratum Corneum Natural Moisturizing Factor Concentration Increase After Birth

To model the rate at which NMF changes in the immediate postnatal period, the NMF data obtained in the first 4 days after birth (1 measurement per neonate) were grouped in 12-hour intervals, according to the time after birth (in hours) at which a measurement was performed.

The average NMF data were then fitted to a simple exponential function as a function of time t (in hours) after birth:

$$NMF_{fit}(t) = NMF_{2w} \cdot e^{-\frac{t_{NMF}}{t}}$$

The model assumes that the NMF content at birth (t = 0) is 0. NMF_{2w} is the average NMF content at 2 weeks after birth, at which the MF content should converge. The time constant t_{NMF} is the fit parameter that determines how fast the NMF content in the SC builds up to NMF_{2w}.

Prediction of FLG Genotype Based on Natural Moisturizing Factor Values

Data normality was assessed using the Shapiro-Wilk test, and the statistical significance of differences in pSC-NMF between *FLGwt* and *FLGmut* groups at various time points after birth was analyzed using the Mann-Whitney *U* test for non-normally distributed variables.

Receiver operating characteristics (ROC) were calculated to determine the ability to predict *FLGmut* status based on measured pSC-NMF concentrations at 0 to 4 days after birth and at 2, 4, 8, 6, and 12 months after birth.

The maximum Youden index was used to define the optimal NMF threshold for predicting *FLGwt* and *FLGmut* status. The Youden index is defined for each point on the ROC curve as J = sensitivity + specificity − 1.[24]

Prediction of Neonates With FLG Wild Type Based on Parental Natural Moisturizing Factor Values

The results of the neonate *FLGmut* analysis and parental NMF measurements were used to establish a parental NMF threshold, such that if both parents scored above this threshold, their newborn was predicted to have *FLGwt* with maximum sensitivity and a specificity of 100%.

Results

Of the 321 infants included in the STOP-AD study, 260 completed it. Of these, 253 patients had buccal swabs used for *FLG* genotyping. Of the infants, 44 (17.4%) were carriers of LoF-*FLGmut*, one of which was compound heterozygous and the remaining heterozygous. As further detailed in Table 1, not every infant participated at each of the 6 NMF measurement time points. A total of 68 children participated at all 6 time points.

Failed NMF measurements were due to low signal quality, most likely caused by poor contact between the skin and window during measurements or to a malfunction of the instrument.

Table 1
Numerical Breakdown of Study Subjects and Measurements

No. of infants included in the STOP-AD study	321	
No. of infants who completed the study	260	
No. of *FLG*-genotyped infants	253	
FLG wild type	209	
FLG null mutation heterozygous	43	
FLG null mutation compound heterozygous	1	
NMF data of *FLG*-genotyped infants	Valid	Failed
No. of infants at time point: 0-4 d after birth	216	3
No. of infants at time point: 2 wk (measured 11-22 d after birth)	171	0
No. of infants at time point: 4 wk (measured 26-40 d after birth)	210	2
No. of infants at time point: 8 wk (measured 53-67 d after birth)	197	0
No. of infants at time point: 26 wk (measured 180-224 d after birth)[a]	186	1
No. of infants at time point: 52 wk (measured 350-458 d after birth)[b]	194[c]	22
NMF data of parents of *FLG*-genotyped infants		
No. of parent pairs with NMF data of both the mother and the father	150	10

Abbreviations: *FLG*, filaggrin gene; NMF, natural moisturizing factor; STOP-AD, Short-Term Topical Application for Prevention of Atopic Dermatitis.
[a]Spread in measurement time points caused by COVID-19 pandemic restrictions.
[b]Spread in measurement time points caused by COVID-19 pandemic restrictions.
[c]One subject was measured at age 542 days and excluded from this time point.

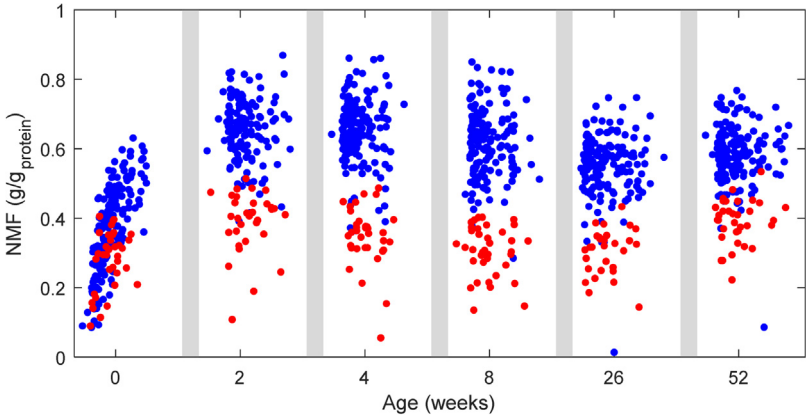

Figure 2. Development of NMF in the first year of life. Blue dots: infants with *FLGwt*. Red dots: infants with *FLGmut. FLGmut, FLG* mutation; *FLGwt, FLG* wild type; NMF, natural moisturizing factor.

Figure 2 reveals the pSC-NMF concentrations in infants measured at the 6 time points in the first year of life.

At birth, NMF is (all but) absent from the SC, after which NMF concentration increases rapidly. Maximum values were reached approximately 2 weeks after birth for both *FLGwt* and *FLGmut*. From week 8, a gradual decrease was apparent, reaching a minimum at week 26 (Fig 2).

The NMF content in pSC in *FLGwt* subjects and *FLGmut* carriers started to diverge in the first few days after birth (Fig 2). The mean neonatal NMF was significantly lower in *FLGmut* carriers than in *FLGwt* subjects (mean [SD]: 0.28 [0.08] vs 0.37 [0.12], $P < .001$). After 2 weeks, there was a distinct separation in NMF concentration according to *FLGmut* status.

At all time points, a statistically significant difference in pSC-NMF concentration was observed between the *FLGwt* and *FLGmut* infants (Table 2).

An exponential fit of the NMF data obtained in the first days after birth and after approximately 2 weeks (see the Methods section) suggests that, on average, 50% of the NMF is formed after 26 hours, and after 1 week, an NMF concentration more than 90% of the NMF concentration at 2 weeks was already formed (Fig 3).

From week 2 after birth, the ROCs for *FLGmut* prediction based on pSC-NMF concentration at 6 time points after birth revealed close to perfect discrimination between *FLGwt* and *FLGmut* infants. Receiver operating characteristics (ROCs) for *FLGmut* prediction based on pSC-NMF concentration measured at 6 time points are shown in Fig 4: (A) 0 to 4 days, (B) 2 weeks, (C) 4 weeks, (D) 8 weeks, (E) 26 weeks, and (F) 52 weeks after birth. The rapid changes in NMF concentration in the first few days after birth act as confounders in distinguishing *FLGwt* from *FLGmut* infants. Nevertheless, it is noteworthy (Fig 2) that in this study, none of the *FLGmut* neonates had an NMF concentration more than 0.41 $g_{NMF}/g_{protein}$ in the first 4 days after birth, whereas 37% of the *FLGwt* infants already had NMF values above this level.

Table 2
Average pSC-NMF Content Values (and SD) at Ages 0 to 4 Days and Ages 2, 4, 8, 26, and 52 Weeks for Newborns With *FLGwt* and *FLGmut*

	FLGmut infants		*FLGwt* infants		
Time	n	Average (SD)	n	Average (SD)	*P* value
0-4 d	38	0.28 (0.08)	178	0.37 (0.12)	<.001
2 wk	33	0.39 (0.09)	138	0.66 (0.09)	<.001
4 wk	37	0.35 (0.08)	173	0.65 (0.09)	<.001
8 wk	38	0.31 (0.07)	159	0.62 (0.10)	<.001
6 mo	31	0.31 (0.07)	155	0.55 (0.09)	<.001
12 mo	34	0.39 (0.07)	160	0.58 (0.08)	<.001

Abbreviations: *FLGmut, FLG* mutation; *FLGwt, FLG* with wild type; pSC-NMF, natural moisturizing factor at the stratum corneum of the palmar eminence.

Table 3 lists the pSC-NMF concentration cutoffs that yielded the best discrimination between *FLGwt* and *FLGmut* infants. This indicates that these cutoffs are age dependent. They were highest at weeks 2 and 4.

Outlier

One infant with *FLGwt* scored NMF values well above the pSC-NMF cutoff at the first 4 time points but had NMF concentrations close to 0 in weeks 26 and 52. We investigated whether this was a measurement artifact. However, the measurements had very good signal quality, but consistently very low signal contributions from the NMF. In addition, an administrative mix-up was ruled out. This infant developed severe AD by week 26, with eczema herpeticum as a complication that also affected the hands and necessitated topical steroid treatment.

The parents had no history of herpetic skin infections. This child was not known to have developed a food allergy.

Parental Natural Moisturizing Factor and Infant FLG Mutation Status

NMF values were obtained from 150 mother-father pairs (Fig 5). A threshold of 0.39 $g_{NMF}/g_{protein}$ was established, such that if both mother and father scored above this threshold, they had an *FLGwt* child (78 cases). All 44 infants with *FLGmut* had at least 1 parent who scored below this threshold (n = 32). In 42 *FLGwt* cases, at least 1 parent had an NMF value below this threshold and the child had *FLGwt.*

Discussion

After birth, the skin adapts by establishing a crucial barrier between the interior of the body and its dry and hostile postnatal environment. The proteolysis of filaggrin within corneocytes seems to begin quickly after birth in response to SC dehydration. This produces amino acids and their derivatives, which are the major constituents of NMF, some of which are highly hygroscopic and serve to bind water in SC. This rapid response of the skin and the fact that NMF makes up as much as 20% to 30% of the dry weight of the SC underline the importance of this process for the adaptation of the body to its new xerotic environment. It also makes clear that one way in which a LoF-*FLGmut* modifies this adaptation is through significantly lower NMF production.

In this study, we provide detailed evidence of the evolution of pSC-NMF during the early days after birth. NMF was very low immediately after birth and increased in the first hours/days, reflecting the rapid development of SC. NMF has been found to develop at varying rates at different body sites in the first year of life, with the cheek slower to stabilize compared with the elbow flexure and nasal tip.[25]

NMF levels plateaued within the first 2 weeks of life NMF increased in the first few days in both *FLGmut* and *FLGwt* newborns

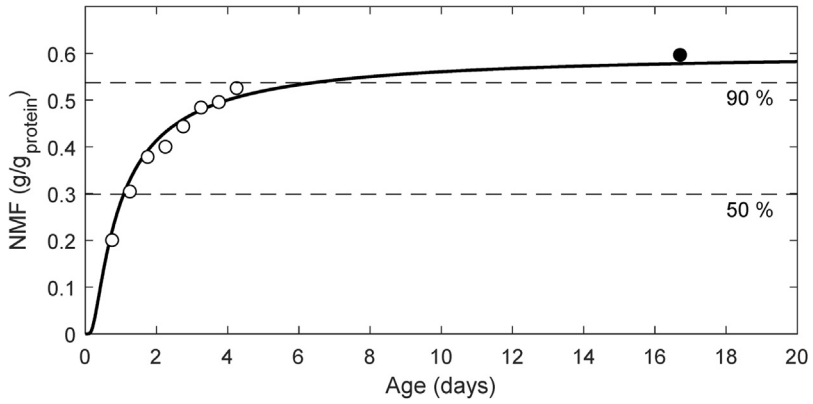

Figure 3. NMF values at birth and at age 2 weeks. Open black dots: mean infant pSC-NMF values in 12 hour time intervals. Black full dot: mean infant pSC-NMF value at 2 weeks. Black line: exponential fit of data. NMF, natural moisturizing factor; pSC-NMF, natural moisturizing factor at the stratum corneum of the palmar eminence.

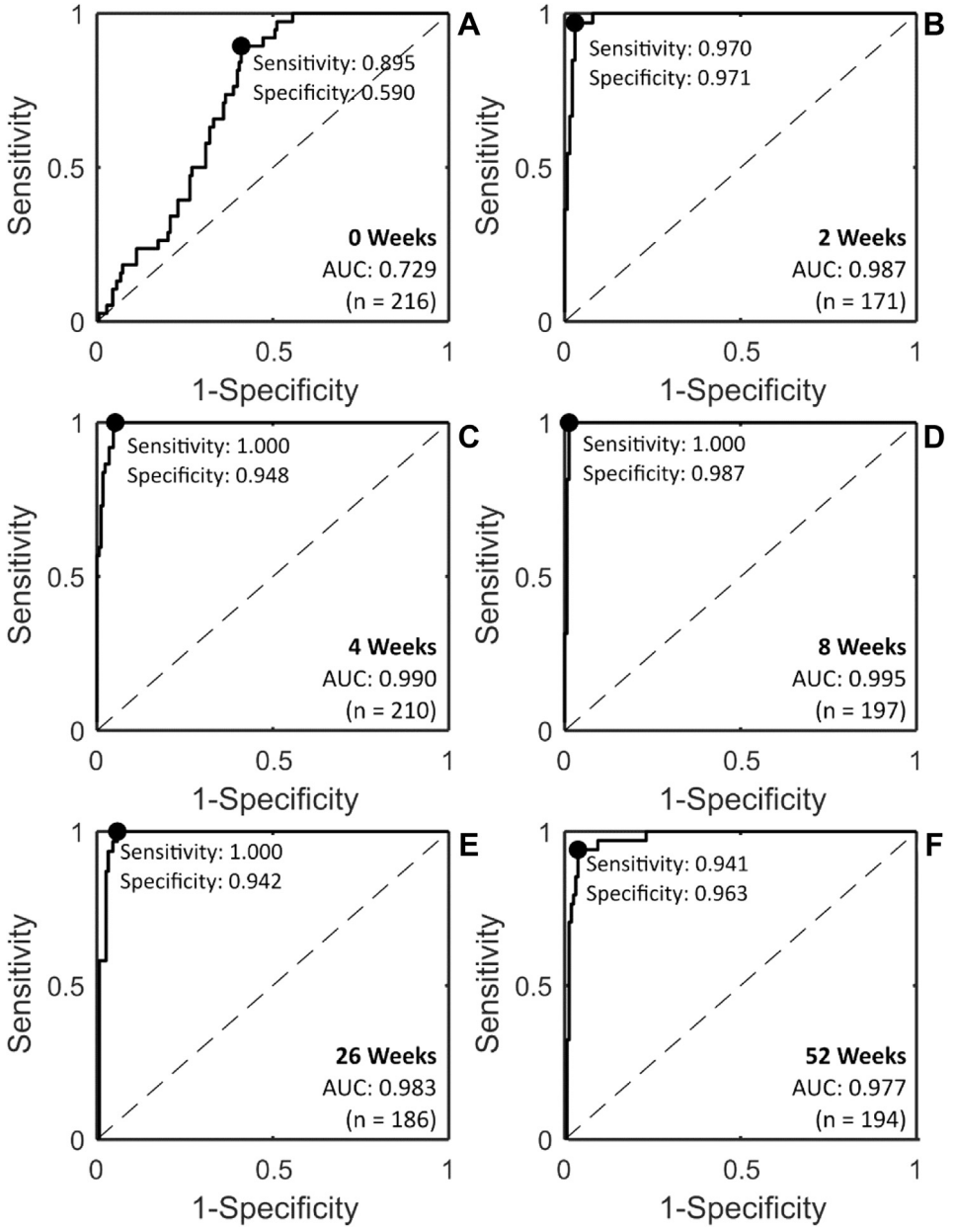

Figure 4. ROCs for *FLGmut* prediction based on pSC-NMF concentration measured at 6 time points: (A) 0 to 4 days, (B) 2 weeks, (C) 4 weeks, (D) 8 weeks, (E) 26 weeks, and (F) 52 weeks after birth. Black dot: maximum Youden index. AUC, area under the curve; *FLGmut, FLG* mutation; pSC-NMF, natural moisturizing factor at the stratum corneum of the palmar eminence; ROC, receiver operating characteristic.

Table 3
pSC-NMF Cutoffs, Based on the Maximum Youden Index, for Optimal Discrimination Between Infants With *FLGwt* and *FLGmut*

Time point	pSC-NMF cutoff [$g_{NMF}/g_{protein}$]	Sensitivity %	Specificity %
2 wk	0.487	97.0	97.1
4 wk	0.487	100	94.8
8 wk	0.403	100	98.7
26 wk	0.433	100	94.2
52 wk	0.457	94.1	96.3

Abbreviations: *FLGmut, FLG* mutation; *FLGwt, FLG* with wild type; pSC-NMF, natural moisturizing factor at the stratum corneum of the palmar eminence.

but plateaued at very different levels, revealing a distinct separation by *FLG* genotype in the early postnatal period.

Fluhr et al[26] and Matsumoto et al[27] used in vivo Raman spectroscopy of the volar forearm to obtain information regarding changes in the molecular composition of the skin in the first year of life. The design of these studies and the small number of included infants did not enable a detailed analysis of the development of NMF concentration in the first days after birth, but the results qualitatively concur with this study in identifying a decrease in NMF concentration at approximately 6 months after birth.

In the first few days after birth, this increase is a confounding factor in the use of pSC-NMF content to discriminate between *FLGwt* and *FLGmut* neonates. Furthermore, 37% of infants with *FLGwt* already had NMF values above 0.41 in the first 4 days after birth, whereas none of the 44 infants with *FLGmut* did. Values above this threshold could identify infants within days of birth who are highly unlikely to have a *FLGmut*. By 2 weeks of age and thereafter, thenar NMF can be used to clearly distinguish subjects by filaggrin genotype with a very high degree of certainty. This could be possible even at 1 week after birth, as more than 90% of the NMF is already formed at that age; however, this remains to be proven in further clinical studies.

Identifying subjects who are at high risk of AD before becoming symptomatic could inform prevention/treatment strategies. In addition to being a strong genetic risk factor for AD, LoF-*FLGmut*s are associated with distinct clinical endotypes or phenotypes of AD, including more severe disease.[1] However, the current methods for filaggrin genotyping require time and laboratory services, with no instant point-of-care measurements available. Our data suggest that NMF measurement is a good surrogate for the *FLGmut* status.

The skin physiology changes significantly during the first year of life.[28] The primary data from the STOP-AD and secondary data related to NMF values suggest that the early postnatal period may be a critical target for AD prevention. We have previously revealed that TEWL, a marker of skin barrier function, increases from birth to 2 months but stabilized thereafter.[29] The NMF in this study and at other body sites in a previous study[25] also stabilized within the first month of life, supporting the earliest possible identification of at-risk infants and the initiation of preventative treatment during this early window.

Infants from this study were participants in an randomized controlled trial to investigate the effect of daily specialized emollient use in the first 8 weeks of life on AD incidence in the first year.[19] The treatment began within 4 days of life, and a reduced incidence of AD in the intervention group was still observed at 12 months and 10 months after the 8-week intervention period, further supporting the early postnatal period as a window of opportunity for AD prevention. Although the STOP-AD trial was powered to differentiate rates of AD in the treatment and control groups blinded to *FLG* status, it also suggested a greater reduction in AD in the high-risk *FLGmut* subgroup than in *FLGwt*. Our recent work[20] proposed 2 initiating AD pathogenic pathways: a *FLGmut*-related skin barrier deficiency pathway and an immune function-related inflammatory pathway. It is reasonable to speculate that early emollient use compensates for the additional risk of developing AD caused by filaggrin deficiency. The mechanisms underlying the observed protective effects of early emollient use, including the ingredients involved and the optimal timing and duration of treatment, require further investigation.

STOP-AD recruited based on parental history of atopy, another well-known risk factor for AD,[30] and observed a reduction in AD incidence in both the *FLGwt* and mutation carriers randomized to the intervention. Instead of screening for *FLG* genotype alone, the identification of parental LoF-*FLGmut* carriers could add to the risk profile to create a composite risk score, in which family history is also accounted for. The pSC-NMF concentration can be determined in the parents during antenatal visits. If both scores are above the pSC-NMF threshold, they could be counseled that their child is highly unlikely to have *FLGmut*, and therefore their child does not need to be tested or offered preventive interventions. In this study, that threshold was 0.39 $g_{NMF}/g_{protein}$ which identified 65% of neonates with *FLGwt*, while excluding 100% of the neonates with *FLGmut*.

These findings provide many opportunities to identify neonates with *FLGmut* as early as possible, which can be selected or adapted to

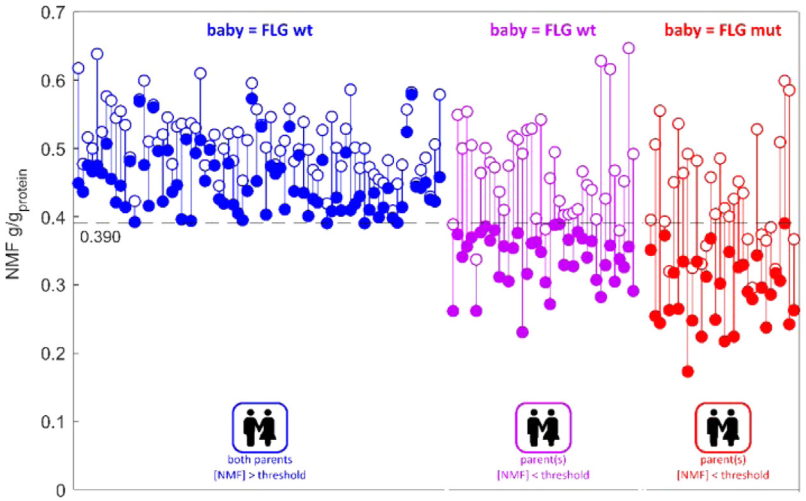

Figure 5. Parental pSC-NMF concentrations. Open circle: parent with the highest NMF score. Closed circle: parent with the lowest NMF score. Blue: both parents with NMF value above the threshold and child with *FLGwt*. Magenta: at least 1 parent with NMF value below 0.39 threshold and child with *FLGwt*. Red: at least 1 parent with NMF value below 0.39 threshold and child with *FLGmut*. *FLGmut, FLG* mutation; *FLGwt, FLG* with wild type; NMF, natural moisturizing factor; pSC-NMF, natural moisturizing factor at the stratum corneum of the palmar eminence.

the best-fit local conditions, enabling targeted preventive treatment of this important subgroup at risk for developing AD.

Introducing an unexpected skincare intervention during a busy neonatal period would add additional demands to new parents. Prenatal parental screening that identifies an infant as a likely carrier of an *FLGmut* could provide parents with a more objective measure of risk, such as shared decision-making and personalized medicine for interventions in newborn infants. Some parents may not wish to undergo antenatal screening and may wait until their child is born.

The limitations of this study are that the STOP-AD was a single-center study using a single licensed emollient product and was not conducted in a multiethnic cohort, limiting generalizability. The infants in this study also had at least 1 parent with a history of atopy which may have affected the NMF cutoffs. The NMFscan is a prototype-dedicated device. On further system development, comparative studies should be performed. Although accurate screening for *FLGmut*s would greatly assist infants who are at both high risk of AD and are likely to disproportionately benefit from barrier-improving emollients at birth, we must recognize that not all *FLGmut* carriers will develop AD and that targeting interventions solely to *FLGmut* carriers would treat those most likely to benefit but would also mean some children who could benefit, even to a lesser extent, would not be treated.

In conclusion, we demonstrated that Raman-derived NMF measurements can be used as a quick, noninvasive, highly accurate surrogate for *FLG* genotyping in parents and newborn infants from day 0. These readings provide opportunities to identify high-risk subgroups before or within days of birth and could inform highly focused early intervention strategies. Future studies should focus on how the determination of *FLG* status can contribute to the estimation of an infant's risk of AD, both separately and in combination with other risk factors. The effect of early emollient use should be evaluated in a larger cohort of *FLGmut* carriers, with concurrent investigation of the mechanisms underlying any protective effects.

Our conclusions are as follows:

1. The pSC-NMF concentration in the SC was very low at birth and rapidly increased in the first 4 days after birth. It reaches a maximum at 2 to 4 after birth.
2. The mean pSC-NMF concentration was significantly lower in infants with *FLGmut* than in infants with *FLGwt*. From the age of 2 weeks and potentially from 1 week, the pSC-NMF concentration can be used as a highly accurate surrogate marker for LoF-*FLGmut*s.
3. Despite the rapid change in pSC-NMF concentration in the first 4 days after birth acting as a confounder, identification of newborns with *FLGwt* with high specificity and moderate sensitivity is possible.
4. Parental NMF measurements can be used to predict infants with *FLGwt* with high specificity and moderate sensitivity.
5. pSC-NMF concentration analysis by in vivo Raman spectroscopy enables various strategies for implementation of identification of infants with *FLGmut* very early after birth. This enables preventative treatment decisions, specifically targeting high-risk groups.

Disclosures

Dr Puppels and Dr Casper are employees and shareholders of RiverD International. Dr Nico is an employee of RiverD International. The remaining authors have no conflicts of interest to report.

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