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REAL-TIME INFRARED SPECTROSCOPIC MEASUREMENT OF NATURAL MOISTURISING FACTOR

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Abbreviations: Atopic Dermatitis, AD; Natural Moisturising Factors, NMF, Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy, ATR-FTIR
The quantification of Natural Moisturising Factor (NMF) is of value to scientists and clinicians with an interest in dry skin disorders such as atopic dermatitis (AD). Hygroscopic amino acids and their derivatives, originating from Filaggrin (FLG) catabolism, represent a predominant component of NMF maintaining the physical permeability barrier of the skin; (Scott et al., 1982) that when deficient are synonymous with xerosis and greater AD severity (Horii et al., 1989, Nouwen et al., 2019). Tape stripping with chromatography is a fully quantitative assessment of NMF ex vivo but time consuming and labour intensive in larger cohorts. In this pilot study, we tested a portable, hand-held Attenuated Total Reflectance - Fourier Transform Infrared (ATR-FTIR) spectrometer as an alternative in vivo measure of NMF, by modelling chemometric absorption using a single, quantitative composite value obtained by established ex vivo laboratory assay. The spectroscopic model was verified by examining known scenarios of reduced NMF abundance in the skin such as the established FLG pathophysiology in AD.

A cohort of 26 participants with healthy skin (n=15) or a history of AD (n=11; 2 with mild active disease) were recruited and completed the single study visit. Written, informed consent was obtained and approval granted by the University of Sheffield Research Ethics Committee (uREC ref: 021945). Four sampling data points across the antecubital fossa and forearm were split equally between model calibration and validation, each consisting of four baseline ATR-FTIR measurements performed contiguous to 3 serial tape strips collected in duplicate (n=6) for laboratory NMF analysis (Supplementary Figure S1). On average, the three predominant components of NMF analysed ex vivo – total free amino acid pool (fAA), pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA) - were significantly reduced in the AD group compared to healthy skin (Supplementary Table S1). Transepidermal water loss (TEWL) and capacitance measurements to assess barrier function, were similar between groups, and the
proportion of FLG loss-of-function (LOF) variants were 20% and 36% respectively. A plot of ex vivo versus in vivo modelled NMF is presented by Figure 1a and b. Using a six-factor predictive model, the coefficient of determination for calibration (R²=0.73) and validation (R²=0.70) sampling data points indicate satisfactory accuracy and precision (±0.35µmoles mg⁻¹) denoted by the root mean square error of calibration (RMSEC). A plot of model loading – the strength of association between wavenumber absorption and latent factor (Figure 1d) – corresponded to an in vitro NMF profile collected by the same spectrometer (Fig 1e). Similar outputs were obtained by Amide I (1640cm⁻¹) and II (1540cm⁻¹) normalisation (Supplementary Table S2). To verify the spectroscopic technique, NMF was modelled before and after bathing the antecubital fossa in an independent cohort of volunteers (n=5, Supplementary Figure S2a); with on average, a 67% reduction in NMF induced by a 20-minute water soak (p=0.0036). Furthermore, the main study cohort (n=26) was stratified two ways (healthy skin / AD and wild type / FLG LOF mutation carriers) and modelled NMF abundance compared, to assess the inherited and acquired FLG defect (Kezic et al., 2011). In all scenarios, similar changes in absorbance were observed that matched an in vitro NMF spectrum (Supplementary Figure S2b-h). These regions were indicative of the carboxylate (-COO⁻) asymmetric or symmetric stretch (1540/1400 cm⁻¹) and methylene group (CH₂) vibrations around 1480cm⁻¹ (Takada et al., 2012). Interestingly, 1340cm⁻¹ is assigned to the hydroxyl group (C-)OH bending mode of serine, an abundant amino acid within the SC (Nakagawa et al., 2011). At the antecubital fossa but not the forearm (Supplementary Figure S3), in vivo modelled NMF was on average 0.64µmol mg⁻¹ lower in AD compared to healthy skin; with all values measured in FLG LOF mutation carriers being below the wild type mean, regardless of AD history (Figure 2a and b). This discrimination between groups was supported by a Receiver Operating Characteristic area under the curve (AUC) of 0.81 and 0.83 respectively (Figure 2c and d).
This methodology was limited to the discrimination of subclinical AD and requires further validation due to the absence of more active disease. Compare this to in vivo Raman Spectroscopy (RS) where more comprehensive classification of FLG genotype by NMF (AUC 0.94) was reported in adults with moderate-severe disease (O'Regan et al., 2010). Our work is ongoing to replicate this ATR-FTIR methodology in a more diverse AD cohort of greater severity, but a key limitation may be the shallow sampling depth (approximately 1.5μm) of the evanescent wave (Brancaleon et al., 2001) whereas RS permits composite NMF profiling across the full SC depth without the requirement of tape stripping. This surface constraint may also render the current ATR-FTIR methodology susceptible to patients washing or applying topical treatments prior to measurements in the clinic. On the flip side, it can be argued that ATR-FTIR is comparatively the more affordable technology that permits the rapid sampling of multiple anatomical sites, with a real-time NMF output displayed by the device.

In summary, we provide preliminary evidence to suggest that measurement of NMF in vivo using ATR-FTIR is robust and comparable to an established ex vivo technique. Considering the portable device used with no sample preparation required, this methodology has the potential to offer new opportunities of clinical research where laboratory access is not feasible. The technology has many potential uses; knowledge of FLG variant status may predict a patient’s response to emollients (Danby et al., 2022) or systemic immunosuppressives (Roekevisch et al., 2017) indicative of future personalised treatment strategies. Our data is also suggestive of attenuated NMF in patients generally clear of symptoms (Engebretsen et al., 2018). Tracking this defect longitudinally with further novel measures of subclinical inflammation, may be of clinical value for monitoring remission following treatment of active disease (Byers et al., 2018). Another use is in predisposed neonates who possess a skin barrier defect long before the onset of clinical AD (Horimukai et al., 2016). There is evidence to
suggest that low NMF associates with skin barrier breakdown at birth (Chittock et al., 2016). Therefore, as presented here in adults with unaffected skin, the hypothesis that NMF abundance may also be discriminative in neonates and be predictive of AD onset either alone, or in conjunction with other biomarkers, is an intriguing proposition yet to be determined.

DATA AVAILABILITY STATEMENT
No large datasets were generated by this study.

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CONFLICTS OF INTEREST STATEMENT
All authors have none to declare.

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AUTHOR CONTRIBUTIONS

Conceptualization: JC, MJC, SD; Data Curation: MJC, SD; Formal analysis: JC, SD; Funding Acquisition: MJC, SD; Investigation: JC; Methodology: JC, SD; Project Administration: JC, SD; Resources: MJC, SD; Supervision: MJC, SD; Writing-original draft preparation: JC; Writing-review and editing: JC, MJC, SD.
REFERENCES


FIGURE LEGENDS

Figure 1: PLS chemometric modelling of surface NMF in the mid infrared spectral region. (a) Plot of *ex vivo* quantified versus *in vivo* ATR-FTIR modelled NMF (the sum of fAA, PCA and UCA) for calibration and (b) validation data sets (see Supplementary Materials and Methods for further details). \( R^2 \) = coefficient of determination denoting linear regression model goodness-of-fit. Respective residual plots inset. Individuals with active AD are shaded red. (c) Image of the spectrometer used. (d) Loading plot associating wavenumber absorption to latent factor (6 in total, colour coded) with (e) an *in vitro* NMF absorbance spectrum presented for reference.

Figure 2: *In vivo* modelled NMF discriminates AD and *FLG* null genotype from controls. Cohort stratification \( (n=26) \) to compare mean *in vivo* NMF at the antecubital fossa between (a) healthy skin / AD and (b) wild type (WT) / *FLG* LOF mutation carriers. Only the model validation data points are presented (see Supplementary Materials and Methods for further details). Individuals with active AD are shaded red. *p* values denote the result of an unpaired student’s *t* test. A Receiver Operating Characteristic (ROC) curve of modelled NMF is presented below the corresponding graph (c) AD/Healthy: area under the curve=0.81, 95% CI, 0.63-0.99, \( p=0.008 \); (d) *FLG*/WT: area under the curve=0.83, 95% CI, 0.66-0.99, \( p=0.01 \).
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