# INVESTIGATING BIOMARKERS OF SKIN BARRIER DEVELOPMENT AND BREAKDOWN ASSOCIATED WITH EARLY-ONSET ATOPIC DERMATITIS

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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## DECLARATION OF AUTHORSHIP

*I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not been previously presented for an award at this, or any other, university.*

**SIGNED**..... **DATE**: 28th February 2022

## PUBLICATIONS AND CONFERENCE ABSTRACTS

Chittock J, Brown K, Cork MJ and Danby SG. *Quantification of natural moisturizing factors at the skin surface using a portable infrared spectrometer device: a pilot, calibration model.* Br J Dermatol 2018. The 10th George Rajka International Symposium on Atopic Dermatitis, 11-13 Apr 2018, Utrecht, Netherlands.

Chittock J, Brown K, Wigley A, Kilby J, Cork MJ and Danby SG. *An investigation of protease activity at non lesional sites in Atopic Dermatitis*. 2017. British Society for Investigative Dermatology Annual meeting, Manchester, UK.

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*. Br J Dermatol. 2016;175(4):713-20.

# ABSTRACT

Skin barrier breakdown is a key pathogenic mechanism in Atopic Dermatitis (AD); a common inflammatory skin disease that negatively impacts the health and wellbeing of children and their families. From birth, a significant period of stratum corneum (SC) adaptation to terrestrial life coincides with an increased risk of developing AD, suggesting that pathological skin changes are taking place that precede and may predict early disease development. To address these questions, a portfolio of non-invasive tests was employed to better understand healthy skin barrier development from birth in a cohort study of 115 infants (Chapter 2). An infrared spectroscopic method to quantify Natural Moisturising Factor (NMF) abundance was piloted in adults ($n$=26) for assessment of the inherited and acquired FLG barrier abnormality (Chapter 3). An observational cohort study (NCT03143504) was then performed in 177 infants to explore differential skin barrier developmental trajectories and biomarkers of breakdown from birth associated with AD (Chapter 4). A transitioning biophysical and biochemical environment at the skin surface from birth was characterised by changes in desquamatory protease activity and NMF abundance that correlated with skin health. A primary *FLG* barrier defect was strongly associated with early onset disease and conferred a significant reduction in NMF and water content at the skin surface by four weeks of age. Accounting for parental atopic disease, these biomarkers were predictive of AD development by 12-months of age. Given its inherent fragility, this study highlights the need for careful infant skin care practices that gently support healthy barrier development from birth. Although exploratory, these findings suggest that a portfolio of tests used in a healthcare or community setting, has the potential to improve current risk evaluations as to whether a baby will develop AD.

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I am grateful to Dr Alison Cooke and Professor Dame Tina Lavender for granting me access to work with the OBSeRvE study cohort.

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And finally, thank you to my lovely family for sticking by me during thick and thin. I could not have done it without you. My only wish is that my Dad could still be here to witness me finally submitting this work.

## COVID-19

Overall the impact of COVID-19 on my studies was minimal. By March 2020 when nationwide restrictions were first introduced in the UK, the Skin Testing for Atopic eczema Risk (STAR) study (results Chapter 4) had completed recruitment and all follow up visits. Due to University laboratory closures, the work associated with this study (*FLG* genotyping and chemometric NMF modelling) was delayed until permission was granted to return on site. It was a relief that I was able to take a leave of absence from my studies over this period and I am very grateful to the medicine PGR team and Research Services for organising and approving this.

# CARBON FOOTPRINT

The department of Infection, Immunity and Cardiovascular Disease that hosted this research work is actively involved with the Green Impact scheme; an initiative designed to make research environments more sustainable. The IICD green impact team was recently presented with a gold award for a number of activities including the improvement of laboratory recycling waste in the department and the provision of reusable face masks throughout the pandemic.

Part of this thesis included the development and use of a spectroscopic method for the rapid measurement of NMF, with the ultimate aim of this technique replacing more laboratory-based methods of analysis based on tape stripping, that uses flammable solvents and generates contaminated waste.

The nature of our work (in person study visits) means that some travelling is unavoidable. To mitigate this impact where possible, participants were actively recruited from the local community surrounding the University of Sheffield for this work. In the event of further studies being conducted based on this work, more environmentally friendly modes of transport should be considered, such as the use of taxi companies with electric fleets to transport researchers/participants to the study visits.

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| | |
|---|---|
| AMP | Antimicrobial peptide |
| AD | Atopic dermatitis |
| EDC | Epidermal differentiation complex |
| FAAs | Free amino acids |
| FCεR1 | High-affinity receptor for IgE type 1 |
| FLG | Filaggrin |
| FTIR | Fourier Transform Infrared Spectroscopy |
| IV | Ichthyosis vulgaris |
| IgE | Immunoglobulin E |
| IFN-γ | Interferon gamma |
| KC | Keratinocyte |
| KIF | Keratin intermediate filament |
| KLK | Kallikrein |
| LB | Lamellar body |
| LEKTI | Lymphoepithelial Kazal-type Trypsin Inhibitor |
| LOF | Loss of function |
| NMF | Natural moisturising factor |
| PAR2 | Protease-activated receptor 2 |
| PCA | Pyrrolidone carboxylic acid |
| PLS | Partial Least Squares regression |
| SC | Stratum Corneum |
| SCH | Stratum Corneum Hydration |
| SCORAD | Scoring atopic dermatitis |

| | |
|---|---|
| TJ | Tight junction |
| TNFα | Tumour necrosis factor alpha |
| TSLP | Thymic stromal lymphopoietin |
| TEWL | Transepidermal water loss |
| UCA | Urocanic acid |

# CHAPTER 1: INTRODUCTION

## 1.1 SKIN BARRIER STRUCTURE AND FUNCTION

The skin is the largest organ of the human body and performs a series of protective functions in response to the surrounding environment. Defence against heat loss, UV light, chemical entities and injury is achieved by its structure and homeostatic mechanisms such as thermoregulation and permeability barrier function. Antimicrobial protection is provided by sensory mechanisms within the epidermis connecting the innate to the adaptive immune system.

### 1.1.1 The structure of the epidermis

Classified as a stratified squamous epithelium, the epidermis represents the outermost layer of skin that overlays the dermis. A schematic of the epidermis and its predominant cell types is provided by Figure 1.1. Its structural basis is four distinct layers of keratinocytes that differ in both morphology and function, situated above a basement membrane. The epidermis is avascular, therefore the most metabolically active cells of this layer are found directly above this basement membrane where nutrients and oxygen diffuse readily from the capillaries of the dermis (1). At its apex, a terminally differentiated, cornified end product is synthesised that contacts the surrounding environment.

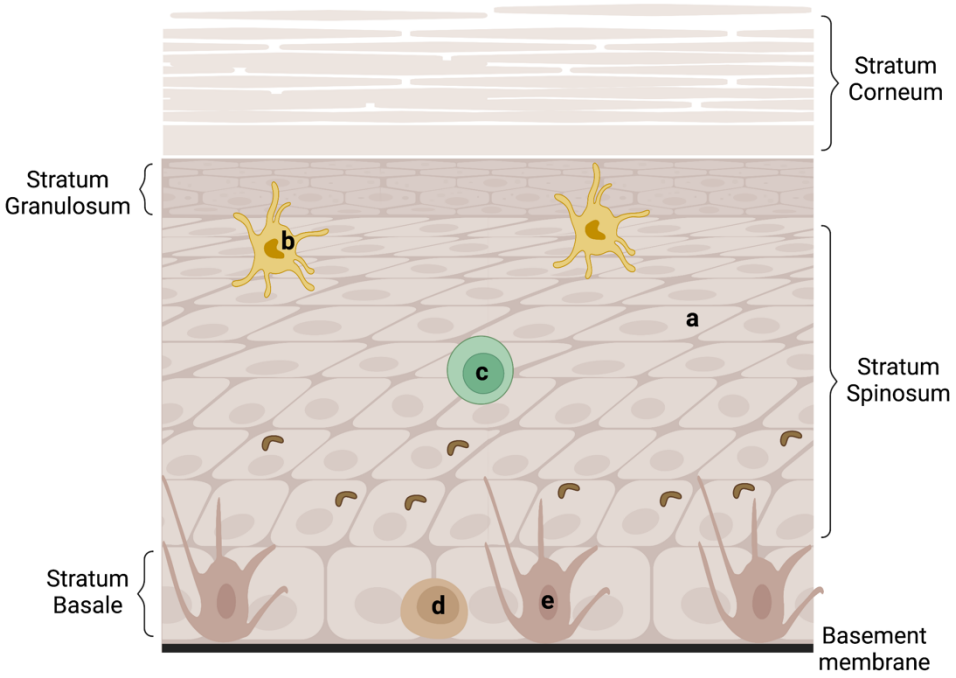


**Figure 1.1**: Cells and layers of the epidermis. (a) Keratinocytes are the predominant cell type and form the basis of its structure. (b) Langerhans cell and (c) CD8+ T cells are resident immune cells that sense and coordinate response to pathogen invasion. (d) Merkel cells function as touch receptors (e) Melanocytes synthesise melanosomes that are engulfed by surrounding keratinocytes and pigment the skin with melanin.

The **stratum basale** (SB) is a columnar layer of un-differentiated stem cells that secrete an extracellular matrix (ECM) to form the basement membrane. The SB is referred to as the germinal layer as asymmetric, mitotic division generates daughter cells to maintain a proliferative basal layer, and supply differentiating keratinocytes to form the overlying layers of the epidermis (1). Expression of keratins commences in the SB to form keratin intermediate filaments (KIF) that are anchored to the basement membrane by hemidesmosomes (2).

In the **stratum spinosum** the KIF in combination with tubulin and actin form a cytoskeleton of 'spinous' morphology (3). In this layer, expression begins of essential proteins for cornified envelope formation such as transglutaminase -1, -5 (3). These keratinocytes are bound together by adhesive desmosomes, structurally composed of cadherin, armadillo and plakin protein families with desmoplakin interlocking the KIF (Figure 1.2). Also situated here are resident dendritic Langerhans cells that coordinate an innate immune response to invading pathogens through antigen uptake and presentation to the adaptive immune system (1, 4).

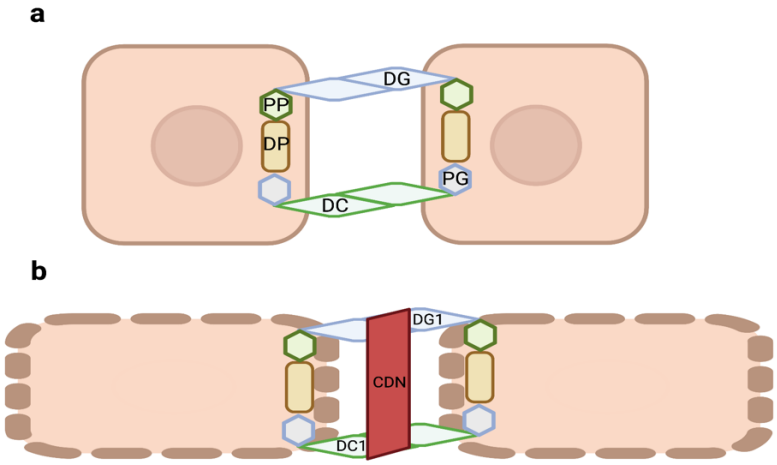


**Figure 1.2**: Structure of the desmosome and corneodesmosome. (a) Schematic of the desmosome cell to cell protein adhesions that anchor the plasma membranes to the cytoskeleton. They are comprised of (1) the cadherins desmoglein (DG) and desmocollin (DC); (2) the armadillos plakoglobin (PG) and plakophilin (PP); and (3) the plakin desmoplakin (DP) that binds KIF. (b) Schematic of the corneodesmosome structure embedded in the cornified envelope with extracellular components desmoglein-1 (DG1), desmocollin-1 (DC1) reinforced by corneodesmosin (CDN). Figure adapted from Ishida-Yamamoto *et al* (5).

The **stratum granulosum** or granular layer is formed by 3-5 layers of keratinocytes entering a process of cornification. Here, the cells de-nucleate and are engulfed by the cornified envelope. Keratohyalin granules are present containing the large, insoluble, histidine-rich protein profilaggrin. In response to increasing intracellular $Ca^{2+}$ levels, dephosphorylation and proteolytic cleavage of profilaggrin generates the monomeric matrix protein filaggrin (6). Mouse models and *in-vitro* studies have associated the serine proteases matriptase, prostasin and kallikrein-5 with this process, but the exact underlying mechanisms in humans remains unclear (7-9). Coupled with increased keratin expression, filaggrin (FLG) aggregates KIF in a cross-linked formation promoting the collapse and flattening of the cell. The ordered structure of the SG - rich in keratins and FLG - forms a tough, impermeable physical barrier, reinforced by cellular links such as adherens junctions, desmosomes and tight junctions (2, 3, 10). This forms an integral part of the skin barrier in conjunction with the cornified layer situated above.

Finally, the process of cornification completes to form the outermost layer of the epidermis termed the **stratum corneum** (SC) or horny layer; the principal component of the skin barrier. It is formed by 15-30 compact layers of terminally differentiated, anuclear corneocytes. Being the interface to the surrounding environment, the SC provides protection from excessive water loss, environmental stressors and allergen penetration that is collectively termed skin barrier function.

### 1.1.2 The stratum corneum is central to the skin barrier

Far from being dormant, the skin barrier is a biologically active amalgamation of protein and lipid structures, chemicals and proteolytic enzymes residing in the two outermost layers of the epidermis (Figure 1.3). As the central component of the skin barrier, a simple analogy is to compare the structure of the SC to a brick wall. Here the corneocytes (bricks) are surrounded by a lipid matrix (mortar) that binds the construction together (11). Professor Cork and colleagues (12) extended this model further to include corneodesmosomes (Figure 1.2). These modified desmosomes contain the extracellular components desmoglein -1, desmocollin -1 and corneodesmosin, that bind the cornified envelope and act like structural iron rods. Alongside tight junction proteins claudin -1 and occludin, they confer biomechanical rigidity and protection from mechanical stress (10, 12).

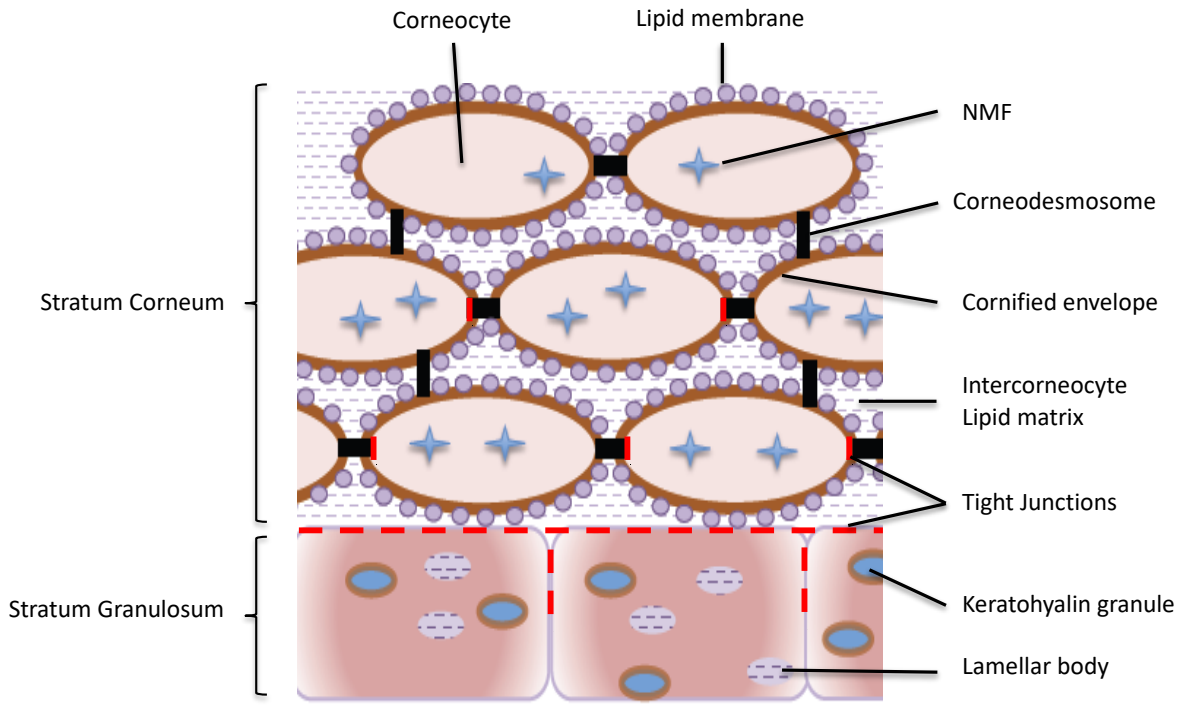


**Figure 1.3:** Structural components of the skin barrier. Corneocytes of the stratum corneum (SC) are engulfed by a cornified envelope and lipid matrix with corneodesmosomes providing cellular support. Natural Moisturising Factors are chemicals with humectant properties to maintain cell elasticity and support permeability barrier function in conjunction with tight junctions and the intercorneocyte lipid matrix.

Encasing each corneocyte, the cornified envelope is formed by structural protein and lipid components. The protein base of envoplakin and periplakin provides anchorage to corneodesmosomes. Reinforcement is provided by involucrin, loricrin, filaggrin, elafin and small proline-rich proteins that are covalently cross-linked by isopeptides via the action of transglutaminases (3, 13). Attached to this protein scaffold is a ω-hydroxyacylsphingosine envelope that connects to an intercorneocyte lipid matrix; a lamellar membrane composed of ceramides (50%), cholesterol (25%) and free-fatty acids (15%) (14). The most abundant species here being a combination of phytosphingosine and 4-hydroxysphingosine bases with hydroxylated and non-

hydroxylated fatty acid groups, and free fatty acids in a C24:0 / C26:0 structural conformation (15). These lipids of the intercorneocyte matrix are delivered to the SC via a unique epidermal organelle; the lamellar body (LB) (16). Packaged inside the LB are phospholipid, glucosylceramide, sphingomyelin lipid precursors and cholesterol sulphate, in addition to enzymes required for their subsequent processing such as beta glucocerebrosidase, acidic sphingomyelinase, secretory phospholipase A2 and neutral / acidic lipases (17). Upon LB delivery to the SC / SG interface, these lipid precursors are secreted into the intercorneocyte space, undergo hydrolysis, and are arranged into the ordered lipid matrix to form the structural basis of the permeability barrier (17).

### 1.1.3 The permeability barrier regulates water loss from the skin

Permeability barrier function is the homeostatic mechanism conferred by the SC, to prevent excessive transcutaneous water loss (inside-out barrier) while guarding against exogenous chemicals and allergens penetrating the skin (outside-in barrier). Its ordered structure prevents the penetration of molecules greater than 500 Daltons in size. The extensive hydrophobic lipid matrix allows small lipophilic substances of <150 Daltons (such as phenols) to penetrate, while also regulating the simple diffusion of water from within corneocytes to maintain optimal levels of hydration (15, 18). As shown by Imokawa and Hattori, treatment with acetone/ether to remove the lipid matrix from the intercorneocyte space induces xerosis and a significant decrease in skin hydration measured by conductance (19). Subsequent lipid replenishment in the form of cholesterol esters, sphingolipids and free fatty acids, reversed this experimental damage and recovered water content in the SC (20).

Water retention is key, but the permeability barrier is a dynamic system. Under steady state conditions a water concentration gradient exists, with movement from the wetter deeper SC layers towards the dryer skin surface (21). Regulating this movement are three structural routes: transcellular-corneocyte diffusion related to corneocyte size and thickness; (22, 23) paracellular diffusion through the "torturous" hydrophilic route of the lipid matrix; (24) and paracellular diffusion through tight junction (TJ) transmembrane proteins rich in claudin-1, claudin -4 and occludin that protect against lethal water loss by forming a barrier to water, ion and molecule movement in the upper stratum granulosum (25, 26). A skin barrier deficient in TJ proteins is also more permeable to albumin, associating these structures with the outside-in barrier against the penetration of irritants and allergens (26, 27).

Transepidermal Water Loss (TEWL) is the passive flux of water vapour from the skin surface. It is commonly measured using non-invasive probes to assess permeability barrier status *in vivo*. Accordingly, removing the SC by tape stripping proportionally increases TEWL, and confirms that inside-out permeability barrier function resides in the SC (28). Somewhat more controversial is the use of TEWL to assess the outside-in barrier to exogenous substances (29). In support of this application are studies that correlate TEWL with the *in vitro* and *in vivo* percutaneous absorption of both hydrophilic and lyophilic substances at various anatomical sites (30-32). In a rodent model, dye penetration assays using toluidine blue or biotin confirm the respective roles of the SC (outside-in) and tight junctions (inside-out) in permeability barrier function (33).

Another biochemical component of the inside-out permeability barrier is Natural Moisturising Factor (NMF). NMF is found in the SC and composed of free amino acids [FAA] (48%), organic acids (25%), lactate (10%), urea (8%) and inorganic ions (5%) (21, 34). During terminal differentiation, FLG is catabolised to form a pool of intracellular FAAs that accounts for >70% found in the SC (35). The cysteine proteases bleomycin hydrolase and calpain 1, in addition to the cysteinyl aspartate protease caspace-14, have all been implicated in this processing from experimental work in mice (36, 37). FAAs such as glutamine and histidine are catabolised to form 2-pyrrolidone-5-carboxylic acid (PCA) and urocanic acid respectively, with PCA constituting the major organic acid fraction of NMF (38). These molecules act as osmolytes, drawing in and retaining water within corneocytes to maintain optimal cell hydration, shape and SC elasticity (34). Further evidence to support this is found in xerotic skin conditions such as Ichthyosis Vulgaris (IV) where the permeability barrier fails and there is excessive water loss from the skin surface. In this scenario of xerosis, comparatively low levels of extractable FAA are found at the skin surface, and a linear relationship exists between their abundance and SC hydration measured by conductance (39).

But FLG is not the sole source of NMF in the SC. Less abundant surface NMF components such as urea, lactate and inorganic ions are derived from eccrine sweating (40, 41). The physiological level of lactate and potassium in healthy subjects is linearly related to SC hydration, stiffness and pH (42). The effect that urea exerts on the skin barrier has been investigated by its topical application to human and rodent skin. Not only is it a potent humectant that maintains SC hydration, but it can restore

disturbed permeability barrier function by stimulating expression of key proteins central to terminal differentiation (43).

### 1.1.4 The microbial barrier resists pathogen invasion

To penetrate the epidermis, pathogens must first adhere to its surface. To combat this, the SC has many tools to guard against colonisation such as the acid mantle, reduced moisture content, desquamation, and a cooler surface temperature (44). An acidic skin pH is suboptimal for the growth of pathogenic bacteria such as *Staphylococcus aureus,* allowing colonisation by beneficial resident microflora (45, 46). For example, the skin commensal *Staphylococcus epidermidis* not only competes for nutrients and space, but it also interacts with the host's inflammatory response to pathogens and stimulates expression of antimicrobial peptides (47). Furthermore, *Staphylococcus epidermidis* and *Staphylococcus hominis* both express autologous antimicrobial peptides with the ability to selectively kill *S aureus* (48). So, an acidic skin surface promotes a more balanced microflora equipped to defend its niche, but what are the molecular mechanisms underlying this acidification of the SC?

Throughout the SC, a pH gradient exists between the neutral inner layers and the more acidified superficial layers; the so-called acid mantle (49, 50). Historically it has been proposed that SC pH is derived from exogenous mechanisms such as microbial metabolism, sebaceous glands and sweat. More recently though, favour has shifted towards endogenous biological mechanisms being responsible for acidification including: (a) free-fatty acid synthesis from phospholipids during lipid matrix generation; (51) (b) the sodium/hydrogen antiporter-1 acidifying membrane domains

at the SC/SG interface; (51) and (c) the deamination of histidine to urocanic acid (UCA) as part of the filaggrin-derived NMF pathway (52). Questions have been asked of the latter's contribution to SC pH, as urocanic acid is primarily intracellular, and histidase deficient mice have a normal, acidified skin-surface pH (53). Nevertheless, the loss of NMF is associated with a more alkaline SC in skin diseases such as Ichthyosis Vulgaris and AD (50, 54). Overall this suggests multiple biological pathways contribute to SC acidification with contingency if one mechanism fails (53).

### 1.1.5 Desquamation is fundamental to continuous SC renewal

To maintain its structural integrity, the SC is continuously renewed by the proliferating cells of the stratum basale (3). Complete renewal takes approximately one month, and in order to maintain a constant SC thickness, corneocytes are shed from the skin surface by the process of desquamation. As keratinocytes progress through terminal differentiation, they begin to express the kallikreins (KLK); a family of 15 extracellular serine peptidases possessing trypsin-like or chymotrypsin-like protease activity. It is the coordinated degradation of corneodesmosomes by KLK5 (trypsin-like) and KLK7 (chymotrypsin-like) proteolytic activity commencing in the stratum compactum that is the biological process underlying desquamation in the model proposed by Caubet and colleagues (55). Since the model's conception, KLK6, KLK14 and the aspartic proteinase cathepsin D have also been attributed to desquamation due to spatial locality to corneodesmosomes and the ability to degrade desmoglein -1 *in vitro* (56, 57).

Structural analysis of the serine protease chymotrypsin using x-ray crystallography revealed the active site is formed by a catalytic triad of amino acids Histidine 57, Aspartic acid 102 and Serine 195 orientated in close proximity. The function of this arrangement is a charge relay system generating a nucleophilic Serine 195 able to break the amide bonds of its protein substrate (58). In the case of chymotrypsin, this would be large aromatic residues such as Tyrosine at the P1 position that bind the enzyme's hydrophobic specificity pocket (59).

The most abundant serine protease with chymotrypsin-like activity in the skin is KLK7 (60). It has been shown *in vitro* at acidic pH that KLK7 degrades Desmocollin-1 and Corneodesmosin; with KLK5 required for Desmoglein-1 proteolysis and complete degradation of the extracellular corneodesmosome structural components in the SC (55). There is evidence that Corneodesmosin is a target of both KLK7 and KLK5, with processing of this 52-56kDa protein occurring throughout terminal differentiation to generate a 15kDa fragment present in non-cohesive corneocytes at the skin surface (61).

In order to maintain homeostatic control and guard against aberrant desquamation, this synergy of protease activity requires strict orchestration through a number of mechanisms, the first being sequential activation (Figure 1.4). Located on chromosome 19q13.3-4, the expression of KLK1, KLK4 KLK5, KLK6, KLK7, KLK9, KLK10, KLK11, KLK13 and KLK14 commences in the SG (62, 63). They are synthesised as inactive zymogens that require proteolytic cleavage of the N-terminal domain to become biologically active. Members of the KLK family itself can fulfil this role of processing in a proteolytic

cascade; KLK5 can cleave pro-KLK7; pro-KLK14 and autoregulate its own inactive zymogen, and KLK14 - an abundant source (up to 50%) of trypsin-like protease activity in the SC (64) - can cleave and activate pro-KLK5 (65). A role for the serine protease mesotrypsin has also been implicated in this proteolytic cascade of activation, (66) as has the matrix metalloproteinase-20 (67).

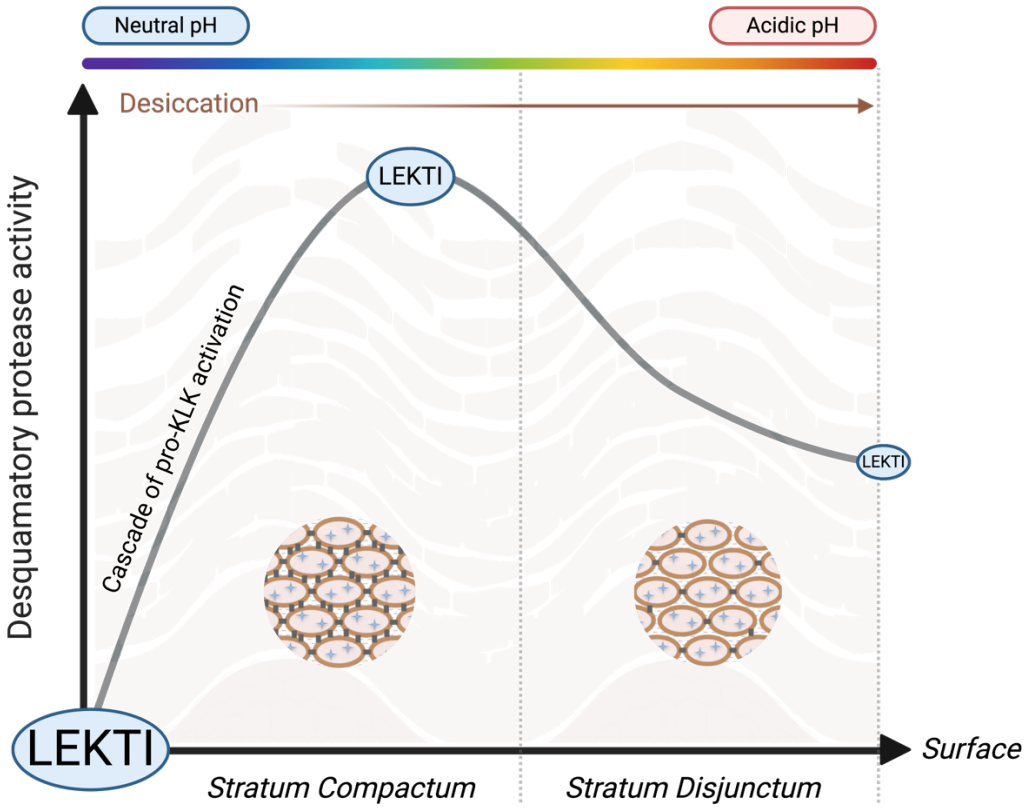


**Figure 1.4:** Multiple mechanisms control the rate of desquamation. Upon secretion with their inhibitor LEKTI at the SG / SC interface, the pro-KLK zymogens undergo a cascade of activation that initiates desquamation. This is balanced by strong LEKTI inhibition that weakens with acidification. Increasing dryness towards the skin surface also regulates proteolysis. The net result is greater rates of proteolysis in the deeper stratum compactum with corneodesmosome degradation largely complete by the stratum disjunctum. LEKTI: Lymphoepithelial-Kazal-type-related inhibitor.

Once zymogen activation is complete, a second mechanism of control is inhibition. Located on chromosome 5q32, *SPINK5* encodes Lymphoepithelial-Kazal-type-related inhibitor (LEKTI) that co-localises with KLK5 and KLK7 to block their activity (68). *In vitro* work has reported that LEKTI expression commences in the upper spinous layers in N-glycosylated form. Then it is rapidly cleaved by furin in a post-endoplasmic reticulum compartment to yield multidomain fragments capable of inhibiting KLK5, -7 and -14 activity (69-71). Immunofluorescence and immunoelectron microscopy of tissue samples from healthy donors report that these active LEKTI fragments are compartmentally packaged with KLK5 and KLK7 into lamellar granules, and delivered to the SG / SC interface by secretory granulocytes (71, 72). The comparatively earlier expression and processing of LEKTI to its KLK targets is designed to provide an additional layer of proteolytic control (71). Inhibition is also provided by structural components of the SC. The persistence of tight junction structures alongside corneodesmosomes at the cell peripheries of stratum disjunctum indicate that they provide physical protection against premature degradation by desquamatory proteases (73). Furthermore, cholesterol sulphate is a potent inhibitor of trypsin and chymotrypsin activity that may account for the hyperkeratosis associated with X-linked ichthyosis (74).

A third layer of control is provided by the acid mantle (49, 50). Due to KLK5 and KLK14 having a neutral-alkaline pH optimum, zymogen activation is initiated in the deeper SC layers. This is balanced by a strong KLK5-LEKTI inhibitory complex in this alkaline environment that weakens towards the acidic outer layers (70). The net result is a coordinated rate of corneodesmosome degradation that nears completion by the

upper stratum compactum (73, 75). In contrast, the activation of pro-KLK7 by KLK5 has a more acidic-neutral optimum of pH5-7 (65). This delay in KLK7 activation suggests a more prominent role in the final stages of desquamation, such as the destruction of desmocollin -1 in the stratum disjunctum (55, 75). Despite this pH discrepancy for activation, both KLK5 and KLK7 are able to cleave corneodesmosin and desmocolin-1 at a similar rate in the pH range 5-7 (55).

Removal of the acid mantle disrupts homeostasis through hyperactive desquamatory protease activity. In a murine model, Hachem and colleagues applied topical superbases to induce a short and longer-term rise in skin pH (76, 77). Consequently, a rapid increase in serine protease activity was associated with a concurrent reduction in SC integrity and cohesion through loss of desmoglein -1. Electron microscopy confirmed immature lamellar bilayers attributed to a protease-induced inhibition of β-glucocerebrosidase activity. All negative effects were normalised by a serine protease inhibition, highlighting a central role for SC pH in regulating desquamatory protease activity (76, 77).

And finally, the degree of skin hydration can exert a regulatory effect on desquamatory protease activity. Water content and environmental humidity are able to modulate rates of KLK7 proteolysis in the SC (78, 79) in line with its water concentration gradient (21). Interestingly, using transmission electron microscopy and electron energy-loss spectroscopy on porcine skin, undegraded corneodesmosomes in the lower SC have been identified as water rich ion channels (80). These spatial increases in water

content could manifest as localised changes in KLK7 activity that coordinate corneodesmosome destruction in the stratum compactum.

During the final stages of desquamation there are structural changes in the stratum disjunctum that facilitates corneocyte shedding. Using imaging techniques, Lin and colleagues show that the space once occupied by corneodesmosomes is replaced by hydrophilic intercorneocyte lacunar domains that expand due to water uptake and dehydration. This dynamic enables the cornified envelope to thicken that furthers lacunar widening. Coupled with the co-localisation of acid ceramidases to split the lamellar bilayers, the process of exfoliation is completed (75).

## 1.2 THE NEONATE SKIN BARRIER

From the safety and warmth of the uterus where it is enveloped by amniotic fluid, upon birth, the skin of the neonate rapidly adapts to a dry, ex-uterine environment. Here, as is the case for adults, the neonate SC must provide an immediate barrier to detrimental water loss, allergen penetration and microbial attack upon its introduction to terrestrial life. The vernix caseosa - a hydrophobic barrier composed of water (80.5%) lipid (8-10%), and protein (8-10%) (81) that covers the skin surface upon birth - assists in this transition by providing continuity between the two environments; first by protecting the developing foetus from amniotic fluid *in utero*, and then by complementing the dry adaptation by hydrating the skin, providing a source of free amino acids, and supporting the development of the acid mantle (82, 83). But not all term neonates are born with a protective covering of vernix, (84) so unaided, does the SC form an intact barrier immediately following birth? A first glimpse of the historical evidence hints that in final-trimester neonates at least, this is indeed the case, as at a gestational age of ≥34 weeks the epidermis is structurally complete (85, 86). Permeability barrier function appears competent (30, 87) and the SC is of equivalent thickness to adults shortly after birth (88). But this apparent maturity assigned by histological analysis hides an inherent fragility and susceptibility in its structure and function, that is only corrected by an extended period of adaptation to life in a dry environment.

1.2.1 The cellular structure of the neonate-infant SC is immature compared to adults

In line with the evolution of modern imaging and molecular techniques, a number of contemporary studies have analysed the structure of the neonate-infant SC *in vivo* and *in vitro*, and reported subtle differences compared to adults (Table 1.1).

| Study | Technique | Age group | Neonate-infant SC | Adult SC |
|---|---|---|---|---|
| Fluhr *et al* 2014 | SEM | 5-6 weeks | Corneocyte size = 646.3μm$^2$<br>Irregular, poorly defined corneocytes<br>Non-uniform corneodesmosomes | Corneocyte size = 895.5μm$^2$ |
| Stamatas *et al* 2010 | CLSM<br>FS | 3-24 months | SC thickness = 7.3μm<br>Corneocyte size = 949.5μm$^2$<br>Increased cell proliferation | SC thickness = 10.5μm<br>Corneocyte size = 1077.6μm$^2$ |
| *Naoko *et al* 2013 | LM | 1,3,6 months | Decreasing corneocyte size with age | Not compared |

**Table 1.1:** Structural differences between the adult and neonate-infant SC. SEM: Scanning Electron Microscopy; CLSM: Confocal Laser Scanning Microscopy; FS: Fluorescent Spectroscopy; LM: Laser Microscopy. Data presented from Fluhr *et al*, (89) Stamatas *et al*, (90) and Naoko *et al* (91) * denotes longitudinal study design.

At birth the SC surface is morphologically disorganised, characterised by poorly defined, asymmetric corneocyte clusters, that become more ordered during the first year (89). These corneocytes are smaller reflecting higher proliferation rates (89, 90). In agreement with this greater cell turnover, Naoko and colleagues applied laser microscopy to *ex vivo* SC samples collected by tape stripping to provide evidence of corneocytes becoming progressively smaller from birth (91). At age 5-6 weeks compared to adults there are differences in the spatial orientation of corneodesmosome fragments, with a lack of central cell-to-cell attachments between corneocyte layers in neonates (89). Differences in SC thickness also exist, with a

thinner SC composed of fewer corneocyte layers belonging to the infant (90). As postnatal age increases, these differences between infant and adult skin become less marked (89, 90). Collectively, these studies evidence the subtle structural differences at birth that persist throughout the early years of life as the barrier matures to adult-like status. The question is, do these structural anomalies reflect altered SC function during this timeframe?

### 1.2.2 Skin permeability barrier function at birth: complete or not?

The status of skin permeability barrier function at birth has been addressed by a number of studies. There is evidence it becomes competent with increasing gestational age, as at full-term, water loss and chemical permeability is low (30, 85). But in order to make firm conclusions, a comparison to healthy adults is required where homeostasis has been attained. Many studies have addressed this using TEWL, but contrasting results means this is a contentious issue; with barrier function reported to be weaker, equal to, or stronger in term infants compared to adults (87, 92-95). Methodological constraints may account for these observed discrepancies, as TEWL is sensitive to differences in skin care and the environmental conditions measurements are performed in (96). For example, the routine washing of neonates shortly after birth may bias the finding of 'weakened' permeability barrier function (higher TEWL) compared to adults, due to the skin absorbing water before measurements are taken (94). When capturing a TEWL measurement, the subject must remain still and calm in order to obtain robust flux data; a significant challenge when assessing young infants in a busy hospital setting where climate may not be adequately controlled. Therefore, although TEWL is an excellent minimally invasive, *in*

*vivo* measure of permeability barrier function in adults, these methodological constraints suggest it is not an ideal clinical tool that can be widely used in infant research.

### 1.2.3 The functional parameters of the neonate-infant SC are in flux

The skin of full-term neonates at birth is both dry and more alkaline indicating that homeostasis has not yet been attained (97, 98). The structural and functional consequences of a neutral skin pH at birth has been investigated using a rat model. In this environment of greater alkalinity, although TEWL is normal, the SC is more fragile, with compromised integrity and cohesion its distinguishing feature compared to older rats where acidification is complete (99). Here, using a combination of electron and confocal microscopy, the authors present incomplete lamellar membranes and lower corneodesmosome density through reduced expression of corneodesmosin and desmoglein 1, as key structural defects. A central mechanism to this being increased bulk serine protease activity (99). Accordingly, as the acid mantle forms a few days following birth, it correlates with the activation of lipid processing machinery that initiates the inside-out acidification of the SC from the SG interface (100). Interestingly, during this transitional acidification phase there was also a concomitant decrease in bulk serine protease activity alongside an increase in corneodesmosome density (99). This data in rats suggests that proteolytic components of desquamation are in flux, due to modulation by pH throughout acid mantle formation. It also suggests a temporary lack of buffering capacity as the SC extracellular spaces progressively acidify throughout its entire depth.

In contrast to the rat SC that acidifies after a few days, the human SC can take up to one month to stabilise around a physiological pH of 5.0, representing a comparatively extended period of skin barrier fragility (92, 98, 101). Furthermore, during this period, the skin hydrates, becomes smoother, and SC cohesion changes according to anatomical site (92, 93, 97, 98, 101). The status of permeability barrier function is less clear during these first few weeks of life, as at first glance, the cross-sectional study design suggests there is no change in TEWL (92). But this is misleading, as currently, perhaps the best evidence of TEWL status in the days following birth is provided by a longitudinal study conducted in a large cohort of 1903 neonates (102). Here, TEWL on average increased from 7.3 $g/m^2/hr$ at birth to 10.9 $g/m^2/hr$ at eight weeks old. This trend is corroborated by smaller studies of similar design, (91, 93) and reflects a significant weakening of permeability function during this transitional phase of skin development. Furthermore, environmental exposures that modulate TEWL could be exerting their effect during this time, such as the use of harsh detergents (103, 104) and exposure to house dust mite allergens (105).

In summary, there are numerous structural differences between neonate-infant and adult skin that are reflected by altered function throughout early life. A thinner epidermis characterised by irregular corneocyte morphology, differences in spatial corneodesmosome organisation and increased proliferation rates exist alongside fluctuations in TEWL, hydration and pH, suggesting mechanisms underlying terminal corneocyte differentiation have not yet reached homeostasis. When comparing study designs, cross sectional may well be more cost effective and less onerous for participants and investigators alike, but are less equipped to detect more subtle

changes over time. For example, it is the longitudinal study design that reported the rise in TEWL and reduction in corneocyte size throughout early life (91, 93, 102).

## 1.3 ATOPIC DERMATITIS

For reasons unknown the world is becoming more allergic. Adult IgE reactivity to common allergens is progressively increasing, (106) as is the worldwide incidence of eczema, asthma and rhinoconjunctivitis in children (107). Today the global prevalence of the allergic skin manifestation atopic dermatitis (synonyms: atopic eczema or eczema [AD]) is estimated to be around 10% of adults and around 20% of children (108, 109). It is a chronic lifelong condition, (110) that represents a significant financial burden for society, patients and caregivers across the world through money spent on prescriptions, travel to appointments, specialised caring at home, and loss of earning through days off work (111-114). In the UK, the cost of treating mild-moderate AD is estimated to be around £462.99 million over 5 years (115).

But money cannot be the only consideration. Yes, this is a treatable skin disease that will not directly cause mortality, but it profoundly affects lives in other ways. For example, AD is associated with other chronic, debilitating forms of atopy and poses a significant cardiovascular risk. Patient, caregiver and family quality of life is poor; with chronic itch, painful stinging skin and lack of sleep being common problems that correlate with greater disease severity. Patients are embarrassed and ashamed of their skin, yearning for a better acceptance of their condition and wanting to fit in with their peers (116-118). Children suffer from behavioural problems and are bullied (119, 120). Often misunderstood by the general public as “just dry skin”, the chronic, unpredictable nature of AD means there is a wide range of disease phenotypes. At the mild end of the spectrum, itchy, localised xerosis and erythema precede full body manifestation, pain, weeping wounds and recurrent skin infection in its most severe

form, that is difficult to control. These patients lack confidence and are often unable to work and form meaningful relationships. This ultimately has a detrimental effect on mental wellbeing, culminating in anxiety, depression and increased suicide risk (121, 122). A summary of these physical and mental comorbidities is presented by Table 1.2 with an estimate of the proportion of patients affected.

| Comorbidity | Prevalence (%) | |
|---|---|---|
| | AD | Control |
| Asthma | 25.5 | 11.7 |
| Allergic rhinitis | 34.9 | 14.2 |
| Food allergy | 15.1 | 3.6 |
| Clinician-diagnosed anxiety | 7.5 | 4.4 |
| Clinician-diagnosed depression | 19.5 | 11.2 |

**Table 1.2:** Physical and mental health comorbidities associated with AD. Prevalence reported by Hua and Silverberg (123), Silverberg and Simpson (124) and Thyssen *et al* (125).

In a clinical setting, AD is described as is a persistent, relapsing, inflammatory disease characterised by chronic xerosis, pruritus, and a susceptibility to skin infections. It forms part of the atopic diathesis; often manifesting alongside asthma, allergic rhinitis and food allergy, underlying a common pathogenesis shared by these conditions. Somewhat surprisingly though, allergy is not always present with AD. Allergic or extrinsic AD (ADe) is associated with higher total serum IgE and predisposes to allergic comorbidities in children (126). Non-allergic or intrinsic AD (ADi) is clinically indistinguishable from ADe and accounts for approximately 20-30% of patients, (126, 127) but total serum IgE measurements are similar to healthy controls (128). The absence of a systemic $T_H2$ cell allergic response in ADi, challenges the long-standing

historical view that it is a disease solely of an immunological imbalance (129). It also does little to explain the chronic xerosis that is ever present in patients.

So if allergy is not the sole orchestrator, what additional pathological mechanisms give rise to AD? Family history is a significant risk factor and points to a strong genetic basis for the disease (130, 131). But despite this observation a large proportion of AD heritability today remains unexplained. To address this need, genome-wide association studies (GWAS) have been employed as a powerful tool to locate genetic regions associated with AD. For example, a recent meta-analysis by Paternoster *et al* in 21,000 AD cases and 95,000 controls brought the total number of risk loci identified to 31, associating impaired epidermal differentiation (1q21.3), the $T_H2$ abnormality (5q31.1) and autoimmunity (5p13.2, 14q13.2) with disease (132). In total these risk signals account for <20% of all AD heritability, and with the majority being intragenic, a significant challenge here is determining their functional significance in AD pathogenesis.

The current most significant genetic risk factor with functional consequence found to date encodes a structural protein that resides in the skin itself. Loss-of-function (LOF) mutations in the gene encoding the epidermal barrier protein Filaggrin (FLG), located on chromosome 1q21.3, represents the most widely replicated risk factor for AD in European populations (133, 134). Since this pivotal finding soon after the turn of the 21$^{st}$ century, (135) the focus of AD research has shifted towards the defective skin barrier and its role in disease pathogenesis. In patients with AD, the structure and function of both involved and uninvolved sites is dysfunctional compared to healthy

skin. These barrier abnormalities confer increased permeability to exogenous substances and water, resulting in a dry skin phenotype susceptible to allergen penetration and cutaneous inflammation. Even environmental exposures can modulate AD risk, as living in a polluted urban environment, (136, 137) the use of harsh soaps (138, 139) and colonisation by *S.aureus* (140-142) can all exacerbate skin barrier breakdown and stimulate inflammation. Therefore, the modern pathogenic model for AD describes the interactions between a primary skin barrier defect, a spectrum of immune hyperactivity and negative environmental stressors that give rise to active disease (Figure 1.5).

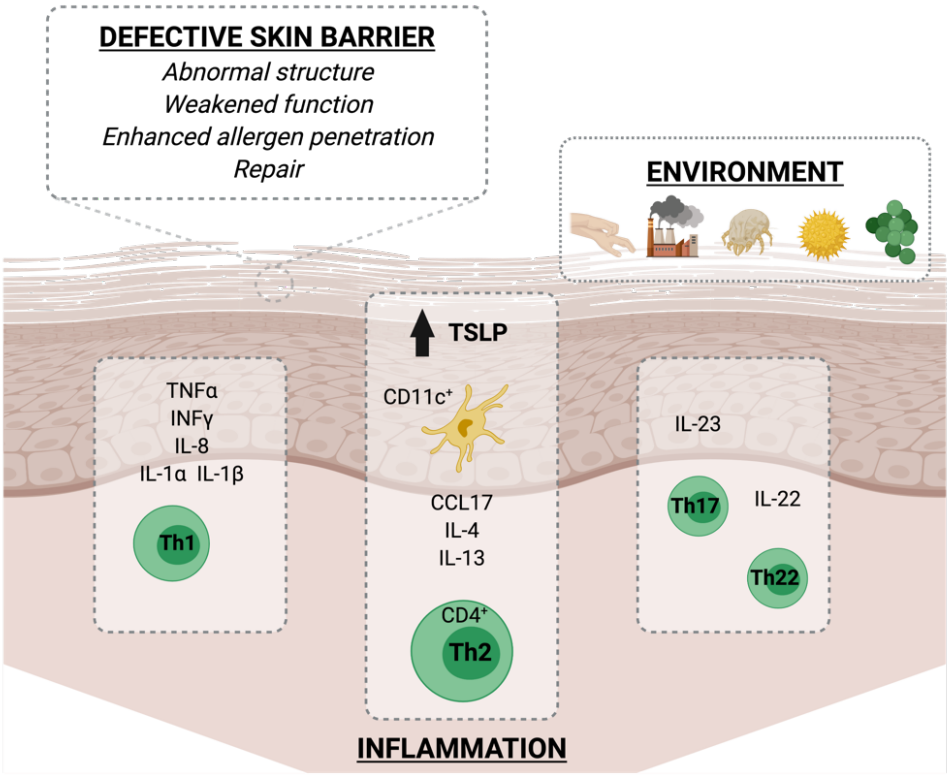


**Figure 1.5:** The pathogenesis of AD. A dysfunctional skin barrier, environmental exposures and genetic predispositions combine to drive $T_H1$, $T_H2$, $T_H17$ and $T_H22$ skin inflammation. A defective skin barrier exacerbated by persistent scratching that permits the penetration of allergens and its colonisation by pathogens such as *S.aureus*. Pollution can modify disease risk and severity. In response to disruption, the epidermis initiates its repair through the expression of cytokines that contribute to the inflammatory environment in AD. For example, elevated expression of Thymic Stromal Lymphopoietin (TSLP) activates $CD11c^+$ dendritic Langerhans cells to drive $T_H2$ polarisation of $CD4^+$ lymphocytes. IL: interleukin; TNFα: tumour necrosis factor alpha; INFγ: interferon gamma; CCL: C-C motif chemokine ligand.

A valuable preclinical tool used in AD research to better understand basic pathogenic mechanisms of disease are animal models. For example, the hapten oxazolone, when repeatedly applied to hairless mice, mimics AD-like lesions and replicates the significant permeability barrier defect, $T_H2$ dominant infiltrate and elevated serum IgE associated with disease (143). To simulate barrier disruption, allergen penetration and sensitisation, tape stripping combined with ovalbumin patch application has been utilised in BALB/c mice (144). In this disease model, repeated allergen exposure promoted skin thickening with marked inflammatory cell infiltrate, elevated $T_H1$ / 2 cytokine and chemokine production with increased total and allergen specific IgE. To mimic attenuated FLG expression in the epidermis, the flaky tail mouse (partial FLG loss) has been employed (145) and a *Flg*$^{-/-}$ mouse engineered (146) to simulate disease-associated null mutations associated with AD. Both FLG models demonstrate an enhanced hapten-induced allergic immune response, with the *Flg*$^{-/-}$ mouse also displaying a dry, scaly phenotype with reduced levels of hygroscopic NMF and increased SC fragility.

### 1.3.1 Skin breakdown in AD

#### *1.3.1.1 Multiple defects manifest as weakened barrier function*

Both the inside-out and outside-in permeability barrier reside in the SC (28, 30-33) conferred by corneocyte, lipid and tight junction structural components (22-25). It is therefore inevitable that multiple defects in structure (summarised in Table 1.3) manifest as weakened permeability barrier function (elevated TEWL) at both non-lesional and lesional sites, that gives rise to the underlying xerotic phenotype associated with AD (147-149). Such is the significance of this permeability barrier defect in AD patients, that TEWL and hydration (SCH) correlate with disease severity (147, 150).

| Barrier component | Defect | Functional significance | References |
|---|---|---|---|
| Cornified envelope | Reduced / intermittent expression of FLG, INV, CDSN<br>Altered LOR expression<br>Increased K6 and K16<br>Increased K5 | High skin pH<br>Fragile SC<br>Low SCH<br>Hyperkeratosis<br>Immature SC | Suarez-Farinas *et al* 2011<br>Jensen *et al* 2004 |
| Lipids | Increase in short chain CER<br>Decreased bulk lipids<br>Decreased A-SMase activity | High TEWL<br>Low SCH | Janssens *et al* 2012<br>Imokawa *et al* 1991<br>Jensen *et al* 2004 |
| Tight Junctions | Reduced expression claudin -1, -23 | Increased permeability | De Benedetto *et al* 2011 |
| Desquamation | Increased expression of KLK 5, -6, -7, -8, -10, -11, -13, -14<br>Increased protease activity | High TEWL<br>Thinner SC | Komatsu *et al* 2007<br>Morizane *et al* 2012<br>Voegeli *et al* 2009 |
| Microbial | Reduced commensals | Pathogenic *SA* colonisation | Nakatsuji *et al* 2017 |

**Table 1.3:** Skin barrier breakdown in AD. CER: ceramides; A-SMase: Acid sphingomyelinase; K6: Keratin 6; K16: Keratin 16; K5: Keratin 5; FLG: Filaggrin; INV: Involucrin; CDSN: Corneodesmosin; LOR: Loricrin; KLK: Kallikrein; *SA*: *Staphylococcus aureus*; SCH: Stratum corneum hydration; TEWL: Transepidermal Water Loss; SC: Stratum Corneum. Data presented from Suarez-Farinas *et al*, (151) Jensen *et al*, (148) Janssens *et al*, (152) Imokawa *et al*, (153) De Benedetto *et al*, (27) Komatsu *et al*, (154) Morizane *et al*, (155) Voegeli *et al*, (156) and Nakatsuji *et al* (48).

Histological analysis has confirmed incomplete late terminal differentiation associated with AD. In patients at both lesional and non-lesional sites compared to controls, key structural building blocks of the cornified envelope – Filaggrin and Involucrin – are poorly expressed, or in the case of Loricrin and Corneodesmosin; virtually absent (148, 151, 157). This weakened structural integrity of the cornified envelope confers fragility in the face of mechanical stress (146, 158-160). An inherent barrier to complete differentiation is the disproportional abundance of proliferating keratinocytes. Expression of proliferation markers Keratin 6 and 16 is greater in AD skin and accounts for the hyperkeratosis that manifests at lesional sites (148, 157). Greater rates of proliferation signal the persistence of immature, non-differentiated keratinocytes in the SC, as supported by the greater expression of basal cell marker Keratin 5 throughout the nucleated epidermis in AD skin (148).

A significant disease-associated defect is also present in the lipid lamellae component of the SC. Bulk SC lipids are decreased by around 44-54%, with a 32-36% reduction in ceramides alongside reduced activity of acid sphingomyelinase; a key enzyme for generating free ceramides prior to processing into the lipid lamellae (148, 153). Not only lacking in quantity, but these ceramides also differ in structural composition and are enriched in shorter carbon chain species (152, 161). This shift to a shorter carbon chain length is associated with a less orthorhombic (ordered) lipid lamellae organisation and greater TEWL (103, 152). Weakened permeability barrier function is further compounded by the loss of key tight junction proteins - Claudin -1 and -23 - that regulate the paracellular movement of ions and water and form part of the inside-out, and outside-in barrier (27, 33).

*1.3.1.2 Filaggrin: a missing link in AD pathogenesis?*

Loss of function (LOF) mutations in the gene encoding profilaggrin cause Ichthyosis Vulgaris; (IV) an inherited skin disorder of incomplete keratinocyte differentiation (162). This study uncovered two common LOF mutations - the single nucleotide polymorphism R501X (rs61816761) and the 4-base pair deletion 2282del4 (rs558269137) - in multiple families of UK and European decent with IV. Clinical similarities such as xerosis and palmar hyperlinearity exist between IV and AD, therefore a subsequent candidate gene approach uncovered the same common *FLG* LOF mutations predispose the development of AD (135). The four most prevalent *FLG* null mutations are summarised by Table 1.4.

| Name | RefSNP ID | Mutation | Consequence | MAF (%) |
|---|---|---|---|---|
| R501x | 61816761 | G > A | Stop | 7.7 |
| 2282del4 | 558269137 | ACTG deletion | Frameshift | 6.8 |
| R2447x | 138726443 | G > A | Stop | 1.4 |
| S3247x | 150597413 | G > T | Stop | 2.8 |

**Table 1.4:** Common *FLG* null mutations that predispose to AD. Minor allele frequencies (MAF) are reported by Margolis *et al* (163) in a white US population.

Since this first finding in 2006, more recent meta-analysis of case control studies report that R501X and 2282del4 confer a 3-fold risk of AD; (OR: 3.12; 95%CI, 2.57-3.79) are associated with more severe, persistent, treatment-resistant forms of disease; and increase the risk of developing asthma (OR: 3.29; 95%CI, 2.84-3.82) (134, 163). Interestingly, one study found that maternal inheritance of these alleles exerts an additive effect on AD risk in IgE sensitised mothers, indicating a strong gene-environment effect in their offspring (164). Intragenic copy number variation also

modulates AD risk, with a protective effect associated with a higher number of FLG monomer repeats (165).

These *FLG* LOF mutations negatively affect skin barrier structure and function in a dose-dependent manner. Histological analysis shows the complete absence of keratohyalin granules, a reduction in KIF density, and the loss of FLG monomers in homozygous IV patients carrying two LOF mutations, with residual expression remaining in heterozygotes (159, 162). An increased proportion of Ki-67 positive proliferative cells with subsequent greater number of SC layers is also found (159). In line with these structural abnormalities, skin barrier function is profoundly compromised through reduced SCH, elevated TEWL, delayed barrier recovery, increased pH and reduced cohesion compared to wild type controls (159). Greater inside-out permeability to water is attributed to a paracellular abnormality as a consequence of immature lamellar bilayers (159).

In agreement with these findings in IV, there is strong evidence that AD patients carrying *FLG* LOF mutations also possess a significant defect in skin barrier structure and function. Observations such as increased skin roughness and scaling, differences in lipid profiles, elevated TEWL, decreased SCH, elevated pH and reduced SC integrity have all been reported in uninvolved FLG-deficient skin compared to healthy controls (158, 166, 167). To expand on these deficiencies further, both the flaky tail and *FLG* null mouse models of AD have been utilised to investigate the pathogenic consequences of inherited FLG loss from the epidermis. These mouse models exhibit a dry, scaly, more fragile primary skin phenotype, characterised by hyperkeratosis, the loss of

keratohyalin granules and FLG monomers from the cornified layer (146, 168). Both the inside-out, and outside-in skin barrier of these animals, measured by TEWL and dye penetration respectively, is significantly compromised (145). A defining feature of dry skin conditions is low levels of extractable NMF (39). AD is no exception to this in that SC NMF levels are significantly reduced compared to healthy skin (40, 146, 169). Given that monomeric FLG is its primary source, NMF levels in AD patients is indicative of corresponding *FLG* genotype (169, 170).

Although undoubtedly significant, *FLG* LOF heritability by itself does not fully explain AD pathology. This is demonstrated by a German study in children that found population attributable risk of AD from *FLG* LOF mutations to be 13.5% and the penetrance at 38.5%, meaning just carrying a *FLG* LOF mutation does not guarantee active disease (171). Nevertheless, after many years of research focused on the powerful dysregulated $T_H2$ type immune response, the discovery of *FLG* LOF mutations represents a pivotal paradigm shift towards a primary skin barrier defect underlying the pathogenesis of AD.

*1.3.1.3 Proteolytic barrier breakdown in AD*

Although a key part of normal skin barrier homeostasis, there is a growing body of evidence relating dysregulated protease activity to the primary skin barrier defect in AD. This is provided by the autosomal, recessive, inflammatory disease Netherton Syndrome (NS); a condition similar to severe AD in its clinical presentation and a predilection to food allergy, that suggests a shared pathogenesis between the two diseases. NS is caused by mutations in *SPINK5* encoding the serine protease inhibitor

LEKTI (172). Loss of functioning LEKTI culminates in increased KLK5, KLK7, KLK14 and elastase-2 epidermal protease activity alongside a clinical phenotype of scaling, erythroderma and severe pruritis. This cocktail of unrelenting protease activity aggressively degrades corneodesmosomes to the extreme whereby the SC becomes detached from the SG. Coinciding with this structural abnormality is a marked permeability barrier defect (173-175).

With these similarities to AD in mind, a candidate gene approach has associated a single nucleotide polymorphism (rs2303067) in *SPINK5* with AD (176). As is the case in NS, although far less profound, the functional consequences of this frequent, non-conservative E420KK LEKTI variant are elevated KLK5, KLK7 and elastase 2 protease activity, depleted SC cohesion through reduced expression of DSG1, and increased expression of TSLP compared to wild type controls (177). Although performed in healthy indivduals, (177) this study suggests a genetic basis for a protease defect in AD that accelerates barrier breakdown and disease development. This is supported by Vasilopoulos and colleagues proposing an additional risk locus; a gain in function insertion located in *KLK7* (178).

Unique to AD, a modulating effect of skin pH on protease activity has been reported by the flaky tail mouse model. Here, under steady-state conditions, elevated skin pH induces mRNA expression of KLK5, 7 and 14 to increase bulk serine protease activity assessed by *in situ* zymography (179). A similar modulation of serine protease activity is observed in the oxazolone-induced mouse model of AD, (143) an effect reversed by re-acidification of the SC (180). In general agreement with this pH mechanism, albeit

using more aggressive experimental methodology, neutralisation of the murine epidermis using topical superbases elicited a more pronounced protease-induced barrier defect. Application of 1,1,3,3-tetramethylguanidine or 1,8-diazabicyclo [5,4,0] undec-7-ene resulted in sustained serine protease hyperactivity, DSG1 degradation and loss of corneodesmosomes, in conjunction with a lipid processing defect (76, 77). Considering the evidence that eczematous skin has a higher pH, (181, 182) these findings together suggest a protease-mediated mechanism of skin barrier breakdown that warrants further investigation in human AD cohorts.

Animal models have provided a rich source of mechanistic evidence, but currently, the degree of SC protease hyperactivity in AD patients is unclear, limiting the translatory potential of this basic research to clinical treatments. In human subjects, perhaps the most comprehensive account of dysregulated protease activity related to AD is provided by Voegeli and colleagues. Here, the authors used fluorescent peptide substrates to profile a range of SC proteolytic activities in patients with mild-to-moderate disease compared to non-lesional skin and healthy controls (156). Increased proteolysis attributed to plasmin, urokinase, leukocyte elastase, trypsin-like, chymotrypsin-like and tryptase-like activity was noted in active AD compared to healthy skin, and was associated with SC thinning, and reduced skin barrier function measured by TEWL, skin-surface pH and SCH (156). Protein quantification in the same subjects found elevated expression of KLK7, KLK11 and plasmin in active AD, accounting for in part, the observed increases in proteolytic activity (183). A similar small study found no evidence for elevated trypsin, and chymotrypsin-like protease activity in the uninvolved, dry skin of patients with established AD, despite a general trend for

widespread increased Kallikrein protein expression in the SC (154). These findings provide preliminary pathogenic evidence relating a synergy of SC protease hyperactivity to accelerated barrier breakdown in AD once cutaneous inflammation is established. Not addressed by these studies however is the potential modulating effect of disease severity on protease activity, nor the primary subclinical protease defect that may exist before disease onset. Nevertheless, this preliminary work supports the finding that remedying part of this proteolytic imbalance through topical inhibition, may prove efficacious for the treatment of established AD and chronic itch (184).

### 1.3.2 Inflammation in AD

A current model of AD course (185) places non-allergic or intrinsic AD as the first phase of disease, with allergen sensitisation and elevated IgE denoting progression to extrinsic AD (phase 2) Phase 3 is a more chronic, autoallergic stage. The latter phases are associated with an increased risk of further allergic and cardiovascular comorbidities (Figure 1.6).

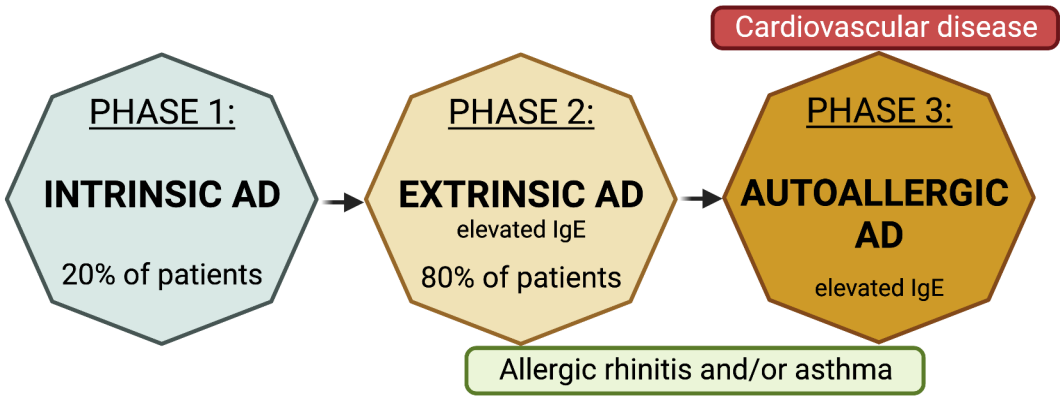


**Figure 1.6:** The course of AD. Genetic susceptibility to a skin barrier defect and subclinical inflammation promotes non-allergic intrinsic AD (phase 1). Allergen penetration and sensitisation denotes progression to allergic AD (phase 2). Autoallergic AD (phase 3) represents a more chronic stage of disease. Figure adapted from Danby *et al* (185).

Acute lesional AD is mediated by the infiltration of $CD4^+$ T cells of the adaptive immune system that produce cytokines to drive cutaneous inflammation and orchestrate the dysregulated immune response. Skin resident dendritic cells present antigens to naïve T cells that upon recognition, differentiate into distinct T helper (Th) subsets classified by their cytokine expression. In AD, the Th2 cell subset predominates, but there is also evidence of a Th1, Th17 and Th22 inflammatory milieu, highlighting the heterogeneous

immunological profile associated with disease pathogenesis and course (151, 157, 186). Compared to psoriasis, there is an attenuated up-regulation of Th17 axis genes associated with IL-17 such as CCL20 in both acute and chronic phases of established AD; but their increased expression is more strongly associated with early disease onset, with IL-26 correlating with barrier dysfunction in infants (157, 187). Th22 and Th1 genes such as IL-22 and IFN-γ respectively are expressed in acute AD with their upregulation noted in more chronic disease (157). Interestingly, the induction of Th1 immune genes such as interferon gamma receptor 1, CXCL (C-X-C Motif Chemokine ligand) -9 and CXCL10 in infants is lacking compared to adults, highlighting further differential immune pathogenesis over time (157, 187).

#### *1.3.2.1 $T_H2$ axis is central to AD pathogenesis*

The inside-out model of AD pathogenesis refers to the broad spectrum of dysregulated inflammation that forms the basis of acute lesional disease and drives the production of allergic IgE (129, 188). Central to this is a strong $T_H2$ polarised immune signature; evident in innate skin resident keratinocytes and dendritic cells, (189, 190) infiltrating and circulating lymphocytes of the adaptive immune system, (191, 192) and serum (193). The alarmin TSLP expressed by keratinocytes is an essential initiator of Th2 signalling and allergic skin inflammation (194). In the early development of eczematous reactions by allergy patch testing, expression of the cytokine interleukin (IL)-4 predominates (195) that stimulates the production of chemokines (CCL17 / CCL22) by resident dendritic cells (196) to augment the infiltration of activated Th2 cells (197). Further overexpression of IL-4 and IL-13 by these activated T cells in lesional skin promotes B cell class switching to produce IgE and promote

eosinophilia (198-200). Further evidence underpinning IL-4 and IL-13 in disease pathogenesis is provided by transgenic mouse model of spontaneous pruritic skin inflammation (201) and the discovery of a missense mutation predisposing to high patient IgE levels in Japanese and German populations with AD (202, 203). It has recently emerged that Itch, the hallmark symptom of AD, is attributed to IL-31 expression by activated Th2 cells (204).

Full thickness skin biopsies from AD patients coupled with molecular techniques is a valuable tool for capturing inflammatory profiles and cellular infiltrate at various stages of disease. Using this methodology, a spectrum of cutaneous inflammation has been reported in non-lesional AD compared to heathy skin. Here, $T_H2$ type cytokines and chemokines predominate with increased expression of IL-13, CCL5, CCL11, CCL17, CCL18, CCL22 alongside MX-1 ($T_H1$) and IL-22 ($T_H22$) (151). Interestingly, the degree of this subclinical inflammation correlates with disease severity, suggesting a pathogenic role for non-lesional skin in the development of active lesions, and confirming that normal-appearing AD skin is far from healthy (151). Transitioning from non-lesional to acute and then chronic AD is associated with a dose dependant increase in expression of $T_H1$, $T_H2$, $T_H17$ and $T_H22$ type chemokines and cytokines, correlating with greater numbers of infiltrating T cells, myeloid cells, dendritic cells and Langerhans cells (157, 205).

*1.3.2.2 Skin barrier defects trigger cutaneous inflammation*

In response to barrier disruption, the epidermis initiates its subsequent repair through the expression of proinflammatory cytokines. After tape stripping to experimentally damage the SC, an increase in TEWL and thickness is accompanied by epidermal expression of Keratin-16, TNF-$\alpha$ (tumour necrosis factor alpha), IFN-$\gamma$ (interferon gamma), IL-8 and IL-10 (206). Prolonged scratching as a consequence of unrelenting KLK7 or cathepsin S activity, precedes the development of skin lesions characterised by lymphocyte infiltration and $T_H1$ cytokines (207, 208). This stressed epidermal barrier undergoing repair, shares similarities with the subclinical barrier defect and inflammation encountered in non-lesional AD compared to healthy skin (148, 151). When skin barrier disruption becomes more chronic - as is the case in *FLG* LOF mutation carriers - there is increased epidermal expression of IL-1$\alpha$ and IL-1$\beta$, an observation supported by fatty acid deficient mice (54, 209). Therefore, a damaged skin barrier alone is sufficient to autonomously initiate a proinflammatory environment that signals disease onset and forms the foundation of heightened $T_H1$ cytokine levels found in lesional disease (205, 210, 211). This evidence associates skin barrier dysfunction to $T_H1$ type inflammation, but how does it relate to the hallmark $T_H2$ inflammatory environment found in diseased skin?

One answer may reside in the innate expression of TSLP by keratinocytes. Highly expressed in AD (212) and following barrier disruption, TSLP stimulates $CD11c^+$ dendrocytes and polarises naïve $CD4^+$ T cells to produce $T_H2$ cytokines (192, 213). There is also evidence that TSLP is an essential inflammatory mediator in the allergic sensitisation to ovalbumin through disrupted skin (214). Likewise, in the mouse model

of NS, aberrant KLK5-mediated cleavage of PAR2 primes cutaneous $T_H2$ inflammation through increased expression of TSLP (173-175, 215, 216). Elevated TSLP expression is also associated with barrier disruption as a consequence of FLG loss from the epidermis (179, 212). Here, animals with hyperkeratosis, acanthosis and permeability barrier dysfunction are predisposed to cutaneous $T_H2$ inflammation, elevated IgE and a reduced threshold to irritants and allergens (179, 212, 217). In this scenario, antigens such as ovalbumin can penetrate more readily to exacerbate a background dermal inflammatory infiltrate of lymphocytes, mononuclear cells and eosinophils through further induction of the $T_H1$, $T_H2$, $T_H17$ axis that drives allergen specific IgE production (146, 218, 219). This data together demonstrates how barrier dysfunction of multiple forms can facilitate antigen penetration and prime disease associated inflammation. Interestingly, TNF-$\alpha$ is required for TSLP induction in skin explants, providing a mechanism that connects the proinflammatory response related to barrier damage to the proallergic arm of the immune system (220).

*1.3.2.3 The acquired skin barrier defect*

In a self-perpetuating pathogenic loop, the heightened inflammatory microenvironment of AD in return can disrupt the skin barrier and exacerbate its breakdown. *In vitro* experimental models that simulate disease-associated inflammation, have informed that the cytokines IL-4, IL-13 and IL-25, (143, 221, 222) in addition to IL-22, (223) and IL-31 (224) can all knockdown FLG expression and subsequently reduce levels of NMF. This is confirmed in patients with functional *FLG* expression, as disease severity exerts a similar effect on skin barrier structure and function independent to LOF mutations (225, 226). Additional structural SC components supressed by cytokines IL-4 and IL-13 include the lipid lamellae, (227, 228) and the cornified envelope proteins (151, 229-231). These cytokines can also stimulate expression of KLK7, (155, 229) accounting for the increased chymotrypsin-like protease acitivity reported in patients with active disease (156). Therefore, not only can a significant barrier defect be inherited, these studies together provide evidence on how it can be acquired as a consequence of disease inflammation.

## 1.4 SKIN BIOMARKERS FOR AD

AD is a chronic, unpredictable disease, often waxing and waning between periods of flare and remission. In these circumstances it is primarily managed through reactive topical anti-inflammatory therapy to treat clinical lesions. Control is then maintained by the proactive use of emollients and topical anti-inflammatories that modify disease course by treating the barrier defect and subclinical inflammation (211, 232) to prolong the subsequent time-to-flare and reduce severity (233-235). This treatment strategy emphasises the pathogenic synergy between a barrier defect and subclinical inflammation that steers the natural disease course.

### 1.4.1 Using biomarkers to understand the natural course of AD

Considering its pivotal role in AD pathogenesis, the skin barrier promises a rich source of potential biomarkers to provide information on triggers, severity and prognosis associated with its breakdown (236). Non-lesional skin being far from healthy due to its altered structure, hyperproliferation, compromised function and subclinical inflammation, (147, 148, 151, 157) is an ideal point of enquiry due its close relationship with disease onset and severity (151, 152, 211). Using non-invasive techniques such as tape stripping to provide SC samples for laboratory analysis, (156, 170, 237) patient cohorts can be easily screened at non-lesional sites for biomarkers related to skin barrier breakdown. A better understanding of these pathogenic signals in uninvolved skin may provide valuable information on disease course and help combat the unpredictable nature of AD.

Dysregulated proteolytic activity is one such candidate for biomarker exploration. Animal models have demonstrated the consequences of protease hyperactivity in AD and NS such as spontaneous lesions, accelerated corneodesmosome degradation and increased TSLP expression, suggesting a central role in disease pathogenesis through premature skin barrier breakdown and the synthesis of $T_H2$ type inflammation (173-175, 207, 215, 216). But robust mechanistic evidence in patients is lacking, particularly on how risk genotypes may translate to increased proteolysis within the SC. In addition, clinical phenotypes associated with protease hyperactivity in AD are largely unknown (Figure 1.7).

Filaggrin and its related breakdown product NMF, offer another intriguing avenue for biomarker research. In contrast to desquamatory proteases, mechanisms of low NMF and the clinical phenotypes associated with FLG risk genotypes are comparatively better understood. Low levels of NMF components PCA and UCA in the SC, has recently been linked to the *FLG* null genotype and AD severity, suggesting that in children at least, an NMF defect can be acquired and modulated through the degree of cutaneous inflammation present as measured by SCORAD (170, 225). But this documented relationship with disease severity albeit significant, is weak, and may indicate other factors that modulate NMF in the skin. Another reason for this weak association with disease severity could be it is lacking data from the largest pool of NMF residing in the SC; free amino acids (34).

A direct consequence of the primary NMF defect may be increased protease activity. AD is disease of elevated skin pH (181, 182) due to *FLG* LOF mutations or the onset of inflammation (54, 143, 166). As SC desquamatory protease activity is pH and desiccation sensitive, here is a potential mechanism of barrier breakdown that requires further investigation (Figure 1.7).

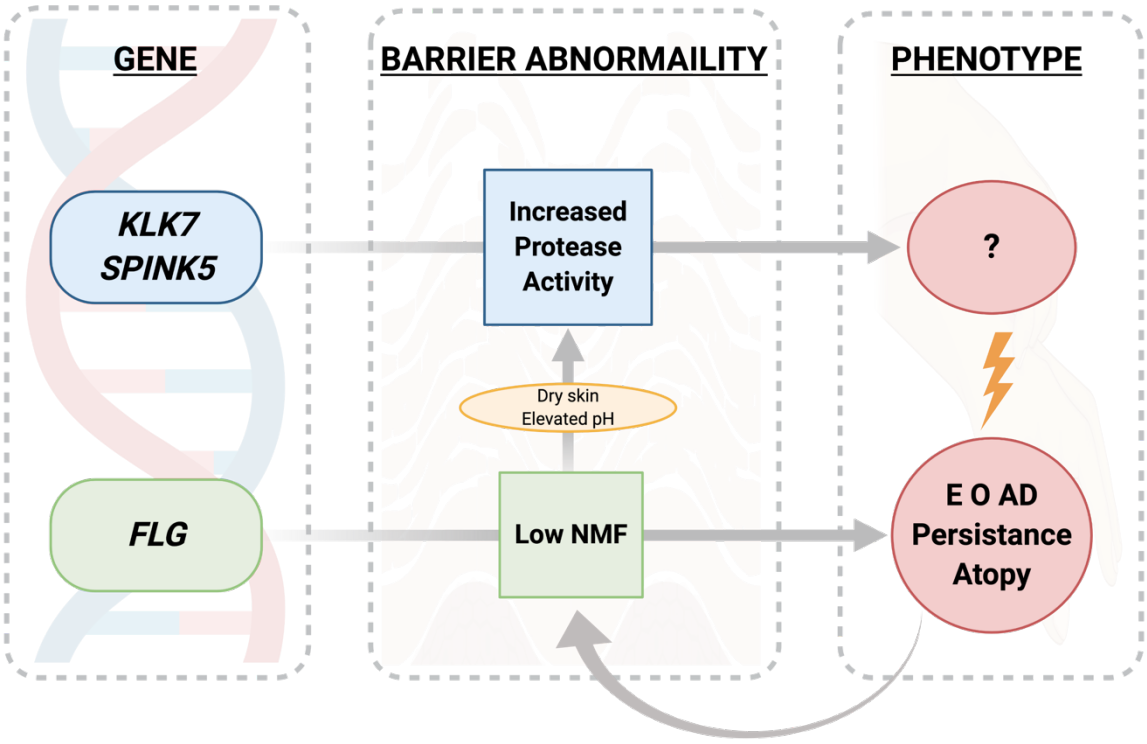


**Figure 1.7:** Mechanisms of skin barrier breakdown related to increased protease activity and low NMF. The functional consequence of risk mutations predisposing to increased desquamatory protease activity and the resulting clinical phenotypes in AD are largely unknown. In contrast, a primary NMF defect as a consequence of *FLG* LOF mutations (inherited) or active disease (acquired) is associated with dry skin and elevated pH that may drive protease hyperactivity in uninvolved skin. E O AD: early-onset AD.

### 1.4.2 Using biomarkers to understand AD development

Neonates are not born with visible signs of AD, but the majority of cases develop during the first year of life (238, 239). It is therefore perhaps of no coincidence that this extended period of skin barrier 'optimisation' and fragility coincides with a significant risk of developing AD. Currently it is unknown exactly why a child will develop active disease, but there are significant epidemiological clues emerging from population-based studies. By far the best predictor a clinician or caregiver has is parental AD (130, 131). Environmental exposures such as climate, living in an urban environment, and even the geographical location of your mother's birth can modulate AD risk (137, 240, 241). Given these strong gene-environment signals, it is plausible to put forward the following pathogenic scenario: that neonates genetically predisposed to AD, are subjected to negative environmental stressors that further weaken an already fragile skin permeability barrier vulnerable to breakdown (Figure 1.8). This culminates in a greater barrier defect by 8 weeks of age and an 8-fold increased risk of developing AD by one year (102). With this in mind, it is also plausible to think that one may be able to pick up further mechanisms of skin barrier breakdown throughout the neonate period that exacerbate loss of permeability barrier function and predate the onset of clinical AD.

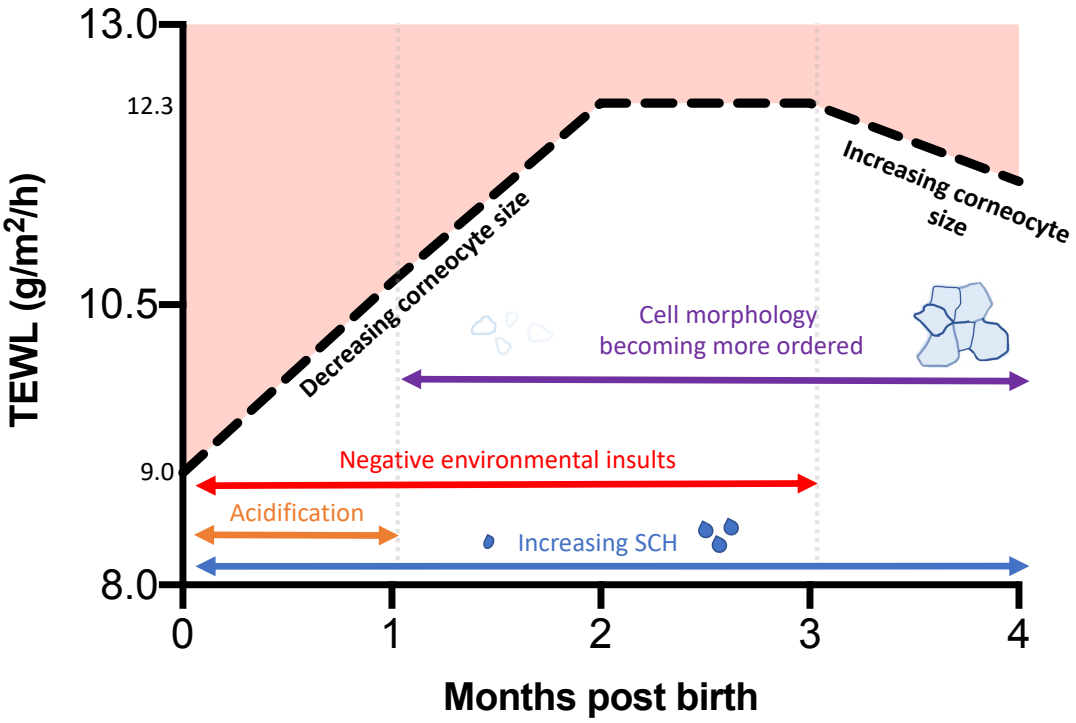


**Figure 1.8:** The relationship between neonate-infant skin barrier development and AD risk. Throughout the first 3 months of life when dynamic structural and functional SC changes are taking place, the skin barrier is more susceptible to environmental stress, culminating in elevated TEWL at 3 months of age. Infants at risk of AD are less able to adapt to these environmental challenges and have weaker permeability barrier function both at birth and 2 months old (upper-quartile TEWL, shaded red) TEWL values reported by Kelleher at al., 2015 (102).

### 1.4.3 Endophenotype stratification is a future goal for AD

Although xerosis, pruritis and flexural erythema are common features in AD, the 4 major and 23 minor observations contained within the Hanifin and Rajka diagnostic criteria tells us that disease phenotypes can be heterogenous in nature (242). A route cause of this is its multifactorial pathology, therefore, a truer illustration of AD may be a series of subtypes or endophenotypes that reflect distinct underlying molecular mechanisms of disease. Central to this endophenotype subclassification are biomarkers; a series of biological tools that can be objectively measured and provide information on progression from predisposing genotype to clinical phenotype (236). As our knowledge of disease pathogenesis continues to grow, preventing the natural disease course using a biomarker / endophenotype approach to stratify patient cohorts and treat accordingly, is a future goal for current AD research in an era of personalised medicine (236, 243).

## SCOPE OF THESIS

Breakdown of the skin barrier is a key component of AD pathogenesis. Although the unaffected skin of patients appears healthy, underlying structural defects render it functionally inadequate. The evidence suggests this facilitates allergen penetration and primes subclinical inflammation to signal disease onset and severity. Despite being key components of a healthy, biologically active skin barrier at physiological levels, there is growing evidence that both protease hyperactivity and low levels of NMF within the SC contribute to barrier breakdown in adult AD.

As cases of AD continue to rise in children around the world, focus has shifted to prophylactic interventions from birth to support barrier development throughout a period of fragility as it adapts to terrestrial life. The hypothesis here being that disease onset in infancy can be delayed or even halted by correcting the barrier defect in predisposed individuals. But in contrast to adults, the infant skin barrier is not well characterised. There are unanswered questions on its rate of development and the mechanisms of breakdown preceding disease onset during infancy, that if addressed, will provide a better understanding of this somewhat unpredictable disease. Furthermore, the identification of at-risk individuals with greater precision may facilitate the appropriate intervention to disrupt the natural course of AD

STUDY OBJECTIVES:

To perform a series of clinical studies in infants and adults to better understand normal skin barrier development from birth and investigate its potential breakdown in subjects predisposed to AD by:

- Adapting non-invasive laboratory assays based on tape stripping to monitor protease activity and NMF development as biomarkers of terminal corneocyte differentiation in a longitudinal birth cohort. Compare findings to healthy adult controls to elucidate how the neonate SC differs to adult skin.

- Developing and validating the use of Infrared Spectroscopy as a non-destructive *in vivo* method of NMF quantification for assessing skin health and the inherited or acquired FLG defect.

- Investigating differential trajectories of skin barrier development from birth in infants that do and do not develop disease. Assessing the predictive potential of skin barrier biomarkers measured in a community setting at birth and 4-weeks, for the development of AD by 12 months of age.

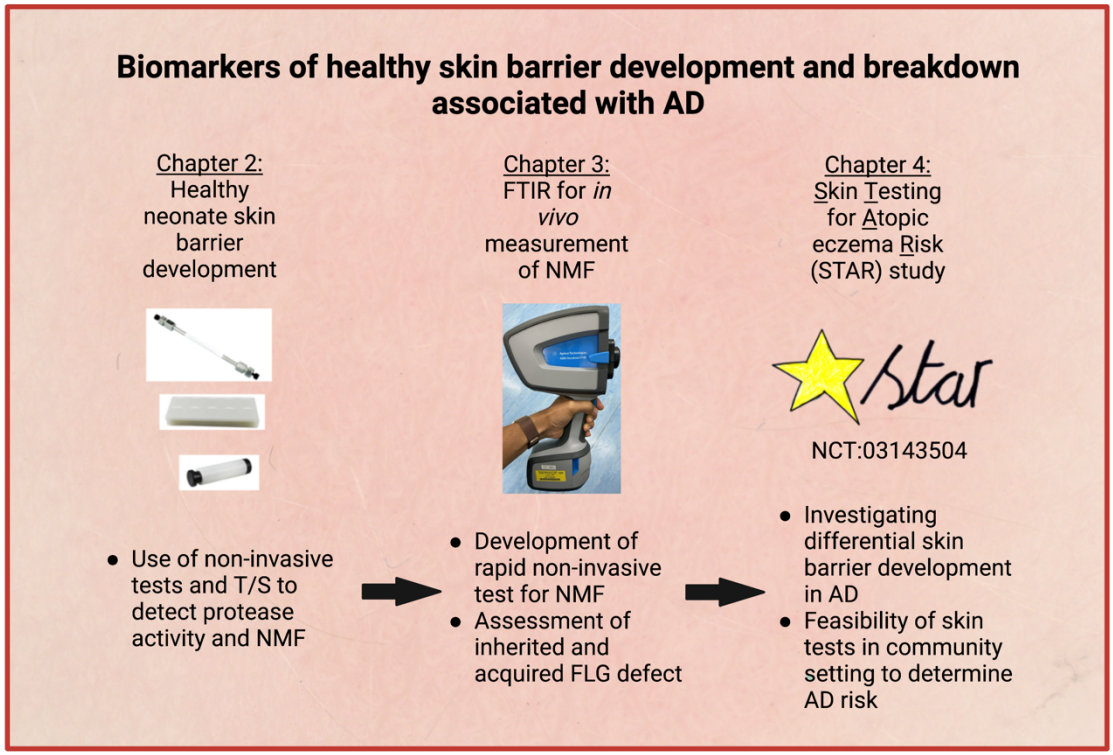


**Figure 1.9:** Biomarkers of skin barrier development and breakdown associated with AD. Healthy skin barrier development was initially assessed over 4 weeks by non-invasive measures and tape stripping (Chapter 2). The logistic and practicality issues raised led to the development of FTIR methodology for the measurement of NMF in adults (Chapter 3) that was piloted from birth to track skin barrier development and breakdown longitudinally (1 year) in relation to AD development (Chapter 4).

# CHAPTER 2: DEVELOPMENT OF STRATUM CORNEUM CHYMOTRYPSIN-LIKE PROTEASE ACTIVITY AND NATURAL MOISTURISING FACTORS FROM BIRTH TO 4 WEEKS OF AGE COMPARED TO ADULTS

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***What's already known about this topic?***

From birth the acidification and hydration of the infant stratum corneum to adult levels suggests transitory mechanisms underlying differentiation and desquamation.

***Study aims***

To assess the feasibility of non-invasive skin tests and tape stripping to better understand healthy neonate skin barrier development.

***Study objectives***

To perform a longitudinal cohort study with measurements performed at birth and repeated at 4 weeks to monitor the biophysical (TEWL, SCH, pH) and biological (protease activity, NMF) properties of the developing neonate SC.

***What does this study add?***

Superficial chymotrypsin-like protease activity and natural moisturising factors (NMF) increase from birth to 4 weeks of age and differ to adults. Impaired barrier function at birth is accompanied by elevated chymotrypsin-like protease activity and reduced NMF, highlighting why certain infants are predisposed to epidermal barrier breakdown and the development of atopic dermatitis (AD).

***What is the translational message?***

Our data reinforce the need for infant skincare regimens from birth that protect and support normal barrier development. Targeted skincare strategies to ameliorate heightened chymotrypsin-like protease activity and low NMF may be an important preventative measure for neonates at increased risk of developing AD.

## AUTHOR CONTRIBUTIONS

This chapter reports a collaborative project between the University of Sheffield and the University of Manchester and presents the author-accepted version of the manuscript published in the British Journal of Dermatology (2016) DOI 10.111/bjd.14568. Skin samples collected from infants recruited by the Oil in baby SkincaRE (OBSeRvE) study conducted in Manchester were used to assay protease activity and NMF during the neonate period. In conjunction with biophysical measurements collected from infants randomized to the "no treatment" arm of the trial, skin barrier development from birth could be monitored and reported by this manuscript. For comparison, a mechanistic study of the skin barrier in healthy adults was conducted in Sheffield to place the infant measurements into context.

*Study conceptualisation*: JC/SD; *Methodology*: JC/SD/MJC; *Data collection*: AC/JC/AW/KB; *Formal analysis*: JC; *Project administration*: AC/JC/SD; *Supervision*: SD/MJC/SV/TL; *Funding acquisition*: SD/MJC/AC/TL.

JC authored the manuscript and was responsible for all stages of submission through to acceptance.

**ABSTRACT**

From birth, the functional properties of the neonatal epidermal barrier mature whereby the stratum corneum (SC) hydrates and the skin surface acidifies. The identification of a thinner infant SC compared to adults suggests underdeveloped mechanisms underlying differentiation and desquamation. The aim of this study was to assess the functional properties of the neonatal SC from birth, in conjunction with the quantification of superficial chymotrypsin-like protease activity and filaggrin-derived natural moisturising factors (NMF). A total of 115 neonates recruited to the oil in baby skincare (OBSeRvE) randomised controlled trial underwent a full evaluation of the SC at birth (<72 hours old) and at 4 weeks of age ($n$=39, no oil control group) using minimally invasive instrumentation and methodology. A cohort of 20 unrelated adults was recruited for comparison. At birth NMF levels correlated with SC hydration ($r$=0.50) and skin-surface pH ($r$=-0.54). From birth to 4 weeks, transepidermal water loss (TEWL), superficial chymotrypsin-like activity and filaggrin-derived NMF significantly elevated. Impaired epidermal barrier function at birth (>75th percentile TEWL) was accompanied by significantly elevated chymotrypsin-like protease activity and reduced levels of NMF. In conclusion, the biophysical, biological and functional properties of the developing neonatal SC are transitional from birth to 4 weeks of age and differ significantly to adults. The presence of impaired barrier function with elevated chymotrypsin-like protease activity and reduced NMF at birth suggests why certain infants are predisposed to epidermal barrier breakdown and the development of atopic dermatitis (AD).

## INTRODUCTION

The developing infant epidermal barrier demonstrates significant structural and functional immaturity as it transitions to adult-like status throughout the first year of life (244). For example from its sub-optimal condition reported at birth, the neonatal stratum corneum (SC) rapidly hydrates and the skin-surface acidifies to adult levels by around day 28 (92, 98, 245-247). The application of novel methodology to infant skin research has revealed a 30% thinner, more disorganised SC with increased cell turnover, characterised by smaller, poorly defined, irregular corneocyte clusters and non-uniformly distributed corneodesmosome artifacts compared to adults (89, 90). Considering the significant influence of SC hydration and pH on epidermal barrier homeostasis, (76, 78, 248) these observations combined suggest that infant mechanisms of differentiation and desquamation are either underdeveloped or poorly regulated.

It is perhaps of no coincidence that this potentially vulnerable transitional period of infant epidermal barrier maturation coincides chronologically with the onset (<1 year of age) of skin manifestations such as atopic dermatitis (AD); (238, 239) an inflammatory disease arising from mechanisms of epidermal barrier breakdown exacerbated by negative environmental triggers (185). One such example of a potential unexplored, negative environmental stressor on normal, full-term infant epidermal barrier maturation is the use of natural oils to treat dry skin; a practice routinely recommended by midwives despite the absence of supporting clinical evidence (249). To this end, the recently published OBSeRvE (Oil in Baby SkincaRE) randomised controlled trial investigated the effect of natural oils on the infant SC throughout the

first month of life (250). Using this valuable OBSeRvE study birth cohort, an opportunity arose to perform an ancillary study evaluating the biophysical and biological properties of the neonatal epidermal barrier at birth in a substantial number of subjects (*n*=115), with repeat measurements pursued at 4 weeks in the no oil control group (*n*=39) to monitor its early development and investigate early signals of barrier breakdown during this critical period. Of particular interest, superficial chymotrypsin-like protease activity and the level of filaggrin-derived NMF was quantified *ex-vivo* to elucidate their role in desquamation maturation and the development of infant barrier function. In an effort to put the infant results obtained into context, a comparison to an unrelated, healthy adult cohort is presented. Finally, using elevated TEWL at birth as a predictive factor for the development of AD by 1 year, (102) an exploratory analysis was performed to investigate the relationship between desquamatory chymotrypsin-like protease activity and NMF with impaired barrier function in neonates.

## MATERIALS AND METHODS

OBSeRvE study birth cohort

A total of 115 healthy, full term (≥$37^{+0}$ weeks gestation) neonates were recruited at Saint Mary's Hospital, Central Manchester NHS Foundation Trust, between September 2013 and June 2014 in accordance with the main Oil in Baby SkincaRE (OBSeRvE) pilot, assessor-blinded, randomised controlled trial protocol (250). Ethical approval for the OBSeRvE study was provided by the Greater Manchester East Research Ethics Committee (13/NW/0512). Infants randomised to the no oil control group represented the returning infant cohort at 4 weeks of age ($n$ = 39). All infant assessments were performed at Saint Mary's Hospital shortly after birth (<72 hours old) before discharge from the postnatal ward and repeated at 4 weeks of age.

Healthy adult cohort

An unrelated cohort of adults with healthy skin ($n$=20) was recruited from the local community between January and April 2015 by Sheffield Dermatology Research, at the University of Sheffield, UK. Volunteers in this cohort had no medical history of skin conditions or atopy and refrained from using any topical products for at least 7 days prior to the single assessment day. The NHS Trent Multicentre Research Ethics Committee approved this study component (04/MREC/70).

Sample size

The infant cohort size for the OBSeRvE study (242) was set at 100 to allow 30 babies per intervention group accounting for a 10% loss-to-follow up. This was considered sufficient for a feasibility trial of this nature. For the unrelated cohort of adults, twenty

participants were recruited for comparison to infant skin as reported by Fluhr *et al* by cross sectional study design (92).

Biophysical assessment of the epidermal barrier

Study sites for both cohorts were defined as: 1) the left volar forearm, midway between the antecubital fossa and the wrist; and 2) the left thigh, midway between the patella and groin. The biophysical properties of the infant epidermal barrier were assessed as previously reported by the OBSeRvE study (250). Healthy adult assessments were conducted at Sheffield Dermatology Research in room conditions maintained at 20.60 ±0.62°C, and 35.71 ±6.51% relative humidity following an initial acclimatisation period of 20 minutes. A series of minimally invasive techniques were employed for assessment including: a single Transepidermal water loss (TEWL) measurement using an AquaFlux AF200 condensing chamber probe (Biox Systems Ltd., London, UK); skin-surface pH and capacitance measurements performed in triplicate (CK electronic GmbH, Cologne, Germany); and tape-stripping – the application and removal of 3 consecutive D-squame discs from a single site using a plunger to consistently apply a standard 225 g/cm$^2$ of pressure to each disc (CuDerm, Dallas, USA). Infrared densitometry using a SquameScan 850A (Heiland electronic, Wetzlar, Germany) was utilised to quantify the mass of SC removed by tape-stripping (251). Following infrared densitometry, all D-squames collected were stored at -80°C before further analysis.

Determination of chymotrypsin-like protease activity and natural moisturising factor levels

Superficial chymotrypsin-like protease activity was assayed *ex-vivo* (156, 252) from pooled, forearm-collected D-squame discs 1-3 using substrate MeOSuc-Arg-Pro-Tyr-AMC (Peptide Protein Research Ltd, Southampton, UK). Superficial levels of filaggrin-derived natural moisturising factors (NMF) were quantified *ex-vivo* from pooled, thigh-collected D-squame discs 1-3, by combining o-Phthaldialdehyde derivatization (42) (free amino acids [FAA] see Appendix Figure 6.1, page 164) and High Performance Liquid Chromatography (2-Pyrrolidone-5-carboxylic acid [PCA] and urocanic acid [UCA]) (253). A Shimadzu HPLC system comprising of a LC-20AD XR pump, SIL-20A XR autosampler and SPD-M20A diode array detector (Shimadzu, Kyoto, Japan) combined with Phenomenex Aqua® 5µm C18 125Å column (Phenomenex, Macclesfield, UK) at a flow rate of 0.8 ml $min^{-1}$ was used for analysis. Protease activity (nU / µg), and NMF (the sum of FAA, PCA and UCA [nmol / mg]) were normalised relative to the mass of SC removed by tape stripping. The limit of quantification (LOQ) for this methodology was as follows: protease = 0.0017 nmol / ml 7-Amino-4-methylcoumarin (AMC); FAA = 0.024 nmol / ml; PCA = 0.025 nmol / ml and UCA = 0.005 nmol / ml. Samples falling below the quantification threshold were assigned a value 0.5x the LOQ for statistical analysis.

Data analysis

All data was collated in excel and statistical tests were executed using GraphPad Prism v6.0b (GraphPad Software Inc., La Jolla, USA). Infant and adult means (TEWL, SCH, skin surface pH, SC mass, protease activity and NMF) and quartile means (protease activity

and NMF only) at birth were compared using a 1-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* test. Correlation analysis was performed through calculation of Pearson coefficients (r). The significance threshold was set at $p$=<0.05. All measurements were included for statistical analysis.

## RESULTS

Biophysical properties of the neonatal stratum corneum (SC) are transitional from birth

Table 2.1 presents the fluctuations in transepidermal water loss (TEWL), capacitance and skin-surface pH that occur on the neonatal forearm SC from birth through to 4 weeks of age. Of the 115 neonates recruited, 98% had no visible vernix caseosa (VC) present, and 1.7% had minimal coverage at the first assessment (<72 hours old). No visible VC was present at any of the test sites throughout the study. In newborn infants (<72 hours old), TEWL (12.14 ±2.31g/$m^2$/h) was comparable to adult skin (12.64 ±3.09g/$m^2$/h) suggesting competent epidermal barrier function (inside-out) at birth. Subject age at the point of TEWL assessment ranged from 6.8 hours to 55.18 hours. Correlation analysis revealed no relationship between neonate age and TEWL (Pearson coefficient r=0.04ns data not shown). However, during the first 4 weeks of infant life, TEWL overall significantly increased (25/35 individuals) representative of weakened epidermal barrier function during this period. Capacitance measurements as an indirect assessment of SC hydration, increased significantly from birth (17.66 ±4.55 relative capacitance units [RCU]) through early infancy (41.79 ±9.65 RCU). From birth, the process of skin surface acidification was complete by 4 weeks of age. Newborn infant SC was both drier (-13.81 ±4.55 RCU), and more alkaline (+1.15 ±0.51 pH units) than adult skin. Infrared densitometry confirmed that comparable SC mass (SC cohesion) was removed by tape stripping to 3 discs in each group. These trends in skin barrier development were confirmed by analysing the OBSeRvE untreated group only (*n*=35) that had measurements taken at both timepoints (see Appendix Table 6.1, page 166). A family history of AD is associated with early disease onset during

infancy (254). As 32% of the birth cohort reported a family history of AD (father, mother or sibling with a clinical diagnosis of AD), the influence of this risk factor on the developing epidermal barrier was investigated. Cohort stratification of biophysical measurements according to a family history of AD at birth and at 4 weeks generated no significant differences in our results to report.

| | Birth | Mean difference (95% CI) | Infant (4 wks) | Mean difference (95% CI) | Healthy adult |
|---|---|---|---|---|---|
| **Subjects (*n*)** | 115 | - | 35∞ | - | 20 |
| **Age** | 28.11 (±11.32) Hours | - | 30.7 (±2.35) Days | - | 24.65 (±6.67) Years |
| **Sex (% male)** | 57 | - | 64 | - | 25 |
| **Family history of AD** | 37/115 | - | 13/39 | - | 0/20 |
| **TEWL (g/m²/h)** | 12.14 (±2.31) | 1.23 * (0.05, 2.42) | 13.38 (±3.02) | 0.73 ns (-0.97, 2.44) | 12.64 (±3.09) |
| **SC Hydration (RCU)** | 17.66 (±4.55)§ | 24.13 **** (21.30, 26.97) | 41.79 (±9.65) | 10.32 **** (6.21, 14.43) | 31.47 (±6.90) |
| **Skin-surface pH** | 5.93 (±0.51)§ | 0.94 **** (0.73, 1.16) | 4.98 (±0.34) | 0.20 ns (-0.11, 0.52) | 4.78 (±0.42) |
| **SC cohesion (µg / 3 discs)** | 292.43 (±77.17) | 12.22 ns (-23.47, 47.90) | 304.65 (±88.65) | 29.23 ns (-22.58, 81.04) | 275.42 (±62.45) |

**Table 2.1:** Cohort demographics and the biophysical properties of the developing infant forearm stratum corneum (SC) at birth and 4 weeks of age, compared to adults. Statistical significance was determined using a 1-way analysis of variance (ANOVA) combined with Bonferroni's *post-hoc* analysis. *$p$ = <0.05, ****$p$ = <0.0001, ns: not significant. RCU: relative capacitance units. AD: atopic dermatitis. ∞39 infants were randomised to the OBSeRvE no oil control group, but 4 infants were loss-to-follow-up. A significant difference in SC hydration and skin-surface pH (****$p$ = <0.0001) was also found between birth and adult cohorts. Mean±SD presented.

Development of superficial chymotrypsin-like protease activity and natural moisturising factor (NMF) levels from birth

Superficial chymotrypsin-like protease activity at birth (1.03 ±0.69 nU/μg) was equivalent to that observed in healthy adults (0.84 ±0.53 nU/μg) suggesting that this component of SC desquamation was fully developed (Figure 2.1a). In contrast, Figure 2.1b shows that the level of filaggrin-derived NMF at birth (243.25 ±209.68 nmol/mg) was significantly lower than the healthy adult cohort (1693.26 ±708.86 nmol/mg). In neonates at birth, the level of filaggrin-derived NMF significantly correlated with TEWL ($r$=-0.38), SC hydration ($r$=0.50) and skin-surface pH ($r$=-0.54, Table 2.2). From birth, a significant increase in superficial chymotrypsin-like protease activity (1.70 ±0.93 nU/μg) and NMF levels (2330.25 ±1415.04 nmol/mg) occurred by 4 weeks of age. As was the case previously, this trend was confirmed by analysing the untreated infant cohort only ($n$=35), with 69% (protease) and 96% (NMF) of individuals showing an increase in this group (see Appendix Table 6.1, page 166). With regards to NMF, all components quantified followed this trend of up-regulation (Table 2.3). Infants with a family history of AD showed no significant difference in chymotrypsin-like protease activity or NMF levels at birth and 4 weeks of age to those reported here.

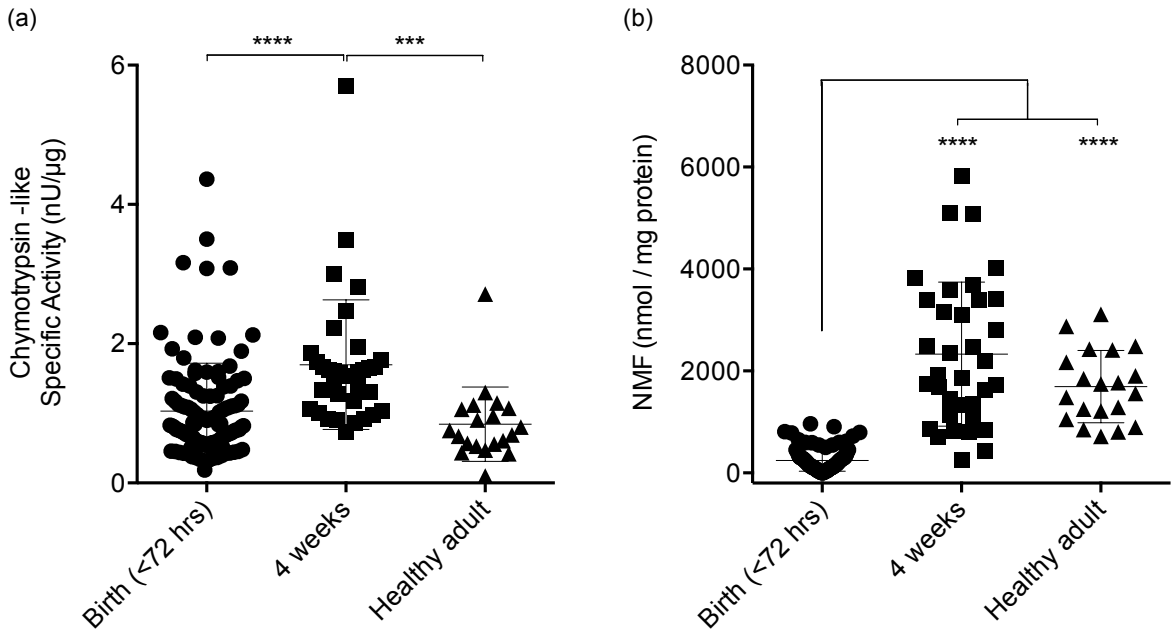


**Figure 2.1:** Development of superficial chymotrypsin-like protease activity and filaggrin-derived natural moisturising factors (NMF) from birth. (a) Quantification of *ex-vivo* chymotrypsin-like protease activity, and (b) NMF levels, from collected D-squames discs in neonates at birth ($n$ = 115), repeated at 4 weeks of age ($n$ = 35) compared to an unrelated healthy adult cohort ($n$ = 20). Significantly elevated chymotrypsin-like protease activity was observed at 4 weeks compared to birth (mean difference: 0.67; 95% Confidence interval [CI]: 0.33, 1.01; ****$p$ = <0.0001) and adults (mean difference: 0.86; 95% CI: 0.36, 1.35; ***$p$ = <0.001). Compared to birth, significantly elevated levels of NMF was observed at 4 weeks (mean difference: 2087; 95% CI: 1759, 2415; ****$p$ = <0.0001) and in adults (mean difference: 1450; 95% CI: 1038, 1862; ****$p$ = <0.0001). A significant difference was also found in NMF between 4 weeks and adults (*$p$ = <0.05). Significance was determined using a 1-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* analysis. Mean ± SD presented.

| | | TEWL ($g/m^2/h$) | SC Hydration (RCU) | Skin-surface pH |
|---|---|---|---|---|
| **Chymotrypsin-like protease activity** | **Birth (*n* =115)** | 0.26 ** (0.08, 0.43) | -0.30 ** (-0.46, -0.12) | 0.25 ** (0.07, 0.42) |
| | **Infant 4wks (*n* =35)∞** | 0.12 ns (-0.22, 0.44) | -0.39 * (-0.64, -0.06) | 0.10 ns (-0.26, 0.40) |
| **Natural moisturising factor (NMF)** | **Birth (*n* =115)** | -0.38 **** (-0.53, -0.21) | 0.50 **** (0.35, 0.63) | -0.54 **** (-0.66, -0.40) |
| | **Infant 4wks (*n* =35)∞** | -0.23 ns (-0.53, 0.11) | -0.10 ns (-0.42, 0.24) | -0.13 ns (-0.44, 0.21) |

**Table 2.2:** Correlation between the biophysical and biological properties of the SC in infants at birth (<72 hours old) and 4 weeks of age. Pearson correlation coefficient (with 95% confidence interval) presented. *$p$ = <0.05, **$p$ = <0.01, ****$p$ = <0.0001, ns: not significant. RCU: relative capacitance units. ∞39 infants were randomised to the OBSeRvE no oil control group but 4 infants were loss-to-follow-up.

| NMF component (nmol / mg) | Birth | Infant (4 wks) | Healthy adult |
|---|---|---|---|
| **Free amino acids** | 205.60 (±175.01) | 1829.08 (±1120.26) | 1408.05 (±538.82) |
| **2-Pyrrolidone-5-carboxylic acid** | 23.57 (±24.01) | 320.80 (±188.08) | 224.44 (±132.43) |
| **Urocanic acid** | 14.09 (±14.65) | 180.38 (±123.93) | 60.78 (±55.78) |

**Table 2.3:** Composition of filaggrin-derived NMF in infants at birth (<72 hours old), 4 weeks of age and healthy adults.

Impaired epidermal barrier function at birth is accompanied by elevated chymotrypsin-like protease activity and reduced levels of NMF

Recently the presence of impaired epidermal barrier function (elevated TEWL) at birth and at 2 months has been presented as a predictive factor for the development of AD by 1 year of age (102). In this study at birth, superficial chymotrypsin-like protease activity and filaggrin-derived NMF correlated with TEWL (r=0.26 and -0.38, Table 2.2), therefore stratification of these biological components according to TEWL was performed as an exploratory analysis to further characterise impaired epidermal barrier function at birth (Figure 2.2). TEWL was grouped as follows: 1-25$^{th}$ percentile ≤10.45g/m$^2$/h; 26-50$^{th}$ percentile = ≤12.14 g/m$^2$/h; 51-75$^{th}$ percentile = ≤13.34 g/m$^2$/h. Figure 2.2 demonstrates that in neonates with the highest TEWL at birth (76-100$^{th}$ percentile, ≥13.35 g/m$^2$/h) there also co-exists significantly elevated chymotrypsin-like protease activity (1.41 ±1.04 nU/μg) and reduced levels of filaggrin-derived NMF (139.80 ±114.40 nmol/mg) compared to individuals within the lower percentiles. Included in this subgroup of neonates with the highest TEWL were five individuals with 118-209% higher chymotrypsin-like protease activity than the group mean (Figure 2.2). Neonates with a family history of AD were present across all TEWL percentile groups in equal proportions.

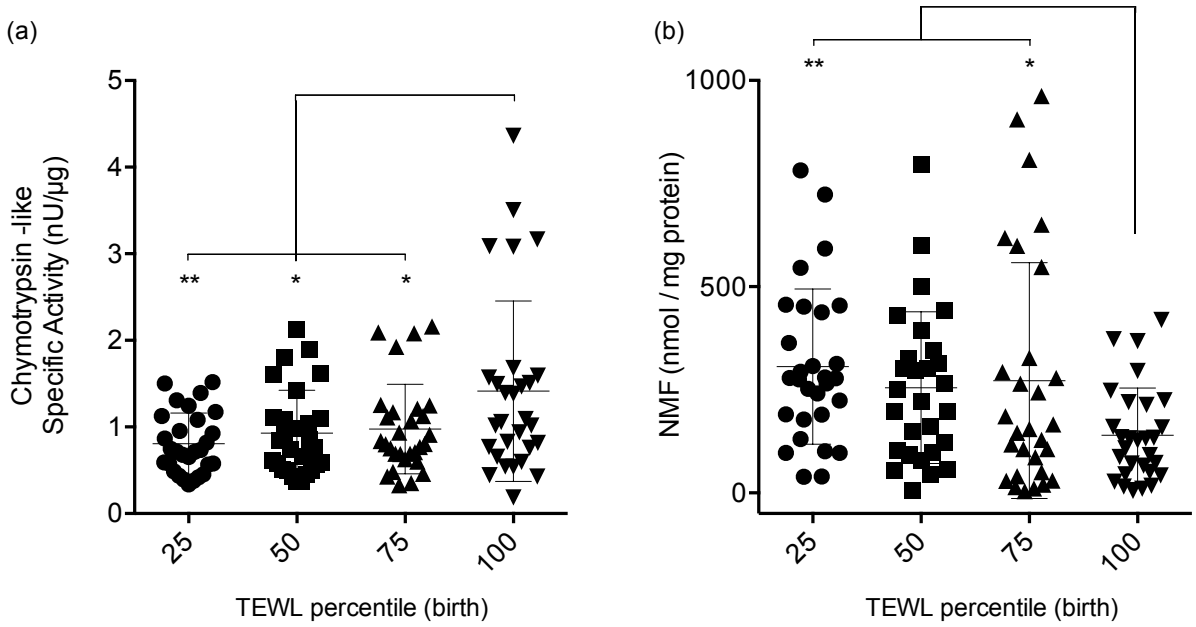


**Figure 2.2:** Impaired epidermal barrier function at birth is accompanied by elevated chymotrypsin-like protease activity and reduced levels of filaggrin-derived NMF. Stratification of superficial (a) chymotrypsin-like protease activity, and (b) NMF levels at birth in accordance with TEWL ($n$ = 115). Co-existing in neonates with the highest TEWL at birth ($n$=29, upper percentile: 76-100$^{th}$) was significantly elevated superficial chymotrypsin-like protease activity and reduced levels of filaggrin-derived NMF compared to the lower percentiles (1-75$^{th}$, $n$=86). Significance was determined using a 1-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* analysis. Mean±SD presented.

## DISCUSSION

Using minimally invasive technology applied to a substantial, full-term healthy birth cohort, this study reports that the biophysical and biological properties of the neonatal epidermal barrier are transitional from birth through to at least 4 weeks of age. During this period where epidermal barrier function declines, SC hydration increases and the skin surface acidifies, an elevation in superficial chymotrypsin-like protease activity (attributed to kallirein-7 [KLK-7]) and filaggrin-derived NMF was also observed. At 4 weeks of age, rather than reaching maturity, chymotrypsin-like protease activity and NMF increased beyond the levels exhibited by healthy adults. Thus, our data supports the view that infant skin is functionally immature compared to adults with undeveloped mechanisms of desquamation and differentiation. This reinforces the need for infant skin care regimens from birth that protect and support normal barrier development (255). Accounting for neonates with a reported family history of AD had no significant effect on our measurements, suggesting that this risk factor does not manifest as impaired barrier function during the first 4 weeks of life. The observation of weakened epidermal permeability barrier function occurring at some point between leaving hospital and 4 weeks following birth, highlights the potential vulnerability of infant skin to environmental stressors and supports the strategy of epidermal barrier enhancement from birth in high-risk individuals as a preventative AD measure (256).

At birth, both the presence of the vernix caseosa (VC) and the active secretion of sebaceous lipids (sebum) at the neonate skin surface have the potential to affect the observations reported by this study. The VC is a protective, hydrophobic layer

comprising of water (80.5%), lipids (8-10%) and proteins (8-10%), (81) reported to be visibly present in around half of neonates at birth (84). Previous studies have identified that VC retention maintains SC hydration and supports acid mantle development, (82) contributed to in part by providing a source of free amino acids to the superficial SC (83). A complementary role for the VC in neonatal epidermal barrier maturation is an intriguing topic, but as no visible vernix was observed at the test sites in neonates participating in our study at the point of assessment (<72 hours), no exploratory analysis to address this question could be performed. Sebum levels on the forehead rapidly increase following birth (245) more markedly in females, (257) thought to result from a flood of maternal androgens during labour (258). Experimental work in a murine model demonstrated that the topical application of sebum proves detrimental to structural surface SC lipids, subsequently elevating TEWL, reducing hydration, and initiating a proinflammatory cascade (259). An interesting question therefore remains as to the effect of excess surface sebum on the infant epidermal barrier that warrants further investigation.

The status of epidermal barrier function at birth in healthy, full-term neonates compared to adults remains inconclusively resolved. For example, reported in the literature are independent studies demonstrating reduced, (93) equivalent (92) or elevated (94) TEWL at birth compared to adults when measured using open-chambered evaporimeters. The results presented here using a closed chamber-condenser system suggest neonatal epidermal barrier function in healthy, full-term neonates is competent when assessed throughout the immediate days following birth. One interesting aspect of our study was that forearm TEWL increased significantly in

our infant cohort from birth during the early weeks of life, an observation replicated by larger studies, (102) and at different anatomical sites such as the thigh and buttocks (93). This elevation in TEWL seemingly persists longitudinally towards the second year of life as the barrier matures to adult like status (260).

One potential mechanism of weakened epidermal permeability barrier function at 4 weeks of age is the concomitant increase in chymotrypsin-like activity reported from birth by this study. In skin diseases such as AD whereby a thinner SC signals epidermal barrier breakdown, impaired permeability barrier function co-exists and correlates with hyperactive desquamatory protease activity (156, 252). Therefore, the increase in chymotrypsin-like activity at the surface of the developing neonatal epidermal barrier could provide a valid explanation for the elevated TEWL, structural differences and immature desquamatory mechanisms observed in infants compared to adult skin (89, 90, 260). Considering the regulatory effect of SC pH and hydration on desquamatory proteases, (76, 156) the authors' hypothesised a period of protease activity maturation occurring in conjunction with the normalisation of barrier function to adult levels during the first 4 weeks of life (92, 98, 245-247). In contrast to this, surface chymotrypsin-like activity at birth was found to be already mature, and its significant rise beyond adult levels over the period studied occurred independently from the acidification and hydration of the SC. Moving forward, the mechanisms underlying elevated SC protease activity throughout this neonatal period requires further investigation. Furthermore, in the interest of a more complete picture of neonatal desquamation maturation, the activity of additional proteases such as kallikrein-5 (KLK-5) requires clarification (55). The quantification of KLK-5 was not possible under

the remit of this study. This was due to a paucity of available samples for laboratory analysis, as a consequence of the ethical restrictions applied to tape stripping in neonates.

Within the SC, a pool of NMF derived from filaggrin proteolysis, maintains barrier function through its hydrating and acidifying properties; (6) a mechanism confirmed by this study in neonates through correlation analysis at birth. It is therefore perhaps not unexpected that the observed increase in SC hydration and skin-surface acidification from birth to 4 weeks of age was accompanied by a significant 9-fold rise in NMF reported here on the infant thigh. Generation of NMF is regulated by environmental humidity, and the transitioning from *in utero* to a drier, terrestrial environment at birth, signals the activation of filaggrin proteolysis within the neonatal SC (248). As the latter stages of cornification proceeds, NMF production is indirectly dependent on KLK-7 activity through caspace-14 activation, (261) providing yet another potential insight into the concomitant rise in chymotrypsin-like activity from birth. But our NMF findings at the infant SC surface are not uniformly replicated in the literature. For example, in consensus with this study are Visscher and colleagues, who demonstrated a significant rise in free amino acids from birth when quantified *ex-vivo* from tape strips in infants at 1 month of age (83). Fluhr *et al*., (92) using raman confocal microscopy to quantify PCA, serine, glycine, histidine, lactic acid, urea and trans-UCA, identified a similar trend but at up to a SC depth of 5μm only. Interestingly bulk profiling performed by the same authors uncovered a greater NMF pool in neonates aggregating at 5-25μm depth (92). Once again this could reflect an inhibitory action of the prenatal environment on the developing profilaggrin-filaggrin-NMF pathway

leading to the accumulation of NMF within the lower SC layers in neonates. In disagreement with the significant rise in surface NMF levels from birth presented here is an investigation utilising attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) methodology to quantify free amino acids, urea, lactic acid, PCA (93). Here the authors declare no significant NMF differences in infants compared to adults but provide no descriptive results as supporting evidence for this conclusion.

Using elevated TEWL at birth as a predictive factor for the development of AD by 1 year, (102) presented here is the suggestion that altered superficial chymotrypsin-like activity and low NMF are early signals underlying epidermal barrier breakdown in predisposed individuals at birth. Of course it is entirely possible that this observation reflects the greater presence of *FLG* mutations in this sub-cohort of neonates with elevated TEWL; (179, 262) a point we could not address due to the omission of DNA sample collection by this study. Nevertheless, although undoubtedly significant in disease pathogenesis, *FLG* loss-of-function mutations do not provide the full mechanistic insight of epidermal barrier breakdown in AD as neatly demonstrated by Kelleher and colleagues (102). One limitation of this study is that our exploratory analysis is purely speculatory, and no follow up of infants to determine a clinical diagnosis of AD was sought due to the limited cohort size. Only subsequent, well designed longitudinal feasibility studies can provide a definitive insight into the questions raised by this investigation such as the pathogenic relationship between protease activity, NMF and AD onset in infancy. Thus, in an era of AD management whereby intervening or modifying the natural course of the disease is a primary aim, clinical strategies aimed at ameliorating these identified early mechanisms of barrier

breakdown may prove a valuable preventative measure in neonates at increased risk of developing AD (236, 243).

Valuable lessons were learned through conducting the OBSeRvE study. For example, it was noted there is limited space on the neonate forearm for SC sampling by tape stripping. As Fourier Transform Infrared (FTIR) spectroscopy is a suitable tool for the *in vivo* measurement of NMF, a logical next step would be to develop and validate this methodology for use in clinical studies. A benchtop FTIR device was also found to be inflexible and impeded recruitment due to the requirement of moving families from the maternity ward to the study room. In response to this a portable FTIR device was introduced for future work.

**ACKNOWLEDGEMENTS**

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# CHAPTER 3: INFRARED SPECTROSCOPY FOR THE *IN VIVO* MEASUREMENT OF NATURAL MOISTURISING FACTORS DISCRIMINATES BETWEEN CLINICAL PHENOTYPES IN ATOPIC DERMATITIS

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***Study aims***

To develop and validate a novel portable methodology for the rapid *in vivo* assessment of NMF at the skin surface.

***Study objectives***

To perform a cohort study in adult participants with and without AD in order to:

- Use chemometrics to model NMF from absorbance spectra collected using FTIR spectroscopy.
- Simulate known scenarios of reduced NMF abundance in the skin to compare the FTIR technique to more established laboratory methodology.

## AUTHOR CONTRIBUTIONS

This chapter presents the author-approved version of a manuscript ready for submission to a Dermatology journal. It is a methodology paper detailing the use of FTIR spectroscopy for the rapid measurement of NMF.

*Study conceptualisation*: JC/SD/MJC; *Methodology*: JC/SD; *Data collection*: JC; *Formal analysis*: JC; *Project Administration*: JC/SD; *Supervision*: SD/MJC; *Funding Acquisition*: SD/MJC.

JC authored the manuscript and was responsible for writing and submitting an ethics application to the University of Sheffield Research Ethics Committee.

**ABSTRACT**

The relative abundance of skin-derived Natural Moisturising Factors (NMF) is indicative of Filaggrin (FLG) genotype, Atopic Dermatitis (AD) severity and the condition of the permeability barrier. They are routinely analysed by *ex vivo* laboratory assay, however this is a time consuming and labour-intensive process. As an alternative, this study evaluated an *in vivo* infrared spectroscopic method for the rapid measurement of NMF in subjects with AD or healthy skin. Chemometric modelling of NMF by Partial Least Squares (PLS) regression calibrated absorption in the fingerprint spectral region obtained using a portable Fourier Transform Infrared (FTIR) device against quantitative NMF obtained by standard *ex vivo* laboratory analysis. The four common European *FLG* loss of function (LOF) mutations were screened. Acceptable PLS model accuracy was noted for both calibration ($R^2$=0.73) and validation ($R^2$=0.70) data sets. Cohort stratification revealed a clinically relevant deficiency in modelled NMF at the antecubital fossa in AD and *FLG* LOF mutation carriers. Receiver Operating Characteristic curves supported this discrimination of clinical phenotypes confirming suitability for assessing the inherited and acquired FLG defect associated with AD. Independent replication of these preliminary findings is required, but the rapid, portable, non-destructive nature of this methodology makes it suitable for any clinical setting.

## INTRODUCTION

The quantification of Natural Moisturising Factors (NMF) is of value to scientists and clinicians around the world with an interest in Atopic Dermatitis (AD) and cosmetic research alike. Synthesised in the lower Stratum Corneum (SC) through the catabolism of Filaggrin (FLG) monomers during terminal differentiation, the predominant components of NMF – free amino acids (fAA), pyrrolidone carboxylic acid (PCA) and the less abundant urocanic acid (UCA) - are powerful humectants integral to maintaining the physical permeability barrier of the skin (35). Confronted by a comparatively dry environment at the SC surface, these chemicals act to preserve optimal levels of corneocyte hydration, that in turn, maintains skin plasticity, limits water loss and regulates the rate of desquamation (34, 38, 79). In its absence, NMF deficiency is synonymous with xerosis, *FLG* genotype, greater disease severity in AD and the suboptimal functioning of the permeability barrier (39, 170, 225, 263, 264). Recent evidence in children suggests that *FLG* mutation status may predict the degree of skin barrier recovery following 6-weeks topical corticosteroid treatment, highlighting a novel use of NMF quantification in AD (265).

Its extraction from tape strips and subsequent analysis by High Performance Liquid Chromatography (HPLC) is a fully quantitative assessment of NMF *ex vivo*. Although this technique is extensively used, it requires laboratory access and is both time consuming and labour intensive when applied to larger cohorts, thus impeding its widespread use in clinical research. As an alternative, *in vivo* vibrational spectroscopy can assess the molecular composition of the skin to reveal components of its biochemical structure, including NMF (21, 253). Techniques such as Confocal Raman

Microspectroscopy has been employed to discriminate *FLG* genotype, (266) whereas Fourier Transform Infrared (FTIR) Spectroscopy – arguably a more widely accessible technology - is yet to be explored in AD. Here we trial a portable, hand-held FTIR spectrometer as a research tool to rapidly measure NMF by modelling its chemometric absorption profile against quantitative values obtained by *ex vivo* laboratory assay. A preliminary evaluation of the *in vivo* model is achieved by replicating known clinical and environmental scenarios of reduced NMF abundance in the skin.

## MATERIALS & METHODS

### Participants

A cohort study was designed to compare surface NMF levels between volunteers with either AD or healthy skin using *in vivo* FTIR spectroscopy as a novel method of quantification. Volunteers were recruited from the local community of the city of Sheffield, UK between November 2017 and April 2019. A diagnosis of AD was made using the UK working party criteria (267). Healthy volunteers had no history of skin disease. An additional cohort of five healthy volunteers was recruited to investigate the effect of a short water soak (20 minutes, 1ml distilled water warmed to 37°C contained by an open chamber) on NMF. All volunteers were asked not to apply any topical products or shower the morning of the study visit. Ethical permission for this study was granted by the University of Sheffield Research Ethics Committee (uREC ref: 021945) and informed consent was obtained from each participant prior to taking part.

### Sample size

As this was an exploratory, proof of concept study, and no pertinent data on clinically relevant differences was available, no formal sample size calculation was performed. The size of the study was based upon Kezic *at al* (170) that piloted NMF measurement by HPLC as a biomarker of *FLG* genotype and reported a mean difference between wild type and heterozygous *FLG* mutation carriers of 1.14μmol $mg^{-1}$.

### Skin assessments

All skin assessments were performed during a single visit to our dedicated, climate-controlled skin barrier suite located at the University of Sheffield. Room conditions

were maintained at 20±2°C and 38-50% relative humidity. The volar aspect of the forearm and the antecubital fossa were the designated study sites. The Eczema Area and Severity Index (EASI) score was employed as a measure of AD severity. Transepidermal Water Loss (TEWL) was assessed using an AquaFlux AF200 closed chamber condensing device (Biox Systems Ltd, London, UK). Skin capacitance was measured using a Corneometer CM825 probe (CK electronic GmbH, Cologne, Germany). Volunteers acclimatised to the room conditions for 20 minutes prior to assessment.

Infrared Spectroscopy

A portable 4300 Handheld Fourier Transform Infrared (FTIR) spectrometer with mercury cadmium telluride detector (Agilent Technologies, Santa Clara, USA) was equipped with a 3-bounce / 2-pass diamond Attenuated Total Reflectance (ATR) accessory to collect absorption spectra at the skin surface in the mid infrared region from 32 scans at 4$cm^{-1}$ resolution.

NMF laboratory analysis *ex vivo* by tape stripping

Adapted from a published assay, (253) SC collected by tape stripping the skin surface (22mm discs, ts1-3, 6 discs in total per sampling data point, see Figure 3.1) was cut and pooled in 750µl methanol. Samples were then subjected to an ultrasonic bath (20 mins) agitated at 4°C (20 mins) filtered using a 0.2µm syringe filter and dried. Distilled water (200µl) was used to resuspend samples before analysis. Isocratic elution of pyrrolidine carboxylic acid (PCA peak at 210nm) and urocanic acid (UCA peak at 270nm) was performed in 0.1M phosphate buffer (pH 2.75) containing 1% acetonitrile

using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with Synergi Hydro RP column (Phenomenex, Macclesfield, UK). 25µl of sample was injected in duplicate. The same extract was used to quantify free amino acids (fAA) by o-phthalaldehyde derivatization in duplicate (see Appendix Figure 6.1, page 164). Quantification of each NMF component was achieved by standard curve interpolation. The sum of all NMF components was calculated (tNMF) and normalised relative to the amount of SC removed by tape stripping (251).

FLG genotyping

Genomic DNA was extracted from buccal swabs using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The four common European mutations were screened by Taqman (R501X and 2282del4) or sequencing (R2447X and S3247X) using established primer and probe sets (268).

Chemometric modelling

To confirm regions of IR absorption by NMF components *in vitro*, chemicals were purchased from Sigma (Merck Life Science UK Ltd., Dorset, UK) dissolved in water at the following mol%: Serine 31%; Glycine 16%; PCA 13%; Histidine 8%; Citrulline 6%; Ornithine 6%; Threonine 6%; UCA 4%; Arginine 3%; Alanine 3%) and analysed using the same spectrometer. For the *in vivo* quantification of NMF by FTIR, Partial Least Squares (PLS) regression modelling using the chemometrics software package Microlab Expert (Agilent Technologies, Santa Clara, USA) was employed to calibrate infrared absorption across the fingerprint spectral region (1090-1653cm$^{-1}$) against quantitative tNMF. For each volunteer, four sampling data points were entered into the model, split

equally into calibration and validation sets (Figure 3.1). Four spectral repeats were averaged for each individual sampling data point. Prior to modelling all spectra were normalised relative to Amide III at 1245cm$^{-1}$ (269).

Statistical analysis

All study data was collated in Excel. An unpaired student's t test was used to compare means (TEWL, SCH, SC mass, *ex vivo* and *in vivo* modelled NMF) between clinical groups. The coefficient of determination assessed the linear regression model fit of *ex vivo* and *in vivo* NMF. Discrimination of AD phenotype and *FLG* LOF genotype by *in vivo* modelled NMF abundance was explored using binary logistic regression and Receiver Operating Characteristic (ROC) curve. All tests were performed using GraphPad prism 9 (San Diego, California, USA).

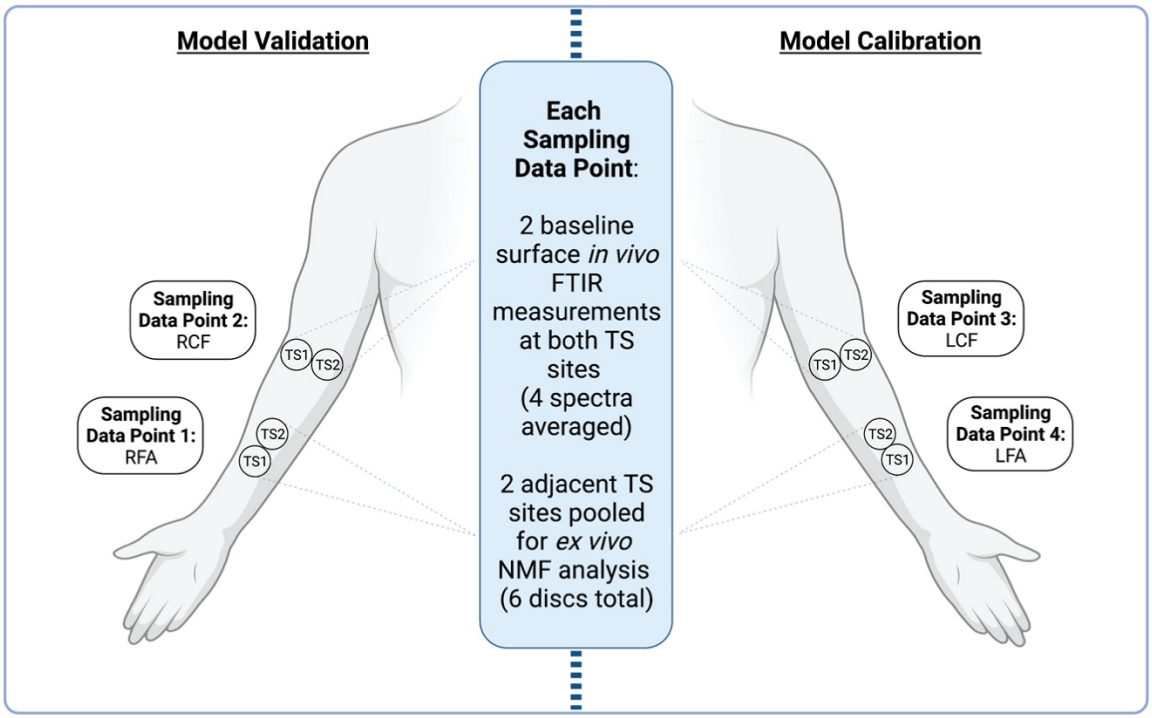


**Figure 3.1:** Overview of the model build. TS: tape stripping site (discs 1-3 collected); RCF/LCF: Right/left antecubital fossa; RFA/LFA: Right/left forearm. FTIR: Fourier Transform Infrared Spectroscopy.

## RESULTS

A total of 26 participants with healthy skin (*n*=15) or AD (*n*=11) were recruited and completed the single study visit (Table 3.1). On average, all three components of NMF (fAA, PCA and UCA) quantified by *ex vivo* laboratory analysis from tape strips (discs 1-3) were significantly reduced in the AD group compared to healthy skin (Table 3.1). No significant differences in Transepidermal Water Loss (TEWL) or capacitance (a measure of SC hydration) were observed between groups indicating comparable skin permeability barrier function. This can be attributed to mild disease in the AD cohort (2/11 individuals with active disease) and an even distribution of *FLG* loss-of-function (LOF) mutations in each group.

| | Healthy | AD | *p* value |
|---|---|---|---|
| ***n*** | 15 | 11 | |
| **Age (years)** | 37 ±14 | 36 ±13 | - |
| **Sex (% female)** | 66 | 45 | - |
| **[1]TEWL (g/m$^2$/hr)** | 13.17 ±3.05 | 14.52 ±4.21 | 0.37 |
| **[1]Capacitance (units)** | 33.71 ±6.83 | 29.48 ±7.12 | 0.14 |
| **[2]EASI score** | - | 2.53 ±0.39 | - |
| **[3]*FLG* LOF (%)** | 3/15 (20) | 4/11 (36) | - |
| *R501x / wt* | *1/3* | *1/4* | |
| *2282del4 / wt* | *1/3* | - | |
| *R2447x / wt* | *1/3* | *1/4* | |
| *S3247x / wt* | - | 1/4 | |
| *2282del4 / R2447x* | - | 1/4 | |
| **[1+]tNMF (µmoles mg$^{-1}$)** | 1.28 ±0.67 | 0.77 ±0.25 | **0.02** |
| **[1+]fAA (µmoles mg$^{-1}$)** | 1.05 ±0.53 | 0.66 ±0.22 | **0.04** |
| **[1+]PCA (µmoles mg$^{-1}$)** | 0.18 ±0.11 | 0.08 ±0.03 | **0.01** |
| **[1+]UCA (µmoles mg$^{-1}$)** | 0.05 ±0.03 | 0.03 ±0.01 | **0.03** |
| **[4]SC mass (mg$^{-1}$)** | 0.47 ±0.08 | 0.45 ±0.05 | 0.63 |

**Table 3.1:** Study cohort characteristics. Mean ±SD presented [1]Averaged across all sampling data points per person (see Materials and Methods); [2]Whole body EASI score averaged from two individuals with active AD; [3]Carrying at least one *FLG* LOF allele with specific genotypes listed below; [4]Cumulative mass of SC removed by tape stripping (discs 1-3) determined by densitometry averaged across all sampling data points; [+]*ex vivo* laboratory quantification of fAA: free amino acids; PCA: pyrrolidone carboxylic acid; UCA: urocanic acid from tape strips (discs 1-3). tNMF is the sum of these three components.

Model calibration and validation

Using a FTIR device to collect spectra from the skin surface *in vivo*, a PLS chemometric model was built to calibrate absorption across the fingerprint spectral region (1090-1653cm$^{-1}$) against quantitative *ex vivo* tNMF obtained by tape strip laboratory analysis. All spectra were normalised relative to Amide III at 1245cm$^{-1}$ prior to modelling, albeit similar model outputs were obtained by normalisation at 1640cm$^{-1}$ and 1540cm$^{-1}$

corresponding to Amide I and II respectively (see Appendix Table 6.2, page 167). A plot of *ex vivo* versus *in vivo* modelled tNMF is presented in Figure 3.2a and 3.2b. Using a six-factor predictive model, the observed coefficient of determination for both calibration ($R^2$=0.73) and validation ($R^2$=0.70) data sets indicate an acceptable degree of accuracy, with precision (±0.35μmoles $mg^{-1}$) denoted by the root mean square error of calibration (RMSEC). A similar value (±0.33μmoles $mg^{-1}$) was noted for the root mean square error of cross validation (RMSECV). A plot of model loading – the strength of association between wavenumber and predictive factor – shows that absorption across the full spectral region contributes to the NMF model, with peaks at around 1580, 1480, 1400 and 1340$cm^{-1}$ suggestive of a greater influence at these wavenumbers (Figure 3.2c). These regions of interest share similarities with an *in vitro* NMF FTIR spectrum (Fig 3.2c), implying the model is detecting changes in absorption related to NMF and its relative abundance in the skin.

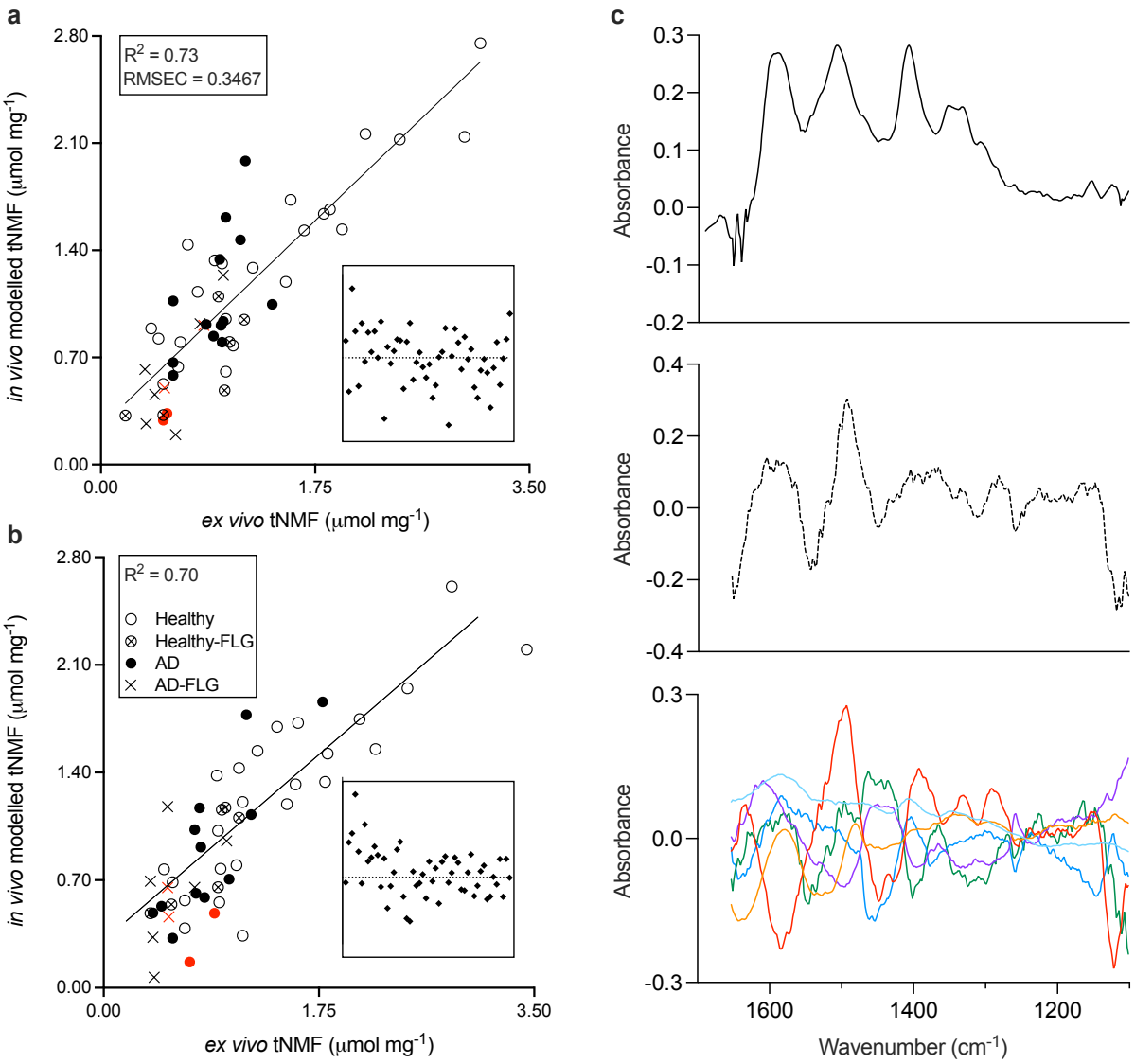


**Figure 3.2:** PLS chemometric modelling of surface NMF in the mid infrared spectral region. (a) Plot of *ex vivo* quantified (discs 1-3 collected by tape stripping) versus *in vivo* FTIR modelled tNMF (the sum of fAA, PCA and UCA) for calibration and (b) validation data sets (see Materials and Methods for further details). $R^2$ = coefficient of determination. RMSEC = Root Mean Square Error of Calibration. Respective residual plot inset. Individuals with active AD are shaded red. (c) Loading plot correlating wavenumber absorption to predictive factor (6 in total, colour coded) with cumulative plot denoted by the dashed line above. Absorbance spectrum of an *in vitro* NMF solution (black solid line) is overlayed for reference.

*Comparing the sensitivity and reproducibility of both methods*

For each method of NMF assessment, a breakdown of each experimental repeat obtained from the validation sampling data points (1 and 2) across the full cohort is presented by Table 3.2. Both methods reliably reported higher tNMF at the antecubital fossa compared to the forearm. Overall, greater intra-measurement variability was associated with the four *in vivo* FTIR repeats. This finding was not unexpected due to sampling differences (two laboratory repeats of one extracted sample compared to four unique FTIR spectra collected) and can be considered an acceptable trade-off for the comparable ease and speed of the FTIR methodology.

| | **Forearm (data point 1) tNMF** | | **Antecubital fossa (data point 2) tNMF** | |
|---|---|---|---|---|
| | ***In vivo*** | ***Ex vivo*** | ***In vivo*** | ***Ex vivo*** |
| **Mean** | 0.90 ±0.53 (0.81) | 0.99 ±0.48 (0.87) | 1.14 ±0.63 (1.08) | 1.16 ±0.76 (0.96) |
| **Repeat 1** | 0.91 ±0.53 (0.83) | 0.99 ±0.48 (0.87) | 1.24 ±0.58 (1.13) | 1.16 ±0.76 (0.96) |
| **Repeat 2** | 0.89 ±0.51 (0.68) | 0.99 ±0.49 (0.88) | 1.22 ±0.64 (1.12) | 1.16 ±0.78 (0.95) |
| **Repeat 3** | 0.87 ±0.49 (0.85) | - | 1.01 ±0.64 (0.96) | - |
| **Repeat 4** | 0.93 ±0.59 (0.82) | - | 1.08 ±0.66 (1.01) | - |

**Table 3.2:** Comparing the sensitivity and reproducibility of both NMF quantification methods. Mean ±SD and (median) of *in vivo* FTIR versus *ex vivo* quantified tNMF for each experimental repeat across the full cohort (*n*=26) at the forearm and antecubital fossa. Repeat 1 = 1$^{st}$ spectrum collected / 1$^{st}$ laboratory repeat; Repeat 2 = 2$^{nd}$ spectrum collected / 2$^{nd}$ laboratory repeat; Repeat 3 = 3$^{rd}$ spectrum collected; Repeat 4: 4$^{th}$ spectrum collected. Repeats collected as part of the validation sampling data points (see Materials and Methods for further details).

Preliminary model evaluation

The model was then verified in three ways by replicating known scenarios of reduced NMF reported in the literature. First, as it is highly soluble in water, NMF was modelled before and after bathing the antecubital fossa, in a small, independent cohort of volunteers (*n*=5). As expected, soaking with water for 20 minutes induced a significant 67% reduction in modelled tNMF (Figure 3.3a). Mean FTIR spectra and mean difference spectra (baseline minus post-soak) revealed changes in absorption related to this decline in NMF at around (1) 1580, (2) 1480, (3) 1400, (4) 1340 and (5) 1280cm$^{-1}$ (Figure 3.3c and d). Next, to investigate the potential clinical relevance of this methodology, the main study cohort (*n*=26) was stratified in two ways to compare healthy skin to AD; and wild type to *FLG* LOF mutation carriers. In line with the results of the water soak, mean FTIR spectra and mean difference spectra obtained from these clinical phenotypes revealed similar patterns in absorption between groups (Figure 3.3e-h). These regions correspond with an *in vitro* NMF absorbance spectrum (Figure 3.3b), reinforcing their relationship with fluctuating NMF abundance in the skin.

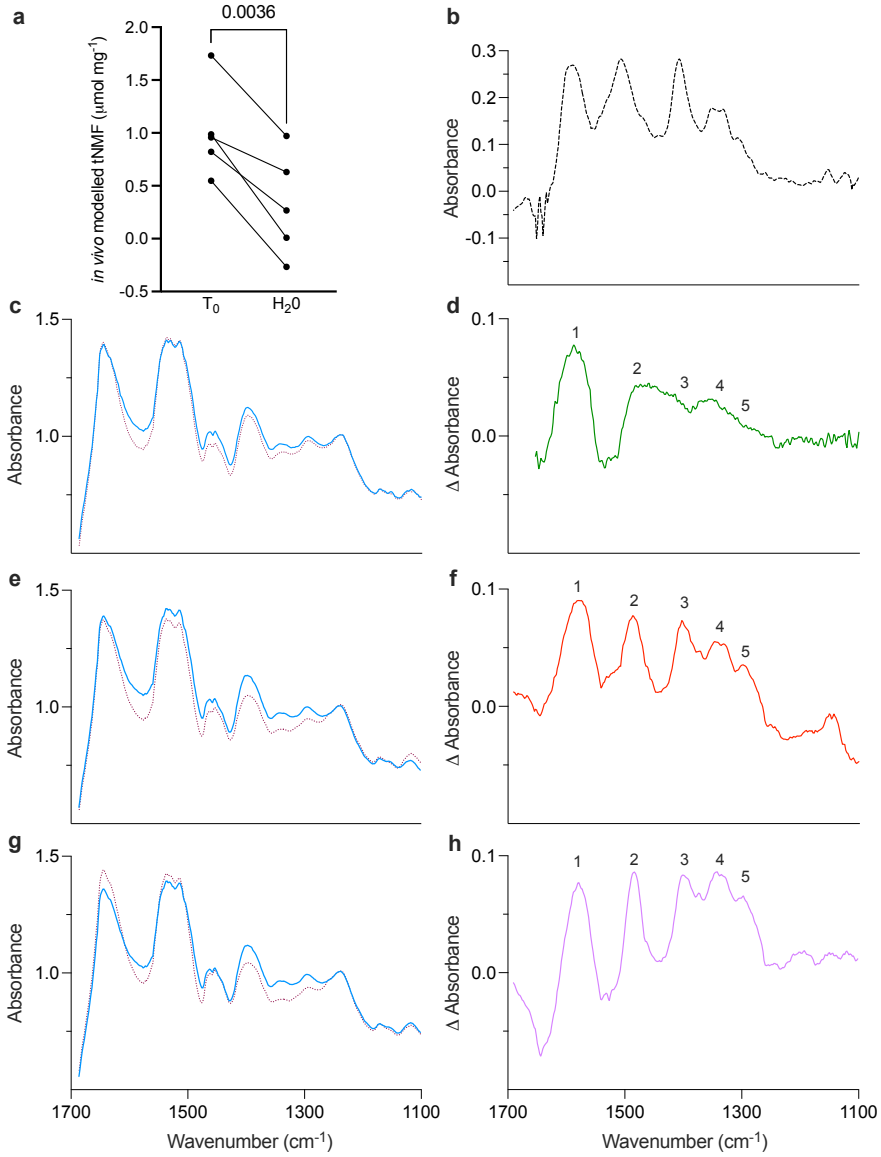


**Figure 3.3:** Correlating spectral regions with NMF abundance. (a) in vivo modelled tNMF before ($T_0$) and after ($H_20$) soaking the antecubital fossa with water (20 minutes) in an additional cohort of five healthy volunteers. A significant reduction in tNMF was observed using a paired students t test (c) Mean FTIR spectra and (d) mean difference spectra ($T_0$-$H_20$) showing the change in absorbance following the water soak. (e) Mean spectra and (f) mean difference spectra (*n*=26) at the antecubital fossa obtained from healthy (blue line) and AD subjects (red dotted line). (g) Mean FTIR spectra and (h) mean difference spectra (*n*=26) at the antecubital fossa obtained from wild type (blue) and *FLG* LOF mutation carriers (red dotted line). Consistent changes in absorption were found at (1) 1580cm$^{-1}$ (2) 1480cm$^{-1}$ (3) 1400cm$^{-1}$ (4) 1340cm$^{-1}$ and (5) 1280cm$^{-1}$ that correlate with an *in vitro* absorption profile of NMF (b).

*Modelled tNMF discriminates between clinical phenotypes*

At the antecubital fossa there was a significant difference between means compared, with modelled tNMF being 0.64µmol $mg^{-1}$ lower in the AD group (Figure 3.4a) compared to healthy skin (-1.07 to 0.21 95%CI) and 0.60µmol $mg^{-1}$ lower in the *FLG* LOF mutation carrier group (Figure 3.4b) compared to wild type (-1.11 to 0.09 95% CI). This discrimination of clinical phenotypes at the antecubital fossa was supported by Receiver Operating Characteristic curve analysis (Figure 3.4a and b lower panels) of *in vivo* modelled tNMF (AD/Healthy: area under the curve 0.81, 95% CI, 0.63-0.99, $p$=0.008; FLG/WT: area under the curve 0.83, 95% CI, 0.66-0.99, $p$=0.01). It should be noted that these findings were not repeated at the forearm with modelled tNMF being 0.20µmol $mg^{-1}$ lower in the AD group compared to healthy skin and 0.17µmol $mg^{-1}$ lower in the *FLG* LOF mutation carrier group compared to wild type (see Appendix Figure 6.3 page 168). This indicates a predilection towards the antecubital fossa for the discrimination of clinical phenotypes in AD. Similar model outputs were obtained by normalisation at 1640$cm^{-1}$ and 1540$cm^{-1}$ corresponding to Amide I and II respectively (see Appendix Table 6.2, page 167).

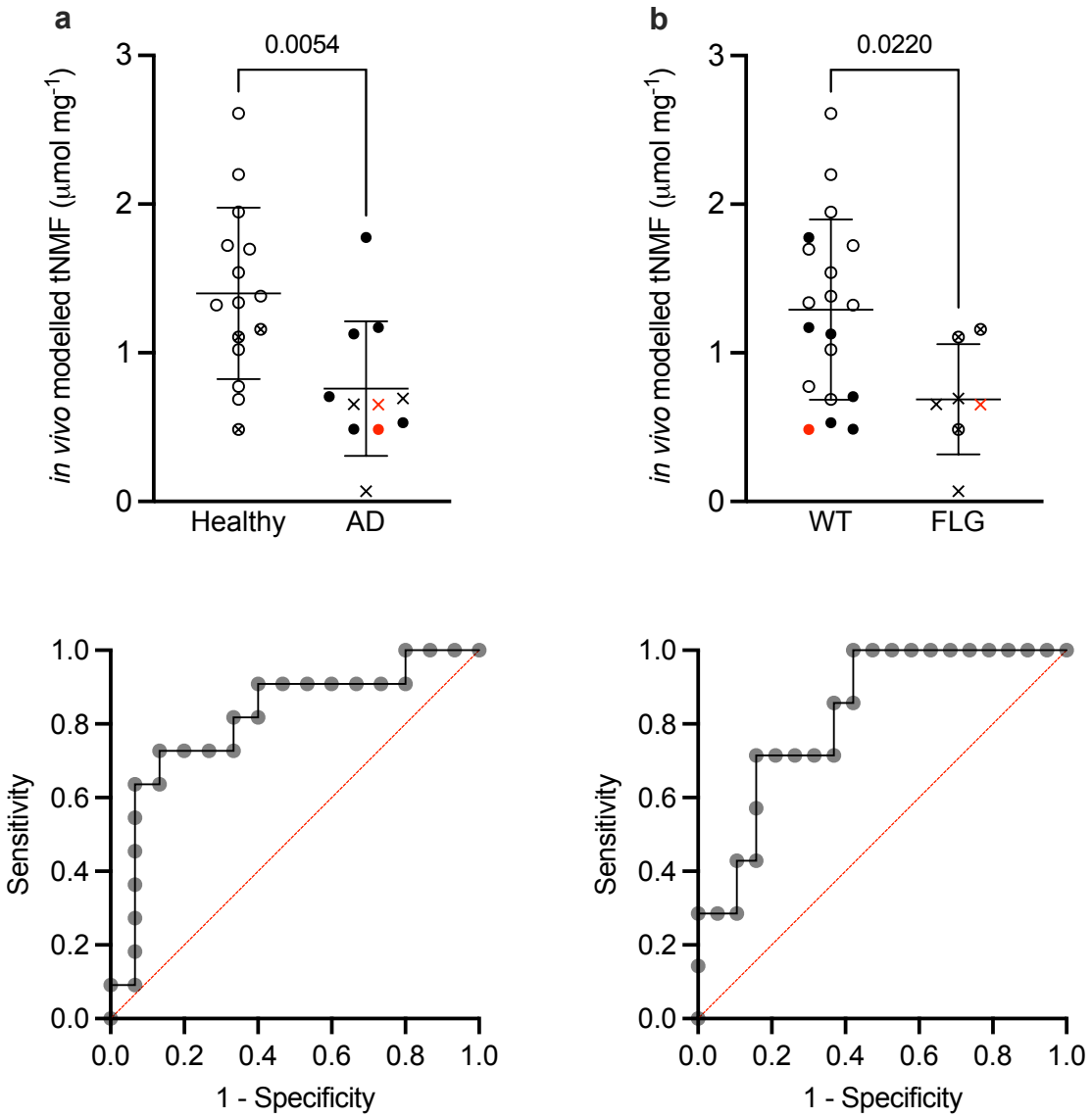


**Figure 3.4:** *In vivo* modelled tNMF discriminates between clinical phenotypes in AD. Cohort stratification (*n*=26) to compare mean *in vivo* tNMF at the antecubital fossa between (a) healthy skin / AD and (b) wild type (WT) / *FLG* LOF mutation carriers. Only the model validation data points are presented (see Materials and Methods for further details). Please refer to Figure 3.2 for key. Individuals with active AD are shaded red. *p* values denote the result of an unpaired students t test. A Receiver Operating Characteristic curve obtained by simple logistic regression of modelled tNMF is presented below the corresponding graph (AD/Healthy: area under the curve 0.81, 95% CI, 0.63-0.99, p=0.008; FLG/WT: area under the curve 0.83, 95% CI, 0.66-0.99, p=0.01).

## DISCUSSION

The *ex vivo* analysis of NMF from tape strips is a fully quantitative, minimally invasive, validated laboratory technique, widely used in skin barrier research. It has been employed to monitor SC development from birth, (83, 270) characterise the unique barrier defect in AD (271, 272) and investigate detrimental environmental exposures to the skin (273, 274). By comparison, relatively few studies have looked towards non-destructive spectroscopic techniques to estimate NMF abundance in the context of disease pathogenesis. Here we provide preliminary evidence to suggest that *in vivo* FTIR NMF measurements are both robust and comparable to the established *ex vivo* technique in its ability to discriminate between clinical phenotypes and assess the inherited or acquired FLG defect associated with AD (170, 225). Considering the study was performed using a portable device that can provide rapid measurements at the skin surface with no sample preparation required, this methodology has the potential to open new avenues of research to any clinical setting when tape stripping is not a feasible option.

By using the full fingerprint region, our study reports key frequencies of IR absorption that are consistent with both the structure and abundance of NMF components in the skin. For example, the 1400cm$^{-1}$ and 1580cm$^{-1}$ wavenumber regions correspond to the symmetric (269) and asymmetric (253) stretching modes of the carboxylate (-COO$^{-}$) functional group present in free amino acids and their derivatives. Another region of interest at around 1480cm$^{-1}$ was also identified by our study that may relate to methylene group ($CH_2$), C-N and $NH_2$ vibrations (275). It cannot be ruled out that this $CH_2$ signal represents both lipid and protein fractions of the SC, but decreased

absorption in this region was observed here after soaking, suggesting the removal of water-soluble components that contribute to its absorption intensity. Interestingly, the 1340cm$^{-1}$ spectral region has been assigned to the hydroxyl group (C-)OH bending mode of serine, (276) the most abundant amino acid in the SC (21).

To evaluate the FTIR methodology, the model output was verified by simulating known scenarios of reduced NMF abundance in the skin. In this regard, comparable results were obtained to the *ex vivo* HPLC assay reporting lower NMF associated with nonlesional AD compared to controls, *FLG* LOF mutations and soaking (170, 225, 277). In line with Raman Spectroscopy, the FTIR NMF measurements were predictive of *FLG* genotype - albeit inferior at patient classification - that may relate to the former's ability to assess NMF across the full SC depth (266). Another contributory factor to the improved sensitivity and specificity of Raman to discriminate genotypes could be its use in patients with greater disease severity; (169) a proven modulator of NMF abundance in the SC that would enhance the primary *FLG* defect (225). When Raman Spectroscopy is used in a similar cohort to ours in that it is free of active disease, a comparable predictive AUC for *FLG* genotype is reported (278). Overall, it can be argued that FTIR offers greater flexibility, as it discriminated at the skin surface using four simple measurements (approximately 1 minute per spectrum) and allows multiple anatomical sites to be quickly and easily assayed by the same device during a single study visit.

One limitation to the accuracy of the *in vivo* FTIR NMF model is the omission of sweat-derived components such as lactic acid and urea from the *ex vivo* laboratory calibration that represents up to 20% of total NMF in the skin (248). These molecules share structural similarities to FLG derived NMF, therefore it is anticipated they contribute to the FTIR absorption at the key frequencies reported here. It may be concluded though in our study cohort at least, that this contribution is minimal, as there was no clear tendency for the *in vivo* model to overestimate NMF at the antecubital fossa, a site more prone to sweating. Although a disease-associated sweating dysfunction has recently been reported in AD, (279) the decision was made to omit lactate and urea from the present study to focus on model evaluation against the more established FLG pathophysiology. A second limitation is that FTIR can only analyse surfaces. This may render the methodology susceptible to subjects washing or applying topical treatments to their skin prior to analysis. As NMF depth profiling with FTIR is only possible in conjunction with tape stripping, this study focused on non-destructive surface measurements in the first instance to maximise its potential as a tool for clinical research. Due to the small cohort size and absence of more severe cases, a third limitation of this study was the inability to replicate the known reduction of NMF by AD severity (225). Overall, as this study reports preliminary findings only, replication with an independent data set is required to work towards unlocking the full clinical translatory potential of the FTIR technique.

By comparing AD skin to healthy controls, an intriguing disease-associated reduction in NMF was noted, suggestive of subclinical inflammation not only in unaffected skin, but in patients with a history of disease that are generally clear of symptoms. This has been evidenced before at a greater SC depth on the forearm, (280) and is supported here by NMF assessment at the antecubital fossa, a site more commonly prone to AD lesions. By tracking this NMF defect longitudinally with disease course in conjunction with further novel measures of subclinical inflammation, (281) the evidence suggests it may be of clinical value for monitoring remission following the successful treatment of clinical disease (211). Another potential utility of the *in vivo* FTIR methodology is related to the knowledge that neonates who later go on to develop AD possess a skin barrier defect long before the onset of clinical disease (282). There is evidence to suggest that low NMF associates with skin barrier breakdown at birth (283). Therefore, as is the case 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. This is one of the research questions addressed by the following chapter describing an observational cohort study that uses skin testing from birth to monitor skin barrier development and AD risk.

**ACKNOWLEDGEMENTS**

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# CHAPTER 4: SKIN BARRIER DEVELOPMENT AND ITS ASSOCIATION WITH EARLY-ONSET ATOPIC DERMATITIS: A LONGITUDINAL COHORT STUDY

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### *Study aims*

To better understand healthy skin barrier development from birth and its breakdown associated with AD over the first year of life.

### *Study objectives*

Use skin testing to perform a longitudinal, observational cohort study from birth in order to:

- Monitor skin health (TEWL) in conjunction with the biological (protease activity, NMF) and biochemical (water and surface lipids by FTIR) properties of the developing infant SC to 12 months of age.
- Identify any differential skin barrier development associated with AD.
- Assess the feasibility of skin testing in a healthcare and community setting to inform on AD risk from birth.

## AUTHOR CONTRIBUTIONS

This chapter reports a collaborative project between the University of Sheffield and the University of Manchester entitled: A longitudinal investigation of skin barrier development from birth and the validation of early predictors of Atopic Eczema risk: the skin testing for Atopic Eczema risk (STAR) study. It is registered on clinicaltrials.gov under project reference NCT03143504. The chapter is in manuscript-drafted form for submission to a Dermatology journal.

*Study conceptualisation*: JC/SD/MJC/AC/TL; *Methodology*: JC/SD/MJC/AC/TL; *Data collection*: JC/LK/AP/KB/AC; *Formal analysis*: JC; *Project administration*: JC/SD/AC; *Supervision*: SD/MJC/AC/TL; *Funding acquisition*: SD/MJC/JC/AC/TL.

JC was a named co-investigator on the project, co-authored the funding proposal and study protocol, was responsible for the REC submission, contributed to study site authorisation / training and authored the manuscript.

**ABSTRACT**

A diagnosis of Atopic Dermatitis (AD) is most common during infancy; however, it is unclear whether differential skin barrier development defines this period and signals disease onset in predisposed individuals. To address this question, a longitudinal observational cohort study was conducted to monitor skin barrier maturation from birth in conjunction with AD development, and a preliminary evaluation of skin measurements for the prediction of disease risk by 12-months was made. Infants were screened for the four common European Filaggrin (FLG) loss-of-function (LOF) mutations. A total of 128 families completed the study and 20% of infants developed mild disease by 12-months of age. Significant changes in skin barrier function measured by Transepidermal Water Loss (TEWL), desquamatory protease activity and molecular composition assessed by Fourier Transform Infrared Spectroscopy (FTIR) were observed longitudinally over the study period, but only subtle evidence of differential skin barrier development was noted between infants that did, and did not develop AD. Common *FLG* LOF mutations were strongly associated with early onset disease and conferred a significant reduction in Natural Moisturising Factors and water content at the skin surface by four weeks of age. Accounting for parental disease, these parameters alongside a greater lipid/protein ratio and reduced Kallikrein-7 activity at birth, were predictive of AD development by 12-months of age. Measured in ambient conditions, TEWL was not associated with disease risk at any stage of the study. These findings suggest that a portfolio of tests used in a healthcare or community setting, has the potential to improve current risk evaluations as to whether a baby will develop AD.

## INTRODUCTION

Skin barrier breakdown is a significant component of Atopic Dermatitis (AD) pathogenesis. Although the uninvolved skin of patients appears clinically healthy, underlying defects in its molecular structure render it functionally inadequate and susceptible to subclinical inflammation that corresponds with disease severity (148, 151). A less ordered lipid structure, protease hyperactivity and low levels of Natural Moisturising factors (NMF) within the Stratum Corneum (SC) are all hallmarks of the barrier abnormality at non-lesional sites (152, 252, 284). These parameters associate with weakened permeability barrier function signified by elevated Transepidermal Water Loss (TEWL).

For reasons unknown the prevalence of AD in younger children is increasing worldwide (285). Infants predisposed to the development AD are not born with clinical signs but are at high risk of a diagnosis before their first birthday (238, 239, 286). Over this time the infant SC is structurally and functionally immature, suggesting an at-risk period of skin barrier fragility as it adapts to a terrestrial environment (90, 260). Considering the pathological evidence from established adult AD, mechanisms of skin barrier breakdown may therefore define a differential developmental trajectory from birth that predates active disease. Modifying factors here may include climate, (287) the home environment and parental skin care practices, but their interaction with the developing skin barrier is unclear.

In a medical era where AD prevention is a key objective, the challenge of pinpointing at risk individuals for early intervention is a troublesome prospect. One of the best predictors as to whether a baby will develop AD is parental atopic disease, but when using this metric for screening it is estimated that around 40% of cases may be missed altogether (243). On the flip side, a significant proportion of infants may receive an unnecessary intervention, burdening new parents when time is scarce and mental health challenged (288). We therefore require additional predictive tools for use in a maternity or community setting to assist in this process of risk evaluation. The use of TEWL for example is one possibility, but this measurement may be unsuitable due to strict environmental requirements (289).

In order to address these research questions, a longitudinal study was designed to better understand skin barrier development from birth, and to pilot non-invasive measures of lipid structure, protease activity and NMF alongside TEWL for predicting AD risk. By improving the early detection of at-risk infants, the introduction of an appropriate medical intervention can be facilitated, that will empower parents to take measured actions from birth to prevent the possible emergence of AD in their baby.

## MATERIALS AND METHODS

### Study Design and setting

A longitudinal observational cohort study was performed to monitor skin barrier development in infants from birth to 4-weeks and 12-months of age (Figure 4.1). By recording the lifetime prevalence of AD, the predictive utility of skin measurements for disease risk by 12-months could be preliminary assessed. Ethical permission was granted by the Preston North West NHS Research Ethics committee (16/NW/0848) and informed parental consent was obtained. The study, entitled - A longitudinal investigation of skin barrier development from birth and the validation of early predictors of Atopic Eczema risk: the skin testing for Atopic Eczema risk (STAR) - was registered on clinicaltrials.gov under reference NCT03143504. This chapter was written in accordance with the STROBE reporting guidelines for observational studies (290).

### 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. A complete list of study eligibility criteria can be found in Table 4.1.

### Sample size

A recruitment target of 180 neonates was considered both sufficient and ethical to explore the primary objective of skin barrier development from birth and assess the feasibility of skin testing in neonates. Assuming between 15-30% of participants

develop AD by 12-months of age, these 27-54 cases of disease will allow the secondary endpoints on AD risk to be addressed. Based on a SD of 2.31g/$m^2$/hr from OBSeRve, (250) this provides adequate power to detect a difference in TEWL of 2g/$m^2$/hr between babies that do and do not develop AD (22 babies required for 80% power). This was based upon a clinically relevant difference in TEWL reported by Kelleher *et al* at birth (102).

Skin assessments

All skin measurements were performed on the maternity ward shortly after birth (≤72 hours old), and at the family home (4-weeks and 12-months old). The volar aspect of both forearms, the right antecubital fossa and the right thigh were the designated study sites. The neonatal skin condition score was calculated at birth (291) in addition to a visual inspection of dryness and erythema conducted at each time point. ***Skin Barrier Function***: Transepidermal Water Loss (TEWL) was assessed using an AquaFlux AF200 closed chamber condensing device (Biox Systems Ltd, London, UK). A single reading was obtained from each study site. A measurement was not attempted if the infant was distressed. ***Infrared Spectroscopy***: A portable 4300 Handheld Fourier Transform Infrared (FTIR) spectrometer equipped with mercury cadmium telluride detector and one bounce/one pass diamond Attenuated Total Reflectance (ATR) accessory (Agilent Technologies, Santa Clara, USA) was used to collect absorption spectra at the skin surface in the mid infrared region from 32 scans at $4cm^{-1}$ resolution. A single spectrum was obtained from each anatomical site and visually checked for quality. Due to poor signal quality and inconsistent measurement performance of the one bounce/one pass ATR at birth, a prototype three-bounce/two-

pass diamond ATR accessory was successfully trialled and introduced by the time participant number 035 was recruited. All subsequent participants were analysed using this new accessory due to its superior performance. For consistency, all prior participants (001-034) were excluded from the spectroscopic endpoints at birth. For quantitative skin parameters, peak intensities related to total lipid (peak 2850 $cm^{-1}$ $CH_2$ stretching mode), sebum (peak 1740 $cm^{-1}$ C=O stretching mode) and water (peak 1640 $cm^{-1}$ $H_20$ deformation) were baseline corrected and normalised to Amide II at 1540$cm^{-1}$ to account for contact pressure (292). Lipid structure (lateral organisation and packing) was measured using the peak 2850$cm^{-1}$ centre of gravity (152). Negative peak intensities were excluded from the analysis and outliers checked for spectra quality. The use of a commercial baby wipe was piloted for the removal of sebum from the skin surface prior to an additional FTIR measurement being taken. ***Tape stripping***: D squame discs (CuDerm, Dallas, TX, USA) 14mm in diameter were used in triplicate to collect *ex vivo* skin samples for desquamatory protease analysis (left forearm) and NMF quantification for *in vivo* modelling using FTIR (left forearm and right antecubital fossa).

Desquamatory protease activity

Caseinolytic and chymotrypsin-like protease activity was assayed at two independent sites from three pooled consecutive tape strips (discs 1-3) using substrates EnzCheck® (Invitrogen, Paisley, UK) and MeOSuc-Arg-Pro-Tyr-AMC (Peptide Protein Research Ltd, Southampton, UK). Specific enzyme activity was calculated by normalisation to protein removed estimated by densitometry (see Appendix Figure 6.2 page 165).

FTIR *in vivo* NMF modelling

As previously described, a chemometric Partial Least Squares regression model was built to calibrate IR absorption across the fingerprint spectral region (1090-1653cm$^{-1}$) against quantitative *ex vivo* tNMF (the sum of free amino acids, pyrrolidone carboxylic acid and urocanic acid) obtained by laboratory analysis of tape strips collected at four weeks and 12-months old. Sampling sites were randomly allocated to model calibration and validation sets. Spectra were normalised to Amide II (1540cm$^{-1}$) prior to modelling. The final spectroscopic model was validated in an independent subset of infants.

Filaggrin genotyping

Saliva was collected at the 12-month study visit using an Oragene OG-250 sampling device and genomic DNA extracted by the PrepIT L2P kit (DNA Genotek, Ottawa, Canada). The four common European Filaggrin loss-of-function (LOF) mutations were screened by Taqman (R501X and 2282del4) or Sanger sequencing (R2447X and S3247X) using established primer and probe sets (268).

Outcome measures

The primary study outcomes were the change in (a) skin barrier function; (b) molecular composition determined by FTIR; and (c) desquamatory protease activity from birth to 4-weeks and 12-months of age. Secondary outcomes included the incidence of AD (either General Practitioner or Investigator diagnosed using the UK working party criteria) and Filaggrin LOF mutations by 12-months of age. The Eczema Area and Severity Index (EASI) score was utilised as a measure of severity (293). Study completion was assigned following submission of the 12-month exit questionnaire by

the family. Additional study outcomes on infant skin care practices and parental satisfaction captured by diary, questionnaire and semi-structured interview, will be reported by subsequent manuscripts.

Statistical analysis

Study data was captured in FileMaker Pro (Claris, London, UK) and collated in Excel. Normality was checked using a Q-Q plot and data log transformed where appropriate. A matched, mixed model, one-way analysis of variance (ANOVA) or nonparametric equivalent was used to compare means (TEWL, FTIR and protease activity) over time to address the primary outcomes on skin barrier development. The equivalent two-way ANOVA was used to compare means over time between infants that developed disease to those that did not to address the secondary outcomes on AD risk. A matched, mixed model, analysis of covariance (ANCOVA) was employed to explore the relationship between TEWL, temperature and AD development over time. Forward multiple logistic regression modelling of parameters associated with an AD diagnosis by 12-months was performed using the log likelihood ratio test ($p$=<0.05) for selection controlling for sex and gestation period. Withdrawn participants were included for skin barrier development outcomes but excluded from the analysis of AD risk. All statistical tests were completed using either GraphPad prism 9 (San Diego, California, USA) or IBM SPSS statistics (version 27; Armonk, NY, USA). The Microlab Expert software package was used for the chemometric modelling of NMF *in vivo* (Agilent Technologies, Santa Clara, USA).

| Inclusion criteria | Exclusion criteria |
|---|---|
| ***Baby***<br>Healthy, full-term ≤72 hours old | Admission to neonatal unit<br><br>Major congenital malformations or limb defects, Illness, social issues or logistical reasons that will prevent comfortable participation by the family<br><br>Currently participating in another clinical study<br><br>The baby is to be adopted |
| ***Mother***<br>Age ≥18 years old<br><br>Singleton pregnancy booked in to give birth at the maternity unit<br><br>Lives within a 5-mile radius of the University of Sheffield | Unable to give informed consent<br><br>Carrying a baby with known chromosomal abnormality or syndromic diagnosis |

**Table 4.1:** Study eligibility criteria

## RESULTS

A total of 689 eligible families were approached on the maternity ward and provided with a study Participant Information Sheet. Of the 180 full-term neonates successfully recruited shortly after birth (mean age 33.61±17.66 hours at first assessment), 128 completed the study at 12-months (Figure 4.1). After obtaining informed consent, 52 participants withdrew from the study due to either screening failures following enrolment (*n*=3); retraction of parental consent (*n*=9); or were loss to follow-up after three failed contact attempts to book a scheduled home visit (*n*=40). Mean age at the 4-week and 12-month assessment was 33.51±6.72 days and 12.02±0.75 months respectively. Twenty-five infants (20%) developed AD by 12-months of age (Table 4.2). Of these participants, 19 were general practitioner confirmed cases and 6 were diagnosed at 12 months using the UK working party criteria (267). The proportion of infants carrying at least one common *FLG* loss-of-function mutation stood at 13% (13/99) with a higher prevalence in the AD group (35%) compared to those that did not develop disease (8%). Of note was the high occurrence of parental atopy (74%) suggesting a cohort of neonates highly predisposed to the development of AD. At 12-months of age the mean whole body EASI score was 1.5 in the 18/25 infants with clinical signs, suggesting mild disease that was well controlled by the study endpoint.

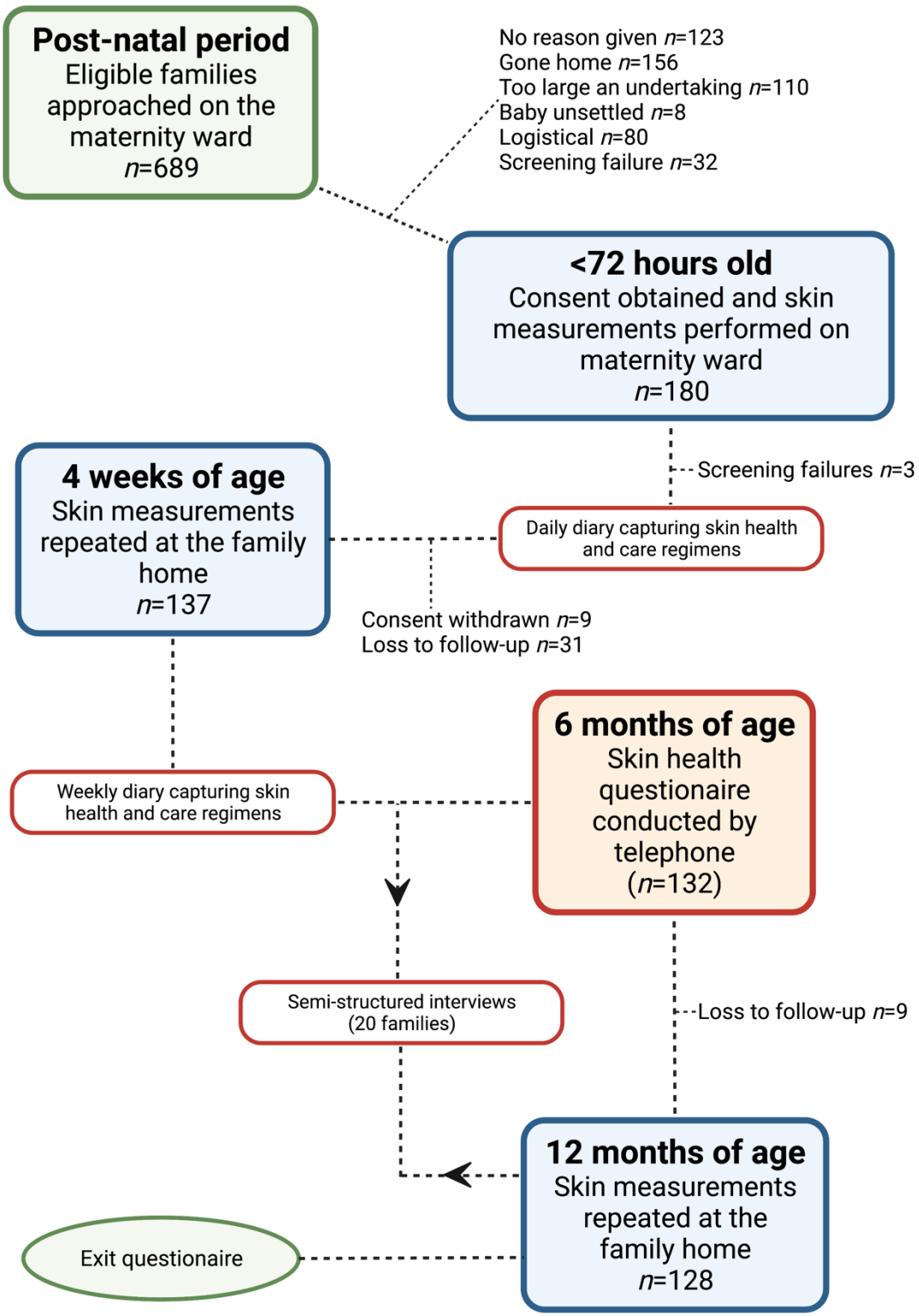


**Figure 4.1:** Participant pathway

| | No AD | Confirmed AD |
|---|---|---|
| ***n*** | 103 | 25 |
| **Sex** (%male) | 51 (50) | 10 (40) |
| **Birth weight** (g) | 3480±408 [2330-4380] | 3477±480 [2620-4540] |
| **Gestation** (days) | 280±9.2 [260-299] | 282±9.3 [262-290] |
| **Mode of delivery**<br>**Caesarean section** (%) | 42 (41) | 9 (36) |
| **Season of birth** (%)<br>Autumn<br>Winter<br>Spring<br>Summer | <br>32 (31)<br>13 (13)<br>26 (25)<br>32 (31) | <br>11 (44)<br>6 (24)<br>3 (12)<br>5 (20) |
| **Age at assessment**<br>Birth (hours)<br>4-weeks (days)<br>12-months | <br>33.38±16.84 [9-88]<br>33.16±7.11 [25-64]<br>12.01±0.76 [11-15] | <br>39.72±21.57 [29-95]<br>34.52±4.54 [29-45]<br>12.03±0.74 [11-14] |
| **NSCS** | 3.46±0.72 | 3.44±0.51 |
| **Mean Dryness score**<br>Birth<br>4-weeks<br>12-months | <br>1.25±0.41<br>1.18±0.35<br>1.08±0.24 | <br>1.24±0.39<br>1.22±0.39<br>1.02±0.07 |
| **Mother's Ethnicity** (%)<br>White<br>Black African<br>Asian<br>Mixed<br>Other | <br>88 (85)<br>6 (6)<br>5 (5)<br>1 (1)<br>3 (3) | <br>20 (80)<br>-<br>3 (12)<br>1 (4)<br>1 (4) |
| [#]**Parental atopy total** (%)<br>AD<br>Asthma<br>Allergic rhinitis | (71)<br>31<br>29<br>57 | (84)<br>12<br>7<br>18 |
| [+]***FLG* LOF mutations** (%) | 6/79 (8) | 7/20 (35) |
| [§]**EASI score** | - | 1.5±1.5 [0.2-5.8] |

**Table 4.2:** Cohort characteristics stratified by AD diagnosis. [+]Number of babies carrying at least one common European loss-of-function mutation. [#]At least one parent reporting atopic disease. [§]Average whole-body score assessed at the 12-month visit in infants with active disease (*n*=18). NSCS: neonate skin condition score (range=3-9). Visual dryness (mean of forearm, antecubital fossa and thigh) scored on a three-point scale (1= no dryness; 2=dry skin; 3= very dry skin). Mean±SD and range presented where appropriate.

Skin barrier development from birth

As a biomarker of permeability barrier function, TEWL proved to be highly variable at all developmental time points. The large standard deviations (SD) and increased frequency of plotted data points greater than 1.5 times the interquartile range (Figure 4.2a) likely reflected its measurement in the wide range of ambient temperatures encountered by the study (Figure 4.2b). For example, a greater cohort SD of 8.89g/$m^2$/hr was observed than previously noted at birth in more climate-controlled conditions (250). 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 both temperature ($p$=0.002) and 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 4.2c).

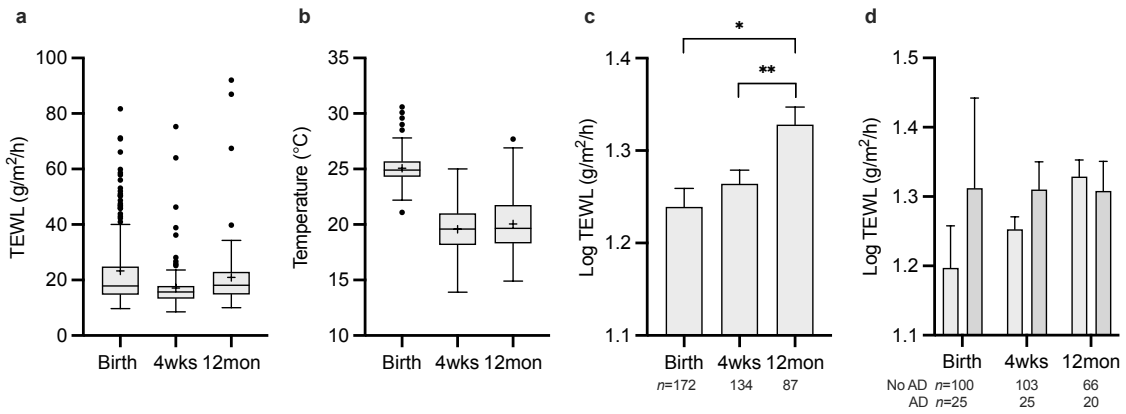


**Figure 4.2:** Development of skin barrier function from birth measured in ambient conditions. (a) A wide range of raw TEWL values at each timepoint was observed in accordance with (b) the highly variable room temperatures encountered throughout the study. Warmer conditions were experienced on the maternity ward. (c) Controlling for temperature, a significant weakening of skin barrier function from birth was observed to 12-months of age, albeit (d) no significant differences in TEWL were noted between infants that do (darker shading) and do not develop AD. A matched, mixed model ANCOVA found a significant effect of time ($p$=0.002) and temperature ($p$=<0.001) but not AD group on 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 patterns were observed between anatomical sites across time (see Appendix Figure 6.4, page 169).

When comparing averaged FTIR spectra collected at the skin surface over the course of the study, clear differences in absorption can be found between the age groups (Figure 4.3). Absorption peaks at 2850cm$^{-1}$ ($CH_2$ stretch), 1740cm$^{-1}$ (C=O stretch), and below 1600cm$^{-1}$ (-$COO^-$ symmetric and asymmetric stretch) relate to respective changes in SC lipids, lipid esters and NMF that warranted further investigation. To track these developmental changes in surface NMF, an FTIR method using Partial least Squares regression modelling was validated for use in infants (see Appendix Figure 6.5, page 171). Cleansing the skin using a commercial baby wipe to remove the potential

interference of sebum prior to a second FTIR measurement being taken proved unsuccessful with inadequate sebum removal and was not pursued further in the analysis (see Appendix Figure 6.6, page 172).

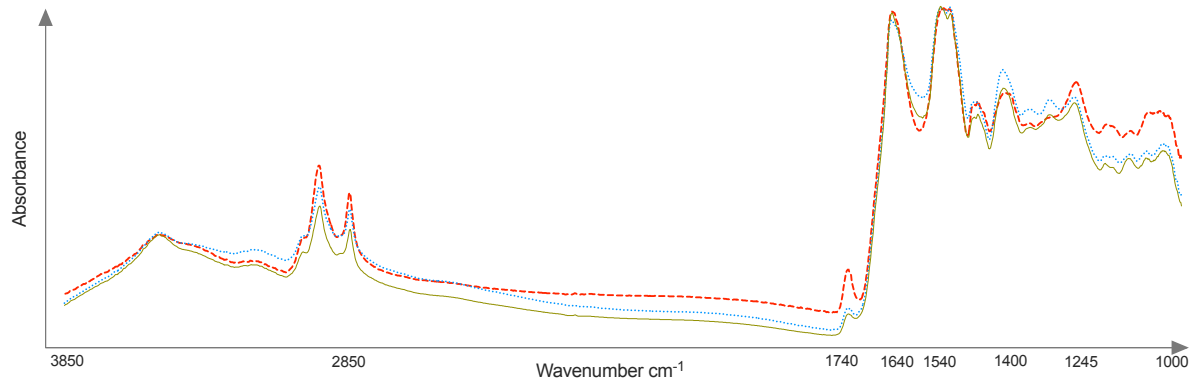


**Figure 4.3:** Skin surface development from birth. Mean FTIR spectra show clear differences in absorption between developmental time points at birth (red dashed line) four weeks (blue dotted line) and 12-months of age (green solid line). Peaks at 2850cm$^{-1}$ ($CH_2$ stretch), 1740cm$^{-1}$ (C=O stretch), and below 1600cm$^{-1}$ (-$COO^-$ symmetric and asymmetric stretch) relate to lipid, lipid esters and NMF respectively. The mean of both forearms, the right antecubital fossa and right thigh is presented for each time point.

A summary of development over the first year of life is presented by Figure 4.4. Like TEWL, variability was high indicated by large SD, but there were several further significant changes in biomarkers measured at the skin surface from birth. Parameters derived from tape stripping such as caseinolytic protease activity dramatically altered (Figure 4.4a) with a 5-fold increase by 4-weeks of age suggesting a rapid rise in the rate of desquamation over this period from birth. Supportive of this was the 1.4-fold rise in chymotrypsin-like protease activity attributed to Kallikrein-7, (Figure 4.4b) a key component of skin shedding (294) and indicative of a greater rate of cell turnover at this age. An increase in FTIR modelled tNMF abundance from birth was accompanied

by a greater 1640/1540cm$^{-1}$ peak ratio at 4-weeks indicative of higher water content (Figure 4.4c and d). Surface lipids (peak 2850/1540cm$^{-1}$ and 1740/1540cm$^{-1}$) decreased from birth alongside an improvement in lipid structure (peak 2850cm$^{-1}$ centre of gravity). Although very little visible vernix caseosa (VC) was encountered at the study sites overall, these lipid observations are presumably due to a combination of VC traces and greater sebum deposition at birth (Figure 4.4e-g). Although there were no significant changes in investigator-observed skin dryness from birth, an inverse relationship with spectroscopically determined water content was noted across all timepoints (Figure 4.4h). Cohort stratification by season of birth did not reveal any significant changes in skin barrier development throughout the first 4-weeks of life (data not shown). Between 4-weeks and 12-months of age there were further reductions in NMF, water and lipid abundance at the skin surface in conjunction with increased TEWL.

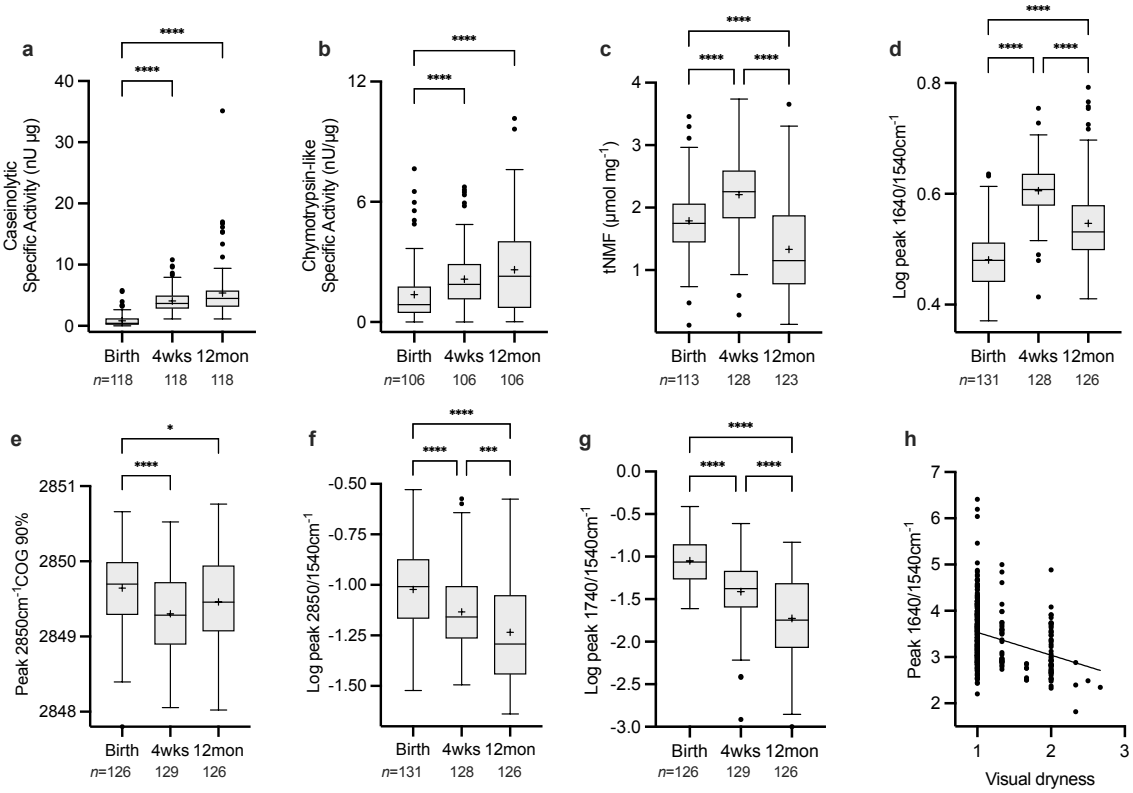


**Figure 4.4:** Skin barrier development from birth. Mean change in surface (a) Caseinolytic and (b) Chymotrypsin-like protease activity, (c) *in vivo* modelled tNMF, (d) water content (e) lipid structure, (f) total lipids and (g) lipid esters from birth to 4-weeks and 12-months of age. Protease measurements were obtained from one repeat on the left forearm only. For skin parameters determined spectroscopically, a single measurement was attempted from each forearm, the right antecubital fossa and right thigh and averaged to provide one value per participant / per timepoint as comparable patterns were observed between anatomical sites across time (see Appendix Figure 6.4, page 169). Infants with a minimum of 2/4 successful readings are presented. A Friedman test with asterisks denoting significance following a Dunn's multiple comparison test to compare timepoints was used for panels (a) and (b) only. For all other panels, a matched, mixed model ANOVA was performed with asterisks denoting significance following a Tukey post-test to compare all timepoints (*$p$=0.05). Log transformations were used where indicated. (h) A significant association was noted between an investigator-observed dryness score and spectroscopically measured skin water content over all timepoints (r=-0.29, ***$p$=<0.0001) using the spearman rank test.

Subtle differential skin barrier development exists between infants that do and do not develop AD

For TEWL and FTIR, similar developmental trajectories were observed across anatomical sites between infants that do and do not develop AD across all time points studied (see Appendix Figure 6.7, page 173). With this in mind, the mean of these body sites was calculated to provide one measurement parameter per infant, per timepoint, with the goal of achieving greater precision to address the secondary endpoints of AD risk. In general overall, variation was high indicated by large SD and few differences in skin barrier maturation were observed between infants that did and did not develop AD. However subtle patterns were present prior to the onset of clinical disease (Figure 4.5). For example, a matched mixed model ANOVA reported an overall significant effect of infant subgroup (AD versus no AD) on FTIR lipid peak 2850/1540cm$^{-1}$ (Figure 4.5f). Here, surface lipids at birth were 27% greater in infants that developed disease, a trend corroborated by lipid esters (Figure 4.5f-g). Chymotrypsin-like protease activity (attributable to KLK7) at birth was on average 40% lower in infants that developed AD (Figure 4.5b). Although no difference in visual dryness was apparent by 4-weeks of age (Table 4.2), spectroscopically modelled NMF and water content was on average 15% and 7% respectively lower in infants that developed AD, indicating a dryer skin surface at this developmental point (Figure 4.5c and d). This finding was likely influenced by the strong inherited NMF defect evident at this timepoint (see Appendix Figure 6.5, page 171). Although TEWL was elevated at birth in infants that developed AD, no significant differential permeability barrier function was noted at any stage, representing comparable skin barrier health between the two infant subgroups throughout the study (Figure 4.2d).

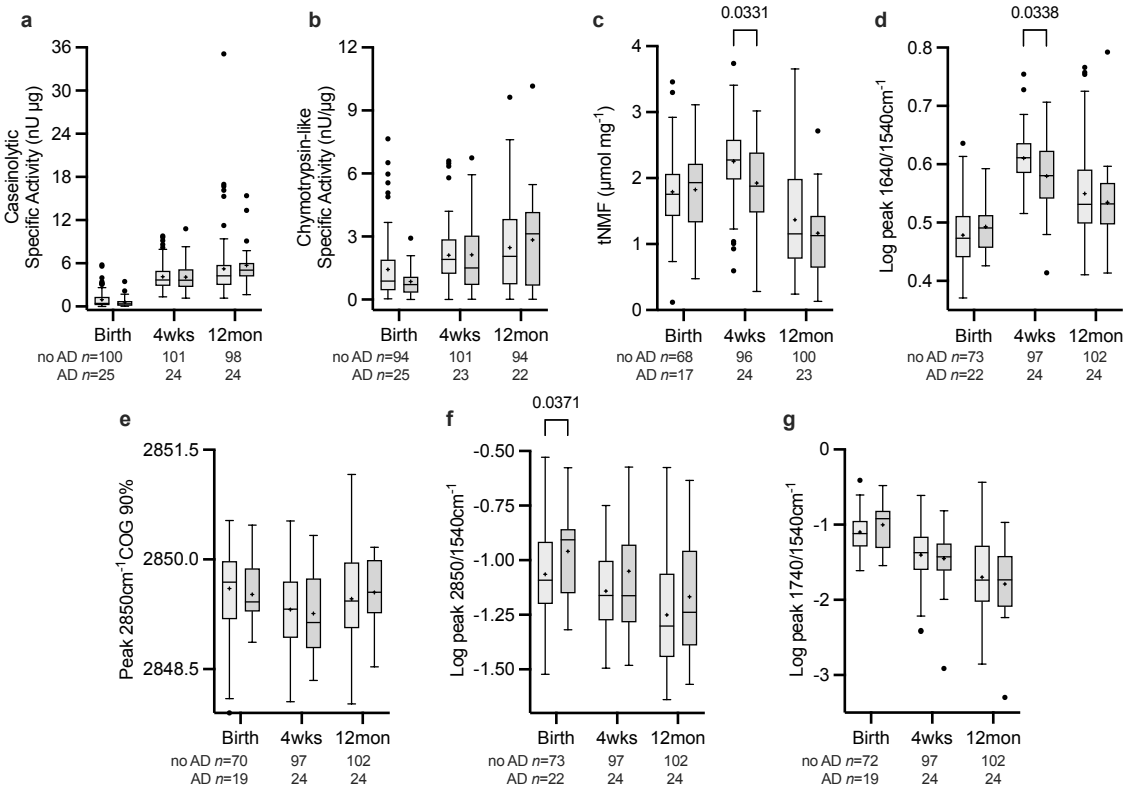


**Figure 4.5:** Subtle differences in skin barrier maturation exist between neonates that do and do not develop AD by 12-months of age. (a) Mean surface caseinolytic (b) chymotrypsin-like protease activity (c) *in vivo* modelled tNMF (d) water content (e) lipid structure (f) total lipid and (g) lipid 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 attempted from each forearm, the right antecubital fossa and right thigh and averaged to provide one value per participant / per timepoint as comparable patterns were observed between anatomical sites across time (see Appendix Figure 6.7, page 173). Infants with a minimum of 2/4 successful readings are presented. A matched mixed model ANOVA reported an overall significant effect of infant subgroup (AD versus no AD) on 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.

Factors predictive of disease risk by 12-months of age

To model parameters associated with an AD diagnosis by 12-months of age, logistic regression was employed controlling for sex and period of gestation (Table 4.3). Starting with 1st degree atopy alone, the forward selection of independent variables using a log likelihood ratio test threshold of $p$=<0.05 for inclusion, revealed that 4/7 skin measurements offered additive predictive value for evaluating disease risk (model 1-4) indicated by an increased aggregate area under the curve (AUC). Modelling 1st degree atopy alone was not predictive of an AD diagnosis (AUC=0.61ns, data not shown). By comparison, increased surface lipids (FTIR peak 2850/1540cm$^{-1}$) at birth, reduced KLK-7 activity (birth), a drier skin surface (4 weeks) and low NMF (4 weeks) were signals associated with disease development. Individuals carrying at least one common *FLG* loss of function mutation (model 5) were almost 6 times more likely to develop AD by 12-months of age and was the single, greatest predictor of disease measured in the cohort (AUC=0.73). A second round of forward selection was attempted to sequentially assess the four skin parameters identified when combined with *FLG* LOF mutations. One model was selected to have additive value that included KLK7 activity at birth (model 6, AUC=0.78). This model at a 50% cut off correctly classified 30% and 99% of cases with and without AD respectively. In contrast, TEWL was not associated with AD development at any timepoint. For the spectroscopically derived data, the predictive aspect of the modelling was improved by setting a more stringent threshold for data inclusion, but this significantly decreased the number of neonates included in the analysis at birth (see Appendix Table 6.3, page 174). The period of gestation also featured in this modelling, with each additional day before labour conferring 10% increased odds of disease (see Appendix Table 6.3, page 174).

| | Model 1 | Model 2 | Model 3 | Model 4 | Model 5 | Model 6 |
|---|---|---|---|---|---|---|
| **No AD *n* =** | 73 | 94 | 96 | 97 | 79 | 74 |
| **AD *n* =** | 22 | 25 | 24 | 24 | 20 | 20 |
| **Parameter** | **2850/1540cm$^{-1}$ (B)** | **KLK7 (B)** | **tNMF (4W)** | **1640/1540cm$^{-1}$ (4W)** | ***FLG*** | ***FLG*** |
| | 1.71 (1.05-2.93) 0.04 | 0.58 (0.31-0.92) 0.05 | 0.42 (0.19-0.90) 0.03 | 0.23 (0.07-0.71) 0.01 | 5.94 (1.61-23.22) 0.008 | 6.24 (1.55-27.60) 0.01 |
| | - | - | - | - | - | **KLK7 (B)** |
| | | | | | | 0.58 (0.29-0.96) 0.07 |
| **Sex (M)** | 0.57 (0.19-1.61) 0.30 | 0.66 (0.25-1.71) 0.39 | 0.66 (0.24-1.71) 0.39 | 0.82 (0.30-2.20) 0.70 | 1.05 (0.34-3.28) 0.93 | 1.05 (0.32-3.53) 0.93 |
| **Gestation** | 1.02 (0.97-1.10) 0.41 | 1.03 (0.98-1.08) 0.34 | 1.01 (0.96-1.06) 0.72 | 1.02 (0.97-1.07) 0.52 | 1.04 (0.98-1.10) 0.22 | 1.04 (0.98-1.10) 0.17 |
| **1$^{st}$ atopy** | 2.00 (0.56-9.48) 0.32 | 2.88 (0.87-13.21) 0.12 | 1.94 (0.57-9.00) 0.33 | 2.10 (0.62-9.78) 0.28 | 5.34 (0.90-104) 0.13 | 7.96 (1.25-166) 0.07 |
| **AUC** | 0.68** | 0.69** | 0.67* | 0.69** | 0.73** | 0.78** |

**Table 4.3:** Forward selection modelling by logistic regression of parameters associated with AD risk by 12-months of age. Meaned spectroscopic parameters from infants with a minimum of two successful readings (out of four possible body sites) are presented. Odds ratio (95% confidence interval) and *p* value shown. B=birth and 4W=four weeks of age. 1$^{st}$ atopy=at least one first degree relative with atopic disease. AUC=area under the curve.

## DISCUSSION

From birth, a significant period of skin barrier maturation (90, 260) coincides with an increased risk of developing AD (238, 239, 286). By performing a portfolio of skin tests at the bedside and family home in a longitudinal birth cohort, this study identified subtle, differential developmental trajectories between healthy infants and those that developed AD before their first birthday. A primary *FLG* defect was strongly associated with early onset disease and conferred a significant reduction in NMF, with a drier skin surface by four weeks of age. In conjunction with reduced chymotrypsin-like protease activity and increased surface lipids at birth, these parameters were of additive value for predicting a diagnosis of AD when accounting for a family history of atopy; a metric used to identify high risk populations for prophylactic intervention (256, 295).

Developmentally, this study reports that during the foremost weeks of life, components of the skin barrier are undergoing a period of change that extends to the conclusion of the first year. From birth the SC acidifies and becomes more hydrated (92, 93, 98, 245, 283). Here, using FTIR to measure absorbance at 1650cm$^{-1}$ attributed to the bending mode of water, (296) a significantly more hydrated skin surface was reported at four weeks of age. This is in agreement with observations using Raman Spectroscopy quantifying greater water content throughout the entire depth of the infant SC compared to adults (260). A mechanism underlying this evolution towards more hydrophilic conditions could be changing NMF abundance; from low levels at birth increasing with postnatal age (83, 270, 283). A similar trend was observed for desquamatory proteases such as KLK-7, that can be modulated through changes in biochemical environment (79). As a biomarker of skin health, it is also apparent that

TEWL increases over this initial period (93, 102, 283) and continues to weaken as more steady state conditions are reached beyond four weeks, supporting an extended period of permeability barrier optimisation from birth compared to adults (260). Although very little visible vernix caseosa (VC) was noted in this birth cohort, bulk surface lipids and esters measured by FTIR peaks 2850cm$^{-1}$ and 1740cm$^{-1}$ respectively, were also found to decrease over this developmental timeframe. Being rich in triglycerides, wax esters and squalene, any residues would increase the lipid/protein ratio compared to the background SC signal (297). In addition, a more disordered lipid organisation observed from birth may reflect the increased proportion of branched chain fatty acids in VC (297). The gradual decline in lipid esters reflects the level of sebum at the neonate skin surface after birth (93, 257, 258).

A secondary outcome measure was the incidence of AD; allowing this study to retrospectively assess whether signals of skin barrier dysfunction from birth were associated with a diagnosis of AD by 12 months of age. Although a small, exploratory cohort, at 20% the number of AD cases was generally consistent with prevalence estimates in UK children, (298) and justified the recruitment target of 180 babies for addressing predictive risk. All active cases we saw were classed as almost clear or mild when assessed by EASI at the scheduled 12-month study visit (299) that may reflect a bias towards recruitment of early-onset/resolving-transient disease with improved clinical prognosis (300, 301). In two recent UK cohorts, between 2-14% of infants suffered from moderate-severe AD, suggesting the early onset trajectory is predominantly a mild disease (302, 303).

This study alongside others reports the significance of common European *FLG* LOF mutations in early AD development. In a recent Scandinavian cohort of 1836 infants, these risk alleles represented 25% of disease cases diagnosed by 12-months conferring a 4-fold increased risk (304). In a UK birth cohort, they accounted for 17% of cases with a 2 to 4-fold elevated risk of disease (300). Here by comparison, they represented 35% of AD cases and conferred a 6-fold increased risk. In this study, a consequence of the inherited *FLG* defect was reduced NMF abundance at 4-weeks when assessed by FTIR that was predictive of loss of function mutations and AD development. This finding is supported by an equivalent spectroscopic measurement of NMF - Raman Microspectroscopy – that is also predictive of *FLG* genotype (AUC=0.79-0.83) but at an earlier timepoint (1-4 days) after birth (278). The capacity of Raman to assess the full SC depth may be a key difference here compared to FTIR that is limited to the penetration of the evanescent wave (1-2μM). Considering the gradual accumulation of NMF from very low initial levels at birth shown by our *ex vivo* analysis, it is plausible that around four weeks of age is the optimum time to differentiate *FLG* endotypes at the skin surface using the FTIR technique. In contrast to a primary *FLG* abnormality, we saw no clear evidence of a secondary (disease-associated) acquired NMF defect, once again probably representative of a cohort devoid of overt inflammation (225, 284). Another outcome of NMF loss is skin dryness, an observation supported here by a significantly lower FTIR peak 1640/1540$cm^{-1}$ ratio at 4-weeks of age in infants that developed disease. Clinical dryness manifests in LOF mutation carriers at 3-months old and predisposes to AD development by 6 months (305, 306). Interestingly, a factor modulating infant xerosis is increasing gestational age (306) that was associated with a diagnosis of AD here.

Weakened permeability barrier function at birth is associated with early onset AD (282, 307). Here, although a similar trend of elevated TEWL was observed at birth, overall our data was unclear, with no significant difference between infant subgroups noted even when AD cases were established at the end of the study. This likely reflects its association with disease severity; (302) with a clear TEWL abnormality observed in more severe infant disease cases by 12 months of age (284). Aside from minimal clinical severity, a contributory factor here could also be the omission of TEWL measurements from the cheek or face, a common site of symptomatic onset (282, 307). 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 a suitable period of acclimatisation (96). The diverse ambient temperatures encountered and the subsequent lack of precision, suggests that 25 babies with AD lacked sufficient power to obtain a reliable population estimate when TEWL was attempted in unstandardised conditions.

The study produced some novel results. One skin parameter that modelled independently from the pronounced inherited *FLG* defect was reduced KLK7 activity at birth. The increased expression and activity of desquamatory proteases are a hallmark of AD, (154-156, 308) but here, proteolysis was attenuated in predisposed babies at birth, highlighting a difference between development and established disease. Relevant to this finding is increased Serine protease inhibitor (Serpin) A12 abundance attributed to the VC of infants that go on to develop AD by 24 months old (309). Being an inhibitor of desquamatory proteases, including KLK7, (310) that is expressed in the adult epidermis, (311) Serpin A12 activity may explain the initial

reduction in chymotrypsin-like proteolysis observed in this subset of predisposed infants while the VC is retained. Differential VC/sebum deposition, or parental skincare intervention may explain the association of increased surface lipids detected by FTIR at birth with a diagnosis of AD. In mice, the topical application of sebum induces lipid barrier disruption and proinflammatory inflammation, (259) while the use of oils for neonate massage by caregivers may inadvertently delay normal infant skin barrier maturation (250). Natural oils such as olive, rich in oleic acid content has been reported to damage skin barrier structure and function in adults when applied topically to the skin over a 4-5 week duration (312).

A strength of this project was its pragmatic design, balancing scientific objectives alongside consideration of the families involved over a longer-term study duration. To maximise participant retention, portable equipment was used enabling data collection at the bedside and in the community; skin tests were restricted to the surface layers; and follow up was ceased at 12-months to capture the majority of AD cases over a minimum timeframe. Even with these careful measures in place, 27% of the recruited families either withdrew or were loss to follow up, highlighting a significant challenge faced by a study of this nature. An observational design is also prone to unintended sources of confounding. Here for instance, although the study objectives were intended for evaluation in a general population, recruitment was biased towards families with a history of atopic disease, limiting the interpretation of the results. At 74% the prevalence of parental atopy was high compared to a more general population estimate of 40-50% (130, 313). Therefore, our rate of AD was more akin to a high-risk population that our immediate findings are more pertinent to (303). By employing

fixed study timepoints, clearer evidence of skin barrier breakdown may have been missed. Variation was high in the skin surface measurements employed across the study. Analysing deeper SC layers that are less prone to environmental factors may help to reduce this and increase precision. In addition, a small window at birth or four weeks may not be the ideal time to detect clear pathological changes many weeks prior to the development of disease, especially when dynamic changes are taking place as the skin adapts to terrestrial life over this period. Moving forward for comparison, a matched cohort of infants with more severe clinical disease can be recruited and assessed using the same skin tests to place our longitudinal data into context.

In conclusion, although this was primarily an exploratory investigation, the evidence suggests that this portfolio of tests has the potential for improving current risk evaluations for early-onset AD when used in a healthcare or community setting from birth. With regards to quantifying NMF abundance by FTIR, here is a technique with the potential to discriminate FLG endotypes at the bedside as a rapid alternative to laboratory genotyping. A further, more definitive exploration accounting for severe disease cases is required, but if successful, not only may high risk infants be better targeted for intervention, it has the potential to identify those at increased risk of atopic morbidity such as food allergy (300-302).

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# CHAPTER 5: FINAL DISCUSSION

Development of the neonate-infant skin barrier

From birth the structural and functional properties of the neonate SC are not in a steady state. This study and others have observed a rapid acidification of the skin surface in conjunction with a near doubling in hydration over the first four weeks of life (93, 98, 245). Compared to adults, the infant epidermis is 20% thinner with greater cell turnover attributed to increased rates of proliferation at the basal layer. The SC is also thinner, with smaller corneocytes, irregularly orientated corneodesmosin fragments and a more alkaline skin surface pH indicating higher rates of proteolysis or uncoordinated desquamation from birth (89, 90, 92, 314). However, we reported that *ex vivo* chymotrypsin-like activity at the skin surface attributable to the abundant KLK-7 (60) was stable and equivalent to adults. Compare this to newborn rats where increased serine protease activity is associated with a neutral skin pH (99). As an alkaline environment is optimal for proteolytic activity of this nature, this may indicate compensatory mechanisms in humans such as a stronger LEKTI/KLK-7 inhibitory complex functioning at the neutral pH sustained over a comparatively longer period of acid mantle formation (70). Both the vernix caseosa (VC) and sebum contain the serine protease inhibitor cholesterol sulphate, (315, 316) that may also function to attenuate the activity of KLK-7 immediately after birth (74).

Over time, a significant elevation in activity was noted during the first month of life suggestive of greater rates of desquamation, but the reasons underlying this evolution remain unclear. One functional explanation could be the dynamic increase in SC hydration over this period supported by a greater FTIR 1640/1540$cm^{-1}$ peak ratio, that

modulates the activity of proteases such as KLK-7 that are sensitive to alterations in water content (79). In the SC of older infants, higher conductance values and increased water abundance has been observed compared to adults (260). A similar study associated increased hydration with elevated bulk protease activity in infant skin – a finding supported here through the longitudinal evolution of increased proteolysis from birth to 12 months - that may account for the thinner SC noted by *in vivo* confocal laser scanning microscopy (317). An additional mechanism underlying transitional protease activity could be differential enzyme abundance within the SC, a point not addressed by this study but could be achieved by ELISA in extracted infant TS samples (154, 183).

A plausible contributor to the rapid rise in skin hydration over this period is the concomitant increase in hygroscopic NMF. In the neonatal rat SC, FLG proteolysis forms this potent pool of humectants in response to the drier environment encountered shortly after birth (35, 38). In agreement, this study and others observed a rise in SC NMF over the first month of life; (83, 270) from very low levels encountered at birth to an abundance greater than adults by four weeks of age reported here. We also observed a significant rise in TEWL as noted by others at different anatomical sites (93, 102). Permeability barrier function is provided by the SC, therefore elevated TEWL at this age can be attributed to fewer cell layers and smaller corneocyte size accelerating the passage of water and vapour flux from the skin surface (90, 314, 318). This is supported by an inverse relationship between TEWL and SC thickness in infants, (319) and the SC becoming progressively thinner from birth through to 6 months (320). Moreover, a computational modelling approach has revealed increased

permeability of infant skin to caffeine, providing novel insight to the outside-in penetration of exogenous substances (321). We found longitudinally that a trend for weakened barrier function continues to the child's first birthday, highlighting an extended period of skin barrier optimisation from birth compared to adults (260).

Using FTIR spectroscopy, this study reported a gradual reduction in total lipids ($2850cm^{-1}$) and lipid esters ($1740cm^{-1}$) at the skin surface throughout infancy, that may be of sebaceous origin. For example, a mixture of triglycerides, wax esters and squalene forms part of the vernix caseosa (VC), that protects the foetus *in utero* during the final trimester of pregnancy and may still be present at the skin surface after birth (297). Sebum is composed of these lipid species and covers the neonate skin surface at an equivalent level to adults shortly after birth, due to the transplacental flood of maternal androgens during labour stimulating production (257). Both VC and sebum are occlusive and associated with moisture retention (322, 323) that may aid the extended functional adaptation to the extra-uterine environment by preventing excessive water loss. In support of this, we observed a lowered skin surface water content and an increase in TEWL from 4 weeks to 12 months of age with a concomitant drop in surface lipids determined by FTIR. In contrast to this benefit, excess sebum is associated with skin barrier damage in mice and may partially account for the increased sensitivity of infant skin when homeostasis is not attained (259).

Throughout infancy, the skin is more susceptible to dryness, erythema and infection owing to its structural and functional immaturity compared to adults. This reinforces the need for careful infant skin care practices that gently support healthy skin barrier

development from birth (324). For example, being more permeable, the use of harsh surfactants should be avoided that may cause irritation. Furthermore, a preference to neutral-acidic synthetic cleansers combined with suitable emollient use to support acid mantle development and combat dryness, may help guard against aberrant desquamatory protease activity, unwanted removal of NMF and premature barrier breakdown.

Biomarkers of infant skin barrier breakdown in relation to AD development

Since the beginning of this project, undoubtedly the landscape of infant AD research has changed due to new sources of evidence. First, less than a decade ago, healthcare professionals were seriously considering the benefits of emollient use from birth to disrupt and alter the natural disease course due to promising pilot work (256, 295). This strategy is supported in adults with established disease, whereby regular emollient therapy is a key component of AD treatment to strengthen the barrier defect and modify disease course by prolonging periods of flare remission (235, 325). But the main Barrier Enhancement for Eczema Prevention (BEEP) study in 1394 UK newborn participants failed to replicate its promising preliminary findings in a multicentre randomised controlled trial (303). This was corroborated by a similar study conducted in Scandinavia – the skin emollient and early complimentary feeding to prevent infant atopic dermatitis (PreventADALL) trial – albeit this was a general population intervention as opposed to targeting those at high risk of disease (326). Evidence on this topic is continually evolving due the emergence of new studies, however a recent meta-analysis concluded no benefit of infant emollient interventions on preventing

disease development (327). This is in contrast to a similar review supporting an emollient intervention delaying the onset of AD in high-risk individuals (328).

A second change was the recent retraction of the high-profile publication by Kelleher *et al*., that provided strong evidence in a large infant cohort of a primary barrier defect (elevated TEWL) at birth and two months preceding the onset of disease by 12 months of age, even when controlling for *FLG* LOF mutations (102). Back in 2015, the emergence of the Kelleher study soon after dissemination of the initial BEEP findings the year before, provided strong support for the rationale of BEEP and the consensus from established disease and animal models (102, 256). These two publications were also key rationale for the work contained in this thesis; building on this prior knowledge to (a) investigate mechanisms of barrier breakdown related to weakened permeability barrier function in predisposed infants and (b) to find out if these skin biomarkers also signal the onset of disease in this demographic, to better identify individuals for future medical intervention. Taken together, this new evidence brings into question the role of skin barrier breakdown in the pathogenesis of infantile AD, and poses an interesting question; is the absence of a prominent skin barrier defect prior to clinical AD development a plausible reason for the unexpected results of the BEEP study? Although small in size, it is anticipated that the findings from our cohort of infants that developed mild disease by their first birthday can assist in placing this new evidence into context.

*Transepidermal Water Loss*

Permeability barrier function resides in the SC, with elevated TEWL correlating with AD severity (147) and the progression to further atopic conditions such as food allergy (271). The $T_H2$ cytokines associated with acute AD, IL-4 and -13, can reduce expression of multiple structural SC components such as Keratin -1 and -10, (329) FLG, Loricrin, Involucrin and ceramides that exacerbates the permeability barrier defect in AD (221, 228, 230). For example, elevated TEWL is associated with a reduction in SC free fatty acid chain length in conjunction with a less dense lipid organisation (330) and reduced FLG expression (NMF) in patients (284). When performed in uncontrolled ambient conditions, this observational study found TEWL readings to be variable, with an unclear association between weakened permeability barrier function and AD development by 12 months of age. The evidence in the literature is also inconclusive, and like TEWL status at birth, could be attributed to the significant methodological heterogeneity between affiliated studies reporting on this matter. For example, two Asian pilot studies are in support of a primary defect when assessed at the face or forearm shortly after birth (282, 307). With no corresponding data in Caucasians for comparison, elevated TEWL measured on the forearm seemingly develops beyond 3 months of age (306, 331). An article by Barents *et al* reporting the sensitivity and specificity of their logistic regression modelling, found elevated TEWL to be a relatively weak predictor of disease onset when measured in Caucasian infants greater than 6 months of age (331). Its successful detection, association with disease and predictive utility, may therefore be dependent on factors such as population, timing, anatomical site and the influence of further unknown environmental exposures that confer increased risk of disease. Even when AD was established at 12 months of age, no

permeability barrier defect was evident that reflects the mildness of the cohort and lack of skin lesions at the point of assessment. Compare this to once overt moderate-severe disease in babies is established with $T_H2$, -17 and -22 centred cutaneous inflammation, where a significant permeability barrier defect exists at clinically unaffected sites on the volar forearm (187, 284).

*Natural Moisturising Factors*

When quantified in conjunction with skin barrier measurements performed in more environmentally controlled conditions, this project initially revealed that in neonates at birth, low levels of NMF associate with high TEWL. It was therefore plausible that the FLG / NMF biological pathway warranted further investigation in relation to early AD onset. Although ultimately successful, the tape stripping NMF assay in neonates proved laborious with logistical constraints impeding its potential translation to a community setting. Moreover, there are space issues limiting multiple tape strip sampling on a neonate forearm, therefore a non-invasive FTIR assay for the purpose of rapid NMF quantification without the requirement of a laboratory, was pursued. By successfully developing and validating this methodology first in adults, corresponding evidence was subsequently found at the neonate skin surface associating reduced NMF with dryness in *FLG* LOF mutation carriers, prior to the onset of clinical disease. In neonates four weeks of age, the FTIR methodology was moderately predictive of *FLG* genotype and AD onset by 12 months. For reasons unknown these clinical associations were not replicated either at birth or 12 months of age. This may represent the importance of measurement timing; with the dynamic adaptation of the SC and the latter introduction of parental skin care interventions possibly confounding more accurate

NMF quantification at these developmental milestones. However, the evidence suggests this technique offers potential as a rapid, non-invasive test suitable for use in a community setting, as a possible alternative to buccal swab collection and laboratory genotyping for the detection of these at-risk individuals.

*The acquired NMF defect*

Compared to a prominent primary deficiency at 4 weeks, no clear evidence of an acquired FLG defect was noted once disease was present at 12 months. Compare this to adults, whereby a significant reduction in NMF abundance at the skin surface was noted in subjects largely free of visible disease compared to healthy controls (Chapter 3). In established disease models, the $T_H2$ type inflammatory axis in AD weakens the skin barrier through attenuating key components of its infrastructure and function. Reduced expression of the EDC structural proteins Loricrin and Involucrin; (230) inhibited fatty acid elongase 3 and 6 (ELOVL3, -6) expression increasing the proportion of short chain SC lipids; (332) reduced desmoglein -1 expression and corneodesmosome density; (229) and elevated skin-surface pH, (333) are all mediated by this pathogenic mechanism. The FLG / NMF differentiation pathway is no exception, as experimentally in keratinocytes, the cytokines IL-4, IL-13, and severe disease in infants that lack common predisposing mutations can significantly reduce FLG expression in the SC (221, 334). Furthermore, a report by Kezic *et al*., confirmed a lower NMF abundance in 40 children with AD compared to controls; a cohort that included moderate-severe disease cases, as evidence of this acquired defect (225). Therefore, on one hand, our infant cohort may not have been of sufficient size, or clinically diverse enough to corroborate this finding, with mild disease reflecting a lack

of cutaneous $T_H2$ inflammation to attenuate FLG. This was supported by our logistic modelling in that there was no additive benefit of NMF to genotyping for the prediction of AD at 4 weeks of age. However, in overt severe paediatric AD compared to adults, there is contrasting evidence from skin biopsies of near normal EDC expression – including FLG – as a consequence of more prominent $T_H17/T_H22$ immune activation lacking in $T_H1$ type inflammation (187). With some evidence of IFN-γ downregulating FLG and bleomycin hydrolase expression *in vitro*, (335, 336) this suggests that an acquired NMF defect may develop over time with disease persistence and chronicity.

But investigating inflammatory biomarkers in infants represents a significant challenge when relying on the traditional laboratory methodologies available to researchers. Skin biopsies in neonates are not viable due to ethical challenges; they cause pain, are prone to scarring and pose an infection risk, meaning parents are unlikely to grant consent for research purposes alone. To navigate this obstacle, investigators are moving towards less-invasive tape stripping (TS) for sample collection to profile cutaneous barrier and immune expression related to diseased and healthy skin. In adult patients, TS to twenty discs removed the entire SC, with expression of FLG and corneodesmosin from this sample being directly comparable to PCR and immunofluorescence results from an equivalent 2mm biopsy (337). Clausen *et al.*, reported clinically relevant increases in $T_H2$ chemokine/cytokine expression such as CCL-17, -22, -27 and TSLP when comparing AD to healthy control skin (338). In infants around one year of age, McAleer and colleagues used TS combined with proteomic analysis to report skewed SC expression of multiple biomarkers related to $T_H2$ inflammation, innate immune activation and angiogenesis in moderate to severe cases

of disease (284). Profiling mRNA expression using real-time PCR in a similar clinical population revealed increased expression of innate immune, $T_H2$ and $T_H17/T_H22$ genes, while lipid mediators were reduced in non-lesional and lesional AD compared to healthy controls (334). Encouragingly, this down regulation of lipid mediators fatty acid 2-hydroxylase (FA2H) and fatty acyl-CoA reductase 2 (FAR2) in infant TS samples was in accordance with previous work in biopsies (187, 334). Moreover, a consistent trend between these TS studies was the upregulation of TARC (C-C motif chemokine ligand 17 [CCL-17]) in the diseased SC of both infants and adults. Elevated levels of this chemokine in the serum of AD patients is reported to be a biomarker of disease severity (193). Therefore, in future, monitoring the expression of these SC biomarkers using TS may prove a valuable tool for investigating immune or barrier abnormalities from birth. However, this novel TS methodology is not without significant limitations. Many inflammatory markers are below the limit of detection compared to traditional biopsies due to not sampling the underlying dermis (334). In addition, the two studies performed in infants at around one year of age collected between 8 and 16 tape strips for their respective analysis (284, 334). With a thinner stratum corneum present in infants from birth compared to adults, it is unknown whether TS to this number of discs, or to a similar depth for an equivalent analysis, is a feasible option in neonates and infants <1 year old. Over the course of this project, tape stripping to 3 discs depth caused minimal distress to the babies and was generally deemed acceptable by their parents.

*Desquamatory protease activity*

Using these 3 d-squames collected by TS, this project assayed the activity of desquamatory proteases in relation to disease onset. Elevated SC protease activity is a hallmark of AD at both non-lesional and lesional anatomical sites (154, 156, 339). When measured in environmentally controlled conditions in conjunction with biophysical markers of SC development (Chapter 2), this study initially reported that infants with the greatest desquamatory protease activity also possessed the driest, most alkaline and weakest permeability barrier at birth. Vogeli *et al*., reported similar observations in acute eczematous skin, therefore the relationship between elevated protease activity and disease development was further investigated by a longitudinal cohort study (156). This work produced unexpected but novel results. Firstly, desquamatory protease hyperactivity was not associated with a diagnosis of AD at any stage, including when clinical disease was established at 12 months. Skin proteolytic activity attributed to KLK7 in adults is at its highest in active lesions due to disease-associated cytokines such as IL-4 and -13 increasing protein expression and abundance in the SC (155, 156, 183). As our infant cohort was of mild severity only, lacking in clinical signs of disease, it suggests that heightened proteolysis is an acquired consequence of cutaneous inflammation (155, 156, 308) as opposed to a primary barrier abnormality reported by experimental models driving disease (179, 207, 340). Secondly, it was neonates with the lowest initial chymotrypsin-like activity at birth that developed AD. This observation is supported by proteomic analysis of the vernix caseosa that described upregulated expression of the Kallikrein inhibitor Serpin A12 in neonates that developed AD by 24 months; advocating a novel mechanism for the attenuated rates of proteolysis at birth in these at-risk individuals (309). Further examples of protease-

inhibitor dysfunction exist in AD with contrasting biological effects. On one hand a non-synonymous frequent polymorphism (E420K) in *SPINK5* has been associated with AD that impedes the formation of LEKTI fragment D6D9 and subsequently enhances Kallikrein and Elastase-2 proteolytic activity (176, 177). Loss of function mutations in *SPINK5* cause Netherton syndrome; (172) a condition characterised by severe eczema-like lesions with increased KLK5 activity driving PAR2 mediated TSLP expression that accounts for the atopic nature of the disease (173). This trypsin-like protease was not specifically quantified by our project and may relate to inflammatory changes in the skin that precede the development of AD. Conversely, reduced serine protease activity is associated with LEKTI or skin-derived antileucoproteinase (Elafin) overexpression and corneodesmosin retention in hyperkeratotic patient lesions and skin equivalent models (341, 342). With the abundance of endogenous Elafin being detectable in the SC of term and preterm neonates using TS and mass spectrometry, (343) using this technique in conjunction with protease substrates demonstrating an improved affinity for their Kallikrein targets, (294) will allow protease-inhibitor interactions to be better explored in relation to SC development and AD risk.

Limitations

This project produced novel results, but there are limitations to consider. This was a single centre study performed in Sheffield, that recruited largely Caucasians and is not representative of a general population. Local advice from healthcare professionals and consistent referral to secondary care may have influenced the study findings, such as the lack of skin barrier breakdown / disease severity observed from birth. Being an observational study of exploratory nature, topical product use was not controlled for, in that no direction was given prohibiting their use. Although data on parental skin care practices was collected by this study, it was not analysed as part of the key outcome measures reported here, with the intention of future analysis and dissemination by study collaborators in subsequent manuscripts.

The lack of more diverse skin types recruited to the study meant that skin barrier development from birth could not be explored in skin of colour, as the evidence suggests that differences in SC thickness, corneocyte size, ceramide content and proteolytic activity exist between skin phototypes (344). For example, common *FLG* mutations that predispose to AD are less prevalent in darker skin groups where NMF does not follow the same attenuated profile when comparing healthy to non-lesional and lesional skin (345). To further explore this, and the feasibility of community skin testing to inform AD risk, future studies must include a wider range of ethnic groups more representative of the UK population.

One way to address this problem of under representation in future would be to include public and patient involvement when designing the research study. Although not

engaged at the design stage here, this study collected parental views and opinions on skin testing that will inform any future work by the group involving young families. Overall, the methodology used by this study was well accepted by the parents involved. Healthcare professionals such as GP's and midwives will also be consulted in future, with the former being able to provide valuable insight to the clinical presentation of infantile eczema in the local community.

In contrast to the first birth cohort studied (Chapter 2), TEWL was measured in the latter cohort (Chapter 4) at ambient temperatures in contrast to published recommendations (289). Building on the results of the first study, the second longitudinal investigation was designed in this way to reflect the strength of TEWL as a biomarker of disease development reported by Kelleher and colleagues; (102) with a fresh examination of its utility in a community setting providing a benchmark for the additional skin tests to be assessed by. Moreover, by assessing participants at their home, the intention was to aid study recruitment and retention. But the ambient room conditions affected precision; we encountered a 10°C range at birth with an SD of 8.89g/$m^2$/hr across the whole population, compared to the 2.31 g/$m^2$/hr previously noted in climate-controlled conditions. Accounting for temperature by ANCOVA, did replicate the reported increase in developmental TEWL to 4 weeks of age and beyond in later infancy, (93, 102, 260) but a relationship with AD risk was unclear. This is in contrast to elevated TEWL being associated with increased disease risk in infants when performed in room conditions maintained within a 3-5°C range (282, 307, 331). Therefore, in retrospect, by using the machine in this way, it limited continuity between the two populations studied and may have impeded detection of a significant

barrier defect preceding disease onset, if one was indeed present to capture in this population. As this pragmatic study was primarily an investigation of novel methodology to monitor skin barrier development from birth, it was not powered or even originally intended to provide a definitive answer on the diagnostic utility for AD. If TEWL is later found to be suitable for this purpose, its dependence on climate is an important practical consideration that may impede its widespread clinical use.

Due to the interference of surface sebum, the study was also unable to clearly investigate the presence of a SC lipid abnormality using FTIR, as a key biomarker of infant skin barrier breakdown (152). More severe infant disease is characterised by deteriorated lipid lamellae structure, in conjunction with reduced expression of key mediators such as ELOVL3; diacylglycerol o-acyltransferase 2 (DGAT2); FAR2 and FA2H (187). Experimentally, the $T_H2$ cytokine IL-4 inhibits ceramide synthesis via decreased mRNA expression of acid sphingomyelinase and glucocerebrosidase (227, 228). Therefore, as previously discussed, due to the lack of overt severe disease and clinical inflammation in our cohort, it can be presumed that an acquired defect in lipid structure would also have been undetectable from birth.

Clinical Implications and Future work

It can be argued that the subtle evidence of skin barrier dysfunction noted was proportional to the severity of the cohort. It is well accepted that in established AD, TEWL is elevated compared to controls; but the degree of this skin barrier defect is dictated by greater disease severity (147, 148, 302, 346). This is also the case for protease activity and NMF components at non-lesional sites in adults (see Appendix Figure 6.8, page 175). For context, the investigations reporting a link between early TEWL and a future AD diagnosis do not present data on severity, and may contain a greater number of more severe cases (282, 307, 331). Given the clear barrier abnormality present once clinical disease is established, a key strategy here would be to recruit and assess a cohort of infants with more severe disease from the Dermatology clinic, to assist with placing our developmental findings into context (187, 284, 334).

It is reported that around 80% of all AD cases are of mild severity in children between 1-5 years of age (347, 348). A closer inspection of the BEEP and Enquiring About Tolerance (EAT) infant cohorts also reveals a high prevalence of mild disease, further placing our cohort and findings into perspective (302, 303). For example, if components of a prominent acquired barrier abnormality were not established by 12 months of age due to mild disease, it is unlikely these biomarkers will be predictive of onset. Likewise, the evidence of a more subtle developmental trajectory preceding disease in this study, provides a plausible contributory factor for the null findings of BEEP and PreventALL; in that a significant barrier defect was not sufficiently present across all early-onset AD cases for a population-level intervention to be successful. In

light of these findings, 20 babies with early onset AD were not enough, with insufficient power to distinguish a more subtle skin barrier defect between cases and controls in the face of predominantly mild disease. As primarily an investigative study, piloting novel applications and measures of skin barrier function, a cohort greater than the 180 babies recruited was not ethical or feasible. Moving forward, by screening more babies it can be determined whether the diagnostic potential of this portfolio of tests is (a) applicable to a more general population including non *FLG* LOF mutation carriers; and (b) can be overall improved by accounting for individuals with greater disease severity to detect a greater acquired barrier defect. Assessing more babies however to pick up AD cases of greater severity will be no small task; as the BEEP study screened 4963 families to pick up just 19 cases of moderate, severe or very severe AD cases by 24 months measured by EASI (303). The EAT study cohort reported over 80% of its AD cases as mild when measured by SCORAD (302). It is these infants though of early-onset persistent disease of greater severity that are at elevated risk of food allergy (300-302). The precise detection of these individuals from birth remains an unmet clinical need that could be fulfilled by the contribution of these diagnostic skin tests performed in a community setting from birth. If detectable, accounting for inflammatory biomarkers such as the $T_H2$ chemokine CCL-17 in conjunction with monitoring significant environmental exposures that modify disease risk, (240) may ultimately be required to more accurately predict early disease onset in a general infant population.

In European infants, common LOF *FLG* mutations confer a significant increased risk of an AD diagnosis within the first year of life (304, 305). It was also the case in this study, and reinforces a role in the pathogenesis of early disease onset in this demographic. Animal models such as the flaky tail mouse have provided a rich source of mechanistic evidence on how a primary epidermal FLG deficiency manifests as a weakened permeability barrier primed for allergic inflammation and lesional AD (218, 219). In infant *FLG* LOF mutation carriers, weakened permeability barrier function (dry skin and elevated TEWL) manifests in early life prior to clinical disease development (304, 305). To support this, we can add that a subsequent NMF defect also exists at the skin surface in these individuals that is moderately predictive of genotype. Further work is required to understand the mechanisms underpinning transition from risk genotype to disease development – such as early exposure to hard water (349) – but an alternative approach to prevention strategies in a general population based on the emerging evidence, may be a personalised medicine approach through the early identification and correction of the inherited FLG defect from birth in this clinical endophenotype (236). Mounting evidence in adults and children suggest that these individuals also demonstrate differential levels of skin barrier repair in response to topical treatment regimens for AD (265, 350).

With further refinement, if measuring NMF abundance by FTIR proves a suitable diagnostic technique for predicting risk by rapid *FLG* genotyping at the bedside, also key to this personalised approach would be the selection of a suitable emollient for intervention; as it is becoming progressively clear that not all emollients exert equal effects on the skin barrier. For example, compared to simple paraffin-based

emollients, more advanced formulations containing humectants have a proven ability for barrier repair, including correcting the NMF defect (351). This is in stark contrast to the widely used emollient Aqueous cream, that has been associated with irritant reactions in children with AD (352). A randomised controlled trial has recently been conducted in high-risk infants to investigate the use of an emollient that contains NMF components arginine and PCA in the prevention of AD from birth (353). Due to impeded recruitment and loss to follow up issues, its findings were inconclusive and further highlights the significant challenges faced when performing studies in this demographic.

Caution must be exercised though, as in addition to commonly used emollients not protecting against eczema development in infants, there is emerging evidence that their early use from birth is associated with the development of food allergy (FA). In this study, (346) the authors reported a dose-dependent relationship between application frequency at 3 months of age with increased FA risk and hypothesised that emollients may facilitate allergen penetration from caregivers' hands, or weaken the developing skin barrier sufficiently to allow epicutaneous transfer and sensitisation (354). In the case of olive oil – the most frequently applied emollient with which this FA association was made (354) – a high composition of the natural penetration enhancer oleic acid, is associated with damage to the adult skin barrier (312) and may delay normal SC maturation when used for infant massage from birth (250). With evidence mounting of more complex emollient formulations enhancing SC structure and protecting from skin irritation, (355) the appropriate choice of emollient to treat

dry skin in infancy should also support healthy skin barrier development to guard against an increased potential risk of FA.

Conclusions

By assessing biomarkers of skin health, terminal differentiation and molecular structure in neonates to further characterise development, this study builds on prior evidence to confirm the biophysical, biological and biochemical properties of the infant SC are in flux compared to adults. This fragility over the first year of life helps explain why infant skin is more susceptible to dryness, erythema and infection, reinforcing the need for careful infant skin care practices that gently support healthy skin barrier development from birth.

This inherent fragility coincides chronologically with increased AD risk; therefore it was hypothesised that differential skin barrier development defined by breakdown, precedes, and is predictive of disease onset from birth. An early AD diagnosis was associated with a primary *FLG* defect; so by developing and validating FTIR for the measurement of NMF over the course of this project, here is a technique that offers some diagnostic potential at the bedside, as a rapid alternative to laboratory genotyping for the detection of this clinical endotype from birth. Furthermore in adults, this methodology can inform on the acquired NMF defect in AD, paving a way for the non-invasive monitoring of subclinical inflammation following treatment regimens. In infants from birth, owing to mild disease, barrier breakdown was subtle and skin testing alone was of limited additive utility for predicting disease onset in a high-risk population. This was an exploratory investigation only and further work is necessary, but these predictive biomarkers may only be of clinical use to reliably identify more moderate-severe cases of AD from birth that are at risk of further atopy. Although a common disease, the prominence of mild cases in early-onset AD,

combined with the associated subtle barrier defects noted here, suggests one plausible reason for the null findings of recent high-profile prophylactic emollient studies to correct barrier dysfunction and prevent or delay disease onset from birth.

# CHAPTER 6: APPENDIX

## 6.1 MATERIALS AND METHODS - SUPPORTING INFORMATION

### 6.1.1 Quantification of free amino acids by o-phthalaldehyde assay

Validation of the plate-based assay described by Nakagawa et al (42) for the quantification of free amino acids using o-phthalaldehyde derivatisation.

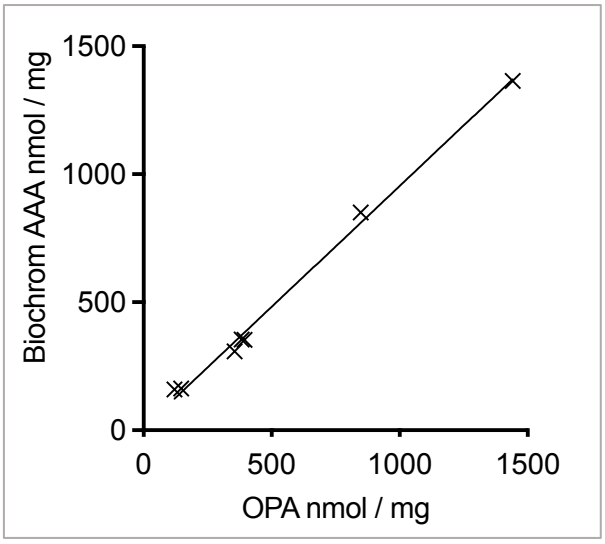


**Figure 6.1:** Relationship between two methods of free amino acid quantification. NMF was extracted from SC samples collected by tape stripping from seven healthy adults by the method described (Chapter 3 Materials and Methods). Free amino acid content from each sample was measured by amino acid analyser (Biochrom Ltd, Cambridge, UK) and o-phthalaldehyde derivatisation, normalised to protein content measured by densitometry and plotted for comparison (nmol/mg). Linear regression confirmed excellent agreement between the two methods ($R^2$=0.995). Due to comparative speed and ease of the plate-based o-phthalaldehyde assay, it was selected to quantify free amino acids from clinical samples collected by tape stripping.

6.1.2 Assay normalisation: Relationship between infrared absorbance (850nm) and protein content (µg) on 14mm discs collected by tape stripping

The following figure describes a repeat of the work reported by Voegeli *et al.*, to validate the use of densitometry for the quantification of SC mass removed by tape stripping using 14mm d squame discs.

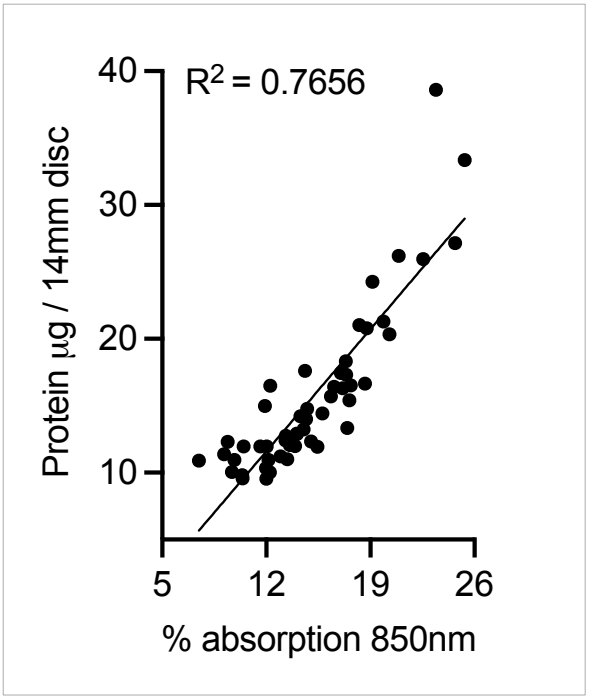


**Figure 6.2:** Relationship between infrared absorbance and protein content on 14mm discs. SC samples collected by tape stripping (13 discs) was performed in four adults with healthy skin and absorbance at 850nm was measured using an infrared densitometer (SquameScan™, Heiland Electronic, Wetzlar, Germany). Total protein was extracted from each disc using 1M NaOH for one hour with agitation and neutralised with 1M HCL. Protein quantification was performed using a QuantiPro™ BCA assay kit and bovine γ-globulin standard (Merck Life Science UK Ltd., Dorset, UK). Plot of absorption vs extracted protein presented. Linear regression determined a good agreement between the two methods ($R^2$ =0.7656). The subsequent equation y=1.305x-4.07 was used to calculate SC mass removed by tape stripping from absorbance measurments for the purpose of infant protease and NMF assay normalisation.

## 6.2 CHAPTER 2 - SUPPLEMENTARY RESULTS

| | Birth | 4 weeks | Mean diff (95% CI) | *p* value |
|---|---|---|---|---|
| **TEWL (g/m$^2$/h)** | 12.61(±2.3) | 13.38(±3.0) | 0.82(-0.33,1.96) | 0.1570 |
| **SCH (RCU)** | 16.14(±3.8) | 41.79(±9.7) | 25.65(22.21,29.08) | <0.0001 |
| **Skin-surface pH** | 6.05(±0.6) | 4.98(±0.3) | -1.05(-1.26,-0.84) | <0.0001 |
| **SC cohesion (∫g/3discs)** | 267(±67) | 305(±89) | 37.6(-1,77) | 0.0577 |
| **Chymotrypsin-like activity (nU/∫g)** | 1.12(±0.7) | 1.70(±0.9) | 0.58(0.17,0.99) | 0.0068 |
| **NMF (nmol/mg)** | 221(±198) | 2330(±1415) | 2109(1623,2595) | <0.0001 |

**Supplementary Table 6.1:** The biophysical and biological properties of the developing infant forearm stratum corneum (SC) from birth to 4 weeks of age. In contrast to Chapter 2 showing the full cohort, here, the OBSeRvE no treatment group is presented only (*n*=35). This group has measurements taken at birth and again at 4 weeks to assess skin barrier development longitudinally over time. Statistical significance was determined using a paired students t test. RCU: relative capacitance units.

## 6.3 CHAPTER 3 - SUPPLEMENTARY RESULTS

### 6.3.1 PLS model outputs according to mode of normalisation

Comparable results were observed for each different mode of Amide normalisation

| | **1640cm$^{-1}$ normalisation** | | | | **1540cm$^{-1}$ normalisation** | | | |
|---|---|---|---|---|---|---|---|---|
| | Healthy | AD | WT | FLG | Healthy | AD | WT | FLG |
| **Calibration ($R^2$)** | 0.72 | | | | 0.71 | | | |
| **Validation ($R^2$)** | 0.72 | | | | 0.71 | | | |
| ***in vivo* tNMF FA site 1 (µmol mg$^{-1}$)** | 0.97 | 0.70 | 0.91 | 0.71 | 0.97 | 0.71 | 0.90 | 0.77 |
| ***in vivo* tNMF CF site 2 (µmol mg$^{-1}$)** | 1.38 | 0.74** | 1.27 | 0.66* | 1.37 | 0.73** | 1.25 | 0.71* |

**Table 6.2:** Comparison of model outputs using alternative Amide normalisation modes prior to modelling. FA: forearm; CF: antecubital fossa. Asterisks denote the result of a paired students t test (Healthy compared to AD; WT compared to FLG).**$p$=<0.01, *$p$=<0.05.

## 6.3.2 *In vivo* modelling of NMF abundance by FTIR

There was no discrimination of clinical phenotypes by NMF measured on the forearm

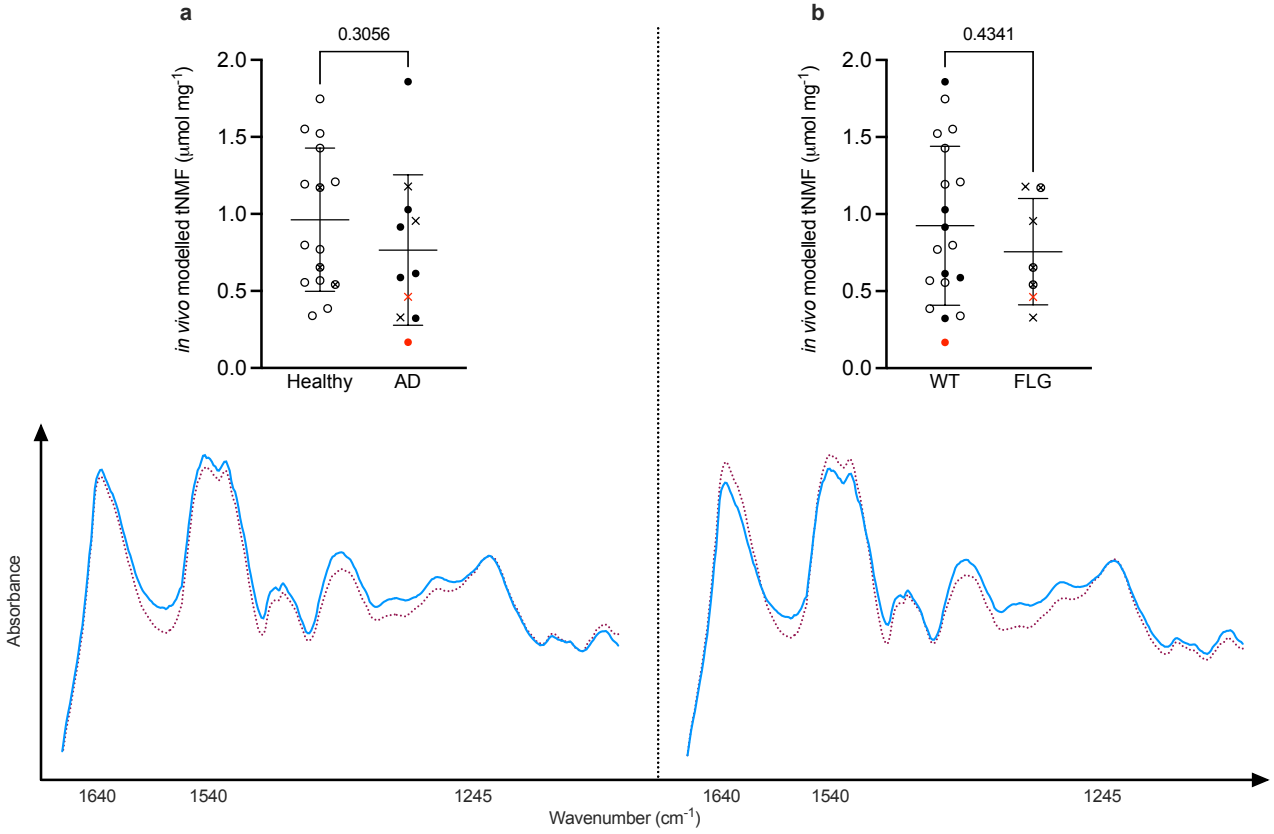


**Figure 6.3:** Evaluation of *in vivo* modelled surface tNMF at the forearm. No significant differences in mean tNMF was found at the forearm using an unpaired students t test for (a) healthy compared to AD and (b) wild type compared to *FLG* LOF mutation carriers. Corresponding mean FTIR spectra shown below each graph. Blue line = healthy (left) and wild type (WT-right). Red dotted line = atopic dermatitis (AD-left) and *FLG* LOF mutation carriers (right). Please refer to Figure 3.2 for key.

## 6.4 CHAPTER 4 - SUPPLEMENTARY RESULTS

### 6.4.1 Skin barrier development from birth to 12 months in infants

Comparable changes in TEWL and the skin parameters measured by FTIR were observed on the forearm, antecubital fossa and thigh.

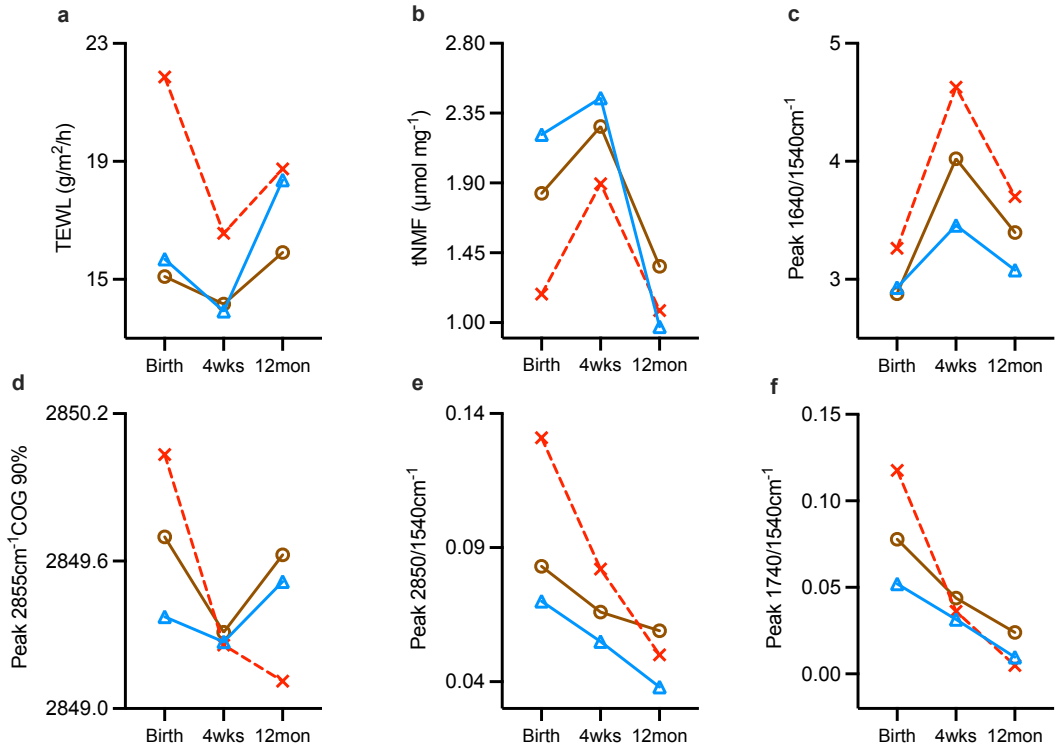


**Figure 6.4:** Different anatomical sites show comparable development at the skin surface from birth. Median change over time in (a) TEWL, (b) *in vivo* modelled tNMF, (c) water content, (d) lipid structure, (e) total lipid and (f) triglycerides at the forearm (brown circle), right antecubital fossa (red cross) and right thigh (blue triangle). A single TEWL or FTIR reading from each anatomical site was collected per infant. Forearm shows the average of the right and left side (FTIR only).

6.4.2 *In vivo* modelling of NMF by infrared spectroscopy in infants

Fourier Transform Infrared (FTIR) spectroscopy is a suitable tool for the chemometric analysis of NMF at the skin surface. Here, using an *ex vivo* skin sample collected by tape stripping (discs 1-3) and analysed for NMF by laboratory assay, the fingerprint spectral region (1090-1654cm$^{-1}$) of complimentary FTIR spectra could be calibrated to provide an instantaneous *in vivo* estimation of NMF. A plot of *ex vivo* versus *in vivo* modelled tNMF in 20 infants assessed at the forearm and antecubital fossa showed acceptable within-model accuracy for calibration and validation sampling points (Figure 6.5a and b). Next, in an independent subset of infants of the same age (*n*=21) the *in vivo* model was further validated at the same anatomical sites (Figure 6.5c). Again, an acceptable degree of model accuracy was observed ($R^2$=0.65) confirming its suitability for assessing NMF abundance in infants. By using this model to spectroscopically determine NMF in the wider STAR cohort, at 4-weeks of age, reduced levels of NMF at skin surface (mean difference 0.67 µmol mg$^{-1}$, 0.29-1.04 95% CI) proved to be moderately predictive of *FLG* genotype (Figure 6.5d and e).

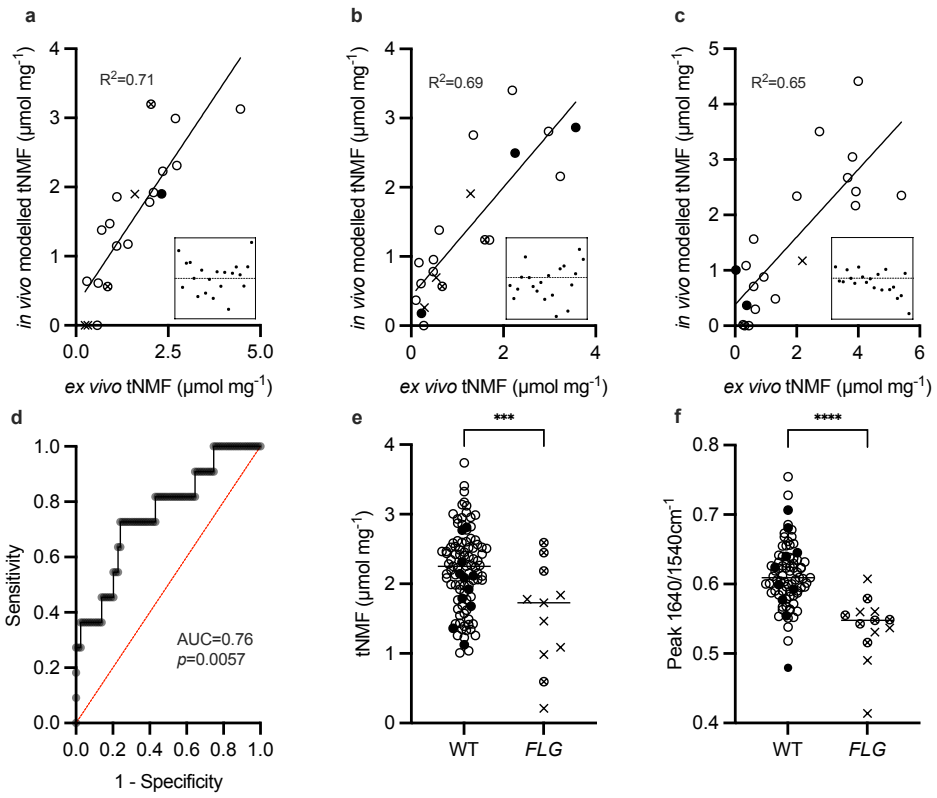


**Figure 6.5:** *In vivo* modelling of NMF at 4-weeks and 12-months of age by infrared spectroscopy. A single, skin surface *in vivo* FTIR spectrum collected at 4-weeks (forearm) and 12-months (antecubital fossa) was calibrated against an equivalent *ex vivo* laboratory analysed sample of pooled surface d squames (discs 1-3) using partial least squares regression to model a spectroscopic estimation of NMF abundance. (a) Plot of *in vivo* modelled versus *ex vivo* quantified tNMF (the sum of free amino acids, pyrrolidone carboxylic acid and urocanic acid) in a subset of infants for model calibration and (b) model validation sampling sites (*n*=20). (c) The NMF model was further validated in an independent cohort of infants (*n*=21). (d) In the wider STAR cohort (*n*=110) *in vivo* modelled tNMF at 4-weeks of age was moderately predictive of *FLG* genotype. On average, infants carrying at least one common *FLG* LOF mutation had significantly reduced levels of (e) *in vivo* modelled tNMF and (f) water content at the skin surface by 4-weeks of age. Open circle = no AD; filled circle = confirmed AD; cross = confirmed AD carrying a *FLG* LOF mutation; open circle with cross = no AD carrying a *FLG* LOF mutation. $R^2$ = the coefficient of determination. AUC= area under the curve following simple logistic regression using modelled tNMF as the independent variable. Asterisks denote the result of an unpaired student t test (***$p$=<0.001).

6.4.3 FTIR measurements and sebum

In an attempt to remove the interference of sebum from the skin surface, a commercial baby wipe was used prior to a further series of FTIR measurements being performed. Using the peak height at 1740cm$^{-1}$ as a measure of lipid ester deposition, (292) this procedure of wiping the skin proved unsuccessful for the complete removal of sebum at each time point studied. The decision was therefore made to discard the collection of post wipe measurements from the analysis of key FTIR study objectives.

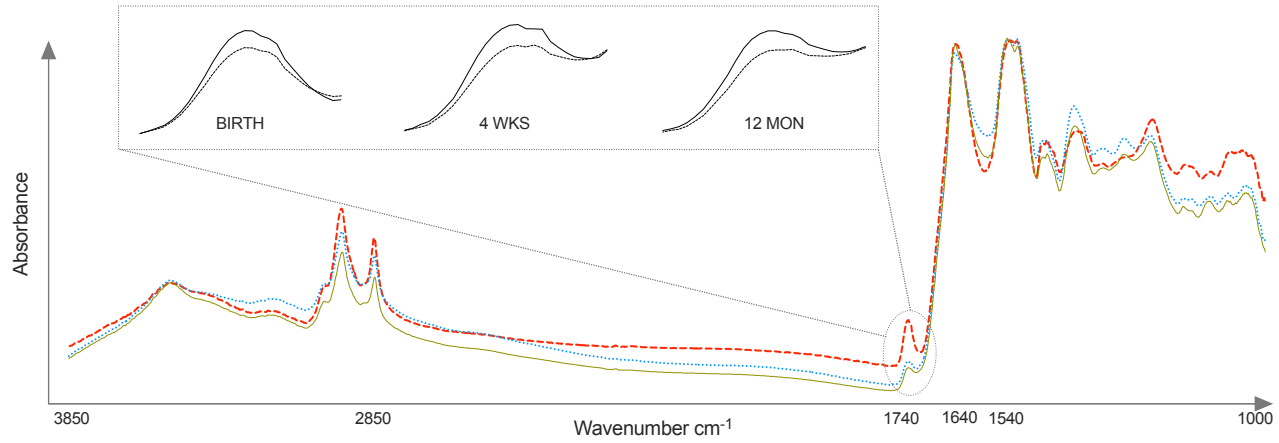


**Figure 6.6:** Mean FTIR spectra collected at the skin surface show clear differences in absorption between developmental time points at birth (red dashed line) four weeks (blue dotted line) and 12-months of age (green solid line). The mean of both forearms, the right antecubital fossa and right thigh is presented for each time point. Assessing the mean peak height at 1740cm$^{-1}$ (C=O vibration of triglycerides) both before (solid line inset) and following the use of a commercial baby wipe (dotted line inset) revealed the incomplete removal of sebum from the skin surface at each time point.

6.4.4 Consistent developmental changes were observed at each anatomical site between infants that did and did not develop AD

For both TEWL and FTIR measurements, the median difference between infants that did and did not develop AD was generally consistent across time at all the anatomic sites studied. With this in mind, the mean of these body sites was calculated to provide one measurement per infant, per timepoint, with the goal of achieving greater precision to address the secondary endpoints of AD risk.

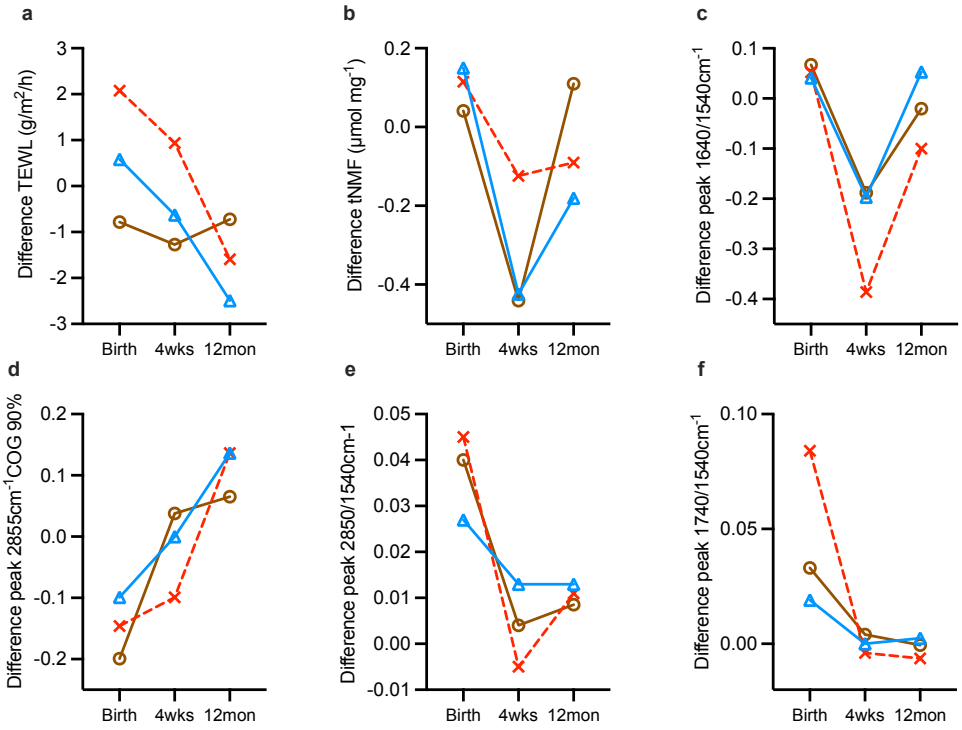


**Figure 6.7:** Anatomical sites show comparable differences over time between infants that did and did not develop AD at the skin surface. Median change (AD-no AD) over time in (a) TEWL, (b) *in vivo* modelled tNMF, (c) water content, (d) lipid structure, (e) total lipid and (f) triglycerides at the forearm (brown circle), right antecubital fossa (red cross) and right thigh (blue triangle). A single TEWL or FTIR reading from each anatomical site was collected per infant. Forearm shows the average of the right and left side (FTIR only).

6.4.5 Logistic regression modelling of FTIR parameters associated with AD in infants

In an attempt to improve the quality of the spectroscopically derived data, the threshold for inclusion was tightened to model parameter means collected only from infants with a successful FTIR reading at every anatomical site (4/4) (Table 6.2). Using this refined criterion, modelling the 2850/1540cm$^{-1}$ peak (lipid/protein) ratio at birth offered the greatest overall prediction of AD (model 1, AUC=0.86). This model at a 50% cut off correctly classified 33% and 95% of cases with and without AD respectively. The period of gestation also featured in this model, as each additional day before labour conferred a 10% increased odds of disease. Further modelling to combine these variables with *FLG* was attempted but not possible due to regression model convergence failure.

| | **Model 1** | **Model 2** |
|---|---|---|
| **No AD *n* =**<br>**AD *n* =** | 55<br>15 | 91<br>23 |
| **Parameter** | **2850/1540cm$^{-1}$ (B)** | **tNMF (4W)** |
| | 2.05 (1.05-4.48) 0.05 | 0.44 (0.20-0.96) 0.04 |
| | - | - |
| | - | - |
| **Sex** | 0.48 (0.12-1.78) 0.28 | 0.58 (0.20-1.07) 0.30 |
| **Gestation** | 1.09 (1.01-1.19) 0.03 | 1.10 (0.86-1.21) 0.64 |
| **1$^{st}$ atopy** | 6.12 (0.92-125) 0.11 | 6.98 (1.30-130) 0.07 |
| **AUC** | 0.86*** | 0.72** |

**Table 6.3:** Forward selection modelling by logistic regression of parameters associated with AD risk by 12-months of age. Meaned spectroscopic parameters from infants with four successful readings (from four possible body sites) are presented. Odds ratio (95% confidence interval) and *p* value shown. B=birth and 4W=four weeks of age. 1$^{st}$ atopy=at least one first degree relative with atopic disease. AUC=area under the curve.

## 6.5 CHAPTER 5: FINAL DISCUSSION - SUPPLEMENTARY RESULTS

### 6.5.1 Relationship between TEWL, protease activity NMF and disease severity

The degree of the skin barrier defect at non-lesional sites is dictated by greater disease severity.

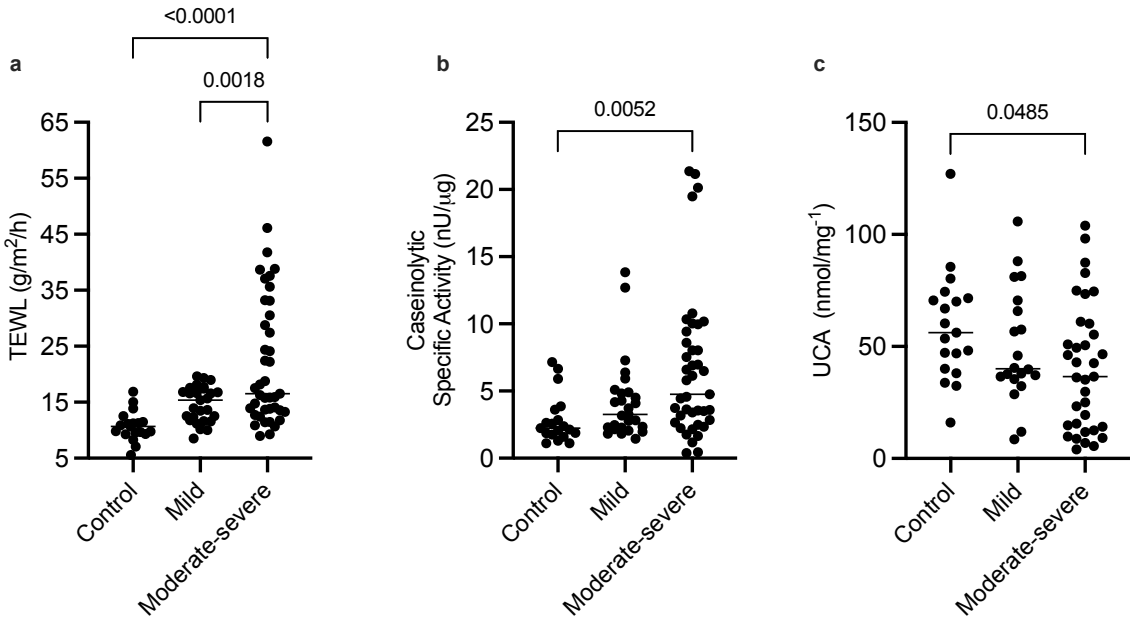


**Figure 6.8:** Relationship between TEWL, protease activity and NMF with disease severity in adults. A cohort of subjects with mild (*n*=27) or moderate-severe AD (*n*=41) assessed by objective SCORAD was compared to healthy controls (*n*=19) with no history of atopic disease, using the methodology described throughout this project. (a) Baseline TEWL and (b) caseinolytic protease activity (discs 1-3) was found to be significantly elevated, while urocanic acid significantly reduced (UCA discs 4-6) in subjects with moderate-severe disease compared to healthy controls. *p* values denote the result of a Tukey's post-test following a one-way analysis of variance (ANOVA).

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