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Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin

Mogbekeloluwa Oluwadamiloju Danso-Eweje
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Mogbekeloluwa Oluwadamiloju Danso-Eweje
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Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin

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Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin

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Contents

6 CHAPTER 1
Introduction

30 CHAPTER 2
Altered expression of epidermal lipid bio-synthesis enzymes in atopic dermatitis skin is accompanied by changes in stratum corneum lipid composition

62 CHAPTER 3
TNF-α and T_{h2} cytokines induce atopic dermatitis-like features on epidermal differentiation proteins and stratum corneum lipids in human skin equivalents

98 CHAPTER 4
Exploring the potentials of nurture: 2nd and 3rd generation explant human skin equivalents

132 CHAPTER 5
Explant cultures of atopic dermatitis biopsies maintain their epidermal characteristics in vitro

150 CHAPTER 6
An ex vivo human skin model for studying skin barrier repair

182 CHAPTER 7
Summary and Perspectives

196 CHAPTER 8
Nederlandse samenvatting
List of publications
Curriculum Vitae

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CHAPTER 1

Introduction
HUMAN SKIN

The skin is the principal contributor of i) protection of the body’s interior from evaporative water loss to the external environment and ii) hindrance of the infiltration of allergens, toxins and pathogenic microbes (1, 2). It also functions to regulate the body’s temperature and water loss and is composed of three main layers (listed from outside to inside): the epidermis, dermis and subcutaneous fat tissue (hypodermis).

Epidermis
Keratinocytes are the main cell type located in the epidermis. Other cell types are also located in this layer, such as melanocytes, Langerhans cells and Merkel cells. The epidermis is stratified into four main layers i.e. stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC) with the SB connected to the basement membrane which separates the epidermis from the dermis (figure 1). The keratinocytes migrate from the SB to the SC during the process of differentiation (3). Keratinocytes that migrate to the SC become cellular skeletons without nuclei and organelles. These are referred to as corneocytes. These cells are surrounded by a cornified envelope with a monolayer of lipids covalently attached to it. In between these terminal differentiated dead cells the extruded lipids form a very dense lipid organization.

Figure 1: Cross section of skin
The skin is mainly divided into the epidermis, dermis and subcutaneous fat layer. The epidermis is composed of four main layers: stratum basale, stratum spinosum, stratum granulosum, stratum corneum. Keratinocytes are the predominant cell type of the epidermis. Other cell types present in the epidermis include melanocytes and Langerhans cells (LC). The dermis is composed of connective tissue and fibroblasts. Adapted from Janssens et al., 2012 (4).
**Epidermal permeability barrier in native human skin**

The SC provides the skin permeability barrier. The SC is 10-15µm in thickness and comprises of a multilayered structure of the flattened corneocytes surrounded by an intercellular lipid matrix. The lipid envelope serves as a boundary between the hydrophilic corneocytes and the lipophilic lipid matrix. This organization of the SC is often referred to as a “brick” and “mortar” structure in which the corneocytes are the bricks and the lipids, the mortar (5-7). The lipid matrix is enriched with ceramides (CERs), cholesterol (CHOL) and free fatty acids (FFAs) that are present at approximately equimolar levels (8-10). The presence of the intercellular lipid domain is vital in maintaining the permeability barrier of the skin as a number of studies report the intercellular SC lipids as the transport route for compounds through the SC (11, 12). This therefore highlights the important role of the SC lipids in maintaining an intact skin barrier (11-13). The SC lipids are organized in lamellae stacks (figure 2). Two lamellar phases have been identified with repeat distances of approximately 13nm and 6nm referred as the long periodicity phase (LPP) and short periodicity phase (SPP) respectively (14-16). The LPP is considered to be important for the skin barrier function as shown by *in vitro* studies using lipid membranes as SC substitutes (17). The lateral organization of the lipids is in the plane perpendicular to the lamellar phases. In this plane, in human SC the lipids adopt mainly an orthorhombic packing, but hexagonal domains are also present (18-22).

Within the epidermal strata, tight junction (TJ) proteins are known to contribute to the inside-outside barrier. They form i) an intercellular barrier between the epidermal cells ii) function to control the selective movement of water and ions through the epidermis (23) and iii) regulate cell proliferation and differentiation (24, 25). Most epidermal tight junctions are located in the stratum granulosum (26) where they form a barrier for molecular tracers (27, 28) e.g. Claudin-1, ZO-1 and occludin.
Figure 2: Lipid organization in human stratum corneum

The stratum corneum (SC) is the topmost layer of the epidermis consisting of corneocytes surrounded by an organized intercellular lipid matrix. The lipids are organized in two lamellar phases: the long periodicity phase (LPP) and the short periodicity (SPP) phase with a repeat distance of 12nm and 6nm respectively. Perpendicular to the lamellar organization, the lipids are organized in a lateral lipid organization. The lipids can be arranged in a liquid (disordered), hexagonal (less dense and ordered) and an orthorhombic (very dense and ordered) organization. This image is adapted from Thakoersing et al., (29)
Epidermal lipid synthesis

De novo FFA synthesis
This process is initiated by carboxylation of acetyl co-enzyme A (CoA) to malonyl CoA and covalently bond malonyl-CoA C2 units by acetyl-CoA carboxylase and fatty acid synthase respectively (30). This biosynthesis produces palmitoyl-CoA (FFA C16) which can be elongated by elongases (ELOVL1-7) (31-33) or unsaturated to mono-unsaturated FFAs (MUFA) by steroyl CoA desaturase (SCD) (34). The elongation of FFAs by elongases (ELOVL) takes place in the endoplasmic reticulum and occurs via two carbons per cycle (32, 35) resulting in chain lengths up to FFA C36 (figure 3). These elongases include ELOVL1 and ELOVL4 which elongate FFAs with carbon chain length of C20-C26 and ≥C26 respectively (31, 36-38). ELOVL 3 elongates FFA C18-C20 whereas ELOVL6 elongates FFA C16 to generate FFA C18. FFA C24 is the most abundant FFA in human SC. In addition, FFAs with an additional hydroxyl group are present in human SC (39). The synthesised FFAs can be used in CER synthesis and then stored in the lamellar bodies (LBs) or transformed into phospholipids. During lamellar body extrusion at the SG-SC interface, secretory phospholipase A2 converts phospholipids back to FFAs (40).

De novo CER synthesis
CERs are composed of a FFA and a sphingoid base chemically linked by an amide bond. CERs account for 50% (by weight) of the total lipid species in the SC (41, 42) and are key to i) forming and maintaining the epidermal barrier and ii) regulating cellular signalling in human epidermis (cell proliferation, apoptosis and differentiation) (43, 44). De novo CER synthesis begins at the endoplasmic reticulum by the condensation of L-serine with palmitoyl-CoA to generate an 18 carbon sphingoid base (45-47). This reaction is catalysed by serine palmitoyltransferase (SPT) producing 3-ketosphinganine which is reduced to dihydrosphingosine. Dihydrosphingosine is subsequently N-acylated to a FFA by ceramide synthase (CerS) to produce dihydroceramides (figure 3). Six CerS (CerS1-6) have been identified in mammals and each CerS displays preferences towards FFA chain length and saturation (48-50). Dihydroceramides are then dehydrogenated by dihydroceramide desaturase (DES) to form CERs. CERs are transported to the Golgi apparatus where they are converted to sphingomyelin and glucosyl CERs and thereafter transported into the LBs by transporters such as ABCA12 (51, 52). The lamellar bodies remain and accumulate in the keratinocytes. When the keratinocytes reach the SG-SC interface, the lamellar bodies are extruded into the intercellular regions. During this process sphingomyelin and glucosyl-CERs are converted back to CER species by acid-sphingomyelinase (aSmase) and β-glucocerebrosidase (GBA) respectively (53).

Fifteen CER subclasses have been identified in human SC and can be classed according to the fatty acid and sphingosine moiety (54). The variation in fatty acid moiety results in non-hydroxy CERs, α-hydroxy CERs, ω-esterified (EO) CERs and 1-O-Acyl CERs (figure 4). In addition to the variation in CER subclasses, CERs also differ in their chain length ranging from 34 to 72 carbon atoms in healthy human SC (8, 55).
Figure 3: Illustration of some important stages in the free fatty acids and ceramide synthesis
Arrows show the conversion of lipids catalysed by enzymes which are displayed close to the arrows. Abbreviations: ELOVL: elongase, CerS: ceramide synthase, GBA: glucosylcerebrosidase, aSmase: acid-sphingomyelinase, SCD: stearoyl CoA desaturase, ωh: omega-hydroxy, GCS: Glucosylceramide synthase.
CERs are composed of a fatty acid and a sphingoid base chemically linked by an amide bond. Fifteen CER subclasses have been identified in human SC and can be classed according to the fatty acid moiety and sphingoid base. The fatty acid moiety includes non-hydroxy CERs, α-hydroxy CERs, ω-esterified CERs and 1-O-Acyl CERs, the sphingoid base includes sphingosine (S), phytosphingosine (P), 6-hydroxy-sphingosine (H), dihydrosphingosine (dS) and dihydroxy-sphinganine (T). Adapted from Rabionet et al., (54)
ATOPIC DERMATITIS

Epidermal barrier dysfunction in AD
AD skin (lesional and non-lesional) displays barrier impairment as shown by increased trans-epidermal water loss (TEWL) and increased penetration of compounds, allergens and pathogens through the skin (outside-inside barrier) (56-59). In addition to the permeability barrier, the antimicrobial barrier is also compromised in AD patients resulting in the colonization by pathogenic flora such as *Staphylococcus aureus* and reduction in antimicrobial peptide levels (60-61). The barrier defects in AD skin have been reported to stem from increased epidermal proliferation, abnormal epidermal differentiation and SC lipid abnormalities (4, 56, 63-65). Factors contributing to the barrier dysfunction in AD include: mutations in skin barrier related genes (e.g. filaggrin), inflammation and the environment.

Filaggrin mutations
In 2006, Palmer et al., (66) reported that 20% of AD patients carry “loss-of-function” mutations in *FLG*. According to Irvine et al., 23 variations of *FLG* mutations have been identified in Europe and 26 in Asia some of which occur in both cohorts (67). R501X and 2282del4 are the most prevalent in AD patients in Europe. These two mutations have been suggested to predict more severe loss of function of *FLG* due to their proximal location within *FLG* (68-71). *FLG* mutations are a high predisposing factor for the development of atopic diseases including AD, allergic rhinitis, contact and food allergies and asthma (72). *FLG* is located on chromosome 1q21 in the epidermal differentiation complex (73). Filaggrin (filament aggregation protein), is derived from a 400kDa polyprotein profilaggrin (encoded from *FLG*) which is phosphorylated and cleaved during keratinocyte differentiation to 10-12 filaggrin molecules. The filaggrin monomers align and aggregate keratin bundles and thus contribute to the strength, compactness and integrity of the corneocytes in the SC (74).

In the follow up process filaggrin is degraded into its constituent amino acids (arginine, glutamine and histidine) and amino acid derivatives by caspase 14 and other enzymes (75). These amino acids can be further degraded into natural moisturizing factors (NMFs) such as 2-pyrrolidone-5-carboxylic acid (PCA) and trans-urocanic acid (UCA) which hydrate the SC, modulate immune function and protect against ultraviolet radiation (76-78). NMFs also affects SC pH which may be important for cohesion of SC, antimicrobial defense, permeability barrier homeostasis, serine proteases and ceramide metabolism (79, 80).

INFLAMMATION IN AD

Acute AD
The enhanced penetration of exogenous allergens into the skin immediately activates Langerhans cells which then acquire an antigen presenting phenotype, reduce their capacity to capture antigens and instead express receptors which allow their migration to the draining lymph nodes e.g. CCR7 receptor. The Langerhans cells produce non-cytotoxic cytokines e.g. IL-4, IL-5, IL-10 and present the allergen peptides to naive CD4+ T-cells which drives the differentiation of the T-cells towards a T\(_\text{H}2\) response (81, 82). The T\(_\text{H}2\) driven T-cells preferentially produce cytokine such as IL-4, IL-5, IL-6, IL-9, IL-31 and IL-13. T\(_\text{H}12\) pathway
has also been shown to be activated in acute AD lesions (83). The T\(_{h2}\) cytokines, IL-4 in particular, initiate a cascade of T\(_{h2}\) transcriptional factors (GATA-3, STAT-6 and c-maf), which further drive the differentiation of naive T-cells to the T\(_{h2}\) pathway, and inhibit the expression of T\(_{h1}\) transcription factors (84, 85). IL-4 and IL-13 are responsible for recruitment of T\(_{h2}\) cells, initiation of IgE synthesis and act as growth and differentiation factors of lymphocytes, mast cells and basophils (86, 87). Thymus-and activation-regulated chemokine (TARC), which is produced by Langerhans cells, initiates the process of the recruitment of CD4+ T\(_{h2}\) cells into the epidermis and activation of eosinophils to maintain the T\(_{h2}\) phenotype (88, 89). Keratinocytes also play a role in the inflammation in AD skin by producing chemokines and cytokines including thymic stromal lymphopoietin (TSLP) which activate dendritic cells to prime naive T-cells to a T\(_{h2}\) immune pathway (90). The activation of T\(_{h2}\) immune pathway leads to the formation of early AD skin lesions displayed by erythematous papules, exudation, excoriation and pruritus with the latter being linked to IL-31 (91). These epidermal and inflammatory characteristics described are significantly higher in AD lesional skin compared to non-lesional AD skin (figure 5).

**Chronic AD**

In chronic AD, there is a switch in the flavor of T-cells from T\(_{h2}\) to T\(_{h1}\) phenotype and therefore the lesions contain less T\(_{h2}\) cytokines and higher levels of IL-11, IL-12, IFN-\(\gamma\) and GM-CSF (92). IL-12 enhances the expression of IFN-\(\gamma\) and IL-18 and inhibits the effect of T\(_{h2}\) cytokines in order to maintain the T\(_{h1}\) response (93, 94). There is also an increase in number of Langerhans cells and inflammatory dendritic epidermal cells (IDECs) in the epidermis and macrophages in the dermis. The skin in chronic lesions is remodeled due to chronic inflammation and tissue damage. Chronic AD skin shows fibrotic papules and lichenification (figure 5) (95).
Figure 5: Inflammation pathways in atopic dermatitis skin

In non-lesional AD skin, T\textsubscript{H}2 cells expressing the skin homing receptor CLA migrate into the skin where they are able to engage Langerhans cells (LCs) and mast cells (MCs) in order to facilitate the development on a T\textsubscript{H}2 immune response. The disrupted barrier in AD non-lesional skin which enhances the entry of allergens, microbes and pathogens into the skin result in the release of cytokines by the keratinocytes (KCs) such as thymic stromal lymphopoietin (TSLP) which further facilitate T\textsubscript{H}2 differentiation. This is the initiation of the acute phase which is associated with increased T\textsubscript{H}2 cells. The foreign bodies are recognized and processed by LCs which then prime T\textsubscript{H}0 cells to T\textsubscript{H}2 cells. In chronic AD, T\textsubscript{H}1 cells are more predominant and the skin is also infiltrated by inflammatory dendritic epidermal cells (IDECs), macrophages and eosinophils. Levels of cytokines such as IL-11, IL-12, IFN-\gamma and GM-CSF are increased (95, 96).

EPIDERMAL CHARACTERISTICS OF AD SKIN

Morphology
Acute AD lesions show epidermal spongiosis (intercellular edema) and in the dermis infiltration of inflammatory cells (lymphocytes and macrophages). Chronic lesions show hyperkeratosis, epidermal hyperplasia, collagen deposition, less spongiosis, dermal thickening and dermal infiltration of inflammatory cells (97, 98).

Epidermal protein expression
The expressions of some early and late epidermal differentiation proteins show a different pattern of expression in AD. Table 1 outlines those relevant to this thesis.
Table 1: Expression of epidermal differentiation proteins in healthy and atopic dermatitis skin. SC: stratum corneum, SG: stratum granulosum, SS: stratum spinosum (56).

<table>
<thead>
<tr>
<th>Epidermal differentiation protein</th>
<th>Healthy human skin</th>
<th>Non-lesional AD skin</th>
<th>Lesional AD skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratin 10</td>
<td>Suprabasal layers</td>
<td>Suprabasal layers</td>
<td>Mainly in SG and upper SS. Reduced in the lower SS</td>
</tr>
<tr>
<td>Involucrin</td>
<td>Upper SS and entire SG</td>
<td>Middle and upper SS, entire SG</td>
<td>Entire SS and SG</td>
</tr>
<tr>
<td>Loricrin</td>
<td>Upper SG</td>
<td>Entire SG</td>
<td>Entire SG and upper SS in an uneven pattern</td>
</tr>
<tr>
<td>Filaggrin</td>
<td>SG and transition to the SC</td>
<td>Thinned expression in SG</td>
<td>Thinned expression in SG. Absent in transition to the SC</td>
</tr>
<tr>
<td>Keratin 6</td>
<td>Absent</td>
<td>Absent</td>
<td>Suprabasal layers</td>
</tr>
</tbody>
</table>

SC lipid composition in AD

AD skin is characterized by abnormalities in the lipid composition, distribution and organization. The impaired barrier function in AD has been linked to significant changes in the SC lipid composition and organization in AD skin. The total level of SC lipids is decreased in both lesional and non-lesional AD skin (63, 99) and has been suggested to occur partly because of disturbed lamellar body maturation, and delayed and incomplete extrusion of lamellar bodies (100, 101). With regards to the lipid classes, a reduction in the relative SC CER levels especially in CER EOS and CER/CHOL ratio in non-lesional and lesional AD skin (63, 102) has been reported. Specifically, when focusing of CERs, alterations in CER subclass composition further emphasizes the impaired barrier in AD skin. For example, the level of very long chain CER EOS, total EO CERs, CER/CHOL ratio, CER NS, AH and CER NP is drastically reduced and correlates with decreased skin barrier function in both lesional and non-lesional skin (65, 103). However, the studies were performed long before the importance of filaggrin mutations in these patient group was reported. Jungersted et al., reported a reduction in CER EOS, EOH and AH in non-lesional AD skin which was independent of the presence of FLG mutations (104). These studies were further confirmed by Janssens et al., and van Smeden et al., who report similar results in non-lesional only and in both non-lesional and lesional skin respectively. (64) (4, 105). These studies also show no correlation with FLG mutations and the changes in lipid composition in AD skin. Additional research demonstrates that the chain length of both CER and FFAs contribute to the barrier function in AD skin as analyzed by TEWL. The CERs with a total chain length of 34 carbon atoms
i.e. C34 CERs (short chain CERs) are increased in non-acyl CER subclasses in non-lesional AD skin and to a greater extent in lesional skin (4, 104, 105). This is accompanied by decreased mean CER chain length in both lesional and non-lesional skin (105). The relative level of very long chain FFA (≥24 carbon atoms) is reduced in AD skin whereas shorter chain FFA (16 and 18 carbon atoms) and MUFAs are increased in AD. These changes were also more pronounced in lesional skin than in non-lesional AD skin.

**SC lipid organization in AD**

The lipids in AD SC have been reported to adopt a more hexagonal organization when compared with control subjects indicating a less dense lateral lipid organization. This change in lipid organization correlates with TEWL values but not with FLG mutations (4, 106). The FFA chain length, has also been shown to be related to lipid organization as a positive correlation, was observed between the FFA chain length and the lateral lipid organization in AD skin.

The lamellar lipid organization is altered in non-lesional AD SC. Small angle x-ray diffraction (SAXD) analysis indicates that the repeat distance of the lamellar phases show the tendency to be shorter in a subgroup of patients. This may be caused by the reduced lipid chain length in AD. In addition, AD patients without a 1st and 3rd order LPP peak show a significant reduction in the total acyl CERs in comparison with other AD patients in which these peaks were present (107). This suggests that acyl CERs are important for the proper formation of the LPP in AD.
CHAPTER 1
MODELLING AD
IN VIVO AND IN VITRO: ANIMAL MODELS AND HUMAN SKIN EQUIVALENTS

In vivo mouse models

Mouse models have been used primarily to model diseases due to the ease of manipulation, cost effectiveness and availability of genetically manipulated strains. The Nc/Nga mouse was described as the first spontaneously occurring model of AD (108). Other AD mouse models developed can be categorized into three groups. Firstly, transgenic models that are genetically engineered to express AD features e.g. flaky tail (FLGft) mice. FLGft mice have a frameshift FLG mutation causing abnormal profilaggrin expression and absence of filaggrin in the cornified envelope (109). Other examples include those that overexpress cytokines involved in AD pathogenesis e.g., IL-4, IL-13, IL-31 and TSLP (110-113). The second group are AD induced models by epicutaneous (EC) application of sensitizers and allergens. This can occur by repeated EC sensitization on tape stripped skin or on genetically modified mice (e.g. BABL/c mice) using house dust mite, haptens and allergens (114-117). Thirdly, there are AD mouse models that spontaneously develop AD-like lesions e.g. Nc/Nga mice and NOA mice. The Nc/Nga mice develop AD like disease under conventional conditions that expose them to environmental aeroallergen (82). The NOA mice are characterized by hair loss, ulcerative skin lesions, high IgE and mast cell accumulation. However, this model lacks classical histological features of human AD (118).

These mouse models have contributed to the current understanding of AD pathogenesis especially in the investigation of the role of specific genes in the development of the disease. However, mouse skin does not fully mimic the epidermal morphology, barrier and SC lipid properties of the human skin and are therefore limited in the modelling of human AD (119). This creates the need for an applicable and suitable substitute to human skin in understanding AD pathogenesis and testing of prospective therapeutics such as 3 dimensional human skin equivalents.

In vitro human skin equivalents (HSEs)

HSEs are derived from human keratinocytes and fibroblasts (primary or immortalized cell lines). In a controlled culture environment the cells develop into a fully stratified epidermis including the SC. Two types of In vitro human skin equivalents are used in this thesis i.e. the Leiden epidermal model (LEM) and the Explant HSE (Ex-HSE). The LEM is generated by seeding keratinocytes onto an inert filter and the Ex-HSE by implanting a human skin biopsy onto a collagen matrix populated with fibroblasts (figure 6) (120, 121). Other HSEs include i) the full thickness HSE generated by seeding keratinocytes onto a collagen matrix populated with fibroblasts and ii) fibroblast derived matrix HSEs made by seeding keratinocytes onto a dermal matrix produced by human fibroblasts. HSEs contain similar features to human skin such as the presence of lamellar bodies and keratohyalin granules in the SG and expression of epidermal differentiation proteins (K10, filaggrin, loricrin) (29, 122-124).

Many SC properties in HSEs also mimic human SC. For example, the presence of the LPP, main SC lipids including all CER subclasses and all CER and FFA chain lengths. Some differences are also found between HSEs and human skin such as the abundance of a hexagonal over orthorhombic lateral packing, relative
increase of MUFAs, presence of unsaturated CERs, premature expression of involucrin and higher SC permeability (29, 125).

The main pathological alteration in AD that have been described are the presence of FLG mutations and the elevated immune response. *In vitro* HSEs that mimic AD skin can be developed by generating HSEs in which the keratinocytes have been altered by introducing FLG mutations and/or creating culture conditions mimicking the cytokine/chemokine cocktail present in AD skin (126). Several methods have been adopted to introduce the effect of FLG mutations in HSEs such as the use of short hairpin RNA and small interfering RNA. As a result of the employment of these techniques, the protein expression of filaggrin is reduced because FLG mRNA expression has been reduced in the keratinocytes. Apart from mimicking AD by reducing FLG, this method also aids the understanding of the role that filaggrin plays in the development of AD. Various FLG knockdown studies have utilized both primary and immortalized cell lines (N/TERT, NHEK). Some studies have shown that the absence of FLG results in impaired keratinocyte differentiation, increased epidermal uptake of compounds and less dense lateral lipid organization (127-129). However van Drongelen et al., shows no effect of FLG knockdown on epidermal morphogenesis, differentiation and SC lipid properties in N/TERT cells (129). *In vivo* studies by Janssens et al., also show no correlation between FLG and changes in lipid composition and organization in AD skin (4). Other methods in which the immune microenvironment in AD skin can be mimicked include i) supplementation of upregulated cytokines in AD skin in HSE culture medium and ii) co-culture of immune cells with HSEs. This will be further discussed in chapter 3.
Figure 6: Establishment of human skin equivalents (HSEs)

a) Keratinocytes are isolated from fresh human skin. The Leiden epidermal model (LEM) is generated by seeding the isolated keratinocytes onto an inert filter. Under optimized culture conditions the keratinocytes proliferate and differentiate to form a fully stratified epidermis. b) A dermal equivalent is generated from collagen matrix populated with fibroblasts that are isolated from the dermis. Explant HSE (Ex-HSE) is generated by implanting a human skin biopsy onto the prepared dermal equivalent. The keratinocytes migrate unto the dermal substrate to form a stratified epidermis which is called the outgrowth. *This Image was provided and adapted from Biomimiq.com.*
AIM AND OUTLINE

The aim of this research is to unravel the interplay between inflammation and lipid barrier properties in AD skin. This insight may aid the development of an HSE that mimics epidermal characteristics of AD that can be used for screening purposes of new compounds for the treatment of AD.

In Chapter 2 we focus on the changes in the expression of enzymes involved in the lipid metabolism in the skin of AD patients and examine whether the observed changes correlate with changes in the lipid composition. To examine the effect of inflammation, the expression of these enzymes and lipid composition was analyzed in both non-lesional and lesional AD skin. These findings served as a benchmark for AD features, which were reproduced in HSEs, described in chapter 3. Chapter 3 describes the effect of cytokines supplemented to the medium on the epidermal morphogenesis, cell proliferation and differentiation using the LEM. To elucidate this, the culture medium was supplemented with T₄,₂ cytokines and TNF-α (individually or in combination). The effect of these cytokines on the lipid properties in LEMs was elucidated by investigating the expression of enzymes involved in the lipid metabolism in relation to the SC lipid composition and organization.

The expansion of small epidermal tissue by generating Ex-HSEs is crucial for analyses that require larger amounts of epidermal tissue, which cannot be harvested from diseased skin. A method to expand the material derived from Ex-HSEs by passaging the outgrowth is described in Chapter 4. This chapter describes the effect of passaging on epidermal morphogenesis and lipid barrier properties of the derived Ex-HSEs. This method was also applied in generating an AD-HSE where biopsies from AD patients with and without FLG mutations are used to establish Ex-HSEs. The epidermal characteristics in the AD-Ex-HSE were compared to the original AD skin in order to compare the reproducibility of the AD characteristics in AD Ex-HSE in vitro. The results of this study are described in chapter 5.

Chapter 6 introduces an ex vivo human skin barrier repair model developed by removing the SC from ex vivo skin using a reproducible cyanoacrylate stripping technique and in vitro culture of the stripped skin. The epidermal characteristics of the stripped/cultured ex vivo skin are described in this chapter. This model can be potentially used in the testing of formulations to aid skin barrier repair.
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Altered expression of epidermal lipid bio-synthesis enzymes in atopic dermatitis skin is accompanied by changes in stratum corneum lipid composition


* Contributed equally to this study

Manuscript prepared for submission
Abstract

The barrier dysfunction in atopic dermatitis (AD) skin is associated with stratum corneum (SC) lipid abnormalities including reduction of global lipid content, shorter ceramide (CER) as well as FFA chain length and reduced CER levels. However, the underlying causes of these changes has not been fully investigated. In the present study, we analyzed in AD patients and controls the expression of enzymes involved in the biosynthesis of free fatty acids (FFAs) and CERs in relation to the SC lipid composition.

These enzymes include β-glucocerebrosidase (GBA), acid-sphingomyelinase (aSmase), ceramide synthase 3 (CerS3) involved in CER synthesis and stearoyl CoA desaturase (SCD), elongase 1 (ELOVL1) and ELOVL6 involved in FFA synthesis. The results reveal an altered expression of SCD and ELOVL1 in AD lesional skin. This was accompanied by functional changes displayed by increased unsaturated FFAs (SCD) and reduced FFA C22-C28 (ELOVL1) in AD lesional skin. The CER composition in AD lesional skin showed corresponding changes such as increased CER AS and NS (aSmase) and decreased esterified ω-hydroxy CERs (CerS3). This study suggests that changes in the lipid composition in AD skin could arise from alterations in the expression of key enzymes in SC lipid synthesis and inflammation may influence the expression of these enzymes.
Introduction

Lesional and non-lesional skin in atopic dermatitis (AD) patients display inside-out barrier dysfunction (i.e. increased trans-epidermal water loss-TEWL) and outside-in impairments (increased penetration of compounds, such as allergens and pathogens through the skin) (1-4). Contributing factors to the development of AD include mutations in skin barrier related genes (e.g. filaggrin), skin inflammation and influences from the environment. The skin barrier is mainly located in the topmost layer of the skin the stratum corneum (SC). This layer is composed of corneocytes surrounded by a lipid matrix in a “brick” (corneocytes) and “mortar” (lipids) arrangement. The composition and organization of SC lipids is of utmost importance in maintaining the skin barrier. Cholesterol (CHOL), ceramides (CERs) and free fatty acids (FFAs) are the main lipid classes present in human SC. In healthy SC these are organized in lipid layers. Within these layers the lipids adopt predominantly a very dense orthorhombic lateral organization (5-9), see supplementary figure S1.

In AD lesional and non-lesional skin, the lipid abnormalities contributing to the barrier dysfunction arise from reduction in SC lipid levels in relation to protein content, reduced relative CER content (CER/CHOL ratio and CER levels) and a shorter mean lipid chain length. An increase in the fraction of unsaturated fatty acids has also been observed in lesional skin, while the fraction of hydroxy fatty acids is decreased (10-16). Particularly, the chain length reduction correlated excellently with a reduced skin barrier (17). The above mentioned lipid abnormalities in AD skin have been suggested to result from changes in the expression or activity of enzymes involved in SC lipid synthesis. The reduced lipid/protein ratio could originate from impairments in the secretion of lipids from lamellar bodies leading to an overall reduction of extracellular lipids (18, 19). Lipid synthesis enzymes such as β-glucocerebrosidase (GBA) and acid-sphingomyelinase (aSmase) catalyze the last step in CER subclass synthesis from glucosylceramides and sphingomyelin, respectively into CERs (20) and the activity of these enzymes depends on pH (21, 22). The increase in pH observed in AD skin may alter the activity and/or expression and degradation of GBA and aSmase and contribute to reduced CER content in AD and/or an imbalance in the level of CER subclasses (1, 23). Inflammation cytokines upregulated in AD have also been shown to affect CER and FFA composition in in vitro studies (24, 25). In relation to the lipid chain length reduction observed in AD skin, enzymes which are necessary to synthesize the (ultra) long FFA chains (ELOVL1 and ELOVL4) or acylate the ultra-long FFA chains (≥C26) to the sphingoid backbone during biosynthesis of CERs (CER synthase 3 (CerS3)) may play a role (24). ELOVL1 elongates FFAs with carbon chain length of C20-C26 and ELOVL4 elongates those longer than C26 (26-29). In addition, ELOVL6 is primarily responsible for the elongation of FFAs with a chain length of C16 and C18. Changes in the expression of stearoyl CoA desaturase (SCD) which functions to produce unsaturated FFAs from saturated FFAs (30) may contribute to the increased level of unsaturated FFAs in AD (31).

This study aims to investigate whether the expression of CER and FFA biosynthesis enzymes are altered in AD skin compared with control skin. To determine whether changes in enzyme expression can be related with changes in lipid composition, we also examined lipid composition in the same group of patients and compared them with control skin. The expression of enzymes examined in this study include GBA, aSmase, CerS3, SCD, ELOVL1 and ELOVL6.
This study demonstrates that changes in the expression of CerS3, GBA, aSmase, SCD and ELOVL1 occur in AD skin and are more pronounced in lesional skin. The changes in protein expression in AD skin are accompanied by corresponding changes in lipid composition compared to control skin. The changes in lipid composition in SC of AD skin are in accordance with changes previously reported (15, 17).
Materials and methods

Study set-up and population
The study was approved by the ethical committee of the Leiden University Medical Centre and conducted according to the Declaration of Helsinki Principles after written informed consent from all the study subjects. Prior to the study, blood was collected in heparin coated tubes from study participants for isolation of DNA and genotyping. 8 Caucasian subjects without any history of dermatological diseases (24.3 ± 3.1 years; 5 female) and 20 Caucasian AD patients (27.0 ± 6.4 years; 12 female) were included. The AD study population consisted of 8 patients with filaggrin mutations (5 with heterozygous and 3 with homozygous FLG mutations) and 12 patients without filaggrin genotype mutations (see filaggrin mutation analysis below, results in supplementary table S1). The subjects did not apply dermatological or cosmetic products on their forearms one week prior to the study. The subjects were acclimatized for 45 minutes in a temperature and humidity controlled room after which the measurements were performed. All measurements were performed within a single day per subject, on the ventral forearms in the acclimatized room. The area of skin to be studied (non-lesional and lesional skin) was carefully selected by a dermatologist at the start of the study. At the selected areas, tape stripping, attenuated total reflectance fourier transformed infrared spectroscopy (ATR-FTIR) measurements were performed as described below. At the end of the study day, two 4mm biopsies were harvested from the ventral fore-arm of 7 patients close to the skin region used for the measurements (see below). This included one biopsy from non-lesional skin and one biopsy from lesional skin. On the study day, only 10 out of the 20 patients included in this study had lesional skin areas. From 3 control subjects, one biopsy was harvested from the ventral forearm on the study day.

SCORAD
In order to determine disease severity, local-SCORAD of non-lesional and lesional skin and overall SCORAD was determined by a dermatologist (see supplementary table S1) in the patient population according to the scoring method of Stalder and Taieb (32).

Immunofluorescence staining
The harvested biopsies were embedded in paraffin and cut at 5µm thickness. Immunohistochemical analysis of the following lipid biosynthesis enzymes was performed: GBA, aSmase, CerS3, SCD, ELOVL1 and ELOVL6. The primary and secondary antibodies are listed in supplementary table S2.

Immunofluorescence analysis was performed on both lesional and non-lesional biopsies from 7 AD patients compared to biopsies from 3 controls. Within this patient group, 3 of the patients showed heterozygous FLG mutation (R501X, 2282del4) and 4 patients a wild type filaggrin phenotype. The severity of AD measured by overall SCORAD ranged from 11.7 - 43.5. The sliced sections were stained as described previously (33, 34). Briefly, the sections were heat treated in sodium citrate buffer (pH 6), blocked with normal horse serum (Vector laboratories Burlingame, CA) and incubated with the primary antibody overnight at 4°C. Afterwards, sections were incubated with the appropriate secondary antibody (Rhodamine Red-X or
Cy3, Jackson immunoresearch Laboratory, USA) for 1 hour at room temperature and mounted using DAPI Vectashield (Vector laboratories Burlingame, CA).

**Filaggrin mutation analysis**

The controls and AD patients were screened for 4 of the most prevalent mutations observed in European Caucasians (R501X, R2447X, 2282del4, S3247X). The mutations were determined using blood samples and subsequent DNA isolation as described previously (35). In brief, using Ficoll density centrifugation, peripheral blood mononuclear cells (PBMCs) were isolated. The dry cell pellets were kept at -80°C until DNA isolation. Using the DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands), DNA was isolated according to the manufacturer’s instructions. Filaggrin mutation analysis revealed a patient distribution of 40% with mutations (25% heterozygous (R501X and 2282del4), and 15% homozygous (2282del4, S3247X)) and 60% wild type patients, see supplementary table S1.

**Tape stripping procedure**

Poly (phenylene sulfide) tape (Nichiban, Tokyo, Japan) was used to harvest SC lipids sequentially from control skin, lesional and non-lesional skin on the ventral fore arm (stripped area 4.5cm²). The tape was applied to the area of interest with a pressure of 450g/cm² using a D-squame pressure instrument (Cuderm Corp., Dallas, TX). The tape was removed in a fluent stroke with alternating directions for each successive strip. Each tape was punched into a circular area of 16mm in diameter and placed in a separate glass vial containing 1mL chloroform/methanol (2:1, all organic solvents were obtained from Biosolve BV, Netherlands) and stored at -20°C under argon until the lipids were extracted.

**Lipid extraction**

Lipids were extracted from tapes 6-9 of each study subject. A modified Bligh and Dyer extraction was performed on each tape separately (36, 37) as described previously (17). In brief, the tapes were incubated in 3 different ratios of chloroform: methanol: water (2:1:0; 1:1:0; 1:2:0.5) for one hour each. Afterwards, the extracts were pooled and a 0.25M KCl solution was added to the pooled extract. The extract was washed with water and the organic layer was collected after full separation of the water and organic layer. The final organic layer from the 4 tapes per subject was pooled and dried under N₂ gas at 40°C and reconstituted in 50µL chloroform: methanol: heptane (2.5:2.5:95). The samples were stored under argon at -20°C until analysis by liquid chromatography-mass spectrometry (LC-MS).

**CER and FFA analysis by LC-MS**

*CER Species:* 5 µl of each lipid sample (4 tapes/50µL, at 40°C) was injected and separated in an analytical normal phase column (PVA-bonded column; 100 x 2.1mm i.d., 5µm particle size, YMC (Kyoto, Japan)) under a flow rate of 0.8 ml/min and using a binary solvent system of heptane (100%, solvent A) and heptane/isopropanol/ethanol (50:25:25, solvent B) in an ACQUITY quaternary UPLC system (Waters, Milford, MA). The UPLC was coupled to a mass spectrometer (Xevo, TO-S, Waters, Milford, MA) in atmospheric pressure chemical ionization (APCI; ion sabre II) and positive scan mode with a scan range of 350-1200 amu. The
temperature of the source heater and capillary voltage was set at 150°C and 3.5kV respectively. Deuterated CER NS (D47) was used as an internal standard. The CER analysis was performed using Target Lynx software version 4.1 and the nomenclature used throughout this article is according to Motta et al., and Masukawa et al., (38, 39). A detailed analyses of this method will be published elsewhere (Boiten et al., in preparation).

**FFA species:** 5 μl of each lipid sample (4 tapes/50µL, at 40°C) was injected and separated in a UPLC HSS T3 column (50 x 2.1mm i.d., 1.7μm particle size, Waters, Milford, MA) under a flow rate of 0.8 ml/min and using a binary solvent system of 0.5mM ammonium acetate in water (solvent A) and 0.5mM ammonium acetate in water/isopropanol/acetonitrile (5:70:25, solvent B) in the UPLC and mass spectrometer system described above. The UPLC was coupled to the mass spectrometer in electrospray ionisation (ESI), negative scan mode with a scan range of 200-600 amu. The temperature of the source heater and capillary voltage was set at 150°C and 1kV respectively. FFA Deuterated-C24 was used as an internal standard. The data was corrected for chain length and matrix effect which is described in the supplementary materials and methods.

**Stratum corneum protein content**
The Squamescan 850A (Heiland electronic, Wetzlar, Germany) was used to relate the absorption of the tape strip to the amount of protein removed by each strip. The poly (phenylene sulfide) tape was applied using a range of pressures between 0-450g/cm². This pressure range was used in order to vary the amount of stratum corneum removed per area. The absorption of 20 tape strips from 4 volunteers was measured by Squamescan and each tape strip was incubated overnight in 1mL of 1M KOH in 10X Hanks buffered saline solution (HBSS, life technologies Europe B.V., Bleiswijk, Netherlands). The pH of each tape strip solution was adjusted to 7/8 with 12M HCl. Thereafter, protein content was determined using Microplate BCA Protein Assay (Reducing Agent Compatible, Thermofischer scientific Inc., IL, USA) according to the manufacturers instruction. A calibration curve was obtained by plotting the protein level of each tape against the squamescan values measured at an optical density at 562nm. Using the calibration curve, the optical density at 562nm (squame scan) from each tape strip of the patients and controls was calculated.

**Lateral organization and conformational order of SC lipids**
ATR-FTIR spectroscopy was utilized to analyze the lateral organization and conformational order of the lipids in all study subjects. FTIR spectra of the SC (from the same location as used for analysis of lipid composition) were collected as a function of depth after two tape stripping’s using a Varian 670-IR spectrometer (Agilent technologies, Santa Clara, CA) until 12 tape strips were collected. The spectrometer was equipped with i) a broad band mercury-cadmium-telluride (MCT) detector and ii) an external sample compartment containing an attenuated total reflection (ATR) accessory (GladiATR, Pike, Maddison, WI) having a single reflection diamond. The sample compartment was constantly purged with dry air. Each spectrum was collected as an average of 200 scans at a resolution of 2cm⁻¹. The peak position of the CH2 symmetric stretching vibration and the band width from the second derivative CH2 scissoring region was determined as described previously (8, 40) using Resolutions Pro 4.1 software (Agilent technologies, Santa Clara, CA).
The scissoring bandwidth was calculated at 50% of the maximum peak height. An increase in width of this band indicates that more lipids adopt a dense lipid organization (an orthorhombic lateral packing). The position of the CH\textsubscript{2} symmetric stretching vibration was also determined as a measure for the conformational disordering of the lipids. A high value of the stretching vibrations indicates a liquid phase, whereas a value $<2850$ cm$^{-1}$ indicates a crystalline phase. Mean values of 5 measurements were calculated per subject for each parameter (measurements were performed using tape strips 2-10 so as to analyze lipids within the same depth as with the lipid composition analysis).

**Data analysis**

Statistical analysis was performed with GraphPad prism 5 (GraphPad software, CA, USA). When comparing two groups, non-parametric Mann-Whitney tests were performed and $p<0.05$ indicates statistical significant data (*$p<0.05$, **$p<0.01$, ***$p<0.001$). In order to analyze the correlation between two parameters, a bivariate analysis was performed and the Spearman’s ρ correlation coefficient was calculated. In total 7 patients from the AD patient population and 3 controls were selected for immunofluorescent analysis (supplementary table S3). This subset of subjects representatively reflect the range of AD severity of the entire study population The overall SCORAD from this AD patient subpopulation ranged from low (11.7-25.8) to high (29.8-43.5), whereas SCORAD of controls was always 0.
Results

Expression of SCD and ELOVL1 is altered in AD lesional skin

In order to investigate the underlying factors that may contribute to the change in barrier properties in AD skin, we analyzed the expression of specific enzymes involved in the biosynthesis of the lipids in SC and related the expression of these enzymes with lipid composition. Stearoyl CoA desaturase (SCD) is an important enzyme in the formation of unsaturated FFAs from saturated FFAs. In control skin, SCD is only expressed in the stratum basale, while in AD non-lesional skin it is expressed in the stratum basale and in 1-2 lower layers of the stratum spinosum. Lesional AD skin shows SCD expression in more epidermal cell layers (figure 1, see supplementary figure S2 for images without DAPI staining). This change in SCD expression is observed in all AD biopsies analyzed.

The elongation of FFAs is an important step in the process of SC FFA synthesis. FFA elongases such as ELOVL1 and ELOVL6 are involved in this process. Specifically, ELOVL1 is an important enzyme in elongation of FFAs with chain lengths ranging from C20-C26. The expression of ELOVL1 in control and AD non-lesional skin is visualized as a gradient from stratum basale to stratum granulosum with the most pronounced expression in the granular and upper spinous layer. However, in the majority of the biopsies in AD lesional skin (4 out of 7), this gradient in ELOVL1 expression is lost and the expression of ELOVL1 was reduced and equal across the epidermal layers (figure 1). ELOVL6 which elongates C16 to C18 also shows a gradient expression in the epidermis with the highest expression in the stratum granulosum in control skin. The expression of ELOVL6 remained unchanged in all AD lesional and non-lesional skin biopsies (figure 1). No differences in the expression of SCD, ELOVL1 and ELOVL6 were noticed between patients with filaggrin mutations (homozygous and heterozygous) and wild type patients (data not shown).
Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin

Figure 1: Expression of SCD and ELOVL1 are changed in AD lesional skin.

Immuno-histochemical staining of stearoyl CoA desaturase (SCD), ELOVL1 and ELOVL6 in one control, non-lesional and the corresponding lesional skin from two AD patients. SCD is expressed in the stratum basale in control skin and extends to suprabasal layers of the epidermis in AD non-lesional and to even more epidermal cell layers in lesional skin. ELOVL1 in control and AD non-lesional skin is visualized as a gradient from stratum basale to stratum granulosum with the most pronounced expression in the granular and upper spinous layer. In most patients, ELOVL1 expression was reduced and equal across the epidermal layers in lesional skin. ELOVL6 also shows a gradient expression in the epidermis with the highest expression in the stratum granulosum in control skin. This remained unchanged in all AD lesional and non-lesional skin biopsies. Representative images are shown with 20x magnification, scale bar 50µm. The overall SCORAD of AD 1 images is 25.8 and AD2 (non-lesional and lesional) 41.3. NL: non-lesional, L: lesional. See supplementary figure S2 for images without DAPI staining.

Different expression pattern of late stage CER synthesis enzymes in AD lesional skin

The expression of CerS3, aSmase and GBA was also analyzed by immunofluorescence staining. Firstly, the highest expression of CerS3 is observed as a one cell layer band along the stratum granulosum in all control and AD non-lesional biopsies with a weaker expression in deeper epidermal layers. Its function has been linked to the production of ultra-long chain CERs with acyl chain lengths of C26 and above (29). The highest expression pattern of CerS3 in AD lesional skin is seen in the 2-4 upper cell layers in various sections along the entire biopsy. This occurred in 5 out of 7 of the biopsies analyzed (figure 2, see supplementary figure S3 for images without DAPI staining). Secondly, aSmase which is involved in the synthesis of CER AS and CER NS (20, 41) shows a gradient expression through the epidermis with the highest expression in the stratum granulosum in control and non-lesional AD skin (2 cell layers, figure 2). In AD lesional skin, the gradient in expression of aSmase is lost as the expression of aSmase is very similar in all epidermal layers. The changes observed in aSmase expression occurred in all analyzed AD biopsies. Thirdly, the GBA expression in control human skin is at the interface between the stratum granulosum and SC and hardly no staining occurs in
the deeper epidermal layers. In AD non-lesional and lesional skin of patients with low SCORAD (overall SCORAD: 11.7-25.8; local SCORAD: 3-6), no differences were observed in the expression of GBA as the expression was similar to control (figure 2, left lesional image). However, in patients with higher SCORAD (4 patients, overall SCORAD: 31.8-43.5; local SCORAD: 6-8), GBA was expressed in 2-3 cell layers along the stratum granulosum and stratum spinosum in lesional skin (figure 2, right lesional image). The expression of GBA in AD non-lesional skin with high SCORAD was also similar to the control skin (figure 2, left non-lesional image). In addition, these changes in GBA, aSmase and CerS3 expression were not affected by FLG mutations as the reported changes were observed in both wild type AD patients and patients with homozygous and heterozygous FLG mutations (data not shown).

**Figure 2:** Expression of CerS3, aSmase and GBA are changed in AD-lesional skin.
Immuno-histochemical staining of CER synthase 3 (CerS3), acid-sphingomyelinase (aSmase) and glucocerebrocidase (GBA) in control, atopic dermatitis (AD) non-lesional and corresponding lesional skin. The highest expression of CerS3 is observed as a one cell layer band along the upper stratum granulosum cell layer and a weaker expression in deeper epidermal layers in all control and AD non-lesional biopsies. The highest expression extended to 2-4 cell layers in the epidermis in lesional AD skin. aSmase also shows a gradient expression which peaks at the stratum granulosum in control and non-lesional AD skin. The gradient expression is no longer present in lesional AD skin as aSmase is similarly expressed in the entire epidermis. GBA is expressed in control and non-lesional AD skin at the interface between the stratum granulosum and SC. Changes in GBA expression was only observed in lesional skin of patients with overall SCORAD ranging between 31.8-43.5 where GBA expression was extended to 2-3 cell layers along the stratum granulosum and stratum spinosum. Representative images are shown with 20x magnification, scale bar 50µm. The overall SCORAD of the left AD images (non-lesional and lesional) ranges from 11.7-25.8 and for the right AD images (non-lesional and lesional) 31.8-43.5. NL: non-lesional, L: lesional. See supplementary figure S3 for images without DAPI staining.
Changes in the expression of FFA bio-synthesis enzymes is accompanied by changes in FFA composition in AD skin

To relate the changes in enzyme expression with the consequences at a functional level, the CER and FFA composition was analyzed by LC-MS. The amount of SC removed (total protein content) from both control and AD skin was determined to examine whether the lipids analyzed were harvested at similar depths in SC. There was no significant difference between the amount of SC removed between control and non-lesional and lesional AD skin (data not shown).

When we focus on the degree of unsaturation of the FFA, in comparison with control skin, non-lesional AD skin showed no significant change in the level of unsaturated FFAs (p=0.16), although a higher expression of SCD was observed in non-lesional skin. In AD lesional skin, a significant increase in unsaturated FFAs was observed compared with control skin (p<0.01, Figure 3a). This may coincide with the higher expression of SCD observed in lesional skin.

The FFA chain length distribution was also examined with respect to the FFA specifically the chain length distribution of both saturated and unsaturated FFA are shown in supplementary figure S4. Since ELOVL1 is particularly involved in the elongation of FFA with chain length of C20-C26 we calculated the total level of FFA C22-C28 from the chain length distributions (saturated and unsaturated). No significant difference was observed in the levels of FFA C22-C28 in AD non-lesional skin (p=0.27). However, there was a decrease in the FFAs C22-C28 in AD lesional skin in relation to control skin (p<0.05, Figure 3b). As ELOVL4 is an important enzyme to elongate the ultra-long FFA chain from C26 and longer, we also calculated the FFAs ≥ C26 and observed a similar trend: The level of FFAs ≥ C26 were significantly reduced in AD lesional skin compared to control skin (p=0.04, Figure 3c). FFAs with chain length of C16-C18 (saturated and unsaturated) was significantly increased in AD lesional skin (p<0.05, figure 3d) compared to control skin.

As previous in vitro studies indicated that specific changes in FFA composition contributes to a less tight lateral packing in the SC, the lipid organization in the SC was examined using ATR-FTIR (31). To obtain this information we focused on the CH₂ scissoring and the CH₂ symmetric stretching vibrations of the lipid hydrocarbon chains. These vibrations provide information on the density of the lipid packing and the conformational disordering of the lipids respectively. An increase in width of the CH₂ scissoring band width at half maximum in the infrared spectrum indicates that more lipids adopt a dense lipid organization (an orthorhombic lateral packing) (40). A high value of the CH₂ symmetric stretching vibrations (>2852 cm⁻¹) indicates a liquid phase, while a value <2850 cm⁻¹ indicates an ordered phase (42, 43). The lipids in AD non-lesional and lesional skin showed less ordering as the CH₂ symmetric stretching vibrations of the lipid hydrocarbon chains was significantly increased in relation to control lipid organisation (p<0.001, supplementary figure S5a). Similarly, the lipids present in AD lesional skin also show a less dense lateral packing compared to control skin as the CH₂ scissoring band width was significantly reduced in both groups compared to the control (p<0.01, supplementary figure S5).
Figure 3: Changes in FFA composition in control, lesional and non-lesional AD skin.
A) Box plots (Whiskers: min to max) showing the relative level of unsaturated FFA. Unsaturated FFAs are significantly increased in AD lesional skin. B) Box plots illustrating relative abundance of FFA with chain length of C22-C28 (saturated and mono-unsaturated). These FFAs were significantly decreased in AD lesional skin. C) Illustrates the level of FFA with chain length ≥C26 (saturated and unsaturated) which is also significantly reduced in AD lesional skin. D) The relative abundance of FFA with chain length C16-C18 (saturated and unsaturated) as illustrated by box-plots is significantly increased in AD lesional skin.

Changes in CER synthesis enzyme expression are accompanied by changes in CER composition in AD skin

12 subclasses of CERs in human SC are derived from the linkage of 3 possible fatty acid chains (non-hydroxy fatty acid [N], α-hydroxy fatty acid [A] and esterified ω-hydroxy fatty acid [EO]) to 4 different sphingoid bases (dihydrosphingosine [dS], sphingosine [S], phytosphingosine [P], 6-hydroxysphingosine [H]). The nomenclature of CERs described above is illustrated in supplementary figure S6.

As a gradient of the expression of aSmase was not observed in lesional skin, we determined the levels of CER [NS] and [AS], since aSmase catalyzes an important final step in the synthesis of these CER subclasses. The levels were not significantly increased in AD non-lesional skin however, a significant increase in the level of CER [NS] and [AS] was observed in AD lesional skin (p<0.05, figure 4a).

Since we observed changes in expression of CerS3 that is particularly important for the synthesis of the ultra-long chain CERs, the level of [EO] CERs (EOS, EOP, EODS, and EOH) was also investigated. [EO] CERs are known to be important for the skin barrier function and in our cohort accounts for 7.17 ± 2.06% of CERs.
in control skin. This is significantly decreased to 5.20 ± 1.87% in AD lesional and 5.15 ± 1.70% in non-lesional skin (p<0.05, figure 4b). It may be clear that no significant differences in [EO] CER level was observed between lesional and non-lesional AD skin. As the FFAs with a chain length of C16 and C18 was increased and a common synthetic pathway of CERs and FFAs has been demonstrated in a previous study, we also examined whether the fraction of CERs with a very short chain length (CER C34) was increased (17). The fraction of CER C34 in lesional skin was 1.67 ± 1.06% which was significantly higher than in control skin (0.35 ± 0.27%, p<0.001, data not shown). When comparing phytosphingosine based CERs with sphingosine based CERs we also observed a highly significant correlation (r=-0.76, p<0.0001, Figure 4c). This result suggests that a relative increase in the sphingosine CER subclasses observed in AD skin is at the expense of the CER classes with a phytosphingosine base. No significant change was noticed in the level of CERs with a dihydrosphingosine or 6-hydroxy sphingosine base.

Figure 4: Changes in CER composition in AD skin.
A) Relative level of CER [AS] and [NS] is significantly increased in lesional skin in relation to control skin. B) Levels of [EO] CERs in control, AD lesional and non-lesional skin. [EO] CERs are significantly decreased in both lesional and non-lesional AD skin. C) Correlation plot of relative level of phytosphingosine CERs versus sphingosine CERs which shows a positive pearsons correlation. The pearson correlation coefficient is displayed in the lower right corner. Control skin is indicated by , AD non-lesional skin by and AD lesional skin by . The values from patients with filaggrin mutations and wild type are represented by filled symbols and open symbols respectively.
Discussion

This study analyses the expression of enzymes involved in the biosynthesis of FFAs and CERs in relation to their final product in SC of AD skin. We especially focused on 3 enzymes involved in FFA synthesis: SCD, ELOVL1 and ELOVL6 and 3 enzymes involved in CER synthesis i.e. GBA, aSmase and CerS3 (see supplementary figure S7).

Lipid synthesis and inflammation

In a previous study focusing on lesional and non-lesional skin of AD patients, it was shown that the average SC lipid chain length in each patient in lesional skin was reduced compared to non-lesional skin in the same patient. This was caused by a reduction in average chain length of both CERs and FFAs. Furthermore, the level of [EO] CER subclasses was also reduced.

In this work we observe that overall changes in the expression of the enzymes were more pronounced in lesional skin than in non-lesional skin in each of the patients analysed and that changes in enzyme expression correspond with the changes in lipid composition. Although the reported changes in lipid composition are all relative, the changes in enzyme expression were in most cases associated with changes in lipid composition in the SC. As clear differences between lesional and non-lesional skin are noticed, the expression of enzymes involved in lipid metabolism may not only be dependent on genetic background of the patients, but inflammation and environmental factors (e.g. dry air, stress, using soaps, pH) may also play an important role (24, 25, 44-46). AD skin lesions are associated with increased levels of TH1, TH2 and TH17 cytokines (47). IL-4 has been proposed to inhibit barrier homeostasis as it reduces the mRNA levels of aSmase and GBA. It also reduces the CER levels in barrier disrupted human skin equivalents (48). In addition, IFN-γ reduced the mRNA expression of ELOVLs and CER synthases in cultured human keratinocytes and in epidermal sheets (24). The CER [NS] chain length was analysed in detail and it was shown that after supplementation of IFN-γ the levels of longer chain CER [NS] were more drastically reduced than those of the shorter CER [NS] (24). Other cytokines such as IL-31 and TNF-α alter the protein expression of ELOVL1 and CerS3 and reduce the level of EO CERs and FFAs with chain length ≥C24 (24, 46).

Enzymes involved in FFA synthesis

In AD lesional skin, the expression of SCD is observed in an increased number of viable cell layers in the epidermis. SCD-1 plays a crucial role in the biosynthesis of unsaturated FFAs C16:1 and C18:1 (26). We observe a significant increase in unsaturated FFAs in AD lesional skin. This suggests that the increase in SCD expression may have a functional effect on the FFA composition and is responsible for the increased level of unsaturated fatty acids. However, changes in the enzymatic activity of SCD cannot be excluded as a contributing factor. In vitro studies using lipid membranes demonstrate that higher levels of unsaturated FFAs contribute to a higher level of conformational disordering and an increase in the level of lipids forming a hexagonal lateral packing (31). Therefore the increased degree of FFA unsaturation and reduction in FFA chain length may contribute to the reduced density of packing and increase in conformational disordering in the lipid matrix of AD skin (17).
The distribution of ELOVL1 expression clearly differs in AD lesional skin. As ELOVL1 elongates FFA with chain length between C20-C26, the change in its expression may contribute to the reduction in FFAs C22-C28 observed in AD lesional skin. We also observe a similar trend in the level of FFAs (SFA and unsaturated FFA) ≥C26 produced by ELOVL4. These very long FFAs were significantly reduced in AD lesional skin compared to control subjects. This indicates that the reduction in level of FFAs C22-C28 is probably not influenced by a higher activity and/or expression of ELOVL4. Unfortunately, the expression of ELOVL4 could not be examined as no suitable antibodies were available. The expression of ELOVL6 which contributes to the biosynthesis of FFA C16 and FFA C18 is unaffected in AD skin (17). However, there is an increase in these short chain FFAs (unsaturated and saturated) in AD lesional skin. The level of FFAs can be influenced by elongases (particularly ELOVL1) and CerS which utilise FFAs in the synthesis of CERs. As there is an increase in the short chain CERs (i.e. CER C34 and CER C36), this accumulation of FFA C16 and C18 may not be due to a reduced expression or activity of ceramide synthases responsible for n-acylating sphingoid bases to FFA C16 and FFA C18 (49). Reduced ELOVL1 expression which is accompanied by reduced FFA C22-C28 may therefore be one of the underlying factors for accumulation of short chain FFA C16 and FFA C18, mainly being the unsaturated FFAs. The expression of CerS3 which n-acylates sphingoid bases to very long chain FFA (≥C26) was only affected in AD lesional skin. In relation to CerS3 expression, a significant reduction in [EO] CERs was observed in AD lesional and non-lesional skin in agreement with previous studies (15, 50). The level of very long chain FFA (≥C26) between non-lesional and lesional skin was not statistically different. This may contribute to the absence of a significant difference in [EO] CER levels between non-lesional and lesional skin.

Enzymes involved in CER synthesis
When we focus on enzymes involved in the last step of biosynthesis of CERs, we observe that the gradient in expression of aSmase in the epidermis with a stronger expression in stratum granulosum is lost in lesional skin. As an increased level of CER [AS] + CER [NS] was observed, a reduced expression of aSmase in lesional skin was not expected since one of the precursors is sphingomyelin. The abundance of CER [AS] + CER [NS] relative to the abundance of the remaining CER subclasses depends on the relative expression and enzyme activity of GBA and aSmase (51). It has been reported that there is an increase in the pH in non-lesional and lesional AD skin (20, 52), and the optimal pH for the activity of GBA and aSmase are 5.2 and 4.5-5, respectively. Therefore, the activity of both GBA and aSMase may be affected in AD skin (21, 53, 54). For aSmase this has been reported (1) and previous studies have shown no change in GBA activity in AD non-lesional SC (55, 56). However, this is not reported for AD lesional skin where the most drastic changes in CER profile are observed. As the expression of GBA is more localized at the interface between the viable epidermis and the stratum corneum, while the expression of aSmase is also present in deeper epidermal layers, the activity of the GBA may be more affected by an increased pH than that of aSmase. This would result in a higher relative level of CER [NS] + CER [AS], as sphingomyelin is only a precursor of CER [NS] and CER [AS] which is what is observed in AD lesional skin. The level of [P] based CERs and [S] based CERs showed a very significant spearman correlation. Since [S] based CERs (NS and AS) are increased in AD lesional skin, the results suggest a relative feedback mechanism between the syntheses of [S] and [P] CERs in AD skin however this requires further analysis beyond the scope of this study.
Even though the changes in CER composition and lipid chain length described in this study and from previous studies are represented as a relative values, similar results were observed in studies which analyse the absolute amounts of lipids in AD skin (10, 12, 57). This further emphasises a relationship between lipid composition and lipid enzymes expression described in this study.

In conclusion, this study points out two major findings. Firstly, the changes in the lipid composition in AD skin could partly arise from changes in the expression of key enzymes in SC lipid synthesis such as SCD, ELOVL1, aSmase, GBA and CerS3. Secondly, inflammation seems to influence the expression of these enzymes as the changes in expression are mainly observed in AD lesional skin.
References

17. van Smeden J, Janssens M, Kaye E C J, et al. The importance of free fatty acid chain length for the


50. van Smeden J, Janssens M, Gooris G S, et al. The important role of stratum corneum lipids for the cutaneous barrier function. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of


Supplementary Figures

Figure S1: Lipid organization in human stratum corneum
The stratum corneum (SC) is the topmost layer of the epidermis which comprises of corneocytes surrounded by an organized intercellular lipid matrix. In the lateral lipid organization, the lipids can be arranged in an orthorhombic (very dense and ordered), liquid (disordered) and hexagonal (less dense and ordered) organization. (Adapted from Thakoersing et al., (1))

Figure S2: Expression of SCD and ELOVL1 are changed in AD lesional skin.
Immuno-histochemical staining of stearoyl CoA desaturase (SCD), ELOVL1 and ELOVL6 in one control, non-lesional and the corresponding lesional skin from two AD patients (as shown in figure 1) without DAPI staining. Representative images are shown with 20x magnification, scale bar 50µm. NL: non-lesional, L: lesional.
Figure S3: Expression of CerS3, aSmase and GBA are changed in AD-lesional skin. Immuno-histochemical staining of CER synthase 3 (CerS3), acid-sphingomyelinase (aSmase) and glucocerebrocidase (GBA) in control, atopic dermatitis (AD) non-lesional and corresponding lesional skin (as shown in figure 2) without DAPI staining. Representative images are shown with 20x magnification, scale bar 50µm. NL: non-lesional, L: lesional.
**Figure S4**: Relative abundance of FFA subclasses (saturated and unsaturated)

a) Bar plots showing the chain length distribution of saturated free fatty acids (FFAs) and b) unsaturated FFAs in healthy, AD non-lesional and AD lesional skin. The total amount of all FFAs was set to 100%. There is a significant trend in the decreasing abundance of saturated FFA C24:0-FFA C30:0 in lesional AD skin (p<0.0001). In the chain length distribution of unsaturated FFA, there is a trend wise increase in the level of unsaturated FFA C16:1-FFA C30:1 in non-lesional and lesional AD skin (p<0.05 and p<0.001 respectively).
Figure S5: Lipid organization parameters in AD patients and control subjects
A) Position of the CH\textsubscript{2} symmetric stretching vibration. The lipids present in AD lesional skin are less ordered than in non-lesional and control skin as shown by a significant increase in the CH\textsubscript{2} symmetric stretching vibrations of the lipid hydrocarbon chains (p<0.001). B) CH\textsubscript{2} scissoring bandwidth in the FTIR spectrum. AD non-lesional and lesional skin show less dense lateral packing compared to control skin as the CH\textsubscript{2} scissoring band width was significantly reduced in both groups compared to the control (p<0.01). ***p<0.001, **p<0.01.

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Figure S6: Nomenclature of stratum corneum ceramides
The ceramides in human stratum corneum comprise of one sphingoid bases which is either i) dihydrosphingosine (dS) ii) sphingosine (S) iii) phytosphingosine (P) or iv) 6-hydroxy sphingosine (H)) chemically linked to a fatty acid chain. The fatty acids are i) a non-hydroxy fatty acid (N), ii) an α-hydroxy fatty acid (A) or iii) esterified ω-hydroxy fatty acid (EO)). These result 12 ceramide subclasses in human stratum corneum.
**Figure S7:** Illustration of late stage free fatty acids and ceramide synthesis

ELOVL: elongase, CerS: ceramide synthase, GBA: glucosylcerbrosidase, aSmase: acid-sphingomyelinase, SCD: stearoyl CoA desaturase, ωh: omega-hydroxy, GCS: Glucosylceramide synthase. The enzymes investigated in this study are highlighted in red (2-6).
### Supplementary tables

#### Table S1: Local, overall SCORAD and filaggrin mutations of AE patients

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**Table S2: Primary and secondary antibodies for immunohistochemical staining**

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Table S3: Number of subjects and techniques applied

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Supplementary materials and methods

Liquid chromatography-mass spectrometry (LC-MS)

FFA standards ranging from C14-C24 were used to correct for the effect of chain length on the MS response. In addition, the effect of the tape matrix on the MS response was corrected by using the ratio of integrated FFA (C16-30) from extracted human stratum with and without tape strip matrix. As reported earlier, endogenous FFAs C16:0, C17:0, C18:0, C16:1 and C18:1 were also present in the blank tape strips and therefore interfere with the analysis (7). An exogenous compound (358 amu) present only within the tape but not in SC correlated excellently with the FFAs present in the tape ($R^2$ values range from 0.92-0.98). Using this compound peak, it was possible to calculate the level of exogenous C16:0, C17:0, C18:0, C16:1 and C18:1 in each tape strip and subsequently correct the FFA profile for these levels. The FFA analysis was performed using Target Lynx software version 4.1.
References


TNF-α and $T_2^H$ cytokines induce atopic dermatitis-like features on epidermal differentiation proteins and stratum corneum lipids in human skin equivalents

Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease in which the skin barrier function is disrupted. In this inflammatory AD environment, cytokines are up-regulated, but the cytokine effect on the AD skin barrier is not fully understood. We aimed to investigate the influence of T\textsubscript{h}2 (IL-4, IL-13, IL-31) and pro-inflammatory (TNF-α) cytokines on epidermal morphogenesis, proliferation, differentiation and stratum corneum lipid properties. For this purpose, we used the Leiden epidermal model (LEM) in which the medium was supplemented with these cytokines.

Our results show that IL-4, IL-13, IL-31 and TNF-α induce spongiosis, augment TSLP secretion by keratinocytes and alter early and terminal differentiation-protein expression in LEMs. TNF-α alone or in combination with T\textsubscript{h}2 cytokines decreases the level of long chain free fatty acids (FFAs) and ester linked ω-hydroxy (EO) ceramides, consequently affecting the lipid organization. IL-31 increases long chain FFAs in LEMs but decreases relative abundance of EO ceramides. These findings clearly show that supplementation with TNF-α and T\textsubscript{h}2 cytokines influence epidermal morphogenesis and barrier function. As a result, these LEMs show similar characteristics as found in AD skin and can be used as an excellent tool for screening formulations and drugs for the treatment of AD.
Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with clinical manifestations of erythema, dryness, and pruritus (1, 2). The epidermal barrier is disrupted in both lesional and non-lesional AD skin and characterized by the presence of T$_h$1, T$_h$2 and T$_h$22 immune responses (3, 4). The interaction between cytokines and the skin barrier is not yet fully understood. Therefore, this study aims to determine whether cytokines influence lipid and protein synthesis in the epidermis and as a consequence affect skin barrier lipid composition and organization.

A fully differentiated epidermis consists of a proliferating stratum basale (SB), differentiating stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC). In the basal layer, keratinocytes synthesize proteins such as keratin 5 and 14, while differentiating keratinocytes synthesize proteins such as keratin 10 (K10), filaggrin, involucrin and loricrin. The latter three proteins are associated with the cornified envelope, a surrounding layer of the corneocytes, located in the SC (5, 6). Filaggrin especially plays an important role in AD because the occurrence of AD is strongly associated with mutations in the filaggrin gene encoding the barrier protein filaggrin (7-10). The skin barrier lipids, which are located within the SC also play a role in AD as changes in their chain length distribution correlate with an impaired skin barrier (11). The SC contains a highly organized lipid matrix located in the intercellular spaces between the corneocytes. The organization and composition of the SC lipid matrix is crucial for a competent skin barrier (12, 13). The main lipid classes in human SC are ceramides (CER), free fatty acids (FFAs) and cholesterol. Twelve CER subclasses have been identified in human SC with broad chain length distributions observed with FFA and CERs (14-16). SC lipids form ordered crystalline lamellar phases with repeat distances of 13nm (long periodicity phase, LPP) and 6nm (short periodicity phase, SPP) (17) (figure S1). Within the lamellae, the lipids form mainly, an orthorhombic (dense) lateral packing in human SC, while a fraction of lipids are organized in a hexagonal (less dense) lateral packing (18, 19) (figure S1). The CER subclass distribution of AD skin varies from healthy skin (20-24). In addition, AD skin shows i) a decrease in very long chain CER, ii) reduction in long chain FFAs levels and iii) increased level of unsaturated FFAs (25-27). These changes are more prominent in lesional than in non-lesional AD skin and correlates with a less dense SC lipid lateral packing and reduced skin barrier function.

It is hypothesized that a disrupted barrier in AD makes the skin vulnerable to penetration of allergens/irritants through the skin, causing a T$_h$2 immune response in the epidermis. Keratinocytes respond to this by secreting thymic stromal lymphopoietin (TSLP). TSLP activates dendritic cells (28) which further enhances the immune response by proliferation and differentiation of CD4$^+$ T-cells. TSLP is also considered to be a link between AD and the development of asthma/atopic march (29).

The aim of this study was to investigate the direct role cytokines play in the development of pathophysiological changes occurring in AD skin focusing on both lipids and epidermal differentiation proteins. Therefore, we used 3-dimensional (3D) tissue engineered human skin equivalents, namely the Leiden epidermal model (LEM), since it harbors a fully differentiated epidermis (30). The culture medium was supplemented
with IL-4, IL-13, IL-31 (TH2 cytokines) and TNF-α (pro-inflammatory cytokine) singly or a combination during generation of the LEMs. If the supplemented LEMs mimic several features of AD skin, the in vitro skin models can be used to examine pharmacologic interventions for AD. This is due to the need for in vitro skin models that mimic important features of AD skin due to large variations in skin architecture between the commonly used mice skin and human skin and limitations in the number of drugs that can be tested at a time during a clinical study.

Our results show that cytokines contribute to the changes in epidermal morphology, differentiation and skin barrier lipids observed in AD. This demonstrates that the LEM is an excellent model for studying the pharmacological intervention of topical formulations for the treatment of AD in in vitro settings.
Materials and methods

Cell culture
Normal human keratinocytes (NHK) were isolated from human adult breast skin donors undergoing cosmetic surgery (52; Supplementary materials and methods). The skin was collected after written informed consent from the donors and handled according to the Declaration of Helsinki Principles.

Generation of the Leiden epidermal models (LEM s)
LEM s were generated similarly as described by El Ghalbzouri et al with some modifications (Supplementary materials and methods) (30).

Morphology and Immunohistochemistry
Harvested LEMs were fixed in 4% paraformaldehyde (Lommerse Pharma, Oss, The Netherlands), dehydrated and embedded in paraffin. 5μm sections were cut, deparaffinized and rehydrated through graded ethanol series and stained with haematoxylin (2mg/ml) and eosin (4mg/ml). Immunohistochemical analysis of filaggrin, K10, ki67, loricrin, ELOVL1 and CerS3 is described in supplementary materials and methods. The primary and secondary antibodies used are listed in Table S2. The qPCR and western blot analysis is described in the supplementary materials and methods.

Enzyme-linked immunosorbent assay (ELISA)
The culture medium at the end of the culture period was collected and stored at -20°C till use. TSLP secretion was quantified using a TSLP quantikine protein ELISA kit (R&D systems, Abingdon, United Kingdom) according to manufacturer’s instructions. Statistical significance was performed with paired student t-test comparison between the control and other culture condition groups.

SC isolation and lipid extraction
The SC from LEM was isolated as described by De Jager et al (53). Lipids were extracted and pooled from three LEM SC using the modified Bligh and Dyer procedure (54) with the addition of 0.25M KCl to extract polar lipids (55). All extracts were dried under a stream of nitrogen, reconstituted in chloroform: methanol (2:1) and stored at -20°C. For LC-MS analysis the lipids were dried under a stream of nitrogen and dissolved in chloroform:methanol:heptane (2.5:2.5:95) at 1mg/ml concentration. The SC from LEMs were weighed before and after extraction in order to determine the total intercellular lipid and protein content.

High performance thin layer chromatography (HPTLC)
Extracted lipids were analyzed by HPTLC as described previously (56). The solvent system used for separation is listed in Table S2. Co-chromatography of a standard lipid mixture was performed to identify the various classes of lipids consisting of cholesterol, palmitic acid, stearic acid, tricosanoic acid, behenic acid, arachidic acid, cerotic acid, lignoceric acid (Sigma) and CER EOH, NS, NP, EOS and AP (Evonik, Germany, according to the terminology of the ceramide nomenclature of Motta et al and Masukawa et al used
throughout this article, (31, 57) (Table S3). The liquid chromatography mass spectrometry analysis is described in the supplementary materials and methods.

**Fourier transformed infra-red spectroscopy and Small angle X-ray diffraction**

For FTIR and SAXD, SC samples were hydrated for 24 hours at room temperature over a 27% (w/v) NaBr solution.

**FTIR:** the hydrated SC was measured as described previously ((58) supplementary materials and methods). The phase transitions of the SC lateral lipid organization was analyzed with a temperature range of 0°C and 90°C at a rate of 0.25 °C/min.

**SAXD:** measurements were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble) at station BM26B. The SC samples were measured as described before ((32); supplementary materials and methods).
Results

Cytokines influence epidermal organization and differentiation

First, we examined the morphology of LEMs supplemented with cytokines. In control LEMs, all epidermal strata are present. LEMs supplemented with IL-4, IL-13 or IL-31 also show the presence of all epidermal strata. However, spongiotic lesions i.e. inter-cellular oedema of keratinocytes, is present within the lower layers of the epidermis. LEMs supplemented with TNF-α only, show spongiosis and flatter suprabasal keratinocytes than the control. In LEMs stimulated with TH2 cytokines, with or without TNF-α, spongiosis and a comparable thickness of the viable epidermis was observed as with single supplementations (figure 1a).

Next, the influence of these cytokines on epidermal differentiation-protein expression was examined by immunohistochemical (IHC) staining. K10 is expressed in all suprabasal layers in control LEMs (figure 1a). However, in LEMs with single supplementations of TH2 cytokines or TNF-α, the SB and sections of the lower SS do not express K10. The combination of TH2 cytokines with or without TNF-α, show also this delayed K10 expression (figure 1a). In control and cytokine stimulated LEMs, filaggrin is expressed in the SG and the interface between the SG and the SC. However, all cytokine stimulated LEMs show reduced filaggrin staining than control LEMs (figure 1a). qPCR and western blott analysis also show that these cytokines significantly reduce mRNA and protein expression of filaggrin (figure S2, figure 1b respectively). The expression of loricrin was observed in the SG in both control and cytokine treated LEMs but cytokine stimulated LEMs show discontinuous or patchy expression of loricrin compared with control LEMs (figure 1a). TH2 cytokines and TNF-α reduced loricrin mRNA expression (38-68%), however, not significantly (figure S2). Loricrin protein expression was only clearly reduced with IL-13 or a combination of TH2 cytokines. This is different from the effect of cytokines on filaggrin expression.
Figure 1: TNF-α and T_{h}2 cytokines alter morphology and epidermal protein expression in LEMs. A) Cross sections of LEMs cultured in the presence or absence of cytokines. 20x magnification; Scale bar: 50µm. The black dotted line demarcates the viable epidermis from the stratum corneum. Overall morphology was assessed with haematoxylin and eosin staining and immunohistochemical analysis was performed for K10, filaggrin and loricrin. Staining of the stratum corneum is considered background/non-specific. B) Filaggrin western blot of cytokine stimulated and control LEMs (HS-Human skin; C-control; All(+)TNF-α- combination of IL-4, IL-13, IL-31 and TNF-α; All(-) TNF-α- combination of IL-4, IL-13, IL-31).
Cytokines increase basal cell proliferation and TSLP secretion of keratinocytes

After analyzing the expression of epidermal differentiation proteins in cytokine supplemented LEMs, we further examined basal keratinocyte proliferation by Ki67 staining. Compared with control LEMs, basal cell proliferation increased significantly with the supplementation of IL-4, IL-31 or TNF-α, whilst IL-13 did not affect basal cell proliferation (figure 2a). The T\_h2 cytokine mixture with or without TNF-α significantly increased basal keratinocyte proliferation compared with control LEMs. LEMs with increased basal proliferation also show delayed K10 expression and reduced filaggrin expression except for supplementation of IL-13.

Supplementation of single cytokines or a combination enhanced significantly medium TSLP levels (p<0.05; figure 2b) compared with the control. There was no significant difference in TSLP levels between supplementation of single cytokines and a combination of only T\_h2 cytokines (p>0.05-0.95). In addition, TSLP secretion was not significantly different in LEMs when comparing the mixture of only T\_h2 cytokines, with the mixture of T\_h2 cytokines and TNF-α (p=0.27).
Figure 2: Basal cell proliferation, TSLP secretion and SC lipid composition (HPTLC) of cytokine supplemented LEMs.
A) Proliferation index (PI) in LEMs supplemented with cytokines singly or in combination. The PI was determined as the percentage of Ki67 positive nuclei compared to total basal cells. B) TSLP secretion by LEMs analyzed by ELISA. C) SC lipid composition analysed by HPTLC of cytokine supplemented LEMs individually or in combination. The data represents the mean and standard deviation of 3 independent experiments. Statistical significance is indicated from student t-test between the control and other culture condition groups (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). All(+): TNF-α-combination of IL-4, IL-13, IL-31; All(-):TNF-α-combination of IL-4, IL-13, IL-31; HPTLC: High performance liquid chromatography. See tableS3 for CER nomenclature according to Motta et al., 1993 (31)

TNF-α and IL-31 affect SC lipid composition in LEMs
After profiling the effect of these cytokines on epidermal morphology and protein expression, we focused on the SC lipid barrier properties. The main SC lipid subclasses CER, cholesterol and FFAs are present in the LEM irrespective of cytokine addition (figure 2c). In addition, the total amount of inter-cellular SC lipids did not change with cytokine stimulation (Table S4). TNF-α singly or in combination with T_h2 cytokines significantly increased the percentage of CERs and reduced cholesterol levels in LEMs (Table S4). We further analyzed the lipids with liquid chromatography coupled to mass spectrometry (LC-MS), examining CER subclasses and chain length distribution of saturated FFAs (SFA) and mono-unsaturated FFAs (MUFA). We focused on the even FFA chain lengths since the odd chain lengths in total contributed < 5% of the total FFA content.
No significant changes were induced in the total SFA and MUFA with cytokine treatment (data not shown). However, when focusing on SFA chain length distribution in LEMs stimulated with either TNF-α or the combination of Th2 cytokines with TNF-α, we observe an interesting trend where the relative abundance of SFA ≥ 20 carbon atoms (C20:0) is reduced compared with control LEMs. Although the individual FFA chain lengths were not significantly decreased, this trend in the reduction of the relative abundance of SFA ≥ C20:0 is significant (p<0.01; figure 3a). The presence of IL-31 in the culture medium increased the relative abundance of SFA ≥ C20:0 compared with control LEMs. Although the changes in individual chain lengths are minor, the trend in the persistent increase in SFA ≥ C20:0 is significant (p<0.05; figure 3c, figure S5, absolute values in Table S5). There was no significant difference in the levels of MUFA in LEMs treated with IL-31, TNF-α or combination of Th2 cytokines and TNF-α, compared with control LEMs (figure 3b and 3d). IL-4, IL-13 and the combination of Th2 cytokines did not affect the chain length distribution of SFA and MUFA (figure S4, absolute values in Table S5 and S6). The mRNA and protein expression of the enzyme elongase 1 (ELOVL1) which is involved in the production of long chain FFAs was analyzed by qPCR and immunofluorescence respectively. The mRNA abundance in LEMs treated with TNF-α and IL-31 was approximately two times lower than the control LEMs although this reduction was not statistically significant (figure S6a). However, ELOVL1 mRNA levels with the combination of Th2 cytokines and TNF-α was slightly higher compared to control LEMs (figure S6a). ELOVL1 protein expression is present in the entire epidermis with the highest expression in the SG and upper SS in the control and IL-31 supplemented LEMs. This high expression of ELOVL1 at the SG and upper SS is absent with TNF-α singly or in combination with Th2 cytokines compared with the control (figure 3e).

The LC-MS CER profile shows 12 CER subclasses in control, IL-31 and TNF-α supplemented LEMs (figure 4a,b,c). The four CERs with higher masses in the upper part of the mass spectrogram belong to the EO subclasses (ester linked ω-hydroxy CER subclasses). The α-hydroxy (A) and non-hydroxy (N) CER subclasses have shorter chains and lower masses (non-EO CERs). The total peak area ratios of EO CERs:non-EO CERs was significantly reduced in TNF-α and IL-31 supplemented LEMs compared with the control (p<0.05; figure 4d, absolute values in Table S7) demonstrating a lower relative level of EO CERs. Ceramide synthase 3 (CerS3) is responsible for linking the very long acyl chains to the sphingoid base and is particularly involved in the synthesis of EO CERs. A two fold reduction in CerS3 mRNA abundance was observed in LEMs supplemented with IL-31 and TNF-α compared with the control, although the reduction was not statistically significant (figure S6b). However, LEMs with the combination of Th2 cytokines and TNF-α show similar CerS3 mRNA levels as in the control LEMs (figure S6b). CerS3 on protein level is expressed in the entire epidermis with the highest expression at the SG and upper SB in the control. The treatment with IL-31 resulted in a reduction in the expression of CerS3 in suprabasal layers. However, LEMs treated with TNF-α alone or in combination with Th2 cytokines showed only CerS3 expression in the granular layer (figure 4e).
Figure 3: TNF-α reduces and IL-31 increases long chain FFAs.

Saturated FFAs chain length distribution in LEM supplemented with A) TNF-α, a combination of IL-4, IL-13, IL-31 and TNF-α referred to as All(+)TNF-α C) IL-31. Unsaturated FFAs chain length distribution in LEM supplemented with B) TNF-α, All(+)TNF-α and D) IL-31. E) Immunofluorescence analysis for ELOVL1, 20x magnification, Scale bar 50µm. The white dotted line demarcates the viable epidermis from the stratum corneum. The data represents the mean and standard deviation of 3 independent experiments. Statistical significance is indicated from a 2-way ANOVA test between the control and other culture condition groups (*p ≤ 0.05, **p ≤ 0.01).
**Figure 4:** TNF-α and IL-31 reduce the ratio of EO to non-EO CERs in LEMs.

Two dimensional multi-mass LC-MS chromatograms of CERs subclasses and CER chain length distribution of LEM supplemented with A) TNF-α B) IL-31 or C) control. The retention time is displayed on the x-axis and the mass on the y-axis. D) Ratio of very long EO CERs:non-EO CERs in control, IL-31 and TNF-α supplemented LEMs. E) Immunofluorescence analysis of CerS3, 20x magnification; Scale bar 50µm. The white dotted line demarcates the viable epidermis from the stratum corneum. The data represents the ratios from three independent experiments. LC-MS:Liquid chromatography-mass spectrometry, CERs:ceramides

**TNF-α changes SC lipid organization in LEMs**

The effect of inflammation on the lipid organization remains unknown, therefore the lamellar organization in SC was examined using small angle X-ray diffraction (SAXD). Diffraction profiles of both control and cytokine supplemented LEM show four diffraction peaks representing the first, second and third order peak of the LPP and cholesterol respectively (referred to as 1, 2, 3 and *, figure 5a,b). Supplementing LEMs with TNF-α or T\(_2\) cytokines and TNF-α combined, significantly reduced the LPP repeat distance (p=0.06 and p=0.02 respectively; figure 5c) compared with the control LEMs. All other supplementations did not significantly affect the LPP repeat distance.

Using FTIR, the lateral organization of the SC lipids was examined as a function of temperature from the \(\text{CH}_2\) rocking vibrations of the FTIR spectrum. In an orthorhombic (dense) lipid packing, the \(\text{CH}_2\) rocking
region consists of vibrations at 719 cm\(^{-1}\) and 730 cm\(^{-1}\), whilst a hexagonal (less dense) lateral organization results in a vibration at 719 cm\(^{-1}\). All LEMs show a strong vibration band at 719 cm\(^{-1}\) and a weak band at 730 cm\(^{-1}\) between 0°C and 20°C. This demonstrates that below 20°C the lipids mainly form a hexagonal lateral packing and small orthorhombic domains. Above 20°C, a single peak is observed at 719 cm\(^{-1}\) showing only hexagonal lateral packing (figure S3a,b).

The CH\(_2\) stretching vibration provides information about the conformational disordering of the lipids. Crystalline packing results in low conformational disorder and symmetric stretching frequencies below 2850 cm\(^{-1}\). High conformational disorder is observed in a liquid phase with symmetric stretching frequencies \(\geq 2852\) cm\(^{-1}\). The midpoint temperature of the transition (MTT) from crystalline to liquid phase was determined as described by Thakoersing et al (32) (figure S3c). The addition of TNF-\(\alpha\) significantly reduced the MTT (p=0.03). The CH\(_2\) stretching band position at 320°C did not statistically differ from the control after the various supplementations (figure S3d).

**Figure 5:** TNF-\(\alpha\) reduces the repeat distance of the LPP and the lipid mid-transition temperature in LEMs.

A) The X-ray curve of control, single cytokine supplemented LEMs and B) LEMs supplemented with a combination of cytokines. C) Repeat distance of the LPP for each culture condition. D) The mid-transition temperature (MTT) from the CH\(_2\) stretching position of the FTIR spectrum. MTT is calculated as the average of the wavenumber at the beginning and end of the transition (see figure S3c). The MTT is therefore the temperature at this calculated wave number. The data represents the mean and standard deviation of 2-3 samples (*p \(\leq 0.05\)). All(+)TNF-\(\alpha\)- combination of IL-4, IL-13, IL-31 and TNF-\(\alpha\); All(-)TNF-\(\alpha\)- combination of IL-4, IL-13, IL-31.
Discussion

In the present study we showed the effect of Th2 cytokines and TNF-α on epidermal and SC lipid characteristics. As the concentrations of IL-4, IL-13, IL-31 and TNF-α in AD skin remains unknown, the cytokine concentrations added to the LEMs was based on a dose dependent study (data not shown). We selected the cytokine concentrations that induced the highest TSLP levels whilst maintaining viable keratinocytes. For the first time we show that Th2 cytokines and TNF-α induce both, epidermal and SC lipid changes characteristic of AD skin. These conclusions are based on the following findings.

Firstly, TNF-α singly or in combination with Th2 cytokines induces modulation in barrier lipid properties, observed in AD lesional skin. The abundance of FFAs ≥ C20:0 was observed to be reduced in lesional skin compared to healthy skin (26). TNF-α singly or in combination with Th2 cytokines caused a coordinated reduction in the relative abundance of saturated FFAs ≥ C20:0. This effect on FFA chain length distribution is larger with a combination of Th2 cytokines and TNF-α than TNF-α alone. ELOVL1 plays a role in the synthesis of long chain FFAs (33, 34). Our results show that TNF-α alone or in combination with Th2 cytokines reduce ELOVL1 protein expression and this change may consequently affect epidermal permeability (34). This reduction of ELOVL1 expression on protein level was not observed on mRNA level with TNF-α in combination with Th2 cytokines. Since mRNA levels only predict 40% of protein abundance (35), we suggest that this cytokine combination may mainly influence translational and/or post-translational mechanisms of ELOVL1 rather than the transcription process.

A reduced level of EO CER subclasses and lower average CER chain length is observed in AD skin which strongly correlates to SC barrier function (25, 27, 36). The mRNA expression of CerS3 was not significantly altered with TNF-α. However, we observe reduced CerS3 protein expression with IL-31 and TNF-α followed by a reduction in the ratio of EO CER:non-EO CER levels. As a result, the effect of IL-31 and TNF-α on CerS3 expression and CER composition may therefore be involved in CER changes in AD. Furthermore, IFN-γ has also been suggested to decrease long chain CER NS via down regulation of ELOVL1 and CerS3 mRNA expression in a 3 dimensional epidermal model, suggesting its involvement in CER synthesis in AD and psoriasis (37). As LEMs do not desquamate, the lipid composition of the LEMs is a combination of the entire culture period. This includes the first 10 days of culture without supplementation of cytokines. Therefore, the effects observed in this in vitro model may be an underestimation of the in vivo situation. Sawada et al., 2013 reports that TNF-α increases and IL-4 decreases the total CER levels in epidermal equivalents (38). We observe an increase in CER levels with TNF-α but no effect with IL-4. These differences may arise from differences in the experimental set-up. In the studies of Sawada et al., cytokines were added to the cultures after they were fully generated, however, in our study, the cytokines were added immediately after a stratified epidermis has been produced. The lipid synthesis may therefore differ based on the time point of IL-4 stimulation. Hatano et al., using human epidermal sheets showed that TNF-α alone does not affect the CER/protein ratio after 3 days of treatment, which is a much shorter period as used in the present study (39).
Secondly, TNF-α alone or combined with T\textsubscript{h}2 cytokines significantly reduces the repeat distance of the LPP and TNF-α alone reduces the MTT in comparison to control. The changes in lipid organization can easily be explained by a reduced chain length of the lipids for the following reasons i) shorter lipid chains result in lower number of carbon atoms bridging the lamellae leading to a shorter LPP and ii) a reduction in saturated long chain FFAs is known to reduce the MTT (40), contributing to a higher trans epidermal water loss (26).

Thirdly, we show that TNF-α and T\textsubscript{h}2 cytokines significantly augment TSLP secretion compared to the control LEMs, as observed in AD (41). Previous studies show that the combination of T\textsubscript{h}2 cytokines and TNF-α induces TSLP production in full thickness human skin explants, however, TNF-α alone has no effect on TSLP levels in their model system (42). The levels of TSLP produced are also higher than observed in our study with LEMs. We suggest that i) the presence of dendritic cells in full thickness skin and ii) a direct effect from the presence of fibroblasts since they have been shown to express TSLP mRNA \textit{in vitro} (43) may contribute to these differences. Therefore, LEMs may provide a good model to examine the direct influence of cytokines on keratinocytes.

Fourthly, stimulation of LEMs with IL-4, IL-13, IL-31 and TNF-α induce spongiosis in LEMs similarly as observed in AD (44). We also show that IL-31 influences epidermal cohesion in addition to its known effects in regulating keratinocyte differentiation and pruritus in AD (45, 46). IL-4 and IL-13 have also been reported as inducers of spongiosis in epidermal models (47).

Lastly, cytokines examined in this study delay early differentiation and increase proliferation in LEMs in a similar pattern as observed in AD skin. However, no increase in the thickness of the viable epidermis was observed contrasting the \textit{in vivo} situation (48, 49). This may result from a high epidermal turnover rate (7 days) in LEMs as compared to 30 days in native human epidermis (50). In addition, the delayed K10 expression also confirms the proliferation increase, as the undifferentiated cells are escaping to the lower stratum spinosum. T\textsubscript{h}2 cytokines, in our experimental conditions, less prominently affect the lipids, but rather reduce the expression of barrier proteins, filaggrin and loricrin. We suggest that the T\textsubscript{h}2 cytokines may affect the permeability barrier by reducing filaggrin and thus reduce NMF levels resulting in increased SC pH. This higher pH can lead to alterations of several enzymes, such as serine proteases and induce alterations in permeability barrier homeostasis, SC integrity and cohesion (51).

We show a clear relationship between the effect of cytokines on proliferation and differentiation. However, these changes in proliferation and differentiation do not reflect in SC lipid composition. This suggests that cytokine induced changes in epidermal differentiation and SC lipid synthesis are influenced by different pathways. We conclude from this study that supplementation of LEMs with cytokines (individually or in synergy) induces several important features of AD skin \textit{in vivo} on lipid and protein level. Therefore, these cytokine supplemented LEMs may serve as an excellent candidate for the testing of prospective drugs and formulations for the treatment of AD.
Acknowledgements

The authors thank the personnel at the DUBBLE beam line (BM26) ESRF for their support with the X-ray measurements. This research was partly financially supported by Dutch Technology Foundation STW (grant no. 10703) and the C.E.R.I.E.S Research Award received by J.A. Bouwstra in 2011. The synthetic CERs were kindly provided by Evonik, Germany.
References


**Supplementary figures**

**Figure S1**: Lateral and lamellar organization in human stratum corneum

The stratum corneum (SC), the outermost part of the epidermis consists of dead cells (corneocytes) embedded in a lipid matrix. The intercellular lipids are organized in layers or lamellae which consists of two lamellar phases. Firstly, the long periodicity phase (LPP) having a repeat distance (d) of 13nm and secondly, the short periodicity phase with a repeat distance of 6nm. The lateral organization is perpendicular to the lamellar organization. The lipids can be arranged in three different patterns: orthorhombic (very dense and ordered), hexagonal (less dense and ordered) and liquid (disordered) organization. Adapted from Thakoersing et al (1)
Figure S2: TH2 cytokines and TNF-α down regulate mRNA expression of barrier proteins. Normalized mRNA expression of A) filaggrin and B) loricrin. Statistical significance is indicated from student t-test comparison between the control and other culture condition groups (n=3±SEM, *p ≤ 0.05, **p ≤ 0.01).
Figure S3: $T_{m2}$ cytokines and TNF-α do not affect the SC lateral lipid organization in LEM. The rocking vibrations in the FTIR spectra providing information on the lateral lipid organization in control and cytokine treated conditions is shown as a function of temperature (A and B respectively). At lower temperatures (0°C), the lipids mainly form hexagonal packing (vibrations at 719 cm$^{-1}$) and a small population of lipids forms orthorhombic domains (additional vibration at 730 cm$^{-1}$). The orthorhombic phase disappears around 20°C in both control and cytokine treated conditions. C) Illustrates the procedure to calculate the midpoint of the crystalline to liquid transition examined by the CH$_2$ stretching vibrations of the lipids. The midpoint transition temperature is calculated by taking the average of the last wave number at the beginning ($W_{N_{start}}$) of the transition and the first wave number at the end of the transition ($W_{N_{end}}$). The midpoint temperature is therefore the temperature at this calculated wave number. D) CH$_2$ stretching peak position at 32°C derived from the FTIR spectrum. All (+) TNF-α: IL-4/IL-13/IL-31/TNF-α; All (-) TNF-α: IL-4/IL-13/IL-31.
Figure S4: Supplementation of Th2 cytokines singly or in combination does not alter FFA chain length in LEMs. Sum of saturated and mono-unsaturated FFA with chain length < and ≥ 20 carbon atoms in LEMs supplemented with A) IL-4 B) IL-13 C) combination of IL-4, IL-13 and IL-31, referred to as All(-) TNF-α and the control. The data represents the mean and standard deviation of 3 independent experiments.

Figure S5: FFA composition in TNF-α and IL-31 stimulated LEMs. Sum of A) saturated and B) mono-unsaturated FFAs with chain lengths < and ≥ 20 carbon atoms in LEMs stimulated with TNF-α, combination of IL-4, IL-13, IL-31 and TNF-α referred to as All(+) TNF-α, IL-31 and the control. The data represents the mean and standard deviation of 3 independent experiments.
Figure S6: IL-31 and TNF-α influence mRNA expression of ELOVL1 and Cers3 in LEMs. TNF-α reduces a) ELOVL1 and b) Cers3 mRNA expression in LEMs and IL-31 reduces CerS3 expression compared with the control. Although the reduction in mRNA levels are not statistically significant these findings support the reduction in protein expression observed by immunohistochemistry. The ELOVL1 mRNA expression with All(+)TNF-α or IL-31 supplementation does not correlate with the protein expression as ELOVL1 protein expression is lower with All(+) TNF-α compared to control, while in case of IL-31 the ELOVL1 protein expression is similar to the control. As published in literature mRNA abundances only partially predict protein levels since only ~40% of the variation in the concentration of protein can be explained by mRNA abundances (2). Therefore, the differences between mRNA abundances and protein levels in All(+)TNF-α may result from changes translational and/or post-translational mechanisms of lipid synthesis proteins rather than the transcription process. In the case of IL-31, the half-life of proteins/mRNA may be an important determining factor that influences protein levels as the mRNA levels may be low but the protein may be stable and translated efficiently. The data represents the mean and standard deviation of three independent experiments measured in duplicate. Statistical significance was determined by a 2-way ANOVA statistical analysis.
Supplementary tables

**Table S1: Primary and secondary antibodies for immunohistochemical staining**

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<tr>
<td>(IF)</td>
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*IF: immunofluorescence*
### Table S2: Solvent system used for barrier lipids analysis by thin layer chromatography

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<td>2</td>
<td>Chloroform/Acetone/Methanol (76:8:16)</td>
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<td>3</td>
<td>Hexane/Chloroform/Acetone/Methanol (6:80:12:2)</td>
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<td>4</td>
<td>Hexane/Chloroform/Hexyl acetate/Acetone/Methanol (6:80:0.1:10:4)</td>
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### Table S3: Ceramide nomenclature according to Motta et al (3)

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Table S4: Total amount of stratum corneum lipids in cytokine stimulated LEMs per dry weight of stratum corneum (mg/mg SC) and percentage distribution of free fatty acids, ceramides and cholesterol.

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<th>Cholesterol (%)</th>
<th>Ceramides (%)</th>
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<td>16.8 ± 1.9</td>
<td>20.6 ± 1.8*</td>
<td>62.6 ± 0.1*</td>
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<td>All(+)TNF-α</td>
<td>0.27 ± 0.09</td>
<td>18.0 ± 2.3</td>
<td>20.3 ± 3.1*</td>
<td>61.7 ± 2.3*</td>
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<tr>
<td>Control</td>
<td>0.26 ± 0.03</td>
<td>18.3 ± 0.4</td>
<td>23.0 ± 1.7</td>
<td>58.7 ± 1.4</td>
</tr>
</tbody>
</table>

SC barrier lipids were fractionated and the bands of the FFA, CER and CHOL were quantified by HPTLC and photodensitometry respectively (sum of FFA, CER and CHOL=100%). The data represents the mean and standard deviation of 3 independent experiments. Statistical significance is indicated from student t-test comparison between the control and other culture condition groups (*p ≤ 0.05). Cytokine supplementation in LEMs did not significantly alter the total amount of stratum corneum lipids per dry stratum corneum weight. SC: Stratum corneum.
Table S5: Absolute amount of saturated FFAs varying in chain length per dry weight of stratum corneum (µg/mg SC) in cytokine stimulated LEMs (analyzed by LC-MS).

<table>
<thead>
<tr>
<th>FFA chain length</th>
<th>IL-4</th>
<th>IL-13</th>
<th>IL-31</th>
<th>TNF-α</th>
<th>All(+) TNF-α</th>
<th>All(-) TNF-α</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFAC16:0</td>
<td>3.3 ±1.1</td>
<td>3.4 ±1.4</td>
<td>3.6 ±1.6</td>
<td>4.6 ±2.3</td>
<td>5.6 ±2.5</td>
<td>4.2 ±1.1</td>
<td>4.8 ±2.3</td>
</tr>
<tr>
<td>FFAC18:0</td>
<td>3.4 ±1.1</td>
<td>3.5 ±0.5</td>
<td>3.4 ±0.6</td>
<td>4.9 ±1.2</td>
<td>4.4 ±1.8</td>
<td>4.3 ±0.9</td>
<td>4.1 ±0.9</td>
</tr>
<tr>
<td>FFAC20:0</td>
<td>1.3 ±0.6</td>
<td>1.5 ±0.5</td>
<td>1.4 ±0.3</td>
<td>1.6 ±0.2</td>
<td>1.3 ±0.8</td>
<td>1.6 ±0.8</td>
<td>1.7 ±0.4</td>
</tr>
<tr>
<td>FFAC22:0</td>
<td>2.2 ±0.5</td>
<td>2.6 ±0.5</td>
<td>2.6 ±0.4</td>
<td>2.7 ±0.5</td>
<td>2.0 ±0.8</td>
<td>2.8 ±0.6</td>
<td>3.0 ±0.2</td>
</tr>
<tr>
<td>FFAC24:0</td>
<td>8.4 ±0.4</td>
<td>9.5 ±1.9</td>
<td>9.4 ±0.8</td>
<td>9.2 ±2.4</td>
<td>7.6 ±3.1</td>
<td>10.7 ±1.9</td>
<td>11.0 ±0.4</td>
</tr>
<tr>
<td>FFAC26:0</td>
<td>4.6 ±0.5</td>
<td>4.8 ±1.5</td>
<td>5.1 ±0.5</td>
<td>4.9 ±2.5</td>
<td>4.0 ±2.7</td>
<td>5.6 ±1.6</td>
<td>5.9 ±1.0</td>
</tr>
<tr>
<td>FFAC28:0</td>
<td>0.5 ±0.4</td>
<td>0.6 ±0.5</td>
<td>0.5 ±0.4</td>
<td>0.4 ±0.5</td>
<td>0.2 ±0.2</td>
<td>0.4 ±0.3</td>
<td>0.5 ±0.4</td>
</tr>
<tr>
<td>≥FFAC30:0</td>
<td>0.1 ±0.0</td>
<td>0.1 ±0.0</td>
<td>0.1 ±0.0</td>
<td>0.1 ±0.0</td>
<td>0.1 ±0.1</td>
<td>0.1 ±0.0</td>
<td>0.1 ±0.0</td>
</tr>
</tbody>
</table>

In LEMs stimulated with either TNF-α or the combination of TH2 cytokines with TNF-α, we observe a trend where the relative abundance of SFA ≥ 20 carbon atoms (C20:0) is reduced compared with control LEMs (p<0.05 and p<0.01 respectively). The data represents the mean and standard deviation of 3 independent experiments. SC: Stratum corneum
Table S6: Absolute amount of unsaturated FFA chain lengths per dry weight of stratum corneum (µg/mg SC) in cytokine stimulated LEMs (analyzed by LC-MS).

<table>
<thead>
<tr>
<th>FFA chain length</th>
<th>IL-4</th>
<th>IL-13</th>
<th>IL-31</th>
<th>TNF-α</th>
<th>All(+) TNF-α</th>
<th>All(-) TNF-α</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFAC16:1</td>
<td>0.6 ± 0.2</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>1.2 ± 0.8</td>
<td>0.9 ± 0.6</td>
<td>0.8 ± 0.4</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>FFAC18:1</td>
<td>3.0 ± 1.1</td>
<td>4.2 ± 2.3</td>
<td>3.2 ± 1.0</td>
<td>6.1 ± 4.9</td>
<td>7.3 ± 5.6</td>
<td>4.2 ± 2.8</td>
<td>5.6 ± 3.1</td>
</tr>
<tr>
<td>FFAC20:1</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>1.3 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>FFAC22:1</td>
<td>0.4 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.6 ± 0.3</td>
<td>1.0 ± 1.2</td>
<td>0.4 ± 0.3</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>FFAC24:1</td>
<td>2.4 ± 0.3</td>
<td>9.5 ± 1.9</td>
<td>9.4 ± 0.8</td>
<td>9.2 ± 2.4</td>
<td>7.6 ± 3.1</td>
<td>10.7 ± 1.9</td>
<td>11.0 ± 0.4</td>
</tr>
<tr>
<td>FFAC26:1</td>
<td>2.4 ± 0.5</td>
<td>2.8 ± 1.8</td>
<td>2.0 ± 1.1</td>
<td>2.8 ± 1.1</td>
<td>4.1 ± 3.1</td>
<td>2.6 ± 1.8</td>
<td>2.8 ± 1.4</td>
</tr>
<tr>
<td>FFAC28:1</td>
<td>1.4 ± 0.6</td>
<td>1.6 ± 1.1</td>
<td>1.2 ± 0.7</td>
<td>1.4 ± 0.7</td>
<td>2.1 ± 1.5</td>
<td>1.4 ± 1.0</td>
<td>1.6 ± 1.0</td>
</tr>
</tbody>
</table>

There is no significant difference in the levels of MUFA in cytokine treated LEMs compared with control LEMs. The data represents the mean and standard deviation of 3 independent experiments. SC: Stratum corneum.
Table S7: Absolute amount of EO CERs and non-EO CERs in cytokine stimulated LEMs per dry weight of stratum corneum (µg/mg SC; analyzed by LC-MS).

<table>
<thead>
<tr>
<th>LEM</th>
<th>EO CERs (µg/mg SC)</th>
<th>Non-EO CERs (µg/mg SC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-31</td>
<td>11.2 ± 4.2*</td>
<td>100.3 ± 4.2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>19.0 ± 2.8*</td>
<td>156.2 ± 2.8</td>
</tr>
<tr>
<td>Control</td>
<td>27.6 ± 9.9</td>
<td>125.1 ± 9.9</td>
</tr>
</tbody>
</table>

IL-31 and TNF-α significantly reduce the levels of EO CERs in comparison with the control LEMs. The data represents the mean and standard deviation of 3 independent experiments (*p<0.05). SC: Stratum corneum.
Supplementary materials and methods

Cell culture
The skin was collected after written informed consent from the donors and handled according to the Declaration of Helsinki Principles. Keratinocytes were cultured in a monolayer with the Dermalife K medium complete kit (Lifeline Cell Technology, Walkersville, MD, USA) supplemented with 1% penicillin/streptomycin (Sigma, The Netherlands) until 60-70% confluent. The keratinocytes were harvested by trypsin digestion. First passage keratinocytes were used to generate LEM.

Generation of the Leiden epidermal models (LEMs)
Keratinocytes (0.2 x 10^6 cells/filter) were seeded into cell culture inserts (Corning Transwell cell culture inserts, membrane diameter 12 mm, pore size 0.4 µm; Corning Life Sciences, Amsterdam, The Netherlands) and cultured submerged in supplemented Dermalife medium until confluent. Subsequently, the LEMs were submerged for 1 day in CnT-02-3DP5 medium (CellnTec, Bern, Switzerland) and serum free medium containing 0.1µM L-carnitine (Sigma), 86µM insulin (Sigma), 10µM L-serine (Sigma), Isoproterenol 1µM, 53nM selenious acid (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany), supplemented with 1 µM α-tocopherol acetate (Sigma), 25mM ascorbic acid (Sigma) and a lipid mixture of 7 µM arachidonic acid (Sigma), 30 µM linoleic acid (Sigma) 25 µM palmitic acid (Sigma) with 0.024 µM bovine serum albumin (Sigma). After this period the cultures were lifted to the air-liquid interface for 7 days. On day 7 of air exposure, IL-4, IL-13, IL-31 and TNF-α (R&D systems, Abingdon, United Kingdom, 30ng/ml, 30ng/ml, 15ng/ml and 3.5ng/ml respectively) or a combination of the interleukins in presence and absence of TNF-α (5ng/ml each) was supplemented to the medium until harvest, for 11 days.

Morphology and Immunohistochemistry
For both staining procedures (with amino-ethylcarbazole and immunofluorescence), the sections were deparaffinised, rehydrated with ethanol series and washed with phosphate-buffered saline (PBS). The sections were incubated in sodium citrate buffer (pH 6) for 30 minutes at 95°C for antigen retrieval. After cooling, the sections were blocked for 20 minutes with normal horse serum and incubated with the primary antibody diluted in 1% PBS/bovine serum albumin (BSA) overnight at 4°C.

Staining procedure with amino-ethylcarbazole: Sections were washed in PBS and incubated with the secondary antibody for 30 minutes, washed with PBS and incubated with ABC reagent for 30 minutes. The sections were further washed with PBS and 0.1M sodium acetate buffer, after which they were incubated for 30 minutes in amino-ethylcarbazole (Sigma) dissolved in sodium acetate buffer (0.02g/100mL) (Sigma) with 0.1% hydrogen peroxide and washed finally with water. Incubation with normal horse serum, ABC reagent and secondary antibody was performed with the RTU vectastain Elite ABC reagent kit (Vector laboratories Burlingame, CA). All sections were counterstained with haematoxylin.
Immunofluorescence staining: Sections were washed in PBS and incubated with the secondary antibody for 30 minutes and again washed with PBS. The sections were mounted using DAPI Vectashield (Vector laboratories Burlingame, CA).

RNA isolation, cDNA synthesis and qPCR
The LEMs firstly underwent a proteinase K treatment and then RNA was isolated using the RNeasy kit (Qiagen) according to manufacturer’s instructions. cDNA was produced with the iScript cDNA synthesis kit (BioRad, the Netherlands) according manufacturer’s instructions. PCR reactions were based on the SYBR Green method (BioRad) with the use of CFX384 system (BioRad). Expression analysis was performed using CFX software using the ΔΔCt method using the reference gene β-2-microglobulin. Data represent mean and standard deviation of 3 different experiments.

Protein isolation and Western blot
Proteins were isolated from LEMs using Tissue Protein Extraction Reagent (T-PER) which was supplemented with protease inhibitor cocktail and HALTTM phosphatase inhibitors (Thermo Scientific, Etten-Leur, the Netherlands). Protein extracts were loaded onto a 10% SDS-PAGE gel and blotted on a polyvinylidene difluoride membrane (Thermo Scientific). 10% Elk Milk (Campina, The Netherlands) in phosphate-buffered saline-T (0,1% Tween) was used as a blocking buffer. The sample was incubated overnight at 4°C with primary antibody after which they were incubated with the secondary antibody, horseradish peroxidase-conjugated anti-rabbit (Thermo Scientific, 1:2500). Proteins were detected using Supersignal West Femto ECL (Thermo Scientific/Pierce).

Fourier transformed infra-red spectroscopy
SC samples were hydrated for 24 hours at room temperature over a 27% (w/v) NaBr solution. A Varian 670-IR FTIR spectrometer (Agilent technologies, Santa Clara, CA), equipped with a broad-band mercury cadmium telluride (MCT) detector, cooled with liquid nitrogen was utilized to acquire spectra. The sample was sandwiched between silver bromide (AgBr) windows and spectra collected in transmission mode and derived from the addition of 256 scans at 1 cm⁻¹ resolution every 4 minutes (frequency range of 600-4000 cm⁻¹, temperature range of 0°C and 90°C at a rate of 0.25°C/min). Varian resolutions Pro software (Agilent technologies, the Netherlands) was used to process the spectra.

Small angle X-ray diffraction
All measurements were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble) at station BM26B. SAXD patterns were detected with a Frelon 2000 CCD detector at room temperature for 10 min using a microfocus of 25µm X 25µm. The X-ray wavelength and sample-to-detector distance were 0.1127nm and 24cm respectively. The samples were hydrated for 24 hours at room temperature over a 27% (w/v) NaBr solution. The scattering intensity (I, arbitrary units) was measured as a function of the scattering vector q (in nm⁻¹). Q is defined as q= (4πsinθ)/λ, in which θ is the scattering angle and λ is the wavelength. From the positions of a series of equidistant peaks (qₙ), the periodicity (d) of a lamellar phase
was calculated using the equation \( d = \frac{2n\pi}{q} \), \( n \) being the order of the lamellar phase. At least 3 samples per condition were measured.

**Liquid chromatography mass spectrometry (LC-MS)**

*FFA species:* LC-MS analysis of FFA species was performed according to van Smeden et al (4). Injection volume of extracted lipids (1mg/ml) was set at 10 µl into a LiChroCART Purospher STAR analytical column (55 x 2 mm i.d. Merck, Darmstadt, Germany) under a flow rate of 0.5 ml/min using a binary gradient solvent system of ACN/H₂O (90:10, solvent A) and methanol/heptane (90:10, solvent B). An Alliance 2695 HPLC system (Waters, Milford, MA, USA) was used in reverse phase. The HPLC was connected to a TSQ Quantum Mass Spectrometer (TSQ Quantum, Thermo Finnigan, San Jose, CA) measuring in Atmospheric-pressure chemical ionization (APCI) negative mode (scan range of 200-600 amu). The source heater temperature and heated capillary was set at 450°C and 250°C respectively and the discharge current at 5µA. The analysis was performed using Xcalibur software version 2.0. The ceramide analysis is described in the supplementary materials and methods.

*CER species:* 1 µl of each lipid sample (40°C) was automatically injected and separated in an analytical normal phase column set at 55°C (ACQUITY HSS Cyano; 21. x 100mm; 1.8µm particle size; Waters, Milford, MA) under a flow rate of 0.8ml/min and using a binary solvent system of heptane (100%, solvent A) and heptane/isopropanol/ethanol (50:25:25, solvent B). An ACQUITY quaternary UPLC system (Waters, Milford, MA) was coupled to a mass spectrometer (Xevo, TQ-S, Waters, Milford, MA) in atmospheric pressure chemical ionization (APCI; ion sabre II), positive scan mode with a scan range of 350-1200 amu. The temperature of the source heater was set at 625°C, capillary voltage at 3.5kV. The analysis was performed using Target Lynx software version 4.1.
References


Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin
CHAPTER 4

Exploring the potential of nurture: 2\textsuperscript{nd} and 3\textsuperscript{rd} generation explant human skin equivalents

Abstract

Explant human skin equivalents (Ex-HSEs) can be generated by placing a 4mm skin biopsy onto a dermal equivalent. The keratinocytes migrate from the biopsy onto the dermal equivalent, differentiate and form the epidermis of 1st generation Ex-HSEs. This is especially suitable for the expansion of skin material from which only small fragments of skin can be harvested e.g. diseased skin. We evaluated whether 2nd and 3rd generation Ex-HSEs can also be generated from a single skin biopsy whilst maintaining the epidermal properties of 1st generation Ex-HSEs and native human skin. 2nd generation Ex-HSEs were produced by placing a biopsy from the 1st generation Ex-HSE onto a new dermal equivalent.

Likewise, the 3rd generation Ex-HSEs were generated from a 2nd generation Ex-HSE biopsy. We show for the first time that Ex-HSEs can be passaged to the 2nd and 3rd generation and display similar epidermal morphology and expression of differentiation markers as in native human skin and 1st generation Ex-HSEs except for involucrin. The 2nd and 3rd generation Ex-HSEs also show many similarities with 1st generation Ex-HSEs in lipid properties e.g. presence of all lipid classes, similar fatty acid chain length distribution and lamellar lipid organization. However, some differences arise in increased level of hexagonal lateral packing and a change in ceramide profiling. The changes in specific lipid classes was also accompanied by changes in the expression of the enzymes responsible for their synthesis. The expansion of skin biopsies to the 2nd and 3rd generation Ex-HSEs could be a promising method to expand valuable epidermal tissue to analyze morphological and differentiation parameters in the native epidermis.
Introduction

Human skin equivalents (HSEs) are useful tools for studying the interplay between biological processes in the skin including modeling skin diseases (1-3), wound healing (4-6), cutaneous irritation and toxicity tests (7-9). A novel method of generating HSEs involves placing full-thickness 4mm skin punch biopsies onto a fibroblast populated collagen matrix i.e. dermal equivalent, forming the so called 1st generation explant human skin equivalent (Ex-HSE) (3, 10, 11). We previously demonstrated that the epidermis of 1st generation Ex-HSEs show comparable epidermal stratification and differentiation as the original ex vivo skin although not all the stratum corneum (SC) lipid properties could not be reproduced in Ex-HSEs (12). Following this study, the next step was to investigate whether the amount of tissue generated from the 1st generation Ex-HSEs can be further expanded by growing a 2nd generation and even a 3rd generation Ex-HSE. In addition, since the free fatty acids play an important role in the changes in lipid organization in HSEs, we analyze for the first time in Ex-HSEs, the SC fatty acid chain length distribution and degree of saturation quantitatively. Furthermore, we examine the expression of some key enzymes involved in SC lipid synthesis because these factors contribute significantly to the SC skin barrier.

Expansion of epidermal tissue by producing up to three generations of Ex-HSEs can be crucial for analyses that require larger amounts of epidermal tissue, which cannot be harvested from patients with skin diseases. These analyses may include quantitative protein analysis or combining different techniques to study various aspects of diseased skin. By generating a 2nd generation Ex-HSE the total epidermal surface area can be expanded by 40 times compared to the original 4mm skin biopsy (table S1). The 1st generation Ex-HSE was generated from the original biopsy harvested from ex-vivo skin. The 2nd generation was produced by harvesting a biopsy from the 1st generation Ex-HSE and implanting this onto a fresh dermal substrate. This approach can be repeated using a biopsy from the 2nd generation Ex-HSE to establish a 3rd generation Ex-HSE (figure S1). During such organ culture, the keratinocytes migrate from the implanted biopsy onto the dermal equivalent, proliferate, differentiate and form an epidermis.

In native human skin, the SC, which is the outermost layer of the epidermis, consists of corneocytes embedded in a lipid matrix and this structure plays an essential role in the skin permeability barrier. The main lipid classes present in native human SC include free fatty acids (FFAs), ceramides (CERs) and cholesterol (CHOL). These lipids form two lamellar phases with repeat distances of approximately 6nm and 13nm referred to as short periodicity phase (SPP) and long periodicity phase (LPP) respectively (13). Within the lipid lamellae, the lipids in native human SC are mainly organized in a dense orthorhombic packing although a fraction of lipids also adopt a hexagonal packing (figure S2) (13-15).

The epidermal stratification, differentiation pattern and SC lipid properties from the 2nd and 3rd generation Ex-HSEs were analyzed in order to investigate whether these properties are similar to those in the 1st generation of Ex-HSE. In addition, the differentiation and barrier properties were also compared to the native human skin.
Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin

Materials and methods

Isolation of human dermal fibroblasts

Adult human breast or abdominal skin tissue was obtained from cosmetic surgery after written informed consent, according to the Declaration of Helsinki Principles. Dermal fibroblasts were isolated as described earlier (16) and cultured in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Netherlands) supplemented with 5% fetal bovine serum (FBS; HyClone, UT, USA) and 1% penicillin/streptomycin (Sigma, Zwijndrecht, Netherlands) (17). Passages 2-5 were used for the experiments.

Generation of human skin equivalents

Dermal equivalents were generated as described earlier (16, 18). Briefly, 1mL of a 1mg/mL collagen solution was pipetted into filter inserts (Corning Life sciences, Amsterdam, Netherlands). After polymerization, 3mL of fibroblast populated (0.4 x 10^5 cells/mL) collagen (2mg/mL) was pipetted on the polymerized collagen. After polymerization, the collagen was cultured submerged (1 week) in DMEM supplemented with 5% FBS, 25mM ascorbic acid (Sigma, Zwijndrecht, Netherlands) and 1% penicillin/streptomycin.

The various generations of Ex-HSEs were generated as described in detail in figure S1. These cultures consist of an explant and its outgrowing area. The explant is defined as the skin biopsy placed onto the dermal equivalent and the outgrowth is defined as the keratinocytes that after migration, proliferate and differentiate to form the main part of the Ex-HSE. After the generation of dermal equivalents, full thickness (FT) 4mm fat free biopsies obtained from breast skin (referred to as the 1st generation explants, age 17-49), were gently pushed onto the dermal equivalents. Subsequently, the HSEs were cultured at the air-liquid interface and cultured for two days with DMEM and Ham’s F12 (Invitrogen, The Netherlands) (3:1 v/v), 0.5µg/mL insulin (Sigma, Zwijndrecht, Netherlands), 0.5µM hydrocortisone (Sigma, Zwijndrecht, Netherlands), 1µM isoproterenol (Sigma, Zwijndrecht, Netherlands), 1% penicillin/streptomycin, 25mM ascorbic acid and 5% FBS. During the next two days, the HSEs were cultured in a similar medium as mentioned above with some changes. These included 1% FBS, 0.053µM selenious acid (Johnson Matthey, Maastricht, The Netherlands), 10mM L-serine (Sigma, Zwijndrecht, Netherlands), 10µM L-carnitine (Sigma, Zwijndrecht, Netherlands), 1µM α-tocopherol acetate (Sigma, Zwijndrecht, Netherlands), 25mM ascorbic acid and a lipid mixture of 3.5µM arachidonic acid (Sigma, Zwijndrecht, Netherlands), 30 µM linoleic acid (Sigma, Zwijndrecht, Netherlands) and 25µM palmitic acid (Sigma, Zwijndrecht, Netherlands). For the remaining culture period, the HSEs were cultured with the same composition of the medium with two changes: i) 7µM arachidonic acid and ii) no FBS. The culture medium was refreshed twice a week and the HSEs were cultured for 21 days at 37°C, 90% relative humidity and 7.3% CO₂. At the end of the culture period, the dermal equivalent was covered with a keratinocyte outgrowth from the biopsy (1st generation Ex-HSE). During the harvest of the 1st generation Ex-HSE, a biopsy from the 1st generation outgrowth was placed onto a new dermal equivalent. This biopsy on the new dermal substrate is referred to as the 2nd generation explant and served as the start of the 2nd generation outgrowth. The Ex-HSE was cultured for another 21 days generating the 2nd generation outgrowth. During harvest, the 2nd generation outgrowth was further passaged to
produce the 3rd generation Ex-HSE and cultured for 21 days. The fibroblast donor used to generate a series of 1st-3rd generations Ex-HSEs was kept the same.

**Morphology and Immunohistochemistry**

Ex-HSEs were fixed in 4% (w/v) paraformaldehyde (Lommerse Pharma, The Netherlands), dehydrated and embedded in paraffin. For morphological analysis, 5μm sections were stained with haematoxylin (2mg/ml) and eosin (4mg/ml). Immunohistochemical analysis for keratin 10 (K10), Ki67, filaggrin, loricrin, involucrin, steroyl-CoA desaturase (SCD) and ceramide synthase 3 (CerS3, analyzed by immunofluorescence) expression was performed as described in supplementary materials and methods.

**Lipid extraction/analysis**

The SC was isolated from native human epidermis and HSEs as previously described (19). The lipids were extracted from the SC using a modified Bligh and Dyer procedure (20). SC extracts from the outgrowth of 2-3 Ex-HSEs of the same skin donor were pooled. The lipid composition of the explants could not be determined due to insufficient amount of material. Extracts were dried under a stream of nitrogen, reconstituted in chloroform: methanol (2:1) and stored at -20°C. Lipid analysis by high performance thin layer chromatography (HPTLC) and liquid chromatography coupled to mass spectrometry (LC-MS) is described in supplementary materials and methods.

**Fourier transformed infra-red spectroscopy (FTIR) and small angle X-ray diffraction (SAXD)**

**FTIR:** Isolated SC sheets were hydrated at room temperature over a 27% NaBr solution for 24 hours. The SC was sandwiched between AgBr windows and mounted into a customized heating/cooling cell. A Varian 670-IR FTIR spectrometer (Agilent technologies, CA, USA), equipped with a broad-band mercury cadmium telluride detector cooled with liquid nitrogen was used to acquire spectra. The spectra were collected in transmission mode, as a co-addition of 256 scans at 1cm⁻¹ resolution. Each spectrum was collected for 4 minutes at a 1°C interval from 0°C - 90°C, with frequency range of 600-4000cm⁻¹ and deconvoluted using half-width of 4cm⁻¹ and an enhancement factor of 1.7. Bio-Rad Win-IR Pro 3.0 software (Biorad laboratories, MA, USA) was used to analyze the spectra.

**SAXD:** All measurements were performed at the European synchrotron radiation facility (ESRF, Grenoble) at station BM26B. SAXD patterns were detected with a Frelon 2000 charged-coupled device detector using a microfocus of 25μm x 25μm. The X-ray wavelength and sample-to-detector distance were 0.1033nm and 24cm respectively. The scattering intensity (I, arbitrary units) was measured as a function of the scattering vector q (nm⁻¹) defined as q=4πsinθ/λ, in which θ is the scattering angle and λ is the wavelength of the x-rays. From the positions of a series of equidistant peaks (qₙ), the periodicity (d) of a lamellar phase was calculated using the equation d=2nm/qₙ, n being the order of a diffraction peak attributed to the lamellar phase.
**Statistical analysis**

Statistical outcomes were determined using GraphPad Prism 5 (GraphPad Software Inc, CA, USA). Two-way ANOVA tests were performed to analyze the FFA chain length distribution and paired student t-tests for mid-point transition temperature and LPP repeat distance.
Results

2nd and 3rd generation Ex-HSEs show a viable epidermis executing early and late epidermal differentiation programs

To determine the morphology and differentiation of the epidermis of the Ex-HSEs, the middle region, representing the main part of the outgrowths, was investigated. The original biopsies could always be passaged till the 2nd generation (age: 17-49 years). However, from 2 out of the 4 skin donors (age: 17 and 18 years), the 3rd generation Ex-HSE could be cultured.

The 2nd and 3rd generation Ex-HSEs display a fully stratified epidermis (figure 1). The expression of the early (keratin 10 (K10)), and late (filaggrin, loricrin and involucrin) differentiation markers was investigated to examine whether their expression changed during passaging. The outgrowths of the 2nd and 3rd generation showed the presence of K10, filaggrin and loricrin similar to the 1st generation and native human skin (figure 1, for explants see figure S3). Early and late differentiation programs were executed as demonstrated by the expression of K10 in all suprabasal epidermal layers and filaggrin and loricin in the stratum granulosum (SG). The expression of involucrin differed in the 2nd and 3rd generation outgrowths compared to the 1st generation. The 2nd generation outgrowth shows an intense involucrin staining in the SG, which extends weakly to the upper and lower stratum spinosum (SS) and 3rd generation outgrowths show evenly distributed involucrin expression over the entire epidermis. However, in the 1st generation outgrowth, involucrin expression is most pronounced in the SG and a weak staining is observed in the upper SS (figure 1).
Orthorhombic lateral packing is gradually lost with increasing generations of Ex-HSEs

Our previous studies showed that the 1\textsuperscript{st} generation Ex-HSEs do not fully maintain SC lipid properties as in native human skin (12). Therefore, we investigated whether passaging to the 2\textsuperscript{nd} and 3\textsuperscript{rd} generation results in SC lipid properties similar to the 1\textsuperscript{st} generation. The lateral packing was examined in the Ex-HSEs by collecting FTIR spectra from 0°C-90°C, focusing on lipid chain CH\textsubscript{2} rocking vibrations. A hexagonal lipid organization is depicted by a single peak at 719 cm\textsuperscript{-1} and an orthorhombic lipid organization by two peaks at 719 cm\textsuperscript{-1} and 730 cm\textsuperscript{-1} in the FTIR spectrum.

\textbf{Figure 1:} Cross sections of the outgrowth of 2\textsuperscript{nd} and 3\textsuperscript{rd} generation Ex-HSEs in comparison with the 1\textsuperscript{st} generation Ex-HSEs and native human skin that are stained with haematoxylin/eosin (HE). Immunohistochemical staining of keratin 10 (K10), filagrin (FLG), loricrin (LOR), involucrin (INV). 20x magnification, scale bar represents 30μm.
At 0°C, 2nd generation explants show two contours at 719 cm⁻¹ and 730 cm⁻¹. The 730 cm⁻¹ peak disappears at 24.6±4.2°C. The lateral organization in the 3rd generation explants could not be detected due to a low IR signal. The 2nd and 3rd generation outgrowths only show a hexagonal packing (figure 2), as only the 719 cm⁻¹ contour is present in the spectrum. When comparing the lateral packing in the 2nd generation explant with the 1st generation explants and native skin, the 1st generation explant and human skin also show two peaks with the 730 cm⁻¹ peak more intense indicating a higher level of orthorhombic packing. The 730 cm⁻¹ peak disappears at 47.3±3.1°C and 35.0±7.1°C for human skin and the 1st generation explant, respectively (data not shown for human skin). When focusing on the outgrowth, the FTIR spectrum of the 2nd generation outgrowth exhibits only one peak, reflecting only a hexagonal lateral packing, while the 1st generation outgrowth shows a small fraction of lipids in an orthorhombic packing which disappears at 30±8.5°C.

We also investigated the CH₂ symmetric stretching frequency to determine whether the conformational ordering of the lipid chains underwent changes during culture. In a crystalline organization, the chains are fully extended and the conformational order is high with CH₂ symmetric stretching frequencies below 2850 cm⁻¹. CH₂ symmetric stretching frequencies at 2852 cm⁻¹ and higher indicate disordered chains, which is characteristic for a liquid phase. From the thermotropic response curves, the mid-point temperature at which the lipid domains change from a crystalline state to a liquid state was determined (for detailed explanation, see figure S4). This is referred to as “Mid-point transition temperature” (MTT). The MTT is significantly lower in the 2nd generation explants and outgrowths than in native human skin (p<0.05 and p<0.01 respectively, table S5). The CH₂ stretching frequency at skin temperature (32°C) was also higher in the 2nd generation outgrowths in comparison to native human skin (table S5, p<0.05). These results indicate an increased conformational disordering in the 2nd generation Ex-HSEs compared to native skin. However, the CH₂ stretching frequency at 32°C and MTT of the 2nd generation Ex-HSE was similar to the 1st generation. The MTT and CH₂ stretching frequency at skin temperature of the 3rd generation outgrowth was comparable to the 2nd generation outgrowth (table S5). However, this information could not be obtained for the 3rd generation explant due to low IR signal.
Figure 2: FTIR spectra showing the CH$_2$ rocking vibrations of 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ generation explants and outgrowths as a function of temperature (0°C-90°C). A hexagonal lipid organization is characterized by the presence of one peak at 719 cm$^{-1}$ at the CH$_2$ rocking band and an orthorhombic lipid packing by two peaks at 719 cm$^{-1}$ and 730 cm$^{-1}$. Generation (gen), not determined (N.D.).

**LPP is present in 2$^{nd}$ and 3$^{rd}$ generation Ex-HSEs**

The lamellar lipid organization was examined in the explants and their corresponding outgrowths using SAXD. The diffraction profiles show three diffraction peaks denoted by 1, 2 and 3, indicating the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ order diffraction peak of the LPP (figure S6). In addition, the diffraction profiles show the presence of crystalline CHOL domains similar to the phase separated CHOL in all Ex-HSEs and native human skin. No statistical significance in repeat distance of the LPP was observed between the 2$^{nd}$ and 3$^{rd}$ generation explants and outgrowths in comparison to the 1$^{st}$ generation (table S6).

**2$^{nd}$ and 3$^{rd}$ generation Ex-HSEs contain the main SC lipid classes in native human skin**

Since we observed changes in the lipid organization of the outgrowth, we examined the lipid composition because changes in the lipid composition are causative for changes in lipid organization. Firstly, we focused on the three main lipid classes, CERs, CHOL and FFAs (figure 3a). The main SC lipid classes were present
in the 2nd and 3rd generation outgrowths and no significant changes were observed in the band intensity of CERs and CHOL in the 2nd and 3rd generation compared to the 1st generation outgrowth and human skin. Compared to native skin, the intensity of the FFAs was reduced in the 2nd and 3rd generation outgrowths as was also observed in the 1st generation outgrowth (figure 3b, p<0.05). Secondly, the 2nd and 3rd generation show all CERs present in human SC as detected by HPTLC. The relative intensities of the ester linked ω-hydroxy (EO) CERs, CER EOS and CER EOP in the 2nd generation were similar to the 1st generation. However, CER EOS and CER EOP relative intensities in the 2nd generation were significantly higher than in native human skin (EOS: p<0.01 and EOP: p<0.05, figure 3c). No significant differences were observed in the relative intensity of the other CER subclasses when comparing the 2nd generation Ex-HSEs with the 1st generation and native human skin. Since CerS3 is involved in the synthesis of EO CERs (linking the sphingoid base to the ω-hydroxy fatty acid), its expression in the epidermis was analyzed by immunofluorescence. CerS3 is expressed weakly in supra-basal layers and intensely in the SG in native human skin (figure 3d). However, all Ex-HSE generations show an intense staining of CerS3 in all suprabasal layers.
**Figure 3:** SC barrier lipids and ceramide synthase 3 (CerS3) expression in 1st, 2nd and 3rd generation outgrowths and native human skin. 

- **a)** Lipid profiles from human skin and outgrowths of Ex-HSEs. Lane 1: Native human skin, Lane 2: 1st generation outgrowth, Lane 3: 2nd generation outgrowth, Lane 4: 3rd generation outgrowth. 
- **b)** Relative intensity of the lipid bands of the main SC lipid classes. 
- **c)** Relative distribution of the various CER subclasses. 
- **d)** CerS3 expression in Ex-HSEs and native human skin. 20x magnification, scale bar 50µm. Cholesterol (CHOL), free fatty acids (FFA), ceramides (CER). CERs are named according to the nomenclature by Motta et al., and Masukawa et al., (35, 36) (see table S4, paired student t-test, *p<0.05, **p<0.01). The data is presented as the mean ± standard deviation from four independent experiments.
The 2nd generation Ex-HSEs show a substantial level of unsaturated FFAs

HPTLC results revealed that the total FFA level was lower in the 2nd generation Ex-HSE compared to native human skin. To analyze the FFA composition in more detail, we examined the chain length distribution and saturation of the FFAs using LC-MS as this was not done in our previous study focusing on the 1st generation of Ex-HSE (12). The FFA chain lengths with C16-C18 were excluded from the analysis to avoid false positive results from sebaceous lipids and subcutaneous fat from native human skin.

Analysis of saturated FFAs (SFAs) and mono-unsaturated FFAs (MUFAs) showed that the 2nd generation contains relatively more MUFAs than SFA in contrast to native human skin (significance p<0.05, figure 4a, b) where the SFAs are always highly abundant compared to MUFAs. The abundance of MUFAs in the 2nd generation was not significantly different to that in the 1st generation.

The SFAs chain length in the 2nd generation outgrowths displays a normal distribution. The even chain length FFAs are more abundant than the odd chain lengths with FFA C24:0 and FFA C26:0 as the most predominant chain lengths. The relative level of long chain (C19:0-C22:0) SFAs was significantly higher in the 2nd generation than in native human skin (figure 4c; p<0.05). Furthermore, the consistent reduction in very long chain SFAs (C24:0-C28:0) in the 2nd generation was significant compared with native human skin (figure 4c; p<0.01). The chain length distribution of 2nd generation SFAs was similar to the 1st generation with the exception of ultra-long chain FFAs (≥C30:0). These were significantly reduced in the 2nd generation compared with the 1st generation (figure 4c).

The chain length distribution of MUFAs in the 2nd generation also shows that even chain lengths are more abundant than odd chain lengths with FFA C20:1 and FFA C24:1 as the most abundant. The relative level of MUFAs with chain lengths C19:1 till ≥C30:1 was significantly higher in the 2nd generation than in native human skin (figure 4d; p<0.001). No significant difference was observed in the MUFA chain length distribution between the 1st and 2nd generation.

The expression of stearoyl-CoA desaturase (SCD), a key enzyme in FFA unsaturation was analyzed. SCD is expressed in the stratum basale (SB) in native human skin. The 1st and 2nd generation outgrowths show SCD expression in the SB and SS while the 3rd generation expresses SCD in the entire viable epidermis (figure 4e).
Figure 4: The relative level of a) saturated FFAs (SFAs) and b) mono-unsaturated FFAs (MUFAs) and the chain length distribution of c) SFAs and d) MUFAs in the native human skin, 1st generation outgrowths and 2nd generation outgrowths. e) expression of stearoyl-CoA desaturase (SCD) in ExHSEs and native human skin. In a and b the SFAs and MUFAs are displayed as a percentage of the total FFAs. c and d show the variations in the relative levels of the various SFAs chain lengths and MUFAs respectively in relation to the total FFA level (i.e. Total FFAs = SFAs+MUFAs = 100%). *p<0.05, **p<0.01, ***p<0.001. The data represented is shown as the mean ± standard deviation of four independent experiments. 20x magnification, scale bar represents 50μm. Native human skin (NHS), generation (gen).
In this study, we investigated whether the tissue generated from the 1st generation Ex-HSEs can be further expanded by growing a 2nd generation and even a 3rd generation Ex-HSE. This method of generating epidermal tissue can be potentially useful for analysis that require larger amounts of skin. For example, in studies that involve skin diseases, the expansion of skin tissue eliminates the need to harvest several skin biopsies and provides a means to study the progression of skin diseases in vitro which cannot be performed in vivo e.g. investigating the invasive behavior of skin cancers.

The results show that it is possible to passage outgrowth from the 1st generation Ex-HSEs to establish 2nd and 3rd generations, which maintain morphology, differentiation process and lamellar lipid organization similar to the 1st generation. This shows that 40x (2nd generation, table S1) and 60x (3rd generation, table S1) expansion of 4mm biopsies can be achieved, which allows extensive characterization of the epidermal tissue and the SC barrier properties which cannot be performed with only a 4mm biopsy. However, only 50% of human skin biopsies could be used to establish the 3rd generation. This may be attributed to differences between donors influencing keratinocyte proliferation and differentiation, including age, genetic factors and lifestyle. Due to donor confidentiality, information regarding lifestyle and genetics could not be obtained. However, skin donors aged 17-18 years could be passaged to the 3rd generation and other skin donors ranging from 33-49 years could not. We also compared Ex-HSEs generated from younger skin donors (17-18 years) and an older skin donor (49 years old). In young donors, a similar proliferation index (PI) maintained from the 1st till the 3rd generation. In Ex-HSEs generated from the older skin donor (49 years), there is a 4x reduction in PI in the 2nd generation compared to the 1st generation Ex-HSEs (data not shown). This may contribute to the inability of this donor to be passaged towards the 3rd generation and is in line with previous studies in which age is associated with proliferation capacity and passaging of keratinocytes in vitro (21, 22). However, in order to fully explore these differences, the outgrowth of more old and young donors should be studied.

We observed that the expression of K10, filaggrin and loricrin were maintained in the 2nd and 3rd generation. However, involucrin expression gradually shifts to the SS and finally to the SB in the 3rd generation. This finding may result from changes in the STAT5a/PPAR-γ pathway as this pathway regulates involucrin expression in differentiating human keratinocytes (23). Suprabasal expression of involucrin in the epidermis has also been reported in other HSEs suggesting that culture conditions may influence the expression of involucrin in vitro (24, 25).

The studies presented here indicate that orthorhombic lipid organization is reduced when increasing the number of generations. Therefore the orthorhombic packing cannot be reproduced in vitro under conditions described in this study. Only a hexagonal lateral packing was observed in the 2nd and 3rd generation outgrowths, similar to other epidermal and full thickness HSEs (12, 25-27). The abundant formation of a hexagonal packing in culture may be explained by: a reduction in SC FFAs levels and in their chain lengths or by an increase in the relative levels of MUFAs (28).
The 2nd generation outgrowth showed a normal SFA chain length distribution although the relative level of SFAs was lower, and MUFAs higher than in native human skin. Furthermore the ultra-long chain SFAs (≥C30:0) were lower in the 2nd generation than the 1st generation outgrowths and human skin. This may contribute to the absence of the orthorhombic lateral packing in the 2nd generation outgrowths. The 3rd generation outgrowth was not analyzed by LC-MS due to its close similarity in lipid organization with the 2nd generation outgrowth. We expect similar lipid composition in both the 2nd and 3rd generation outgrowths since the lipid composition highly determines lipid composition. In contrast to the lateral packing, the LPP repeat distance can be maintained in all three Ex-HSE generations. Studies using model lipid systems also suggest that increased levels of MUFAs mainly affects the lateral packing while the lamellar phases are not changed (28). The increased MUFAs content in the 2nd generation Ex-HSEs and in other HSEs (29) in relation to native human skin might be related to the increased expression of SCD. In 2nd generation Ex-HSEs, the level of MUFAs is increased, especially the long chain MUFAs (C24:1-C28:1), whereas long chain SFAs levels (C24:0-C28:0) are decreased in comparison with native human skin. This suggests that FFA processing in the Ex-HSEs is skewed towards unsaturation probably due to increased SCD expression and/or activity (29). SCD-1 generates C16:1 and C18:1 from C16:0 and C18:0, respectively, after which most probably the unsaturated FFAs are elongated (30). We therefore hypothesize that in Ex-HSEs, most FFAs are first unsaturated and thereafter elongated. In the absence of measuring the abundance of C16:0 and 18:0 FFAs, the average chain length of the FFA was similar between the native human skin and the Ex-HSEs (data not shown), which suggests that the main difference in FFA profile is unsaturation.

The relative intensities of EO CERs in the HPTLC plates were increased in 1st until 3rd Ex-HSE generations and in other HSEs compared to native human skin (29). The formation of CERs in keratinocytes is partly dependent on ceramide synthases (CerS) which n-acylate FFAs to a sphingoid base. An increase in CerS activity or expression, especially CerS3 (which synthesizes very long chain CERs) may contribute to the increased EO CER levels (31). PPARβ/δ and PPARγ may also play a role in the increased EO CERs in the outgrowths, because PPARβ/δ and PPARγ activation stimulates CerS3 expression in human keratinocytes and increases EO CERs in the hairless mouse (32, 33). Furthermore, increased ω-acyl transferase (generates EO CERs from acid condensation of linoleic acid to ultra-long CERs) expression and the availability of very long chain FFAs could influence the levels of EO CERs in the outgrowths (34).

Investigation of the lipid properties with regards to age were also compared. Our data suggest that the average FFA chain length in 1st generation Ex-HSEs is not affected by age as no differences in average chain length were observed. However, the level of MUFAs relative to the total FFA is higher in 1st generation Ex-HSEs from the older skin donor (71%) compared to younger donors (40%). This is also reflected in the lipid organization of the 1st generation outgrowth. A higher fraction of lipids form an orthorhombic lateral packing in the 1st generation Ex-HSEs from the young donors than the lipids in the 1st generation Ex-HSE from the old donor (figure S7). Therefore, age might contribute to the lipid composition of Ex-HSEs. However, further studies are required to fully explore this.
In addition to the presented Ex-HSEs, we also examined outgrowth cultures derived from isolated human epidermal sheets. Before culturing, the epidermis of the native human skin was separated by dispase treatment and the epidermal sheet was placed onto the dermal equivalent. 2\textsuperscript{nd} and 3\textsuperscript{rd} generation HSEs were also generated and with similar epidermal differentiation as in Ex-HSEs (figure S5) as well as the lipid properties (data not shown).

We conclude from this study that expansion of 4mm skin biopsies on a dermal equivalent to the 2\textsuperscript{nd} or 3\textsuperscript{rd} generation, depending on the skin donor, can serve as a method to expand valuable skin material in order to analyze morphological and differentiation parameters as in the native epidermis. Some aspects of the lipid organization and composition of native human skin is not reproduced in 2\textsuperscript{nd} and 3\textsuperscript{rd} generation Ex-HSEs, which should be considered when performing barrier function studies.
Acknowledgements

The authors thank the personnel at the DUBBLE beam line (BM26), ESRF for their support with the x-ray measurements. This research was financially supported by Dutch Technology Foundation STW (grant no. 10703).
References


Supplementary figures

Figure S1: Generation of Ex-HSEs. A 4mm full thickness biopsy from native human skin is placed onto a fibroblast populated collagen matrix. This allows the epidermal cells to migrate and cover the surface of the collagen matrix and establish the 1st generation Ex-HSE (a). A small fragment of the 1st generation Ex-HSE is placed onto a new fibroblast populated collagen matrix to generate the 2nd generation Ex-HSE (b). A similar procedure is repeated with the 2nd generation Ex-HSE to generate the 3rd generation Ex-HSE (c). Generation (gen), explant (exp), outgrowth (out).
The stratum corneum (SC), the outermost part of the epidermis consists of corneocytes which are surrounded by an organized intercellular lipid matrix. The lipids are organized in two lamellar phases: the long periodicity phase (LPP) and the short periodicity (SPP) phase with a repeat distance of 12nm and 6nm respectively. The lipids are also organized in the plane perpendicular to the lamellar organization which is referred to as lateral lipid organization. The lipids can be arranged in a liquid (disordered), hexagonal (less dense and ordered) and an orthorhombic (very dense and ordered) organization. This figure is adapted from Thakoersing et al., (1)

Figure S2: Lipid organization in human stratum corneum
Figure S3: Immunohistochemical staining of the explants from 1st, 2nd and 3rd generation Ex-HSEs that are stained for keratin 10 (K10), filaggrin (FLG), loricrin (LOR), involucrin (INV) and stearoyl-CoA desaturase (SCD). Scale bar represents 30μm, 20x magnification.
Figure S4: a) The stretching vibrations of the FTIR spectra from a representative Ex-HSE explant is shown as a function of temperature. In a crystalline packing, the chains are fully extended, the conformational order of the chains are high and this reflected by low CH$_2$ stretching frequencies less than 2850 cm$^{-1}$ (peak centre). Disordered chains, which is characteristic for a liquid phase have CH$_2$ symmetric stretching frequencies of 2852 cm$^{-1}$ and higher. b) Illustrates the procedure used to calculate the midpoint of the crystalline to liquid transition using the CH$_2$ stretching vibrations of the lipids. The midpoint transition temperature (MTT) is calculated as the mean of the last wavenumber at the beginning ($W_{N_{\text{start}}}$) of the transition and the first wave number at the end of the transition ($W_{N_{\text{end}}}$). The wavenumbers at the beginning and the end of the transition are determined by linear regression. The temperature at this calculated wavenumber is referred to as the MTT.
Figure S5: Immunohistochemical staining of the outgrowth of 1st, 2nd and 3rd generation epidermal sheet outgrowth HSEs for keratin 10 (K10), filagrin (FLG), loricrin (LOR), involucrin (INV) and stearoyl-CoA desaturase (SCD). Scale bar represents 30μm, 20x magnification.
Figure S6: Representative diffraction pattern of SC from 1st, 2nd and 3rd generation explants and outgrowth. The 1st, 2nd and 3rd order of the LPP are indicated as 1, 2 and 3 respectively. The peak denoted by 2 in the diffraction pattern of native human SC is not only based on the 2nd order of the LPP, but also the 1st order SPP demonstrated by the increased width of this peak (2). Consequently, the repeat distance of the LPP and SPP in native human SC cannot be calculated directly from this diffraction profile. Crystalline cholesterol is indicated by (*). Generation (gen), explant (exp), outgrowth (out).
Figure S7: FTIR spectra showing the CH$_2$ rocking vibrations of 1st generation outgrowths as a function of temperature (0°C-90°C) from young and old skin donors. A hexagonal lipid organization is characterized by the presence of one peak at 719cm$^{-1}$ at the CH$_2$ rocking band and an orthorhombic lipid packing by two peaks at 719cm$^{-1}$ and 730cm$^{-1}$. 
## Supplementary tables

### Table S1: Epidermal outgrowth in Ex-HSEs

<table>
<thead>
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<th>Ex-HSE</th>
<th>Average outgrowth area (cm²)</th>
<th>Increase in epidermal material (compared to 4mm biopsy)</th>
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<tr>
<td>Original biopsy</td>
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<td>0</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; generation</td>
<td>2.543</td>
<td>20x</td>
</tr>
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<td>40x</td>
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<td>60x</td>
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<td>Antibodies</td>
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<td>----------------------------------</td>
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<tr>
<td><strong>Primary antibodies</strong></td>
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<tr>
<td>Mouse cytokeratin 10 Ab-2</td>
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<td>FLG01</td>
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<td>Rabbit Loricrin</td>
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<td>1:1200</td>
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<td>Mouse involucrin</td>
<td>SYS</td>
<td>1:1200</td>
</tr>
<tr>
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<td>Rabbit ceramide synthase 3 (IF)</td>
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<td>1:75</td>
</tr>
<tr>
<td>Rabbit Ki67</td>
<td>SP6</td>
<td>1:800</td>
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| **Secondary antibody**           |        |          |                              |
| Biotinylated universal antibody, |        |          | Vector laboratories          |
| anti-rabbit/mouse IgG            |        |          | Burlingame, CA, USA          |
| Rhodamine Red-X                  |        | 1:300    | Jackson immunoresearch       |
| (Goat anti-rabbit) (IF)          |        |          | Laboratory, USA              |
Table S4: Ceramide nomenclature

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<th>Eluent</th>
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<tr>
<td>1</td>
<td>Dichloromethane/Ethylacetate/Acetone/Methanol (88:8:4:1)</td>
<td>40</td>
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<tr>
<td>2</td>
<td>Chloroform/Acetone/Methanol (76:8:16)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Hexane/Chloroform/Acetone/Methanol (6:80:12:2)</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>Hexane/Chloroform/Hexyl acetate/Acetone/Methanol (6:80:0.1:10:4)</td>
<td>95</td>
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</table>

The ceramides in human stratum corneum contain one of these 4 sphingoid bases (dihydrosphingosine (dS), Sphingosine (S), phytosphingosine (P) and 6-hydroxy sphingosine (H)) and one of three acyl chains (non-hydroxy fatty acid (N), α-hydroxy fatty acid (A) and esterified ω-hydroxy fatty acid (EO)). These result in the 12 ceramide subclasses known to be present in human stratum corneum.

Table S5: Midpoint transition temperature and CH₂ symmetric stretching at 32°C in Ex-HSEs

<table>
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<th>Midpoint transition temperature (°C)</th>
<th>CH₂ symmetric stretching (cm⁻¹) at 32°C</th>
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<tr>
<td></td>
<td>Explants</td>
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<tr>
<td>Ex-HSE</td>
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<tr>
<td>1st generation</td>
<td>60.7 ± 6.4</td>
</tr>
<tr>
<td>2nd generation</td>
<td>58.5 ± 0.7 *</td>
</tr>
<tr>
<td>3rd generation</td>
<td>N.D.</td>
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<tr>
<td>Native human SC</td>
<td>73.5 ± 1.8</td>
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Paired student t-test was performed to determine significance between the native human SC and the Ex-HSEs (**p<0.01, *p<0.05), not determined (N.D.).
Table S6: Repeat distance of the LPP in the 1st - 3rd generation explants and outgrowths.

<table>
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<th>Outgrowth</th>
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<td>LPP repeat distance (d)</td>
<td>LPP repeat distance (d)</td>
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<tr>
<td>Ex-HSE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st generation</td>
<td>12.1 ± 0.3nm</td>
<td>12.1 ± 0.4nm</td>
</tr>
<tr>
<td>2nd generation</td>
<td>11.9 ± 0.5nm</td>
<td>11.8 ± 0.4nm</td>
</tr>
<tr>
<td>3rd generation</td>
<td>11.6 ± 0.6nm</td>
<td>11.4 ± 0.4nm</td>
</tr>
</tbody>
</table>

Note: LPP in native human skin is ~13nm (2)
Supplementary materials and methods

Morphology and Immunohistochemistry
The primary and secondary antibodies are listed in table A2. The sections were incubated in sodium citrate buffer (pH 6) at 95°C for antigen retrieval. After cooling, the sections were blocked with normal horse serum (Vector laboratories Burlingame, CA) and incubated with the primary antibody diluted in 1% PBS/bovine serum albumin (BSA) overnight at 4°C.

Staining procedure with amino-ethylcarbazole: Sections were incubated with the secondary antibody (Vector laboratories Burlingame, CA) for 30 minutes and thereafter with the ABC reagent (Vector laboratories Burlingame, CA) for 30 minutes. The sections were washed with 0.1M sodium acetate buffer and subsequently in amino-ethylcarbazole (Sigma) dissolved in N,N-dimethylformamide (1g/250mL) (Sigma) with 0.1% hydrogen peroxide. All sections were counterstained with haematoxylin.

Immunofluorescence analyses: Sections were incubated with the secondary antibody for 1 hour and mounted using DAPI Vectashield (Vector laboratories Burlingame, CA).

Lipid analysis
High performance thin layer chromatography (HPTLC): Extracted lipids were analyzed by HPTLC as described previously (3). The solvents and running distances are provided in table A3. Co-chromatography of a standard lipid mixture was performed to identify the different lipid classes. The standard mixture contained free fatty acids (cerotic acid, tricosanoic acid, behenic acid, arachidonic acid, stearic acid, lignoceric acid, palmitic acid), cholesterol (Sigma) and ceramides (EOS, EOP, NS, NP, AS and AP, Cosmoferm, The Netherlands). The ceramide nomenclature is according to the terminology of Motta et al.,(4) and Masukawa et al.,(5) (table A4).

Reverse-phase liquid chromatography mass spectrometry (LC-MS): LC-MS analysis of FFA species was performed according to van Smeden et al., (6). An Alliance 2695 HPLC (Waters Corp. Milford, MA) was attached to a TSQ Quantum mass spectrometer (Thermo Finnigan, San Jose, CA, USA) which measured in atmospheric-pressure chemical ionization (APCI) mode. The analysis was performed in negative ionization mode with a scan range of 200-600 amu. The source heater temperature was set at 450°C, and heated capillary at 250°C and the discharge current at 5 μA. The total sample lipid concentration was 1mg/mL with an injection volume of 10 μL. FFA separation was achieved using a LiChroCART Purospher STAR analytical column (55 x 2 mm i.d. Merck, Darmstadt, Germany) under a flow rate of 0.6 mL/min using a binary gradient solvent system of acetonitrile/H₂O (90:10) to methanol/heptane (90:10). The analysis was performed using Xcalibur software version 2.0. The FFA chain lengths with C16-C18 were excluded from the analysis to avoid false positive results from sebaceous lipids and subcutaneous fat from native human skin.
References


CHAPTER 5

Explant cultures of atopic dermatitis biopsies maintain their epidermal characteristics \textit{in vitro}

Abstract

Atopic dermatitis (AD) is a common inflammatory skin disorder characterised by various epidermal alterations. Filagrin (FLG) mutations are a major predisposing factor for AD and much research has been focused on the FLG protein. Human skin equivalents (HSEs) might be useful tools for increasing our understanding of FLG in AD and to provide a tool for the screening of new therapies aimed at FLG replacement.

Our aim is to establish an explant HSE (Ex-HSE) for AD by using non-lesional skin from AD patients wild-type for FLG or harbouring homozygous FLG mutations. These Ex-HSEs were evaluated as to whether they maintained their in vivo characteristics in vitro and whether FLG mutations affected the expression of various differentiation markers. FLG mutations did not affect the outgrowth from the biopsy for the establishment of Ex-HSEs. FLG expression was present in healthy skin and that of AD patients without FLG mutations and in their Ex-HSEs but was barely present in biopsies from patients with FLG mutations and their corresponding Ex-HSEs. AD Ex-HSEs and AD biopsies shared many similarities, i.e., proliferation and the expression of keratin 10 and loricrin, irrespective of FLG mutations. Neither KLK5 nor Lekti expression was affected by FLG mutations but was altered in the respective Ex-HSEs. Thus, Ex-HSEs established from biopsies taken from AD patients maintain their FLG genotype-phenotype in vitro and the expression of most proteins in vivo and in vitro remains similar. Our method is therefore promising as an alternative to genetic engineering approaches in the study of the role of FLG in AD.
Introduction

Atopic dermatitis (AD) is one of the most frequently occurring inflammatory skin diseases and is characterized by various epidermal alterations, including disrupted skin barrier function. Currently, AD affects 15-30% of Caucasian children and 2-10% of adults (1). Clinically, the skin from AD patients is characterized by dry, red and pruritic skin with possible chronic or relapsing eczematous lesions. Since the discovery of filaggrin (FLG) gene mutations as a major predisposing factor for AD, much research has been focused on this protein (FLG) in the context of AD (2).

The role of FLG in the formation of the cornified envelope and as a precursor for the natural moisturizing factor (NMF) is well established (3-5). However, the relationship between the presence of FLG mutations and the skin barrier defects in AD is currently unclear. In previous studies, reduced FLG protein expression attributable to mutations in vivo or FLG knockdown (FLG-KD) in vitro appears not to affect various skin barrier properties (6-9).

In order to study AD pathogenesis and the role of FLG in AD development, various model systems have been used, including murine models and in vitro three-dimensional human skin equivalents (HSEs). The murine models include the flaky tail (ft) mouse, which contains a homozygous frameshift mutation in the FLG gene. These ft mice are FLG-deficient and are therefore frequently used as a model for studying the role of FLG in the skin barrier (10-13). However, recent studies have shown that additional mutations in the Tmem79/matt gene are the cause of the barrier defects in ft mice (14, 15).

Because of the morphological and functional differences between mouse and man, in vitro skin equivalents might serve as an useful alternative (16). The establishment of HSEs in vitro involves FLG-KD in keratinocytes to reduce FLG protein expression, thereby mimicking the FLG mutations as seen in vivo in AD patients (9, 17, 18). Other approaches include the supplementation of cytokines to mimic lesional AD skin (19, 20). Whereas these studies have used manipulated keratinocytes, either by genetic engineering or through cytokine supplementation, our aim in the current study is to establish an explant HSE (Ex-HSE) by using the primary keratinocyte outgrowth from non-lesional AD biopsies. Such an HSE will represent more closely AD compared with genetically engineered keratinocytes. This approach should recapitulate features of the original AD biopsies. Earlier studies used this explant approach to establish an HSE for recessive epidermolysis bullosa simplex (REBS) or squamous cell carcinoma (SCC) and these have showed that specific characteristics of these skin disorders, e.g., disturbed differentiation in the SCC skin equivalents persist in vitro (21, 22). Based on these findings, we reason that AD biopsies that are wildtype for FLG (FLG+/+) or that harbour a homozygous FLG mutation (FLG-/-) can be used to establish HSEs. Here, we present such an HSE, which has been characterised for the expression of various epidermal markers for epidermal differentiation, proliferation and desquamation. The results presented in this study indicate that FLG mutations result in reduced FLG protein expression in vivo and in vitro and in the in vitro maintenance of the expression of various epidermal markers from the original AD biopsy.
Materials and methods

Healthy volunteers and AD patients
The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the Leiden University Medical Centre. All subjects (healthy volunteers and AD patients) were recruited by public advertisement and gave their written informed consent. Three healthy Caucasian volunteers without a history of dermatological disorders and six Caucasian AD patients (age range: 20-42; 2 male and 7 female) were included. Of the AD patients, three were wildtype (AD FLG+/+) and three had a homozygous FLG mutation (AD FLG-/-); the FLG genotyping analysis is described below. No dermatological products were applied onto the forearms for at least 1 week prior to the study. From each individual/patient, two biopsies from non-lesional skin from the inner forearm were taken; one biopsy was used to evaluate the original status and the other biopsy was used to establish the outgrowth skin model.

FLG genotyping
Heparinised blood was drawn from healthy volunteers and AD patients. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation. Dry cell pellets were stored at -80°C until DNA isolation. From the PBMCs, DNA was isolated by using the DNeasy Blood and Tissue kit according to the manufacturer’s instructions (Qiagen, Venlo, The Netherlands). All individuals were genotyped for the four most prevalent mutations found in European Caucasians (2282del4, R501X, S3247X and R2447X), which cover approximately 93% of the currently known FLG mutations that have been detected in Western Europe (23), according to the method described earlier (24). After determination of the FLG genotype of the participants, three wildtype healthy volunteers, three wildtype AD patients and three AD patients with a homozygous FLG mutation were selected for the study.

Fibroblast culture
For the isolation of human dermal fibroblasts, dermis was obtained by overnight incubation of freshly obtained skin in dispase II solution (Roche Diagnostics, Almere, The Netherlands). To isolate the fibroblasts, the dermis was incubated for 2 h in a solution containing collagenase II (Invitrogen, Breda, The Netherlands) and dispase II (ratio 1:3 and 3 ml/g dermis) at 37°C for 2 h. The cells were filtered by using a 70-μm cell strainer (BD Biosciences, Breda, The Netherlands) and cultured in fibroblast medium at 37°C and 5% CO₂ until subconfluency. Fibroblast medium consisted of Dulbecco’s modified Eagle’s medium (DMEM; Gibco/Invitrogen) supplemented with 5% fetal bovine serum (FBS; HyClone/Greiner, Nurtingen, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Culture medium was refreshed every 3 days. Fibroblast at passages two to five from a single donor were used for the experiments.

Explant human skin equivalents
Fresh punch biopsies (4 mm) from the inner forearm from healthy volunteers and from AD patients were trimmed and rinsed three times in sterile phosphate-buffered saline (PBS). Thereafter, one biopsy was immediately fixed in 4% paraformaldehyde and one biopsy was placed on dermal equivalents that were
constructed as described earlier (25-27). In short, rat-tail collagen was seeded with 1.25 \times 10^5 fibroblasts/ml and incubated for at least 1 day and maximally 7 days under submerged conditions.

All explants were cultured at the air-liquid interface under serum-free conditions for 3 weeks. The medium used during this period was composed of DMEM and Ham’s F12 medium (3:1 ratio) supplemented with 0.5 μM hydrocortisone, 1μM isoproterenol, 0.1 μM insulin (Sigma-Aldrich, Zwijndrecht, The Netherlands), 100 U/ml penicillin and 100 μg/ml streptomycin, 53 μM selenious acid, 10 mM l-serine, 10 μM l-carnitine, 7 μg/ml β-dextrin, 1 μM DL-α-tocopherol-acetate, 100 μg/ml ascorbic acid phosphate and a lipid supplement containing 2.4 \times 10^5 M bovine serum albumin (Sigma-Aldrich), 25 μM palmitic acid, 30 μM linoleic acid and 7 μM arachidonic acid.

**Immunohistochemistry**

After the culture period, Ex-HSEs were fixed in 4% paraformaldehyde, dehydrated and paraffin-embedded. The morphology of the biopsies and skin equivalents was examined in 5-μm-thick sections by using haematoxylin and eosin (HE) staining.

Immunohistochemical analysis was performed in the central part of the outgrowth to detect markers specific for keratinocyte proliferation, basement membrane formation and epidermal differentiation. For immunohistochemical analysis, 5-μm-thick sections were cut, deparaffinised and rehydrated, followed by heat-mediated antigen retrieval. After the blocking of non-specific binding with PBS containing 1% BSA and 2% normal human serum (NHS; Sanquin, Leiden, The Netherlands), primary antibodies were incubated overnight at 4°C. The used primary antibodies were raised against: Ki67 (1:100; clone MIB1, DAKO, Glostrup, Germany), Collagen type IV (1:75; clone MAB1430, Chemicon, Melbourne, Victoria, Australia), keratin 10 (K10, 1:100; clone DE-K10, Labvision/Neomarkers, Fremont, Calif., USA), loricrin (1:1000; clone AF62, Covance, Rotterdam, The Netherlands), kallikrein-related peptidase 5 (KLK5, 1:400; polyclonal, Santa Cruz, Heidelberg, Germany), lympho-epithelial Kazal-type-related inhibitor (Lekti, 1:20; clone 1C11G6, Invitrogen, Breda, The Netherlands). Following incubation with the appropriate secondary antibody, the streptavidin-biotin-peroxidase system (GE Healthcare, Buckinghamshire, UK) was used according to the manufacturer’s instructions with 3-amino-9-ethylcarbazole (AEC) for visualisation. The sections were briefly counterstained with haematoxylin and sealed with Kaiser’s glycerin. For the collagen type IV staining, a protease treatment with a 0.025% protease solution (Sigma, Zwijndrecht, The Netherlands) was performed prior to incubation with the primary antibody.

**Immunofluorescence**

For immunofluorescence analysis, 5-μm-thick paraffin sections were cut, deparaffinised and rehydrated, followed by heat-mediated antigen retrieval. Hereafter, non-specific binding was blocked by using PBS containing 1% BSA and 2% NHS followed by overnight incubation at 4°C with the primary antibody for FLG (1:1000; Covance, Rotterdam, The Netherlands). Subsequently, sections were incubated with the appropriate secondary antibody (conjugated to Rhodamine Red, 1:300; Jackson ImmunoResearch, Amsterdam,
The sections were mounted with Vectashield containing 4,6-diamidino-2-phenylindole (DAPI) for visualisation of the nuclei (Vector Laboratories, Amsterdam, The Netherlands).

**Proliferation index**

The proliferation index was determined by an independent researcher who counted the number of Ki67-positive nuclei in a total number of 100 basal cells (x 100 %) at three locations per slide for three different donors and their corresponding skin models. Data represent means + standard error of the mean (SEM).

**Statistical analysis**

Statistical analysis was conducted by using GraphPad Prism, version 5.04. The statistical differences between biopsy groups, between Ex-HSE groups and between the biopsies and their corresponding Ex-HSEs were analysed by using two-way analysis of variance.
Results

Explant cultures of AD biopsies with FLG mutations display normal epidermal morphogenesis

To evaluate whether AD biopsies could be used to establish Ex-HSE and whether FLG loss-of-function mutations affected epidermal regeneration, 4-mm biopsies from non-lesional AD skin were placed onto a fibroblast-populated collagen matrix. The AD patients from whom this skin was taken were either wildtype (AD FLG+/+) or had homozygous FLG mutations (R501X or 2282del4, AD FLG-/-).

After culture for 21 days at the air-liquid interface, keratinocytes grew from the biopsies in a lateral fashion and covered over 90% of the collagen matrix (figure 1a). This occurred irrespective of the presence of FLG mutations. The biopsies from healthy volunteers and AD patients displayed the presence of all epidermal layers, namely the stratum basale, stratum spinosum, stratum granulosum (figure 1b-d). We observed reduced granular structures in the location of the stratum granulosum in AD (-/-) biopsies compared to healthy biopsies and AD (+/+) biopsies. The Ex-HSEs established with biopsies from healthy volunteers (healthy Ex-HSE), AD patients without FLG mutations (AD Ex-HSE [+/-]) or with FLG mutations (AD Ex-HSE [-/-]) displayed the presence of all the epidermal layers. However, similar to AD (-/-) biopsies, AD Ex-HSE (-/-) also showed reduced granular structures in the location of the stratum granulosum (figure 1e-g). In addition, the number of viable cell layers was comparable between healthy Ex-HSE, AD Ex-HSE (+/+) and AD Ex-HSE (-/-). FLG expression was present in the granular layer of the epidermis (which could be identified by from healthy volunteers and AD FLG+/+ biopsies, whereas the AD FLG-/- biopsies showed an almost complete absence of FLG, as demonstrated by immunofluorescence staining (figure 1h-j). More importantly, whereas the healthy Ex-HSE and the AD Ex-HSE (+/+) showed pronounced FLG protein expression in the granular layer, the AD Ex-HSE (-/-) displayed an almost complete absence of FLG protein expression, similar to the original biopsies from these patients (figure 1k-m).
Figure 1: Establishment of explant human skin equivalents (Ex-HSEs) by using biopsies from atopic dermatitis (AD) patients and healthy volunteers. a Example of an AD Ex-HSE established by placing an AD biopsy onto a fibroblast-populated collagen matrix. Biopsies from both healthy and AD patients (FLG+/+ [wild-type for filaggrin] and FLG-/-) gave similar results, i.e., coverage of at least 90% of the collagen matrix (dotted line boundaries of the outgrowth, solid line original biopsy). b-g Cross-sections of biopsies and their corresponding Ex-HSEs. All epidermal layers were present in all biopsies and their corresponding Ex-HSEs, irrespective the presence of FLG mutations, as shown by haematoxylin and eosin staining. h-m Immunofluorescent staining for FLG showing reduced FLG expression in the granular layer of AD FLG (-/-) and AD Ex-HSE (-/-). Scale bar 50 μm.
AD Ex-HSEs maintain expression of differentiation markers

After evaluation of the morphology and FLG protein expression, the Ex-HSEs were further characterised for their expression of various epidermal markers. The presence of the basement membrane was assessed by collagen type IV staining. Biopsies from healthy volunteers and AD FLG+/+ and AD FLG-/- patients showed continuous collagen type IV expression at the dermal-epidermal junction indicating the presence of a normal basement membrane (figure 2a-a’). Early differentiation was assessed by keratin 10 staining. Keratin 10 was expressed in all suprabasal cell layers of the epidermis from biopsies from healthy volunteers and AD patients, irrespective of FLG mutations (figure 2b-b’). Terminal differentiation was assessed by staining for involucrin and loricrin. Involucrin expression was present in the granular layer and in the upper spinous layers of all biopsies, irrespective of FLG mutations (figure 2c-c’). However, whereas the biopsies from healthy volunteers and AD FLG+/+ patients displayed continuous loricrin expression in the granular layer of at least two cell layers thick, loricrin expression was present in one cell layer and strongly reduced in the granular layer in the biopsies from the AD FLG-/- patients (arrows, figure 2d-d’’).

All Ex-HSEs showed the formation of a basement membrane as assessed by a collagen type IV staining, similar to their respective original biopsies (figure 2e-e’’). In all Ex-HSEs, keratin 10 expression was present in all suprabasal layers indicating that early differentiation was similar in the Ex-HSEs compared with the original biopsies. Involucrin expression was present in almost all suprabasal layers of all Ex-HSEs, irrespective of the presence of FLG mutations. This earlier expression of involucrin was different in the Ex-HSEs compared with the original biopsies. Expression of loricrin in the Ex-HSEs was present in the granular layer of healthy Ex-HSE and AD Ex-HSE (+/+), similar to their original biopsies. However, the AD Ex-HSE (-/-) displayed a reduced loricrin expression in the granular layer (arrows, figure 2h-h’’) with the thickness of the granular layer staining being 1-2 cell layers thick in contrast to that of the healthy and AD FLG +/+ HSEs with 3-4 cell layers thickness.

To evaluate whether proliferation was affected in the AD Ex-HSEs and/or is influenced by FLG mutations, staining for the proliferation marker Ki67 was performed. The proliferation index in biopsies from healthy volunteers (24.7 ± 0.7), AD FLG+/+ patients (23.7 ± 4.9, p=0.76) and AD FLG-/- patients (21.7 ± 6.2, p=0.49) was similar, whereas the Ex-HSEs showed a proliferation index of 27.5 ± 0.5 (healthy Ex-HSE), 23.7 ± 1.7, p=0.07 (AD Ex-HSE (+/+)) and 28.3 ± 3.5, p=0.71 (AD Ex-HSE (-/-); figure 2i).
Figure 2: Expression of epidermal markers is maintained in AD Ex-HSEs. a–h” Immunohistochemical staining for collagen type IV, keratin 10, involucrin and loricrin in biopsies from healthy volunteers and AD patients (a–d”) and their corresponding Ex-HSEs (e–h”). Collagen type IV expression was continuously expressed at the dermal-epidermal junction in all specimens. Keratin 10 expression was present in all suprabasal cell layers of the biopsies and their corresponding Ex-HSEs. Involucrin
was expressed in the granular layer of the biopsies but the expression was increased in their corresponding Ex-HSEs. Loricrin expression was present in the granular layer of healthy volunteers and in AD FLG+/+ biopsies and their corresponding Ex-HSEs but the expression was reduced in the AD FLG-/- biopsies and in the AD Ex-HSE (-/-); arrows indicate residual loricrin expression. Scale bar 50 μm. i Graph showing that the proliferation index of the initial biopsies (white bars) and Ex-HSEs (black bars) was similar and not affected by the presence of FLG mutations. Data represent means + SEM of three independent biopsies or Ex-HSEs for each condition.

AD Ex-HSEs show alterations in expression of desquamation-related enzymes
To evaluate whether the expression of enzymes involved in the desquamation process were affected in AD biopsies and in Ex-HSEs, immunohistochemical analyses for KLK5 and Lekti were performed. In the biopsies, both enzymes were expressed in the granular layer of the epidermis and were unaffected by the presence of FLG mutations (figure 3a-a”’, b-b’’). In the Ex-HSEs, KLK5 expression was continuously present in the granular layer of the healthy Ex-HSE and AD Ex-HSE (+/+). However, in the AD Ex-HSEs (-/-), KLK 5 expression was reduced and patchy (figure 3c’’). In one case, KLK5 expression was barely present in the granular layer of the AD Ex-HSE (-/-) (data not shown). Compared with the biopsies, Lekti showed a different expression pattern in all Ex-HSEs. In the biopsies, Lekti was expressed in the uppermost two granular layers, whereas in all Ex-HSEs, the expression was present in the granular layer and in the upper layer of the stratum spinosum (figure 3d-d’’).
Figure 3: Expression of desquamation-related enzymes in Ex-HSEs. Cross-sections of the initial biopsies (a-a”, b-b”) and Ex-HSE cultures (c-c”, d-d”). Immunohistochemical staining for kallikrein-related peptidase 5 (KLK5) and lympho-epithelial Kazal-type-related inhibitor (Lekti) in biopsies from healthy volunteers and AD patients (a-a”, b-b”) and their corresponding Ex-HSEs (c-c”, d-d”). Both KLK5 and Lekti are expressed in the granular layer of the biopsies, irrespective of FLG mutations. In the healthy Ex-HSEs and AD Ex-HSEs (+/+), KLK5 expression is present in the granular layer but is reduced in the AD Ex-HSE (-/-) culture. All Ex-HSEs show Lekti expression in the granular layer and in the upper spinous layers. Scale bar 50 μm
Discussion

The objective of the current study was to establish an in vitro EX-HSE for AD. Previous studies used FLG-KD in keratinocytes or cytokine supplementation to mimic AD in vitro. In the current study, we used biopsies from non-lesional skin from AD patients, wildtype for FLG (FLG+/+) or harbouring a homozygous FLG mutation (FLG-/-; R501X or 2282del4). These biopsies were placed onto a fibroblast-populated collagen matrix. Our main purpose was to evaluate whether we could maintain characteristics from the original biopsy in vitro and to evaluate whether FLG mutations played a role in the epidermal morphogenesis of the Ex-HSEs. FLG mutations did not affect the ability to form an Ex-HSE. Earlier studies showed that reduced profilaggrin expression eliminated the granular cell layer by abolishing keratohyalin granules and FLG monomers from the epidermis (28). In the current study, AD biopsies and the generated Ex-HSEs displayed all epidermal layers, including the granular layer. More importantly, the presence of FLG mutations resulted in reduced FLG protein expression in the biopsies and in the outgrowth areas of the corresponding Ex-HSEs.

FLG-KD skin models have previously been described (9, 17). However, no skin models have been presented in which primary FLG-deficient cells from AD patients have been used for their establishment. In addition, to the best of our knowledge, no studies have so far been conducted in which the effect of FLG mutations on the expression of various epidermal proteins in vivo and in vitro has been evaluated within the same patient.

The role of FLG monomers in aggregating keratin filaments, as a component of cornified cell envelope and as a source of free amino acids for the NMF has been well established (29). Furthermore, FLG has been found to be associated with in particular keratins 1 and 10 (4). In the current study, we show that the expression of keratin 10 is similar in all biopsies and Ex-HSEs, irrespective of FLG mutations. These findings are similar to those in which HSEs were generated after FLG-KD in keratinocytes; in these FLG-KD HSEs, no effect on keratin 10 expression was observed (9, 17).

Since the FLG gene is located in the epidermal differentiation complex, mutations in FLG might affect the expression of other genes and proteins located in this complex, e.g., involucrin and loricrin. The expression of involucrin in the biopsies is not affected by the presence of FLG mutations. However, in all Ex-HSE, involucrin is expressed earlier, irrespective of FLG mutations. These observations are in agreement with earlier studies that characterised various in vitro HSEs (27). Loricrin expression is reduced in AD FLG-/- biopsies and in their corresponding Ex-HSEs; this implies that the reduction of FLG protein expression is not compensated by loricrin. Lentiviral-mediated knockdown of profilaggrin in primary keratinocytes has been shown to result in a hyperproliferative epidermis in HSEs (30). However, in the Ex-HSEs established with FLG-/- biopsies, we did not observe an effect of FLG mutations on epidermal proliferation. The observation that FLG mutations, i.e., R501X and 2282del4, do not affect proliferation indicates that such mutations do not affect the presence of (a partly) functional profilaggrin. Other studies have demonstrated the presence of a truncated profilaggrin in the epidermis of human ichthyosis vulgaris patients and in the flaky tail mouse (10, 24, 31, 32).
The processing of profilaggrin is controlled by a complex network of serine proteases, including KLK5 and Lekti(33-36). Although we did not observe an effect of FLG mutations on KLK5 expression in vivo, reduced KLK5 expression was observed in the AD Ex-HSEs (-/-). Since desquamation is absent in HSEs, the underlying mechanisms and the consequences of this reduced KLK5 in the AD Ex-HSEs (-/-) are difficult to address. Lekti loss-of-function mutations are the underlying cause of Netherton syndrome, a rare skin disorder that shares many similarities with AD. Lack of Lekti has been shown to result in premature profilaggrin processing (37), attributable to uncontrolled activity of proteases during the formation of the cornified envelope. However, here we show that the lack of FLG does not affect Lekti expression. The earlier expression of Lekti in the stratum spinosum of Ex-HSEs might result in increased Lekti activation and the subsequent increased inhibition of KLK5 activity, as observed in various HSEs (40). Such a change in Lekti expression in HSEs might therefore (partially) explain the absence of desquamation in vitro.

In conclusion, we present a novel AD-HSE that is established with biopsies from non-lesional AD skin wild-type for FLG or harbouring a homozygous FLG mutation. We have shown that most of the in vivo AD characteristics, including the FLG genotype-phenotype, are maintained in vitro. Therefore, we believe that this approach is a promising alternative to FLG-KD in keratinocytes in order to be able to mimic this in vivo feature of AD in vitro and to improve the study and understanding of the role of FLG in AD. FLG-KD is an effective but rather artificial method for reducing FLG expression in skin models, as opposed to using primary FLG-deficient cells. Furthermore, this Ex-HSE model might aid in the development and evaluation of therapies aimed at FLG protein replacement (38, 39).
References


Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin
CHAPTER 6

An ex vivo human skin model for studying skin barrier repair

Abstract

In the studies described in this paper, we introduce a novel ex-vivo human skin barrier repair model. To develop this, we removed the upper layer of the skin, the stratum corneum (SC) by a reproducible cyanoacrylate stripping technique. After stripping the explants, they were cultured in vitro to allow the regeneration of the SC. We selected two culture temperatures 32°C and 37°C and a period of either 4 or 8 days.

After 8 days of culture, the explant generated SC at a similar thickness compared to native human SC. At 37°C, the early and late epidermal differentiation program was executed comparably to native human skin with the exception of the barrier protein involucrin. At 32°C, early differentiation was delayed, but the terminal differentiation proteins were expressed as in stripped explants cultured at 37°C. Regarding the barrier properties, the SC lateral lipid organization was mainly hexagonal in the regenerated SC, whereas the lipids in native human SC adopt a more dense orthorhombic organization. In addition, the ceramide levels were higher in the cultured explants at 32°C and 37°C than in native human SC. In conclusion, we selected the stripped ex vivo skin model cultured at 37°C as a candidate model to study skin barrier repair since epidermal and SC characteristics mimic more closely the native human skin than the ex vivo skin model cultured at 32°C. Potentially, this model can be used for testing formulations for skin barrier repair.
Introduction

The skin being the largest organ of the body (1.5m² in adults) provides protection for the body’s interior against the external environment. This barrier function is mainly located in the outermost layer, the stratum corneum (SC) (1). The SC provides an excellent barrier against excessive water loss and penetration of pathogens and allergens through the skin (2, 3). The SC is 10-15µm thick with 15-20 corneocyte layers (4, 5) and its organization has been described as “brick-and-mortar” structure (6). The bricks represent the terminally differentiated corneocytes and the mortar the intercellular lipid matrix surrounding the corneocytes (7, 8). The SC lipid composition and organization plays an important role in the barrier function of the skin because the major pathway for penetration of molecules through the SC is via the SC lipid matrix (9-11). The main lipid classes in native human SC are free fatty acids (FFAs), ceramides (CERs) and cholesterol which form two lamellar phases. These include the short periodicity phase (SPP) and the long periodicity phase (LPP) with repeat distances of approximately 6nm and 13nm respectively (12). Within the lipid lamellae, the lipids are mainly organized in a dense orthorhombic packing, although a fraction of lipids adopt a hexagonal packing (13, 14). Within the epidermal strata, tight junction (TJ) proteins are known to contribute to the inside-outside barrier. They form an intercellular barrier between the epidermal cells and function to control the selective movement of water and ions through the epidermis (15) and regulate cell proliferation and differentiation (16, 17).

Atopic dermatitis (AD), dry skin conditions, 1st degree burns and sunburned skin are examples of skin conditions associated with impaired skin barrier function (18-20). In order to develop novel formulations or active components to enhance skin barrier repair, in vitro models are required. Currently, skin barrier repair is mainly studied in animals. This does not provide an optimal situation for translation into humans as the morphology in combination with SC properties of animal skin varies greatly from human skin (21-23). Furthermore, removal of SC from animals causes stress and animal testing of cosmetic products and ingredients have been banned in the European union since 2009. Consequently, the use of in vitro human skin barrier repair models can play an important role in screening formulations (24-27).

Currently, no appropriate in vitro human skin models are available to study skin barrier repair. The available human skin equivalents may offer a possibility however, applying formulations can only be performed during generation of the human skin equivalents. This is very labor intensive and needs dedicated expertise. In addition, an impaired barrier induced by tape stripping cannot be performed with human skin equivalents, due to the poor epidermal/dermal adhesion (28). In the present study, we introduce an ex vivo human skin model to study skin barrier repair. Using a reproducible cyanoacrylate stripping technique to remove SC from ex vivo human skin, we investigated the regrowth of SC in vitro by characterizing the epidermal morphogenesis, differentiation, SC lipid composition and organization. Potentially, this skin barrier repair model can be used for optimizing formulations and active ingredients to study their effect on skin barrier repair. The results show that the stripped skin cultured for 8 days at body temperature (37°C) or skin temperature (32°C) has an actively proliferating and differentiating epidermis resulting in the regeneration of SC in vitro. In addition, the regenerated SC lipids are organized in a crystalline lamellae with the presence of the same SC lipid classes and subclasses as seen in native human SC, with some interesting differences.
Materials and methods

Stripping of SC with cyanoacrylate

Human breast skin was obtained from Caucasian skin donors (aged 25-42 years) after written informed consent and handled according to Declaration of Helsinki principles. The skin was dermatomed at 400µm using a Padgett Electro Dermatome (Model B, Kansas city, KS, USA). 18mm punch biopsies of the dermatomed skin were used as a control. 26mm biopsies from the dermatomed skin were fixed into a custom made stripping device (see Supplementary figure S1). A single droplet of preheated cyanoacrylate (Pattex Gold original, Henkel, Dusseldorf, Germany) at 40°C was spread on a 20mm diameter stainless steel cylinder preheated to 40°C. The cylinder with cyanoacrylate was immediately placed on the skin. Standardized pressure was applied on the cylinder using a 2kg weight. After 2 minutes the cylinder was removed in one stroke and in alternating directions to ensure even removal of the SC from the skin surface. This stripping procedure was repeated until the skin gave a glossy appearance indicating that most of the SC has been removed (4-5 strips were required to remove the stratum corneum). The unstripped skin at the border of the biopsy was removed using a scalpel, yielding stripped biopsies of 18mm in diameter. From each donor, at least one stripped skin biopsy was used as control to analyze the number of corneocyte layers remaining on the stripped skin surface by safranin-O-red staining (described below). Stripped and non-stripped biopsies were cultured as described below.

Culture procedure

Non-stripped biopsies (served as controls) and stripped biopsies were washed thrice in sterile phosphate buffered saline (PBS, Braun, Melsungen, Germany) and placed in transwell filter inserts (Corning Life sciences, Amsterdam, Netherlands). The skin biopsies (referred to as explants) were cultured at air-liquid interface for 4 days or 8 days at 37°C or 32°C, 90% relative humidity and 7.3% CO2. The culture medium contained DMEM and Ham’s F12 (Invitrogen, The Netherlands) (3:1 v/v) supplemented with 0.5µM hydrocortisone (Sigma), 1µM isoproterenol (Sigma), 10µM L-carnitine (Sigma), 10mM L-serine (Sigma), 0.053µM selenious acid (Johnson Matthey, Maastricht, The Netherlands), 0.5µg/mL insulin (Sigma), 1µM α-tocopherol acetate (Sigma), 1% penicillin/streptomycin, 25mM vitamin C (Sigma) and a lipid mixture of 7µM arachidonic acid (Sigma), 30 µM linoleic acid (Sigma) and 0.25µM palmitic acid (Sigma). The medium was refreshed twice a week.

Safranin-O-red staining

The cultured skin explants and the non-cultured (stripped and non-stripped) control biopsies were cryofixed in Tissue-Tek O.C.T.™ (Sakura Finetek Europe B.V., The Netherlands). The sections were stained with Safranin-O as described previously (29). Cryofixed skin of 5µm thickness were stained with 1% (w/v) Safranin-O solution (Sigma) for one minute and thereafter incubated in 2% (w/v) KOH solution for 30 minutes to swell the corneocytes. Five microscopic images (from three donors) per explant or biopsy were taken at 64x magnification.
Morphology and Immunohistochemistry
The skin explants and controls were embedded in paraffin, cut at 5µm thickness and stained with haematoxylin (2mg/ml) and eosin (4mg/ml) for morphological analysis. Immunohistochemical analysis of keratin 10, filaggrin, loricrin, involucrin, Ki67, caspase 3, keratin 6, β-glucosylcerebrosidase, steryl-CoA desaturase and acid-sphingomyelinase expression was also performed on 5μm paraffin sections. The primary and secondary antibodies are listed in Supplementary table S1. For further details see supplementary materials and methods.

Lipid extraction and analysis
SC was isolated from the skin explants using trypsin digestion as described by de Jager et al (30). Briefly, the explants were incubated overnight in 0.1% trypsin solution at 4°C followed by incubation at 37°C for 1 hour after which the SC could be peeled off. Lipid extracts from 2 explants per condition, were pooled for lipid analysis. The SC lipids were extracted by a modified Bligh and Dyer procedure (31, 32). Briefly, liquid-liquid extraction from native human SC and cultured stripped and non-stripped explants was performed using 3 different ratios of a chloroform/methanol/water mixture (1:2:0.5/1:1:0/2:1:0). The extracts from the three mixtures were pooled together after which 0.25M KCl solution was added to the pooled extract. The extract solution was washed with water and then the organic layer was collected after full separation of the water and organic layer. The collected organic layer was dried under a stream of nitrogen gas at 40°C and reconstituted in chloroform/methanol (2:1).

High performance thin layer chromatography (HPTLC): SC lipid composition was quantitatively analyzed by HPTLC (33) described in detail in the supplementary materials and methods.

Fourier transformed infra-red spectroscopy (FTIR) and Small angle x-ray diffraction (SAXD)
FTIR and SAXD measurements were performed as described earlier (34). The SC sheets were hydrated for 24 hours over a 27% NaBr solution prior to measurements. FTIR spectra were collected with a Varian 670-IR FTIR spectrometer (Agilent technologies, CA, USA), containing a broad-band mercury cadmium telluride detector, cooled with liquid nitrogen. SAXD patterns were detected with a Frelon 2000 CCD detector at room temperature for a period of 10 min using a microfocus as described by Bras et al., (35). 3 samples per condition were measured.

Statistical analysis
All statistical outcomes were determined using Graph-pad prism software and a two-tailed student’s t-test to analyze the data.
Results

Stripped ex vivo skin generates SC in vitro

The skin was placed in a customized stripping device and using a customized metal cylinder coated with cyanoacrylate, the SC was removed sequentially (see Supplementary figure S1). The stripping procedure reduced the SC layers in the ex vivo skin explant from 13 ± 2 layers to 4 ± 2 layers (figure 1a,b) as determined by counting the number of corneocyte layers from five different spots of safranin-O stained sections. No difference was observed between the amount of SC removed in the central or boundary areas of the stripped skin. However, less SC at the furrows in the skin was removed compared to the flatter parts of the skin as described previously (36). After the removal of the SC, these biopsies were cultured to generate SC. After 4 days of culturing at 37°C, there was no significant increase in SC layers. However, after 8 days of culturing at 37°C, the SC layers of the stripped skin increased to 10 ± 3 layers (figure 1b). Decreasing the culture temperature to 32°C resulted in similar observations. After 4 days of culture no significant increase in the number of corneocyte layers was observed, but after extending to 8 days, the number of corneocyte layers increased to 10 ± 2 layers. Non-stripped controls were also cultured in the incubator for 4 and 8 days at 37°C and 32°C. After 8 days these non-stripped controls generated some additional corneocyte layers, but this increase was not as pronounced as with the stripped skin (figure 1b). Since our goal was to develop a skin barrier repair model, further analysis was performed using skin explants cultured for 8 days as the number of SC layers generated was closer to that in native human skin.
Figure 1: Stratum corneum (SC) layers and proliferation index in cultured ex vivo skin. (a) Swollen SC cell layers visualized by safranin-O staining. (b) The number of corneocyte layers was counted from safranin-O stained sections. The cyanoacrylate stripping procedure was efficient to remove the SC which was generated after 8 days of culturing at 37°C and 32°C. (c) Proliferation index of keratinocytes determined by Ki67 staining. The non-stripped skin explants cultured at 37°C and 32°C show decreased epidermal proliferation. At 37°C, the stripped explants show similar
proliferation as in native human skin and at 32°C, a 2x higher proliferation than native human skin. Data represent mean ± SEM of four donors. Five microscopic pictures were taken per explant analysis. Proliferation index was calculated from the number of Ki67 positive basal cells out of 100 counted basal cells from five microscopic images. Scale bar: 30µm, 64x magnification. NHS: native human skin, S: stripped, NS: non-stripped, Epi: viable epidermis, SC: stratum corneum, D: dermis

*p<0.05, **p<0.01, ***p<0.001

Skin explants express epidermal differentiation proteins in vitro

Since SC is generated by cultured stripped skin explants, we examined their epidermal proliferation, morphology and epidermal differentiation. The proliferation index was calculated as the percentage of proliferating cells in the stratum basale by Ki67 staining. The proliferation index was drastically reduced in the non-stripped skin explants after 8 days of culture at 37°C and 32°C. In stripped explants cultured at 37°C, the proliferation was similar to native human skin while at 32°C proliferation was 2 fold higher compared to the native human skin (figure 1c). We also observe Ki67 positive cells in the first and second supra-basal layers of stripped explants cultured at 32°C. However, other conditions only showed basal cells positive for Ki67 (data not shown).

When focusing on the number of viable epidermal cell layers, the cultured explants showed similar number of viable cell layers as in native human skin with the exception of the stripped explants cultured at 32°C. These showed a significant increase in the number of viable epidermal cell layers from 6±1 layers in the native skin to 10±1 layers in the stripped skin (p<0.01, data not shown).

The stripped and non-stripped skin explants cultured at 37°C and 32°C show a normally differentiated epidermis. However in stripped explants cultured at 32°C, the terminal differentiation is not completely executed demonstrated by nuclei remnants in the SC (indicated by arrows, figure 2).

The early and terminal epidermal differentiation program was correctly executed in explants cultured at 37°C with the exception of involucrin and to a lesser extent filagrin. Similar to control skin, keratin 10 (K10) was expressed in all suprabasal layers of the epidermis while loricrin and filagrin were expressed in the stratum granulosum. However, the number of cell layers positive for filagrin increased to 2-3 layers in both the stripped and non-stripped explants compared to 1 layer in control skin. Conversely, involucrin expression in the skin explants was observed in all epidermal layers in contrast to the stratum granulosum in native human skin.

When the culture temperature was reduced to 32°C, K10 expression was delayed in the stripped explants as 2-4 epidermal layers were negative for K10 expression (indicated by an arrow, figure 2). Loricrin and filagrin were expressed as in native human skin in both stripped and non-stripped skin explants. However, the number of cell layers positive for filagrin was also increased in both the stripped and non-stripped explants compared to control skin. In addition, involucrin was expressed in all epidermal layers in stripped and non-stripped explants cultured at 32°C (figure 2).
Analysis of keratin 6 (K6) expression in the explants showed that native human skin and non-stripped explants show little or no expression of K6 in the suprabasal layers of the epidermis. Conversely, stripped explants cultured at both temperatures showed a marked increase of K6 expression in the epidermis (supplementary figure S5). In addition, the expression of caspase 3 in the stripped and non-stripped explants cultured at 32°C and 37°C was very similar to native human skin: the expression of caspase 3 is present in the entire epidermis in all conditions (supplementary figure S5).
Figure 2: Morphology and epidermal differentiation in native human skin compared to non-stripped and stripped explants cultured for 8 days. Haematoxylin and eosin staining for morphological overview and immunohistochemical staining for keratin 10 (K10), Loricrin (LOR), involucrin (INV) and filaggrin (FLG). The stripped and non-stripped explants still display a differentiated epidermis at 37°C but at 32°C parakeratosis is observed mainly in the SC of the stripped explants (indicated by arrows). At 37°C, the non-stripped and the stripped explants express keratin 10, loricrin and filaggrin with similar localization as in the native human skin. The expression of involucrin in stripped and non-stripped explants was shifted to all epidermal layers rather than in the stratum granulosum. Non-stripped and the stripped explants cultured at 32°C also express filaggrin and loricrin as in native human skin however, the expression of keratin 10 is delayed and involucrin is expressed in all epidermal layers as with explants cultured at 37°C. Scale bar: 25µm. Images are representative of results consistently observed in three different skin donors. NHS: native human skin, S:stripped, NS: non-stripped

In vitro generated SC from stripped skin shows a similar lamellar lipid organization as native human SC but differences in the lateral packing

The SC lipid properties of the cultured explants were examined in relation to native human SC (the control). The lateral packing was examined using the CH₂ rocking vibrations of the lipid chains in the FTIR spectra from 0°C-90°C. A hexagonal lipid organization is portrayed by a single peak at 719cm⁻¹ and an orthor-
hombic lipid packing by two vibrations at 719 cm\(^{-1}\) and 730 cm\(^{-1}\) in the spectrum. At 0°C, the lipids from native human SC adopt an orthorhombic lateral packing demonstrated by two strong contours at 719 cm\(^{-1}\) and 730 cm\(^{-1}\) (figure 3a). Around 42.5°C \(\pm\) 2.5°C, the orthorhombic packing is converted into an hexagonal packing. This is characterized by a transition from a doublet to a singlet at 719 cm\(^{-1}\). The non-stripped skin explants cultured at 37°C show an orthorhombic lateral packing until 40.5°C \(\pm\) 5.3°C (figure 3b). However, the stripped explants cultured at both temperatures show a strong peak at 719 cm\(^{-1}\) and a small peak at 730 cm\(^{-1}\) at 0°C (figure 3c, e). The orthorhombic packing is only present until 20.7°C \(\pm\) 1.2°C and 29.3 \(\pm\) 4.2°C at 37°C and 32°C, respectively i.e. the lipids are mainly organized in a hexagonal packing with a small population of lipids still forming an orthorhombic packing.

The conformational order of the lipids was also investigated using the thermotropic behaviour of the CH\(_2\) symmetric stretching frequencies in the spectrum. In a crystalline organization (orthorhombic or hexagonal packing) the conformational order is high with CH\(_2\) symmetric stretching frequencies <2850 cm\(^{-1}\). In a disordered organization (liquid phase), the CH\(_2\) symmetric stretching frequencies are \(\geq\) 2852 cm\(^{-1}\). Since the order-disorder transition occurs within a temperature range, the mid-point temperature at which the lipid domains change from an ordered state to a disordered liquid state was determined (37) i.e. “Mid-point Transition Temperature” (MTT). The results show that the MTT of the lipids in the regenerated SC of the stripped skin explants (37°C and 32°C) is significantly lower than in non-stripped cultured controls and native human SC (p<0.05, figure 3f). The CH\(_2\) stretching frequency at skin temperature (32°C) was also increased in the stripped skin explants cultured at 37°C compared to the non-stripped explants (p<0.05, supplementary figure S2). This indicates an increased conformational disorder in the regrown SC of the stripped explants.

The lamellar lipid organization was also examined in the stripped and non-stripped explants in relation to native human skin using SAXD. In native human SC, the diffraction profiles show three diffraction peaks indicating the 1\(^{\text{st}},\) 2\(^{\text{nd}}\) and 3\(^{\text{rd}}\) order diffraction peak of the LPP (depicted by 1, 2 and 3,) and crystalline cholesterol domains, indicated by an asterisk (*). Peak “2” in native human skin represents both LPP (2\(^{\text{nd}}\) order) and SPP (1\(^{\text{st}}\) order). The stripped and non-stripped explants cultured at 37°C show a very similar profile, and exhibit a 1\(^{\text{st}}\) - 3\(^{\text{rd}}\) order diffraction peak of the LPP and crystalline cholesterol (supplementary figure S3). As the 1\(^{\text{st}}\) order peak of the SPP is partly obscured by the 2\(^{\text{nd}}\) order of the LPP, the presence of the SPP should induce a broadening of this peak. However, we could not obtain evidence for this broadening and thus the presence of this phase. In addition, the explants cultured at 32°C show similar diffraction profiles as in explants cultured at 37°C (data not shown).
Figure 3: FTIR spectra showing the CH$_2$ rocking vibrations in the spectra of the stratum corneum (SC) of generated stripped explants as a function of temperature (0°C-90°C). (a) Native human SC and (b) non-stripped explants cultured at 37°C and (d) 32°C, show an orthorhombic lateral organization. The peak intensity of the 730cm$^{-1}$ band is reduced in stripped explants cultured at (c) 37°C and (e) 32°C suggesting a higher population of lipids adopting a hexagonal organization. (f) Mid-point transition temperature (MTT). Data represents mean ± SEM from three donors, n.s.: not significant, *p<0.05, NHS: native human skin, S:stripped, NS: non-stripped
Generated SC from stripped explants contain the main SC lipid classes

The SC generated by the explants after stripping contains the main SC lipid classes CERs, FFAs and cholesterol with some changes in the distribution of these lipid classes as analyzed by HPTLC. The generated SC at 37°C and 32°C showed significantly higher CER levels respectively compared to native human skin (figure 4b, p=0.03 and p=0.06 respectively, for relative values see supplementary figure S4). In addition, the non-stripped controls cultured at both temperatures show a similar trend (figure 4b, p=0.04 and p=0.07, for relative values see supplementary figure S4).

All the CER subclasses detectable by HPTLC in native human skin were present in the generated SC cultured at both temperatures (figure 4c, for relative values see supplementary figure S2). However, some differences exist in the CER distribution of the cultured explants in relation to native human SC. At 37°C the levels of CER NS, EOH and AS/NH in regenerated SC were significantly increased compared to native human SC (p<0.01, p<0.05). Similarly, at 32°C the regrown SC showed significant increase in CER NS and AS/NH (p<0.01, p<0.05). The non-stripped explants cultured at 37°C and 32°C also have significantly more CER EOH and CER NS respectively than native human skin.

We further examined the expression of β-glucosylcerebrosidase (GBA) and acid-sphingomyelinase (aSmase) involved in the synthesis of CERs from CER precursors. The expression of aSmase in the human epidermis shows a gradient from the basal layer to the granular layer with the highest expression seen in the granular layer. In all cultured explants, the expression of aSmase is similar in most epidermal layers (figure 4d). In native human epidermis, the expression of GBA is localized in the interface between the granular layer and SC. In explants cultured at 32°C or at 37°C, the localization of GBA expression is the same as native human epidermis.

Changes in unsaturated FFA levels have been shown to be involved in SC lipid organization. Therefore, we also examined the expression of steroyl CoA desaturase (SCD) which mono-unsaturates FFAs. SCD expression is seen in the basal layer of the epidermis in native human skin but in non-stripped and stripped explants cultured at 32°C and 37°C the expression of SCD is extended to all epidermal layers (figure 4d).
Figure 4: Stratum corneum (SC) lipid composition and epidermal expression of lipid synthesis enzymes in cultured ex vivo skin. (a) The lipids were separated according to the solvent system provided in supplementary table S2. 1-Native human skin, 2-Non-stripped 37°C, 3-Stripped 37°C, 4-Non-stripped 32°C, 5-Stripped 32°C (b) Absolute levels of cholesterol (CHOL), free fatty acids (FFA) and ceramides (CERs) (c) Absolute CER subclasses in cultured ex vivo skin. The SC from the non-stripped and stripped explants contains all CER subclasses in native human SC which are visible by high performance thin layer chromatography (HPTLC). (d) Immunohistochemical analysis of acid-sphingomyelinase (aSmase), β-glucosylceribrosidase (GBA) and steroyl CoA desaturase (SCD). Data represents mean ± SEM from three donors. Scale bar 25µm. NHS: native human skin, S:stripped, NS: non-stripped.
The aim of this study was to establish a human skin model to study skin barrier repair by i) removal of SC from ex vivo skin and ii) generation of the SC during in vitro culturing of stripped skin. We established a reproducible method of SC removal using a cyanoacrylate stripping technique which served as a starting point. After 8 days of culturing the stripped explants at 32°C and 37°C, the number of SC layers regrown was similar to the SC layers before stripping. The recovery of SC to control thickness after stripping in vivo ranges from 3-6 days in the arm and leg (38). In addition, barrier recovery (calculated by recovery of trans epidermal water loss values to baseline) after SC removal by tape-stripping in human facial skin reaches ~90% after 1 week and 100% recovery at 4 weeks (39). Duplan et al., presents an ex vivo human skin model for irritation/repair using SDS and vasointestinal peptide (40). In order to restore native epidermal features, the skin is treated with hydroxydecon in culture. Our present study examines skin barrier repair by removal of stratum corneum and culturing without the addition of a formulation and is thus a different approach.

Epidermal morphology and SC layers at the end of the 8 days culture period at both temperatures are similar to that of native human skin. However, the stripped explants cultured at skin temperature (32°C) exhibit parakeratotic epidermis, a feature also observed after repeated tape-stripping in vivo (41). This may occur from increased proliferation induced by stripping as nuclei are observed only in the lower layers of the SC. Parakeratosis is associated with a hyper proliferative epidermis, delayed K10 expression, increased involucrin expression and increased number of epidermal cell layers positive for filaggrin (41, 42). All of these characteristics are observed in stripped skin explants cultured at 32°C. Stripped explants cultured at 37°C also display increased filaggrin and involucrin expression, but very importantly no parakeratosis and no delayed expression of K10 is observed.

Proliferation (Ki67 expression) of stripped explants cultured at 37°C is similar to native human skin but at 32°C, proliferation is double that of native human skin. However, the number of SC layers generated after 8 days are similar at both temperatures. This may occur from the changes in proliferation index at 4 and 8 days of culture in the explants, as observed in a pilot study. At 4 days of culture, proliferation index in stripped explants cultured at 32°C is around 1% and 30% at 37°C (data not shown). However, after 8 days, the proliferation index is ~40% at 32°C and 12% at 37°C. This data suggests the increase in proliferation in stripped explants as a response to the SC removal is delayed at 32°C compared to 37°C. The number of corneocytes layers in the SC is an accumulative value and depends on the proliferation rate during the whole culturing period as no desquamation takes place.

The SC lipid properties in the stripped explants show several similarities to native human SC. The SC from the stripped and non-stripped explants cultured at 37°C contains the main SC lipid classes. However, we observe a significant increase in the absolute level of CERs in the explants cultured at 37°C and 32°C. Increase of CER levels also occurs in other full-thickness and epidermal human skin equivalents (43). Furthermore, when focusing on the CER subclasses, 10 different CER subclasses can be detected, of which CER NdS/NS and CER AS/NH cannot be separated. The composition of these CER subclasses is very similar to that in native human skin, although some important differences are also noticed. The increase in the level
of CERs was accompanied by a stronger staining for aSmase suggesting its role in the increased CER levels. ASmase catalyzes the conversion of sphingomyelin to ceramides. Sphingomyelin is a precursor of CER NS and AS (44, 45) and both are increased by approximately 2-3 fold in the cultured explants compared to native human SC. The change in environmental conditions and barrier disruption induces stress in keratinocytes. This may result in increased CERs via sphingomyelinases and up-regulation of the activity of serine palmitoyl-transferase and/or ceramide synthases in de novo CER synthesis (46, 47).

Disruption of the SC also results in the loss of the epidermal calcium gradient which is crucial for differentiation (48, 49). The loss of calcium gradient affects the expression of epidermal differentiation proteins including filaggrin and involucrin and may also contribute to the changes in involucrin and filaggrin expression in the cultured skin explants (50, 51). In non-stripped skin, we still observe increased CER levels and altered involucrin expression although there is no barrier disruption. The exact mechanism responsible for these changes in non-stripped explants remains unknown.

SC lipids generated by the stripped explants adopt mainly a hexagonal organization as opposed to an abundant orthorhombic organization in native human SC. The hexagonal lateral organization is also observed in epidermal or full thickness human skin equivalents (52). In these models an increased relative level of MUFAs was detected, which may be responsible for the abundant hexagonal packing (43, 52). Using lipid model systems we observed indeed that increased levels of MUFA enhances the formation of the hexagonal lateral packing (53). Therefore the hexagonal lipid organization in the generated SC in the explants may be attributed to an increased level of MUFAs resulting from increased SCD expression. The effect of increased SCD expression on lateral lipid organization is not observed in the non-stripped skin due to the very low amount of SC layers generated in vitro (1-2 layers) compared to the SC layers on the skin before culturing (13 ± 2 layers).

Some features of the cultured stripped skin can be observed in barrier related skin diseases such as increase in involucrin expression, CER NS, AS and hexagonal lipid organization (54-57). However, there are also aspects that do not resemble diseased skin e.g. increased CER EOS, similar levels of CER NP and filaggrin, loricrin expression as in native skin etc. This suggests that the cultured stripped skin generates some aspects of diseased skin, but is not a perfect model for diseased skin. The cultured stripped model also generates some important features especially seen in human skin equivalents and this might be induced by the culture conditions (48).

We suggest that culture of cyanoacrylate stripped skin explants provides a potential model to study i) skin barrier repair ex vivo and ii) investigating the effect of culture medium composition and environmental conditions during culture on the restoration of the skin barrier. Firstly, the SC lipid properties in regrown SC bear some aspects observed with altered skin barrier function (57-59) including reduced orthorhombic lipid organization and increased lipid conformational disorder of SC lipids. The presence of these characteristics in the cultured stripped explants can provide a system where formulations can be tested to enhance skin barrier repair.
Secondly, in the development of 3D *in vitro* skin models, no human skin equivalent fully mimics the SC lipid properties of native human skin (52, 60, 61). Our model shows some advantages over the available human skin equivalents when applied to optimization of culture conditions to mimic native human SC properties *in vitro*. This is because the culture period is shorter (14-21 days for human skin equivalents) and does not require cell isolation and seeding which saves time and is less labor intensive. It is also possible to examine inter-donor changes in these cultures with human skin.

In conclusion, the removal of SC from *ex vivo* skin and culture for 8 days at 37°C generates an *ex vivo* human skin model which possesses various similarities in epidermal properties to native human skin than at 32°C. Therefore this model has the potential to be used in studying skin barrier repair.
Acknowledgements

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References

Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin


Supplementary figures

Supplementary figure S1: Stripping of stratum corneum (SC) from ex vivo skin. 26 mm skin biopsies of 400 µm thickness is fixed into a custom made cyanoacrylate stripping device. A single droplet of preheated cyanoacrylate (40°C) was spread on a 20 mm diameter stainless steel cylinder which was also preheated to 40°C. The cylinder with cyanoacrylate was immediately placed on the skin together with a 2 kg weight to achieve a standardized pressure for 2 minutes. The cylinder was removed in one move, with alternating directions of 180° to ensure even removal of the SC on both sides. This stripping procedure was repeated until the skin gave a glossy appearance indicating that most of the SC has been removed.

Supplementary figure S2: FTIR spectra showing the CH₂ symmetric stretching frequency at 32°C in the spectra of the stratum corneum (SC) of cultured explants. Data represents mean ± SEM from three donors, *p<0.05. NHS: native human skin, S:stripped, NS: non-stripped
**Supplementary figure S3:** Representative x-ray diffraction pattern of SC from stripped, non-stripped explants and native human skin. 1, 2 and 3 indicate the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} order of the long periodicity phase (LPP) respectively. The “2” peak from native human SC and non-stripped SC represent both the 2\textsuperscript{nd} order of the LPP and the 1\textsuperscript{st} order of the short periodicity phase (SPP). Therefore, the repeat distances cannot be calculated directly from this diffraction profile. Crystalline cholesterol is indicated by (*).
Supplementary figure S4: Relative levels of SC lipids in cultured explants. (a) Cholesterol (CHOL), free fatty acids (FFA) and ceramides (CERs) (b) various classes of CERs. Data represents mean ± SEM from three donors.
Supplementary figure S5: Immunohistochemical staining for keratin 6 and caspase 3. Images were taken at 20x magnification. Scale bar: 25µm
### Supplementary tables

**Table S1: Primary and secondary antibodies for immunohistochemical staining**

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Table S2: Solvent system used for barrier lipids analysis by thin layer chromatography

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<thead>
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<th>Eluent</th>
<th>Composition (v/v)</th>
<th>Distance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dichloromethane/Ethylacetate/Acetone/Methanol (88:8:4:1)</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform/Acetone/Methanol (76:8:16)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Hexane/Chloroform/Acetone/Methanol (6:80:12:2)</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>Hexane/Chloroform/Hexyl acetate/Acetone/Methanol (6:80:0.1:10:4)</td>
<td>95</td>
</tr>
</tbody>
</table>

Table S3: Ceramide nomenclature

<table>
<thead>
<tr>
<th></th>
<th>Non hydroxy fatty acid (N)</th>
<th>α-hydroxy fatty acid (A)</th>
<th>Esterified ω-hydroxy fatty acid (EO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrosphingosine (dS)</td>
<td>NdS</td>
<td>AdS</td>
<td>EOdS</td>
</tr>
<tr>
<td>Sphingosine (S)</td>
<td>NS</td>
<td>AS</td>
<td>EOS</td>
</tr>
<tr>
<td>Phytosphingosine (P)</td>
<td>NP</td>
<td>AP</td>
<td>EOP</td>
</tr>
<tr>
<td>6-hydroxy sphingosine (H)</td>
<td>NH</td>
<td>AH</td>
<td>EOH</td>
</tr>
</tbody>
</table>

The human stratum cornuem(SC) contains 4 sphingoid bases (dihydrosphingosine (dS), Sphingosine (S), phytosphingosine (P) and 6-hydroxy sphingosine (H)) and three acyl chains (non-hydroxy fatty acid (N), α-hydroxy fatty acid (A) and esterified hydroxy fatty acid (EO)). Together, these result in the 12 ceramide subclasses present in human SC.
Supplementary materials and methods

Morphology and Immunohistochemistry

The skin sections (except the sections stained for aSmase) were incubated in sodium citrate buffer (pH 6) for 30 minutes at 95°C for antigen retrieval. The sections stained for aSmase were incubated in sodium citrate buffer (pH 6) for 5 minutes at 110°C in an autoclave (Laboratory autoclave, model A275, Zirbus technology, Germany) for antigen retrieval. Thereafter, all sections were blocked with 2.5% normal horse serum (Vector laboratories Burlingame, CA) for 20 minutes and incubated overnight at 4°C with the primary antibody diluted in 1% bovine serum albumin (BSA) in PBS.

Staining procedure with amino-ethylcarbazole: Subsequently, the sections were incubated for 30 minutes at room temperature with the secondary antibody (Vector laboratories Burlingame, CA) and the ABC reagent (Vector laboratories Burlingame, CA) for 30 minutes and 10 minutes for caspase 3. The sections were washed with 0.1M sodium acetate buffer and incubated in amino-ethylcarbazole (Sigma) dissolved in N,N-dimethylformamide (1g/250mL) (Sigma) with 0.1% hydrogen peroxide for 30 minutes at room temperature. All sections were counterstained with haematoxylin.

Staining procedure for immunofluorescence: Sections were incubated with the appropriate secondary antibody for 1 hour at room temperature and mounted using DAPI Vectashield (Vector laboratories Burlingame, CA).

Proliferation index: Five microscopic images per explant or biopsy (from three donors) were taken at 20x magnification. For each image, the number of basal cells were counted and the proliferation index was calculated as the percentage of Ki67 positive cells within the basal layer of the epidermis.

High performance thin layer chromatography (HPTLC)

To identify the different classes of lipids, co-chromatography of a standard lipid mixture was performed. This included six subclasses of synthetic CERs (EOS, NS, EOP, NP, AS and AP (Evonik, Germany)), FFAs (stearic acid, tricosanoic acid, palmitic acid, behenic acid, arachidonic acid, cerotic acid and lignoceric acid (Sigma)) and cholesterol (Sigma). The ceramide nomenclature is according to the terminology of Motta et al., (1) and Masukawa et al., (2) which is used throughout this article (Supplementary table S3).

Lipid quantification by HPTLC: The standard lipid mixture and extracted lipids were sprayed on a silica gel plate (Merck, Germany) using a Camag Linomat IV device, (Muttenz, Switzerland) within a concentration range of 4µg-30µg. Variation in the amounts of sprayed standard lipids allowed the calculation of calibration curves for lipid quantification. The lipids were separated using organic solvent mixtures at specific running distances (supplementary table S2). The plate was stained with a copper acetate-copper sulfate solution (3:1) and charred at 170°C for 15 minutes. The images were obtained with a GS800 calibrated densitometer (Bio-Rad,CA, USA) and analyzed with quantity-one software 4.6.5 (Bio-Rad, CA, USA). The amount of each lipid class was determined from a non-linear fit of the calibration curve from its synthetic
lipid counterpart. This provided the relative levels of the various lipid classes. The absolute values were provided by weighing the SC before and after extraction.

**Fourier transformed infra-red spectroscopy (FTIR) and Small angle x-ray diffraction (SAXD)**

The SC sheets were hydrated for 24 hours over a 27% NaBr solution and sandwiched between AgBr windows (Pier-optics, Japan). The spectra were collected using a Varian 670-IR FTIR spectrometer (Agilent technologies, CA, USA), equipped with a broad-band mercury cadmium telluride detector, cooled with liquid nitrogen. The spectra were collected in transmission mode, as a co-addition of 256 scans at 1cm\(^{-1}\) resolution during 4 minutes from 0°C-90°C at a frequency range of 600-4000cm\(^{-1}\). The spectra was deconvoluted using enhancement factor of 1.7 and half-width of 5 cm\(^{-1}\) and analysed using Bio-Rad Win-IR Pro 3.0 software from Biorad (Biorad laboratories, MA, USA).

The samples were hydrated as described above and SAXD patterns were detected with a Frelon 2000 CCD detector at room temperature for a period of 10 min using a microfocus as described by Bras et al., (3). From the scattering angle, vector q was calculated from the following equation \(q = (4\pi \sin \theta)/\lambda\). \(\theta\) is the scattering angle and \(\lambda\) is the wavelength of the x-ray.
References


Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin
CHAPTER 7

Summary and future perspectives
A defective permeability barrier exists in both lesional and non-lesional skin areas of AD skin. The impaired skin barrier in AD is characterized by increased trans-epidermal water loss (TEWL), increased permeability and reduced hydration in the upper layer of the skin, the stratum corneum (SC) where the skin barrier is located (1-4). The SC comprises of a multilayered structure of the flattened corneocytes surrounded by an intercellular lipid matrix. The lipids serve as a boundary between the hydrophilic corneocytes and the lipophilic lipid matrix. The SC organization is often described as a “brick” and “mortar” structure in which the corneocytes are the bricks and the lipids the mortar (5).

The lipid matrix mainly consists of ceramides (CERs), cholesterol (CHOL) and free fatty acids (FFAs) that are present at approximately equimolar ratios (6-8). The intercellular SC lipid matrix is a major penetration pathway of most molecules/compounds through the SC. This is the reason why its organization and composition is important for an intact barrier (9, 10). The SC lipids are organized in lamellae stacks with repeat distances of approximately 13nm and 6nm namely: the long periodicity phase (LPP) and short periodicity phase (SPP) respectively (11-13). Within the lipid lamellae, the lipids adopt a well-organized structure, the so called lateral organization. In human SC an orthorhombic packing (dense) is mainly present, while a small fraction of lipids assembles in a hexagonal (less dense) packing (14-18).

In SC of AD patients several changes in the lipid composition have been reported. Some alterations in the lipid composition include: reduction in SC lipid levels in relation to protein content, reduced relative CER content (CER/CHOL ratio and CER levels), shorter mean lipid chain length in lesional skin, increased level of unsaturated fatty acids and decreased level of hydroxy fatty acids. Several of these changes correlated with an increased TEWL(19-25).

AD being a complex chronic relapsing inflammatory skin disease has several interacting pathogenic pathways. These are manifested by filagrin (FLG) mutations, disrupted epidermal barrier, microbe skin colonization and activation of T₃₁,T₂/T₃₁,T₁₇/T₃₂,T₂₂ inflammatory pathways (26-28). AD is also associated with an increased risk of developing other allergic diseases such as asthma, allergic rhinitis and food allergies which is termed as ‘atopic march’ (29). Among the known pathogenic factors suggested for AD, the disrupted epidermal barrier in combination with the abnormal immune response is considered as very significant to the development of AD (2, 30, 31).

In AD a TH₁ and TH₂ balance exists. Acute AD is regulated by T-helper 2 (T₃₂) cells that produce cytokines such as IL-4, IL-13 and IL-5. In chronic AD this switch (T₃₂ → T₃₁) results into higher expression of T₃₁ cytokines such as IL-12 and IFN-γ (32). In relating inflammation in AD to the skin barrier, both T₃₁/T₃₂ cytokines have been shown to modify mRNA and protein levels of lipid synthesizing enzymes (CER and FFA) which result in altered levels of the corresponding lipids (33, 34).
Mouse models have been very valuable in elucidating the pathogenesis and molecular mechanisms involved in AD. Although these are excellent models to study biochemical pathways, there are limits in the translation to AD in humans due to differences in skin architecture between mice and humans (35, 36). As an alternative, validated and robust three dimensional (3D) human skin equivalents (HSEs) are a useful tool in exploring the pathogenesis of AD and validating new therapeutic molecules.
Aim

The aim of this thesis is to develop reproducible HSEs that mimic key characteristics of AD in vitro. Additionally, we also focused on the effect of inflammation in the development of AD barrier characteristics. This was achieved by investigating the following research subjects:

1) Linking lipid synthesis enzyme expression in AD with SC lipid composition and organization
   a. Is the expression of CER and FFA biosynthesis enzymes altered in AD skin compared with native human skin?
   b. Do these changes in enzyme expression (CER and FFA biosynthesis enzymes) affect SC lipid composition and organization in AD skin?
2) Does inflammation play a role in the changes in epidermal morphogenesis, differentiation and lipid properties observed in lesional AD skin?
3) Can epidermal characteristics of AD skin be maintained in AD-HSEs derived from AD skin biopsies in vitro?
4) Is it possible to develop a model for skin barrier repair that mimics several barrier characteristics observed in AD skin?

Inflammation, lipid biosynthesis enzyme expression and SC lipid properties in AD

Using cell and tissue culture, the direct effect of inflammatory cytokines on epidermal morphology and differentiation proteins has been studied (37-40). However, much still remains unknown about the mechanism and direct effect of various chemokines and cytokines upregulated in AD skin on lipid biosynthesis and thus lipid composition and organization in SC. The lipid abnormalities in AD skin have been suggested to result from changes in the expression or activity of enzymes involved in SC lipid synthesis. In the research described in this thesis, a number of enzymes involved in CER and FFA synthesis have been examined. These include β-glucocerebrosidase (GBA), acid-sphingomyelinase (aSmase), CER synthase 3 (CerS3), stearoyl CoA desaturase (SCD), Elongase1 (ELOVL1) and ELOVL6.

GBA and aSmase catalyze the last step in CER subclass synthesis from glucosylceramides and sphingomyelin, respectively into CERs (41). ELOVL1 elongates FFAs with carbon chain length of C20-C26 and ELOVL4 elongates those longer than C26 (42-45). CerS3 acylates the ultra-long FFA chains (≥C26) to the sphingoid backbone during biosynthesis of CERs (34). In addition, ELOVL6 is primarily responsible for the elongation of FFAs with a chain length of C16 and C18 and SCD functions to produce unsaturated FFAs from saturated FFAs (46).

The studies in chapter 2 focus on the expression of lipid biosynthesis enzymes (these include SCD, ELOVL1, ELOVL6, CerS3, GBA and aSmase) as underlying factors that may contribute to the change in barrier properties in AD skin. Besides the protein expression of enzymes in the epidermis, the SC lipid composition was also assessed in the patient cohort. Lesional as well as non-lesional AD skin was examined and compared to a control group. The expression of SCD is increased in AD lesional skin compared to the control.
and non-lesional AD skin. This is associated with the significant increase in the level of unsaturated FFAs in AD lesional skin. Similarly, the pattern of ELOVL1 expression also differs in AD lesional skin compared to control (healthy) skin and non-lesional skin and is accompanied by reduction in FFAs C22-C28 observed in AD lesional skin. A similar trend in the level of FFAs ≥C26 produced by ELOVL4 was also observed. These very long FFAs were significantly reduced in AD lesional skin compared to control subjects. We observed no changes in ELOVL6 expression in AD skin.

The expression of aSmase, CerS3 and GBA (that are involved in CER synthesis) in AD lesional skin was changed in comparison to non-lesional skin and control subjects. The relative levels of lipids associated with these enzymes also showed significant changes, such as increased CER AS and NS (aSmase) and decreased esterified ω-hydroxy CERs which are derived from CerS3 activity.

The AD features described in chapter 2 together with previous studies describing other epidermal AD characteristics provided the benchmark required to characterize an AD-HSE. Using the Leiden epidermal model (LEM) in chapter 3, the direct effect of upregulated cytokines in AD on epidermal morphogenesis, differentiation and SC lipid properties was investigated. Results show that the studied inflammatory cytokines upregulated in AD skin (TNF-α, IL-4, IL-13 and IL-31) affect epidermal morphogenesis, proliferation, differentiation, and SC lipid properties singly or in synergy. TNF-α and Th2 cytokines reduced the expression of keratin 10 (K10), filaggrin and loricrin in LEMs. These cytokines also had an effect on stimulating keratinocytes to secrete thymic stromal lymphopoietin (TSLP) and increased basal cell proliferation. Regarding the SC lipid composition, TNF-α alone or in combination with Th2 cytokines reduces the expression of ELOVL1 in LEMs corresponding with the progressive reduction in saturated long chain FFAs (≥ C20:0). The CER composition was also influenced by IL-31 and TNF-α as these cytokines reduce the ratio of ester linked ω-hydroxy (EO) CER to non-EO CER subclasses and the expression of CerS3 in LEMs. These changes in CER composition are reflected in the repeat distance (d) of the long periodicity phase (LPP) as TNF-α alone or in combination with Th2 cytokines reduce significantly the repeat distance of the LPP. It can be concluded from this study that i) inflammation could be an influencing factor in the development of several important features of AD skin barrier ii) supplementation of LEMs with cytokines (individually or in synergy) may serve as an excellent candidate for the testing of prospective drugs and formulations for the treatment of AD.

**Modelling AD in vitro using skin explants from AD patients**

AD skin biopsies can serve as an alternative way to develop AD-HSEs than using isolated primary keratinocytes in developing 3D HSEs as described in chapter 3. This involves placing full-thickness 4mm skin punch biopsies onto a fibroblast populated collagen matrix i.e. dermal equivalent, forming the so-called 1st generation explant human skin equivalent (Ex-HSE). This technique can be applicable for analyses that require larger amounts of epidermal tissue, which cannot be harvested from patients with skin diseases e.g. quantitative protein analysis or when analyzing the lipid barrier properties. A 1st generation Ex-HSEs can may be even further expanded by growing a 2nd generation and even a 3rd generation Ex-HSE. The 2nd generation can be produced by harvesting a biopsy from the 1st generation Ex-HSE and implanting this onto
a fresh dermal substrate. This approach can be repeated using a biopsy from the 2nd generation Ex-HSE to establish a 3rd generation Ex-HSE. The results from chapter 4 reveal that 2nd and 3rd generation Ex-HSEs display similar epidermal morphology and expression of K10, loricrin and filaggrin as in native human skin and 1st generation Ex-HSEs. The 2nd and 3rd generation Ex-HSEs also show many similarities with 1st generation Ex-HSEs and native human skin in lipid properties e.g. presence of all lipid classes, similar fatty acid chain length distribution and lamellar lipid organization. However, some differences arise in the lipid properties, especially when comparing the 1st generation with the native human skin. These include: increased level of hexagonal lateral packing and an increased level of EO CERs. The expansion of skin biopsies to the 2nd and 3rd generation Ex-HSEs could be a promising method to expand valuable epidermal tissue to produce HSEs with similar morphological and differentiation parameters in the native epidermis. However, the reproducibly does not extend to the SC lipid properties in Ex-HSEs.

Using this technique of developing Ex-HSEs, AD Ex-HSEs were generated using non-lesional AD skin biopsies from wildtype or patients with homozygous FLG mutations. The AD Ex-HSEs were analyzed to examine whether the AD epidermal features can also be maintained in vitro and if FLG mutations have an effect on epidermal morphogenesis. The results described in chapter 5 show that the expression of epidermal differentiation proteins in the original AD biopsies (flaggrin, involucrin, kallikrein 5 and Lekti) was maintained in the AD Ex-HSE and this was unaffected by FLG mutations. Although the expression of loricrin was also maintained in Ex-HSEs, the expression of loricrin was reduced in AD biopsies with homozygous FLG mutations and their corresponding Ex-HSEs. The study reveals that AD-HSEs can be derived from small AD biopsies which maintain most in vivo characteristics of AD skin including the genotypic and phenotypic features of FLG. This may be useful in providing additional material in understanding the role of FLG in AD or FLG replacement therapies and evaluation of prospective therapies directed toward filaggrin replacement in AD patients.

An ex vivo model for skin barrier repair
Currently, no suitable in vitro human skin models are available to study skin barrier repair. An impaired barrier induced by tape stripping cannot be performed with HSEs, due to the poor epidermal/dermal adhesion (28). The study in chapter 6 introduces an ex vivo human skin model to study skin barrier repair mechanisms using a cyanoacrylate stripping technique to remove SC from ex vivo human skin. The regrowth of the SC in vitro (at 37°C and 32°C), epidermal morphogenesis, differentiation, SC lipid composition and organization was investigated. The results show that the stripped skin cultured for 8 days at 37°C or 32°C has an actively proliferating and differentiating epidermis resulting in the regeneration of SC in vitro. The SC lipids generated by the stripped explants adopt mainly a hexagonal lateral organization as opposed to an abundant orthorhombic organization in native human SC. The relative CER levels are also increased in the regrown SC in vitro. The above mentioned SC lipid features of the cultured stripped skin bear similarities with AD (23, 24, 47) and provide a system where formulations can be tested to enhance skin barrier repair. Furthermore, the cyanoacrylate stripped skin explants provides a potential model to investigate the effect of culture medium composition and environmental conditions during culture on the restoration of the skin barrier.
Conclusion

The studies in this thesis describes the barrier defects in AD skin and various techniques to develop AD-HSEs which can be used to better understand the role of several factors in the pathogenesis of AD skin. We show that Inflammation plays a pivotal role in the development of epidermal and SC features of AD skin and that AD epidermal features can be maintained \textit{in vitro} when AD skin biopsies are used to generate Ex-HSEs. These AD-HSEs can also serve as a tool to screen potential therapeutics for AD and skin barrier repair. However, limitations exist in the complexity and full representation of all possible factors known to influence the development of AD e.g. FLG mutations, other aspects of inflammatory microenvironment, microbe colonization etc. Several possibilities exist to overcome these limitations and are discussed below.
Perspectives

Understanding in more detail the barrier defect in AD skin

To obtain more information on the barrier defects in AD, studying the activity and expression of CER and FFA synthesis enzymes (e.g. CerS3, GBA, ELOVL1, ELOVL4 etc.) is of particular interest. These enzymes have been shown to be related to the various changes in lipid composition in AD skin. A quantitative analysis of the expression and activity of these enzymes can be correlated with the lipid composition. For example, a bivariate analysis of the activity of SCD-1 and the level of mono-unsaturated FFA for FFAs and bivariate analysis of the expression of GBA in relation to the glucosylCER and CER levels in AD skin. This could provide more insight into the origin of the changes in the lipid composition observed in AD skin.

The bound lipids in the SC i.e. ω-hydroxy (EO) CERs and ω-hydroxy FFAs are thought to be a relevant factor in the skin barrier function as they form a lipid monolayer around corneocytes by an ester linkage of the ω-hydroxy group to involucrin (48-50). Filaggrin is a known component of the cornified envelope and since these lipids are chemically linked to the cornified envelope, analysis of the level and chain length of bound lipids may be important in understanding the relationship between the barrier defect in AD skin and filaggrin.

Additionally, inflammation has been shown to be a key contributing factor to the changes in expression of these enzymes. in vitro studies which examine the effect of other upregulated cytokines (individually or in synergy) in AD on CER and FFA synthesis enzymes can provide more insight into the interplay between inflammation and SC lipid synthesis in AD skin.

Apart from analyzing the factors that contribute to the barrier of the skin in AD e.g. SC lipids, it is also important to measure the overall quality of the barrier in developed AD-HSEs in relation to control HSEs and native human skin. Studies analyzing TEWL in HSEs in vitro and permeation studies using model drugs are further endpoints that can be used to characterize AD-HSEs.

AD-HSEs can potentially be used to screen formulations that can contribute to barrier repair in AD skin. CER and FFA synthesis enzymes are known to be regulated by various factors including hormones, temperature, pH, presence of other lipids etc. (51, 52). Formulations containing components which can directly promote the activity of CER and FFA synthesis enzymes or create an environment to stimulate these enzymes could be a promising approach to investigate as a treatment for AD.

Improving the SC lipid properties of human skin equivalents

HSEs have been very useful in broadening our understanding of the pathogenesis in AD. However, limitations exist in their uses to mimic the barrier properties of human skin in vitro. Studies from chapter 4 and 5 reveal that Ex-HSEs do not maintain the exact lipid composition and organization in the original biopsy. HSEs show a more hexagonal lipid organization, less FFA and increased mono-unsaturated FFAs (MUFAs) (53, 54). in vitro studies have shown that an increased level of MUFAs can contribute to a more hexagonal
lipid organization (55). The expression of SCD-1 which synthesize MUFAs is increased in HSEs. Therefore inhibition of SCD-1 may result in a reduced level of mono-unsaturated FFAs and lead to a more orthorhombic lateral organization in HSEs. Thyroid hormone, leptin, glucagon, thiazolidinediones are known to repress the expression of SCD-1 and may be introduced in an optimized amount into the culture medium to regulate SCD-1 expression (51).

A temperature gradient exists in the skin between the hypodermis and the SC as the skin surface temperature is 32°C. Since enzyme activity and expression is known to be also temperature dependent, the gradient in temperature may be necessary for the proper function of enzymes involved in epidermal differentiation and lipid synthesis. In vitro culture procedures which can provide such a gradient at the time point where the HSEs are just fully differentiated may support the development of HSEs that fully resemble human skin barrier properties. This could be achieved by culturing HSEs at 37°C from the point of seeding of keratinocytes to air exposure. After 7 days of air exposure where a thin layer of SC has been formed, the HSEs can be cultured in conditions were the culture medium is maintained at 37°C and the surrounding temperature set at room temperature in order to mimic conditions in vivo. Microfluidic systems can also be applied in the culture of HSEs as they can provide a small and complex environment which mimics the in vivo environment for cells.

The humidity during the culture of HSEs (95% humidity) is much higher than what native human skin is exposed to. This may hinder the formation of an intact barrier in HSEs in comparison to human skin and optimization studies that investigate the effect of various levels of reduced humidity are useful to perform. Other factors that may influence the barrier include exposure to daylight and development of the dermal microenvironment that includes immune regulatory cells as seen in vivo. The dermal immune regulatory cells may assist in reversing the activated state of the epidermis in HSEs which is shown by expression of activation associated markers such as Keratin 6, 16 and 17 (53). The introduction of a combination of these factors mentioned above in the development of HSEs may lead to HSEs with similar barrier properties as native human skin.
References

Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin

CHAPTER 8

Nederlandse samenvatting
In constitutioneel eczeem (Atopic Dermatitis, AD) is de huidbarrière aangetast in zowel aangedane als niet-aangedane huid. Een defecte barrière wordt gekenmerkt door verhoogd trans-epidermaal waterverlies (TEWV), verhoogde permeabiliteit en verminderde hydratatie van de buitenste laag van de huid, de hoornlaag, welke de barrière vormt (1-4). De hoornlaag bestaat uit een gelaagde structuur van afgeplatte corneocyten omringd door een intercellulaire lipidematrix. Deze lipiden vormen het grensvlak tussen de hydrofiele corneocyten en de hydrofobe lipidematrix.

De structuur van de hoornlaag wordt vaak vergeleken met de structuur van een stenen muur, waarbij de corneocyten de bakstenen zijn en de lipiden het cement (5). De lipidematrix bestaat voornamelijk uit ceramiden (CERs), cholesterol (CHOL) en vrije vetzuren (FFAs), welke in equimolaire ratios aanwezig zijn (6-8). Verreweg de meeste stoffen komen via de lipidematrix de hoornlaag binnen; vandaar dat de organisatie en samenstelling van deze matrix cruciaal is voor de barrièrefunctie (9-10). De lipiden in de hoornlaag zijn georganiseerd als lamellen, in een repeterend patroon met een interval van afwisselend 13 en 6 nm tussenruimte. Deze worden respectievelijk de lange periodieke fase (long periodicity phase, LPP) en de korte periodieke fase (short periodicity phase, SPP) genoemd (11-13). Binnen deze lamellen vormen de lipiden een geordende structuur: de zogenoemde laterale organisatie. In de menselijke hoornlaag zijn de lipiden voornamelijk in een orthorombische structuur (met hogere dichtheid) geordend, terwijl een klein deel van de lipiden een hexagonale ordening (met lagere dichtheid) vormen (14-18).

In de hoornlaag van AD patiënten zijn verschillende afwijkingen in de lipidensamenstelling gevonden. Deze afwijkingen zijn onder andere: verlaging van de CERs/CHOL verhouding, kortere lipidenketens, verhoogde hoeveelheid onverzadigde vrije vetzuren en een verlaagde hoeveelheid hydroxy-vetzuren. Een aantal van deze afwijkingen zijn gecorreleerd aan verhoogd TEWV (19-25). Dit bevestigt dat de lipiden belangrijk zijn voor de barrièrefunctie van de huid.

AD is een complexe chronische recidiverende huidontstekingsziekte. Er zijn verschillende afwijkingen beschreven die allen een invloed hebben op de kans dat AD zich ontwikkeld en de ernst en mate waarin AD zich manifesteert. Voorbeelden zijn fillagrine- (FLG) genmutaties, verstoring van de huidbarrière, afwijkende microbiële kolonisatie van de huid, en verhoogde activatie van (TH1/TH2/TH17/TH22) onstekingsroutes (26-28). AD wordt ook geassocieerd met een verhoogd risico op andere allergische aandoeningen, zoals asthma, hooikoorts en voedsellergieën (29). Algemeen wordt aangenomen dat een combinatie van een verminderde huidbarrièrefunctie en een verhoogde gevoeligheid van de huid voor ontstekingen (verhoogde immuunrespons) een significante rol kunnen spelen bij de ontwikkeling van AD (2,30,31).

De huid van AD patiënten typeert zich door een verhoogde aanwezigheid van cytokines zoals interleukine (IL) 4, IL-5 en IL-13 (zogenaamde T\textsubscript{H}2 cytokines). Zodra de ziekte chronisch wordt, vindt een verhoogde productie van cytokines zoals IL-12 en interferon (IFN)-γ plaats (zogenaamde T\textsubscript{H}1 cytokines) (32). Cytokines hebben invloed op de enzymen die een rol spelen bij de biosynthese van de lipiden in de huid. Dit resulteert in een afwijkende lipidensamenstelling in de hoornlaag (33,34).

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Development of human skin equivalents to unravel the impaired skin barrier in atopic dermatitis skin
Muismodellen zijn dusver waardevol geweest om de mechanismes van AD-pathogenese te ontrafelen. On- 
danks het feit dat deze modellen geschikt zijn voor het onderzoeken van de biochemische mechanismes is 
de vertaalslag naar AD in mensen lastig te maken, omdat de huidstructuur van muizen en mensen veel van 
elkaar verschillen (35, 36). Gekweekte humane huid, zoals humane huid equivalenten (HHEs), vormt een 
goed alternatief om AD-pathogenese te onderzoeken en mogelijke nieuwe therapeutische aangrijpings- 
punten te ontdekken.
Doel

Het doel van het onderzoek beschreven in dit proefschrift is het reproduceerbaar ontwikkelen van HHEs die AD-kenmerken nabootsen. Vervolgens kan met deze HHEs het effect van een ontsteking op de kantertistieke eigenschappen van de huidbarrière onderzocht worden. Echter, om dit tot stand te brengen, is eerst de biosynthese van lipiden in aangedane en niet-aangedane huid van AD patiënten onderzocht.

In het onderzoek beschreven in dit proefschrift staan de volgende vragen centraal:
1) Bestaat er een verband tussen de expressie van een aantal geselecteerde enzymen die een rol spelen in de lipidenbiosynthese in de huid en de lipidensamenstelling in de hoornlaag van AD patiënten?
2) Speelt een ontsteking in de huid een rol bij de veranderingen in epidermale morfogenese, differentiatie en lipideneigenschappen in aangedane AD-huid?
3) Kunnen de epidermale eigenschappen van AD huid behouden blijven in *in vitro* AD-HHEs, gemaakt op basis van AD huid biopaten?
4) Is het mogelijk om een HHE voor barrièreherstel te ontwikkelen dat verschillende aspecten van de barrière van AD-huid nabootst?

Ontsteking, enzymexpressie van de lipidebiosynthese en de SC lipide-eigenschappen in AD

Door gebruik te maken van HHEs is het mogelijk om effecten van ontstekingscytokines te onderzoeken op de huidbarrièrefunctie (37-40). Dit is van belang omdat het nog niet bekend is welke invloed verhoogde concentraties chemokines en cytokines hebben op i) de lipidensynthese in de huid en ii) de samenstelling en organisatie van de lipiden in het SC. De veranderingen in de lipidensamenstelling komen waarschijnlijk door een verandering in expressie of activiteit van één of meerdere van deze enzymen. We focuseren ons hierbij voornamelijk op de enzymen ß-glucocerebrosidase (GBA), acid-sphingomyelinase (aSmase), CER synthase 3 (CerS3), stearoyl-CoA desaturase (SCD), Elongase1 (ELOVL1) en Elongase6 (ELOVL6).

GBA en aSmase katalyseren de laatste stap in de CER-synthese van respectievelijk glucosylceramides en sphingomyelines naar ceramides. ELOVL1 verlengt vrije vetzuren met een koolstofketen van C20 tot C26, ELOVL4 verlengt de vrije vetzuren die langer zijn dan C26 (42-45). CerS3 koppelt de zeer lange FFA ketens (langer dan C26) aan een sphingosinebase tijdens de synthese van de CERs (34). Daarnaast is ELOVL6 verantwoordelijk voor de verlenging van vetzuren met een koolstofketen van C16 en C18. SCD zorgt dat verzadigde vetzuren met een ketenlengte van 16 en 18 C atomen omgezet worden in onverzadigde vetzuren(46).

In de studies beschreven in hoofdstuk 2 is onderzocht of er een verandering is in de expressie van de enzymen SCD, ELOVL1, ELOVL6, CerS3, GBA en aSmase alsmede een verandering in lipidensamenstelling in aangedane en niet-aangedane huid van AD patiënten. De expressie van deze enzymen en de lipidensamenstelling in AD patiënten is vergeleken met een controlegroep. De SCD-expressie is verhoogd in de aan-
gedane huid en dit gaat gepaard met een significante verhoging van de hoeveelheid mono-onverzadigde vrije vetzuren. De expressie van een tweede enzym, ELOVL1, is verlaagd vergeleken met die in de controle huid en niet-aangedane huid. In dezelfde patiënten werd een reductie in de hoeveelheden vrije vetzuren met een C22- tot C28-koolstofketen waargenomen. Een vergelijkbare waarneming is gedaan voor de ultra-lange vrije vetzuren (ketenlengte groter dan 26 koolstofatomen). Deze vetzuren worden verlengd door ELOVL4; de hoeveelheid ultra-lange FFAs was significant gereduceerd in door AD aangedane huid vergeleken met de controle huid. Er was geen verschil in de expressie van ELOVL6 tussen de AD en controle huid.

De resultaten beschreven in hoofdstuk 2 samen met eerder gepubliceerde studies geven een goed inzicht in enzymatische en lipiden afwijkingen in huid van AD patiënten. In de in hoofdstuk 3 beschreven studies zijn, met behulp van het Leiden epidermale model (LEM), de directe effecten van de verhoogde cytokines, een aspect van huid inflammatie, op de epidermale morfogenese, differentiatie en SC lipidensamenstelling onderzocht. Uit deze studies blijkt dat cytokines (TNF-α, IL-4, IL-13 en IL-31), waarvan de concentraties in de epidermis verhoogd zijn in aangedane huid van AD patiënten, de epidermale morfogenese, differentiatie en SC lipidensamenstelling beïnvloeden. TNF-α en T₄₂ cytokines reduceren de expressie van keratine 10 (K10), fillagrine en loricrine in de LEMs. Deze cytokines hebben tevens effect op thymisch stromaal lymphopoietine (TSLP) secretie door keratinocyten, welke hierdoor een verhoogde proliferatie hebben. Uit de analyses van de lipidensamenstelling blijkt dat TNF-α, met of zonder T₄₂ cytokines, de expressie van ELOVL1 en de hoeveelheid verzadigde lange FFAs (≥ C20:0) verlaagt.

Toevoeging van IL-31 en TNF-α aan het medium beinvloedt ook de ceramide synthese. De verhouding van veresterde ω-hydroxy (EO) CERs (ceramides met zeer lange vetzuurketens, waaraan een linoleate veresterd is) ten opzichte van niet-EO ceramides (de ceramides met een normale structuur) is verlaagd. De verlaagde expressie van CerS3 zal waarschijnlijk bijdragen aan deze verlaagde verhouding. CerS3 is een enzym dat in staat is deze EO CERs te synthetiseren. De verandering in de CER-samenstellingen ging gepaard met een kortere repetitieafstand van LPP. Deze aandt is korter in HHEs gegenereerd met T₄₂ cytokines in het medium. Uit de studies beschreven in hoofdstuk 2 kan geconcludeerd worden dat i) ontsteking de oorzaak kan zijn van belangrijke afwijkingen in aangedane huid van AD patiënten, en ii) de toevoeging van cytokines aan de LEMs een uitstekend model is voor het testen van nieuwe medicatie of formuleringen voor de behandeling van AD.

**In vitro AD model door middel van gebruik van AD-huid explantaten**

AD-huid biopaten kunnen gebruikt worden als een alternatieve manier om AD-HHEs te genereren; als alternatief voor HHEs gegenereerd uit geïsoleerde primaire keratinocyten. Dit onderzoek is beschreven in hoofdstuk 3. Voor deze techniek wordt gebruik gemaakt van biopten met een diameter van 4 mm. Dit biopt wordt aangebracht op een collageenmatrix met fibroblasten (dit compartiment kan vergeleken worden met de dermis). In kweek zal deze epidermis lateraal uitgroeien. Dit vormt dan de 1e generatie van explant humane huid equivalent (Ex-HHEs). Deze techniek kan gebruikt worden voor analyses waarvoor meer weefsel nodig is dan normaliter uit patiëntenmateriaal verkregen wordt, zoals voor eiwitbepalingen, of voor onderzoek naar eigenschappen van de lipidenmatrix in de hoornlaag. Deze techniek kan uitkomst
bieden om de afname van weefsel van patiënten met huidziektes voor onderzoek te minimaliseren. De 1e generatie explant kan gebruikt worden om vervolgens 2e en een 3e generatie Ex-HHEs te maken. De 2e generatie kan worden gemaakt door een biopt van de 1e generatie op een nieuw dermaal compartiment te plaatsen. Dezelfde aanpak wordt gebruikt voor het maken van de 3e generatie Ex-HSE.

De resultaten van hoofdstuk 4 laten zien dat de morfologie van de 2e en 3e generatie Ex-HSEs vergelijkbaar is met die van de 1e generatie. Echter, sommige lipideneigenschappen verschillen, vooral wanneer de 1e Ex-HHEs vergeleken wordt met natieve huid. Ook de expressie van differentiatiemarkers, K10, loricrine en fillagrine, is vergelijkbaar met die van de natieve huid en de 1e generatie Ex-HHEs. In zekere mate, zijn ook de lipideneigenschappen van de 2e en 3e generatie Ex-HSEs vergelijkbaar met die van de 1e generatie Ex-HHE en natieve huid. De belangrijkste verschillen zijn een verhoogde hoeveelheid korte vetzuren, de hexagonale laterale structuur en een verhoging van de EOS CERs. De uitgroei van de huidbiopten naar de 2e generatie en 3e generatie Ex-HSEs kan een veelbelovende methode zijn voor het genereren van waardevol materiaal, echter voor het bestuderen van lipideneigenschappen hebben Ex-HHEs beperkingen.

De hierboven beschreven techniek is ook gebruikt voor het genereren van Ex-HHEs met biopten afkomstig van niet-aangedane huid van AD-patiënten, met en zonder homozygotische FLG-mutatie. Deze AD Ex-HSEs zijn onderzocht om te bepalen of de epidermale eigenschappen van AD-patiënten nog aanwezig zijn en tevens om te bepalen of FLG mutaties een rol spelen in epidermale morfogenese. De resultaten beschreven in hoofdstuk 5 tonen aan dat de expressie van de epidermale differentiatie-eiwitten (fillagrine, involucrine, kallikrein 5 en LEKTI) in de Ex-HHEs van AD-biopten onveranderd is. Hoewel ook de loricrine-expressie in Ex-HSEs onveranderd was, bleek deze verlaagd te zijn in zowel AD biopten met een homozygotische FLG mutatie als ook de corresponderende Ex-HSEs. Deze studie laat zien dat de in vivo FLG mutatie-eigenschappen in het genotype en fenotype behouden blijven in het in vitro model, de Ex-HHEs. Deze kennis kan bijdragen aan de ontwikkeling van een weefselmodel om de rol van FLG in AD of FLG-hersteltherapieën te onderzoeken. Zo’n model zou waardevol zijn om mogelijke nieuwe therapieën, zoals FLG-vervanging, te testen en te evalueren.

Een ex vivo model voor barrièreherstel van de huid
Momenteel zijn er geen geschikte in vitro modellen beschikbaar voor het onderzoeken van huidbarrièreherstel geïnduceerd door topicaal opgebrachte farmaceutisch en/of formuleringen. In het onderzoek beschreven in hoofdstuk 6 wordt een humaan ex vivo huidmodel geïntroduceerd dat gebruikt kan worden om huidbarrièreherstel te meten. Door middel van cyanoacrylata strips wordt het SC van de ex vivo humaan huid verwijderd. Vervolgens wordt de gestripte huid in kweek gebracht bij 37°C en 32°C. Na 8 dagen kweken kan de morfologie van de epidermis, de expressie van differentiatie markers alsmede de lipidensamenstelling en organisatie in het SC worden onderzocht. Uit de resultaten is gebleken dat de gestripte huid een actief prolifererende en differentiërende epidermis heeft, wat resulteert in het genereren van het SC in vitro. De lipiden in het geregenereerde SC vormen voornamelijk een hexagonale laterale packing. Dit is afwijkend van de dominante orthorhombische packing in natieve humaan SC. De absolute hoeveelheid van CERs in het in vitro geregenereerde SC zijn ook verhoogd ten opzichte van de andere lipiden. De absolute
hoeveelheid CERs NS en CER AS/NH is ook verhoogd in het in vitro geregenereerde SC gekweekt bij of 37°C of 32°C. Hieruit kan geconcludeerd worden dat de bovengenoemde karakteristieken van SC lipiden van de gestripte en gekweekte huid de lipidensamenstelling van huid van AD betreffende een aantal aspecten nabootsen (23, 24, 47). Daarom kan dit model mogelijk in de toekomst gebruikt worden om formuleringen te testen die als doel hebben het barrièreherstel te versnellen.
Conclusie

De studies in dit proefschrift zijn gefocussed op de verminderde barrièrefunctie van huid van AD. HHEs zijn gebruikt om de rol van vele factoren in de pathogenese van AD-huid beter te kunnen begrijpen.

We laten zien dat ontsteking een centrale rol speelt in de ontwikkeling van epidermale en SC-kenmerken van AD-huid. De AD epidermale kenmerken in vitro persisteren wanneer AD-huidbiopten gebruikt worden om Ex-HSEs te ontwikkelen. Deze AD-HSEs kunnen vervolgens als model dienen om mogelijke therapieën voor AD en barrièreherstel van de huid te screenen.


List of publications


Curriculum Vitae

Mogbekeloluwa Danso-Eweje was born on the 9th of September 1988 in Warri, Nigeria.

After graduating from Christ the Redeemers College, Sagamu in 2004 she started her A-levels at Oxbridge tutorial College in Lagos. In 2005, she commenced her Bachelor studies in Biomedical science at the University of Sheffield in England and completed it with Honors in 2008. At the same university she also pursued her Master’s degree in stem cell and regenerative medicine and graduated with Distinction in 2009. Her master’s internship focused on the role of fat secreted factors in the differentiation of mesenchymal stem cells under the supervision of Dr. Andy Scott.

In 2010, she started her PhD project under the supervision of Prof. Dr. J.A. Bouwstra and Dr. A. El Ghalbzouri at the division of Drug delivery technology at the Leiden academic center for drug research, Leiden University, the Netherlands. The results of this PhD project are described in this thesis.

She currently works as a scientist at Galapagos N.V, Leiden, a clinical-stage biotechnology company located in the Leiden bio-science park.
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