Lipids as therapeutic targets for barrier repair in skin diseases

Boiten, W.A.

Chapter 1

General introduction and aims

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1.1 How the skin functions as a barrier

Covering our entire body, the skin is the largest human organ and one of the main protective barriers against the outside world. It serves this protective role by forming three distinct layers. The most inner layer is the hypodermis; it consists of fatty adipose tissue and provides insulation and cushioning[1]. The middle layer is called the dermis. Consisting of a fibrous network, the dermis gives mechanical strength to the skin. However, being enervated and vascularized the dermis also has a nutritional and an important immunological function. It holds dendritic cells, mast cells, and a large population of T-cells[2].

The outer layer of the skin is the epidermis, which in turn consists of four different layers. Its main role is to form a physical barrier protecting the body from pathogens, allergens, and irritants, but also from excessive water loss and subsequent desiccation. Formation of the barrier starts at the basal layer, which is a single layer of proliferating keratinocytes. Moving outwards, keratinocytes undergo a terminal differentiation process called cornification[3,4]. From the start of this process complex lipids mixtures are being synthesized and stored in specific organelles called lamellar bodies (LBs). Cells containing these LBs form the granular layer. This is the most outer viable layer because cornification is a terminal differentiation process resulting in the “dead” corneocytes. These cells neither have organelles nor a cellular membrane[3]. In the last stages of differentiation the cellular membrane is replaced by a crosslinked protein structure called the cornified envelop (CE). It is mainly composed of involucrin, loricrin, periplakinand, and envoplakin and construction starts at the cytosolic side of the plasma membrane[5]. At the interface between the granular layer and the corneocytes, the lamellar bodies are excreted into the intercellular space after which its lipid content becomes part of the final barrier.

The outer layer of the epidermis is called the stratum corneum (SC) and forms a physical barrier composed of lipids and corneocytes, which can be represented as a brick and mortar wall, respectively. Corneocytes are connected by a specific cell-cell adhesion called

corneodesmosomes[6]. Although the SC seems inert, numerous enzymatic processes occur. One process driven by enzymatic degradation is desquamation, which is the active shedding of the outer SC layers[7]. In this process the corneodesmosomes are gradually degraded thereby detaching the corneocytes[4]. Within the intercellular space of the corneocytes the lipids form a highly organized structure and are a crucial part of the barrier. In the next section this essential role of the lipids will be further discussed.

1.2 Lipids form the SC its permeability barrier

1.2.1 From precursor to SC lipid

In the human SC three main classes of intercellular lipids occur: ceramides, free fatty acids, and sterols[8]. Yet, it is not excluded that there are minor fractions of phospholipids, squalene, wax-esters and cholesterol esters[9]. However, this minor lipid fraction could originate from sebum lipids. **Figure 1.1** shows an overview of the chemicals structures of the three main lipid classes. Different weight ratios of the lipid classes and different ratios per skin sites have been observed[10,11]. SC lipids synthesises starts at the deeper epidermal layers. *De novo* ceramide synthesis begins at the endoplasmic reticulum by condensation of a serine with a palmitoyl-COA. By multiple enzymatic steps the product is converted to a dihydrosphingosine[12]. To produce a dihydroceramide, acylation with an acyl-COA is performed by one of a group of n-acyltransferases called ceramide synthases (CerS). In human epidermis alpha-hydroxylated and omega-hydroxylated acyl groups become attached as well[13]. Six different CerS are known and each CerS has affinity for specific acyl-COAs with different chain lengths[14]. There are two enzymes known that further modify dihydroceramides. These are dihydroceramide desaturatare 1 (DES1) and dihydroceramide C-4 hydroxylase (DES2) which coverted dihyrdoceramides to ceramides or phytoceramides[15], respectively. The enzyme responsible for production of hydroxy-sphingosine containing ceramides is unknown. After synthesis ceramides are directly glycosylated to glucosylceramides (GluCers) or phosphocholinated to sphingomyelin. This inactivates ceramides as they are bioactive lipids[16,17]. Fatty acids are phosphorylated or converted to triglycerides. These precursor lipids and the enzymes to convert them are stored in the LBs[18]. At the interface between the viable epidermis and the SC the LBs release the precursor lipids and converting enzymes together into the extracellular space[19,20]. Here, the glucose and phosphocholine are detached form the ceramides.

1.2.2 Ceramides

Of all three SC lipid classes, ceramides have the most diverse chemical structure. Different sphingoid bases can be chemical linked to different acyl chains. **Figure 1.1** gives an overview of the subclasses. Four acyl groups have been identified: non-hydroxylated (N), alpha-hydroxylated (A), and omega-hydroxylated (O). The omega-hydroxylated acyl group can be further modified with an esterified linoleate moiety (EO), although other long chain fatty acids have been observed as well[21]. Five different sphingoid bases have been observed: dihydrosphingosine (dS), sphingosine (S), phytosphingosine (P), hydroxysphingosine (H), and a recently discovered two hydroxysphingosine (T)[22]. Together, they can form 20 different ceramide subclasses. They are named according to Motta et al.[23]. It combines the letters identifying the chemical composition of the subclass (i.e. NS = non-hydroxylated acyl coupled to sphingosine). Also a group of ceramides with a linoleate attached to the sphingoid base called 1-O-acyl ceramides have been observed in SC[24]. In human SC the ceramide its sphingoid base chain length can vary between 16 and 28 carbons and the acyl chain can vary from 16 till 34 carbons. However, >28 carbons acyl chains are most common in O and EO chains. The total carbon chain length is indicated after the subclass abbreviation (i.e. NS C44). Odd

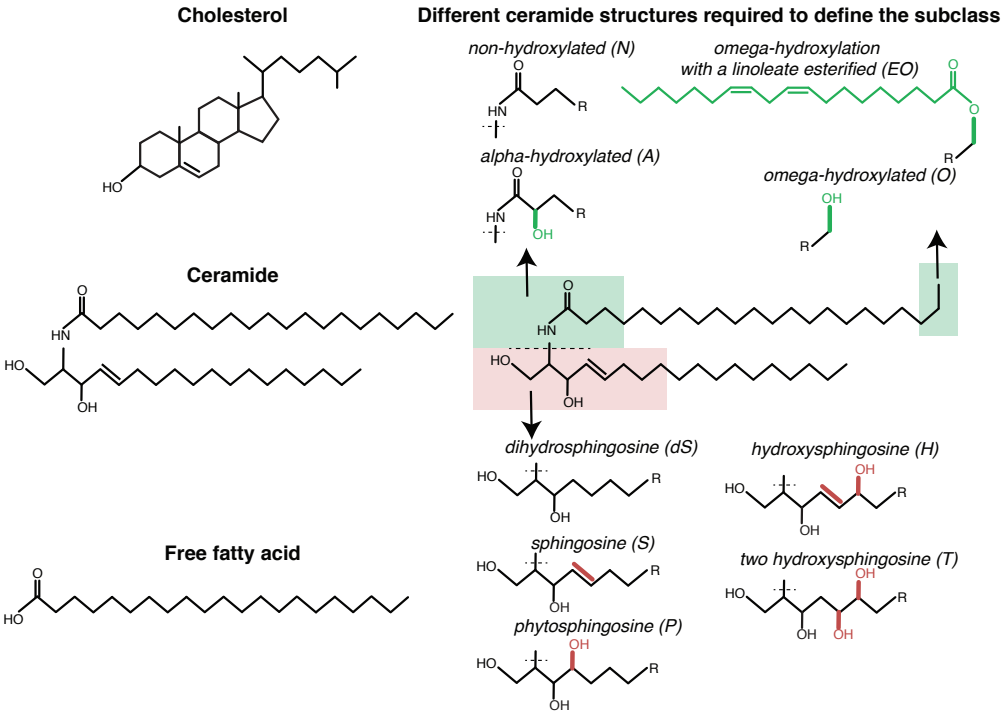

Figure 1.1: *The chemical structures of the three main different SC lipids classes. For the ceramides the different structure of both the acyl-chain and the sphingoid base defining the different subclasses are shown.*

chain lengths and desaturation of the acyl chain are observed in human SC ceramides as well[25]. The total numbers of structurally different ceramides reported ranges from 342 till >1000[22,26]. Yet, when taking both chain length distributions and subclasses into account, more than 5000 different chemical structures are expected. Ceramides will be the main lipid focused of the research described in this thesis.

1.2.3 Free fatty acids and cholesterol

Although not as numerous as the ceramides, the free fatty acids (FFA) in the human SC have a wide variety of chemical structures as well. These carboxylic acids have a chain length distribution reaching from 16 till 34 carbons. Both alpha- and omega-hydroxylated FFA have been observed[27]. As with the ceramides odd number chains lengths and desaturation of the carbon chain can occur. SC fatty acids are longer and more heterogeneous than those in other tissues[28]. The last lipid class is the sterols, consisting mostly of cholesterol and a small fraction of cholesterol sulfate. The biosynthetic precursor of the sterols, squalene, is also present in minor amount. It is though that the observed squalene originates form the sebum lipids, as do the wax esters[29].

1.2.4 Bound lipids

When the precursor lipids are released at the viable epidermis SC interface, part of the lipids becomes covalently attached forming the cornified lipid envelope (CLE)[5]. These lipids are also referred to as the bound lipids and server as an interface between the hydrophilic corneocytes and hydrophobic extracellular lipids matrix. Only a specific group of lipids containing an omega-hydroxyl group is bound. To become bound it is required that they are glycosylated and have esterified linoleate omega-hydroxyl group. Previous studies have shown that the two conjugated double bounds of the linoleate are modified to a hydroxyl-epoxide in two steps[30]. Than the altered linoleate moiety is removed and the GluCer bound to the CE[31]. Lastly, the glucose is removed. It is hypothesized that bound ceramides are converted by ceramidases to bound omega-hydroxyl fatty acids[32,33]. Changes in the bound lipid fraction have also been linked to an altered barrier function[34]. The bound lipids can act as a scaffold for the unbound lipids, arranging the orientation of the lipid matrix and thus have an important function in the SC.

1.2.5 Lipid organization and barrier

Unlike the cell membrane, the extracellular SC lipids form a densely packed lipid barrier. When examining the lateral organization of these lipids, a large fraction is packed into a dense orthorhombic structure and a smaller fraction into a less dense hexagonal structure[35]. The tightness of this packing is important for the barrier function of the SC[36,37]. Perpendicular to the lateral organization, the SC lipids are organized into lamellar stacks. Two different sizes of these lamellar stacks have been observed in human SC. These have repeat distance of 6 or 13 nm andw are called the short and long periodicity phase, respectively[38]. Ceramides with an EO group are essential for formation of the long periodicity phase[39] and for proper barrier function[40]. Changes in the SC lipid composition can affect the lateral and lamellar organization, consequently affecting the barrier function[41,42]. Alterations in the SC lipid composition and organization play a role in skin diseases which is further described in the next section.

1.3 Stratum corneum lipids are a potential target for atopic dermatitis treatment

1.3.1 Atopic dermatitis and inflammation

Atopic dermatitis (AD) is heterogeneous skin disease characterized by lesional skin sites with erythroderma, xerosis, scaling skin[43]. Although mostly seen as a children's disease (observed in 10-25% of the western pediatric population), AD also has a high prevalence under adults of around 7-10%[43-45]. The skin of AD patients exhibits a decreased skin barrier and cutaneous inflammation at both the lesional but also at non-lesional skin sites. Both chronic and acute skin inflammation (flares) occurs. The decreased barrier function is put at the center of AD initiation. Scratching or penetration of endogenous compounds can triggered a release of cytokines, like thymic stromal lymphopoietin (TSLP), by the keratinocytes. These cytokines in-turn activates T helper 2 cells (Th2) which produce interleukin (IL) 4 and 13. These are characteristic inflammatory markers observed in AD lesions and can perpetuate the inflammation by activation of other immune cells and keratinocytes. Originally AD was seen as an atopic disease with typical increased levels of allergen specific IgE. Yet, AD can also occur without increased IgE levels[46]. There are many different immunological paths involved in AD[47-49]. The clinical effects of this skin inflammation are erythroderma, swelling, and pruritus but can also lead to a decreased skin barrier. For instance, IL4 and IL13 decrease the production of and important epidermal protein filaggrin[50].

Filaggrin production starts at the lower epidermis where pro-filaggrin is synthesized and stored in keratohyalin granules. Pro-filaggrin is phosphorylated polymer of 10-12 filaggrin monomers. When the keratohyalin granules are secreted

pro-filaggrin is cleaved into monomers. Filaggrin has multiple roles in the skin barrier. The monomers have a stabilizing effect on keratin filaments and are part of the cornified envelope[4,6]. When filaggrin detaches it is proteolysed into amino acids which are important for acidification and hydration of the SC[51]. Loss of function mutations of the filaggrin gene are known risk factors for AD[52], yet, do not necessarily cause AD[53,54]. Homozygous loss of function causes a dry scaling skin disorder called ichthyosis vulgaris[43]. With its important role in the SC, decreases in filaggrin can play a role in barrier dysfunction. As filaggrin also becomes down regulated by inflammation it plays a role in the continuation of chronical lesions. Inflammation can also decrease skin barrier function by deregulating another important part of the SC barrier, the SC lipids[55].

1.3.2 Stratum corneum lipids and skin barrier function in Atopic dermatitis

A hallmark of AD is an increased transepidermal water loss (TEWL). The TEWL is used as a measure for the stratum corneum its barrier function. The main barrier for water penetration is formed by the SC lipids[56] and increased ceramide content can decrease TEWL[57]. The Increased TEWL of AD patients is an indication that the permeability barrier of the SC lipids is affected. Multiple studies examined the changes in SC lipids of AD patients focusing on the three main lipid classes[37,58-61]. Using thin layer chromatography decreased total ceramide amounts were observed[58,61]. With a detailed analysis using liquid chromatography (LC) coupled with mass spectrometry (MS) ceramides specific compositional changes were reported[60,62,63] like:

1. An increased amounts of ceramides with a total chain carbons length of 34;
2. A decreased mean carbon chain length;
3. Increased amounts of ceramides with sphingoid (S) base;
4. Decreased amounts of EO ceramides;
5. Decreased amounts of phytosphingoid (P) ceramides.

Next to changes in ceramides, the FFA composition of AD patients was affected as well. Increased amounts of unsaturated fatty acids were observed and the mean chain length decreased[59].

Interestingly, the above described alteration in lipid composition were already observed in non-lesional skin, but were greater at lesional sites. Compositional changes correlated to an alter lipid organization and increased TEWL[59]. Changes in lipid composition of model membrane mimicking the SC lipid matrix gave similar changes in organization, supporting the relation between lipid composition and organization[64,65]. That lipid alteration and the subsequent changes in lipid organization already occur in SC of non-lesional skin, shows that healthy appearing skin sites of AD patients already

have a compromised permeability barrier. Restoring the lipid organization by improving the lipid composition and thereby decreasing the barrier defect in AD patients can be seen as viable treatment strategy[66,67].

1.3.3 Treatments of atopic dramatis and barrier repair formulations

As both barrier defects and inflammation are part of the AD pathology, treatment focuses on both. Inflammation is generally countered by using immunosuppressants[68], which are prescribed to counter flares and reduce itching. As indicated above, another treatment strategy for AD is barrier repair or barrier replacement. Mineral oils derivatives and other emollients are commonly prescribed as occlusive agents who can enhance skin hydration[69,70]. Despite that these treatments alleviate xerosis and form an occlusive barrier; they do not actively restore the SC barrier lipids in AD patients. A specific group of formulations aims at restoring the skin barrier by the addition of SC lipids to the formulations, most often ceramides[71,72]. It has been shown that the composition and ratios between the lipids in the formulation is important for short term effects[73,74]. In patients with mild AD, ceramide-containing formulations have shown similar clinical effects as emollients[75,76]. It is hypothesized that by diffusion the formulated lipids can enter the lipid matrix. FFAs can diffuse into the viable epidermis and ceramides remain on the skin for two days in *ex vivo* conditions[77]. Others have shown minor penetration of ceramides[78]. However, treatments with emollient are more long term and a reduced barrier would enhance the absorption of barrier lipids.

A potential barrier-restoring formulation is a mimic of the naturally occurring vernix caseosa (VC). This is the “cheesy” (caseosa in Latin) “varnish” (vernix in Latin) on preterm babies. There it forms a protective layer in addition to the pretermal SC[79]. Studies of the VC had shown that it contains wax esters, triglycerides, squalene, and a selection of SC lipid and has barrier properties of its own[80,81]. Furthermore, a synthetic VC mimicking formulation showed to be more effective than petrolatum in the barrier recovery of mice[82]. Besides being able to deliver barrier lipids, the VC is also a permeable barrier. Having the right balance between occlusiveness and permeability is important for a formulation as excessive water retention and disruption of the calcium gradient can disrupt the proper cornification. The optimal composition of a VC formulation and how human skin acts after VC formulation application are not well known.

1.4 Analyzing the stratum corneum lipids and barrier

1.4.1 Lipidomics and stratum corneum ceramides

Lipidomics is the study of all lipids in a biological matrix, the lipidome. Due to the lipids changes in chain length and head group architecture, the lipidome can consists of very large numbers of different chemical structures[83]. There are two different approaches in lipidomics, targeted and non-targeted. Non-targeted lipidomics aims at identifying as many lipid species in a qualitative manner and targeted lipidomics aims at accurately quantifying a selective group of lipids.

A powerful tool to analyze the lipidome is with MS, which allows separation of the lipids by their mass charge ratio (m/z). When combined with LC further separation can be achieved. Depending on the type of column used in for LC, lipids can be separated by chain length or hydrophilic head group. In non-target lipidomics, MS/MS fragmentation is applied to ascertain the required structural information for identification of large number of lipids within one measurement[84]. With highly validated methods retention time and mass can be used to predict chain length differences for the same lipid class, yet, fragmentation will always be required for identification of unknown compound. For quantitative analysis in targeted lipidomics information is required on the sensitivity of the MS response to the analyte. This requires chemical standards to determine this response sensitivity, severely limiting the number of compounds that can be quantified in a sample. The data resulting from lipidomics can be challenging, due to the large number of compounds in a sample. Advanced statistical tools are required to compare the lipidome between different experimental conditions[85]. One method to reduce the number of variables in the comparison is principal component analysis. This is a form of dimensional reduction of variables to principle components based on the amount of variation between samples.

Lipidomics is an excellent tool to analyze the SC ceramides[86-88]. Due to the large number of different ceramide species and only a limited number of standards, comprehensive quantification of all SC ceramides becomes challenging[89]. Especially, when taking into account that different experimental conditions might change the occurring lipid structures. Then, comprehensive analysis requires a method which combines both qualitative and quantitative analysis. The second chapter of this thesis will demonstrated that by modeling a MS response factor using the chemical structure of the ceramide, these challenges can be tackled. Further challenges are present in validating the sampling and extraction of the SC from the samples. Using lipidomics to acquire an accurate measurement of the SC lipid composition can improve the understanding of the relation between lipid composition and lipid organization.

1.4.2 Analysis of the lipids organization

As the lipid organization is instrumental for a proper barrier function, examining the lipid matrix is detrimental. A powerful tool to gain information about the lateral organization of the lipids is Fourier transformed infrared spectroscopy (FTIR). This can be used to measure the absorption of wavelengths corresponding to the movement in the hydrogen-carbon bonds of lipid carbon tails. In this thesis we focus on the symmetric CH_2 stretching peak position and the CH_2 scissoring region[36,90]. Using attenuated total reflection (ATR)-FTIR, absorption spectra can be obtained *in vivo* by placing an arm on the ATR-crystal[91].

Small angle X-ray diffraction (SAXD) can be used to study the lateral packing of the SC lipids. With SAXD, the scatter of an X-ray beam is used to determine the lamellar phases. A sample is placed in in front of an X-ray detector. A beam is send through the sample scattering the X-ray on the detector. The two repeat distances of the lipid lamellae in the SC both give different repeating maxima in a plot of the scatter intensity verses the angle of the diffraction[38].

1.4.3 Measuring the barrier function

As stated above, the TEWL can be used as a measure of barrier function. To determine the TEWL the evaporation of water from the SC is measured as an indication of the flux through the SC. In this thesis all TEWL measurements have been performed with a closed chamber and condenser system. The condenser prevents the accumulation of water in the chamber and a humidity gradient. In the chamber the change in relative humidity is measured. Using the contact surface of the chamber the water flux in g/cm^2/h is calculated[92]. Although the TEWL can be quickly assessed (<3min), it is highly susceptible to other variations than skin barrier function[93]. Monitoring the TEWL throughout SC barrier recovery after disruption can be used to determine the speed of the recovery process.

1.5 Aims and outline

As delineated above, lipids form an integral part of the SC, are part of AD pathology, and are possible targets for treatment. The aim of the research described in this thesis is to examine the role that ceramides can have as therapeutic targets for barrier repair in skin disorders characterized by an altered SC lipid compositions. This leads to the following goals:

1. To develop a method for extraction and quantification of all SC ceramides;
2. Determine how barrier recovery affects the SC ceramides and if *ex vivo* SC regeneration can be used as a model for clinical testing;

3. Discover how a vernix caseosa based formulation affects barrier recovery *in vivo* and determine how formulations influence the ceramide composition in the SC in healthy and diseased skin;
4. Examine the composition of the bound ceramides in healthy skin, skin models, and diseased skin and compared it to the composition of the related unbound ceramides.

To obtain these goals, research was performed which is described in six separate research chapters of this thesis, further divided into three parts. The first part focusses on analytical methodology and translation of *ex vivo* skin models to the clinical setting. In the following two research chapters, a method to accurately and robustly quantify all SC ceramides is described and changes in regenerated SC of an *ex vivo* skin model are compared to human *in vivo* SC after regeneration, respectively.

- Quantitative analysis of ceramides using a novel lipidomics approach with three dimensional response modelling.
- Compromising human skin *in vivo* and *ex vivo* to study skin barrier repair.

The second part focusses on the effects of a vernix caseosa based formulationon humans which was examined in two clinical studies. First, the effect of this formulation on barrier recovery in healthy volunteers will be presented. Second, the possibility of the formulation as treatment in AD patients will be examined. The findings are decribed in two chapters:

- Applying a vernix caseosa based formulation accelerates skin barrier repair by modulating lipid biosynthesis.
- Emollient monotherapy in moderate to severe atopic dermatitis patients induced stratum corneum lipid properties changes, but did not alleviate disease severity.

The last part focuses on the bound ceramides. Here, a detailed analysis of the bound ceramides is presented with the discovery of a new ceramide subclass. The selectivity in ceramide binding is examined and how the bound ceramide composition was affected by the liver X receptor. Also a detailed comparison of the bound ceramides in healthy and AD patients is presented. This part contains the following research chapters:

- Selectivity in cornified envelop binding of ceramides in human skin and the role of LXR inactivation on ceramide binding.
- The cornified envelope bound ceramide fraction is alter in atopic dermatitis patients

References:

1 Wong, R., Geyer, S., Weninger, W., Guimberteau, J. C. & Wong, J. K. The dynamic anatomy and patterning of skin. *Exp Dermatol* **25**, 92-98 (2016).

2 Matejuk, A. Skin Immunity. *Arch Immunol Ther Exp (Warsz)* **66**, 45-54 (2018).

3 Eckhart, L., Lippens, S., Tschachler, E. & Declercq, W. Cell death by cornification. *Biochimica et biophysica acta* **1833**, 3471-3480 (2013).

4 Houben, E., De Paepe, K. & Rogiers, V. A keratinocyte's course of life. *Skin Pharmacol Physiol* **20**, 122-132 (2007).

5 Kalinin, A. E., Kajava, A. V. & Steinert, P. M. Epithelial barrier function: assembly and structural features of the cornified cell envelope. *Bioessays* **24**, 789-800 (2002).

6 Matsui, T. & Amagai, M. Dissecting the formation, structure and barrier function of the stratum corneum. *Int Immunol* **27**, 269-280 (2015).

7 Rawlings, A. V. Molecular basis for stratum corneum maturation and moisturization. *Br J Dermatol* **171 Suppl 3**, 19-28 (2014).

8 Gray, G. M., White, R. J., Williams, R. H. & Yardley, H. J. Lipid composition of the superficial stratum corneum cells of pig epidermis. *Br J Dermatol* **106**, 59-63 (1982).

9 Lampe, M. A. *et al.* Human stratum corneum lipids: characterization and regional variations. *Journal of lipid research* **24**, 120-130 (1983).

10 Weerheim, A. & Ponec, M. Determination of stratum corneum lipid profile by tape stripping in combination with high-performance thin-layer chromatography. *Arch Dermatol Res* **293**, 191-199 (2001).

11 Farwanah, H., Neubert, R., Zellmer, S. & Raith, K. Improved procedure for the separation of major stratum corneum lipids by means of automated multiple development thin-layer chromatography. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **780**, 443-450 (2002).

12 Gault, C. R., Obeid, L. M. & Hannun, Y. A. An overview of sphingolipid metabolism: from synthesis to breakdown. *Adv Exp Med Biol* **688**, 1-23 (2010).

13 Hama, H. Fatty acid 2-Hydroxylation in mammalian sphingolipid biology. *Biochimica et biophysica acta* **1801**, 405-414 (2010).

14 Levy, M. & Futerman, A. H. Mammalian ceramide synthases. *IUBMB Life* **62**, 347-356 (2010).

15 Mizutani, Y., Kihara, A. & Igarashi, Y. Identification of the human sphingolipid C4-hydroxylase, hDES2, and its up-regulation during keratinocyte differentiation. *FEBS Lett* **563**, 93-97 (2004).

16 Bektas, M. *et al.* Induction of apoptosis by synthetic ceramide analogues in the human keratinocyte cell line HaCaT. *Exp Dermatol* **7**, 342-349 (1998).

17 Wu, B. X., Clarke, C. J. & Hannun, Y. A. Mammalian neutral sphingomyelinases: regulation and roles in cell signaling responses. *Neuromolecular Med* **12**, 320-330 (2010).

18 Wertz, P. Epidermal Lamellar Granules. *Skin Pharmacol Physiol* **31**, 262-268 (2018).

19 Menon, G. K., Lee, S. E. & Lee, S. H. An overview of epidermal lamellar bodies: Novel roles in biological adaptations and secondary barriers. *J Dermatol Sci* **92**, 10-17 (2018).

20 van Smeden, J. *et al.* In situ visualization of glucocerebrosidase in human skin tissue: zymography versus activity-based probe labeling. *Journal of lipid research* **58**, 2299-2309 (2017).

21 Hirabayashi, T. *et al.* PNPLA1 has a crucial role in skin barrier function by directing acylceramide biosynthesis. *Nat Commun* **8**, 14609 (2017).

22 t'Kindt, R. *et al.* Profiling and characterizing skin ceramides using reversed-phase liquid chromatography-quadrupole time-of-flight mass spectrometry. *Analytical chemistry* **84**, 403-411 (2012).

23 Motta, S. *et al.* Ceramide composition of the psoriatic scale. *Biochimica et biophysica acta* **1182**, 147-151 (1993).

24 Rabionet, M. *et al.* 1-O-acylceramides are natural components of human and mouse epidermis. *Journal of lipid research* **54**, 3312-3321 (2013).

25 Thakoersing, V. S. *et al.* Modulation of stratum corneum lipid composition and organization of human skin equivalents by specific medium supplements. *Exp Dermatol* **24**, 669-674 (2015).

26 Masukawa, Y. *et al.* Characterization of overall ceramide species in human stratum corneum. *Journal of lipid research* **49**, 1466-1476 (2008).

27 van Smeden, J. *et al.* Combined LC/MS-platform for analysis of all major stratum corneum lipids, and the profiling of skin substitutes. *Biochimica et biophysica acta* **1841**, 70-79 (2014).

28 Kihara, A. Very long-chain fatty acids: elongation, physiology and related disorders. *J Biochem* **152**, 387-395 (2012).

29 Norlen, L., Nicander, I., Lundh Rozell, B., Ollmar, S. & Forslind, B. Inter- and intra-individual differences in human stratum corneum lipid content related to physical parameters of skin barrier function in vivo. *The Journal of investigative dermatology* **112**, 72-77 (1999).

30 Zheng, Y. *et al.* Lipoxygenases mediate the effect of essential fatty acid in skin barrier formation: a proposed role in releasing omega-hydroxyceramide for construction of the corneocyte lipid envelope. *J Biol Chem* **286**, 24046-24056 (2011).

31 Amen, N. *et al.* Differentiation of epidermal keratinocytes is dependent on glucosylceramide:ceramide processing. *Hum Mol Genet* **22**, 4164-4179 (2013).

32 Elias, P. M. *et al.* Formation and functions of the corneocyte lipid envelope (CLE). *Biochimica et biophysica acta* **1841**, 314-318 (2014).

33 Wertz, P. W., Madison, K. C. & Downing, D. T. Covalently bound lipids of human stratum corneum. *The Journal of investigative dermatology* **92**, 109-111 (1989).

34 Epp, N. *et al.* 12R-lipoxygenase deficiency disrupts epidermal barrier function. *J Cell Biol* **177**, 173-182 (2007).

35 Pensack, R. D., Michniak, B. B., Moore, D. J. & Mendelsohn, R. Infrared kinetic/structural studies of barrier reformation in intact stratum corneum following thermal perturbation. *Appl Spectrosc* **60**, 1399-1404 (2006).

36 Boncheva, M., Damien, F. & Normand, V. Molecular organization of the lipid matrix in intact Stratum corneum using ATR-FTIR spectroscopy. *Biochimica et biophysica acta* **1778**, 1344-1355 (2008).

37 Janssens, M. *et al.* Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *Journal of lipid research* **53**, 2755-2766 (2012).

38 Bouwstra, J. A., Gooris, G. S., van der Spek, J. A. & Bras, W. Structural investigations of human stratum corneum by small-angle X-ray scattering. *The Journal of investigative dermatology* **97**, 1005-1012 (1991).

39 Bouwstra, J. A. *et al.* Role of ceramide 1 in the molecular organization of the stratum corneum lipids. *Journal of lipid research* **39**, 186-196 (1998).

40 Jennemann, R. *et al.* Loss of ceramide synthase 3 causes lethal skin barrier disruption. *Hum Mol Genet* **21**, 586-608 (2012).

41 van Smeden, J., Janssens, M., Gooris, G. S. & Bouwstra, J. A. The important role of stratum corneum lipids for the cutaneous barrier function. *Biochimica et biophysica acta* **1841**, 295-313 (2014).

42 Groen, D., Poole, D. S., Gooris, G. S. & Bouwstra, J. A. Is an orthorhombic lateral packing and a proper lamellar organization important for the skin barrier function? *Biochimica et biophysica acta* **1808**, 1529-1537 (2011).

43 Weidinger, S. & Novak, N. Atopic dermatitis. *Lancet* **387**, 1109-1122 (2016).

44 Mortz, C. G., Andersen, K. E., Dellgren, C., Barington, T. & Bindslev-Jensen, C. Atopic dermatitis from adolescence to adulthood in the TOACS cohort: prevalence, persistence and comorbidities. *Allergy* **70**, 836-845 (2015).

45 Chiesa Fuxench, Z. C. *et al.* Atopic Dermatitis in America Study: A Cross-Sectional Study Examining the Prevalence and Disease Burden of Atopic Dermatitis in the US Adult Population. *The Journal of investigative dermatology* **139**, 583-590 (2019).

46 Tokura, Y. Extrinsic and intrinsic types of atopic dermatitis. *J Dermatol Sci* **58**, 1-7 (2010).

47 David Boothe, W., Tarbox, J. A. & Tarbox, M. B. Atopic Dermatitis: Pathophysiology. *Adv Exp Med Biol* **1027**, 21-37 (2017).

48 Otsuka, A. *et al.* The interplay between genetic and environmental factors in the pathogenesis of atopic dermatitis. *Immunol Rev* **278**, 246-262 (2017).

49 Smith, A. R., Knaysi, G., Wilson, J. M. & Wisniewski, J. A. The Skin as a Route of Allergen Exposure: Part I. Immune Components and Mechanisms. *Curr Allergy Asthma Rep* **17**, 6 (2017).

50 Howell, M. D. *et al.* Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy*

Clin Immunol **124**, R7-R12 (2009).
51 Thyssen, J. P. & Kezic, S. Causes of epidermal filaggrin reduction and their role in the pathogenesis of atopic dermatitis. *J Allergy Clin Immunol* **134**, 792-799 (2014).
52 Boguniewicz, M. & Leung, D. Y. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev* **242**, 233-246 (2011).
53 Sekiya, A. *et al.* Compound heterozygotes for filaggrin gene mutations do not always show severe atopic dermatitis. *J Eur Acad Dermatol Venereol* **31**, 158-162 (2017).
54 Bandier, J., Carlsen, B. C., Rasmussen, M. A., Petersen, L. J. & Johansen, J. D. Skin reaction and regeneration after single sodium lauryl sulfate exposure stratified by filaggrin genotype and atopic dermatitis phenotype. *Br J Dermatol* **172**, 1519-1529 (2015).
55 Tawada, C. *et al.* Interferon-gamma decreases ceramides with long-chain fatty acids: possible involvement in atopic dermatitis and psoriasis. *The Journal of investigative dermatology* **134**, 712-718 (2014).
56 Downing, D. T. Lipid and protein structures in the permeability barrier of mammalian epidermis. *Journal of lipid research* **33**, 301-313 (1992).
57 Tanno, O., Ota, Y., Kitamura, N., Katsube, T. & Inoue, S. Nicotinamide increases biosynthesis of ceramides as well as other stratum corneum lipids to improve the epidermal permeability barrier. *Br J Dermatol* **143**, 524-531 (2000).
58 Yamamoto, A., Serizawa, S., Ito, M. & Sato, Y. Stratum corneum lipid abnormalities in atopic dermatitis. *Arch Dermatol Res* **283**, 219-223 (1991).
59 van Smeden, J. *et al.* The importance of free fatty acid chain length for the skin barrier function in atopic eczema patients. *Exp Dermatol* **23**, 45-52 (2014).
60 Ishikawa, J. *et al.* Changes in the ceramide profile of atopic dermatitis patients. *The Journal of investigative dermatology* **130**, 2511-2514 (2010).
61 Di Nardo, A., Wertz, P., Giannetti, A. & Seidenari, S. Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Derm Venereol* **78**, 27-30 (1998).
62 Ito, S. *et al.* Ceramide synthase 4 is highly expressed in involved skin of patients with atopic dermatitis. *J Eur Acad Dermatol Venereol* **31**, 135-141 (2017).
63 van Smeden, J. *et al.* Skin barrier dysfunction in non-lesional atopic eczema: the role of stratum corneum lipids. *European journal of dermatology : EJD* (2013).
64 Mojumdar, E. H., Kariman, Z., van Kerckhove, L., Gooris, G. S. & Bouwstra, J. A. The role of ceramide chain length distribution on the barrier properties of the skin lipid membranes. *Biochimica et biophysica acta* **1838**, 2473-2483 (2014).
65 Uche, L. E., Gooris, G. S., Beddoes, C. M. & Bouwstra, J. A. New insight into phase behavior and permeability of skin lipid models based on sphingosine and phytosphingosine ceramides. *Biochim Biophys Acta Biomembr* (2019).
66 Meckfessel, M. H. & Brandt, S. The structure, function, and importance of ceramides in skin and their use as therapeutic agents in skin-care products. *J Am Acad Dermatol* **71**, 177-184 (2014).
67 Sahle, F. F., Gebre-Mariam, T., Dobner, B., Wohlrab, J. & Neubert, R. H. Skin diseases associated with the depletion of stratum corneum lipids and stratum corneum lipid substitution therapy. *Skin Pharmacol Physiol* **28**, 42-55 (2015).
68 LePoidevin, L. M., Lee, D. E. & Shi, V. Y. A comparison of international management guidelines for atopic dermatitis. *Pediatr Dermatol* **36**, 36-65 (2019).
69 Rawlings, A. V. & Lombard, K. J. A review on the extensive skin benefits of mineral oil. *Int J Cosmet Sci* **34**, 511-518 (2012).
70 Angelova-Fischer, I., Neufang, G., Jung, K., Fischer, T. W. & Zillikens, D. A randomized, investigator-blinded efficacy assessment study of stand-alone emollient use in mild to moderately severe atopic dermatitis flares. *J Eur Acad Dermatol Venereol* **28 Suppl 3**, 9-15 (2014).
71 Wolf, R. & Parish, L. C. Barrier-repair prescription moisturizers: do we really need them? Facts and controversies. *Clin Dermatol* **31**, 787-791 (2013).
72 Hon, K. L., Leung, A. K. & Barankin, B. Barrier repair therapy in atopic dermatitis: an overview. *Am J Clin Dermatol* **14**, 389-399 (2013).
73 Man, M. M., Feingold, K. R., Thornfeldt, C. R. & Elias, P. M. Optimization of physiological lipid

mixtures for barrier repair. *The Journal of investigative dermatology* **106**, 1096-1101 (1996).
74 Oh, M. J. *et al.* Novel phytoceramides containing fatty acids of diverse chain lengths are better than a single C18-ceramide N-stearoyl phytosphingosine to improve the physiological properties of human stratum corneum. *Clin Cosmet Investig Dermatol* **10**, 363-371 (2017).
75 Koppes, S. A. *et al.* Efficacy of a Cream Containing Ceramides and Magnesium in the Treatment of Mild to Moderate Atopic Dermatitis: A Randomized, Double-blind, Emollient- and Hydrocortisone-controlled Trial. *Acta Derm Venereol* **96**, 948-953 (2016).
76 Lynde, C. W. & Andriessen, A. A cohort study on a ceramide-containing cleanser and moisturizer used for atopic dermatitis. *Cutis* **93**, 207-213 (2014).
77 Zhang, Q. *et al.* Topically applied ceramide accumulates in skin glyphs. *Clin Cosmet Investig Dermatol* **8**, 329-337 (2015).
78 Sjovall, P. *et al.* Imaging the distribution of skin lipids and topically applied compounds in human skin using mass spectrometry. *Sci Rep* **8**, 16683 (2018).
79 Haubrich, K. A. Role of Vernix caseosa in the neonate: potential application in the adult population. *AACN Clin Issues* **14**, 457-464 (2003).
80 Rissmann, R. *et al.* New insights into ultrastructure, lipid composition and organization of vernix caseosa. *The Journal of investigative dermatology* **126**, 1823-1833 (2006).
81 Mikova, R. *et al.* Newborn boys and girls differ in the lipid composition of vernix caseosa. *PLoS One* **9**, e99173 (2014).
82 Oudshoorn, M. H. *et al.* Effect of synthetic vernix biofilms on barrier recovery of damaged mouse skin. *Exp Dermatol* **18**, 695-703 (2009).
83 Lam, S. M. & Shui, G. Lipidomics as a principal tool for advancing biomedical research. *J Genet Genomics* **40**, 375-390 (2013).
84 Sandra, K. & Sandra, P. Lipidomics from an analytical perspective. *Curr Opin Chem Biol* **17**, 847-853 (2013).
85 Checa, A., Bedia, C. & Jaumot, J. Lipidomic data analysis: tutorial, practical guidelines and applications. *Anal Chim Acta* **885**, 1-16 (2015).
86 Kendall, A. C. *et al.* Lipidomics for translational skin research: A primer for the uninitiated. *Exp Dermatol* **27**, 721-728 (2018).
87 Sadowski, T. *et al.* Large-scale human skin lipidomics by quantitative, high-throughput shotgun mass spectrometry. *Sci Rep* **7**, 43761 (2017).
88 Wu, Z. *et al.* Lipidomic platform for structural identification of skin ceramides with alpha-hydroxyacyl chains. *Analytical and bioanalytical chemistry* **408**, 2069-2082 (2016).
89 Masukawa, Y. *et al.* Comprehensive quantification of ceramide species in human stratum corneum. *Journal of lipid research* **50**, 1708-1719 (2009).
90 Krill, S. L., Knutson, K. & Higuchi, W. I. The stratum corneum lipid thermotropic phase behavior. *Biochimica et biophysica acta* **1112**, 281-286 (1992).
91 Damien, F. & Boncheva, M. The extent of orthorhombic lipid phases in the stratum corneum determines the barrier efficiency of human skin in vivo. *The Journal of investigative dermatology* **130**, 611-614 (2010).
92 Imhof, R. E., De Jesus, M. E., Xiao, P., Ciortea, L. I. & Berg, E. P. Closed-chamber transepidermal water loss measurement: microclimate, calibration and performance. *Int J Cosmet Sci* **31**, 97-118 (2009).
93 Rogiers, V. EEMCO guidance for the assessment of transepidermal water loss in cosmetic sciences. *Skin Pharmacol Appl Skin Physiol* **14**, 117-128 (2001).