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Lipids as therapeutic targets for barrier repair in skin diseases

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Lipids as therapeutic targets for barrier repair in skin diseases

Walter Alexander Boiten

Geboren 27 december 1985 te Leiderdorp



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Cover design: (by Walter Boiten) The front depicts an ionmap of my own unbound stratum corneum ceramide fraction, the spine depicts cholesterol. The back depicts the bound ceramide fraction of the stratum corneum lipids.

Lipids as therapeutic targets for barrier repair in skin diseases

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Chapter 1

General introduction and aims

1

General introduction and aims

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 cushioning¹. 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-cells².

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 membrane³. 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 membrane⁵. 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

corneodesmosomes⁶. 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 layers⁷. In this process the corneodesmosomes are gradually degraded thereby detaching the corneocytes⁴. 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 sterols⁸. Yet, it is not excluded that there are minor fractions of phospholipids, squalene, wax-esters and cholesterol esters⁹. However, this minor lipid fraction could originate from sebum lipids. **Figure 1.1** shows an overview of the chemical 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 lipid synthesis 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 dihydrosphingosine¹². 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 well¹³. Six different CerS are known and each CerS has affinity for specific acyl-CoAs with different chain lengths¹⁴. There are two enzymes known that further modify dihydroceramides. These are dihydroceramide desaturase 1 (DES1) and dihydroceramide C-4 hydroxylase (DES2) which convert dihydroceramides to ceramides or phytoceramides¹⁵, 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 LBs¹⁸. 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 from 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 well²¹. 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 SC²⁴. 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

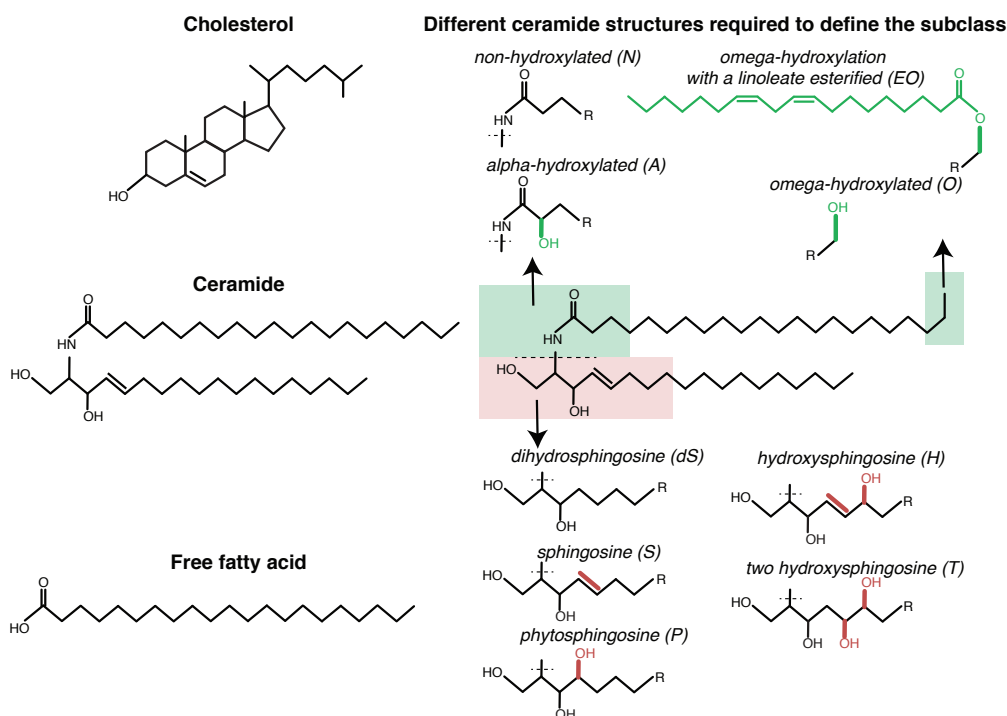


Figure 1.1: The chemical structures of the three main different SC lipid classes. For the ceramides the different structure of both the acyl-chain and the sphingoid base defining the different subclasses are shown.

chain lengths and desaturation of the acyl chain are observed in human SC ceramides as well²⁵. 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 observed²⁷. 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 tissues²⁸. 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 from the sebum lipids, as do the wax esters²⁹.

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 serve 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 bonds of the linoleate are modified to a hydroxyl-epoxide in two steps³⁰. Then the altered linoleate moiety is removed and the GluCer bound to the CE³¹. 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 function³⁴. 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 structure³⁵. 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 and are called the short and long periodicity phase, respectively³⁸. Ceramides with an EO group are essential for formation of the long periodicity phase³⁹ and for proper barrier function⁴⁰. 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 skin⁴³. 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 trigger a release of cytokines, like thymic stromal lymphopoietin (TSLP), by the keratinocytes. These cytokines in-turn activate 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 levels⁴⁶. There are many different immunological paths involved in AD⁴⁷⁻⁴⁹. 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 filaggrin⁵⁰.

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 SC⁵¹. Loss of function mutations of the filaggrin gene are known risk factors for AD⁵², yet, do not necessarily cause AD^{53,54}. Homozygous loss of function causes a dry scaling skin disorder called ichthyosis vulgaris⁴³. 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 chronic lesions. Inflammation can also decrease skin barrier function by deregulating another important part of the SC barrier, the SC lipids⁵⁵.

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 lipids⁵⁶ and increased ceramide content can decrease TEWL⁵⁷. 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 decreased⁵⁹.

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 TEWL⁵⁹. 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 dermatitis 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 immunosuppressants⁶⁸, 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* conditions⁷⁷. Others have shown minor penetration of ceramides⁷⁸. 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 SC⁷⁹. 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 mice⁸². 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 structures⁸³. 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 measurement⁸⁴. 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 conditions⁸⁵. 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 ceramides⁸⁶⁻⁸⁸. Due to the large number of different ceramide species and only a limited number of standards, comprehensive quantification of all SC ceramides becomes challenging⁸⁹. 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-crystal⁹¹.

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 front of an X-ray detector. A beam is sent 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 versus the angle of the diffraction³⁸.

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 $\text{g}/\text{cm}^2/\text{h}$ is calculated⁹². Although the TEWL can be quickly assessed (<3min), it is highly susceptible to other variations than skin barrier function⁹³. 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 formulation on 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 described 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 envelope binding of ceramides in human skin and the role of LXR inactivation on ceramide binding.
- The cornified envelope bound ceramide fraction is altered in atopic dermatitis patients

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Part I

Chapter 2

Quantitative analysis of ceramides using a novel lipidomics approach with three dimensional response modelling

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Background:

Chromatogram of a mixture of ceramide standards



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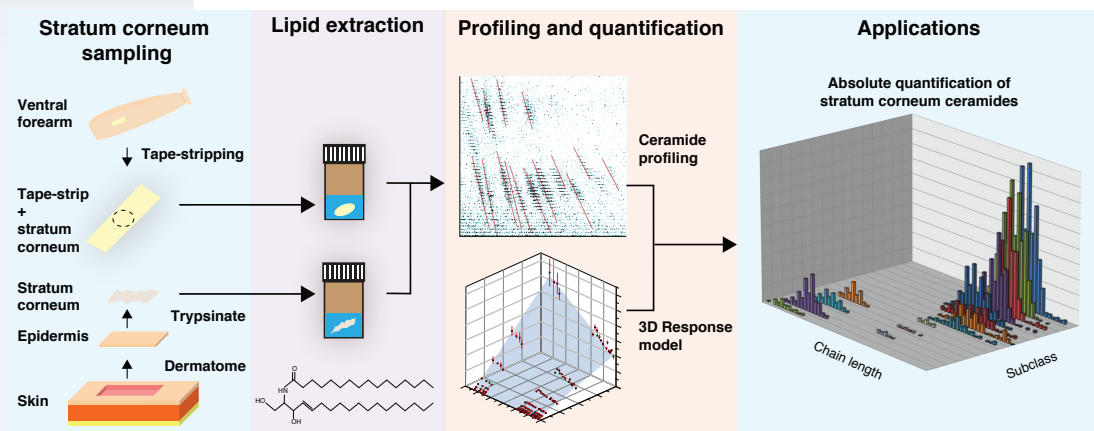
Quantification of ceramides using lipidomics

Abstract

In the outermost layer of the skin, the stratum corneum (SC), ceramides form a diverse and essential pool of lipids. Due to their diversity and the limited availability of synthetic standards it is challenging to quantitatively analyze all SC ceramides independently. We aim to perform a detailed analysis of ceramides on SC harvested from *in vivo* and *ex vivo* skin, therefore, a LC/MS method was developed in which all steps from sample acquisition until data analysis were examined and optimized. Improving extraction efficiency of ceramides resulted in an increase in efficiency from 71.5% to 99.3%. It was shown that sample harvesting by tape-stripping *in vivo* was accurate and precise. A full scan MS method was developed compatible with all sample types, enabling simultaneously qualitative and quantitative data analysis. A novel three dimensional response model was constructed to quantify all ceramides from full scan data using a limited amount of synthetic ceramides. The application is demonstrated on various SC sample types. When *ex vivo* SC was regenerated during human skin culture increases were observed compared with native human skin in the amount of the ceramide sphingosine subclasses, in mono-unsaturated ceramides (which have an *cis*-double bond in the acyl chain), and ceramides with a short C34 carbon chain (ceramides with a total carbon chain of 34 carbon atoms). These changes in ceramide levels are also often encountered in diseased skin.

keywords:

Stratum corneum;
Skin;
Tape-stripping;
Extraction efficiency;
Mass spectrometry;
Stereoisomers;



Graphical abstract

2.1 Introduction

The stratum corneum acts as a barrier against penetration of substances from the environment and protects the body against desiccation. This layer is often represented as a brick and mortar structure, with the bricks being terminally differentiated keratinocytes and the mortar the lipid matrix in between these cells. This lipid matrix is mainly composed of three lipid classes: ceramides, free fatty acids, and cholesterol forming an organization of regular stacked lipid layers. For most compounds the lipid matrix constitutes the penetration route of least resistance through the SC. The SC is a dynamic tissue and the lipid homeostasis plays a pivotal role in maintaining this barrier¹. In several skin diseases lipid metabolism is affected resulting in changes in lipid composition thereby changing the lipid organization and barrier function²⁻⁵.

Ceramides are present in most tissues and have diverse key physiological functions⁶. SC ceramides, however, are the most structurally diverse of all tissues and of the SC lipid classes. Ceramides and their structural diversity have an essential role in the crystalline lipid organization of the SC and changes affect the SC barrier⁷⁻⁹. To understand the role of ceramides in diseased skin and the metabolic processes from which they originate, it is crucial to profile the ceramide diversity and determine the absolute amounts with high accuracy. There are multiple methods to obtain SC for analysis: *In vivo* human SC is often harvested by tape-stripping^{10,11}, whereas SC of *ex vivo* or *in vitro* cultured human skin is often isolated by enzyme digestion¹².

Ceramides are a group of sphingolipids with a sphingosine group covalently linked to an acyl chain by an amide bond. Their name and subclass are based on the sphingosine and acyl chains molecular structure, according to the nomenclature by Motta et al¹³. Till date, 15 different subclasses of SC ceramides have been identified¹⁴. The ceramides either have an, N (non-hydroxy), A (α -hydroxy), O (ω -hydroxy), or EO (ω -esterified linoleic acid) acyl group linked to either, an S (sphingosine), dS (dihydrosphingosine), P (phytosphingosine), H (hydroxyl-sphingosine) sphingosine chain. **Figure 2.1** depicts a general molecular structure of a ceramide with the variations in molecular architecture of the various subclasses. Both the acyl and the sphingosine carbon chain vary in length, resulting in previous reported numbers of 342 to >1000 structurally different SC ceramides^{14,15}.

Initially, SC ceramides were analyzed using high performance thin layer chromatography (HPTLC)^{16,17}. However, in recent years several LC/MS methods have been developed using diverse chromatographic and mass spectrometric techniques^{4,14,18-26} and one comprehensive quantitative method was developed requiring synthetic standards of every subclass¹⁵. Until now there have been no lipidomics approaches to SC ceramide analysis in which both profiling (untargeted) and quantification (targeted) are performed simultaneously. Combining both approaches has the advantage that

unknown lipid species can be detected while simultaneously including them in the quantification. The challenges in combining untargeted and targeted analysis of lipids are the low sensitivity, small linear dynamic range and reproducibility of full scan measurement, next to the challenge of developing a method with a high reproducibility and accuracy required for the quantification of a very large numbers of lipids while using a limited number of standards^{27,28}.

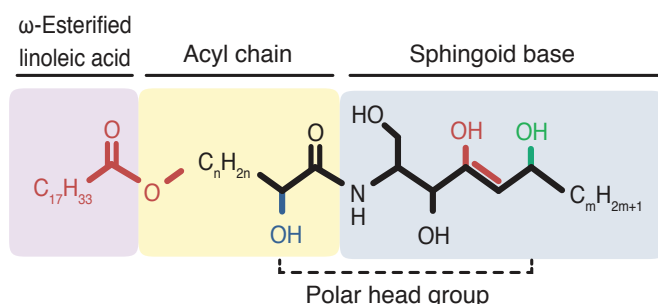


Figure 2.1: General structure of a ceramide.

An overview of the structural variations in SC ceramides: The acyl chain can have an ω -hydroxyl group (O,) an α -hydroxyl group (A), or no hydroxyl group (N). The ω -hydroxyl group can have an esterified linoleic acid group attached (esterified omega, EO). The sphingoid base can

be either unsaturated (referred to as sphingosine, S) or is unsaturated with one hydroxyl group (hydroxyl sphingosine, H), or the sphingoid base is saturated (dihydroxysphingosine, dS) or saturated with an extra hydroxyl group (phytosphingosine, P). Both the sphingoid base and the acyl chain can vary in carbon chain length as shown by m and n , the number of carbon atoms.

In this paper we present a novel method to extract and thereafter simultaneously profile and quantify all known SC ceramides. With this method the area under the curve (AUC) of the chromatogram of any specific ceramide can be used to calculate the molar amount. The latter was achieved by employing full scan MS and using a novel three dimensional (3D) response model based on MS settings, compound properties, and a limited number of synthetic ceramides. Moreover, an extensive analysis and improvement of the SC lipid extraction was performed. **Figure 2.2** provides an overview of the topics described in this paper to achieve these goals.

Sample preparation	Measurement	Data analysis	Application
<u>Extraction</u> - Extraction efficacy - Recovery <u>Sample validation</u> - Sample amounts - Tape-stripping procedure	<u>Method development</u> - MS settings <u>LC/MS method Validation</u> - Linear dynamic range - Matrix effects - Method stability	<u>Processing</u> - Monoisotopic mass integration <u>Corrections</u> - ISTD - Isotopes - Source fragments <u>Data modeling</u> - MS response	<u>Different SC samples</u> - Total amount of ceramides - Disease related parameters - Effects of regenerating SC in culture

Figure 2.2: An overview of the 4 stages focused on for the method development.

The 4 stages are in sequence of preparation and analysis of samples, and the corresponding data analysis. Each stage shows the parameters used to calibrate and validate these procedures.

The method was reproducible and applicable on many different SC sample types, test by analysis of SC harvested by tape-stripping often used in clinical studies, SC from *ex vivo* human skin, and SC isolated from cultured human skin. By making use of the quantitative measurements, biological relevant changes in the amount of a single ceramide, ceramide subclasses, or the total amount of ceramides can be obtained.

2.2 Materials and Methods

2.2.1 Chemicals

Millipore water (18.2mΩ), UPLC grade methanol (MeOH), isopropanol (IPA), and ethanol (EtOH) were purchased from Biosolve (Valkenswaard, the Netherlands). HPLC grade methanol and chloroform (Chl) were from Lab-Scan (Gliwice, Poland), and HPLC grade n-heptane (Hep) from Actu-All Chemicals (Oss, the Netherlands). The synthetic ceramide standards are listed in [Supplemental S2.1](#) and were either purchased from Avanti Polar Lipids (Alabaster AL, USA) or kindly provided by Evonik Industries (Essen, Germany).

2.2.2 Liquid chromatography and mass spectrometry method

All samples were dissolved in Hep:Chl:MeOH (95:2.5:2.5, v:v:v). Before analysis, an internal standard (ISTD) of CER N(24deu)S(18) was added to all samples at 3 pmol per injection. Analyses were performed using an Acquity UPLC H-class (Waters, Milford, MA, USA,) connected to an XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA). Of each sample 5 µl was injected. To monitor short and long term stability, three quality control (QC) samples of SC lipid extract at either 0.12 or 0.06 mg/ml were analyzed after every 10 samples. Samples were separated on a normal phase pva-silica column (5 µM particles, 100x2.1mm i.d.)(YMC, Kyoto, Japan) applying gradient elution starting at 98% mobile phase A (100% heptane) towards 50% mobile phase B (Hep:IPA:EtOH, 50:25:25, v:v:v). A flow rate of 0.8 ml/min was used. A detailed description of the gradient can be found in van Smeden et al.²³ and [Supplemental S2.2](#). MS measurements were performed using atmospheric pressure chemical ionization (APCI) in positive ion full scan MS mode, scanning from m/z 350 to 1200 the flow was directed to the MS between 1.25 and 12.5 min. APCI was used because of the difficult ionization of lipids^{29,30} and its potential to reduce matrix effects³¹⁻³³. Source settings were optimized using a mix of E(18:2)O(30)S(18), A(24)P(18) N(24)S(18), and N(24)S(16). As the molar fraction of (high mass) EO ceramides in SC lipids is relatively low, and to create an increase of response with mass for modelling, it was preferred to gain sensitivity and reduce resolution at high masses. 'Ion energy' and the 'low' and 'high mass resolution' settings were optimized. A maximum peak-width at half maximum of

0.6 amu was accepted. MS tune settings are listed in [Supplemental S2.2](#). To confirm the identified ceramide subclasses 4 ceramides per subclass were fragmented using LC-MS/MS (Scanwave daughter scan) during method development. These fragments were identical to the fragments reported previously^{10,34}.

2.2.3 SC samples acquisition

Three different sample types often encountered in skin research were used: SC of *ex vivo* Skin, *in vivo* tape-stripped SC and in culture regenerated SC. Human *ex vivo* skin from surgery was obtained from hospitals after written informed consent of the skin donors and institutional approval was obtained, all in accordance to the Declaration of Helsinki Principles. Skin of both genders was obtained and the mean age of the subject was 35 ($\pm$ SD 10) years old. The skin was dermatomed at a thickness of 400 μ m. The SC was separated from the epidermis by incubating the dermatomed skin at 4°C overnight on filter paper with trypsin (0.1 % w:v in PBS). Subsequently, the dermatomed skin was kept at 37°C during 1 hour as described by de Jager et al³⁵. Lipids were extracted with the most efficient method described in the results. These *ex vivo* SC lipid extracts are referred to as SC_{ev}. After cyanoacrylate stripping of dermatomed skin, SC was regenerated in culture³⁶. Thereafter, the skin was treated as described above. Lipid extracts from regenerated SC are referred to as SC_{cul}. The third sample type was SC from *in vivo* human skin. This was obtained by a non-invasive tape-stripping procedure⁷. In short, tape-stripping was performed using polyphenylene sulfide tape (Nichiban, Tokyo, Japan) that was placed on the ventral forearm. A weight of 2.5 kg was applied for 5 seconds and the tape was removed in a fluent movement. This procedure was repeated 10 times (tape 0-9). From each tape-strip a circle ($\varnothing$ 1.6 cm) was punched. Circles of tapes 5 to 8 were extracted as specified below. The combined lipid extracts of these 4 tape-strips were reconstituted in 1.5 ml (Chl:MeOH, 2:1, v:v) referred to as SC_{tape}.

2.2.4 Determining, inter/intraday variation, linear dynamic range, and matrix effects.

To prepare calibration curve stock solutions, mixture of synthetic ceramide standards listed in [Supplemental S2.1](#) were dissolved in Hep:Chl:MeOH, (95:2.5:2.5, v:v:v) at concentrations of either 50 or 100 μ M per single ceramide. To determine the ceramide standards linear dynamic range, non-serial exponentially diluted calibration curves with a range from 0.005 to 75 pmol (injected) per ceramide were prepared and measured in randomized order. To determine the intra/interday variation, nine calibration curves were prepared (each from a separate stock solution) and analyzed at 3 different days, three calibration curves per day.

The linear dynamic range and optimal sample amount of SC_{ev} were determined by preparing, in triplicate, SC lipid extract calibration curves containing 6 to 600 μg lipids/ml. These were prepared from a SC_{ev} pool extracted out of SC from 20 donors. The linear dynamic range and optimal amount of tape-strips in a sample was determined by preparing a large pool of SC_{tape} where an aliquot of 1.5 ml represented an average tape-strip sample of 4 tapes. Then, these aliquots were either diluted or concentrated to prepare a calibration curve of tape-strip samples ranging from 0.8-40 tapes/ml. To determine the matrix effects standard addition experiments were performed. The standards were added to the three different sample matrices:

1. The solvent mixture used for samples (H:C:M, 95:2.5:2.5, v:v:v);
2. Samples of extracted SC (SC_{ev}); extracted with modified B&D (300 $\mu\text{g}/\text{ml}$);
3. SC Samples harvested by tape-stripping (SC_{tape}); extracted with modified B&D (20 tapes/ml).

The standards listed in [Supplemental S2.1](#) were added at multiple concentrations (50, 20, 10, 5, 2, 1, 0.5, 0 μM) and added to the sample matrices in triplicate resulting in $n=24$ per matrix. Due to interday variation, standard addition samples were measured at the same day as well with $n=8$ per matrix.

2.2.5 Validation of the tape-stripping procedure

Additional variation due to the tape-stripping procedure was examined. Several variations can be distinguished: I) interindividual variation and II) a combination of intraindividual variation and variation caused by the procedure. Interindividual variation was determined by acquiring samples in 3 different individuals. From each individual seven SC_{tape} samples of the ventral forearms were obtained. These seven were used to prepare a calibration curve (0.8 to 40 tapes/ml). A blank tape extract was included. The calibration curve was used to determine the variation added by the tape-stripping procedure within a single individual, expecting intraindividual variation to be minimal on the forearm. A SquameScan 850A (Heiland Electronic, Wetzlar, Germany) was used to determine the amount of stripped SC of each sequential tape by measuring the absorbance at 850 nm after stripping, corrected for blank tape³⁷.

2.2.6 Extraction efficiency and recovery

A 'modified' Bligh and Dyer (B&D) extraction³⁸ was performed as follows. Per mg of SC or SC tape strip 1 ml of solvent mixture is added, shaken for 1 hour at 120 RPM, and removed. Different ratios of Chl:MeOH:H₂O were used successively for this extraction: 1:2:0.5, 1:1:0, and 2:1:0 v:v:v. After combining the extracts an equal volume of water

and 40 μ l of 0.25 M of KCl per ml water were added. Phases were left to separate overnight at 4°C. The organic layer was collected and the water layer washed with chloroform. The combined organic layers were filtered using a 0.45 μ m PVDF filter (Grace, Deerfield IL, USA). The syringe and filter were washed after filtration with 3 ml of Chl:MeOH (2:1). The combined filtrates were then evaporated at 40°C under a gentle flow of N₂ and samples reconstituted in Chl:MeOH (2:1, v:v).

2.2.6.1 Extraction efficiency:

The extraction efficiency of ceramides from the SC by the 'modified' B&D was examined by extracting *ex vivo* SC sheets twice consecutively. The 'modified' B&D did not result in an adequate and equally extraction of all ceramides (see results section 2.3.3). To improve the extraction efficiency three changes in the extraction procedure were tested: I) changing extraction temperature from room temperature to 40°C, II) adding an additional extraction step of a lipophilic mixture of Hep:IPA (1:1, v:v), or III) the combination of I) and II), see [Supplemental S2.3](#). To determine the amount of ceramides left in extracted SC a more rigorous extraction was performed subsequently. Samples were saponified with 1M KOH in 90% MeOH for 1 hour at 60°C neutralized with 1M HCl and a 'modified' B&D was performed thereafter.

2.2.6.2 Extraction recovery:

To determine the extraction recovery (the amount of lipids lost during the procedure) three samples types at three different sample amounts were prepared:

4. Mixture of 6 ceramide standards at 0.025, 0.25, and 2.5 pmol.
5. SC_{ev} at 15, 75, and 150 μ g.
6. 0.025, 0.25, and 2.5 pmol of six ceramide standards added to 150 μ g of SC_{ev}.

All samples were prepared in six fold of which three underwent an additional extraction procedure. Re-extracted samples were compared to non-extracted samples.

2.2.7 Sample and measurement stability

To determine the sample stability, 6 SC_{ev} samples were analyzed, stored at either 4°C or 40°C for one month, and afterwards analyzed again. The long term stability of the method was determined using QC samples prepared of SC_{ev} and measured over 9 months.

2.2.8 Application of the method

To illustrate the methods application nine different SC samples: 5 SC_{tape}, 3 SC_{ev}, and one SC_{cul} sample, were analyzed and quantified. Three of the tape-strip samples were acquired from one individual who was tape-stripped 33 times at one site. Samples were prepared of tape-strips 5-9, 19-22 and 30-33. All samples were extracted with the most efficient extraction method. Three calibration curves ranging from 0.1-20 pmol (injected) containing the ceramides listed in **Supplemental S.1** were included.

2.2.9 Data analysis and software

All MS data was analyzed and processed using MassLynx and TargetLynx software (V4.1 SCN 843 Waters Inc.). AUCs were calculated from chromatograms of the monoisotopic peak (± 0.5 Da) of the most abundant ion. 3D-area plot was made using OriginPro 8.5. Regression and statistical analysis were performed using GraphPad Prism 5.01 software. A curve weight of $1/x$ is applied to all linear and nonlinear regression, unless explicitly specified. If an outlier was observed a Grubbs test was performed to confirm the outlier. Two-way ANOVA was used to determine intraday variation, matrix effects and extraction recovery. Statistical differences were considered significant with a 0.95 chance of the zero hypotheses being false

2.3 Results

2.3.1 Inter/intraday variation, linear dynamic range and matrix effects

All parameters assessed for the quantitative LC/MS method are described in separate paragraphs below:

2.3.1.1 Inter/Intraday variation

Using the data obtained by measurements of synthetic ceramide calibration curves, the AUCs of chromatograms from the monoisotopic masses were determined, corrected for the ISTD, and plotted against concentration. **Figure 2.3A** depicts the linear range of 9 calibration curves of ceramide N(24)dS(18) measured at 3 different days. To determine the variation within and between days, the slope of these calibration curves were calculated and plotted (**Figure 2.3B**). A 2-way-ANOVA showed there was a significant interday variation, yet, an insignificant intraday variation. Analysis of all other ceramide standards resulted in comparable results (not shown).

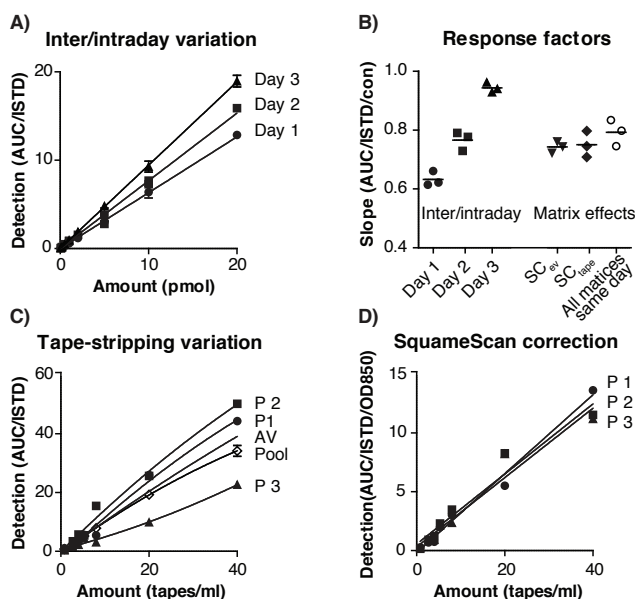


Figure 2.3: Method Calibration

A: The linear range of 9 calibration curves of N(24)dS(18) measured on 3 separate days. Measurements at the same day are plotted as mean $\pm$ SD with a linear regression line. **B:** The response factors (slopes) displaying the intra/interday variation ($\blacksquare$, $\bullet$, and $\blacktriangle$). Slopes of calibration curves added to SCev ($\blacktriangledown$) and SCTape ($\blacklozenge$) measured on separate days. Slopes of calibration curves added to Hep:Chl:MeOH (95:2.5:2.5), SCev, or SCTape measured on one day to assess matrix effects ($\circ$). **C:** SCTape calibration curves: data are the Σ AUC of 30 abundant ceramides in tape-strip samples. Each was prepared from SCTape samples obtained in a separate individual, shown as: $\blacksquare$, $\bullet$, and $\blacktriangle$ (R^2 : 0.986, 0.979 and 0.990). Lines are unweight 2nd order polynomial regressions. A regression through the average of the three curves is plotted as well. A calibration curve prepared from SCTape samples pooled ($\circ$) is shown as mean $\pm$ SD ($n=3$) with unweight 2nd order polynomial regression. **D:** The data of the calibration curves prepared from SCTape samples obtained in a single individual corrected for the SquameScan with an unweight linear regression ($\blacksquare$, $\bullet$, and $\blacktriangle$, R^2 : 0.987, 0.953 and 0.949).

2.3.1.2 Linear dynamic range

For ceramide N(24)dS(18), detector saturation was observed at levels above 20 pmol (**Supplemental S2.4A**) evident as a non-linear response. Detector saturation is observed for all ceramides at a similar AUC, indicating that the upper detection limit is dependent on the detector, not the ionization. Therefore, the upper detection limit was defined as a unit of AUC: 7.5×10^8 . For the lower limits of detection and quantification (LOD and LOQ, respectively) signal to noise (S/N) ratios of 3 and 10 were selected, respectively. Taking into account the interday variation and difference in sensitivity between ceramides, the linear range varies and needs to be assessed for each series of measurements. Typically, when using this full scan MS method low mass ceramides LOQ values are in the 100 fmol range, whereas high mass ceramides (EO ceramides) have values in the 50 fmol range. The upper limit is typically between 10 and 50 pmol.

As the linear range is dynamic, it is important to determine the amount (ng) of SC lipids which is required to adequately quantify all ceramides in SC_{ev}. In calibration curves of SC_{ev} the Σ AUC of 28 abundant SC ceramides was examined. A linear response

was observed over the full range (**Supplemental S2.4B**). An injected amount of 1500 ng of SC lipids proved optimal, resulting in the highest detection with negligible interference of other SC lipids and contaminants. This was different for SC_{tape}, as interference of the glue was observed at high levels in calibration curves of SC_{tape} (**Figure 2.3C**, and **Supplemental S2.4C** for more detail). An optimal sample amount of 20 tapes/ml was selected.

2.3.1.3 Matrix Effects:

Matrix effects, like ion suppression, can become significant when analyzing SC_{ev} and SC_{tape}, thereby hampering quantification. To examine matrix effects, standard addition was performed. The slopes of calibration curves added to SC_{ev} and the SC_{tape} matrices were compared to only solvent (calibration curves used to determine interday variation). Because both SC_{ev} and SC_{tape} matrices contain ceramides, the response factors (slope) of the calibration curve were used to compare them. **Figure 2.3B** depicts the slopes representing the response factors for N(24)dS(18) in all matrices. No significant difference in slopes was observed for any standard added if the calibration curves were measured at the same day. This demonstrated that an increase of the molar amount of ceramide gives the same increase of response in the MS in any of the sample matrixes.

2.3.2 Validation of the tape-stripping procedure

To quantify the amount of ceramide in multiple subjects using the tape-stripping procedure, it is important to distinguish biological variation and the variation caused by the tape-stripping procedure. Three calibration curves of SC_{tape} each obtained from a single individual were analyzed (**Figure 2.3C**). The separate curves and the average of the curves was plotted, the latter was comparable to pooled SC_{tape}. All three individual curves have R²-values >0.979, indicating an unexplained variance of <3.1%, which would represent the intraindividual variation, the variation added by the tape-stripping procedure and measurement error. An apparent interindividual variation was observed, demonstrated by the difference in slopes of the individual curves. The underlying factor of this variation could be explained by the total amount of tape-stripped material. When the data was corrected for the amount of SC stripped (**Figure 2.3D**), the variation between subjects was highly reduced although the methodological variance increased due to the impreciseness of the SquameScan method.

2.3.3 Extraction efficiency and recovery

To determine if all ceramides are efficiently extracted from SC, the SC was extracted twice consecutively using the 'modified' B&D. The Σ AUC of 24 abundant ceramides,

two per subclass, were compared to determine the percentage in the first extract. With the first B&D extraction 86.6% (SD $\pm$ 1.1)(n=9) of the ceramides was extracted. Furthermore, a bias towards the EO ceramide subclasses was observed in the first extraction, exemplified by the ratio non-EO and EO ceramides ([Supplemental S2.5](#)). This indicates that the 'modified' B&D does not extract all lipids from SC equally and may lead to inaccurate quantification of both the relative and absolute level of ceramides. Therefore, the extraction efficiency was optimized by using either I) a second less polar extraction solvent, II) by using a higher temperature, or III) combining both. [Figure 2.4A](#) depicts the percentage of the Σ AUC/ISTD of 22 abundant non-EO ceramides in the 1st extraction compared to the total amount in two consecutive extractions (no EO ceramides were included in this calculation as EO ceramides already had a higher extraction efficiency ([Supplemental S2.5](#)) and EO ceramides degrade during saponification). By either increasing the temperature or adding a more lipophilic step (extraction with Hep:IPA) an increased extraction efficiency was observed. Using both proved superior, as an extraction efficiency of >98% was achieved.

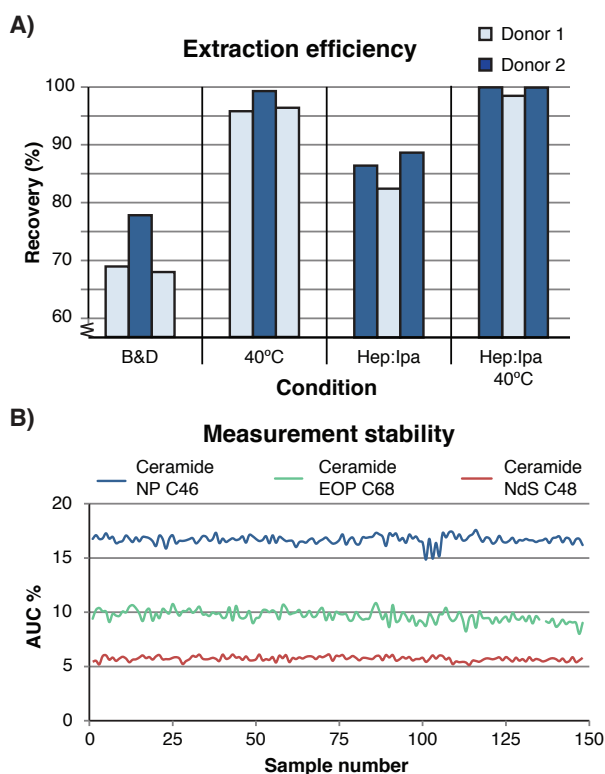


Figure 2.4: Extraction efficiency and measurement stability

A: The extraction efficiency as percentage of the amount extracted during the 1st extraction when two consecutive extractions were performed. Data are shown as: Σ AUC/ISTD of 22 abundant non-EO ceramides in the 1st extract / Σ AUC/ISTD of 22 abundant non-EO ceramides in both the 1st extraction and the 2nd extraction after saponification. Four different condition were examined: modified B&D at room temperature, modified B&D at 40°C, modified B&D with an extra Hep:IPA extraction step, and a modified B&D with an extra Hep:IPA extraction step all performed at 40°C. Two SC donors were used (light and dark blue bars donor one and two, respectively) **B:** Measurement stability over a time period of 9 months (N=149) expressed as the percentage of the AUC from NP C46 and NdS C48 to that of the Σ AUC from 16 non-EO ceramides and the percentage of the AUC from EOP C68 to that of the Σ AUC 8 EO ceramides

The amount of sample lost during the extraction procedure was calculated. In SC_{ev} samples $\sum AUC/ISTD$ 24 abundant SC ceramides were selected to represent all SC ceramides. **Table 2.1** depicts the percentages of these ceramides recovered after re-extraction. The detection was compared to the mean of 3 non-extracted samples. A slight reduction due to the extraction procedure was observed with synthetic ceramides and SC_{ev} . Taking together, the results of standards, SC_{ev} and the perceived error, about 5% of sample is lost due to extraction which was minimally influenced by the amount extracted.

Table 2.1: The recovery of ceramide standards and ceramides in SC_{ev} after a full extraction procured. Data are the mean and SD of the recovery percentage from 3 samples compared to the same samples but not extracted. SC_{ev} extracted were the $\sum AUC/ISTD$ of 24 SC ceramides. SC_{ev} + standards extracted were the $\sum AUC/ISTD$ of 24 SC ceramides in standard addition samples. Standards extracted were ceramide standards. Standards added and extracted were ceramide standards added to SC. High, medium and low amounts of samples of sample (for ceramide standards: 0.025, 0.25, and 2.5 pmol)(for SC_{ev} : 150, 75, and 15 mg).

Sample amount	SC_{ev} extracted (%)		SC_{ev} + standards extracted (%)		Standards extracted (%)		Standards added & extracted (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
High	86.5	1.9	98.6	5.0	98.6	4.6	99.7	4.2
Medium	94.4	6.3	95.3	6.3	96.1	5.9	98.1	7.0
Low	100.6	8.0	92.3	6.6	85.9	5.1	92.3	6.0

2.3.4 Sample and measurement stability

To ensure that SC ceramides are stable during storage at different temperatures, SC_{ev} samples were measured prior and after storage for a month at either at 4 or 40°C in CHl:Hep:MeOH solution. No significant differences were observed when comparing the AUCs of 30 abundant ceramides before and after storage (mean ratio of 0.99). The LC/MS method stability was determined by comparing the AUC of 24 ceramides in QC sample of the same batch measured over 9 months (**Figure 2.4B**), showing the method remained stable during this time period.

2.3.5 Data processing and post processing

Figure 2.5A depicts the 7 steps and corrections required to determine the absolute amount of a ceramide in any sample from full scan data. Prior to data processing, changes in ceramide profiles can be detected by using an ion map (**Figure 2.5B**). The ceramide subclasses that contained isobaric compounds were adequately separated.

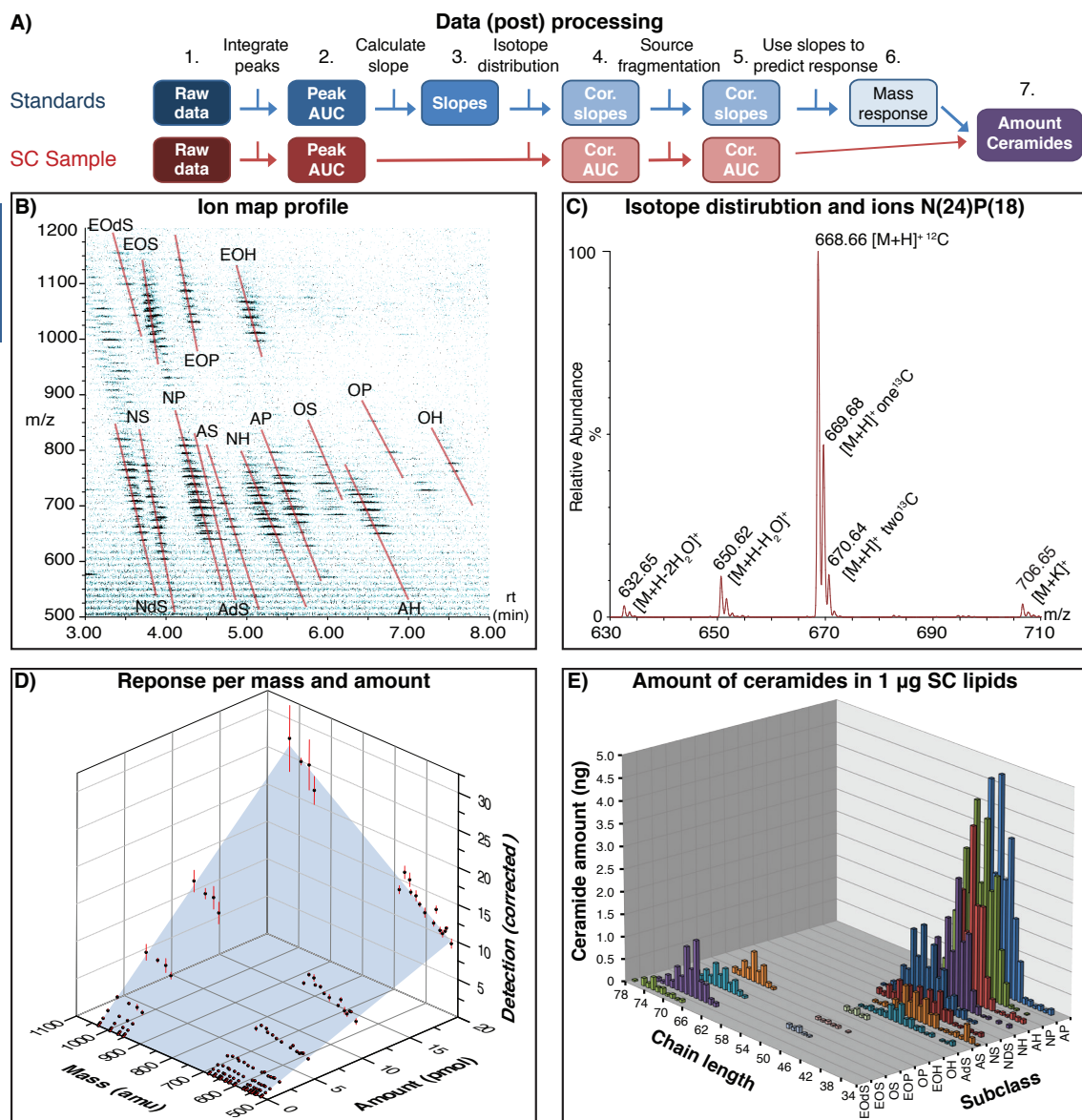


Figure 2.5: Processing and quantification

A: The processing and post-processing of the raw data from SC extract and ceramide standard samples to obtain quantitative data. **B:** Raw data ion map profile of a SCev sample with all identified ceramide classes indicated. **C:** The spectrum of a N(24)P(18) ceramide standard with the isotope distribution and the different adduct and fragments observed for this compound indicated. **D:** The mean $\pm$ SD of the corrected AUC from three calibration curves of ceramide standards. The corrected AUC is plotted over the exact mass and the amount injected. The area depicted is calculated using response factors of the calibration curves and the exact mass. **E:** The amount of the all individual ceramides in ng per μ g of SC lipids of a SCev depicted per subclass and chain length.

In total 374 ceramides were detected. As [R] and [S] configured α -hydroxy ceramide (A) subclasses have a different retention time (**Figure 2.6**), it was concluded that all A subclasses in SC had the α -hydroxyl group of the acyl chain in the [R] configuration.

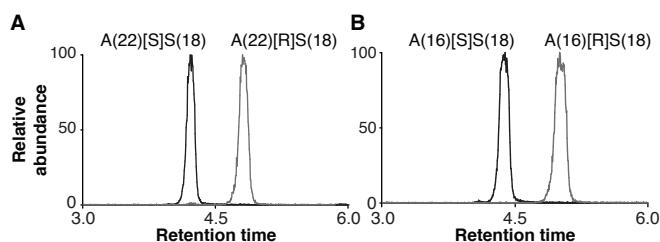


Figure 2.6: Separation of α -hydroxy ceramide stereoisomers

A: The chromatographic separation of ceramide A(22)S(18) with the α -hydroxyl of the acyl chain either in the [R] or the [S] configuration. **B:** The chromatographic separation of ceramide A(16)S(18) with the α -hydroxyl of the acyl chain either in the [R] or the [S] configuration.

Because of the limited amount of available ceramide standards with respect to the large number of ceramides present in SC, a model was made to predict the response factor for any ceramide using two properties: chain length and subclass. First, the AUC of each peak was corrected for the ISTD. Second, as all ceramides analyzed have carbon chain lengths above C32, the abundance of ^{13}C isotopes becomes substantial and dependent on chain length. **Figure 2.5C** depicts the isotope distribution for N(24)P(18). For all ceramides the theoretical abundance of the second, third, and - for very long chain ceramides - the fourth isotopic mass was calculated. This theoretical isotope distribution was verified by analysis of the data from ceramide standard calibration curves (**Supplemental S2.6A and B** provides an example for N(24)S(18)). As the isotope abundance is specific for each ceramide, AUCs were corrected for their specific isotope pattern. Thirdly, ceramides were ionized to proton and potassium adducts: $[\text{M}+\text{H}]^+$, $[\text{M}+\text{K}]^+$, and water loss fragments of the latter two: $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$, $[\text{M}+\text{H}-2\text{H}_2\text{O}]^+$, and $[\text{M}+\text{K}-\text{H}_2\text{O}]^+$ ions (**Figure 2.5C**). The abundance of each ion was examined for all subclasses and chain lengths. The $[\text{M}+\text{K}]^+$ and $[\text{M}+\text{K}-\text{H}_2\text{O}]^+$ ions contribute < 5% to the total peak area and were excluded from the analysis. Abundance of the $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ and $[\text{M}+\text{H}-2\text{H}_2\text{O}]^+$ ions was found I) to be dependent on the head group, II) have a substantial interday variation, and III) was chain length dependent. For low abundant SC ceramides these ions are below the detection levels, yet, contribute to the total ceramide amount and thus crucial for accurate quantification. To predict the relative abundance of every ion for every ceramide, sample of QCs and standards were used. The ratio of ions to the most abundant ion was plotted per subclass over chain lengths and regression was performed (**Supplemental S2.7** is an example of NS). Note that it is required to determine this ratio for each series of measurement due to interday variation in ionization. For all ceramides the non-main ions contributed less than 30%

to the total peak area, except for EOS and OS, where both the AUC of $[M+H]^+$ and $[M+H-H_2O]^+$ were processed.

Due to the MS tuning, it was expected that an increase in response will be observed with an increase in mass and molar amount of the ceramide. To verify this, AUCs of calibration curves from ceramide standards (corrected for ISTD and ion and isotope distribution) were plotted against their monoisotopic mass and against molar amount (**Figure 2.5D**). It was observed that detection increased as expected. For all calibration curves the slope was calculated and plotted against the monoisotopic mass. A linear regression was applied. To evaluate the regression a %-error plot of the response factors to the regression line was made. A residual below 16% and an equal distribution around zero was observed (**Supplemental S2.8 A and B** depict the regression and residual). Using this regression, the response for any ceramides, of any mass, at any amount within the linear dynamic range, independent of subclass, can be predicted with high accuracy and precision (shown as a 3D surface in **Figure 2.5D**). Using this 3D response model and the molecular weight, the exact amount of each observed ceramide in SC_{ev} were calculated (**Figure 2.5E**).

2.3.6 Application of the method

Simultaneous profiling and quantification of SC ceramides was performed in samples of three different origins (SC_{ev} , SC_{cul} , and SC_{tape}). First, qualitative analysis was performed using ion map profiles of the 3 different sample types (**Supplemental S2.9**). The profiles of SC_{tape} were similar to SC_{ev} . In SC_{cul} sample an increase in ceramide S subclasses and ceramides with a 34 carbon chain length was observed, monounsaturated ceramides (MuCer): ceramides with a cis-double bond in the acyl chain were observed as well and included in further analysis. Yet, to express these observations in a more relevant manner in relation to the total lipid amount or amount of SC, further quantitative analysis is required.

All samples were quantified using the method described above. In SC_{ev} and SC_{cul} , ceramides comprised 12% of all lipids extracted from SC. Next, the amount of ceramides in SC_{ev} and SC_{cul} is expressed per mg of SC. SC_{tape} samples were corrected for the amount of tape-stripped material. **Figure 2.7A and B** depicts the total amount of every subclass in all samples. SC_{ev} and SC_{tape} samples had similar subclass distributions and show two distinct patterns of subclasses with either NP and NH increase or AP and AH increased. No changes in subclasses were observed over the depth of the SC determined by tape-stripped at the same site (**Figure 2.7C**). When the amount of the subclasses in SC_{ev} is compared to SC_{cul} , an increased amount of all S class ceramides is observed and a decrease of all P class ceramides. Furthermore, an increased amount of MuCer is observed in SC_{cul} (**Figure 2.7B**).

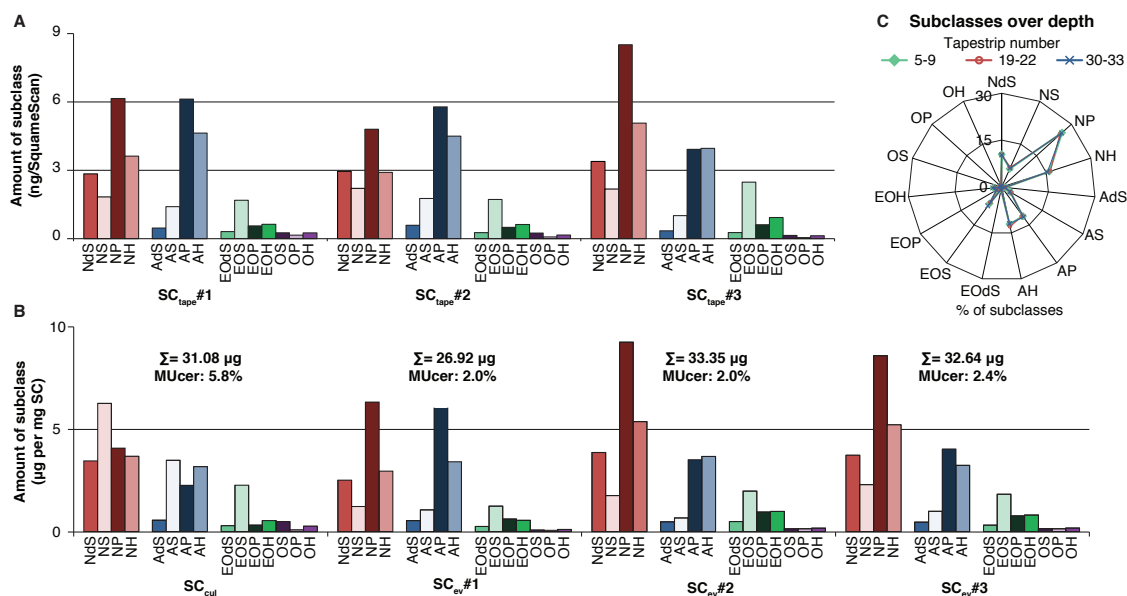


Figure 2.7: Amount of the all ceramide subclasses in different SC samples types.

A: The total ceramide subclasses amount (ng/SquameScan) of 3 SCtape samples obtained in different individuals. **B:** The total ceramide subclasses amount (µg/mg SC) in a SCcul sample and in three SCEv samples. The total ceramides amount in µg and the MUcer percentage are depicted above the graph. **C:** The percentage of the different subclasses in 3 SCtape samples obtained at the same site at different depths of the SC.

In addition to ceramide subclasses, LC/MS permits studying ceramide chain length. The mean chain length of SC ceramides is an important biological parameter. Using the molar amount of the individual ceramides, the mean total ceramide chain length was calculated as well as the mean chain length per individual ceramide subclass (**Figure 2.8A**). Though no difference can be observed between SCEv and SCcul in the average total chain length, clear changes are observed in the mean chain length of individual subclasses being either shorter or longer in SCcul. The total amount of the very short ceramides with a chain length of 34 carbons (C34 ceramides) was found to be increased in SCcul (**Figure 2.8B**). The difference in amount of the C34 ceramides and the average chain length are compared in individual subclasses and inversely correlated. For EOS a longer average chain length was observed in SCcul due to an increase of the very long chain length ceramides EOS(C72).

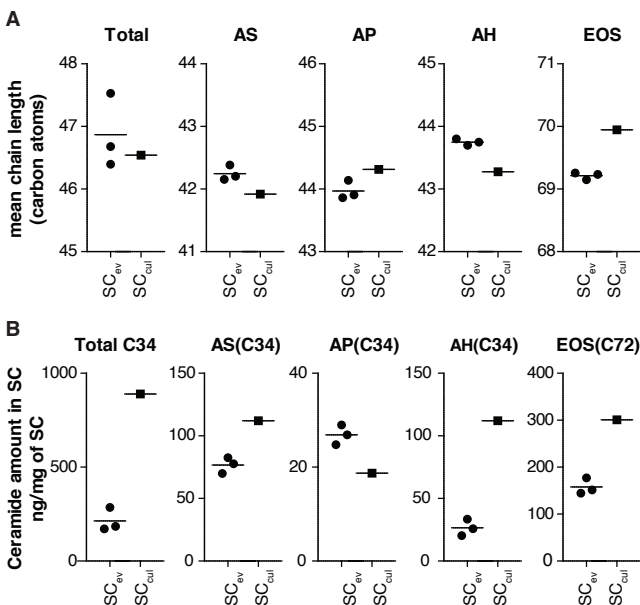


Figure 2.8: The effect of individual ceramides on subclass

A: The mean chain length of the total and the ceramide subclass AS, AH, AP, and EOS in SC_{ev} and SC_{cul}. **B:** The total amount of C34 ceramide ($\mu\text{g}/\text{mg}$ SC) and the amount of ceramide AS(C34), AH(C34) AP(C34) and EOS(C72) (ng/mg SC) in SC_{ev} and SC_{cul}.

2.4 Discussion

In this paper a method for the quantification of all ceramides in different commonly used SC samples using full-scan LC/MS analysis is presented. This analysis can include unexpected changes in the ceramide profile due to experimental conditions, requiring full scan MS measurements to obtain such data. The observed presence of the MuCers in the SC_{cul} sample illustrates the advantage and importance of full scan profiling. To quantify these samples, a 3D response model applicable to full scan MS data and using a limited amount of synthetic ceramide standards needed to be developed. The latter is important as there is only a limited amount of lipid standards available. Most current SC ceramides LC/MS methods are either qualitative^{14,18,19,24,26} semi quantitative^{4,23}, quantify a limited number of ceramides^{20-22,25,39}, or use a single standard for all ceramide species⁴⁰. Until now, only one method has been reported focusing on a comprehensive quantitative analysis of all ceramides in SC, however, it requires a ceramide standard of each subclass and made use of Selected Ion Monitoring and was thus not able to do simultaneous profiling¹⁵. Here a novel 3D response model was constructed to predict the response for ceramides without standards based on two known parameters: chain length and subclass. A standard of every subclass was not required as ion ratios can be calculated from QCs and isotope abundance can be predicted. With APCI as ionization source – instead of the more commonly used electrospray source – changes in sensitivity due to subclasses were expected to be minimal⁴¹ and no significant change in response factors of isobaric compounds of different subclasses were observed. The

equal distribution of the residual error in the response factor model supports these findings. Using chromatograms of the monoisotopic masses of the most abundant ion and thereafter modelling the contribution of the isotope distribution and other ions was preferred over quantification using large and multiple mass ranges chromatograms because: I) it reduces error, II) has less chance of including overlapping ions in the chromatogram (e.g. other ceramide ions or contaminants), and III) the sample processing times are decreased and processing accuracy is increased.

To fully quantify all ceramides in a SC sample it is essential that all ceramides are extracted from the SC matrix. Many methods are used to extract SC ceramides^{4,10,14,15,20,21,24,40}. Yet, to our knowledge none of these publications have reported the extraction efficiency of the ceramides from SC. In the present study, the 'modified' B&D resulted in a low extraction efficiency of 71.5% where not all subclasses were extracted equally as extraction was bias towards EO ceramides. This leads to skewed data even when semi-quantitative/relative data is presented. For this reason, in addition to the development of quantification of ceramides, the extraction method was optimized. It was shown that the extraction can be significantly improved by increasing temperature (reported elsewhere²¹) and further improved by adding a subsequent extraction step of more lipophilic solvents. The typical extraction solvents chloroform and methanol have a relatively high polar index value for optimal extraction of the non-polar ceramides. The combined method resulted in extraction efficiencies ~100%.

Different methods have been used to harvest SC lipids from the skin. Tape-stripping is a non-invasive procedure to acquire SC and preferred over the cupping method^{18,22,39}, where organic solvents are applied on the skin with a risk of skin irritation. Due to its non-invasive character, tape-stripping is easy applicable in persons with skin diseases. Because tape-stripping is sometimes perceived as hard-to-reproduce, special attention was paid to determine non-biological variations in the lipid analysis introduced by the tape-stripping procedure. Our data show that a sample with 4 SC tape-strips results in reproducible data showing mainly interindividual biological variation. This demonstrates that we developed a fast and effective non-invasive method for harvesting *in vivo* SC that can be used for accurate quantitative ceramide analysis. Further, we demonstrate that even when tape-strip samples were obtained at various SC depths, the lipid composition remains similar and that SC_{tape} and SC_{ev} have comparable subclass distributions.

When the absolute amount of ceramides was determined in different sample types biological differences in the samples representative of human skin, SC_{ev} and SC_{tape}, were observed. One group of individuals contained higher levels of subclass AH and AP and the other contained higher levels of subclass NP and NH. Yet, overall amounts of

P or H ceramides were similar, suggesting changes in α -hydroxylation of the SC fatty acids by fatty acid 2 hydrolase (FA2H)⁴². Due to the selectivity of the novel method, our data suggest that the ceramide synthesis has strong stereospecific selectivity toward the [R] stereoisomer of the α -hydroxyl fatty acid, as no [S] stereoisomer was observed in human SC, contrary to findings on FA2H *in vitro* using adipocytes⁴³. When comparing data from our LC/MS method with literature that used HPTLC, the weight fraction of ceramide in a SC lipids extract of ~12 % concurs with that observed in HPTLC analysis by others^{44,45} and by our group (unpublished data).

Nowadays, human skin models provide an important alternative to animal models, and SC_{cul} mimics human SC in many aspects. When the ceramide composition of SC_{cul} was compared with SC_{ev}, evident changes were observed originating from regeneration of SC under culture conditions. The increased amount of MuCers was observed before in human skin equivalents⁴⁶. In addition, changes resembling the ceramide composition of atopic dermatitis and Netherton Syndrome⁴⁷ were observed: a decreased amount of NP and AP with an increased amounts all S subclasses⁵ and an increased amount of C34 ceramides⁷. Potential biological explanations of the latter would be an increased activity of the ceramide synthases 5 and 6^{6,48}, which specifically produce C34 ceramides. Yet, a reduction in activity of several elongases that increases the pool of short chain fatty acids required to synthesize C34 ceramides⁴⁹ could be responsible for these changes as well. An apparent effect of the C34 ceramides is observed on the mean chain length within a subclass. The increase in the absolute levels of S subclass and a reduction in the absolute levels of P subclasses could suggests an imbalanced activity of human-dihydro- Δ 4-desaturase and human-sphingolipid-C4-hydroxylase, responsible for the production of S and P ceramides, respectively^{50,51}. Epidermal ceramides are stored as sphingolipid intermediates (sphingomyelin and glucosylceramides) and converted back to ceramide by acid sphingomyelinase and glucocerebrosidase respectively. Sphingomyelinase specifically converts back NS and AS subclass ceramides⁵². Our data suggest that the increases S subclass amount could also be due to higher activity of sphingomyelinase in the cultured skin. With the above mentioned finding in SC_{cul} we like to illustrate possible applications of this method in examining epidermal lipid processes; related to *in vitro* cultured skin SC experiments as well as clinical studies (using tape-strips). Furthermore, this method can be combined with our reported analysis of cholesterol and FFAs to get an accurate representation of the total free lipid amounts of the SC²³, as both methods use APCI-MS and both are compatible with tape-strips.

The method and model presented here provide an easy and fast way of profiling and quantifying complex ceramide mixtures of various SC extracts and could be of great value to clinical analysis of ceramides in SC of skin diseases, animal models, and cultured skin. Measurements were shown to be reproducible in a large linear dynamic

range and with minimal intraday variation and matrix effects. It is demonstrated that tape-stripping is an accurate and reproducible method for non-invasively SC harvesting.

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2

Supplementals chapter 2

Supplemental S2.1: List of the ceramides standards used with the corresponding supplier

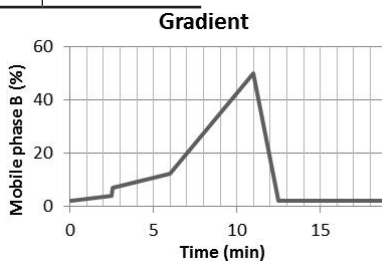
Ceramide	Supplier	Ceramide	Supplier
N(24)dS(18)	Avanti*	A(24)S(18)	Avanti*
N(18)dS(18)	Avanti*	A(R)(22)S(18)	Avanti*
N(16)dS(18)	Avanti*	A(S)(22)S(18)	Avanti*
N(24)S(18)	Evonik**	A(R)(16)S(18)	Avanti*
N(22)S(18)	Avanti*	A(S)(16)S(18)	Avanti*
N(20)S(18)	Avanti*	A(R)(24)P(18)	Avanti*
N(18)S(18)	Avanti*	E(18:2)O(30)S(18)	Evonik**
N(16)S(18)	Avanti*	E(18:2)O(27)S(18)	Evonik**
N(24)P(18)	Evonik**	E(18:2)O(30)P(S18)	Evonik**
N(22)P(18)	Evonik**	E(18:2)O(27)P(18)	Evonik**
N(16)P(18)	Evonik**	N(24deu)S(18)	Evonik**

* Avanti polar lipids (Alabaster AL, USA)

** Evonik Industries (Essen, Germany)

Supplemental S2.2: Settings used for the LC/MS method and gradient

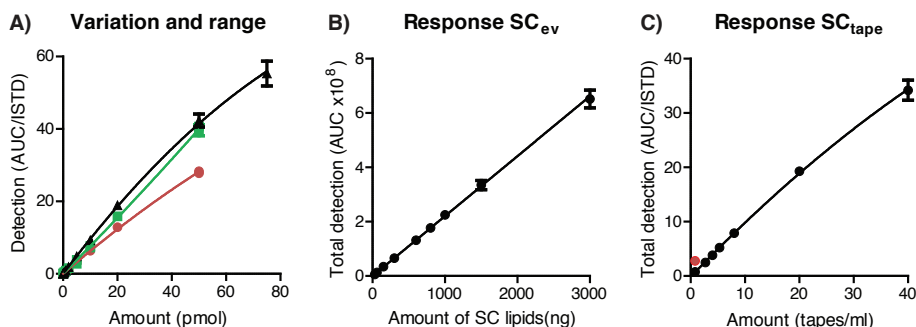
Parameter	Settings	Time (min)	Mobile phase B (%)
Needle current	5 μ A	0	2
Cone voltage	80 V	2.5	4
Offset	55 V	2.55	7
Desolvation gas	600 l/min	6	12
Cone gas	175 l/min	11	50
Nebulizer gas pressure	6.5 bar	12.5	2
Scan range	350-1200 m/z	19	2
Scan speed	0.25 ms		



Supplemental S2.3: Procedures used for extraction of the SC lipids.

For each experiment the extraction procedure used is listed. Four different extraction procedures were performed to increase the efficiency of a three step 'modified' Blight and Dyer (B&D) extraction. For the extraction efficiency a second extraction was performed on the same sample listed as 2nd extraction.

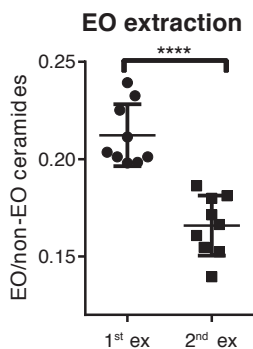
Experiment/samples	1 st extraction			2 nd extraction	
	B&D	40°C	Hep:lpa	Saponification	B&D
Extraction efficiency 1 st test	X	-	-	-	X
Condition 1	X	-	-	X	X
Condition 2	X	X	-	X	X
Condition 3	X	-	X	X	X
Condition 4	X	X	X	X	X
QC	X	-	-	-	-
Matrix effect	X	-	-	-	-
Recovery	X	-	-	-	-
Validation	X	X	X	-	-



Supplemental S2.4: Linear dynamic ranges

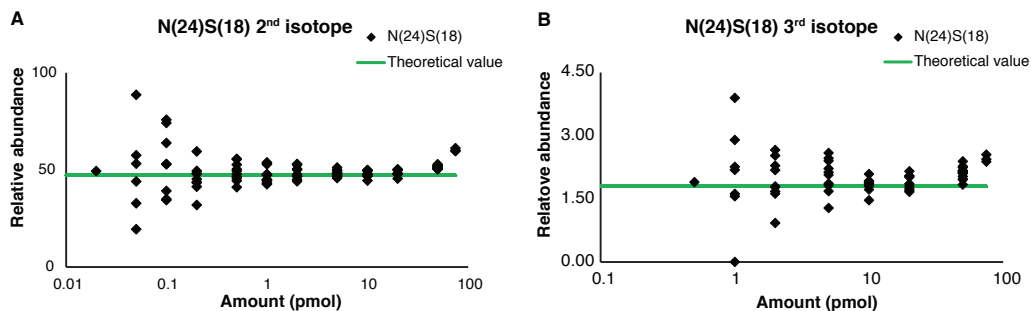
A: 9 calibration curves of N(24)dS(18) measured on 3 different days. Samples measured on the same day were plotted as mean $\pm$ SD with a 2nd order polynomial regression line drawn.

B: Calibration curve of the Σ AUC 28 abundant ceramides. Points are mean $\pm$ SD (n=3) with a linear regression line drawn **C:** The calibration curve of Σ AUC/ISTD 30 abundant ceramides in SC. Points are mean $\pm$ SD (n=3) with a 2nd order polynomial regression line drawn, in red an observed outlier is indicated.



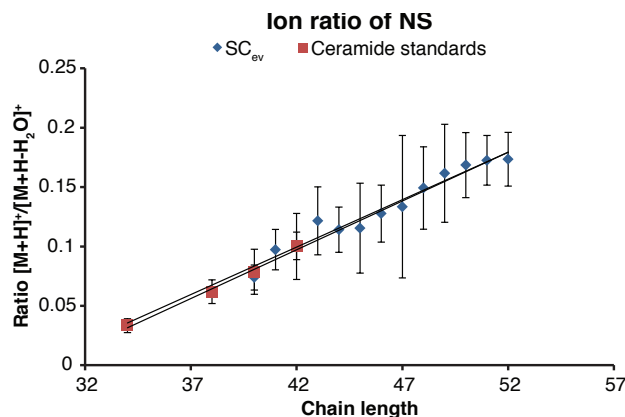
Supplemental S2.5: Extraction of EO ceramide when using a 'modified' Blight and Dyer extraction.

Two 'modified' Blight and Dyer extractions were performed consecutively. The ratio of EO ceramide to non-EO ceramides is determined in both the first (1st ex) and the second extract (2nd ex). A non-parametric student-t test was performed indicating a significant change ($p < 0.0001$).



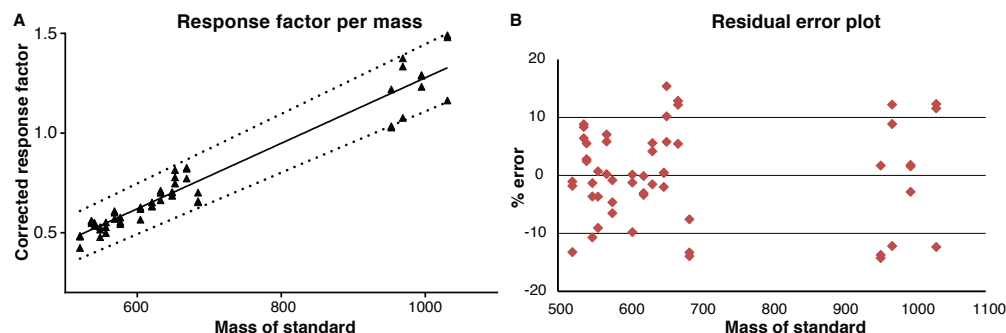
Supplemental S2.6: Checking the isotope distribution of N(24)S(18)

The relative abundance, the red lines are the predicted theoretical abundance values of N(24)S(18) A: The 2nd isotope abundance compared to the main isotope over a large concentration range. B: The 3rd isotope is compared to the main isotope over a large concentration range. At high amount detector saturation can be observed and at low amount an increased error.



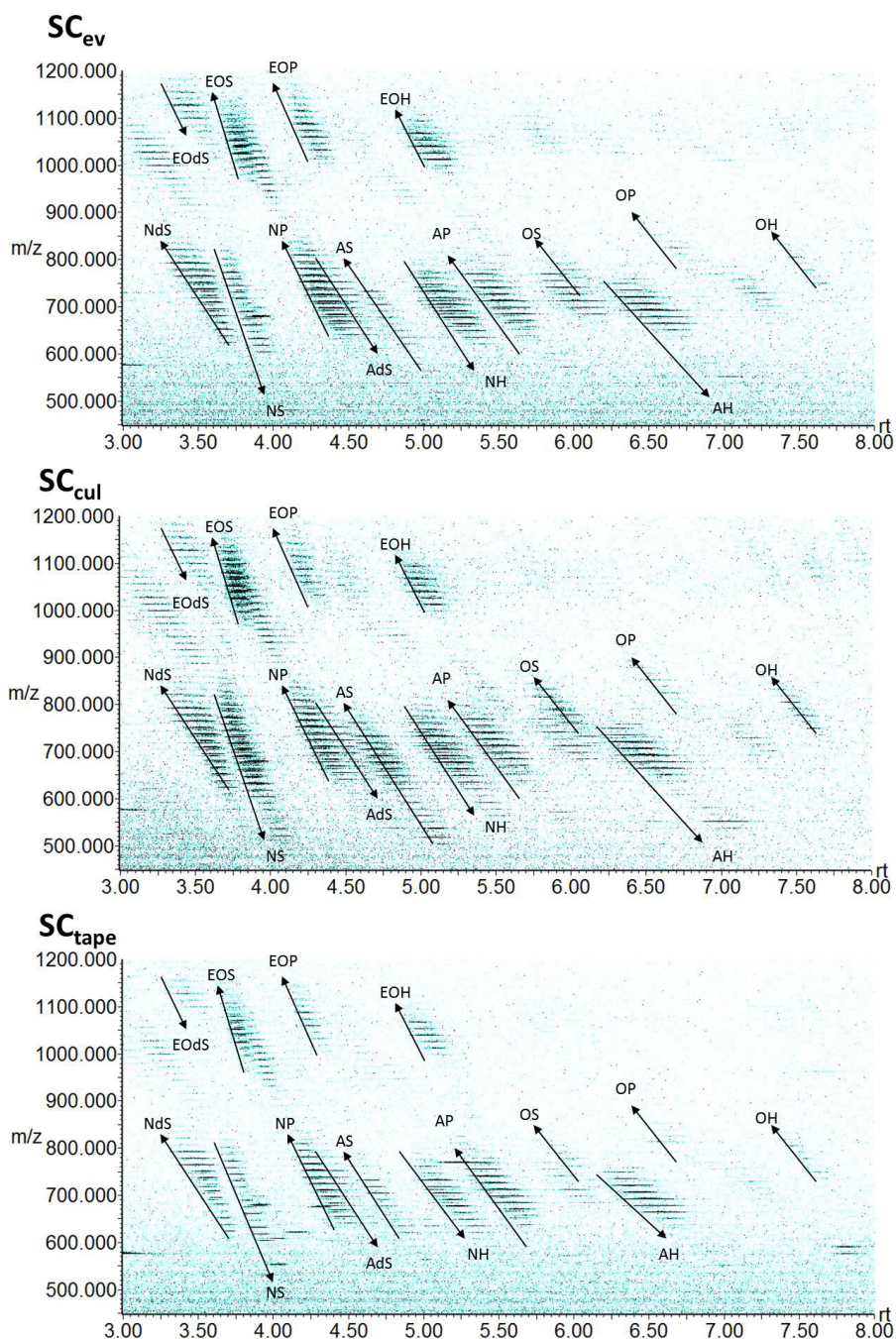
Supplemental S2.7: Ratio of $[M+H]^+$ to $[M+H-H_2O]^+$ ions of N(24)S(18)

Comparing $[M+H]^+$ to $[M+H-H_2O]^+$ in both a ceramide standard mix (red squares) and in SC_{ev} samples (blue diamonds) at different chain lengths. A linear regression is applied on both sets of data points.



Supplemental S2.8: Correct response factor per mass and percentage error plot

A: The slopes of calibration curves corrected for isotope distribution and ion contribution, plotted against the mass of the ceramide corresponding to that calibration curve. A linear regression line is drawn together with the dotted 95% predication lines. **B:** The percentage each point deviates from the regression line at the different masses.



Supplemental S2.9: Profiles of different SC lipid sample types

Representative ion maps of three different SC lipid extracts (SC_{ev} , SC_{cul} , and SC_{tape}). All subclasses are indicated by arrows, spanning over the different chain lengths.

Chapter 3

Compromising human skin *in vivo* and *ex vivo* to study skin barrier repair

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Background

Chromatograms of selected ceramides in samples from *ex vivo* and *in vivo* SC (left and right, respectively)

3

In vivo and *ex vivo* skin barrier repair

Abstract

To study skin with a compromised barrier, regenerated stratum corneum (SC) of cultured *ex vivo* skin can be used as a model. Yet, it needs to be established how close regenerated SC models mimics the lipid composition and lipid ordering of regenerated humans SC *in vivo*. Using a comprehensive ceramide analysis method we examined whether human *ex vivo* regenerated SC showed similar ceramide alterations as human *in vivo* regenerated SC. Both *in vivo* and *ex vivo* regenerated SC had increased percentages of sphingosine ceramide subclasses and decreased percentages of phytosphingosine ceramide subclasses, a reduced mean ceramide chain length, and a higher percentage of unsaturated ceramides. Predictive data modeling using the lipid parameter showed that changes in lipid organization were partly explained by changes in ceramide composition. Overall, regenerated SC *ex vivo* showed more pronounced but similar changes compared to the *in vivo* response. One of the purposes of these skin models is to use them to mimic the compromised skin of inflammatory skin diseases. The altered lipid properties in regenerated SC were comparable to those observed in several inflammatory skin diseases, showing they can be applied as models to study barrier properties in inflammatory skin diseases.

Highlights;

- Ceramide compositions of *in vivo* and *ex vivo* regenerated stratum corneum were affected similarly
- Modeling could partly predict changes in lipid ordering
- Changes in ceramide composition in both models reflects that of inflamed skin

keywords:

Regenerated
Stratum corneum;
Skin;
Human *in vivo*;
Cultured *ex vivo*;
Mass spectrometry;

3.1 Introduction

The main skin barrier function is located in the uppermost epidermal layer, the stratum corneum (SC). This layer consisting of terminally differentiated corneocytes embedded in a lipid matrix¹. A proper lipid organization and lipid composition in this matrix are important for a well-functioning skin barrier²⁻⁴. The SC barrier lipids (e.g. ceramides, fatty acids, and cholesterol) are mainly assembled in a dense orthorhombic lateral packing while a smaller lipid fraction adopts a less dense hexagonal packing^{5,6}. The SC ceramide fraction consists of a specific set of ceramide subclasses defined by their sphingoid base and acyl chain. Both chains can vary in carbon chain length and polar head group, resulting in a wide array of differently structured ceramide species (**Supplemental S3.1**).

Tape-stripping healthy human *in vivo* skin is a method to compromise the SC barrier and can be used as: a model to study several aspects of inflammatory skin diseases⁷, to examine the penetration of compounds through the skin^{8,9}, to increase the bioavailability of topical products at the deeper epidermal layers¹⁰, and to examine the biological processes of skin barrier repair¹¹⁻¹³. One of these processes is restoring the lipid composition and lipid ordering in the SC. However, little is known about the effect of barrier disruption and SC regeneration on the lipid properties of the SC and whether the regenerated SC lipid properties mimic healthy or diseased skin.

An alternative model to study skin barrier repair is using cyanoacrylate stripped *ex vivo* skin which regenerates SC over time in an incubator, this model is named the skin barrier repair (SkinBaR) model¹⁴. Several aspects of this model have been studied and are known to mimic, to some extent, the lipid properties of inflammatory skin diseases^{14,15}. However, it is unknown to what extent the ceramide composition of the SkinBaR model reflects that of tape-stripped and regenerated human skin *in vivo*. In the present study we examine how the SkinBaR model translates to skin barrier repair studies in clinical settings. Therefore, we determined the ceramides composition and lipid ordering in regenerated SC of the *ex vivo* SkinBaR model and in regenerated SC of *in vivo* tape-stripped skin. Using statistical modeling we were able to determine and compare the effects sizes of SC regeneration in the SkinBar model to human *in vivo*, examining different parameters of the ceramide composition. Both *in vivo* and *ex vivo* regeneration altered the ceramide composition and mimicked important aspects of the ceramide composition encountered in atopic dermatitis (AD).

3.2 Materials & Methods

3.2.1 Examining the compromised *in vivo* skin barrier

A clinical study was setups in which 15 healthy Caucasian volunteers between 18-29 years old participated. The study was approved by the medical ethical committee of Leiden University Medical Center and performed according to the Declaration of Helsinki. All volunteers signed written informed consent. To exclude interference with topical products, the volunteers were asked not to use soaps or cosmetics on their ventral forearms during the whole study period and one week prior to the study. At the start of the study the SC was disrupted by tape-stripping an area of 3.5 x 2.5 cm on the ventral forearm using D-Squame tape (CuDerm, Dallas, TX). Tape-stripping continued until trans-epidermal water loss (TEWL) > 60 g/m²/h and the skin had a shiny appearance¹⁶. TEWL was measured using an AquaFlux AF200 (Biox, London, UK). After 16 days of recovery, 21 tape-strips were harvested at the regenerated site and a control site at the same arm using polyphenylene sulfide tape (Nichiban, Tokyo, Japan). The first tape-strip was discarded. An infrared spectrum was recorded after every other tape-strip in order to examine the lipid ordering (see below). The *in vivo* samples obtained from control skin SC and SC regenerated after tape-stripping are abbreviated as Ctrl^{*in vivo*} and Reg^{*in vivo*}

3.2.2 *Ex vivo* human skin (SkinBaR) model

Ex vivo human skin was obtained from a local hospital, used within 12 hours after surgery, and handled according to the Declaration of Helsinki principles. Skin from 3 different donors was used in these experiments. For the SkinBaR model, skin was cleaned, processed, cyanoacrylate stripped, and cultured as described before¹⁴. SkinBaR and *ex vivo* SC was isolated and SC sheets were stored over silica gel under argon atmosphere until use for ceramide extraction or infrared spectroscopy (see below). SC obtained directly from *ex vivo* control skin, cultured SkinBaR skin, and regenerated cyanoacrylate stripped SkinBaR skin is hereafter called Ctrl^{*ex vivo*}, Cul^{*ex vivo*}, and Reg^{*ex vivo*}, respectively. Different numbers of samples for each donor were prepared for each of these conditions.

3.2.3 Ceramide analysis by LC/MS

Lipid extraction, liquid chromatography combined with mass spectrometry (LC/MS) measurements, and quantification of tape-strips and SC sheets was performed as described elsewhere¹⁷. The obtained tape-strips were punched to Ø 16 mm and extracted using a modified 4 step Bligh and Dyer at 40°C. Extracts of tapes 5-8 were combined in one sample. After extraction and evaporation of the extraction solvents,

samples were dissolved in heptane:chloroform:methanol (95:2.5:2.5)(v:v:v). Ceramides were analyzed using an Acquity UPLC H-class (Waters, Milford, MA) connected to an XEVO TQ-S mass spectrometer (Waters, Milford, MA). Processing and post-processing were performed as described before¹⁷. The total molar amount of all ceramides was determined quantitatively and used to calculate the molar percentage of each individual ceramide. The analyzed ceramide subclasses are depicted in [Supplemental S3.1](#). Ceramides standards used for calibration are listed in [Supplemental S3.2](#).

3.2.4 Lateral organization and conformational ordering of the SC lipids

Fourier-transform infrared spectroscopy (FTIR) was used to examine the SC lateral lipid organization and the conformational ordering of the SC lipids of *in vivo* and *ex vivo* skin samples¹⁸. All FTIR spectra were recorded using a Varian 670-IR spectrometer (Agilent Technologies, Santa Clara, CA). For more details see the [Supplemental S3.12](#).

3.2.5 Statistical analyses

SPSS (v23, IBM, New York, NY) was used for group-wise comparisons using linear mixed models (LMMs). LMMs were used because of the advantages of I) analysis of multiple variables and their interactions in one model, II) data pairing (e.g. multiple conditions within one subject) can be examined, III) the ability to handle missing data, and IV) the use of nested variables. LMMs were used to examine differences between the Ctrl^{in vivo} and Ctrl^{ex vivo} and the effect of SC regeneration. The effect of culturing was examined comparing Ctrl^{ex vivo} and Cul^{ex vivo}. [Figure 3.1](#) depicts the different sample groups. For more details see [Supplemental S3.12](#).

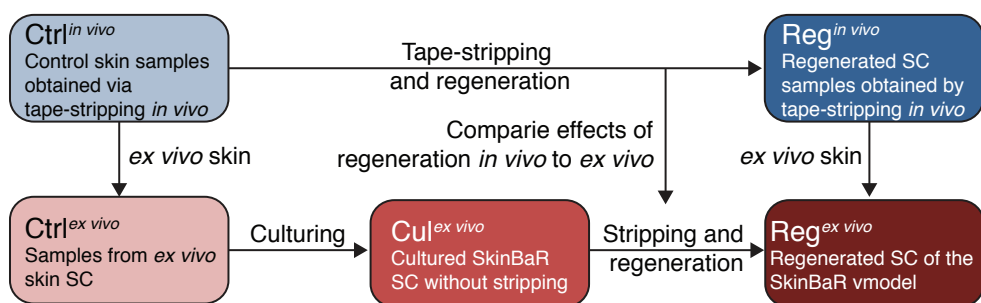


Figure 3.1: Samples and model used to compare regeneration *in vivo* to ex vivo

Five different sample types are indicated: Ctrl^{in vivo} and Ctrl^{ex vivo} are the SC samples either obtained for healthy human volunteers or ex vivo SC, respectively. Reg^{in vivo} and Reg^{ex vivo} are samples from SC regenerated after tape or cyanoacrylate stripping, respectively. Cul^{ex vivo} is SC from non-stripped SkinBaR cultures. The effect of regeneration *in vivo* was determined by the difference between Ctrl^{in vivo} and Reg^{in vivo}. The effect of regenerating ex vivo SC was determined by the difference between Cul^{ex vivo} and Reg^{ex vivo}. The effects of regeneration *in vivo* and ex vivo were compared using linear mixed modelling.

3.3 Results

To examine if SC regeneration induced changes in ceramide profile and whether SC regenerated *ex vivo* was similarly affected by regeneration as *in vivo* skin SC, the SC ceramide of the tape-strips obtained in the *in vivo* study and the *ex vivo* isolated SC were quantified. The following compositional parameters were examined: ceramide subclasses molar percentage (**Supplemental S3.1** explains abbreviations used for ceramide subclasses according to Motta et al.¹⁹), mean carbon chain length (MCL, total number of carbon atoms of both sphingoid base and acyl chain), the percentage of ceramides with a total chain length of 34 carbon atoms (C34 ceramides), and ceramides with a monounsaturated acyl chain (MuCers). We focus on these parameters as they correlated with skin barrier function in previous studies^{3,12,20,21}.

3.3.1 SC regeneration changed the ceramide subclass profile

Figure 3.2A and B show the ceramide profile of all sample groups. Similar trends due to regeneration in both *in vivo* and *ex vivo* SC were observed (Reg^{*in vivo*} vs. Ctrl^{*in vivo*} and Reg^{*ex vivo*} vs. Cul^{*ex vivo*}). Both *in vivo* and *ex vivo* regenerated SC showed increased S ceramide subclass and decreased P ceramide subclass percentages. Using LMMs, differences between *in vivo* and *ex vivo* SC regeneration were compared and statically tested. **Supplemental S3.3** depicts effects size of regeneration *in vivo* for each subclass. This showed that most subclasses levels were affected similar by regeneration, except for subclasses AH and EOH which had only decreased *ex vivo* and NdS and EOS which were only affected *in vivo*. Regenerations *ex vivo* gave similar but larger changes in NS, EOdS, EOP and OS subclasses levels. Culturing (Cul^{*ex vivo*} vs. Ctrl^{*ex vivo*}) affected the ceramide subclass in a similar way as regeneration did. Overall the control conditions Ctrl^{*in vivo*} and Ctrl^{*ex vivo*} had comparable subclass distributions, except for subclass NP, AH, EOdS, EOP, and OH. Changes in subclass AP and NH are common donor variations and EOdS, EOP and OH subclasses occur at sub one molar percentage levels, concluding, that control conditions were similar enough for a reliable comparison of regeneration effect sizes.

The percentage of linoleate esterified omega-hydroxyl acyl ceramides (EO ceramides) changed due to regeneration and was increased *in vivo* and decreased *ex vivo* (**Figure 3.2C** and **Supplemental S3.4**). However, the percentage of ceramides with an omega-hydroxyl chain (O ceramides) increased by *ex vivo* regeneration. The total percentages of ceramides with an omega-hydroxyl chain, either acylated or not, was not affected by regeneration *ex vivo*. EO ceramides are an important factor contributing to the MCL.

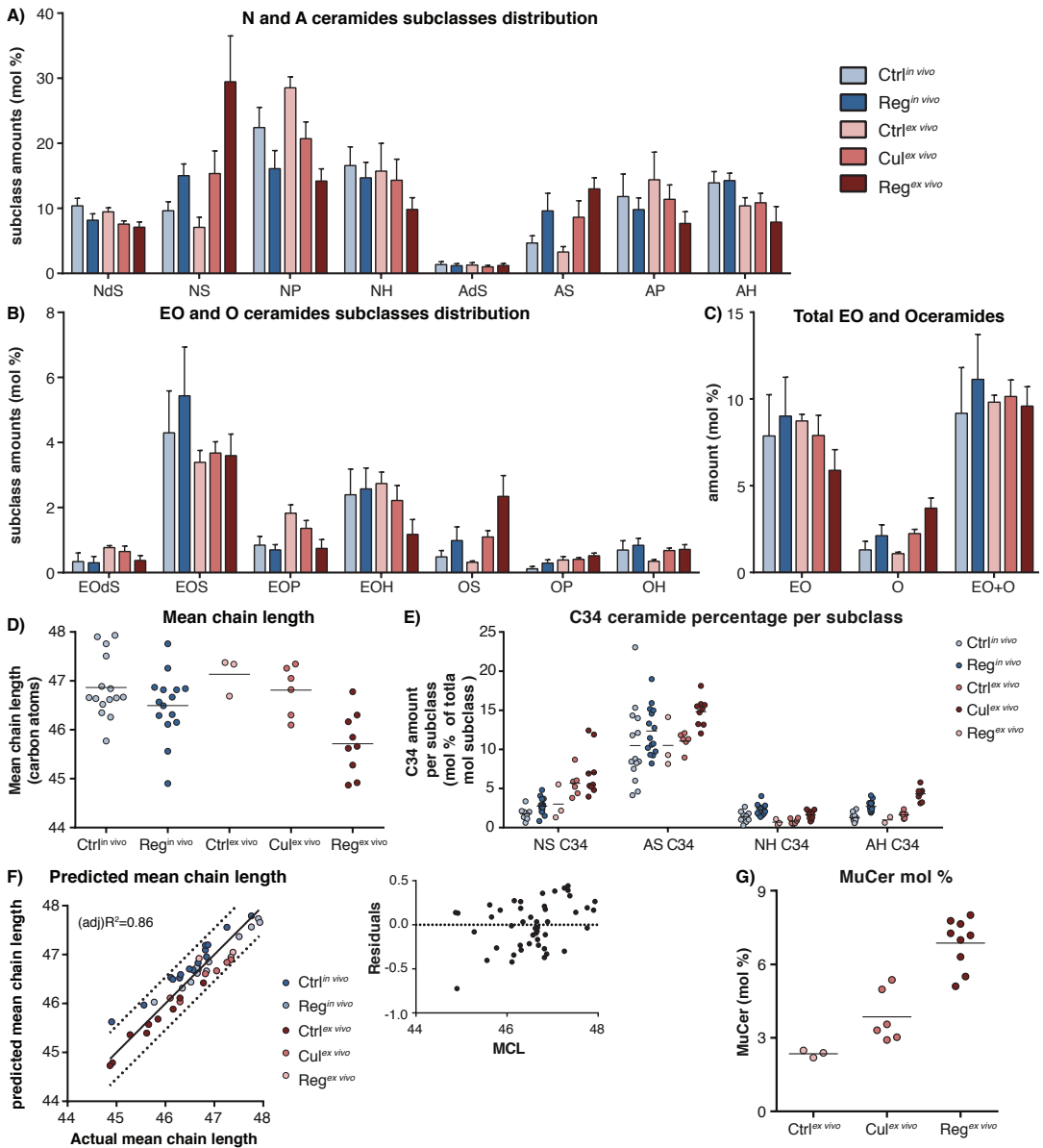


Figure 3.2: Changes in ceramide composition.

A,B: Subclass amount as molar percentage of the total molar amount of ceramides in a sample. **C:** Molar percentage of EO ceramides, O ceramides, and combined compared to total ceramide amount in a sample. Bars are mean +SD (n=15 for in vivo samples, n=3, 6, and 9 for Ctrl^{ex vivo}, Cul^{ex vivo}, and Reg^{ex vivo}, respectively). **D:** Mean carbon chain length of all samples. **E:** The molar percentage of C34 ceramides compared to the total molar amount of its subclass. **F:** The mean chain length plotted against the model predicted mean chain length. Dotted lines are 95% predictive interval around a linear regression (intercept 0:0). To the right the residuals of the model are shown. **G:** Molar percentage of ceramides with monounsaturated acyl chain compared to the total ceramide amount. Lines are the mean of the sample group.

3.3.2 Mean chain length and ceramides with a C34 chain length

Changes in MCL were expected because the EO ceramides percentage increased. The MCL of all ceramides was calculated (**Figure 3.2D** and **Supplemental S3.4**). Regeneration of the SC significantly reduces the MCL. Although, both *in vivo* and *ex vivo* regeneration reduced the MCL, it was significantly more decreased in *ex vivo* SC. Culturing did not significantly alter the MCL compared to Ctrl^{ex vivo}.

A parameter which has been linked to barrier dysfunction as well is the percentage of C34 ceramides. Regeneration and culturing both increased the total C34 ceramides percentage. Effects of regeneration were significantly larger in *ex vivo* than *in vivo* SC, while Ctrl^{in vivo} and Ctrl^{ex vivo} had similar percentages of C34 ceramides (**Supplemental S3.5 and S3.6**). To determine if the increase in C34 ceramide fraction was independent of changes in subclass (the increased S subclasses contain the most C34 ceramides), the C34 percentages within each subclass was determined. **Figure 3.2E** depicts the molar C34 ceramide percentage within subclasses NS, AS, NH and AH. Comparable increases of C34 ceramides were observed for each subclass in both Reg^{in vivo} and Reg^{ex vivo} compared to Ctrl^{in vivo} and Cul^{ex vivo}, respectively (**Supplemental S3.7**). The F-test of the fixed effect for regeneration specifies that regeneration has a significant effect on C34 ceramide percentage independent of sample type or subclass. For ceramide subclasses NP, NdS, AP, and AdS a similar increase was observed but only in *ex vivo* SC (**Supplemental S3.5B**). Due to the limited amount of SC acquired by tape-stripping these ceramide subclasses fall below the quantification limit *in vivo*. Culturing did increase the C34 percentage within subclasses and the percentage did not significantly differ for Ctrl^{in vivo} and Ctrl^{ex vivo}. It can thus be concluded that the C34 ceramide percentage is affected by regeneration independently of subclass and this is comparable between *in vivo* and *ex vivo* SC.

The C34 ceramide percentage together with EO ceramide percentage showed strong correlations to the MCL in previous studies³. A generalized linear model was created to predict the MCL using these parameters as covariates. **Figure 3.2F** displays the model predicted MCL against the calculated MCL and the residuals (model output can be found in **Supplemental S3.8**). The model had an adjusted R² of 0.87, indicating that 87% of the variation in MCL could be explained by the change in EO ceramides and C34 ceramides percentage and this was independent of sample type.

3.3.3 Ceramides with an unsaturated acyl chain

It was previously observed that MuCers were increased in the SkinBar model¹⁷ and MuCers might be affected by regeneration too. Although MuCers were detected in *in vivo* tape-stripped samples, these could not be accurately determined for all samples. The MuCer percentages of in the *ex vivo* groups are depicted in **Figure 3.2G**. It was

observed that MuCers significantly increased in CuI^{ex vivo} samples and even further due to regeneration (**Supplemental S3.9**).

3.3.4 SC regeneration does not affect lipid ordering

The lipid ordering was analyzed using FTIR. For the *ex vivo* samples CH₂ stretching vibrations in the FTIR spectra could be obtained over a temperature range from 0 to 90°C. In order to compare these results to those from *in vivo* studies, the lipid ordering at skin temperature (32°C) was examined using the center of gravity of the CH₂ symmetric stretching vibration peak in the FTIR spectra. A peak position below 2850 cm⁻¹ indicates a conformational ordering of the lipids and higher values indicated disordering. **Figure 3.3A** depicts the stretching peak position for all the samples and **supplemental S3.10** depicts the output of a LMM comparing the sample groups. The CH₂ peak position was not affected by regeneration. For the Ctrl^{ex vivo} samples the peak position was located at wavenumbers of 2849.2 cm⁻¹, respectively, which was significantly higher than in the spectra of the *in vivo* samples.

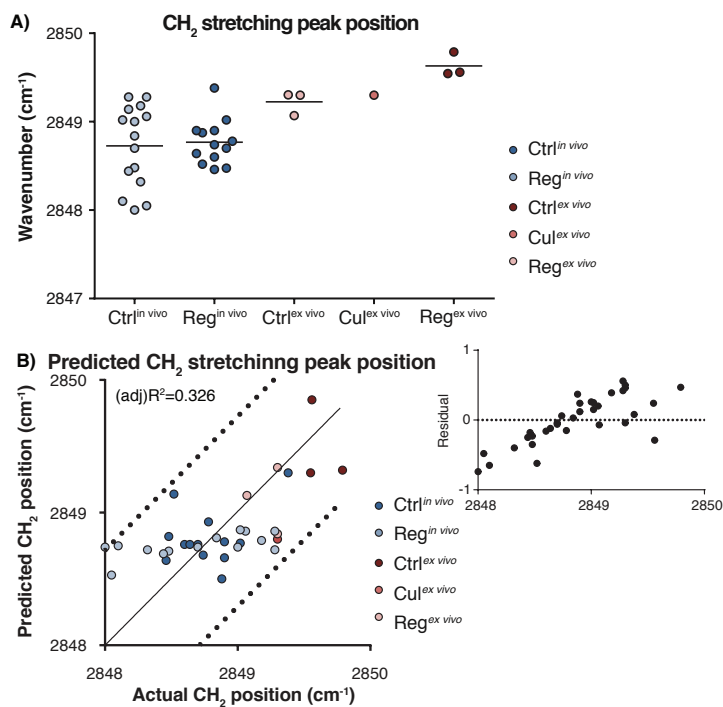


Figure 3.3: CH₂ symmetric stretching vibrations in spectra of *in vivo* skin and SkinBaR models and correlation of predicted values for the CH₂ stretching peak with observed values.

A: The CH₂ stretching peak positions in the FTIR spectra. Lines indicate the mean of the sample group. **B:** The measured CH₂ stretching peak position compared to the model predicted CH₂ stretching peak position. Sample groups are indicated by color and the dotted lines are 95% predictive interval around a linear regression (intercept 0:0). To the right the residuals of the model are shown. The adjusted R² is given.

3.3.5 Using ceramide composition to predict the lipid ordering

Due to the relation between the lipid composition and conformational ordering, the difference between the conformational ordering of the lipids in the SC of the *in vivo* and *ex vivo* samples might be explained by changes in lipid composition. To obtain insight in the contribution of ceramide compositional parameters, a predictive general linear model for the lipid ordering was made (i.e. CH₂ stretching peak position). The following parameters were used I) C34 ceramides percentage, II) EO ceramide percentage, and III) the molar ratio between the sphingoid bases as: dS+P+H/S (excluding EO ceramides). This ratio was shown to correlate with barrier function in a previous study¹². For more details about the subclass ratio, see **Supplemental S3.12**. **Figure 3.3B** depicts the measured CH₂-peak position compared to the predicted position, the inset shows the residual of the data. An adjusted R² of 0.36 that some variation in lipid ordering could already be explained using these ceramide parameters and two variables correlated significantly to the changes in lipid ordering (**Supplemental S3.11**).

3.4 Discussion

Important for the validity of a skin model is the translatability to physiological conditions. Here, we examined the translation to physiological conditions of the previously developed SkinBar model¹⁴ to the human *in vivo* situation with a focus on the SC lipids and SC regeneration.

3.4.1 *ex vivo* regenerations SC causes similar lipid alterations as regeneration *in vivo*

The ceramide composition and lipid properties were altered due to regeneration and these alterations were comparable between *in vivo* and *ex vivo* conditions. Some changes observed were more pronounced in *ex vivo* SC. The processes behind some of the lipid alterations are discussed below.

- I) Both *in vivo* and *ex vivo* regenerated SC showed a decreased molar percentage of phytosphingosine ceramide subclasses and increased percentage of sphingosine ceramide subclasses compared to control SC samples. This indicates an imbalance in activity of the enzymes acid sphingomyelinase and glucocerebrosidase, both involved in post-synthetic modification²² or in alterations in ceramide synthesis. Although the expression of the post synthetic modifying enzymes was not affected in the SkinBar model¹⁴, their activity could have been altered²³;
- II) In agreement previous observations¹⁷, the MuCers percentage was increased

in Reg^{ex vivo} SC. This increase coincides with an increased expression of Stearoyl-CoA desaturase in the SkinBaR model¹⁴. This is a lipid processing enzyme responsible for hydrocarbon chain unsaturation;

- III) The only substantial difference between the *in vivo* model and *ex vivo* model was the change in EO ceramides percentages, which was increased in the Reg^{in vivo} samples and decreased in the Reg^{ex vivo} samples. The total percentages of ceramides with an omega hydroxyl chain (EO and O ceramides subclass) were comparable to *in vivo* SC, indicating that lineolate esterification could have been affected in *ex vivo* regenerated SC.
- IV) In both *in vivo* and *ex vivo* conditions regeneration did not significantly affected the lipid ordering. In the SkinBaR samples this might be due to lesser stripping depth in the present study compared to previous studies. Previously, lipids in the regenerated SC of the SkinBaR model were less ordered when almost all SC was removed¹⁵. The difference in CH₂ stretching peak position between Ctrl^{in vivo} and Ctrl^{ex vivo} was significant and possibly due to a difference in spectrum collection methods. Nonetheless, the ceramide composition could be used to predict part of the variation in the lipid ordering. The residual error does indicate that many other factors influence the peak position

3.4.2 Epidermal proliferation in vivo and ex vivo

Previously, it has been shown that in clinical settings the proliferation rate immediately after barrier disruption is higher than after a recovery period of several days and remained elevated for at least 10 days⁷. This may be caused by an inflammatory response in healthy *in vivo* skin during skin barrier repair that slows down the repair process²⁴. As culturing *ex vivo* already induced similar changes in ceramide composition as regeneration, it can be hypothesized that culture conditions stimulate regeneration. Although, Cul^{ex vivo} samples have a decreased proliferation index they do not shed SC and do not require addition SC generation. Eight days after cyanoacrylate stripping proliferation is at the same level as Ctrl^{ex vivo} skin indicating that barrier repair is still ongoing¹⁴, as it expected to decrease due to culturing. Since barrier function recovery of stripped healthy human skin takes at least 14 days²⁵ (although longer recovery times have been observed in chapter 4 of this thesis), this period and the period of 8 days in the SkinBaR model are sufficient long to study the effect of formulations on skin barrier repair.

3.4.3 Changes ceramide composition of regenerated SC are similar to those in inflammatory skin diseases

It was shown that tape-stripping healthy skin induced parakeratosis⁷, and induced an inflammatory response by secretion of various cytokines^{26,27}. Some of these cytokines also play a role in AD and/or psoriasis^{28,29} and have been shown to induce changes in the lipid biosynthesis in the viable epidermis, such as a reduction in lipid chain length, also observed in the present study^{30,31}. When comparing the lipid properties of regenerated SC to that in SC of inflammatory diseased skin, many similarities were observed. These were I) similar changes in ceramide subclasses composition²⁰, II) a decreased MCL³², III) an increase in percentages of C34 ceramides^{20,32}, and IV) an increased percentage of unsaturated lipids³³. Most of these changes correlated with an impaired skin barrier function^{3,12,20}. Although the degrees of changes vary, many of these alterations in ceramide composition have also been observed in other inflammatory skin diseases like psoriasis, and Netherton syndrome^{31,34}. Additionally, a less dense lipid packing observed in Reg^{ex vivo} SC¹⁵ also corresponds to findings in SC of these inflammatory skin diseases: a higher fraction of lipids adopting a less dense hexagonal lateral packing has been reported³⁵. The present study focused on the composition and organization of the free extractable lipids, yet we acknowledge that the bound ceramides are an important part of the stratum corneum. Analysis of the bound ceramides might be the subject of future studies.

3.5 Conclusion

This study shows that the changes in lipid properties in both the Reg^{ex vivo} and Reg^{in vivo} models are comparable and mimic the lipid properties in inflammatory skin diseases. This concerns changes in ceramide subclass profiles, C34 ceramides, MCL, and conformational ordering. Therefore, the SkinBaR model can be used to predict the *in vivo* response with regards to the lipid composition after application of topical barrier repair treatments aiming to restore the normal ceramide composition. By doing so, the need for clinical studies is reduced.

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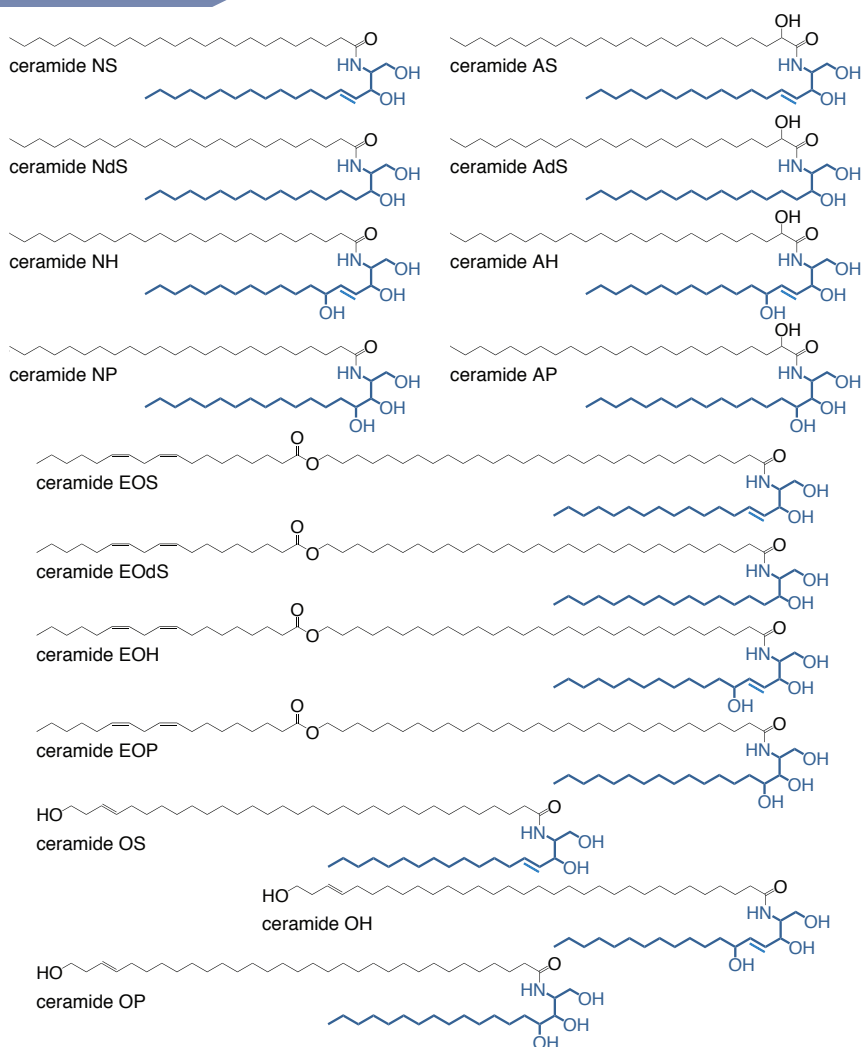
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3

Supplementals chapter 3



Supplemental S3.1: Ceramides subclasses in the stratum corneum lipid matrix

Ceramides consist of a sphingoid base coupled to an acyl chain, which can both vary in molecular structure. ceramides are named according to their molecular structure. The sphingoid base can either be a sphingosine (S), dihydrosphingosine (dS), phytosphingosine (P), 6-hydroxysphingosine (H), whereas the acyl chain is either non-hydroxylated (N), α -hydroxylated (A), ω -hydroxylated (O), or esterified ω -hydroxylated (EO). Both the sphingoid base (blue) and the fatty acid chain (black) can vary in number of carbon atoms.

Supplemental S3.2: Synthetic ceramides that were used as calibrators for LC/MS analysis.

Numbers in brackets indicate the number of carbon atoms in the fatty acid chain and sphingosine chain, respectively.

Ceramides			
N(24)dS(18) ¹	N(20)S(18) ¹	A(24)S(18) ¹	E(18:2)O(30)S(18) ²
N(18)dS(18) ¹	N(18)S(18) ¹	A(22)S(18) ¹	E(18:2)O(27)S(18) ²
N(16)dS(18) ¹	N(16)S(18) ¹	A(16)S(18) ¹	E(18:2)O(30)P(S18) ²
N(24)S(18) ²	N(24)P(18) ²	A(24)P(18) ¹	E(18:2)O(27)P(18) ²
N(22)S(18) ¹	N(16)P(18) ²		N(24deu)S(18) ²

¹ Avanti polar lipids (Alabaster, USA) ² Evonik Industries (Essen, Germany)

Supplemental S3.3: Output of linear mixed models comparing the subclass composition.

Ctrl^{in vivo} was set as the intercept of the model and the value is the mean molar percentage of selected ceramide subclass. The effect size of regeneration, ex vivo and cultured indicates the molar percentage with which the variable changes the intercept. The nested term (reg.(cu.l(evx.))) compares effect size of regeneration in vivo to the effect size of regeneration ex vivo.

	NdS		NS		NP		NH	
	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value
intercept (Ctrl ^{in vivo})	10.391	<0.001	9.647	<0.001	22.408	<0.001	16.588	<0.001
regeneration	-2.220	<0.001	5.387	<0.001	-6.312	<0.001	-1.899	0.015
ex vivo	-0.919	0.173	-2.573	0.249	6.126	0.003	-0.849	0.623
cultured	-1.936	<0.001	8.838	<0.001	-7.988	<0.001	-1.876	0.199
reg.(cul(evx.))	1.791	<0.001	8.173	<0.001	-0.035	0.977	-2.120	0.113

	AdS		AS		AP		AH	
	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value
intercept (Ctrl ^{in vivo})	1.378	<0.001	4.669	<0.001	11.829	<0.001	13.915	<0.001
regeneration	-0.180	0.015	4.935	<0.001	-2.023	0.001	0.353	0.456
ex vivo	-0.094	0.674	-1.382	0.299	2.573	0.150	-3.524	0.002
cultured	-0.294	0.038	5.596	<0.001	-2.951	0.011	0.183	0.842
reg.(cul(evx.))	0.415	0.002	-0.820	0.431	-1.757	0.086	-3.037	0.001

	EOdS		EOS		EOP		EOH	
	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value
intercept (Ctrl ^{in vivo})	0.341	<0.001	4.298	<0.001	0.846	<0.001	2.391	<0.001
regeneration	-0.040	0.241	1.140	<0.001	-0.145	0.024	0.184	0.122
ex vivo	0.429	0.007	-0.905	0.288	0.982	<0.001	0.350	0.435
cultured	-0.162	0.020	0.202	0.598	-0.531	<0.001	-0.634	0.009
reg.(cul(evx.))	-0.197	0.003	-1.137	0.003	-0.406	0.001	-1.116	<0.001

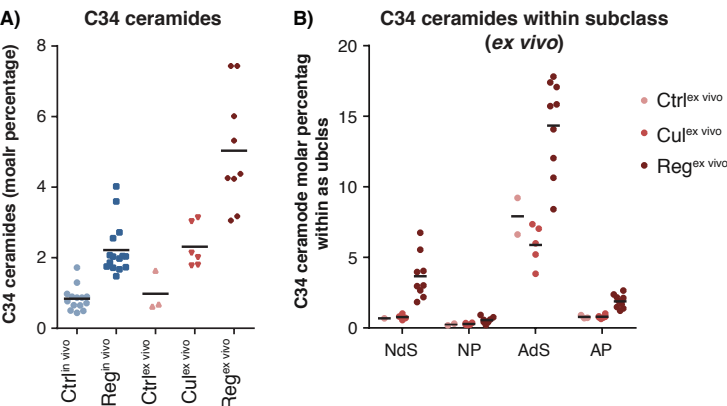
Table S3.3 contiuend

	OS		OP		OH	
	estimate	p-value	estimate	p-value	estimate	p-value
intercept ($\text{Ctrl}^{\text{in vivo}}$)	0.483	<0.001	0.119	<0.001	0.696	<0.001
regeneration	0.501	<0.001	0.174	<0.001	0.145	0.002
<i>ex vivo</i>	-0.164	0.511	0.270	<0.001	-0.349	0.031
cultured	0.830	0.001	0.018	0.761	0.317	0.001
reg.(cul(evx.))	0.694	0.002	-0.063	0.243	-0.098	0.199

Supplemental S3.4: Output of linear mixed models for omega-hydroxylated ceramides and mean chain length

$\text{Ctrl}^{\text{in vivo}}$ was set as the intercept of the model and the effect sizes of the variable are given. The nested term compares effect size of regeneration in vivo to that of regeneration *ex vivo*.

	EO		O		EO+O		MCL	
	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value
intercept ($\text{Ctrl}^{\text{in vivo}}$)	7.876	<0.001	1.298	<0.001	9.174	<0.001	46.9	<0.001
regeneration	1.139	<0.001	0.820	<0.001	1.959	<0.001	-0.4	0.010
<i>ex vivo</i>	0.856	0.545	-0.213	0.559	0.643	0.685	0.3	0.522
cultured	-1.140	0.041	1.202	<0.001	0.063	0.900	-0.4	0.094
reg.(cul(evx.))	-2.840	<0.001	0.597	0.012	-2.245	<0.001	-0.6	0.017



Supplemental S3.5: C34 ceramides percentages

The total amount of all ceramides was set at 100%. **A:** The level of C34 ceramides as percentage of the total ceramide content. **B:** The level of C34 ceramides as molar percentage within each subclass. These four subclasses could only reliably detect in *ex vivo* skin SC samples. Lines are the mean.

	estimate	p-value
intercept ($\text{Ctrl}^{\text{in vivo}}$)	0.84	0.001
regeneration	1.38	<0.001
<i>ex vivo</i>	0.14	0.804
cultured	1.41	0.025
reg.(cul(evx.))	1.26	0.028

Supplemental 3.6: Output of the liner mixed model comparing total C34 ceramide molar percentage

$\text{Ctrl}^{\text{in vivo}}$ was set as the intercept of the model and the effect sizes of the other variable are given. The nested term compares effect size of regeneration in vivo to the effect size of regeneration *ex vivo*.

Supplemental S3.7: Output of the linear mixed models for C34 ceramides within its subclass

The first part of the table specifies the F-test type III effects. Indicating if parameters were significantly changed independent of interaction variables. Here showing that regeneration is significantly affects C34 % within the subclasses, independent of sample type or which subclass is selected. Second part of the table shows the specific estimates of fixed effects with $Ctrl^{in vivo}$ set as intercept. The effect sizes of regeneration, ex vivo, and cultured are indicated with significance. The nested interaction variable indicates if the changes due to regeneration were different for ex vivo samples.

C34 ceramide percentage per subclass F-test of type III effects

Source	F	Sig.		F	Sig.
intercept	298.650	<0.001	ex vivo * subclass	0.401	0.753
regenerated	31.825	<0.001	cultured * subclass	1.083	0.360
cultured	4.477	0.037	reg* subclass	2.425	0.070
ex vivo	0.114	0.737	reg.(cul(evx.))	1.508	0.222
subclass	92.262	<0.001	reg.*subclass(cul(evx.))	0.899	0.445

Parameter estimate	NS C34%		AS C34%		NH C34%		AH C34%	
	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value
intercept ($Ctrl^{in vivo}$)	1.60	0.033	10.58	<0.001	1.40	0.050	1.30	0.055
regeneration	1.15	0.153	1.76	0.008	0.91	0.243	1.42	0.056
ex vivo	1.40	0.340	-0.06	0.963	-0.71	0.623	-0.32	0.843
cultured	3.06	0.015	0.51	0.682	0.20	0.873	0.65	0.647
reg.(cul(evx.))	-0.20	0.868	2.03	0.075	-0.19	0.877	1.28	0.283

	Size	p-value	95% confidence	
			Lower	Upper
Intercept	45.32	<0.001	44.89	45.74
EOpr	0.21	<0.001	0.17	0.26
C34pr	-0.20	<0.001	-0.26	-0.15

Supplemental S3.8: General linear model output predicting mean chain length

A model predicting the MCL was created using EO and C34 ceramide percentage as covariant, EOpr and C34pr, respectively. The following parameters were observed with significance and 95% confidence interval indicated.

	MuCer molar%	
	estimate	p-value
intercept ($Ctrl^{ex vivo}$)	2.35	0.003
regeneration	3.06	<0.001
cultured	1.45	0.030

Supplemental S3.9: Output of the linear mixed models for MuCer in ex vivo SC

$Ctrl^{ex vivo}$ was set as the intercept and the effect of regeneration was nested within cultured. Effect sizes are given.

	Stretching peak position	
	estimate	p-value
intercept (Ctrl ^{in vivo})	2848.73	<0.001
regeneration	0.03	0.799
ex vivo	0.50	0.036
cultured	0.07	0.836
reg.(cul(evx.))	0.30	0.423

Supplemental S3.10: Outcome of the linear mixed models for lipid ordering in the SC matrix

The intercept indicates the mean of the peak position for the stretching vibrations in the Ctrl^{in vivo} sample. The effect size indicates the change of the peak position relative to the intercept.

	Size	p-value	95% confidence	
			Lower	Upper
Intercept	2847.93	0.000	2846.94	2848.92
C34pr	0.30	0.001	0.14	0.46
EOpr	-0.02	0.602	-0.08	0.05
Subratio	0.13	0.017	0.02	0.24

Supplemental S3.11: General linear model output predicting CH₂ stretching peak position of the FTIR spectrum.

A model to predict the CH₂ stretching peak position was made using the molar ratio dS+P+H/S between the sphingoid base of the ceramide (excluding EO ceramides), EO ceramide percentage, and C34 ceramide percentage as covariates. The following parameters were observed with significance and 95% confidence interval indicated.

Supplemental S3.12

Chemicals

For culturing the SkinBaR models the following chemicals and materials were used: Cyanoacrylate (Bison, Goes, the Netherlands), DMEM, Ham's F12, and penicillin/streptomycin were obtained from Fisher Scientific (Waltham, Massachusetts, USA), bovine serum albumin, sodium bromide, ethanol, acetone, trypsin, trypsin inhibitor, selenious acid, hydrocortisone, isoproterenol, L-carnitine, L-serine, insulin, α -tocopherol acetate, ascorbic acid, arachidonic acid, linoleic acid, and palmitic acid were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).

For SC lipid extraction and LC/MS analysis the following solvents were purchased: methanol and chloroform were obtained from Lab-Scan (HPLC grade, Gliwice, Poland), isopropanol and ethanol were bought from Biosolve (UPLC grade, Valkenswaard, the Netherlands), and heptane was acquired from Actua-All (HPLC grade, Oss, the Netherlands). The synthetic ceramide listed in supplemental S3.2 were used as calibrators for LC/MS analysis.

Inclusion and exclusion criteria for in vivo study

Prior inclusion, a dermatologist checked whether the volunteer was eligible for participation. Exclusion criteria were: (history of) dermatological disorders, chronic inflammatory disease, use of systemic drug therapies, abundant hair or unnatural abnormalities on the ventral forearm, (history of) drug abuse, and pregnancy.

Stratum corneum isolation from ex vivo skin

SC was isolated from the ex vivo skin samples by placing the skin overnight in a 0.1% trypsin solution in PBS (pH 7.4) and 1 hour at 37°C. Subsequently, the SC was peeled off, washed in 0.1% trypsin inhibitor solution, and twice in Millipore water.

FTIR analysis

All measurements were performed on a spectrometer equipped with a broadband mercury-cadmium-telluride detector cooled with liquid N₂. Dry air constantly purged the sample compartments. Due to practical reasons, spectra for the *in vivo* SC and the SkinBaR SC were collected in reflection and transmission mode, respectively.

In vivo samples:

In order to measure the lipid organization in the SC on the arms of the volunteers, attenuated total reflection FTIR (ATR-FTIR) was used. The spectrometer was connected to an external ATR accessory with a single reflection diamond (GladiATR, PIKE technologies, Maddison USA). Each spectrum was an average of 150 scans with a spectral resolution of 2 cm⁻¹ measured at skin temperature. The instrument software Resolutions Pro 4.1 (Agilent Technologies) was used for data treatment. The center of gravity of the CH₂ symmetric stretching peak was determined at 90% of the peak height to determine the lateral ordering of the lipids. The centers of gravity 5 spectra were combined (e.g. after tape 2-10, and after tape 12-20) for analysis.

Ex vivo samples:

24 hours prior to data collection, the isolated SC sheets were hydrated over a 27% NaBr solution in D₂O at room temperature. After hydration, the SC sheet was sandwiched between two AgBr-windows and measured in transmission mode. During the measurement, the sample temperature increased from 0 to 90°C. Spectra were recorded during 2 minutes at a 1°C interval, and as a co-addition of 128 scans using 1 cm⁻¹ resolution. The center of gravity of the CH₂ symmetric stretching peak at skin temperature (32°C) was determined as described above.

Statistical analysisLinear mixed models

LMM were used in order to compare the means of several variables regarding CER composition and lipid organization between the five sample groups. The models were setup so that the effect of regeneration *in vivo* could be compared to regeneration *ex vivo*. Three variables were chosen as fixed factors: I) if the sample was *in vivo* or *ex vivo* (Ex vivo), II) if the SC was regenerated or not, and III) if the sample was cultured or not. To accommodate and test for the possibility that regeneration had a different effect *in vivo* compared to *ex vivo*, a separate nested factor for regeneration was created. Nesting was performed to obtain reliable F-test statistics. This regeneration factor was nested in culturing which was nested in *ex vivo*. This because only *ex vivo* skin can be cultured and for *ex vivo* skin only cultured *ex vivo* skin can be regenerated. The resulting term compares the effect size of regeneration *in vivo* (difference between Ctrl^{*in vivo*} and Reg^{*in vivo*}) to the effect of regeneration in the SkinBar model (difference between Cul^{*ex vivo*} and Reg^{*ex vivo*}). The starting point of the models (intercept) was chosen to be the *in vivo* control samples.

w

General linear models

General linear modeling was performed to compare a variable (like MCL) with multiple other variables. These are set as covariates, without interactions, and with prediction of the intercept.

These models predict the amount of change (in MCL) caused by the other parameters (for MCL molar fraction of EO and C34 ceramide) which is added to the intercept. To obtain a predictive model for the CH₂ stretching peak position we chose variables that have previously been used or are expected to affect the lipid ordering. C34 and EO percentage were chosen, as both have been related to lipid organization¹ and they are predictors of the average chain length¹ therefore omitting the mean chain length in the model. Ratios between the subclasses have been shown to affect the lipid organization²⁻⁴. Here, the ratio was chosen which is an expression of sphingoid bases, as EO ceramides are included as a separate factor these were left out of this ratio. The model uses these 3 parameters as covariant.

Supplemental references:

- 1 Janssens, M. *et al.* Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *J. Lipid Res* **53**, 2755-2766 (2012).
- 2 Boiten, W. A. *et al.* Applying a vernix caseosa based formulation accelerates skin barrier repair by modulating lipid biosynthesis. *J Lipid Res* **59**, 250-260 (2018).
- 3 Groen, D., Poole, D. S., Gooris, G. S. & Bouwstra, J. A. Investigating the barrier function of skin lipid models with varying compositions. *European journal of pharmaceuticals and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V* **79**, 334-342 (2011).
- 4 Skolova, B., Kovacik, A., Tesar, O., Opalka, L. & Vavrova, K. Phytosphingosine, sphingosine and dihydrosphingosine ceramides in model skin lipid membranes: permeability and biophysics. *Biochimica et biophysica acta* **1859**, 824-834 (2017).



Part II

Chapter 4

Applying a vernix caseosa based formulation accelerates skin barrier repair by modulating lipid biosynthesis

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Background:

Smoothed chromatogram of the two ceramides in the vernix formulation

4

Barrier repair treatment in healthy volunteers

Abstract

Restoring the lipid homeostasis of the stratum corneum (SC) is a common strategy to enhance the skin barrier function. Here, we used a ceramide containing vernix caseosa (VC) based formulation and were able to accelerate barrier recovery in healthy volunteers. The recovery was examined over 16 days by monitoring trans-epidermal water loss (TEWL) after barrier disruption by tape-stripping. Four skin sites were used to examine the effects of both treatment and barrier recovery. After 16 days, samples were harvested at these sites to examine the SC ceramide composition and the lipid organization. Changes in ceramide profiles were identified using principal component analysis (PCA). After barrier recovery, the untreated sites showed increased levels of ceramide subclass AS and ceramides with a 34 total carbon-atom chain length, while the mean ceramide chain length was reduced. These changes were diminished by treatment with the studied formulation, which concurrently increased the formulated ceramides. Correlations were observed between SC lipid composition, lipid organization, and TEWL, and changes in the ceramide subclass composition suggesting changes in the ceramide biosynthesis. These results suggest that VC based formulations enhance skin barrier recovery and are attractive candidates to treat skin disorders with impaired barrier properties.

keywords:

*Epidermis,
Clinical study,
Mass Spectrometry,
Lipidomics,
Sphingolipids,
Lipid Organization,
Vernix Caseosa,
Barrier Recovery,
Stratum Corneum,
Treatment.*

4.1 Introduction

Vital functions of the stratum corneum (SC) are preventing excessive water loss from the body and protecting the body from the hostile outside environment by acting as an inside-out and an outside-in barrier, respectively. The SC is generated by a dynamic process in which keratinocytes in the lower layer of the epidermis undergo terminal differentiation and transform into corneocytes¹. During this differentiation process, lipids are synthesized, stored in lamellar bodies and, at the interface between the viable epidermis and SC, these lamellar bodies are secreted in the intercellular regions². The secreted lipids form an extracellular lipid matrix crucial for a proper skin barrier^{3,4}. Three main lipid classes have been identified: cholesterol, fatty acids, and ceramides. The latter constitutes the group of SC lipids that have the most diverse chemical structure. Their building blocks are an array of sphingoid bases and acyl chains chemically linked together by an amide bond, resulting in 16 ceramide subclasses⁵. Both chains (the sphingoid base and acyl chain) can vary substantially in carbon chain length. The composition of the SC ceramide fraction (the ceramide profile) was correlated with and shown to be important for lipid organization and skin barrier function⁶⁻⁸. In the present study, the effect of a ceramide containing formulation on skin barrier recovery, the ceramide composition, and lipid organization were studied.

Through the processes of cornification and desquamation, the SC is continuously rejuvenated^{9,10} and can thereby quickly recover the barrier function after disruption¹¹. An impaired skin barrier function is encountered in several inflammatory skin diseases; one of which is atopic dermatitis (AD). In AD an inadequate recovery of the impaired skin barrier perpetuates the condition. Normalization of the barrier function in AD is crucial to reduce the penetration of irritants and allergens into the skin. Application of lipophilic formulations that act as an additional barrier is a common treatment¹²⁻¹⁵. Another approach aims at normalization of the ceramide composition⁴. Changes in this composition were correlated to the reduced skin barrier function in AD^{8,16,17}. Previously, ceramide containing formulations have been used to study skin barrier repair¹⁸⁻²¹ and it was reported that the ratios between the three main SC lipids in a formulation was important for short term recovery²². Furthermore, it was suggested that long term application could enhance barrier recovery²¹. Nonetheless, it is disputed whether ceramides in a formulation actually normalize the SC lipid composition or remain on the skin surface²³. It has been shown that the orally administered essential fatty acids were incorporated in the epidermis²⁴.

How these formulations accelerate barrier recovery is not clearly understood. Therefore, the primary aim of this study was to examine how treatment with a formulation during barrier recovery affects the SC lipid composition and subsequently lipid organization. This was done by examining the normal physiological responses in

healthy volunteers. In this study, we applied a ceramide containing formulation based on the composition of the vernix caseosa (VC). This is a natural ceramide containing formulation that serves as a protective lipid film covering the fetus in the last trimester of pregnancy and during delivery²⁵. Previously, it was shown that VC like formulations were able to significantly accelerate barrier recovery in mice^{26,27}. This vernix formulation has the advantage that ceramides can be formulated. The formulation is semi-occlusive²⁸. To examine the effect of the formulation on barrier recovery, we used barrier disruption by tape-stripping, as this is an effective way to remove the barrier and induce barrier recovery in healthy skin^{27,29-32}.

Here, it is shown that treatment with the formulation resulted in accelerated barrier recovery of tape-stripped skin compared to the untreated tape-stripped skin. In regenerated untreated SC, an altered ceramide composition was observed. These alterations correlated to a decrease in skin barrier function. When applying the ceramide containing formulation, the ceramide composition was modulated towards a ceramide composition more closely mimicking that of control skin. Combined, these results indicate that barrier disruption induces changes in lipid processing during recovery, related to a decreased barrier function and that these changes in lipid processing can be modulated by application of the VC formulation.

4.2 Materials and methods

4.2.1 Chemicals

The following solvents were used for lipid extraction and analysis: Millipore water (18.2 mΩ), HPLC grade methanol and chloroform from Lab-Scan (Gliwice, Poland), UPLC grade isopropanol and ethanol from Biosolve (Valkenswaard, the Netherlands), and HPLC grade heptane from Actu-All (Oss, the Netherlands). The synthetic ceramides listed in **Supplemental S4.1** were used as calibrators for the LC/MS analysis. Trypsin and trypsin inhibitor for SC isolation were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).

4.2.2 Clinical study design

The clinical study was approved by the Leiden University Medical Center ethical committee and performed according to the declaration of Helsinki. All volunteers signed an informed consent. Eight female and seven male Caucasian volunteers, age 18 to 29 years (mean age 23 years) were recruited and checked by a dermatologist. Volunteers were excluded if they had dermatological disorders or a history of dermatological disorders, suffered from chronically inflammatory diseases, used systemic drug

therapies, had abundant hair or unnatural abnormalities on the ventral forearms, had a history of drug abuse, or if they were pregnant. All enrolled volunteers completed the clinical study.

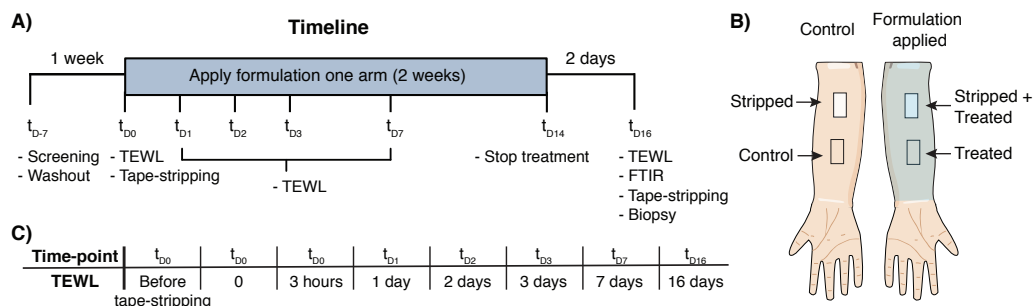


Figure 4.1: Study design

A: Timeline of the clinical study (Time points are indicated as t with days as subscripts). Volunteers were first screened by a dermatologist (t_{D-7}), followed by a one week washout period. At t_{D0} the SC was removed by tape-stripping. The formulation was applied 2x daily for 2 weeks followed by two days washout. The activities and measurements are indicated at each time point (t_{D-7} till t_{D16}). At all indicated time-points TEWL was measured, except for t_{D-7} . **B:** The studied sites on the ventral forearms. On both arms one site was tape-stripped; on one arm formulation was applied. **C:** The time-points at which TEWL was measured to monitor barrier recovery of the stripped sites.

Figure 4.1A depicts the timeline of the study. Before the study commenced, volunteers had a one week washout period in which no soaps or cosmetics were applied on the ventral forearms. At t_{D0} a site of 3.5 by 2.5 cm of SC was removed at both ventral forearms by consecutive tape-stripping using Dsquake tape (Cuderm, Dallas, USA). The tape-stripping continued until the site appeared shiny³³ and a trans epidermal water loss (TEWL) of $> 60 \text{ g/m}^2/\text{h}$ was reached (these sites are further referred to as stripped). TEWL was measured using AquaFlux AF200 (BIOX, London, UK). Thereafter, two fingertip units of a VC like formulation (composition is shown in **Supplemental S4.2**) were applied twice daily for 14 days on one ventral forearm (referred to as treated), followed by a two day washout period. Treatment allocation to either left or right arm was alternated between volunteers. **Figure 4.1B** depicts the 4 sites investigated in this study: control (no treatment, no tape-stripping), treated (treated with formulation), stripped (tape-stripped at t_{D0}), and stripped+treated (tape-stripped at t_{D0} and treated with formulation). At the final time point (t_{D16}) 21 tape-strips, using polyphenylene sulfide tape (Nichiban, Tokyo, Japan), were harvested at all 4 sites. The amount of SC on each tape-strip was determined by measuring the tape-strips with a SquameScan (Heiland Electronic, Wetzlar, Germany). Tape-strip zero was discarded. After every other tape-strip an infrared spectrum of the tape-stripped skin region was made (section 4.2.5). After tape-stripping, a 4 mm biopsy of both recovered

sites was collected near the site where Nichiban tape-strips were harvested to perform Small angle X-ray diffraction (SAXD)(section 4.2.6). During the study, TEWL of all sites was monitored at distinct time points, these measurements were performed before formulation application.

4.2.3 TEWL measurements to determine barrier recovery

To determine barrier recovery of the stripped sites TEWL was monitored. At each time point depicted in **Figure 4.1C**, TEWL was measured at three different sections of each site (**Supplemental S4.3**). The barrier recovery percentage was calculated by defining the average of three TEWL measurements at the non-stripped sites as 100% barrier and TEWL at the stripped sites directly after tape-stripping as 0% barrier. The following equation was used²⁶:

For each volunteer several values were calculated for both the

$$\text{Recovery \%}_{(\text{time-point})} = \frac{(\text{TEWL } T_0 - \text{TEWL } T_{(\text{time-point})})}{(\text{TEWL } T_0 - \text{Average TEWL nonstripped site } T_{(\text{time-point})})} * 100$$

treated and untreated stripped sites: I) the recovery percentage of the site at three sections at each time-point. II) The mean of the three previous values. III) The area under the barrier recovery curve (AUC) of the means over time, using a point-to-point linear fit. IV) Using the same fit, the time it took to reach designated recovery percentages between 5-100%, with increment of 5%. V) The difference in time between the stripped and stripped+treated sites at each of the previously mentioned recovery %. Recovery percentages >90% were excluded, as only 5 volunteers reached these values at both stripped sites, biasing these data.

4.2.4 Quantification of ceramides by LC/MS

Tape-stripped SC can be used to examine the lipid content by mass spectrometry (MS)³⁴⁻³⁶. Samples for ceramide analysis by LC/MS were prepared from tape-strips 5-8 and 17-20, harvested at the 4 sites on t_{D16} . Samples prepared from tape-strips at a depth of 5-8 and 17-20 tapes were analyzed separately to determine if the SC ceramide composition generated in the initial stage of the recovery process (tapes 5-8) was different from that generated in later stages of the recovery process (tapes 17-20). Tape-strips were punched to an area of 2 cm², extracted, analyzed and quantified as described in chapter two of this thesis³⁴. Shortly, a modified 4 step Bligh and Dyer extraction at 40°C was performed. Extracts were analyzed using an Acquity UPLC H-class (Waters, Milford, MA, USA) connected to an XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA). Separation was performed on a pva-silica column (5 µm

particles, 100 × 2.1 mm i.d.)(YMC, Kyoto, Japan). Using the calibrators, a response model was built and used to quantify the ceramides in the analyzed samples. All data were corrected for the cumulative SquameScan value (SQ) of the four tape-strips comprising the corresponding sample. The data resulted in quantitative SC ceramide profiles for each volunteer, at all 4 sites, and at two depths per site. Ceramides are named according to hydrophilic head group (subclass) and chain length (e.g. NS C42, ceramide subclass NS with a total chain length of 42 carbon atoms)³⁷. For an overview of subclasses and ceramide structures see **Supplemental S4.4**.

Quantitative data were used to calculate several parameters. Ceramides EOS C66 and NS C40 (supplied in the formulation) were excluded from these calculations. The following parameters were calculated:

1. Total amount of the individual subclasses (ng/SQ);
2. Total amount of ceramides with a total chain length 34 carbons (ng/SQ);
3. Mean carbon chain length:
4. Percentage of EOCers, as $\frac{\sum \text{each ceramide}(\text{molar amount ceramide} \times \text{carbon chain length})}{\text{total molar amount all ceramides}}$

4.2.5 Lateral organization and conformational ordering of SC lipids

The lateral organization and conformational ordering of the SC lipids were examined by attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR). FTIR spectra were collected using a Varian 670-IR spectrometer (Agilent Technologies, Inc., Santa Clara, CA) equipped with a mercury-cadmium-telluride detector and an external sample compartment containing an ATR accessory (GladiATR, PIKE Technologies, Maddison, WI) with a single reflection diamond. The sample compartment was constantly purged with dry air. Each spectrum was an average of 150 scans, with a spectral resolution of 2 cm⁻¹. Conformational ordering of the lipids was determined using the center of gravity of the peak of the CH₂ symmetric stretching vibrations at 90% of the peak height. The bandwidth from the second derivative of the CH₂ scissoring region was selected as a measure for lateral packing of the lipids and determined as described before^{38,39}. CH₂ stretching vibration peak position values <2850 cm⁻¹ indicate an ordered phase, whereas values above 2852 cm⁻¹ indicate a high degree of disordering being a liquid phase. The second derivative of the CH₂ scissoring region was baseline-corrected between the endpoints of the scissoring region (~1460-1480 cm⁻¹) and the full width at half maximum (FWHM) was calculated³⁹. Values of 5 spectra (tape 2-10 and tape 12-20) per site per volunteer were used for the analyses.

4.2.6 Small angle X-ray diffraction

SC isolated from biopsies harvested at stripped and stripped+treated sites were examined by SAXD. SAXD was used to determine the SC lipids lamellar organization. To isolate SC for SAXD analysis, biopsies were placed overnight in a 0.1% trypsin solution in PBS at 4°C, followed by one hour incubation at 37°C. The SC was peeled off and washed in 0.1 % trypsin inhibitor solution in PBS and twice in Millipore water. Dried SC sheets were stored under argon and over silica gel until use. All SAXD measurements were performed at the European Synchrotron Radiation Facility (Grenoble, France) at station BM26B. SAXD patterns were collected on a Pilatus 1M detector at room temperature for 5 minutes. The sample-to-detector distance was 2m. Prior to data collection, SC samples were hydrated over a 27% (w/v) NaBr solution for 24 hours at room temperature. The scattering intensity I (arbitrary units) was measured as a function of the scattering vector q (in nm^{-1}), defined as: $q = (4\pi \sin(\Theta)) / \lambda$, in which Θ is the scattering angle and λ is the wavelength. From the position of the main peak (referred to as peak 2 with position q_2), the spacing (d) of the lipid lamellae was calculated using $d = 2\pi / q_2$. As in SC this peak position is determined by the structure of the two lamellar phases, a change in the spacing indicates a change in these lamellar phases⁸.

4.2.7 Software and statistical analysis

All LC/MS data were processed using MassLynx and TargetLynx software (V4.1 SCN 843 Waters Inc.). FTIR data were processed and analyzed using Resolutions Pro 4.1 (Agilent Technologies, Inc.). Principal component analysis (PCA) is a commonly used analysis of lipidomics datasets^{40,41}. It is a mathematical dimensional reduction that can effectively visualize variation and the difference between samples in large datasets by reducing all variables to principal components (PCs). PCA was performed using Multibase add-in for excel (NumericalDynamics.Com). For PCA, the quantitative data obtained from the ceramide analyses were normalized to the total amount of ceramides in the samples. The four individual sites (control, treated, stripped and stripped+treated) of each volunteer were defined as PCA samples. All quantified ceramides at both depths were defined as variables of that "PCA sample" (total of 584 variables), thereafter, the mean of each ceramide/variable in all "PCA samples" was centered at zero (subtracting the mean).

Correlation analysis and point-to-points fits were performed using GraphPad Prism (V6.05, Graphpad). Group-wise comparisons were performed using linear mixed models (LLMs) in SPSS (V24 IBM). All measurements within the same subject were treated as repeated measurement. LMMs were used because these models have the advantage that 1) the effect size of individual variables and their interactions can be

examined, and II) they can handle missing data⁴². **Supplemental S4.5** explains how the LMMs were used here. All interactions between fixed variables were included in the first run of a model and when non-significant ($p > 0.05$) taken out one by one. Significance levels are indicated as followed: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.005$) and **** ($p < 0.001$).

4.3 Results

4.3.1 Barrier recovery in healthy volunteers

To determine the effect of the formulation on barrier recovery, changes in skin barrier over time (measured as TEWL and expressed as recovery %) were examined. **Figure 4.2A** shows a typical example of barrier recovery % as function of time for untreated and treated stripped sites of one volunteer. After 16 days, all volunteers had reached a recovery % > 85 at both sites. For each volunteer the AUC was calculated of both the untreated and treated stripped sites (**Figure 4.2B**). A larger AUC implies less barrier dysfunction during the 16 days. Treatment with the formulation significantly increased the AUC, indicating increased recovery.

Yet, the AUC does not take into account the kinetics of the recovery process. To determine if treatment accelerated barrier recovery, differences in time between untreated and treated stripped sites were examined at multiple designated recovery percentages (Δt in **Figure 4.2A**). **Figure 4.2C** depicts the median and interquartile range of Δt at different recovery percentages for all volunteers. A median of zero would indicate no change in recovery time due to treatment. A positive deviation from zero was observed at almost all recovery percentages with a spike at 25%, and a steady increase above 50%. Treatment significantly accelerated barrier recovery ending with a more than two days faster median recovery. As control both the AUC and the Δt were tested for correlation to the TEWL directly after tape-stripping (**Supplemental S4.6**). No correlations were observed, indicating that these parameters were not dependent on the amount of barrier disruption.

4.3.2 Principle component analysis of ceramide profiles

To gain insight into differences between the ceramide profiles of the four different sites PCA was performed on the quantitative ceramide data. **Figure 4.3A** depicts the variable loadings, indicating how strongly each variable (ceramides detected in the samples) correlated to PC1 and PC2. If the value is higher (deviates from center of the graph in x or y position) it has contributed more to the variation explained by PC1 or PC2, respectively. The 10 variables with the highest correlation coefficients are

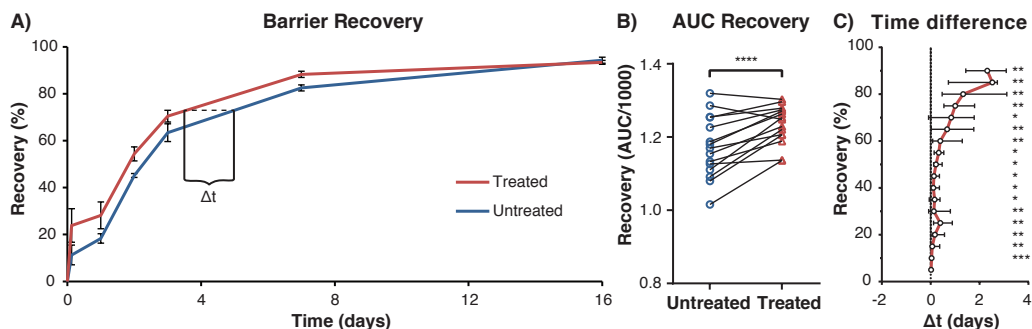


Figure 4.2: The effect of treatment on barrier recovery

A: The typical elapse of barrier recovery over time for untreated and treated stripped sites, for a single volunteer. Points are means ($\pm$ SD) of three measurements within one site. Lines are point-to-point connections. The dotted line indicated the difference in time (Δt) between the untreated and treated curve at a designated recovery %. **B:** The AUCs/1000 for all volunteers. Lines connect data points of the same volunteer. A paired t-test showed significant increase at the treated sites. **C:** The Δt for all volunteers at recovery percentages between 5 and 90 %. The data were non-normally distributed and are shown as median and interquartile range. Deviations from zero were tested with a Wilcoxon signed-rank test.

indicated in the plot. PC1 and PC2 explained a total inertia (variance) of 66.3%. **Figure 4.3B** shows the plot of the “PCA samples”, depicting the PC1 and PC2 scores of each “PCA sample”. Samples were assigned to clusters corresponding to the 4 sites indicated by different colors. Separation between clusters was observed demonstrating that variation between the ceramide profiles corresponded to variation between samples of different sites. Therefore, changes in ceramide profile due to treatment and tape-stripping can be explained by the variable loadings of the PCs.

Clusters of treated sites were separated from untreated sites as depicted in **Figure 4.3B**. By examining plot **4.3A** it shows that this was due to 4 variables primarily. These variables corresponded to the two formulated ceramides (NS C40 and EOS C66), detected in samples of both depths. The PCA demonstrated that a large part of the variation in the ceramide profiles was due to the two ceramides in the formulation. The increase due to formulation application of these naturally occurring ceramides was examined. **Supplemental S4.7** shows that the amount at treated sites was increased at both tape-stripping depths. An LMM of the logarithm of these amounts confirmed these observations (**Supplemental S4.8**).

Next, separation of clusters corresponding to stripped and non-stripped sites was investigated. Variables correlating strongly to PC2 were primarily responsible for the observed separation. (**Supplemental S4.9** shows a complete graphic overview of the PC2 loadings). When examining the loadings of PC2, highly positive correlations were observed for ceramides of subclass AS and, to a lesser extent, ceramides for

subclass NS. Highly negative correlations were observed for ceramides from subclass NP. Changes in ceramide profile were observed between the different sites. In the next section, the results of the PCA were used as leads to investigate if these changes correlated to changes in barrier function and the recovery process.

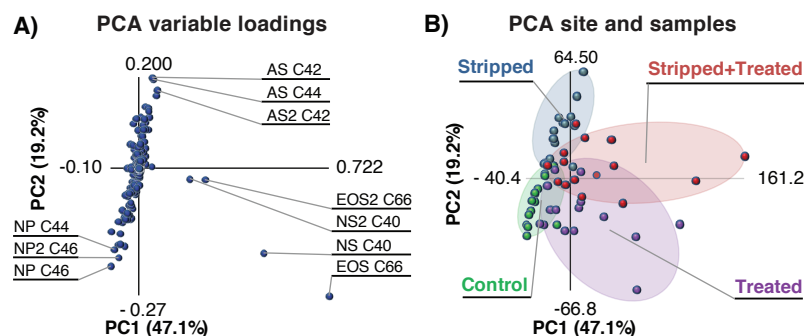


Figure 4.3: PCA of all SC ceramides

A: The PCA loading plot. Inertia per PC is indicated as % variation. Variables are all ceramides quantified in samples obtained from tape-strips 5-8 and samples obtained from

tape-strips 17-20, the latter are indicated with a 2 after the subclass name. **B:** Plot of the PCA samples (4 sites of all volunteers). Samples were clustered per site.

4.3.3 Correlations between ceramide profile and barrier function

To investigate if barrier function correlated to changes in SC ceramide profile, changes in ceramides AS and NS, identified by PCA, were correlated to the TEWL at t_{d16} (**Supplemental S4.10** shows the TEWL at t_{d16}). **Figure 4.4A and B** depicts these correlations for the total amount of subclass AS. At both depths, a significant positive correlation between the amount of subclass AS and TEWL was observed. This illustrates that an increase in subclass AS correlated with a reduced skin barrier function. Next, it was examined whether stripping, treatment, SC depth, or the interaction between these conditions had an effect on the amount of subclass AS using a LMM. The model used stripped, treated, and depth as fixed factors, and subject as random factor. **Table 4.1** depicts the effect sizes obtained with the model. **Figure 4.4C** depicts the mean ($\pm$ SD) of the amount of subclass AS measured at all 4 sites at both depths. Using the model it was determined that subclass AS increased significantly at stripped sites. Treatment or depth alone showed no significant effects compared to the control. However, at the stripped site treatment did have a significant effect. The interaction stripped*treated in **Table 4.1** illustrates this effect. When subjected to stripping and treatment the amount of AS changed by the effect sizes of stripped and treatment, in addition to both effects the interaction term stripped*treated is added. At stripped+treated sites treatment significantly reduced the increase in AS observed due to stripping with 81.2 ng/SQ (treatment + stripped*treated). Similar results were obtained when examining NS C44 (**Supplemental S4.11** and **Table 4.1**). It was decided not to use the total amount

of subclass NS, as this value was influenced by ceramide NS C40 of the formulation. NS C44 is most abundantly present of the NS ceramide subclass members and chosen as a proper representative of subclass NS.

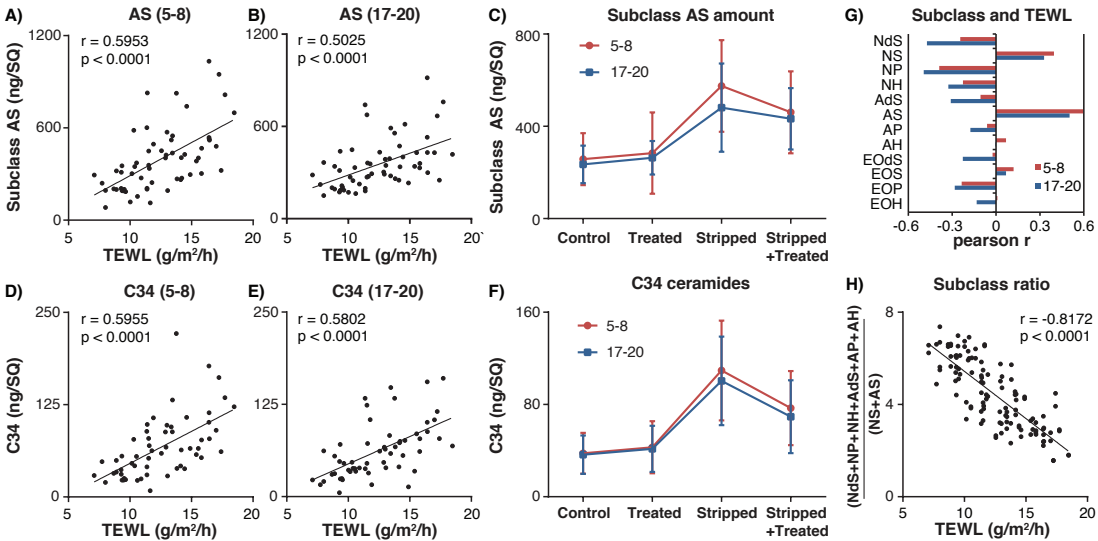


Figure 4.4: The effect of treatment on ceramide groups correlating to barrier function

A, B: Correlations between TEWL and the amount of subclass AS (ng/SQ). The Pearson r and p -value are indicated (A: Samples of tape-strips 5-8 B: Samples of tape-strips 17-20). **C:** The amount subclass AS (ng/SQ) at all sites at depths of both 5-8 and 17-20 tape-strips (mean $\pm$ SD). Effect size was determined with a linear mixed model. **D, E:** Correlations between TEWL and the amount of ceramides with a chain length of 34 carbon atoms (ng/SQ). The Pearson r and p -value are indicated (D: Samples of tape-strips 5-8 E: Samples of tape-strips 17-20). **F:** The amount of ceramides with a chain length of 34 carbon atoms (ng/SQ) at all sites at depths of both 5-8 and 17-20 tape-strips (mean $\pm$ SD). Effect size was determined with a linear mixed model. **G:** Pearson r of the correlation between TEWL and the total amount of each subclass at all sites at depths of both 5-8 and 17-20 tape-strips. **H:** The correlation between TEWL and ratio of subclasses $(\text{NdS} + \text{NP} + \text{NH} + \text{AdS} + \text{AP} + \text{AH}) / (\text{NS} + \text{AS})$ of both depths, excluding NS C40. Pearson r per site were: control=-0.648, Stripped=-0.808, Treated=-0.729, Stripped+Treated=-0.694. All statistical outputs of the LMM are found in **Table 4.1**.

Besides subclasses, ceramide chain lengths, especially ceramides with a total carbon chain length of 34 carbons (C34), are known to correlate to barrier dysfunction¹⁷. Here, a significant positive correlation was observed between TEWL and the total amount of C34 ceramides (summation of amounts of NdS, NS, NP, NH, AdS, AS, AP, and AH with a total of 34 carbons) at both depths (**Figure 4.4D and E**). This demonstrated that higher levels of C34 ceramides correlate with reduced barrier function at day 16. A similar LMM as that of subclass AS and NS C44 was built for C34 ceramides (**Table**

4.1). The total amount of C34 ceramides at the 4 sites at two depths was plotted as the mean $\pm$ SD in Figure 4.4F. By using the model a significant increase at the stripped sites was observed. Comparable to subclass AS, treatment only significantly affected the stripped+treated sites. Treatment reduced the increase in C34 ceramides with 31.6 ng/SQ compared to stripped sites.

Table 4.1: The output of the LMMs per ceramide subgroup

The intercept is the estimated amount at the control site at a depth of 5-8 tape-strips. Estimates are in ng/SQ. The effect size is the amount in ng/SQ with which the variable changes the intercept. Significance levels of all effect sizes and interaction are given.

		Subclass AS		Ceramide NS C44		C34 ceramides	
		Estimate	p-value	Estimate	p-value	Estimate	p-value
	Intercept (control)	267.1	< 0.001	56.6	< 0.001	39.4	< 0.001
Effect size	Treated	+27.3	0.403	+7.2	0.194	+5.6	0.438
	Stripped	+281.5	< 0.001	+56.4	< 0.001	+68.0	< 0.001
	Depth 17-20	-41.3	0.076	-2.1	0.597	-5.2	0.297
Interactions	Stripped*Treated	-108.5	0.020	-19.5	0.013	-37.2	< 0.001

Next, the correlation of each subclass to TEWL was examined (**Figure 4.4G**: Pearson r for each subclass). This correlation underlined the observations with PCA, where subclass NS and AS had a positive correlation and subclass NP a negative correlation to PC2. Both subclasses NS and AS have a distinct synthesis route different from subclasses NP and AP, this suggested a ratio dividing the subclasses as followed: $(NdS+NP+NH+AdS+AP+AH) / (AS+NS)$, referred to as dS+P+H/S. This ratio correlated to TEWL (**Figure 4.4G**) even for the samples of each site independently. Together, these data showed that I) barrier function correlated to the ratio of ceramide subclasses from different synthesis routes, II) certain ceramide subgroups are increased in stripped SC, III) the amount of these subgroups correlated to TEWL, and IV) treatment is able to reduce the increase of these ceramide subgroups that correlate positively to an increased TEWL (AS, NS 44 and C34 ceramides).

4.3.4 Mean carbon chain length and spacing related to the lamellar phases

Previous studies have shown that the main ceramide chain length is an important parameter of the SC ceramide fraction⁴³. For this reason it was decided to study whether a correlation existed between mean chain length and both the EOCer fraction and lamellar spacing. **Figure 4.5A** shows the mean chain length of the ceramides in samples prepared from tapes 5-8 and 17-20. A similar LMM as for subclass AS was

made (Table 4.2). This showed that stripping decreased the mean chain length significantly. Treatment was able to increase the mean chain length restoring the mean chain length at the stripped+reated sites. The increase in mean chain length of the stripped+reated sites could be due to an increase in the fraction of EOCer fraction (Supplemental S4.12 shows this correlation per site). Changes in EOCer fraction due to treatment of the stripped site (stripped – stripped+reated) correlated to changes in the mean chain length due to treatment of the stripped site (Figure 4.5B). Treatment was able to increase the EOCer fraction significantly (Figure 4.5C and Table 4.2 show the EOCer fraction and the output of LMM, respectively).

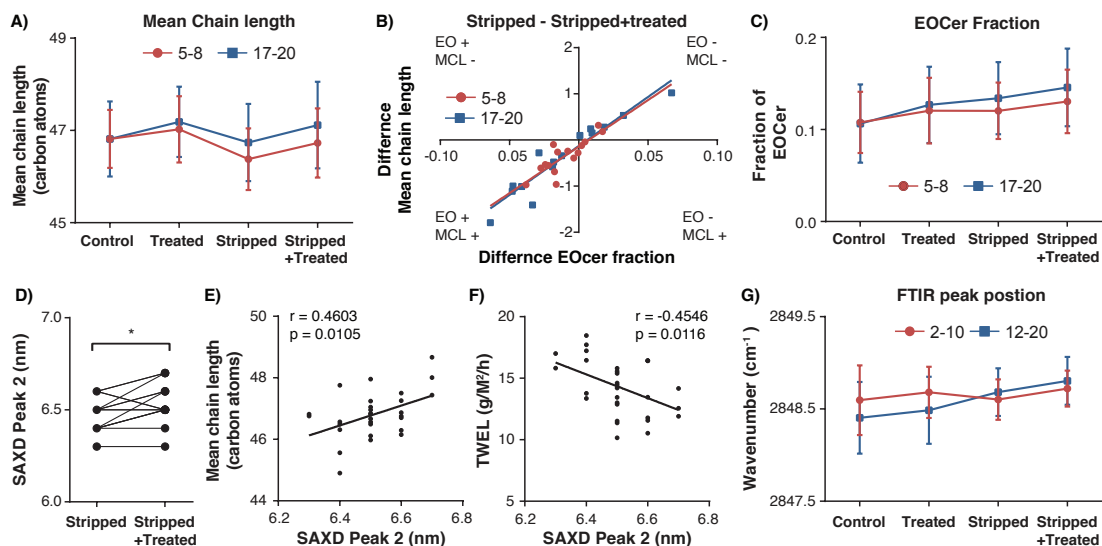


Figure 4.5: Mean chain length and lamellar spacing

A: The mean total chain length (carbon atoms) for the 4 different sites at two depths excluding the ceramides of the formulation in the calculation. Data were compared using a LMM. **B:** The correlation between changes in fraction of EOCer and changes in the mean chain lengths (carbon atoms) at the stripped site due to treatment (amount at the stripped site subtracting the stripped+reated). **C:** The EOCer fraction of the samples at the 4 different sites at two depths. Data were compared using a LMM. **D:** The difference between the SAXD peak 2 position in the stripped and Stripped+reated sites. The differences were tested with a Wilcoxon matched pairs signed rank test. **E:** The correlation between the SAXD peak 2 (nm) and TEWL (g/m²/h) at the end of the study. Pearson *r* and *p*-value are provided. **F:** The correlation between the SAXD peak 2 (nm) and the mean chain length (carbon atoms) of all ceramides. Pearson *r* and *p*-value are provided. **G:** The wavenumber in cm⁻¹ of the stretching peak position. Data were compared using a LMM.

The carbon chain length of the ceramides could influence the spacing of the strong diffraction peak in the diffraction pattern as measured by SAXD. From the position of the strong diffraction peak in the X-ray diffraction curve (peak 2) the corresponding

spacing was calculated (referred to as peak 2 in **Figure 4.5D**). A significant increase in spacing due to treatment was observed and a positive correlation was observed between the mean chain length and the spacing, (**Figure 4.5E**). When comparing the spacing to TEWL, a correlation was observed between an increased TEWL and a decrease in spacing (**Figure 4.5F**). Stripping and treatment thus affected the mean chain length, which correlated to EOCer fraction and the lamellar spacing.

Table 4.2: The output of the LLMs for mean chain length, EOCer fraction, and stretching peak position

The mean chain length and EOCer fraction were calculated excluding ceramides NS C44 and EOS C66. The wavenumber in cm^{-1} of the stretching peak position was calculated using the center of gravity at 90% peak height. As fixed factors stripped, treated and depth were used. The intercept was the control site at a depth of 5-8 tape-strips or for spectra of tapes 2-10.

		Mean chain length		EOCer fraction		Wavenumber cm^{-1}	
		Estimate	p-value	Estimate	p-value	Estimate	p-value
	Intercept	46.76	<0.001	0.107	<0.001	2848.60	<0.001
Effect size	Treated	+0.33	<0.001	+0.014	<0.001	+0.09	0.013
	Stripped	-0.37	<0.001	+0.011	0.007	+0.02	0.622
	Depth	+0.08	0.366	+0.003	0.537	-0.17	<0.001
Interactions	Stripped*depth	+0.29	0.021	+0.012	0.043	+0.26	<0.001

4.3.5 Effects of stripping and treatment on lateral packing.

Next to the lamellar spacing, the lateral packing is important for a proper barrier function. For this reason the effect of treatment and stripping on the lateral packing was examined. All skin sites were studied using ATR-FTIR, where both the CH_2 symmetric stretching vibrations and the CH_2 scissoring vibrations were examined. A LMM was built for the CH_2 symmetric stretching peaks. Stripped, treated, and depth were used as fixed factors and subject as random factor (**Table 4.2**). A significant increase in peak position was observed due to barrier disruption and treatment suggesting a slight increase in disordering of the lipids. The effects were stronger deeper in the SC. However, the peak position at all sites is located at wavenumbers between 2848.46 cm^{-1} and 2848.80 cm^{-1} , indicating an ordered lateral lipid organization at all sites. A similar linear mixed model was built for the CH_2 scissoring peak. We determined the FWHM of the scissoring vibrations. **Supplemental S4.13** depicts the result of this model. At the control site the FWHM was significantly decreased at deeper layers (tape-strips 12-20), yet, the FWHM was 11.3 cm^{-1} or higher, indicative for a large fraction of lipids forming an orthorhombic lateral packing. No deviation from an ordered structure by treatment or stripping was observed.

4.4 Discussion

In this study we showed that a VC based formulation is able to accelerate the barrier recovery of tape-strip disrupted healthy human skin and, for the first time, demonstrate that formulations can act by normalizing the SC lipid composition. Multiple effects of barrier disruption and treatment on barrier recovery, ceramide composition, and lipid organization were examined (Figure 4.6 provides an overview). Using PCA, samples were clustered into groups of treatment and stripped, based on changes in ceramide profile. Moreover, correlations of ceramide levels to PC2 had great similarities to correlations between ceramide levels and barrier function after barrier recovery. Thus, the loading variables for PC2 were a powerful tool to identify ceramides responsible for profile changes related to barrier disruption. At the stripped sites after recovery, multiple changes in the ceramide profiles were observed. Treatment partially normalized these changes in SC ceramide composition caused by barrier disruption. These ceramide profile changes correlated to the barrier function, indicating a relation of the changes in these lipids and an accelerated barrier recovery process.

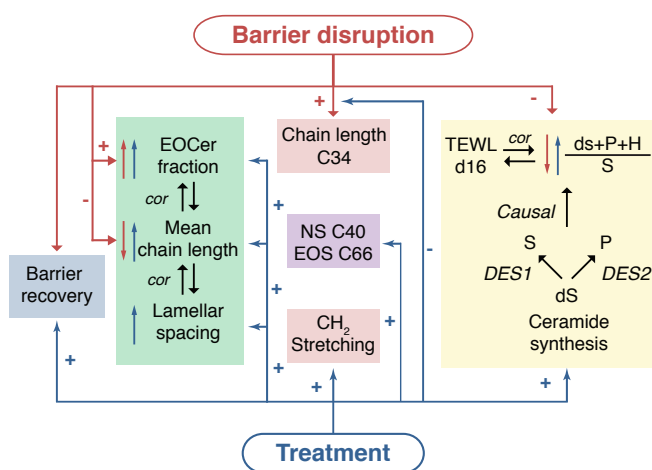


Figure 4.6: An overview of the effects of barrier disruption and treatment.

Negative effects are indicated with an – sign and positive effects with a + sign. Barrier disruption induces barrier recovery and treatment accelerates this process. Changes in the EOCer fraction, mean chain length, and lamellar spacing showed correlations (cor) indicated by the arrows. The changes in lipid composition, organization and subsequent effects are indicated. The ratio $ds+P+H/S$, is mainly determined by the

enzymes human-dihydro- $\Delta 4$ -desaturase (DES1) and human-sphingolipid-C4-hydroxylase (DES2), responsible for the production of sphingosine and phytosphingosine ceramides from dihydrosphingosine ceramides, respectively. The arrows indicate that barrier disruption changed the lipid synthesis decreasing this ratio and treatment had the opposite effect. The correlation of the TEWL at day 16(d16) to the subclass ratio is indicated, yet, the amount of C34 and the SAXD Peak 2 position correlated to TEWL as well.

When examining barrier recovery, two phases could be distinguished; a first steep increase in barrier recovery % after three hours, followed by second gradually diminishing barrier recovery rate. Treatment increased the level of the first phase,

where after three hours a recovery of ~25% was reached compared to ~10% without treatment. However, early effects of formulation on barrier recovery measured by TEWL can be caused by leftover formulation on the skin²⁷, even though leftover formulation was removed prior to TEWL measurement. The second recovery phase was not affected by residual formulation on the skin surface, because the formulation was applied after TEWL measurement. During the second phase, treatment had a pronounced effect on the barrier recovery rate.

One of the aims of the tested formulation was to replenish SC ceramides. Treatment caused significant increase in the formulated ceramides level; even at deeper SC layers. Although, a 2-day washout period would have removed almost all superficial formulation, it cannot be stated with 100% certainty that the applied ceramides measured with LC/MS were incorporated into the SC. Some formulated ceramides could still be present in folds of the SC. In cultured skin, it was shown that ceramides stay in these folds²³. Even though the increase of the formulated ceramides was observed at both depths, tape-strips obtained lipid material from various depth of SC⁴⁴ and, thus, tape-strips at both depths can contain material from these folds. Nevertheless, it has been demonstrated that when treating stripped *ex vivo* SC with deuterated ceramides once, small ceramide amounts were incorporated into the SC lipid matrix after regeneration⁴⁵. Others have shown that dimeric ceramides are able to diffuse into the skin⁴⁶. Together with the relatively large increases of the formulated ceramides, it is likely that a fraction of the applied ceramides did incorporate into the SC lipid matrix and actively enhance barrier recovery.

Multiple studies have used SC lipid containing formulations, either for long or short term treatment¹⁸⁻²¹ and it was shown that the SC lipids ratio in formulations is essential for short term barrier recovery^{22,47}. Visscher et al. tested the effect of 5 days VC treatment on TEWL and hydration of tape-stripped SC, but they observed no changes in TEWL when comparing with untreated stripped SC²⁸. In comparison to our study, the barrier was less extensively disrupted. Therefore, incomplete SC removal may lead to a lower efficacy of the formulation to improve barrier repair. Here, a detailed description of the recovery process is provided and, for the first time, insights are given into changes in the SC ceramide composition due to treatment. Below, a mechanism is suggested on how a VC based formulation affects ceramide synthesis.

At the stripped (non-treated) sites, two prominent changes were observed in the endogenous ceramides: I) the amounts of S subclass were increased and II) C34 ceramides were increased. The amounts of these ceramide groups correlated to a decrease in barrier function. However, from these data it could not yet be deduced if these increases were causal or contingent with a decreased barrier function. A plausible reasoning is that in a direct response to barrier disruption, the epidermal calcium

gradient changed² and a subsequent modulation in gene expression occurred²⁹. These changes in epidermal homeostasis might have affected the SC ceramide processing in two ways:

First, in the epidermal lipid biosynthesis, ceramides containing a dihydrosphingosine are either converted into sphingosine or phytosphingosine containing ceramide^{48,49} (**Figure 4.6**) (biosynthesis of subclass H ceramides has not yet been reported). When defining the ratio between the subclasses as: dS+P+H/S, this ratio could only change if either of the two known lipid biosynthesis pathways changed. The present study showed that this ratio was substantially decreased at the stripped sites, indicating barrier disruption influenced the specific synthesis pathways of subclasses S and P. Importantly, the ratio between the subclasses correlated strongly to TEWL after barrier recovery, which suggests that an optimal ratio is important for an optimal skin barrier repair. Furthermore, this correlation was site independent, thus, at stripped sites with a higher TEWL, synthesis of sphingosine ceramides was preferred over phytosphingosine containing ceramides. Treatment was able to favorably change the subclass ratio towards a lipid composition that correlated to a more efficient skin barrier recovery, by reducing the amount of NS and AS subclasses.

The Second change in lipid biosynthesis due to barrier disruption was the increased amount of C34 ceramide. These ceramides are produced from the sphingoid and acyl building blocks (a C16 or C18 acyl chain and a C16 or C18 sphingoid chain⁵⁰) and do not require additional elongation steps⁵¹. Although these C34 ceramides can be produced as a quick first response to mitigate an impaired SC barrier, C34 ceramides are “short” chain ceramides which have been shown to reduce skin barrier function^{6,52}.

Altogether, the C34 ceramides and the ratio of ceramide subclasses could function as indicators of reduced barrier function and changes in barrier lipid homeostasis. Here, we showed that treatment was able to partly diminish the amount of C34 ceramides and, by diminishing the amount of NS and AS, able to change the subclass ratio and thus the preceding ceramide synthesis. This reduction could be attributed to I) the semi-occlusive effect of the formulation, in which the formulation acted as an additional barrier, II) the presence of excess formulated ceramides reducing the response to replenish the ceramides. As a whole, these data show that the formulation partially normalized the lipid biosynthesis and directed the SC towards a more functional lipid homeostasis. The lipid organization was used to verify if changes in ceramides composition were causal to changes in barrier function.

An important characteristic of the SC ceramide fraction is the mean chain length. The chain length was reduced when the barrier function is impaired e.g.: in stripped sites and AD^{8,17}. Here, treatment was able to restore the mean chain length at stripped sites (excluding the added ceramides). The mean chain length correlated strongly to the

fraction of EOCer and treatment was able to increase the EOCer fraction. At the treated sites, the spacing was slightly increased indicating an increase in the repeat distances of the lamellar phases. Furthermore, the spacing correlated to the mean chain length and weakly correlated to the EOCer fraction (data not shown). Taken together, the data suggest that treatment influenced the lamellar lipid organization through increasing the EOCer fraction thereby increasing the mean change length (**Figure 4.6**).

As far as the lateral packing was concerned our studies showed that a high fraction of lipids formed an orthorhombic packing and this was not affected by stripping. For this reason the fraction of lipids forming an orthorhombic packing could not be increased due to treatment. Changes due to treatment might become more apparent in affected skin of AD patients where the fraction of lipids forming an orthorhombic organization is decreased compared to that in healthy skin⁴³.

To summarize the effects of the VC based formulation on barrier recovery in healthy SC, it was observed that treatment: I) accelerated barrier recovery, II) changed the lipid synthesis to a more favorable lipid composition by decreasing the amount of C34 ceramides and changing the subclass ratio dS+P+H/S, and increasing the fraction of EOCer. III) These changes in lipid composition affected the SC lipid organization and correlated to the SC barrier function. Interestingly, similar increases in S subclass and increases in the fraction of C34 chain length ceramides were observed in AD^{8,17}. An essential application of this formulation could be in a particular group of AD patients, where barrier restoring lipophilic formulations are commonly used⁵³. Moreover, the treatment presented here modulated the alterations in lipid composition observed in AD, towards a healthy lipid homeostasis.

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4

Supplementals chapter 4

Supplemental S4.1: Synthetic ceramides that were used as calibrators for LC/MS analysis.

Numbers in brackets indicate the number of carbon atoms in the fatty acid chain and sphingosine chain, respectively.

Ceramides		
N(24)dS(18) ¹	N(16)S(18) ¹	E(18:2)O(30)S(18) ²
N(18)dS(18) ¹	N(24)P(18) ²	E(18:2)O(27)S(18) ²
N(16)dS(18) ¹	N(16)P(18) ²	E(18:2)O(30)P(S18) ²
N(24)S(18) ²	A(24)S(18) ¹	E(18:2)O(27)P(18) ²
N(22)S(18) ¹	A(22)S(18) ¹	N(24deu)S(18) ²³
N(20)S(18) ¹	A(16)S(18) ¹	
N(18)S(18) ¹	A(24)P(18) ¹	

¹ Avanti polar lipids (Alabaster, USA) ² Evonik Industries (Essen, Germany) ³ISTD

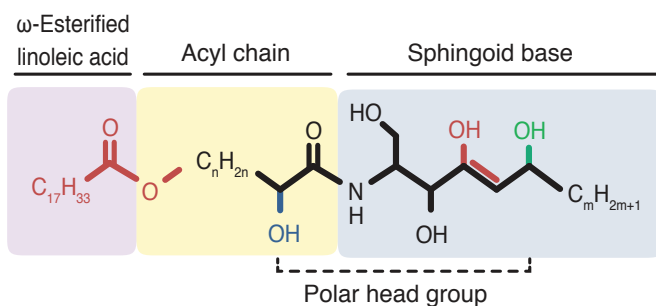
Supplemental S4.2: Ingredients of the formulation

Ingredient	Supplier	Weight %
Super sterol esters Crodalan SWL	(Croda, UK)	49.72% w/w
Crodamol GTCC miglyol	(Croda, UK)	36.98% w/w
Squalane	(Galderma)	6.63% w/w
Cholesterol USP/NF	(Croda, UK)	3.63% w/w
Palmitic acid (FFAC16:0)	(Emery)	0.83% w/w
Stearic acid (FFA 18:0))	(Berg + Schmidt)	0.10% w/w
Ceramide NS (C40)	(Evonik, Germany)	1.18% w/w
Ceramide EOS (C66)	(Evonik, Germany)	0.83% w/w
Oxyplex LM	(Merck, Germany)	0.10% w/w
anti-oxidant for ceramide EOS containing:		
DL-γ -Tocopherol (Vitamin E)	0.025% w/w	
Lecithin	0.025% w/w	
Ascorbyl palmitate	0.020% w/w	
Glycerin monostearate	0.020% w/w	
Glycerin monooleate	0.0025%w/w	
Citric acid	0.0025%w/w	



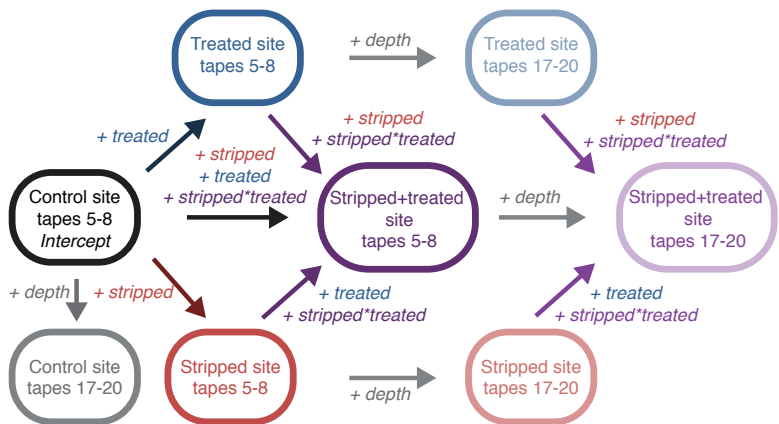
Supplemental S4.3: Indication of TEWL measurement sites

The TEWL measurements at the sites were performed at three sections of the sites. Here the stripped site is shown which can be identified by the erythroderma. TEWL measurements were performed at the sections indicated with black circles. At every time-point TEWL was measured at the right, middle, and left section.



Supplemental S4.4: General ceramide structure

The polar head group consists of a sphingoid base and an acyl chain. There are 4 different sphingoid bases in human SC: the sphingoid (S), a dihydrosphingosin (dS), a 4-hydroxy or phyto-sphingosine (P), and a 6-hydroxy-sphingosine (H). The acyl chain of human SC occurs in 3 different forms. A non-hydroxyl acyl chain (N), an alpha-hydroxyl-acyl, and an omega-hydroxyl-acyl chain (O). In SC a specific type of ceramide occurs where the omega-hydroxyl group becomes esterified with a linoleic acid called the EOCer subclass. Together these form 16 different subclasses. Both the acyl and sphingoid chain can vary in carbon chain length.



Supplemental S4.5: Linear mixed modelling

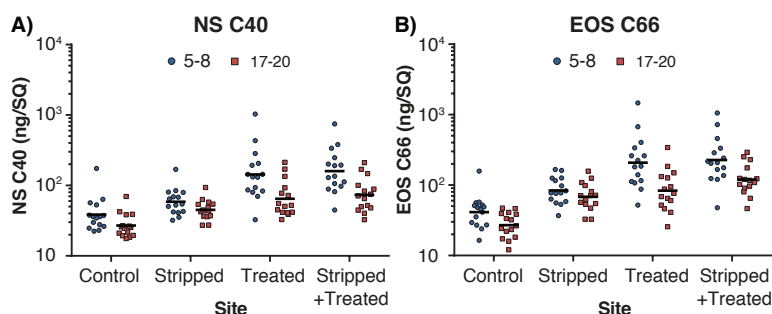
The LLMs used here compare the effects of treatment, stripping, and depth to the control site of the subject. Using subject as a random factor, gives each subjects control site (starting point) its own value, so it can start at any random point. It then determines the effect size of the fixed factors (treatment, stripping, and depth). ‘Fixed’ means that the model will say that all these effects have the same size for each subject and for each time this effect occurs, so also the effect at treatment at the stripped site (difference between stripped and stripped+treated). Because a stripped site can respond differently to treatment, an interaction term is added to the model, in this case *stripped*treated*. In this model the interaction is the effect size of treatment additional to the effects of stripping and treatment (the fixed factors). For most data we were interested in the effect of treatment on the stripped sites. This is the sum of the effect sizes of treatment and *stripped*treated* (illustrated above as the arrow from stripped site to Stripped+treated site). In the schematic above it is shown which steps are required to calculate the predicted values of the mixed model. The effect sizes are indicated in italic. Here, we were not interested in the mean predicted by the model but in the effect sizes of the different factors, as these calculate how the experimental conditions changed and if these were significant.

Supplemental S4.6: Correlations to amount of barrier disruption

The table depict the Pearson *r* and *p*-value of the correlation between the TEWL directly after barrier disruption and AUC of the barrier recovery curve. It also contains the correlations between the difference in TEWLs directly after barrier disruption and the ΔT at different recovery %.

	TEWL start vs AUC	TEWL dif vs 5%	TEWL dif vs 10%	TEWL dif vs 15%	TEWL dif vs 20%	TEWL dif vs 25%	TEWL dif vs 30%	TEWL dif vs 35%	TEWL dif vs 40%	TEWL dif vs 45%
Pearson <i>r</i>	-0.26	0.05	0.15	0.20	0.17	-0.15	-0.26	-0.27	-0.34	-0.43
<i>p</i> -value	0.17	0.85	0.60	0.47	0.54	0.60	0.35	0.34	0.22	0.11

	TEWL dif vs 50%	TEWL dif vs 55%	TEWL dif vs 60%	TEWL dif vs 65%	TEWL dif vs 70%	TEWL dif vs 75%	TEWL dif vs 80%	TEWL dif vs 85%	TEWL dif vs 90%	TEWL dif vs 95%
Pearson <i>r</i>	-0.50	-0.40	-0.34	-0.34	-0.22	-0.09	-0.06	-0.23	-0.11	0.86
<i>p</i> -value	0.06	0.14	0.21	0.22	0.44	0.76	0.82	0.42	0.71	0.06



Supplemental S4.7: LLM of the formulation ceramides

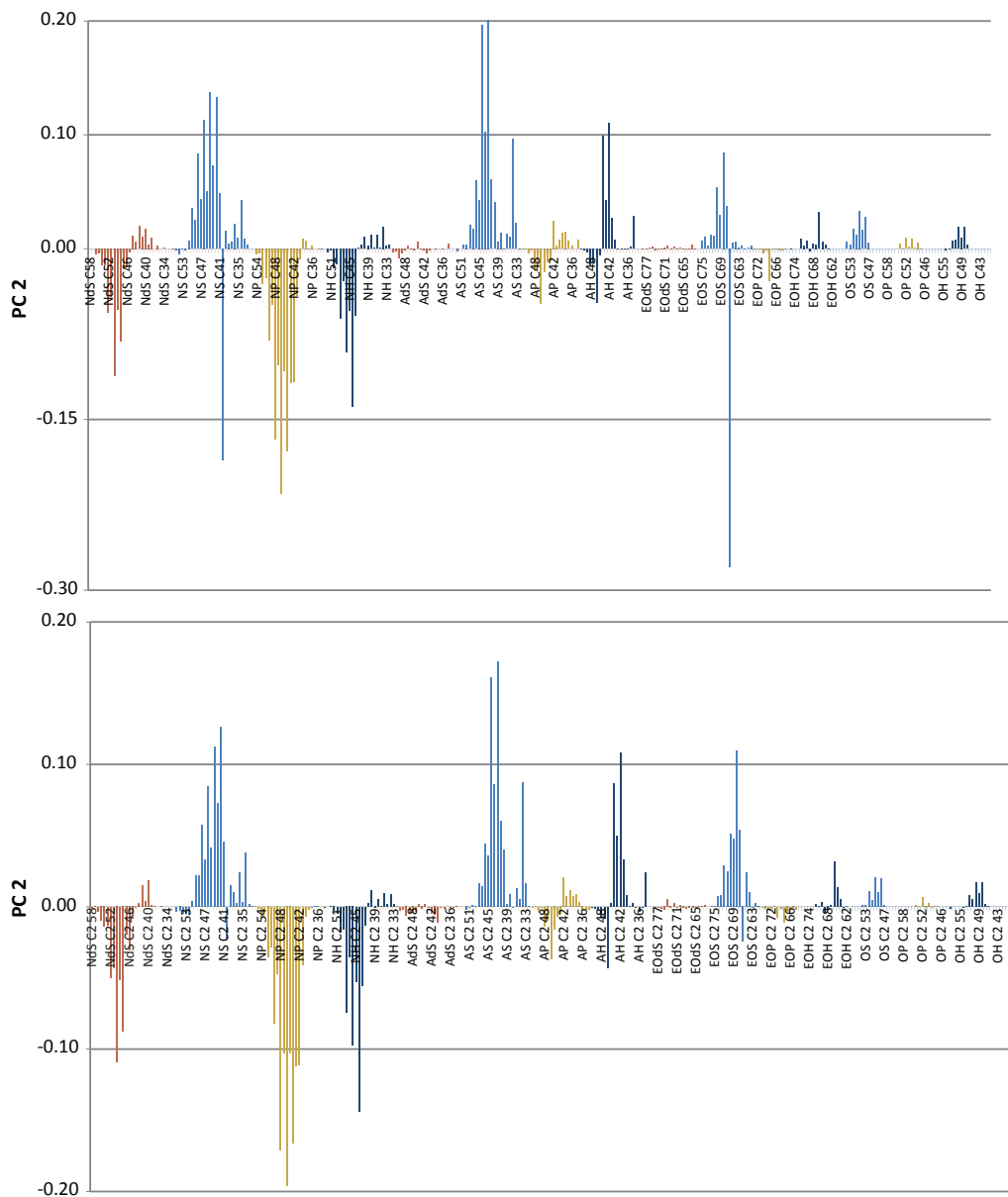
A,B: Plots of the amounts of NS C40 and EOS C66 (log (ng/SQ)) in all quantified samples. Lines are geometric means. Statistical outputs of the LMMs are reported in **Supplemental S4.8**.

Supplemental 4.8: LLM of the formulated ceramides:

The table shows the output of the LLM of the logarithm of the amount of NS C40 (ng/SQ) and EOS C66 (ng/SQ). The effect sizes are the changes to the intercept (control site tape-strips 5-8), due to the depth, stripping, and treatment. The second part of the table shows the amount in ng/SQ. This was calculated by exponentiation of the mean log value outputted by the LLM.

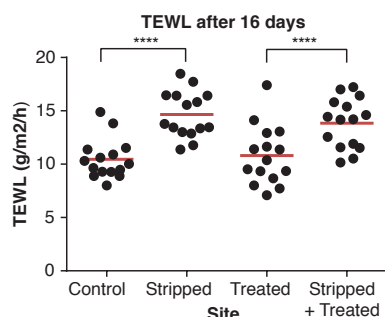
		Log NS C40		Log EOS C66	
		Estimate	p-value	Estimate	p-value
	Intercept	1.575	< 0.001	1.590	< 0.001
Effect size	Depth	-0.135	0.011	-0.135	0.011
	Stripped	+0.204	< 0.001	+0.356	< 0.001
	Treated	+0.580	< 0.001	+0.697	< 0.001
Interactions	Depth * Treated	-0.207	0.006	-0.200	0.006
	Stripped * Treated	-0.152	0.040	-0.260	0.040

Calculated Amount ng/SQ	NS C40		EOS C66	
	Depth 5-8	Depth 17-20	Depth 5-8	Depth 17-20
Control	37.61	27.57	38.93	28.54
Stripped	60.13	44.09	88.32	64.76
Treated	142.92	65.12	193.64	89.64
Stripped Treated	160.87	73.29	241.68	111.87



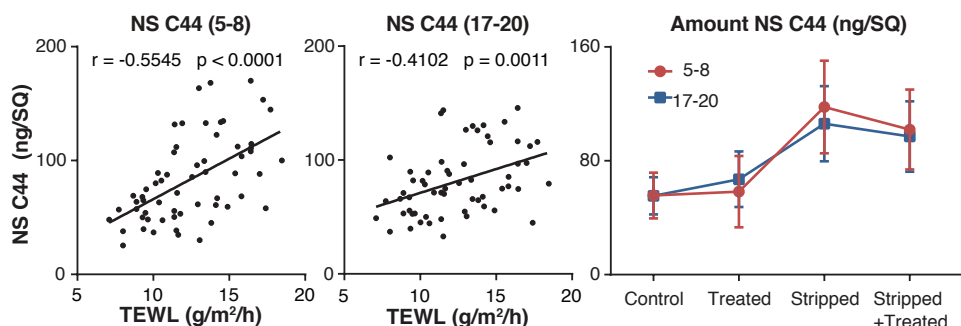
Supplemental S4.9: PC2 plot

The two graphs depict the scores/correlation of all ceramides to PC2. The first graph shows all ceramides detected in samples 5-8 and the second graph all ceramides detected in samples 17-20. Subclasses were given different colors. High positive scores indicate a correlation to the striped sites and a high negative score to the control sites.



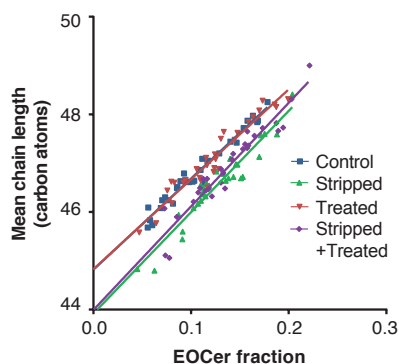
Supplemental S4.10: TEWL at day 16

The TEWL (g/m²/h) of all 4 sites after 16 days of treatment and/or barrier recovery. The red line is the mean value and the significance levels that are indicated were tested with a 1-way ANOVA with Bonferroni post-test.



Supplemental S4.11: NS C44 amount and correlation to TEWL

Pearson r and the corresponding p -value are indicated above the data. The amount in ng/SQ of NS C44 at the 4 site and at both depths.



Supplemental S4.12: Correlation between mean chain length and EOCer fraction

The correlations between the mean chain length and the fraction of EOCer are depicted per site. Each site showed significant Pearson correlations coefficients: control (0.9817), stripped (0.9565), treated (0.9600), and stripped+treated (0.9568).

Supplemental S4.13: LLM of the effects on the lateral lipid packing

The linear mixed models output for the FWHM of the scissoring peak. The intercept is the estimated amount at the control site at a depth of 2-10 tape-strips. Estimates are in cm⁻¹. Significance levels of all effect sizes and interactions are given.

		FWHM	
		Estimate	p-value
Effect size	Intercept	11.86	< 0.001
	Stripped	-0.37	0.19
	Treated	-0.43	0.08
	Depth 12-20	-0.59	0.02
Interaction	Stripped *Treated	0.44	0.23
	Stripped *Depth	0.67	0.07
	Treated *Depth	0.47	0.17
	Stripped *Treated *Depth	-1.32	0.01

Chapter 5

Emollient monotherapy of atopic dermatitis patients induced stratum corneum lipid properties changes, but did not alleviate disease severity

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^{*} Both authors contributed equally to this work

Ready for submission:

Background;

Chromatogram of the common observed alterations in ceramide composition of atopic dermatitis patients: C34 ceramides, S-subclasses, and MuCers.

5

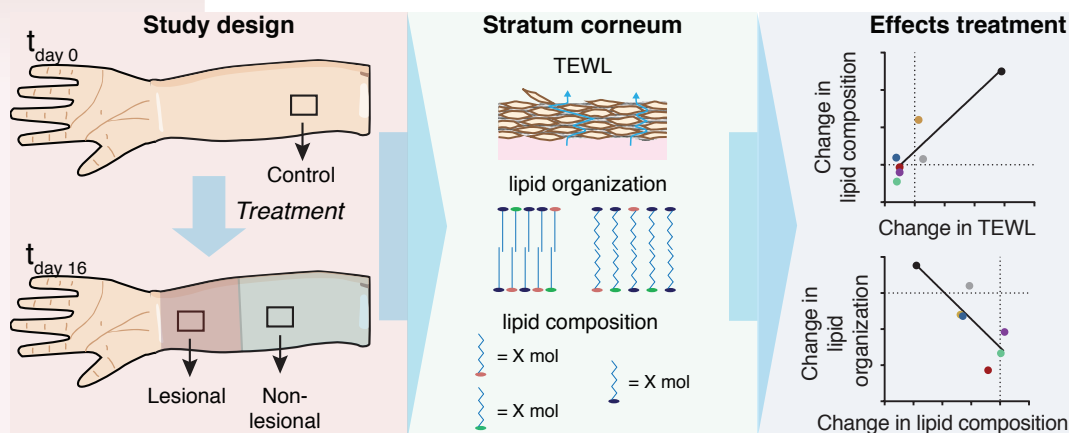
Treatment of atopic dermatitis patients

Abstract

Skin barrier repair emollients are a commonly used treatment in atopic dermatitis (AD). Yet, the underlying mechanistic mode of action is poorly understood. Here, the effects of emollient monotherapy on skin barrier lipids in AD patients were investigated. An intra-individual controlled pilot study was performed in which moderate to severe AD patients applied a ceramides containing emollient for 2 weeks. Changes in stratum corneum (SC) ceramide composition and lipid organization were examined. Disease severity was scored and trans-epidermal water loss (TEWL) was monitored. Five different lipid parameters important for barrier function were measured. After treatment improvements in lipid ordering were observed at non-lesional sites. Other lipid parameters did not show improvements. The investigated lipid parameters did correlate to TEWL. When comparing changes in lipid parameters during treatment, effect sizes correlated. Yet, monotherapy of the forearm with the selected emollient did not prevent lesion formation. Severity at the start of the study did correlate to TEWL at the end of the study, indicating an effect of systemic flares in AD. It can be concluded that local monotherapy with emollient did induce changes in stratum corneum lipid properties of AD patients, yet, did not substantially improve the condition of moderate/severe AD patients.

keywords:

*Epidermis,
Clinical study,
Mass Spectrometry,
Lipidomics,
Sphingolipids,
Lipid Organization,
Vernix Caseosa,
Barrier Recovery,
Stratum Corneum,
Treatment.*



Graphical abstract

5.1 Introduction

Emollients are commonly prescribed for treatment of atopic dermatitis (AD), to counter xerosis, and enhance skin barrier repair^{1,2}. A wide variety of emollients are available and three different mechanisms of action are commonly suggested. Firstly, emollients are thought to function as a supplemental barrier decreasing penetration of irritants, allergens, and pathogens and decreasing trans-epidermal water loss (TEWL). Secondly, they increase stratum corneum (SC) hydration by added humectants. Thirdly, an emollient could replenish SC barrier lipids^{1,3}. The latter are a specific group of barrier repair emollients containing barrier lipids⁴. Of this group the clinical effects on scoring atopic dermatitis (SCORAD), TEWL and skin hydration have been extensively studied⁴⁻⁹. However, the mechanism by which these emollients enhance skin barrier repair and affect the SC lipid composition and organization is unknown. This is of particular interest as specific emollient components could normalize the lipid composition, thereby enhancing barrier repair^{10,11}.

Lipids form an integral part of the SC barrier. This barrier can be represented as a brick and mortar structure, with terminally differentiated keratinocytes (corneocytes) as the bricks, and the lipids as mortar¹². The SC lipid matrix is the main route of penetration for allergens and irritants¹³ and the lipid constitution is important for a proper barrier formation¹⁴. Three main SC lipid classes are known: sterols, free fatty acids, and ceramides. The latter are the most diverse group of SC lipids, characterized by different hydrophilic head groups defining the subclass, and a large variation in hydrocarbon chain length within these subclasses. **Figure 5.1** shows the chemical structure of all subclasses relevant to this chapter.

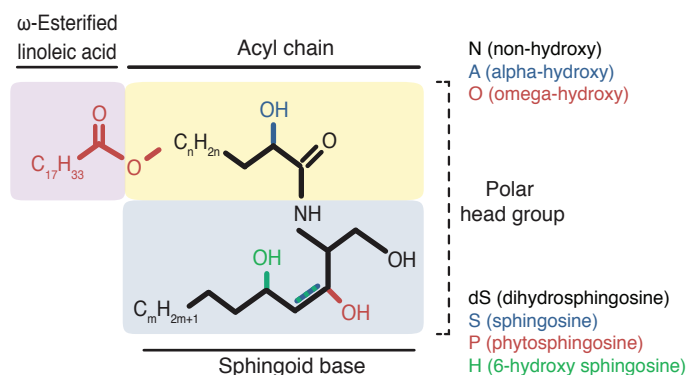


Figure 5.1: Ceramide structure

Ceramides consist of an acyl chain bound to a sphingoid base. Its subclass is defined by these two parts of the ceramide. The ceramides investigated can contain three different acyl chains indicated by the letters N, A, and O and one with a linoleic acid esterified to the omega hydroxyl (EO). There are four different sphingoid bases dS, S, P, and H. The color corresponds to the structural. The name is conjunction of the letters that define the acyl-chain and sphingoid base.

S, P, and H. The color corresponds to the structural. The name is conjunction of the letters that define the acyl-chain and sphingoid base.

In AD alterations in composition and organization of SC lipids have been reported¹⁵ and were correlated to a decreased barrier function^{16,17}. Increased amount of ceramides with a C34 carbon chain were observed, increased S-subclass ceramides, and a decreased mean carbon chain length were observed. A recent study showed that barrier disruption by tape-stripping induced similar lipid alterations as AD in regenerated SC of healthy volunteers. Treatment with a vernix caseosa (VC) based emollient ameliorated the lipid composition of these healthy volunteers¹⁸. Barrier repair in mice was effectively enhanced by a synthetic VC emollient as well^{19,20}. It was hypothesized that this emollient could have a similar beneficial effect in AD patients.

We present a pilot study in which this vernix caseosa based formulation was locally applied by moderate to severe AD patients as monotherapy. Improvement of lipid organization at the non-lesional sites was observed. Despite this, the local SCORAD of these patients indicated aggravated AD at the treated sites. It was observed that the AD severity at the start of the study affected the outcome.

5.2 Materials and methods

5.2.1 Inclusion and ethics

To determine if a synthetic formulation based on the VC improves the SC lipid barrier, a pilot study with 8 AD patients was performed. **Supplemental S5.1** depicts the composition of the synthetic VC formulation. This formulation contained multiple barrier lipids, cholesterol, free fatty acids with chain lengths C16 and C18, and ceramides NS C40 and EOS C66 (named according to Motta et al²¹), added to improve the SC lipid composition and the organization. Institutional approval by the Medical ethical committee of the Leiden University Medical Center was obtained and all experiments were performed according to the 1964 declaration of Helsinki and its later amendments. Before inclusion each patient signed an informed consent. Subjects were included when they suffered from AD for ≥ 2 years and their base-line TEWL was ≥ 15 g/m²/h on non-lesional skin sites. An elevated TEWL was set as requirement, as this was associated with an altered lipid organization¹⁶. This would offer the possibility to determine if the emollient could improve the lipid organization. The data of one subject (subject 7) was excluded, as another treatment was subscribed during the trial.

5.2.2 Study design

The study commenced at t_{d-7} when a photograph was taken and the AD severity scores SCORAD, local SCORAD (ventral forearms), Eczema area and severity index (EASI), and trans-epidermal water loss (TEWL) (AquaFlux AF200, BIOX, London, UK) were

determined. For one week subjects did not receive any systemic treatment or topical treatment on their ventral forearms. The study focus on the difference between the start and end of the study and t_{d0} was set as a non-lesional not treated control. At t_{d0} , TEWL was measured at non-lesional sites of both ventral forearms and the arm with the highest TEWL was selected. A photograph was taken and 12 tape-strips were obtained for ceramide analysis (shortly described below). After every two tape-strips, an infrared spectrum was measured on the stripped site using attenuated total reflectance Fourier transfer infrared spectroscopy (ATR-FTIR, Agilent Technologies, Inc., Santa Clara, CA). In the spectrum the position of the CH_2 symmetric stretching vibrations of the SC lipids was determined (referred to as CH_2 stretching). This is a measure for the SC lipids conformational ordering²².

Thereafter, subjects applied the synthetic VC formulation on both forearms for two weeks, 2x daily. During the study subjects were allowed to treat the rest of their skin with emollients as normal. As no treatment would be a burden on the subjects both forearms were treated with the formulation. To avoid formulation mixing formulation was only applied on both arms. After the treatment period, a two-day washout period commenced. At t_{d16} a non-lesional and lesional sites were selected of the same arm at which t_{d0} a sample was acquired. The TEWL was determined. Of both sides 12 tape-strips were acquired to determine lipid composition and ATR-FTIR measurements were taken. The SCORAD, local-SCORAD, EASI, and a photograph were taken as well. **Figure 5.2** gives an overview of the sites investigated and the timeline of the study.

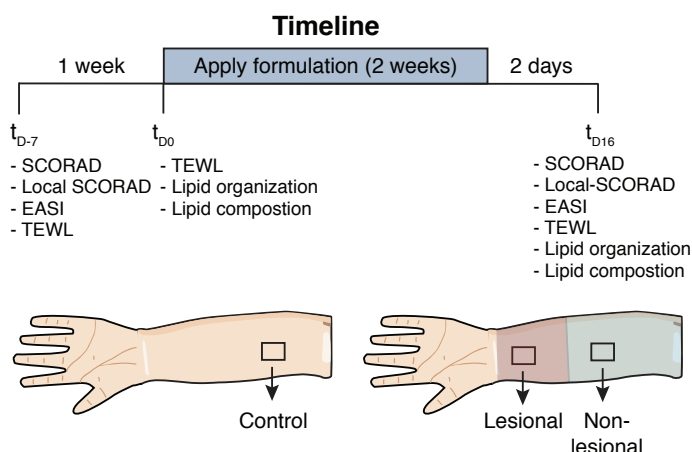


Figure 5.2: Study design and time line

Showing the timeline of the study and sites examined. At each time point the performed tests are indicated. Below the test sites of the arm that were examined are shown. The examined arm was left or right based on the TEWL at t_{d0} .

5.2.3 Tape stripping and lipid analysis

The selected skin sites were tape stripped using polyphenylene sulphide tape (Nichiban, Tokyo, Japan). A D-squame pressure applicator with a one kg weight was used to apply equal pressure to the tape-strip and the removal direction of the tapes was alternated.

Tape-strips were left to rest for more than 3 min and measured using a SquameScan (SQ) (Heiland Electronic, Wetzlar, Germany) and the values corrected for a blank tape. The corrected SQ value is a measure for the amount material on the tape-strip. Thereafter, 2 cm² of the tape was punched out and tapes 5-8 were extracted by a modified blight and dyer as validated elsewhere²³. The extracts of tapes 5-8 were pooled and dissolved at a concentration of 20 tapes/ml. LC/MS analysis and subsequent quantification of the ceramide content of the pooled tape-strip extracts was performed according to Boiten et al.²³. Ceramides were separated using a pva-silica columns 5µm particles 100 X 2.1mm i.d (YMC, Kyoto, Japan) on an Acquity UPLC class (Waters, Milford, MA)). Afterwards, APCI ionization and detection on a XEVO TQ-S (Waters, Milford, MA, USA) was performed. To quantify all ceramides a method specific 3D response model was made to predict the response factor for each ceramide detected above the LOQ. The amount was corrected for the cumulative SQ value of the four tapes used.

5.2.4 Statistical analysis

Statistical analyses were performed using SPSS (V24, IBM). Group wise comparisons were performed using linear mixed modeling with treatment as fixed factor and lesional as a nested factor within treatment (lesional site were always treated and compared to non-lesional). Subject paring was performed by setting subject as a random variable when comparing sites. Correlations between parameters were examined using GraphPad Prism (version 6.05). To determine if correlations were site/treatment independent, the correlations were determined using Linear mixed models without subject paring, with site (control, non-lesional, and lesional) as fixed factors and the parameter of interest as covariant (see **Supplemental S5.2** for SPSS scripts used).

5.3 Results

5.3.1 Affected lipid parameters in atopic dermatitis

The mechanistic effects of emollient treatment on SC lipid parameters were examined in AD patients. Recently, treatment with an emollient of healthy volunteers had shown to affect alterations in lipid parameters also affected in AD¹⁸. Here, five lipid parameters important for barrier function were examined: 1) CH₂ stretching vibration peak position (CH₂ stretching): higher values indicate more disordering in the SC lipids causing a reduced barrier function¹⁷; 2) Mean ceramide carbon chain length (MCL): a longer MCL was correlated to increased barrier function¹⁵; 3) Fraction of ceramides with a 34 carbon chain length (C34 ceramides): this was associated with a decreased barrier function¹⁵; 4) Molar ratio between the ceramide subclasses, dS+P+H/S excluding (esterified)omega hydroxyl-ceramides ((E)O ceramides): this ratio was shown to decrease with a reduced

barrier¹⁸; 5) Fraction of ceramides with a monounsaturated carbon chain (MuCers): increased unsaturation was correlated to decreased barrier function²⁴.

Figure 5.3(A-E) depict the five studied parameters. The ceramides added to the VC formulation (EOS C66 and NS C40) were not used in composition based parameters. After treatment, the CH₂ stretching vibrations decreased significantly at non-lesional treated site compared to control. This indicated that lipid ordering increased. After treatment, no significant changes in MCL, C34 ceramide amount, and MuCer amount were observed. The subclass ratio dS+P+H/S decreased significantly. At treated lesional sites, the MCL was shorter compared to treated non-lesional sites. The CH₂ stretching at treated lesional sites showed no significantly increase compared to the control or non-lesional site. Indicating that after treatment lesional sites had the same lipid ordering as non-lesional sites before treatment.

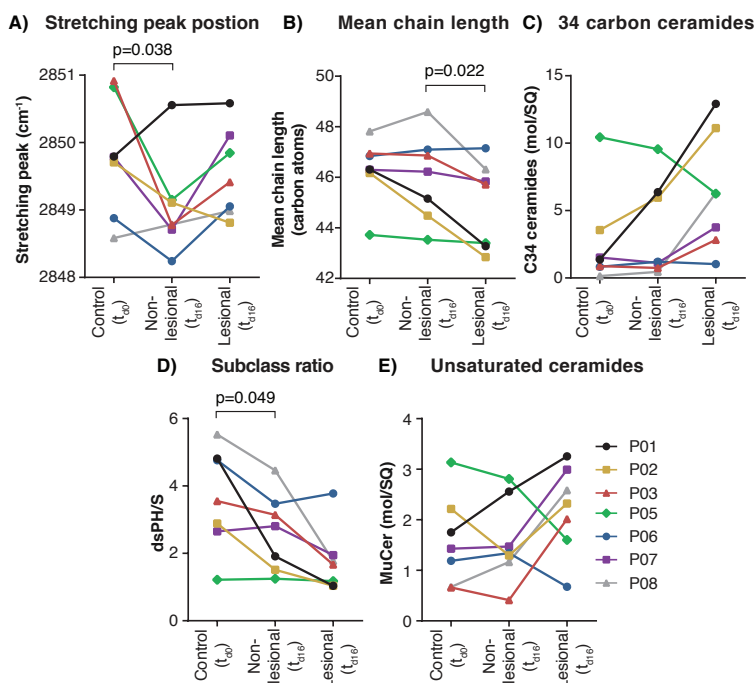


Figure 5.3: Lipid parameters throughout the study

The size of the lipid parameters at the non-lesional control site (t_{d0}), treated non-lesional site (t_{d16}) and treated lesional site (t_{d16}); **A**: Stretching peak position of the SC lipids in cm⁻¹; **B**: Mean total carbon chain length (MCL) of the ceramides; **C**: Amount of ceramides with a carbon chain length of 34 (pmol/SQ*10); **D**: Ceramide subclass ratio as: dS+P+H/S excluding acyl-ceramides (EO ceramides); **E**: Amount of monounsaturated ceramides (nmol/SQ*10). Significant changes are indicated by the p-value. Each subject is indicated by a specific color and points connected by a line.

2.2 Treatments effect on the clinical parameters

Next to pathophysiological changes in SC lipids, the impact treatment had on the disease progression was examined. Therefore, two scores of AD severity were determined at the start (t_{d-7}) and end (t_{d16}) of the study. **Figure 5.42A and B** show the SCORAD and EASI, respectively. No significant change in either score was observed. As treatment was only applied on the ventral for arm, the local-SCORAD of that area was determined. **Figure 5.4C** shows that the local-SCORAD increased significantly. This is further illustrated in **Figure 5.5** which depicts images of each patients arm at three time points and showed more lesional sites at t_{d16} .

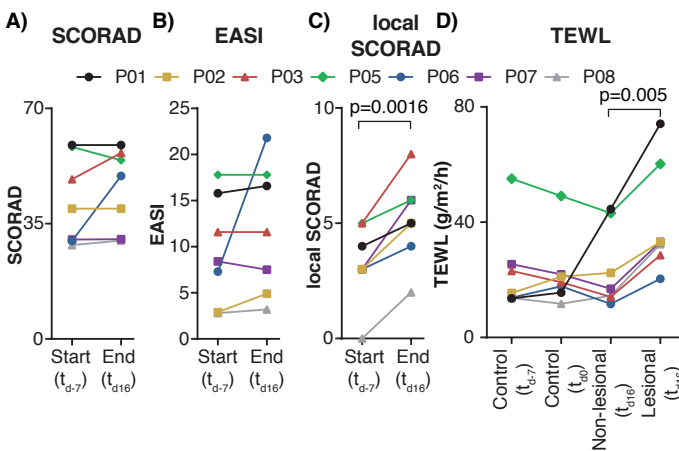


Figure 5.4: Clinical parameter throughout the study

A,B and C: SCORAD, EASI and local-SCORAD, respectively, at the start and end of the study for all subjects. **D:** TEWL throughout the study, for each subject. Non-lesional and lesional sites were both treated and measured at t_{d16} . Lesional sites were significantly different from all other sites (F-test of mixed model $p=0.006$). Each subject is indicated by a specific color and points connected by a line.

During the study the skin barrier function was monitored using TEWL. No significant changes in TEWL occurred between non-lesional control (t_{d-7}), non-lesional control (t_{d0}), and the non-lesional treated site (t_{d16}). In all subjects, TEWL at treated lesional sites (t_{d16}) was significantly higher than at treated non-lesional sites. There were substantial interindividual differences in TEWL values: subject one (P01) had a larger increase in TEWL during treatment than all other patients; Subject 5 (P05) only had lesional sites at the ventral forearm, therefore, had an exceptionally high TEWL value at the control site. Altogether, the emollient treatment effects on barrier function were diverse but beneficial for most patients, i.e. the TEWL of non-lesional sites decreased for most subjects, only the mean difference in TEWL was not significant (**Supplemental S5.3** depicts TEWL changes).

Lipids are crucial for a proper skin barrier. Alteration in the lipid parameters observed here could relate to an altered barrier function. Therefore, TEWL was correlated to the five lipid parameters. All five parameters correlated to TEWL or 1/TEWL (**Table 5.1**). Parameters which decrease with an increase in TEWL had inverse

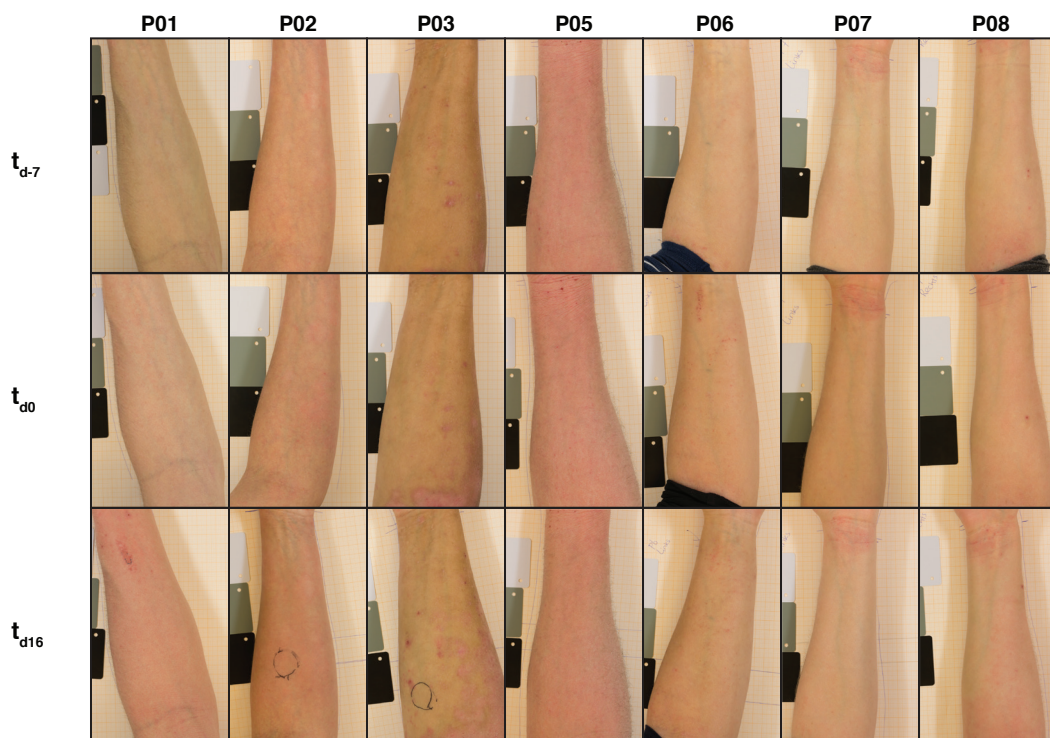


Figure 5.5: Photographs of the studied ventral forearms

Photographs of the examined ventral forearm of each subject at three time points: one week prior the emollient treatment (t_{d-7}), at the start of the treatment (t_{d0}), and 2 days after the treatment period (t_{d16}).

(1/x) correlations; therefore, 1/TEWL was used for these parameters. **Supplemental S5.4** contains correlations between all parameters. The correlations between these parameters indicated that size of the lipid parameters and barrier function correspond; Exemplified by P05 where TEWL was high and the lipid parameters CH_2 stretching, C34 amount and MuCer amount were high, and MCL and molar subclass ratio were low. To further investigate the correlation between lipids and barrier during treatment, the effect size of treatment per subject was studied.

Table 5.1: Correlations of lipid parameters to barrier function

For each parameter the correlation to either TEWL or 1/TEWL is given. The *f*-test *p*-values indicated if the correlation (*cor*) was significant, or that this correlation was different per site (*site*). The slope and *p*-value of each site independently is shown below.

F-test	TEWL (g/m/h)						1/TEWL (g/m/h) ⁻¹			
	Stretching Peak position (cm ⁻¹)		C34 (nmol/ SQ*10)		MuCer (nmol/ SQ*10)		Subclass ratio (dSPH/S)		MCL (carbon atoms)	
	cor.	site	cor.	site	cor.	site	cor.	site	cor.	site
<i>p</i> -value	0.001	0.313	<0.001	0.969	0.002	0.821	<0.001	0.447	<0.001	0.146
	Slope	<i>p</i> -value	Slope	<i>p</i> -value	Slope	<i>p</i> -value	Slope	<i>p</i> -value	Slope	<i>p</i> -value
Control	8.84	0.107	3.327	0.009	11.531	0.061	0.0124	0.001	0.0140	0.002
Treated	15.78	0.023	3.468	0.007	15.254	0.023	0.0173	<0.001	0.0115	0.001
Lesional	22.40	0.006	3.114	0.004	10.147	0.097	0.0105	0.032	0.0538	0.067

5.3.3 Subject specific treatment effects.

As treatment might affect each patient differently, changes during treatment per patient were examined by comparing the treatments effect size. This was calculated as followed: non-lesional control (t_{d0}) subtracted from the non-lesional treated site (t_{d16}). For each clinical and lipid parameter **Figure 5.6A** depicts if treatment had a beneficial effect i.e. towards a better skin barrier or less severe AD. Distinctions between subjects were observed: P01 had no beneficial effects, whereas treatment had the most beneficial effects for P05. Furthermore, some changes in parameters coincided for multiple subjects. If one was beneficial the other was as well (e.g. comparing MCL to EO%, C34 amount to TEWL, and MuCer amount to CH₂ stretching). Effect sizes of these parameters were compared, shown in **Figure 5.6B-E**. These correlations in parameters showed that during treatment parameters were similarly affected, either positively or negatively (**Supplemental S5.5** shows all correlations). Showing that besides correlating to one another in size, changes in lipid parameters within a subject are also related. Changes in MuCer and CH₂ stretching coincided, but did not correlating significantly (**Supplemental S5.6**). Changes during treatment were not dependent on state of the skin at the control time point, exemplified by P05 and P07. They both showed progress but had differences in levels of the parameters at t_{d0} . Altogether, changes were subject dependent, but within a subject dependent on one another.

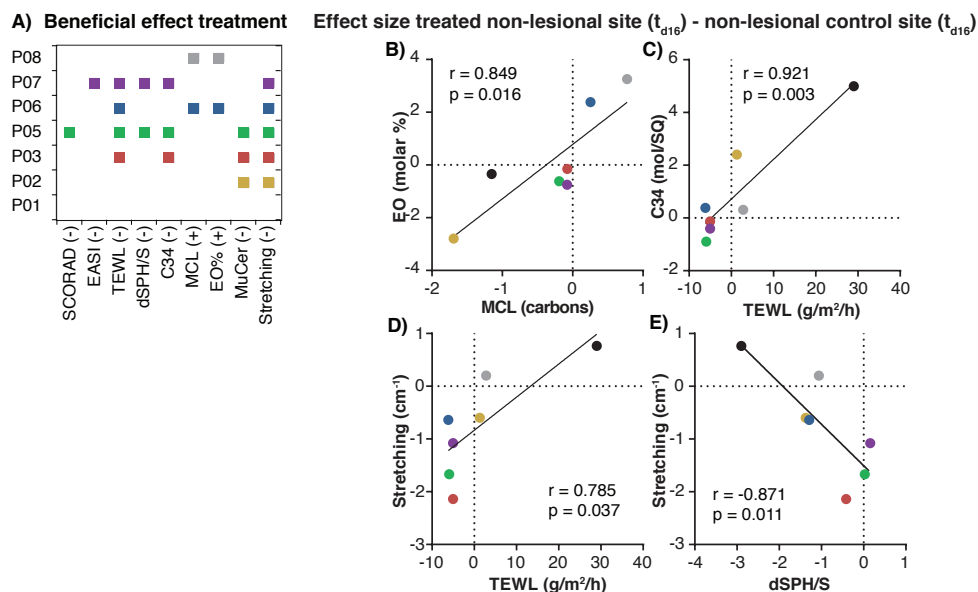


Figure 5.6 Changes in parameters during treatment

A: Plot showing if a change during treatment was beneficial (indicated by a colored square) or non-beneficial (indicated by the absence of a colored square). Each subject is indicated by a different color. The – and + signs indicate if beneficial changes in parameters were positive or negative values. **B, C, D, and E:** Effects size of the changes and how they compared to one another. If both are negative or positive for the same individual, it indicates that the effects were in a similar direction. The line indicates a linear regression, which is an indication that the effect sizes were comparable.

As only the arms were treated, other body sites could influence AD severity through systemically induced flares. To examine if the AD severity at the beginning of the study had an influence on the barrier function at the end of the study, the SCORAD and EASI scores at t_{d-7} were compared with TEWL at t_{d16} (**Figure 5.7**). Significant correlations for both SCORAD and EASI were observed. Although $n=7$, both correlations indicated that the AD severity had a substantial influence on changes in barrier function. No correlations between both AD severity scores at t_{d-7} and lipid parameters at t_{d16} were observed.

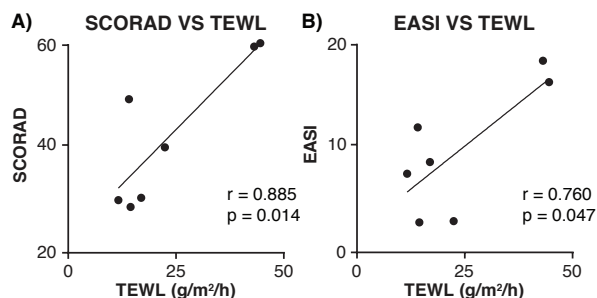


Figure 5.7: Correlation between TEWL and AD severity

A: Correlation between the SCORAD at t_{d-7} and TEWL at t_{d16} . **B:** Correlation between EASI at t_{d-7} and TEWL at t_{d16} . The r and p -values are the Pearson correlation and the significance level. The line is a linear regression.

5.4 Discussion

The goal of this study was to determine how local mono-therapy with a vernix caseosa based emollient affected the SC lipid composition and organization. Changes at non-lesional sites showed that for most subjects the TEWL decreased. This was accompanied by significant increases in CH₂ stretching, indicating that treatment improved the organization of the lipid barrier. However, no direct effect of the emollient on ceramide composition parameters was observed. The effect on lipid organization might be due to combined effects of the ceramide parameters or other emollient ingredients like free fatty acids. These were previously shown to incorporate into the SC lipid matrix^{25,26}. The lipid parameters examined and the correlations between them were comparable to previous AD studies without treatment^{15,16}. Here, we demonstrated that these correlations were also valid when the skin is treated. Moreover, the changes in parameters during treatment correlated. This showed that changes in TEWL correlated to changes in conformational lipid ordering and changes in C34 amount. The CH₂ stretching correlated to the molar subclass ratio, indicating that ceramide subclass composition could affect the conformational lipid ordering.

Previously, we showed that treatment of healthy volunteers with a compromised skin barrier decreased C34 amounts and increased the MCL¹⁸. However, changes induced by barrier disruption and effect of the treatment (treatment effect sizes, C34: 0.69 fmol/SQ*10, MCL: 0.3 carbons), were significantly smaller than changes observed in the present study. Therefore, beneficial effects of the barrier restoring emollient had a small impact compared to the larger changes in SC lipid properties induced by AD. Changes in the five lipid parameters corresponded to changes in TEWL (**Figure 5.6C and D**). Therefore, these lipid parameters could be used as biomarkers for future research into topical AD treatment.

During the study no systemic corticosteroid or immunosuppressants were used. Therefore, a systemic immune response, external factors like weather changes, or formulation sensitization could have had a major impact on the disease state. This could explain why aggravation of the disease state was observed in most patients. Other studies have shown that when the AD severity is first reduced by anti-inflammatory treatment, a barrier restoring formulation was beneficial^{27,28}. That the AD severity at t_{d-7} correlated to the skin barrier at t_{d16} indicates that the disease state influenced the barrier during the study and strengthens the hypothesis that systemic inflammation and related external factors played a role in the outcome of this study. A specific example was subject P01, who started with the highest SCORAD of all subjects but relative normal barrier function and lipid parameters. Both barrier function and lipid parameters changed during treatment to a state comparable to lesional skin. The large variation in lipid parameters between subjects and effects during treatment could,

thus, partially be explained by the AD severity. From the result presented here, it can be concluded that in moderate to severe AD patients, monotherapy with this emollient was insufficient to counter the progression of AD.

For several subjects treatment did show improvement of the lipid parameters. Because observed effect sizes were small and it might take many cycles of cornification and desquamation before the barrier is properly restored, more than two weeks of formulation application would be desirable to sufficiently restore the SC lipid composition and organization of AD patients. Emollient treatment should be combined with anti-inflammatory treatment to counter flares during this period. The results in non-lesional AD skin support the assumption that a vernix caseosa emollients can help restore barrier abnormalities in AD, as it did in healthy volunteers with artificial induced barrier impairment.

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5

Supplementals chapter 5

Supplemental S5.1: Ingredients of the formulation

Ingredient	Supplier	Weight %
Super sterol esters Crodalan SWL	(Croda, UK)	49.72% w/w
Crodamol GTCC miglyol	(Croda, UK)	36.98% w/w
Squalane	(Galderma)	6.63% w/w
Cholesterol USP/NF	(Croda, UK)	3.63% w/w
Palmitic acid (FFAC16:0)	(Emery)	0.83% w/w
Stearic acid (FFA 18:0))	(Berg + Schmidt)	0.10% w/w
Ceramide NS (C40)	(Evonik, Germany)	1.18% w/w
Ceramide EOS (C66)	(Evonik, Germany)	0.83% w/w
Oxyxyn LM	(Merck, Germany)	0.10% w/w
anti-oxidant for ceramide EOS containing:		
DL-γ -Tocopherol (Vitamin E)	0.025% w/w	
Lecithin	0.025% w/w	
Ascorbyl palmitate	0.020% w/w	
Glycerin monostearate	0.020% w/w	
Glycerin monooleate	0.0025%w/w	
Citric acid	0.0025%w/w	

Supplemental S5.2: SPSS scripts

Code used to compare groups with SPSS.

```

MIXED stretch BY Treated Lesional
  /CRITERIA =CIN(95) MXITER(100)
  MXSTEP(10) SCORING(1)
  SINGULAR(0.000000000001)
  HCONVERGE(0, ABSOLUTE)
  LCONVERGE(0, ABSOLUTE)
  PCONVERGE(0.000001, ABSOLUTE)
  /FIXED= Treated Lesional(Treated)
  SSTYPE(3)
  /METHOD=REML
  /PRINT =SOLUTION TESTCOV
  /RANDOM = INTERCEPT |
  SUBJECT(Volunteer) COVTYPE(VC).

```

*Treated and lesional are the fixed factors
 and lesional is nested within Treated
 *Volunteer is used for the pairing per subject.

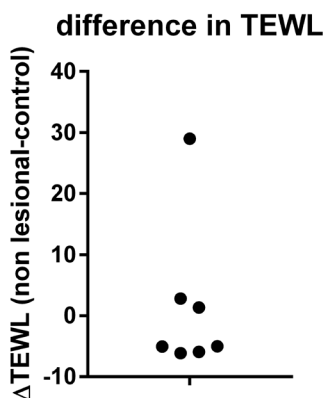
Code used to plot correlations:

```

MIXED MCL BY Site WITH C34
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  MXSTEP(10) SCORING(1)
  SINGULAR(0.000000000001)
  HCONVERGE(0, ABSOLUTE)
  LCONVERGE(0, ABSOLUTE)
  PCONVERGE(0.000001, ABSOLUTE)
  /FIXED=Site C34 Site*C34 | SSTYPE(3)
  /METHOD=REML
  /PRINT=SOLUTION TESTCOV.

```

*Site 1 to 3 are control, non-lesional, and
 lesional and is a fixed factor
 *MCL and C34 are the dependent variable
 and covariate.



Supplemental S5.3: Difference in TEWL

Difference in TEWL between t_{d0} and t_{d16} of non-lesional sites.

Supplemental S5.4: correlation matrix between lipid and barrier parameters

	TEWL		1/TEWL		MCL		Mucer (pmol/sq)	
	pearson r	p-value	pearson r	p-value	pearson r	p-value	pearson r	p-value
TEWL			-0.878	<0.001	-0.806	<0.001	0.725	<0.001
1/TEWL	-0.878	<0.001			0.813	<0.001	-0.765	<0.001
MCL	-0.806	<0.001	0.813	<0.001			-0.670	<0.001
Mucer	0.725	<0.001	-0.765	<0.001	-0.670	<0.001		
EO %	-0.475	0.030	0.537	0.012	0.695	<0.001	-0.323	0.153
C34	0.845	<0.001	-0.810	<0.001	-0.906	<0.001	0.796	<0.001
Ratio	-0.753	<0.001	0.850	<0.001	0.852	<0.001	-0.696	<0.001
Stretching	0.586	0.005	-0.616	0.003	-0.384	0.086	0.466	0.033

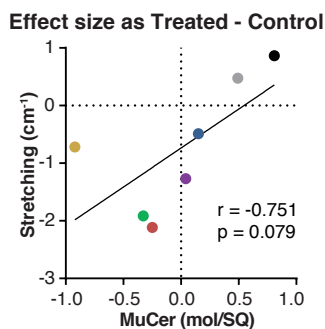
	EO molar %		C34 (pmol/sq)		Subclass ratio		CH ₂ Stretching	
	pearson r	p-value	pearson r	p-value	pearson r	p-value	pearson r	p-value
TEWL	-0.475	0.030	0.845	<0.001	-0.753	<0.001	0.586	0.005
1/TEWL	0.537	0.012	-0.810	<0.001	0.850	<0.001	-0.616	0.003
MCL	0.695	<0.001	-0.906	<0.001	0.852	<0.001	-0.384	0.086
Mucer	-0.323	0.1534	0.796	<0.001	-0.696	<0.001	0.466	0.033
EO %			-0.483	0.0266	0.406	0.0676	-0.403	0.070
C34	-0.483	0.027			-0.819	<0.001	0.387	0.083
Ratio	0.406	0.068	-0.819	<0.001			-0.365	0.104
Stretching	-0.403	0.070	0.387	0.083	-0.365	0.1037		

Supplemental S5.5: Correlation matrix between the changes in parameters during treatment.

	TEWL		MCL		EO%		Mucer	
	pearson r	p-value	pearson r	p-value	pearson r	p-value	pearson r	p-value
TEWL			-0.467	0.291	-0.081	0.863	0.597	0.157
MCL	-0.467	0.291			0.849	0.016	0.392	0.384
EO%	-0.081	0.863	0.849	0.016			0.641	0.121
MuCer	0.597	0.157	0.392	0.384	0.641	0.121		
C34	0.921	0.003	-0.692	0.085	-0.259	0.575	0.334	0.464
Ratio	-0.871	0.011	0.457	0.302	-0.063	0.893	-0.477	0.279
Stretching	0.785	0.037	-0.177	0.705	0.277	0.547	0.640	0.121
SCORAD	0.288	0.531	-0.349	0.443	-0.505	0.248	-0.110	0.814
EASI	0.205	0.660	-0.181	0.698	-0.427	0.339	-0.063	0.893
loc SCORAD	0.373	0.410	-0.138	0.768	0.197	0.671	0.274	0.552

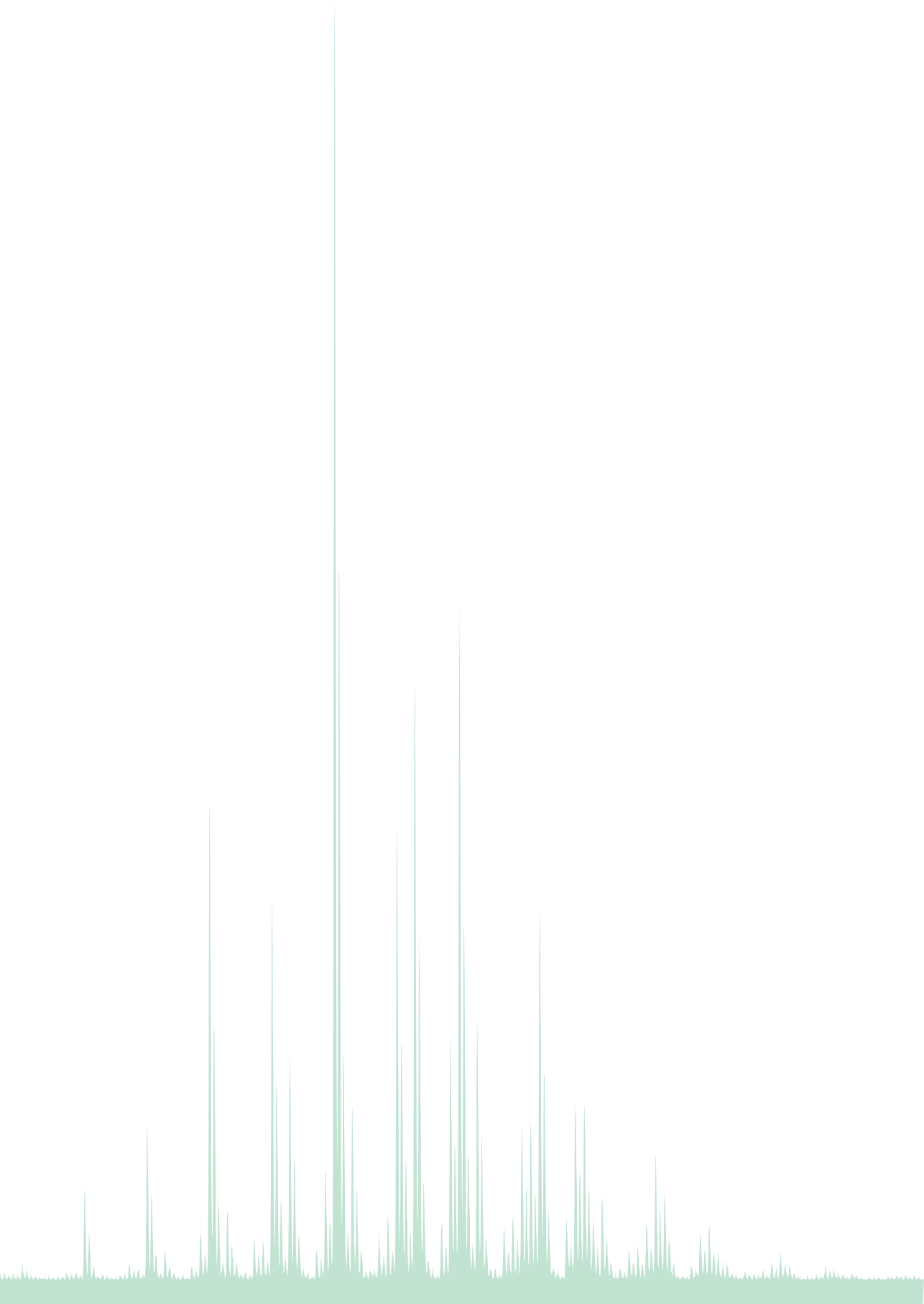
	C34		Ratio		Stretching		SCORAD	
	pearson r	p-value	pearson r	p-value	pearson r	p-value	pearson r	p-value
TEWL	0.921	0.003	-0.871	0.011	0.785	0.037	0.288	0.531
MCL	-0.692	0.085	0.457	0.302	-0.177	0.705	-0.349	0.443
EO%	-0.259	0.575	-0.063	0.893	0.277	0.547	-0.505	0.248
MuCer	0.334	0.464	-0.477	0.279	0.640	0.121	-0.110	0.814
C34			-0.934	0.002	0.744	0.055	0.137	0.770
Ratio	-0.934	0.002			-0.815	0.026	0.114	0.808
Stretching	0.744	0.055	-0.815	0.026			0.088	0.851
SCORAD	0.137	0.770	0.114	0.808	0.088	0.851		
EASI	0.017	0.971	0.233	0.615	-0.110	0.815	0.874	0.010
loc SCORAD	0.365	0.421	-0.518	0.234	0.442	0.321	-0.093	0.842

	EASI		Local SCORAD	
	pearson r	p-value	pearson r	p-value
TEWL	0.205	0.660	0.373	0.410
MCL	-0.181	0.698	-0.138	0.768
EO%	-0.427	0.339	0.197	0.671
MuCer	-0.063	0.893	0.274	0.552
C34	0.017	0.971	0.365	0.421
Ratio	0.233	0.615	-0.518	0.234
Stretching	-0.110	0.815	0.442	0.321
SCORAD	0.874	0.010	-0.093	0.842
EASI			-0.473	0.284
loc SCORAD	-0.473	0.284		



Supplemental S5.6: Correlation between changes in MuCer amount and CH2 Stretching

Changes in stretching peak position and change in MuCer amount during treatment for each patient. The Pearson r and significance are indicated.



Part III

Chapter 6

Selectivity in cornified envelop binding of ceramides in human skin and the role of LXR inactivation on ceramide binding

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Background:

Spectra of the bound ceramides and their unbound counterparts (left bound OS, right unbound EOS)

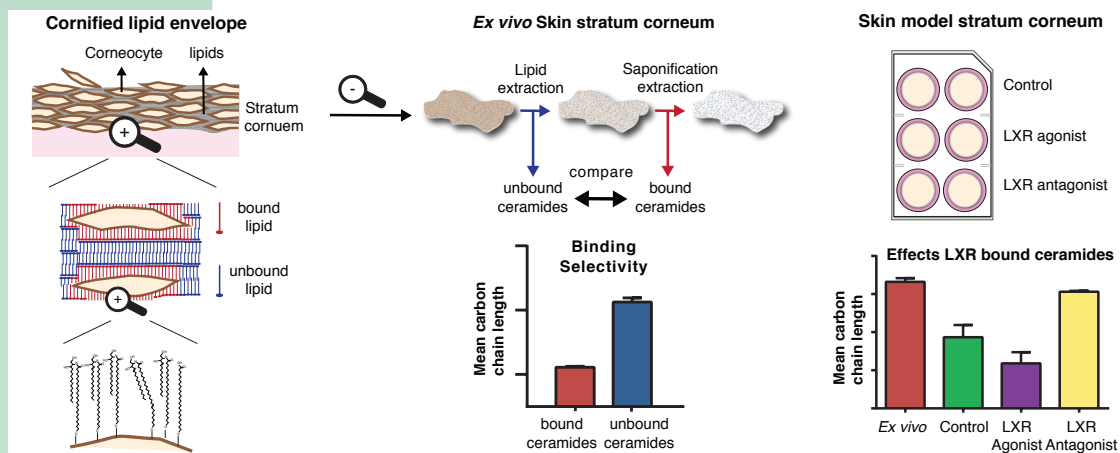
6

Selectivity in cornified envelope binding of ceramides

Abstract

The cornified lipid envelope (CLE) is a lipid monolayer covalently bound to the outside of corneocytes and is part of the stratum corneum (SC). The CLE is suggested to act as a scaffold for the unbound SC lipids. By profiling the bound CLE ceramides, a new subclass was discovered and identified as an omega-hydroxylated dihydrosphingosine (OdS) ceramide. Bound glucosylceramides were observed in superficial SC layers of healthy human skin. To investigate the relation between bound and unbound SC ceramides, the composition of both fractions was analyzed and compared. Selectivity in ceramide binding towards unsaturated ceramides and ceramides with a shorter chain length was observed. The selectivity in ceramide species bound to the cornified envelope is thought to have a physiological function in corneocyte flexibility. Next, it was examined if skin models exhibit an altered bound ceramide composition and if the composition was dependent on liver X-receptor (LXR) activation. The effects of an LXR agonist and antagonist on the bound ceramides composition of a full thickness model (FTM) were analyzed. In FTMs, a decreased amount of bound ceramides was observed compared to native human skin. Furthermore, FTMs had a bound ceramide fraction which consisted mostly of unsaturated and shorter ceramides. The LXR antagonist had a normalizing effect on the FTM bound ceramide composition. The agonist exhibited minimal effects. We show that ceramide binding is a selective process, yet, still is contingent on lipid synthesized.

KEYWORDS:
cornified lipid
envelope,
bound lipids,
ceramide,
quantification,
skin culture,
liver-X-receptor.



Graphical abstract

6.1 Introduction

The outer skin layer, the stratum corneum (SC), consists mainly of terminally differentiated keratinocytes (corneocytes) embedded in an extracellular lipid matrix of ceramides, free fatty acids, and sterols. During the terminal differentiation process, the plasma membrane is replaced by a crosslinked protein structure called the cornified envelope (CE)¹. Covalently bound to the CE is a monolayer of lipids referred to as the cornified lipid envelope (CLE). These lipids act as an interface between the hydrophilic corneocytes and the lipophilic extracellular lipids and are therefore an essential part of the skin barrier^{2,3}. With a reduced CLE mice exhibit severe skin barrier problems⁴.

It has been reported that the CLE consists of ceramides and fatty acids⁵⁻⁷. Ceramides are composed of two parts: a sphingoid base and an acyl chain. The molecular architecture of the ceramide its hydrophilic head group defines the ceramide subclass⁸. The bound ceramides comprise of a subset of the unbound subclasses^{5,6}, **Supplemental S6.1** depicts the structures of these subclasses. This subset has an omega-hydroxylated acyl chain (OCers). The hydroxylated group at the end of the carbon tail facilitates an ester linkage to the CE proteins^{9,10}. It also facilitates the binding of a linoleic acid. Both unbound SC ceramides (in the lipid matrix) and the bound SC ceramides (attached to cornified envelope) originate from the same pool of precursor lipids¹¹.

During the differentiation process the SC lipids are synthesized by keratinocytes¹². In the upper part of the viable epidermis keratinocytes contain specific lipid vesicles called lamellar bodies. They contain the bound and unbound precursor lipids. At the interface between the viable epidermis and SC, ceramide precursors (glycosylated (GlcCers) or phosphocholinated (sphingomyelin) ceramides¹³) are released into the extracellular space together with the enzymes to convert them¹⁴. Simultaneously, formation of the CLE occurs^{15,16}. Enzymes involved in ceramide binding to the CE have been colocated at the viable epidermis SC interface¹⁷. Essential to ceramide binding is that ceramides have I) a linoleate esterified omega-hydroxylated acyl chain (EOCers)¹⁸⁻²⁰ and II) are glycosylated. The esterified linoleate is removed, the GlcCers are bound, and thereafter, they are cleaved to ceramides¹¹. Which ceramides of the pool of precursors are bound and if this is a selective process is not yet reported.

To study which ceramides are selected for CE binding, SC from *ex vivo* skin and from skin models was used. Comparing the composition of both bound and unbound lipid extracts of *ex vivo* SC could elucidate whether ceramide binding is a selective process. Human skin equivalent full thickness models (FTMs) were selected as skin model. These models mimic aspects of normal human skin including SC generation. Yet, in FTMs the SC lipids had an increased fraction of unsaturated ceramides and a reduced mean ceramide chain length^{21,22}. It was hypothesized that the bound lipid fraction of FTMs was altered too. LXR is a nuclear receptor sensitive for sterols and responsible for

multiple lipid processing pathways²³, one of which is regulation of Stearoyl coenzyme A desaturase 1, responsible for the desaturation of fatty acids also used in the epidermal ceramide biosynthesis²⁴. By adding a liver X-receptor (LXR) agonist or antagonist, the lipid metabolism of FTMs can be manipulated²⁵. LXR signaling was hypothesized to affect the bound lipids in FTMs as well.

Recently, a new LC-MS method to simultaneously profile and quantify the unbound SC ceramide fraction was developed and could be applied to discover new bound ceramides²⁶. Quantification of the entire bound ceramide profile provides insight into subclass and chain length distribution and the degree of unsaturation. This method was applied to the bound ceramide fractions of *ex vivo* SC, tape stripped SC, and FTMs. We aimed to examine if ceramide binding is a selective process and if this process is affected in FTM by an LXR agonist or antagonist.

6.2 Materials and Methods

6.2.1 Chemicals

For extraction and analysis of the lipids the following solvents were used: UPLC grade methanol, ethanol, iso-propanol (Biosolve, Valkenswaard, the Netherlands), HPLC grade methanol, chloroform (Lab-Scan Gliwice, Poland), and n-heptane, and milli-Q water (18.2Ω). KCL and NaOH (Merck KGaA, Darmstadt, Germany) were used in the extraction. **Supplemental S6.2** contains a list of standards and the internal standard used for ceramide quantification.

6.2.2 Samples

All human skin was obtained after institutional approval and a written informed consent according to the declaration of Helsinki.

6.2.2.1 Ex vivo SC samples

Skin from two mammoplasties and one abdominoplasty was dermatomed to a thickness of 0.6 mm within 24 hours after surgery. The dermatomed skin was incubated overnight on filter paper containing 0.1% trypsin solution in PBS and thereafter digested for 1 hour at 37°C. SC sheets were peeled off, thoroughly washed with 0.1% trypsin-inhibitor followed by 2x milli-Q water, and were air dried before storage. For each donor, 3 SC samples of each ~3.5 mg SC dry weight were used for lipid extraction and subsequent bound lipid extraction.

6.2.2.2 *In vivo* tape-stripped SC samples

One site on the ventral forearm of the volunteer was tape-stripped using polyphenylene sulfide tape (Nichiban, Tokyo, Japan). Equal pressure was applied on the tapes using a 1 kg weight and a D-squame pressure instrument with additional foam pad and the removal direction alternated. The amount of material on the tapes was determined by detecting the absorption at 850nm using a SquameScan (Heiland Electronic, Wetzlar, Germany), subtracting the absorbance of a blank tape. The first five tapes were discarded and four consecutive tapes were used for the bound lipid extraction. Each tape was extracted individually.

6.2.2.3 *Full thickness models* SC samples

FTMs were generated using isolated human keratinocytes obtained from three different skin donors. Keratinocytes were seeded on a collagen gel populated with fibroblasts and FTMs were generated as described by Thakorsing et al.²². LXR activation or inhibition was induced by supplementation of the agonist T0901317 or antagonist GSK2033, respectively (Sigma Aldrich, Zwijndrecht, the Netherlands). The concentration of the agonist and antagonist in the medium was 500 nM (with an EC_{50} of 50nM and IC_{50} of 32nM, respectively); 0.05 vol% of a 1mM stock in DMSO. For each donor FTMs were generated under four different conditions: FTM_{CONTROL} (normal medium), FTM_{DMSO} (medium + DMSO), FTM_{AGONIST} (Medium + Agonist), and FTM_{ANTAGONIST} (Medium + Antagonist). The FTMs SC was isolated as described above.

6.2.3 *Lipid extractions*

The SC samples were dried under vacuum at 50°C, stored under N₂, and weighted using a microbalance ME5 (Sartorius, Bradford, MA, USA). To obtain the unbound lipids, SC sheets and tape-strips were first extracted using a modified four step Bligh and Dyer procedure²⁶. MilliQ and 0.04 vol% of aqueous 0.25M KCl was added to the combined extracts of the four steps. After phase separation over night at 4°C the organic layer was collected. Thereafter, the hydrophilic layer was washed with chloroform and the organic layers were combined, filtered (0.20µm PTFE filter), evaporated under N₂, and reconstituted in chloroform:methanol (2:1, v:v), 0.4 ml per mg SC dry weight.

After extraction of the unbound lipids, the SC sheets were washed with chloroform and dried under vacuum. A solution of 10% 1M aqueous NaOH and 90% methanol (v:v) was added to the dried samples to detach the bound lipids. For saponification the samples were shaken for 1 hour with 120 rpm at 60°C. To stop the reaction, the pH of the samples was decreased to a final pH between 4-7 using 1M and 0.1M HCl. After saponification tape-strips and SC sheets were further extracted at 40°C

by using subsequently: chloroform:methanol (1:1, v:v), chloroform:methanol (2:1, v:v), and heptane:Isopropanol (1:1, v:v). Thereafter, the combined bound lipid extracts were treated as described above for the unbound lipids. The extraction efficiency and recovery of the bound lipid extraction are described in [Supplemental S6.3](#) and were both above 90%.

Before analysis, the concentration of the unbound and bound lipids extracts was determined by the gravimetric difference of a specific volume before and after drying. For LC-MS analysis, unbound lipid samples were prepared at 0.3 mg/ml of extract and tape-strip extract at 20 tapes/ml. Bound lipids samples were prepared at 0.1 mg/ml and tape-strip extracts at 40 tapes/ml. All samples were dissolved in heptane:chloroform:methanol (95:2.5:2.5, v:v:v) with internal standard added at 0.6 μ M.

6.2.4 LC-MS and LC-MS/MS analysis

All samples were analyzed and quantified using a Waters Acquity UPLC H-class (Waters, Milford, MA, USA) connected to an XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA). Of the samples, 5 μ l was injected and separated on a pva-silica column (5 μ M particles, 100x2.1mm i.d.)(YMC, Kyoto, Japan). Quantitative analysis was performed as described previously²⁶. For identification, LC-MS/MS data were obtained with Scanwave daughter scan mode on the m/z values of interest with fragmentation energies of 40 or 50 eV. [Supplemental S6.4](#) shows the m/z ratios analyzed to identify the omega-hydroxylated dihydrosphingosine (OdS) ceramide.

6.2.5 Quantification

All ceramides that were identified in the bound and unbound fraction of the *ex vivo* SC samples were quantified to pmol and ng injected into the LC. Because the bound lipid extracts contained other materials than lipids (explained below), it was essential to normalize it to SC dry weight. The total amount of material extracted from the SC was calculated by multiplying the concentration of the extract with the volume in which it was dissolved. Either 1.5 μ g (unbound sample) or 0.5 μ g (bound sample) of this material was injected into the LC. The total amount of ceramides detected in this injected fraction of the sample was used to calculate the amount of ceramides in the complete extract (nmol or μ g). The total amount of ceramides in the extract (μ g) was normalized to the SC dry weight in mg. To determine which fraction of ceramides was bound, molar amounts per mg of SC of the unbound and bound ceramides were compared.

As a control for the gravimetric approach to determine the bound lipid

amount extracted, three blank saponified extractions were performed. **Supplemental S6.5** shows the results and discussion of these experiments. It was concluded that in saponified extracts excess non-lipid materials (most probably salts) were present.

6.2.6 Software and statistics

LC-MS and LC-MS/MS data analysis was performed using MassLynx and TargetLynx V4.1 SCN 843 (Water Inc. Milford, MA, USA). Statistical analyses were performed using linear mixed modeling in SPSS version 24 (IBM Corp.). A detailed description of the models is given with the corresponding Supplemental.

6.3 Results

6.3.1 Profiling and quantification

To examine which bound ceramides could be detected by our method, a profile was made by LC-MS. **Figure 6.1A** depicts an ion-map profile of a 0.5 µg *ex vivo* SC bound ceramide extract. Previously observed ceramide subclasses OS, OP, and OH were detected, however, additional compounds were detected as well. The retention times (Rts) of two groups of compounds corresponded to the Rts of GlcCers and had masses corresponding to Glc-OS and Glc-OH subclasses. A part of the GlcCers loses their glucose group during ionization. This was used to confirm the presence of bound GlcCers by detecting OS at the same Rt as Glc-OS (**Supplemental S6.6**). Bound GlcCers were also detected in a tape-strip sample, yet, they are not observed in the corresponding unbound fraction.

One group of compounds had a shorter Rt than that of subclass OS and a mass of 2 amu higher. To confirm that this was the suspected subclass OdS, fragmentation of 3 saturated, 2 unsaturated, and 1 odd number chain length of these compounds was performed (**Supplemental S6.4**). **Figure 6.1B** depicts the fragmentation spectrum of OdS C52:0. The characteristic fragments of dS ceramides were observed (294.3 and 322.3 amu). As comparison a fragmentation spectrum of OS C52:0 is shown in **Figure 6.1C**, depicting a sphingoid fragment two amu lower (292.3 and 320.3 amu). Finally, a group of compounds was observed with the mass of an omega-hydroxyl acyl chain with a dihydroxy-dihydrosphingosine (T). The latter was described by t' kind et al²⁷. Combined with their long Rt we assumed this is ceramide subclass OT. Only including the subclasses OdS, OS, OP and OH, a total of 103 different bound ceramides were detected in *ex vivo* SC bound lipid extracts.

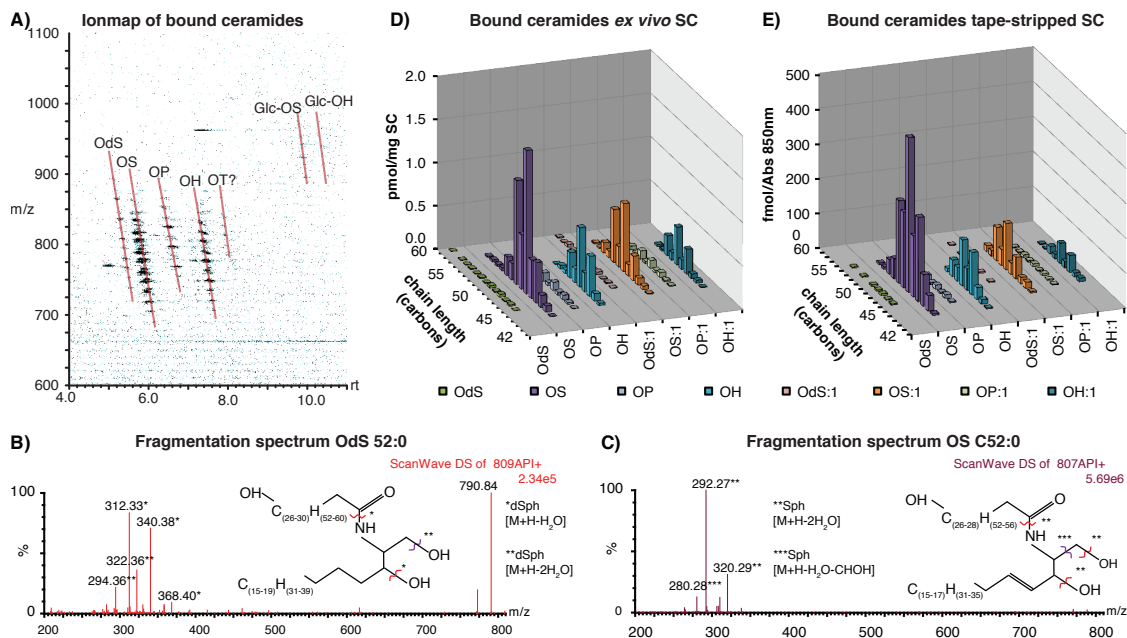


Figure 6.1: Profiling of bound ceramide fraction

A: An ion-map of the bound ceramide fraction of *ex vivo* SC. Each ceramide subclass is indicated by a red line. **B:** Fragmentations spectrum of the compound eluting before OS, *m/z* 808.81 and collision energy of 40eV. **C:** Fragmentations spectrum of OS C52:0, *m/z* 806.80 and collision energy of 50eV. **D and E:** The amount of all quantified bound ceramides shown per subclass and chain length of an *ex vivo* SC samples and a tape-stripped SC sample, respectively.

Thereafter, the profiled bound ceramides were quantified. Next to *ex vivo* SC, a tape strip extract was examined to determine if this method was applicable to this sample type. **Figure 6.1D and E** show the quantitative profiles of a representative *ex vivo* SC sample and a tape-strip sample, expressed as the molar ceramide amount per mg SC or the amount per amount SC stripped (expressed as absorbance at 850nm), respectively. The profile showed that: 1) It is possible to isolate the bound ceramides from tape-stripped SC after extraction of the unbound ceramides; 2) the bound ceramide profile of a tape-strip sample was similar to that of an *ex vivo* SC sample.

To compare the bound and unbound fractions, the total amount of unbound ceramides in the *ex vivo* SC was quantified. Unbound and bound ceramide amounts are provided in **Figure 6.2**. **Figure 6.2A** depicts the total unbound and bound ceramide amounts in μg per mg SC, whereas **Figure 6.2B** depicts the molar percentage of ceramides that were bound. It was observed that: I) there were no large differences between the bound ceramide amounts of the three donors; II) 2.5-4 w% of the SC weight was ceramides; and III) about 20 mol% of the ceramides in the SC was bound.

Table 6.1 depicts the relative molar amount (%) of each bound ceramide subclass to the total bound ceramides amount. In the next section, the bound ceramide fraction and how it relates to the unbound fraction will be examined in more detail.

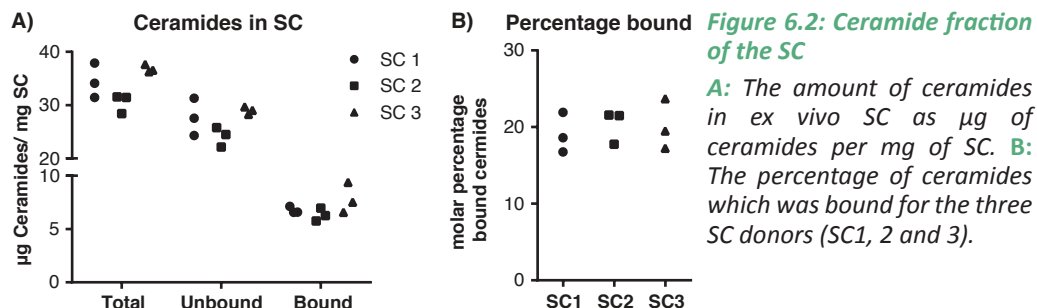


Table 6.1: The relative molar amounts of the bound ceramides per subclass. Given as the mean and SD ($n=3$). The Σ molar amount of all bound ceramides in a sample was set to 100%.

	SC 1		SC 2		SC 3	
Subclass	Molar %	SD	Molar %	SD	Molar %	SD
OdS	1.5	>0.1	2.3	0.3	1.3	>0.1
OS	51.1	0.5	48.6	0.7	43.9	1.3
OP	3.8	0.3	5.1	0.2	3.5	0.1
OH	20.6	0.2	25.3	0.7	17.7	1.0
OdS:1	0.8	0.1	0.9	0.2	1.4	0.3
OS:1	13.3	0.2	9.3	0.1	18.8	1.5
OP:1	2.1	>0.1	2.1	0.2	3.1	0.2
OH:1	6.8	0.3	6.3	0.1	10.3	0.3

6.3.2 Selectivity in ceramide binding

To examine if there is selectivity in ceramide binding, the differences in composition between the bound and unbound ceramide fraction of *ex vivo* SC were determined. It was observed that the bound ceramides contained large amounts of ceramides with a monounsaturated acyl chain (MuCers) and this was higher than the unbound fraction (**Figure 6.3A**). Differences in MuCer % between the 3 donors were observed. Changes for each donor were consistent; if the unbound MuCer% increased, the bound MuCer% increased too. MS/MS fragmentation of the MuCers showed that the double bond was only present in the ceramides acyl chain (**Supplemental S6.7**).

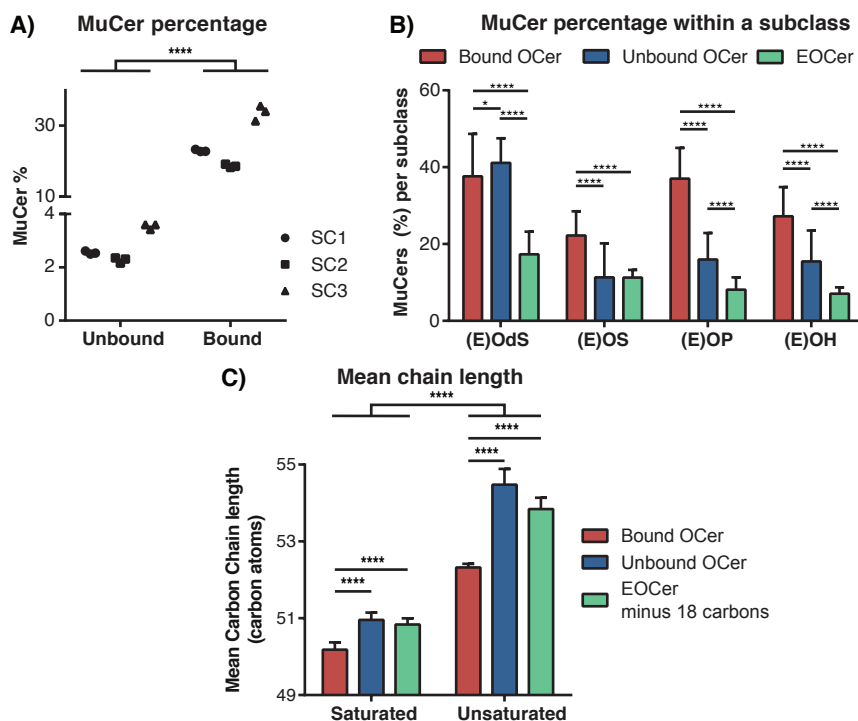


Figure 6.3: Selectivity in ceramide binding for multiple parameters

A: The percentage of MuCers in the bound and unbound ceramides of three SC donors. **B:** The percentage of MuCers within a subclass, comparing the bound OCer, unbound OCer, and EOCer with the same sphingoid base. In a sample, the sum of both the saturated and unsaturated ceramides of a subclass was set as a 100%. **C:** The MCL of the bound and unbound OCers is shown for the saturated and unsaturated ceramides. From the EOCers MCL, 18 carbons were subtracted to compensate for the linoleate moiety. Bars are mean $\pm$ SD of 9 samples, 3 per donor. Parameter estimates of the fixed effects are given in [supplemental S6.8 and S6.9](#).

To compare the bound and unbound ceramides originating from the same GlcCers, the unbound counterparts of the bound ceramides fraction were examined: EOCers and OCers. The unbound ceramides with the highest MuCers content were the EOCer and OCer subclasses ([Supplemental S6.8](#)). Of the bound and unbound ceramides the MuCers percentage within each subclass was determined and plotted per subclass ([Figure 6.3B](#)). Comparing these data with a linear mixed model showed that the bound OCer fraction consisted of a significantly higher MuCers percentage than both the unbound EOCer and OCer subclasses, except for subclass OdS ([Supplemental S6.9](#) depicts the linear mixed model results). In the unbound fraction, subclasses OdS, OP, and OH contained a higher percentage MuCers than their corresponding EOCer subclasses. Comparing the bound and unbound ceramides degree of unsaturation indicates selective binding towards unsaturated ceramides.

Alongside the MuCers percentage, the bound and unbound fractions mean carbon chain lengths (MCL) were calculated. To compare the bound and unbound subclasses, the MCL of the OCer and EOCer subclasses were determined. MuCers are depicted separately because they had a longer MCL than saturated ceramides (linear mixed model results [Supplemental S6.10](#)). To compare the EOCer to the OCer, 18 carbons were subtracted from EOCers MCL to compensate for the esterified linoleates additional 18 carbons. [Figure 6.3C](#) depicts the MCL for the bound OCer, unbound OCer, and unbound EOCer. The MCL of the bound ceramides differed significantly from that of the unbound ceramides. For both the saturated ceramides and MuCers, the MCL was shorter in the bound lipid fraction. The chain length of both unbound OCers and the EOCer-C18 were comparable. These findings on MCL show that there was selectivity toward binding of shorter ceramides to the cornified envelope.

6.3.3 The effect of LXR on the bound lipid fraction in FTMs

Previously, alterations in unbound SC ceramides of FTMs were observed. It was examined if similar changes were observed in the bound ceramide fraction and if the bound ceramides were influenced by the LXR. In [Figure 6.4A](#) quantitative amounts of bound ceramides/mg SC are provided for four FTM conditions: FTM_{CONTROL}, FTM_{DMSO}, FTM_{AGONIST}, and FTM_{ANTAGONIST}. No differences in the total amount of bound ceramides were observed between the various FTM conditions. It was observed that there was a significantly lower quantity of bound ceramides in the FTMs compared to *ex vivo* SC samples. [Supplemental S6.11](#) gives an overview of the statistical tests and output comparing the sample groups. [Supplemental S6.11](#) depicts that in FTMs an oleate was attached to the EOCers instead of a linoleate.

When examining the bound lipid fraction of the FTMs, it consisted predominantly of MuCers ([Figure 6.4B](#)) and even polyunsaturated ceramides (PuCer) were detected. [Table 6.2](#) depicts the relative molar composition of the four different conditions. The percentage of bound MuCers in FTMs was significantly higher than in *ex vivo* SC. By adding an LXR antagonist a significant decrease in the percentage of MuCers was observed. No effect was observed from adding the LXR agonist.

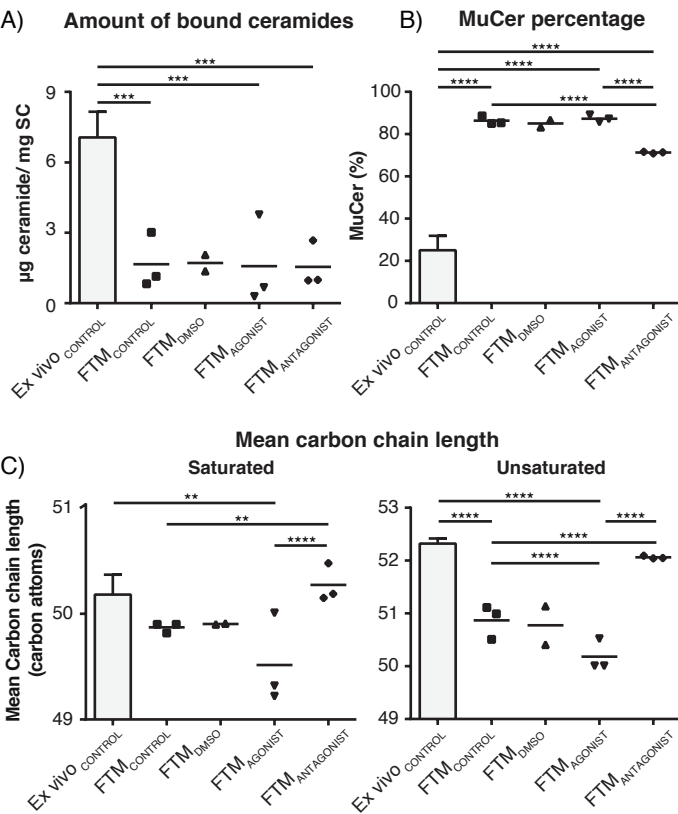


Figure 6.4: Effects of LXR in FTMs on the bound lipids

A: The amount of bound ceramides per mg SC. B: The percentage of the bound ceramides that had a monounsaturated acyl chain. C: The MCL of the saturated and unsaturated ceramides. All depict the ex vivo control as bar (mean +SD n=9), FTMs: CONTROL, DMSO, AGONIST and ANTAGONIST, are shown as individual points and mean. Parameter estimates of the fixed effects are given in [Supplemental S6.10](#).

For FTMs the bound ceramides MCL of saturated and unsaturated bound ceramides was determined and compared to that of ex vivo SC ([Figure 6.4C](#)). In FTMs, a significant decreased MuCers MCL was observed compared to ex vivo SC. The MCL was further decreased by the LXR agonist. However, using the LXR antagonist the MCL is increased to the MCL of ex vivo SC. Due to the lower percentages of saturated ceramides (see [Table 6.2](#)), the MCL of the saturated ceramides was predominantly based on subclass OS. Nonetheless, the same trends were observed as in the MuCers. Concluding, FTMs had an altered bound lipid composition compared to ex vivo SC. An LXR antagonist had a normalizing effect on the FTMs bound ceramide composition, reducing the percentage of MuCers and increasing the MCL.

Table 6.2: The mean and SD ($n=3$ except DMSO which had $n=2$) of the bound lipids relative molar subclass amount in FTMs. For each sample the Σ molar amount of all bound ceramides was set to 100%.

subclass	FTM _{CONTROL}		FTM _{DMSO}		FTM _{AGONIST}		FTM _{ANTAGONIST}	
	Molar %	SD	Molar %	SD	Molar %	SD	Molar %	SD
OdS	n.d.*		0.4	n.a.**	0.6	n.a.**	0.6	0.4
OS	6.8	1.4	6.5	1.1	3.4	1.1	17.5	1.8
OP	0.6	n.a.**	0.8	n.a.**	0.5	0.2	0.9	0.6
OH	2.9	0.6	2.9	0.4	2.8	0.6	8.0	0.2
OdS:1	2.4	0.7	3.5	2.0	3.2	0.2	2.7	0.5
OS:1	53.4	1.2	52.2	3.4	51.6	4.3	42.8	1.2
OP:1	6.2	0.9	6.6	1.7	7.5	0.8	6.1	0.6
OH:1	24.4	3.4	22.7	2.6	25.1	4.3	19.7	2.0
OdS:2	0.3	n.a.**	0.9	n.a.**	0.9	0.0	0.2	n.a.**
OS:2	2.5	0.2	3.1	0.4	3.5	0.1	1.6	1.0
OP:2	0.3	n.a.**	0.2	n.a.**	0.5	n.a.**	0.1	n.a.**
OH:2	1.0	0.3	1.2	0.3	1.8	0.4	0.9	n.a.**

*n.d. not detected

**n.a. not available (only detected in one of the replicates)

6.4 Discussion

Selectivity in ceramide cornified envelope binding was observed by comparing compositions of the unbound EOCer and OCer subclasses to the bound SC ceramide fractions. In FTMs, the ceramide binding was reduced and the bound ceramide composition influenced by LXR. The new ceramide subclass OdS was identified⁵⁻⁷. Furthermore, a ceramide subclass with masses corresponding to an omega-hydroxy acyl dihydroxy-dihydrosphingosine (OT) was observed. It was hypothesized that dihydroxy-dihydrosphingosine exists as a bound ceramide, because these sphingoid bases have been observed attached to the various acyl chains of the unbound lipids (unpublished data).

Using the compositional differences between the bound and unbound ceramides, it was shown that bound ceramides had a higher degree of unsaturation and shorter carbon chains than their unbound counterparts. Both the bound and unbound ceramides are thought to originate from the same pool of precursor GlcCers²⁸. Thus, during the binding process there was selectivity towards MuCers and shorter chain ceramides. Selectivity in ceramide binding conceivably has a physiological importance to provide corneocyte flexibility. Bound lipids form a template for the formation of the SC lipid organization and can align the lipid lamellae parallel to the cornified

envelope^{4,29,30}. Furthermore, the SC and within it the corneocytes, are not rigid structures but require flexibility³¹⁻³³. Atomic force microscopy studies showed that the CLE is less rigid than the internal corneocyte structures^{31,32}. With different hydration states the shape of corneocyte changes³³, requiring flexibility which should also be provided by the CLE. Flexibility of the CLE might be enhanced by a shorter MCL^{34,35} and the presence of MuCers, although it is unknown if these are cis or trans configured either would impact the flexibility.

In FTMs, a lower amount of bound ceramides per mg dry SC was observed than in *ex vivo* SC. Previously published results showed no reduction³⁶. It is likely that the observed reduction is due to the reduction of a precursor in the ceramide binding pathway (**Figure 6.5**). In the unbound ceramides of FTMs a large fraction of EOCers contained an esterified oleic acid (**Supplemental S6.12**)³⁷. Previously, it has been shown that at the viable epidermis SC interface the linoleic acid is converted to an epoxide by 12R-LOX and subsequently to a hydroxy-epoxide by e-LOX^{3,38}. In mice missing either of these enzymes, reduced amounts of bound ceramides were observed, indicating that this process is essential for ceramide binding⁴. Furthermore, in essential fatty acid deficient pigs and mice, linoleic acid of EOCers became substituted with oleic acid, resulted in skin barrier dysfunction^{11,39}. Thus, in FTMs the decreased amount of bound ceramides was likely due to the absence of sufficient amounts of linoleic acid esterified EOCers.

FTMs bound ceramides consisted predominately of MuCers and had a decreased chain length compared to those in *ex vivo* SC. Similar as to the bound ceramides, FTMs unbound ceramides also had an increased MuCer fraction and reduced chain length compared to *ex vivo* SC^{21,25,40}. Hence, changes in FTMs bound ceramide composition were comparable to changes in the free ceramide fraction. It was observed that the LXR could alter the unbound SC lipid composition of FTMs²⁵. Here, it was shown that the bound lipid composition improved upon LXR inhibition compared to the FTM_{control}, reducing the MuCers fraction and increasing the FTMs MCL. Contrastingly, an LXR agonist induced minor changes in the bound ceramide composition, expected as the endogenous substrate would be present in FTMs. Effects of LXR inhibition were comparable to the changes observed in the unbound lipid fraction upon LXR inhibition²⁵. This showed that LXR has down-stream effects and can change the CLE composition.

In this study, bound glycosylated ceramides were observed in human *ex vivo* SC and also in tape-strips samples. Other reported that mice with a deficiency in sphingolipid activator proteins precursor (pSAP^{-/-}) and that in a Gaucher disease mouse model, the glycosylated bound ceramides were increased compared to wild type mice^{41,42}. Yet, to our knowledge bound GlcCers had never been observed in



- healthy human SC samples. The bound GlcCers in tape-stripped SC samples demonstrates that the bound GlcCers are also present in superficial layers of the SC, while GlcCers were not detected in the unbound lipids of tape-stripped samples. Free GlcCers primarily reside at the viable epidermis SC interface^{14,43}. Whether the bound GlcCers escaped cleavage or that they have biological function is not known, yet, it does indicate that ceramide binding and glucosyl-cleavage are two separate

Our data demonstrate selectivity in the binding process for ceramide linkage to the CE. Here, we propose a selective mechanism for ceramide binding based on our and previously published data ([Figure 6.5](#)). We propose the following: 1) Ω -hydroxylation of the acyl chain creating OCers²⁹. 2) Mediated by PNLPA1 and with ABHD5 as a co-factor, a lineolate (C18:2) is bound to the OCers resulting in EOCer^{18-20,45}. 3) By UDP-glucose ceramide glucosyltransferase (UGCG), glucose is attached to the remaining OCers and EOCers and both are stored in lamellar bodies. Glucose attachment is essential for ceramide binding⁴⁴. GlcCers are released into the viable epidermis SC

interface. During this event, enzymes essential for ceramide binding colocalize¹⁷. In the next two steps (4, 5) 12R-LOX and eLOX3 convert the linoleate to a hydroxy-epoxide, yielding a ceramide with a hydroxy-epoxide and glucose attached. Literature reports that both modifications are necessary for ceramide binding, shown by PNLPA1 deficient mice which have abundant free OS and free Glc-OS in the SC, but virtually no bound ceramides¹⁸. 6) Transglutaminase 1 is hypothesized to bind the ceramides to the cornified envelope⁴⁶, but other mechanisms have not been excluded⁴⁷. 7) GBA1 removes the glucose of the bound ceramide¹⁴. We show that this mechanism is selective towards MuCers and short chain ceramides, which further increases in FTMs. Yet, when the lipid synthesis is altered by LXR inhibition, compositional changes in the unbound ceramides were observed in the bound ceramides as well. This indicates that the ceramide binding is selective but still contingent on the lipid synthesis. Although it cannot be specified which part of the binding process is responsible for the selectivity, it can be deduced that it serves an important physiological function by providing flexibility to the corneocytes. Alterations in the binding and composition of the bound lipids could affect the formation of a proper SC lipid barrier.

Acknowledgements:

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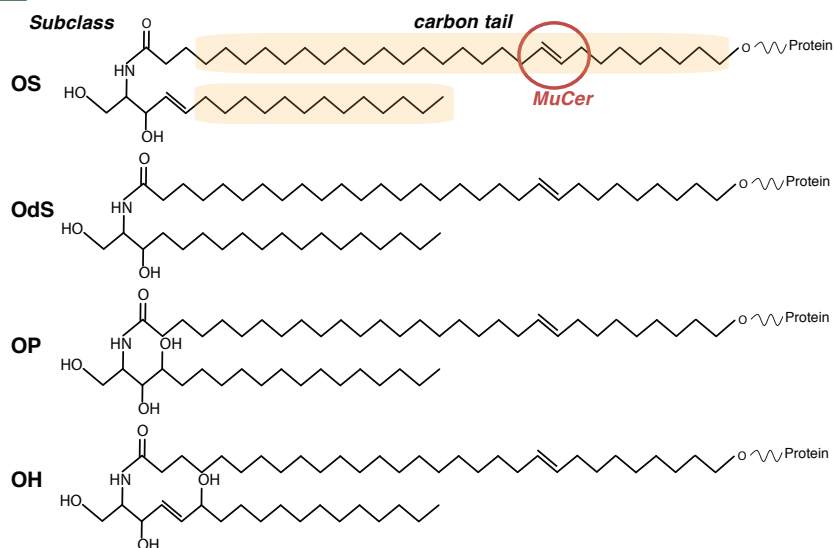
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6

Supplementals chapter 6



Supplemental S6.1: Bound ceramide structures

Ceramides standard used for the quantification of the ceramides and structures of the bound ceramide subclasses. The first one has the, carbon tail, and unsaturation indicated.

Supplemental S6.2: Synthetic ceramides that were used as calibrators for LC/MS analysis.

Numbers in brackets indicate the number of carbon atoms in the fatty acid chain and sphingosine chain, respectively.

Ceramides		
N(24)dS(18) ¹	N(16)S(18) ¹	E(18:2)O(30)S(18) ²
N(18)dS(18) ¹	N(24)P(18) ²	E(18:2)O(27)S(18) ²
N(16)dS(18) ¹	N(16)P(18) ²	E(18:2)O(30)P(18) ²
N(24)S(18) ²	A(24)S(18) ¹	E(18:2)O(27)P(18) ²
N(22)S(18) ¹	A(22)S(18) ¹	N(24deu)S(18) ²³
N(20)S(18) ¹	A(16)S(18) ¹	
N(18)S(18) ¹	A(24)P(18) ¹	

¹ Avanti polar lipids (Alabaster, USA) ² Evonik Industries (Essen, Germany) ³ISTD

Supplemental S6.3: Extraction efficiency and Recovery

To determine the extraction efficiency, four sheets of ex vivo SC from two donors (two sheets per donor) were used. First, the unbound lipids were extracted and the SC washed with chloroform (as described in the methods). Then, two consecutive bound lipid extractions were performed on the SC sheets. To compare both extracts, the first extract was dissolved in 1.4 ml and the second in 0.28 ml. A fifth of the volume was chosen as it was expected that the second extract would only contain a limited amount of ceramides. The samples were measured and the ceramides that could be detected in the second extracted used to determine the extraction efficiency. **Supplemental S6.3 Table 1** shows the percentage of these ceramides that was detected in the first bound lipid extract.

Supplemental S6.3 Table1:

The extraction efficiency as the percentage in the first extract compared to the total of two consecutive saponifications. Values were calculated for each ceramide per SC sample. All value is the summed detection of the listed ceramides. The mean per ceramide, per sample and total mean are given as well.

Extraction Efficiency %	Donor 1		Donor 2		Mean
Ceramide	Sample 1	Sample 2	Sample 1	Sample 2	
OdS C58	69.5	100.0	100.0	100.0	92.4
OdS C56	83.1	100.0	91.5	100.0	93.7
OS C52	98.0	99.2	99.4	99.5	99.0
OS C51	98.2	99.2	100.0	100.0	99.4
OS C50	98.5	99.3	99.5	99.6	99.2
OS C48	98.4	100.0	100.0	100.0	99.6
OP C52	97.8	100.0	100.0	100.0	99.5
OH C52	98.9	99.4	100.0	100.0	99.6
OH C50	99.0	99.6	99.7	99.8	99.5
OH C48	98.6	99.5	100.0	99.8	99.5
All	98.3	99.4	99.7	99.8	99.3
Mean	94.0	99.6	99.0	99.9	98.1

To determine the recovery of the bound lipid extraction, 3 mixtures of the calibration standards (listed in **Supplemental S6.2**) were aliquoted into two 1 ml samples of 2 μ M. One aliquot was extracted by saponification and the other was left untreated. Both were analyzed and the recovery was determined as the difference in detection between the extracted and non-extracted sample relative to the detection in the non-extracted samples. **Supplemental S6.3 Table 2** depicts the recovery as percentage.

Supplemental S6.3 Table 2:

Recovery of the bound lipid extraction, as percentage remaining after extraction compared to a non-extracted sample. The mean of all three samples per ceramide and the total mean are given.

Recovery %				
Ceramide	sample 1	sample 2	sample 3	mean
NdS C42	105.1	88.0	95.9	96.3
NdS C36	99.1	88.5	94.2	93.9
NdS C34	101.0	83.3	98.5	94.3
NS C42	99.7	93.3	90.1	94.4
NS C40	103.5	83.3	87.2	91.3
NS C38	95.6	87.5	93.8	92.3
NS C36	103.1	89.9	98.0	97.0
NS C34	113.2	92.6	95.5	100.5
NP C42	99.6	81.0	89.7	90.1
NP C34	97.5	90.9	105.6	98.0
AS C42	105.2	82.1	92.0	93.1
AS C40	103.8	77.9	93.6	91.8
AS C34	96.3	88.6	93.7	92.9
AP C42	90.5	80.5	94.6	88.5
			mean	93.9

Supplemental S6.4 Overview of observed fragments from multiple OdS Ceramides

Ceramide		OdS C56	OdS C56	OdS C56:1	OdS C56:1
Chem Form.		C ₅₆ H ₁₁₃ O ₄ NH	C ₅₆ H ₁₁₃ O ₄ NH	C ₅₆ H ₁₁₁ O ₄ NH	C ₅₆ H ₁₁₁ O ₄ NH
Select mass.		864.87478	864.87478	862.85913	862.85913
Col (eV)		50	40	50	40
Frag. (m/z)	Chain sph	C22-26	C22-26	C22-26	C20-26
294.3	C20				x
308.3	C21				
322.3	C22	x	x	x	x
336.3	C23				
350.3	C24	x	x	x	x
364.3	C25		x		
378.3	C26	x	x		x
	+18				
312.3	C20				x
326.3	C21				
340.3	C22			x	x
354.3	C23				
368.3	C24	x	x	x	x
382.3	C25		x		
396.3	C26	x	x	x	x
	+2*18				
358.3	C22				-
372.3	C23				
386.3	C24	x	x		x
400.3	C25				
414.3	C26	x	x		x
	-CH2OH				
338	C24		x	x	
352	C25				
366	C26	x	x		

Table S6.4 continued

Ceramide		OdS C52	OdS C52	OdS C52:1	OdS C52:1
Chem Form.		C ₅₂ H ₁₀₅ O ₄ NH	C ₅₂ H ₁₀₅ O ₄ NH	C ₅₂ H ₁₀₃ O ₄ NH	C ₅₂ H ₁₀₃ O ₄ NH
Select mass.		808.81218	808.81218	806.79653	806.79653
Col (eV)		50	40	50	40
Frag. (m/z)	Chain sph	C18-24	C20-24	C18-24	C18-24
266.3	C18	x		x	x
280.3	C19				
294.3	C20	x	x	x	x
308.3	C21	x			
322.3	C22	x	x	x	x
+18					
284.3	C18			x	x
298.3	C19	x			
312.3	C20	x	x	x	x
326.3	C21	x			
340.3	C22	x	x		x
354.3	C23				
368.3	C24	x	x		
+2*18					
274.3	C16				
330.3	C20		x		
344.3	C21				
358.3	C22		x		
-CH2OH					
254	C18	x		x	
268	C19	x			
282	C20	x	x	x	x
296	C21	x			
310	C22	x	x	x	
324	C23				
338	C24			x	x

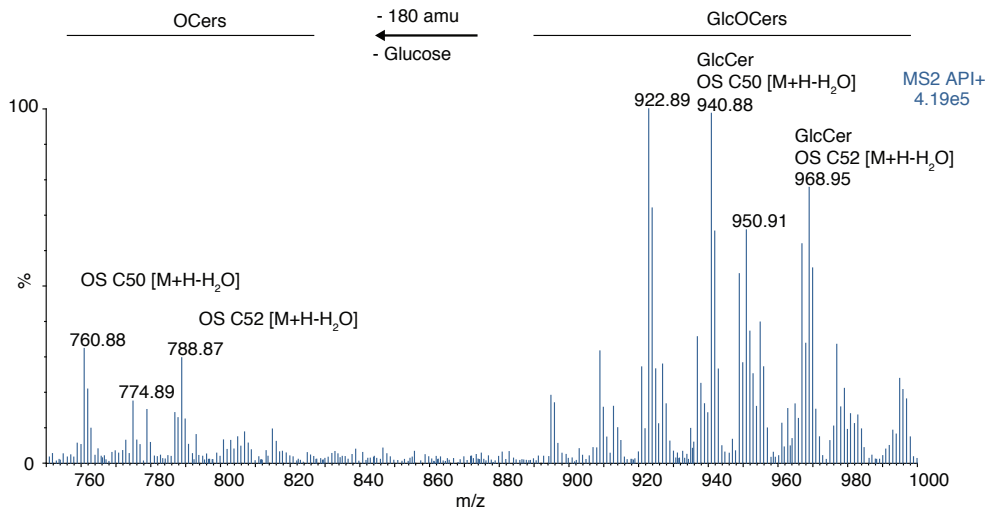
Ceramide		OdS C51	OdS C51	OdS C50	OdS C50
Chem Form.		C ₅₁ H ₁₀₃ O ₄ NH	C ₅₁ H ₁₀₃ O ₄ NH	C ₅₀ H ₁₀₁ O ₄ NH	C ₅₀ H ₁₀₁ O ₄ NH
Select mass.		794.79653	794.79653	780.78088	780.78088
Col (eV)		50	40	50	40
Frag.(m/z)	Chain sph	C17-23	C18-22	18-20	18-22
266.3	C18			x	x
280.3	C19	x		x	
294.3	C20	x	x	x	x
308.3	C21	x	x		
322.3	C22	x	x		x
336.3	C23	x			
+18					
270.3	C17	x			
284.3	C18	x	x	x	x
298.3	C19	x	x		x
312.3	C20	x	x	x	x
326.3	C21	x	x		x
340.3	C22	x	x		x
+2*18					
302.3	C18				x
316.3	C19		x		
330.3	C20				x
344.3	C21		x		
-CH2OH					
240	C17	x			
254	C18	x		x	x
268	C19	x		x	
282	C20	x		x	x
296	C21	x			

Supplemental S6.5

To determine if the process of saponification with a subsequent Bligh and Dyer extraction, will extract unintended material a blank extraction was performed. Three saponification procedures were performed in empty vials. The extraction solvent mixtures were added to the neutralized solvents and the Bligh and Dyer extraction as described in the method section. The obtained organic phases were evaporated and reconstituted in 1.5 ml of chloroform:methanol (2:1). Using the gravimetric approached the dry weight and subsequent concentration of material in the extracts was determined. Supplemental table 3 depicts the concentration of these samples. This showed there was a substantial amount of material extracted. This material is probably NaCl which is produced during the neutralization of the saponification. Although the solvent are chloroform and methanol, these are able to dissolve a fraction of water and salt. Therefore, the lipid concentration of saponified samples could not be determined.

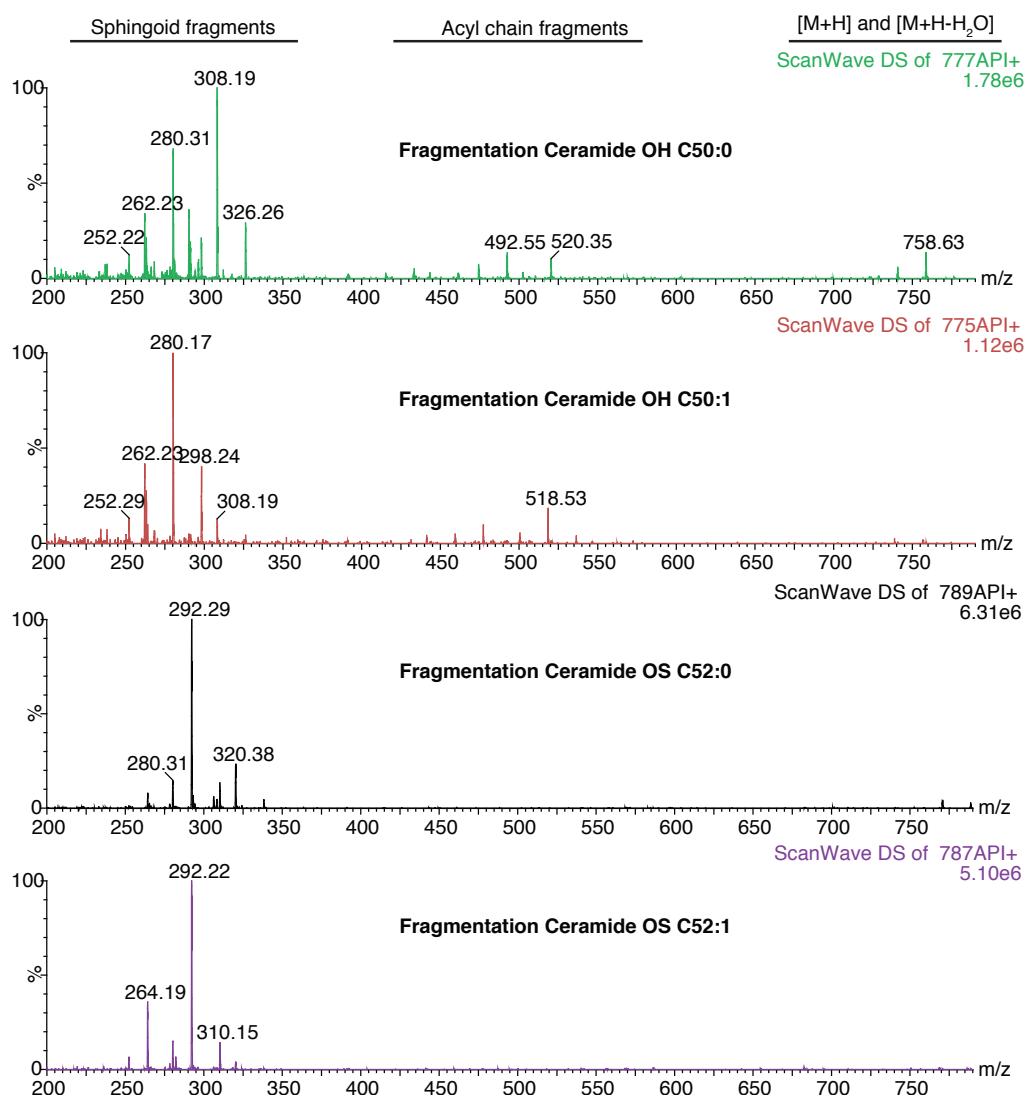
Supplemental S6.5 Table 1: concentration of blank extracts

	Concentration mg/ml
Sample 1	0.155
Sample 2	0.151
Sample 3	0.113



Supplemental S6.6

A smoothed and centered spectrum of the bound glucosylceramide eluting at an Rt of 9.8 min. The m/z of O-GluCers and the m/z of the corresponding OCer were observed at the same Rt. These could be distinguished by a difference of 180 amu (the mass of a glucose).



Supplemental S6.7: Saturated sphingoid chains in bound MuCers

The figure shows unsmoothed fragmentation spectra of both the saturated and unsaturated ceramide of the same subclass with the same total chain length. This is shown for two ceramide of different subclasses. Fragmentation was performed on extracted bound ceramides from ex vivo SC. The same sphingoid fragments were observed in both the saturated and unsaturated ceramide, observe as the same mass (exemplified by m/z 308.19 and 292.22). This showed that the unsaturation was in the acyl chain of the ceramides. As conformation the unsaturated acyl chain was observed in the OH ceramides (520.35 compared to 518.53).

	SC Donor 1		SC Donor 2		SC Donor 3	
Subclass	Molar%	SD	Molar%	SD	Molar%	SD
NdS	2.23	0.44	2.77	1.24	3.39	1.01
NS	0.76	0.11	0.66	1.15	1.22	1.07
NP	0.44	0.33	0.82	0.69	1.11	0.55
NH	1.52	0.17	1.38	0.43	1.60	0.46
AP	1.06	0.34	1.61	0.22	1.72	0.22
AH	1.12	0.17	1.18	0.16	1.23	0.12
EOdS	14.53	1.04	12.41	7.25	16.41	7.24
EOS	10.59	1.11	9.46	2.09	10.91	2.54
EOP	7.06	1.21	5.11	4.23	7.65	4.00
EOH	6.46	0.65	5.59	2.03	6.93	2.17
OdS	41.61	5.44	36.04	7.10	39.86	1.93
OS	8.87	2.19	2.82	11.01	7.85	13.08
OP	14.62	4.10	8.86	9.76	14.77	9.71
OH	12.34	2.10	8.09	10.44	13.77	10.50

Supplemental S6.8: MuCer percentage per subclass in unbound SC lipids

Table depicts the average molar percentage and SD (n=3) of MuCers within a subclass. The total amount in moles of both saturated and unsaturated ceramides of a subclass is set as a 100%. This is shown for 3 different SC donors.

Supplemental S6.9

A linear mixed model was made to compare the percentage of MuCers between bound ceramides and the unbound taking into count the differences between subclasses. The bound ceramide was set as the control condition of which the intercept (mean) is given. The effects size indicates the change in value due to the change in examined group (unbound OCers or EOers). The p-value indicates the probability that the intercept or effect size is non-zero.

MuCer % per subclass		OdS/EOdS		95% Confidence Interval		OS/EOS		95% Confidence Interval	
		Size	p-value	lower	upper	Size	p-value	lower	upper
	Intercept (bound)	37.62	0.008	21.77	53.47	22.25	0.024	6.40	38.10
Effect size	Unbound EOers	-20.30	<0.001	-23.43	-17.17	-10.98	<0.001	-14.11	-7.85
	Unbound OCers	3.50	0.029	0.37	6.64	-10.90	<0.001	-14.04	-7.77

		OP/EOP		95% Confidence Interval		OH/EOH		95% Confidence Interval	
		Size	p-value	lower	upper	Size	p-value	lower	upper
	Intercept (bound)	37.02	0.008	21.17	52.87	27.24	0.016	11.39	43.09
Effect size	Unbound EOers	-28.90	<0.001	-32.03	-25.77	-20.15	<0.001	-23.29	-17.02
	Unbound OCers	-21.05	<0.001	-24.18	-17.92	-11.79	<0.001	-14.92	-8.66

SPSS code used to obtain the result of the model. The code was ran separately for each subclass.

```
MIXED MuPr1 BY Condition2 Subclass
/CRITERIA=CIN(95) MXITER(100) MXSTEP(10) SCORING(1) SINGULAR(0.000000000001) HCONVERGE(0,
ABSOLUTE) LCONVERGE(0, ABSOLUTE) PCONVERGE(0.000001, ABSOLUTE)
/FIXED=Condition Subclass Condition*Subclass | SSTYPE(3)
/METHOD=REML
/PRINT=SOLUTION TESTCOV
/RANDOM=INTERCEPT | SUBJECT(SC) COVTYPE(VC)
/EMMEANS=TABLES(Condition*Subclass) .
```

¹MuPr = the MuCer percentage per subclass

²Condition = Bound, Unbound OCers, and EOers

Supplemental S6.10

A linear mixed model was made to compare the MCL between bound ceramides and the unbound ceramides taking into account if these were saturated or desaturated. The bound ceramides were set as the control condition of which the intercept (mean) is given. The effects size indicates the change in value due to the change in examined group (unbound OCer, EOCer -18 carbons, or if it was unsaturated). The interaction shows the additional change when two different condition occur together additional to the changes of both independent conditions. In other words the MCL of unsaturated unbound OCer was more than 2.08 carbons longer than the saturated unbound OCer. The p-value indicates the probability that the intercept, effect size or interaction is non-zero.

MCL (Carbon atoms)			95% Confidence Interval		
		Estimate	p-value	lower	upper
	Intercept (bound)	50.18	<0.01	49.95	50.41
Effect sizes	Unbound OCers	0.66	<0.001	0.43	0.88
	EOCers	0.77	<0.001	0.55	1.00
	Unsaturated	2.08	<0.001	1.85	2.30
Interaction	Unbound OCers * Unsaturated	0.93	<0.001	0.61	1.25
	Unbound EOCers *Unsaturated	1.41	<0.001	1.09	1.73

SPSS code used to make this model

```
MIXED MCL BY Condition1 saturation
/CRITERIA=CIN(95) MXITER(100) MXSTEP(10) SCORING(1) SINGULAR(0.000000000001)
HCONVERGE(0, ABSOLUTE) LCONVERGE(0, ABSOLUTE) PCONVERGE(0.000001, ABSOLUTE)
/FIXED=Condition saturation Condition*saturation | SSTYPE(3)
/METHOD=REML
/PRINT=SOLUTION TESTCOV
/RANDOM=INTERCEPT | SUBJECT(SC) COVTYPE(VC)
/EMMEANS=TABLES(Condition*saturation).
```

¹Condition = bound, unbound OCer, EOCer -18 carbon

Supplemental S6.11: Output of linear mixed models of FTM with LXR agonist and antagonist.

Output of the linear mixed model comparing the bound ceramides in Ex Vivo_{control} FTM_{Control}, FTM_{DMSO}, FTM_{AGONIST} and FTM_{AGONIST}. For these models only the output for FTM_{DMSO} compared to FTM_{Control} is depicted. The models were ran multiple times with different groups set as intercept: Ex vivo_{control} FTM_{Control} and FTM_{agonist}. Using these 3 runs all groups could be compared.

Supplemental S6.11 Table 1: Comparison of total bound ceramide amount and MuCer percentage.

	Amount ceramides µg/ mg SC		95% Confidence Interval		MuCer		95% Confidence Interval	
	Size	p-value	lower	upper	Size	p-value	lower	upper
Intercept (Ex vivo)	7.058	<0.001	5.277	8.840	25.08	0.001	16.26	33.90
Effect size FTM control	-5.394	0.002	-7.858	-2.929	61.26	<0.001	48.86	73.66
FTM agonist	-5.483	0.002	-7.948	-3.018	62.26	<0.001	49.86	74.66
FTM antagonist	-5.511	0.002	-7.976	-3.046	46.22	<0.001	33.82	58.62

Table S6.11.tabel1 continued

		Amount ceramides µg/ mg SC		95% Confidence Interval		MuCer		95% Confidence Interval	
		Size	p-value	lower	upper	Size	p-value	lower	upper
	Intercept (FTM control)	1.664	0.060	-0.097	3.425	86.33	<0.001	77.61	95.06
Effect size	FTM DMSO	0.698	0.368	-0.938	2.334	-1.82	0.197	-4.73	1.09
	FTM agonist	-0.089	0.893	-1.517	1.338	1.00	0.402	-1.52	3.52
	FTM antagonist	-0.117	0.860	-1.545	1.311	-15.03	<0.001	-17.56	-12.51

		Amount ceramides µg/ mg SC		95% Confidence Interval		MuCer		95% Confidence Interval	
		Size	p-value	lower	upper	Size	p-value	lower	upper
	Intercept (FTM agonist)	1.575	0.072	-0.186	3.336	87.33	<0.001	78.61	96.06
Effect size	FTM antagonist	-0.028	0.967	-1.456	1.400	-16.03	<0.001	-18.56	-13.51

Supplemental S6.11 Table 2: Comparison of total bound mean chain length saturated and unsaturated

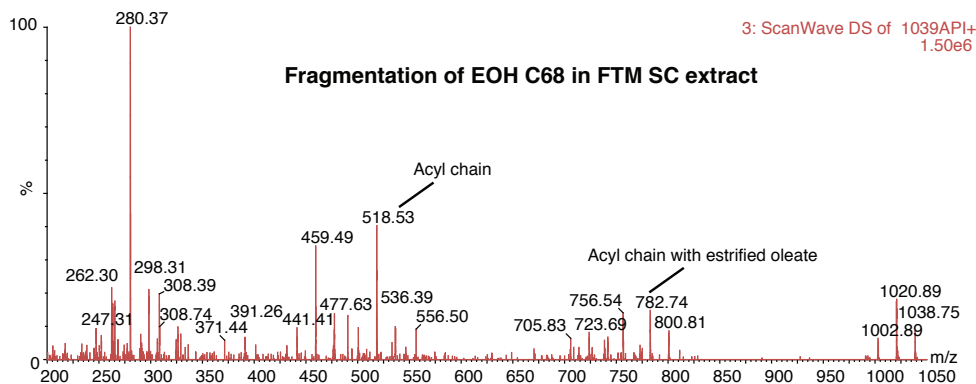
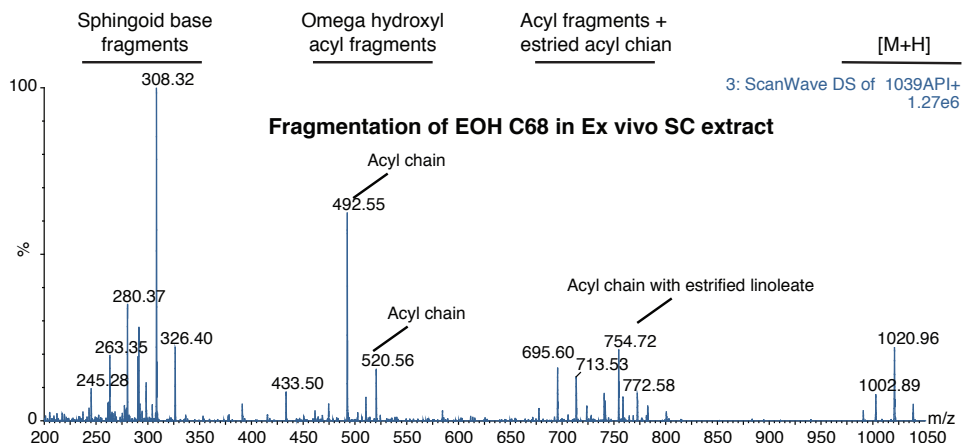
		MCL Sat		95% Confidence Interval		MCL unSat		95% Confidence Interval	
		Size	p-value	lower	upper	Size	p-value	lower	upper
	Intercept (Ex vivo)	50.18	<0.001	49.90	50.46	52.32	<0.001	52.04	52.60
Effect size	FTM control	-0.31	0.113	-0.71	0.09	-1.45	<0.001	-1.85	-1.05
	FTM agonist	-0.67	0.005	-1.07	-0.27	-2.14	<0.001	-2.54	-1.74
	FTM antagonist	0.09	0.612	-0.31	0.49	-0.26	0.170	-0.66	0.14

		MCL Sat		95% Confidence Interval		MCL unSat		95% Confidence Interval	
		Size	p-value	lower	upper	Size	p-value	lower	upper
	Intercept (FTM control)	49.87	<0.001	49.58	50.17	50.87	0.000	50.58	51.16
Effect size	FTM DMSO	0.12	0.406	-0.18	0.43	0.00	0.989	-0.31	0.30
	FTM agonist	-0.36	0.011	-0.62	-0.09	-0.69	<0.001	-0.96	-0.42
	FTM antagonist	0.40	0.005	0.13	0.67	1.19	<0.001	0.92	1.46

		MCL Sat		95% Confidence Interval		MCL unSat		95% Confidence Interval	
		Size	p-value	lower	upper	Size	p-value	lower	upper
	Intercept (FTM agonist)	49.52	<0.001	49.22	49.81	50.18	<0.001	49.89	50.47
Effect size	FTM antagonist	0.76	<0.001	0.49	1.02	1.88	<0.001	1.61	2.15

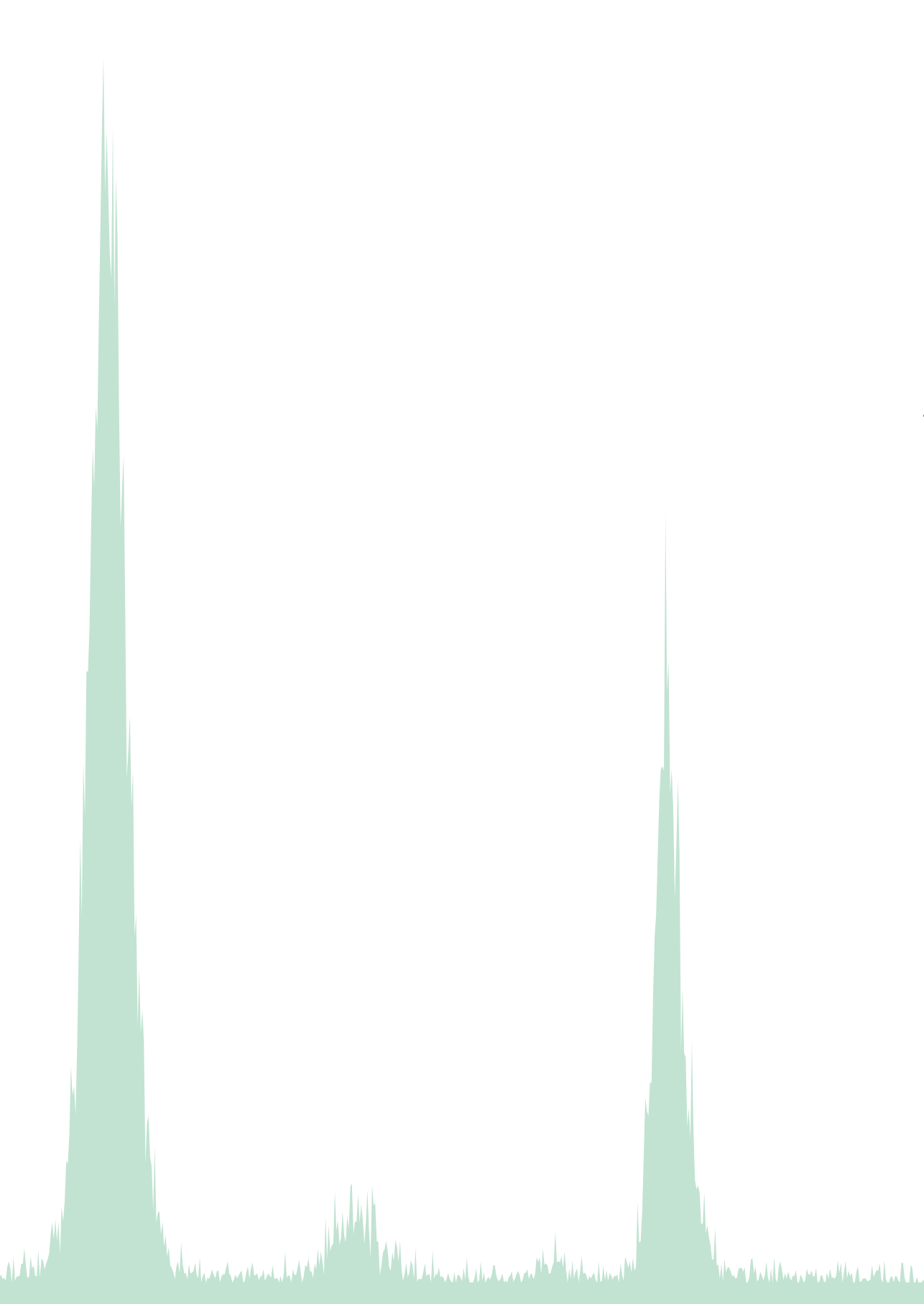
SPSS code used to make the models of supplemental S6.11

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Supplemental S6.12: Oleic acid instead of linoleic acid attached as acyl chain in EOCers.

Fragmentation of EOH C68 (1039 amu) at 40 eV in ex vivo and FTM SC lipid extracts. The characteristic fragments are indicated above the figures. The fragments of both a saturated and unsaturated acyl chain are indicated (also present in supplemental S6.8). The fragments with the linoleate and oleate attached to it are indicated as well.



Chapter 7

The cornified envelope bound ceramide fraction is altered in atopic dermatitis patients

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Background:

Chromatograms of the bound ceramides in healthy (right) and atopic dermatitis (left)

7

Altered bound ceramide composition in atopic dermatitis

7.1 Letter to the editor

The lipids covalently bound to the cornified envelope of corneocytes are essential for the human skin barrier¹.

Together with the unbound lipids they form a matrix regulating the permeability of water and pathogens through the stratum corneum (SC). Three main lipid classes are observed in the SC: sterols, fatty acids, and ceramides. Bound ceramides are a subset of the unbound ceramides and have an ultra-long omega-hydroxyl chain (OCer)^{2,3}. They are biosynthetically related to the unbound linoleate esterified omega-hydroxyl ceramide (EOCers). Ceramide binding is selective, where ceramides with an unsaturated acyl-chain (MuCers) and shorter chains are preferentially bound⁴ (*Chapter 6*). Yet, as both bound and unbound ceramides originate from the same precursors, compositional changes of these precursors can affect both. Previously, changes in the unbound ceramide composition of atopic dermatitis (AD) patients were observed. These changes correlated to a decreased barrier function and altered lipid organization^{5,6}. Changes in bound ceramide composition were observed in autosomal recessive congenital ichthyosis, psoriasis and in atopic dogs and mice⁷⁻¹⁰. It is hypothesized that the bound ceramide composition of AD patients deviates too and contributes to AD pathology. To our knowledge, no detailed LC-MS analysis of bound ceramides in AD patients has been reported.

Here, we applied a quantitative LC-MS method to SC samples of AD patients¹¹. Samples were obtained after institutional approval and written informed consent, according to the declaration of Helsinki. From eight healthy volunteers and sixteen AD patients, a 1.6 cm diameter non-lesional site on the ventral forearm was sequentially tape-stripped. Eight AD patients exhibited lesional sites at the ventral forearm, of which a second tape-strip sample was obtained. Tapes were measured with a SquameScan (Heiland Electronic, Wetzlar, Germany) to determine the amount of SC stripped. Both bound and unbound lipids were extracted from tapes 9-12, as described elsewhere⁴. Before tape-stripping, trans-epidermal water loss (TEWL) was measured to determine the skin barrier function.

LC-MS analysis did not show a decreased bound ceramide amount in AD, contrary to what others observed using thin layer chromatography¹². **Figure 7.1A** depicts the total detected bound ceramide amount corrected by SquameScan. To examine the bound ceramide composition in more detail, ceramides were grouped using three distinguishing features of their molecular structures (**Figure 7.1B**): MuCers percentage, sphingosine subclass (S-subclass) percentage, and mean carbon chain length (MCL). **Supplementary S7.1** explains the linear mixed modeling used to determine the effect-sizes. The models determined changes by AD compared to healthy and the difference of a lesional site compared to the same patients non-lesional site. **Figure 7.1C** and **Supplemental S7.1 Table 1** shows that AD patients had a 6.0% increase of bound MuCers, which further increased by 7.1% at their lesional sites. EOCers had similar increases in MuCers. In the unbound ceramides of AD patient, increased percentages

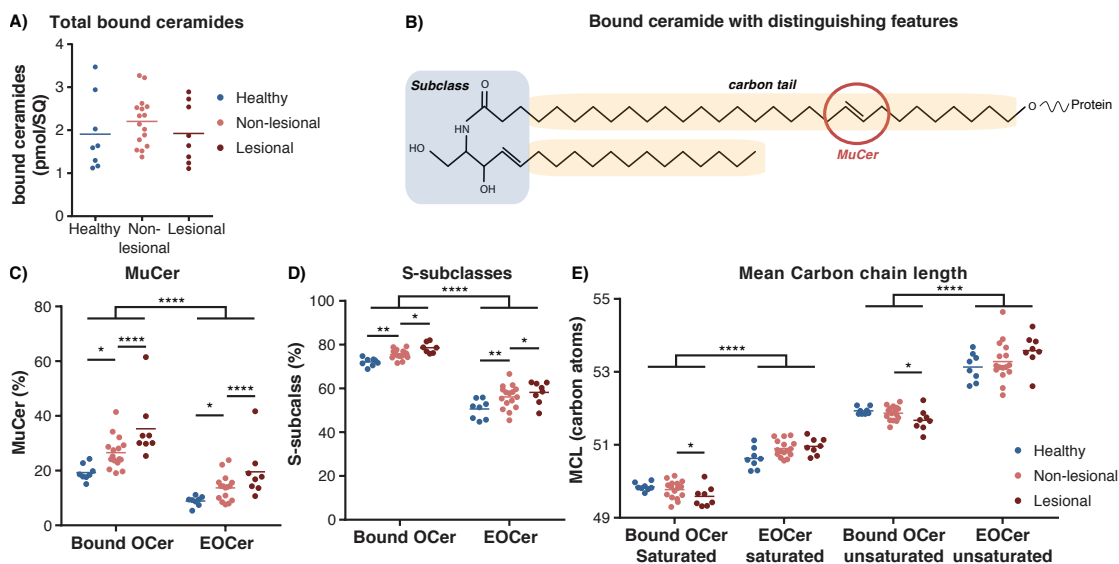


Figure 7.1: Alteration in bound ceramides of atopic dermatitis patients

A: The amount of bound ceramide detected in pmol/SquameScan value in the tape-stripped samples of healthy, non-lesional, and lesional SC. **B:** The structure of a bound omega-hydroxyl ceramide (OCer OS C48:1) with three distinguishing features of the chemical structure indicated: the carbon tail length (red), the hydrophilic head group defining the ceramide subclass (blue), and an unsaturated acyl chain (circled). **C:** The percentage of unsaturation in the bound and unbound EOCers fractions. **D:** The percentage of S-subclass ceramides in the bound and in the unbound EOCers fractions. **E:** The mean carbon chain length in the saturated and unsaturated bound ceramides and the mean carbon chain length of the saturated and unsaturated EOCer. For the EOCers 18 carbons were subtracted compensating for the linoleic acid. The MCL of the saturated and unsaturated ceramides are depicted separately. Significance indications are as followed: * =<0.05, ** =<0.01 ***=<0.005 ****=<0.001, significance of interactions is not indicated and can be found in **Supplemental S7.1 Table 3**.

of S-subclass ceramides were observed^{5,13}. **Figure 7.1D** and **Supplemental S7.1 Table 2** depict that in AD also the bound S-subclass ceramides were increased with 4.5% and an additional 2.1% in lesional skin. Again, these changes were similar in the unbound EOCers.

Examining the chain length distribution, it was observed that at lesional sites relatively more short chain ceramides were bound, thereby depleting short-chain EOCers. To compare MCLs of OCers and EOCers, 18 carbons were subtracted from the latter to compensate for the linoleic acid moiety. The MCL of saturated and unsaturated ceramides differed significantly and were examined as separate parameters but were affected similarly by AD and at lesional skin (**Figure 7.1E**, **Supplemental S7.1 Table 3**). At lesional sites the MCL of bound ceramides significantly decreased, however, non-lesional sites showed no significant decrease. Inversely, the EOCer MCL had an increasing trend in AD. The changes in the MCL due to AD and lesional were inverse and significantly different between the bound OCers and the EOCers. **Supplemental S7.2** shows the saturated ceramides chain length distribution of the bound OCers and EOCers. Although, the overall EOCers amount decreased, short chains EOCers were further depleted. In AD decreased amounts of EOCers are thought to play an role in decreasing barrier function¹⁴.

It was examined if changes in the bound ceramides composition coincided with changes in barrier function. The three distinguishing ceramide features were compared to TEWL. **Table 7.1** shows the Pearson r and corresponding p-value (**Supplemental S7.3** graphically displays the correlations). The increased MuCer percentage correlated to a reduced barrier function. Previously, it was demonstrated that unbound unsaturated lipids can contribute to a more disorganized lipid matrix thereby decreasing the barrier function¹⁵. Although the bound ceramides already have an increased MuCer percentage compared to unbound ceramides, a further increase MuCers percentage could have a destabilizing effect increasing SC permeability. For S-subclass ceramides a similar correlation was observed. To examine the correlation with depletion due to shorter chain ceramide binding, the difference in MCL between the bound OCer and EOCers was calculated. Saturation was included as a separate parameter. The difference in saturated ceramides MCL between the EOCers and OCers correlated to the SC barrier function. It is expected that this could be due to EOCer depletions.

Table 7.1: The correlation coefficients and p-values of bound ceramide composition compared to barrier function.

Four different distinguishing features were used to describe the bound ceramide composition: MuCer percentage, the percentage of S-subclass ceramides and the difference in MCL between the unbound EOCers and bound OCers. The MCL differences of saturated and unsaturated ceramides were examined separately, because MuCers had a higher chain length and an increased MuCer percentage would increase the MCL.

	TEWL (g/cm ² /h)	
	Pearson r	p-value
MuCer (%)	0.731	<0.001
S-subclass (%)	0.692	<0.001
Difference MCL saturated (carbons)	0.561	0.001
Difference MCL MuCer (carbons)	0.231	0.203

Summarizing, AD patients bound ceramide composition contained more MuCers and S-subclass ceramides in lesional and non-lesional sites compared to healthy SC and at lesional sites the chain length was reduced. These alterations in composition correlated to a decreased barrier function. Although the individual alterations correlated, this does not imply a direct causality. It is feasible that all changes in the bound and unbound lipid composition of AD patient combined would compromise the integrity of the lipid organization and thereby decrease the SC barrier function. Nonetheless, it is thought that the bound lipids function as a scaffold for the unbound lipids. Both changes in the precursor lipids synthesis and changes in the binding process could have influenced the bound ceramide composition in AD⁴. The altered state of the bound ceramide composition could impair this scaffold function. Treatment in dogs and mice has shown to increase bound ceramides^{9,10}, making bound ceramides a possible target for barrier repair treatment of AD.

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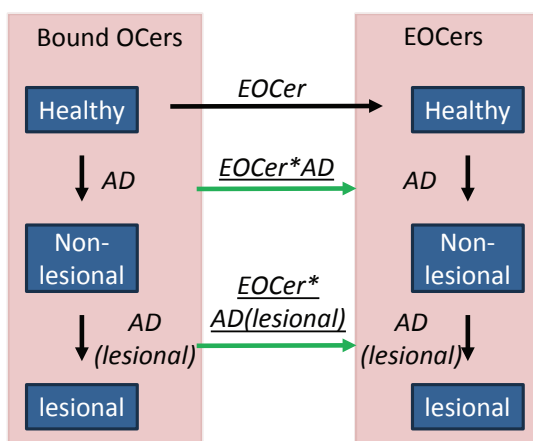
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Supplementals chapter 7

Supplemental S7.1: Statistical analysis

Statistical analysis was performed using linear mixed modeling in SPSS version 24 (IBM corp). Volunteers and patients number were set as random variable (1 to 24); this enables a paired comparison of the non-lesional and lesional sites within a patient. In all models AD was set as a fixed variable, being either 0 or 1, indicating if the skin was healthy or from a patient, respectively. Lesional was set as fixed variable nested within AD: AD(lesional). Hereby, the model takes into account that changes due to lesional can only occur in patients. This gives a separate value which indicates if lesional sites were different compared to non-lesional. A fixed variable named EOCers was made; this indicates if there was a difference between the groups of bound OCers and unbound EOCer.

For the statistical analysis of the MuCer %, a model was made including all specifications described above. Interaction terms between the fixed variables EOCers and AD, and EOCers and the nested lesional effect were tested. These interactions describe if a change in MuCer due to AD or lesional was different in the EOCers compared to the OCers. **Supplemental S7.1 Figure 1** gives a schematic overview of this model. After running the model the interactions had non-significant contributions. This showed that changes in MuCers due to AD and lesional were similar in bound OCers and EOCers. The interactions were excluded for the final model. The following parameter estimates were obtained with the model (**Table S7.1**). The intercept represent the health bound OCer group.



Supplemental S7.1 Figure 1: Schematic representation of the LLM.

Groups are indicated in blue boxes, fixed effects are indicated by in italics and interactions are underlined and in italics

Supplemental S7.1 Table 1: LMM output for the MuCers%.

The table shows the output for the fixed variables. The estimate of the intercept and effect sizes is give with p-value and 95% intervals.

MuCer %	Estimate	p-value	95% confidence interval	
			lower	upper
Intercept (Healthy bound)	20.58	<0.001	16.54	24.62
EOCer	-12.96	<0.001	-15.29	-10.62
AD	6.03	0.015	1.27	10.80
AD(Lesional)	7.07	<0.0001	3.94	10.20

The same model as was used for the MuCers%, was used for the percentage of S-subclass ceramides. Again, a non-significant contribution of interaction was observed, indicating that the changes in S-subclass percentage did not differ for bound OCers and EOCers. After excluding the interactions, the following parameter estimates were observed (**Supplemental S7.1 Table 2**).

Supplemental S7.1 Table 2: Output of the LMM used to compare the % of S-subclasses.

The table shows the output for the fixed variables of the LMM. The estimate of the intercept and effect sizes are given with p-value with 95% intervals.

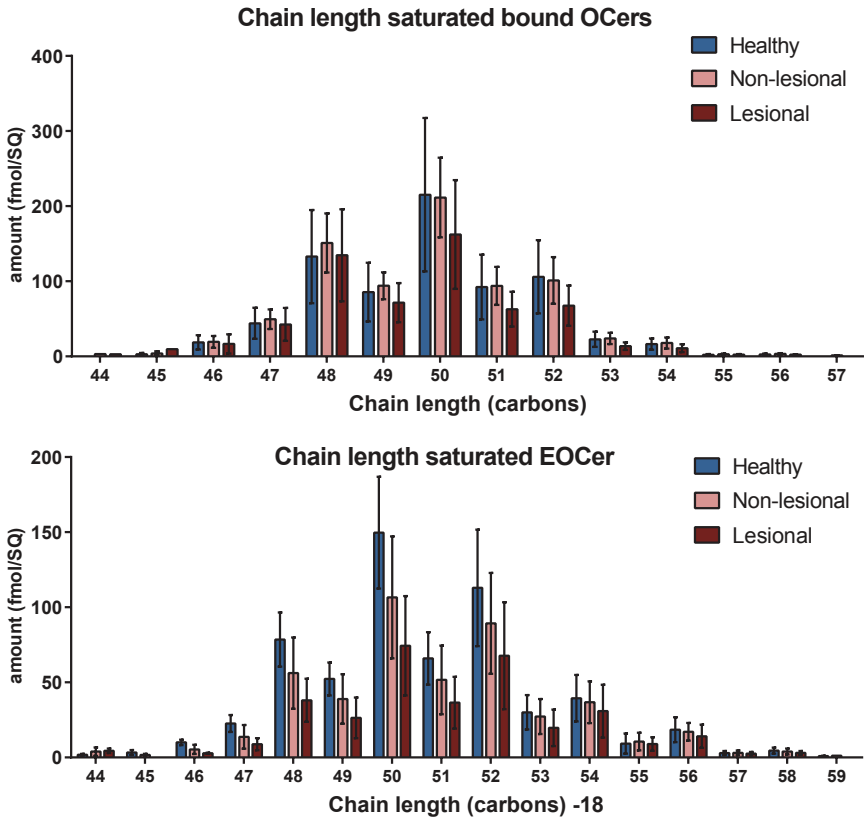
S-Subclass %	Estimate	p-value	95% confidence interval	
			lower	upper
Intercept (Healthy bound)	71.43	<0.001	69.02	73.84
EOCer	-20.18	<0.001	-21.66	-18.70
AD	4.50	0.003	1.67	7.32
AD(Lesional)	2.64	0.010	0.67	4.61

To examine the MCL, a fixed factor for saturation was added to the model. This indicated if there was a difference in MCL between the saturated and unsaturated ceramides. This gives an addition interactions and another level of 3 way interactions. The interaction between AD and lesional with saturation was added and the interaction between saturation and EOCers was added. The 3 way interactions between AD and lesional with EOCer and saturation were added. This describes if the change in MCL due to AD or lesional in either the bound OCer or EOCers was different due to saturation. By running the model it was shown that the 3-way interactions were not significant and they were excluded. The interactions of saturation with AD and lesional were non-significant and were excluded as well. **Supplemental S7.1 Table 3** depicts the results of the final model. The interactions between the EOCers and AD, and EOCers and lesional were significant. This showed that the change in MCL due to AD and lesional were different in the bound OCers compared to the unbound EOCers. Furthermore, the interaction between EOCer and saturation was significant. This showed that the change in MCL between OCers and EOCers were different between saturated and unsaturated ceramides.

Supplemental S7.1 Table 3: Output of the LMM for the MCL data.

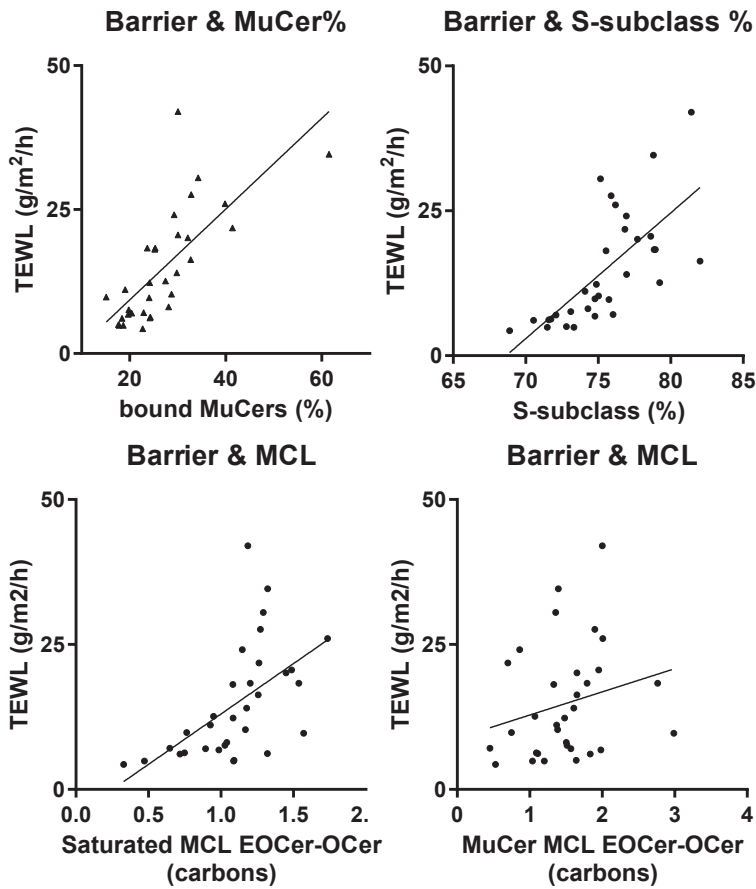
The table shows the output for the fixed variables of the linear mixed model. The estimate of the intercept and effect sizes is give with the p-value and 95% intervals.

			95% confidence interval		
		Estimate	p-value	lower	upper
	Intercept	49.84	<0.001	49.66	50.03
Interactions	AD	-0.07	0.511	-0.28	0.14
	AD(Lesional)	-0.23	0.008	-0.40	-0.06
	EOCer	0.80	<0.001	0.59	1.01
	Saturation	2.09	<0.001	1.97	2.22
	AD*EOCer	0.28	0.016	0.05	0.51
	AD(Lesional)*EOCer	0.37	0.002	0.15	0.60
	Saturation* EOCer	0.38	<0.001	0.20	0.57

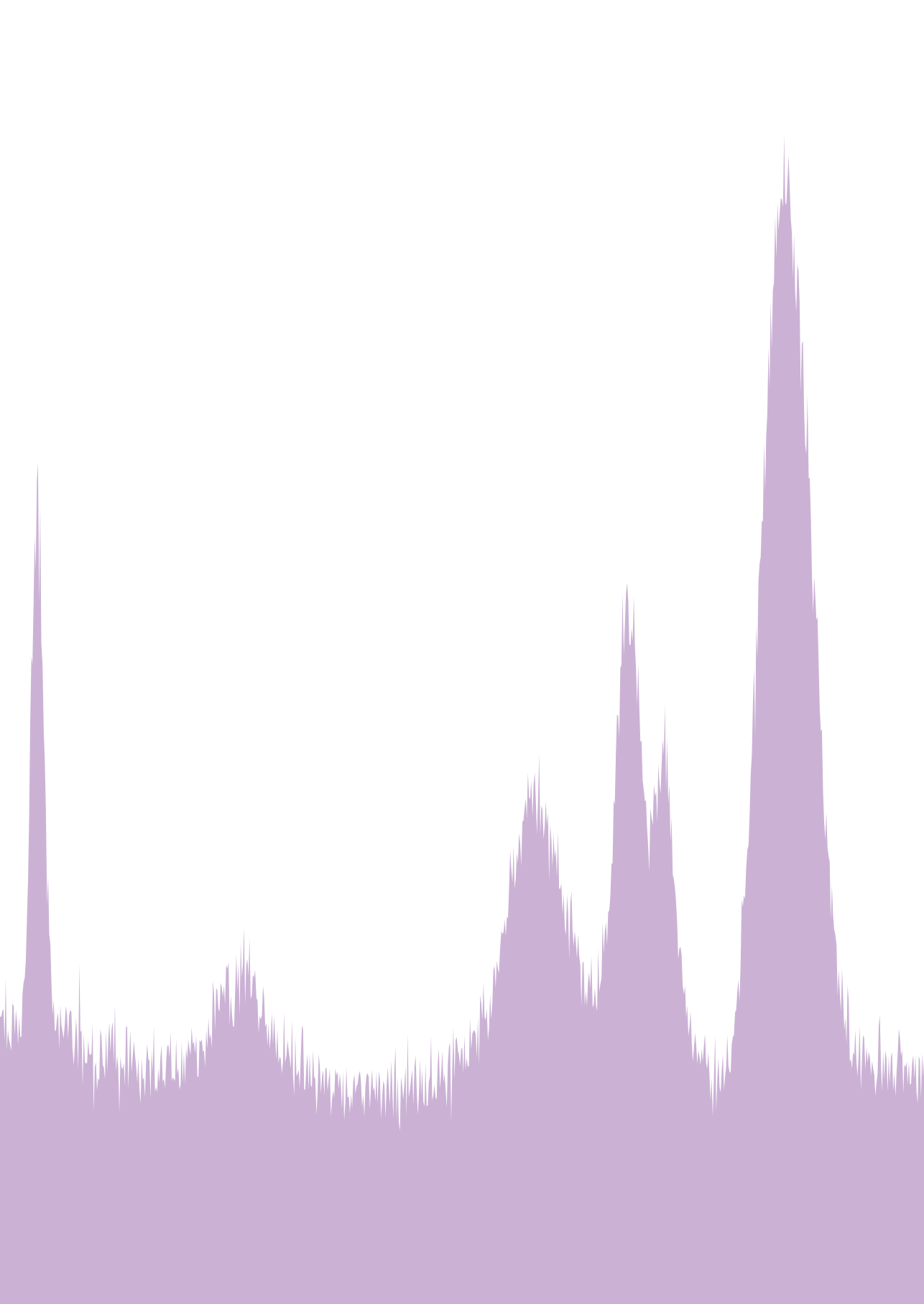


Supplemental S7.2: Comparing the chain length distribution

The carbon chain length distribution of the bound OCers and unbound EOCers is depicted. For the EOCers 18 carbons were subtracted to compensate for the linoleic acid. The amount is given in fmol per amount of stripped SC measured as SquameScan. The data is depicted as mean $\pm$ SD (Healthy $n=8$, Non-lesional $n=16$, lesional = 8).

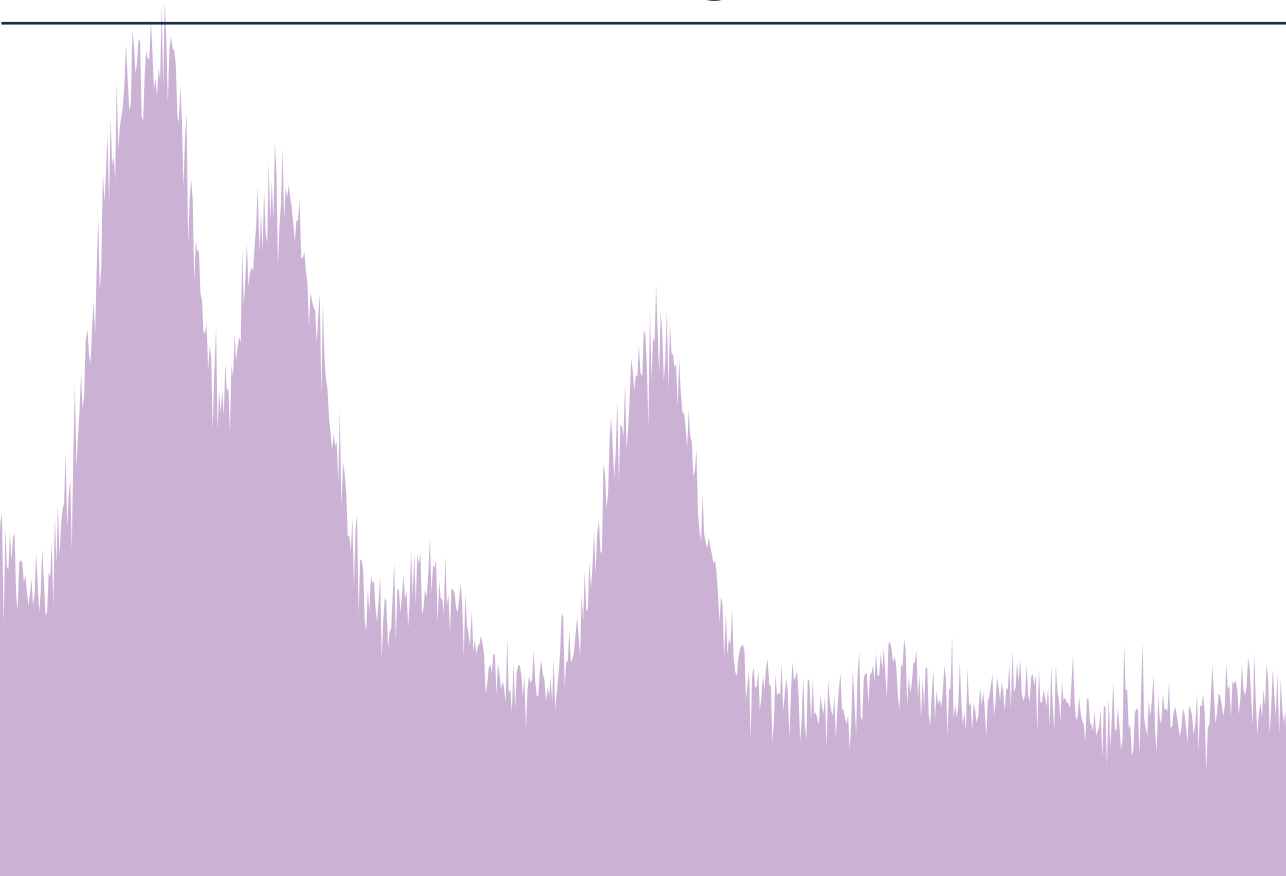


Supplemental S7.3: Graphical depiction of correlations with barrier function.



Chapter 8, 9, and Appendices

Summary, Discussion, Prospects,
Conclusion, Nederlandse
samenvatting, and CV



8.1 Chapter one: Introduction

The skin is one of the human body's most important barriers. In the common skin disease atopic dermatitis (AD) (prevalence of around 7-10% in the western population¹) this skin barrier function is reduced. This leads to an increased chance for irritants, allergens, and pathogens to penetrate the skin. These penetrating substances, in-turn causes an inflammatory response leading to itching and red skin. Moreover, this also leads to a deregulated barrier formation and back to a damaged skin barrier function. These events drive the formation of skin lesions characteristic for AD and a reduced skin barrier is a crucial part of AD pathology.

The outer part of the skin forms a physical barrier called the stratum corneum (SC). Consisting of lipids and terminally differentiated keratinocytes called corneocytes, it represents a brick and mortar wall like structure. Lipids are an integral part of the skin barrier. They form a highly organized solid matrix occupying the space between the corneocytes and form the permeability barrier. The SC contains three major lipid classes: ceramides, free fatty acids, and sterols. Of these lipids, ceramides are the most structurally diverse group. They consist of sphingoid base attached to acyl group, both of which can differ in structure. **Figure 8.1** depicts the different structures of the ceramides hydrophilic head group which determines the name and subclass. Part of the SC ceramides become covalently attached to the outside of the corneocytes². The role of these ceramides is not well understood, it is known however that unbound ceramides are important for the lipid organization³ and consequently for the skin barrier⁴.

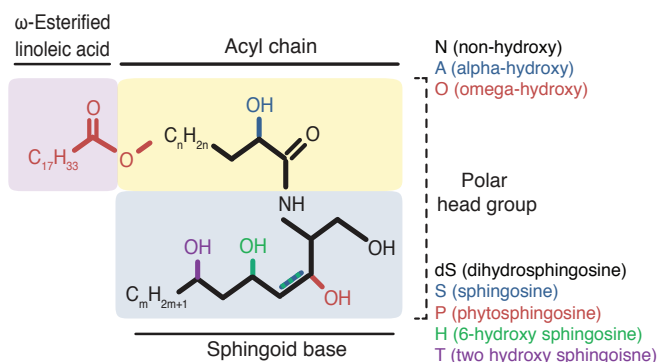


Figure 8.1: A composite structure of all different ceramide subclasses in stratum corneum.

The different structures of the hydrophilic head group are indicated with different colors. The names according to Motta et al.⁵, of the structural differences has the same color.

AD skin is also characterized by SC lipid alterations at both lesional and non-lesional skin sites. The changes in lipid composition correlate to a reduced lipids ordering and a decreased barrier function measured as trans-epidermal water loss (TEWL)^{6,7}. In AD the ceramide amount is reduced⁸ and the following alterations in composition were observed^{6,9}:

- The ceramides with a total chain length of 34 carbon atoms were increased (C34 ceramides);
- The ceramides with a sphingosine base (S) were increased;
- Linoleate esterified omega-hydroxyl ceramides (EOCers) were reduced.

A common way to treat AD patients is the use of lipophilic formulations that target the skin barrier¹⁰. Such formulations can act as an (partly) occlusive barrier but can also contain humectants to enhance hydration. It is hypothesized that formulations containing barrier lipids could replenish and modify the SC lipid composition. A natural formulation containing SC lipids is the vernix caseosa (VC), a film covering preterm infants. Synthetic VC formulations containing barrier lipids have been developed and were more effective than occlusive formulations in restoring the barrier of mice after disruption¹¹.

The goal 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. Multiple aspects of the lipid composition still needed to be studied. A detailed study of the bound ceramide composition in AD SC has not been reported. A comparative study of the relation between bound and unbound ceramides will paint a complete picture of all SC barrier ceramide alterations in AD. Knowing these alterations is essential when wanting to improve the SC barrier by treatment focusing on ceramides. Yet, how formulations

influence barrier repair and if they affect the SC ceramide composition is still unknown. More detailed understanding of these processes could help to optimize the composition of barrier repair formulations. As the VC is a complex lipid mixture, testing differences in composition clinically becomes impractical. Skin models are an attractive alternative to study and compare different formulations. However, a more detailed understanding of how models translate to the clinical situation is required. To be able to compare the molar ceramide amount in skin models and *in vivo* samples, a new method needed to be developed which could comprehensively and accurately quantify SC ceramides. The inquiries above led to the following goals for the research presented in this thesis:

1. Develop a method for extraction and quantification of all SC ceramides;
2. Determine how barrier recovery affects the SC ceramides and if *ex vivo* regeneration of the SC 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. Determine 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.

8.2 Chapter two: Quantification of ceramides using lipidomics

To achieve the first goal we focused on developing a lipidomics platform to quantitatively analyze all SC ceramides in *ex vivo* SC and *in vivo* tape-stripped SC samples. It is important to extract all ceramides from the SC in an equal manner to prevent wrongful comparisons. By addition of a lipophilic extraction step and increased extraction temperature the ceramides extraction efficiency was improved to ~99%. To quantify all extracted ceramides a model was created to predict the mass spectrometer response for ceramides which had no standards available. This model was based on the ceramides source fragmentation, isotope distribution, and molecular mass. Calibrations were used to determine the relation between mass charge (m/z), response (AUC/internal standard), and amount (pmol). To show the application of the method, in cultured generated SC was analyzed in which changes compared to *ex vivo* SC could be observed. Comparable quantitative ceramide subclass profiles were obtained between tapes and *ex vivo* SC. No difference in ceramide profile was observed in samples of tape-stripping acquired at different SC depth. These findings enabled the comparison of human *in vivo* and *ex vivo* SC samples required for the second goal.

8.3 Chapter three: In vivo and ex vivo skin barrier repair

A study was designed to determine how results obtained in cultured human *ex vivo* skin models translate to human *in vivo* skin. In this study the effect of SC regeneration on the ceramide composition and lipid organization was examined as well. Both *in vivo* and *ex vivo* SC showed similar changes when regenerated:

- The molar percentage of the following ceramide subclass was affected;
 - sphingosine (S) ceramide subclasses increased;
 - phytosphingosine (P) ceramide subclasses decreased;
 - 6-hydroxysphingosine (H) ceramide subclasses had a minor decrease;
- Molar percentage of C34 ceramides increased independent of subclass amount;
- The mean carbon chain length (MCL) of the ceramides in the SC decreased.

These changes were larger in *ex vivo* regenerated SC than *in vivo* regenerated SC. These changes were already observed in the cultured *ex vivo* SC without regeneration. Although the lipid organization was not significantly affected by regeneration, differences in lipid organization between groups could partly be explained by the differences in ceramide composition. Barrier recovery in skin models was representative for barrier recovery *in vivo* as the ceramide composition responded similarly. Moreover, cultured skin SC and regenerated skin had ceramide alterations comparable to AD skin SC, indicating that the model is applicable for testing formulations aiming at AD treatment. A synthetic vernix caseosa based formulation was developed and further tested *in vivo* which will be described next.

8.4 Chapter four: Barrier repair treatment in healthy volunteers

As the effect of formulation application during barrier recovery on the ceramide composition had not been studied before, our aim was to study these effects using a VC formulation in healthy volunteers. An intra-individual comparison study of barrier recovery after tape-stripping with and without VC treatment was performed. Treated sites had a median recovery which was more than 2 days faster than untreated sites. When comparing the ceramide compositions after 16 day of barrier recovery, treatment had reduced increases in both C34 ceramides and ceramide subclass AS which were observed at disrupted sites. The amount of both C34 ceramides and AS subclass after recovery correlated to the TEWL after recovery. The EOCer fraction and MCL were less affected by treatment, but correlated to an altered lipid organization. Changes in the ratio between ceramide subclasses indicate that during barrier recovery

the lipid synthesis was altered when applying a VC formulation. The effects of the VC formulation on barrier recovery and ceramide composition make it an attractive candidate formulation to be tested in AD patients.

8.5 Chapter five: Treatment of atopic dermatitis patients

A pilot study was performed to determine if the VC formulation had barrier improving effects in AD patients. Moderate to severe AD patients received monotherapy with the VC formulation. Improvement of the lateral lipid organization was observed after treatment; yet, the SC ceramide composition did not show significant improvement. The treated sites worsened in clinical scoring. Lesional treated sites had a larger deviation from untreated control than non-lesional treated sites. Changes in lipid parameters at non-lesional sites during treatment correlated, indicating that these parameters were affected in a similar manner within subjects. Overall treatment did not improve SC ceramide composition or barrier function. Systemic inflammatory responses were not countered by barrier formulation monotherapy and could have had a larger influence than the treatment. Longer formulation treatment combined with treatment countering flares would more clearly show the effects of formulation on the ceramide composition. Formulation treatment could also affect the bound lipids. However, first the relation between the unbound and bound ceramide composition needed to be examined.

8.6 Chapter six: Selectivity in cornified envelope binding of ceramides

To obtain detailed information on the bound ceramides composition, LC-MS and MS/MS analysis of the bound ceramides composition in human skin was performed. A new omega-hydroxy dihydrosphingosine ceramide subclass was discovered. As the bound and a part of the unbound ceramides originate from the same precursors, the relation between bound and unbound ceramides composition was examined. Selectivity in ceramide binding towards ceramides with an unsaturated acyl chain (MuCers) and with a shorter MCL was observed. It is hypothesized that this selectivity makes the cornified envelope more flexible. The bound lipid composition of the SC from full thickness skin models (FTM) was also examined. A decreased amount of bound ceramides was observed, which is thought to be due to a lack in linoleate esterified omega-hydroxy ceramides. FTMs had high levels of MuCers and a shorter MCL than human *ex vivo* skin SC. Inactivation of the liposensor Liver-X-receptor had a normalizing effect on bound ceramide composition, yet, did not increase ceramide binding. These studies gave more

insights into the relation between the bound and unbound ceramide composition and showed that this relations could be affected by different skin conditions. This leads to the question if these relations are also affected in AD skin SC.

8.7 Chapter seven: Altered bound ceramides in atopic dermatitis

It was expected that the bound ceramide composition of AD patients could be altered, as the unbound ceramide composition also deviates. To study the bound ceramide in AD, tape-strip samples of healthy volunteers were compared to tape-strip samples of non-lesional and lesional sites of AD patients. Increased amounts of MuCers and S-subclass ceramides were observed in the bound lipids of AD patient compared to healthy control. These changes were more pronounced at lesional sites. Furthermore, binding of shorter chain ceramides seemed to deplete the shorter chain unbound EOCers, which are biosynthetically related to the bound ceramides. The alteration in bound ceramide composition of AD patients correlated to TEWL, indicating that they might play a role in the decreased barrier function of AD patients and could be a target for treatment.

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9

Discussion, prospects and conclusion

9.1 Changes in SC ceramide composition

In the research described in this thesis emphasis is put on the ceramide composition in SC from different skin sample types.

Although the studied skin samples had undergone different treatments or processes, similar alterations in ceramide composition were observed when compared to control. In this discussion I elaborate on when these changes occur, what they are, what their role might be, how they may be applied as biomarkers, and what future research would be required. Examples of processes or disease altering the ceramide composition described in this thesis are:

- SC of *ex vivo* skin that has been cultured;
- SC which has been generated after barrier disruption either *in vivo* or *ex vivo*;
- SC of AD patients at both lesional and non-lesional sites, with more pronounced changes at lesional sites.

A common denominator of these samples is that the skin has been exposed to a form of stress where the epidermis deviates from its homeorhesis of cornification and desquamation. This causes the epidermis to respond in a similar way producing characteristic changes in ceramide composition. The changes observed in all sample types were:

- Alteration in amounts of non-EO ceramide subclasses,
 - Increased S subclass ceramides,
 - Decreased P subclass ceramides,
- Decreased H subclass ceramides,
- Increased amount of C34 ceramides,
- Increased amount of Mucers.

Previously, similar alterations in ceramide profile have been observed in Netherton syndrome skin SC¹ and in SC of human skin equivalents². In the chapters of this thesis we show that these alterations also correlated to TEWL indicating a role in the barrier function. That alterations in

bound lipids were observed in AD patients and skin models indicates that these might be affected as well.

On alteration, that of the MuCers, is not commonly reported. A new analytical method with increased sensitivity showed that MuCers are present in native SC (chapter 2, 3, 5, and 6). They are selectively bound to the cornified envelope. Higher amounts of MuCers had previously been detected in human skin equivalents³ and in chapter three we show that they also increase in SC of cultured *ex vivo* skin. MuCers had not yet been implicated in diseased skin SC. In chapter seven we show that MuCers also increase in SC of AD skin compared to that in healthy control skin in both the bound and unbound fraction. The additional double bond could affect the way that the carbon tails of the lipids interact, thereby affecting the lipid organization. Increased unsaturation levels are a possible parameter contributing to the higher degree of disorder in the lipids of AD patients. Further investigation of the unsaturated acyl chain its role in barrier function and when it becomes unsaturated is required.

9.2 Predicting the relations between ceramides and barrier function

9.2.2 Modeling the mean chain length

Models can be used to predict a parameter dependent on multiple other parameters. This can be used to find quantitative relations between multiple parameters. Below, it is explained that selected lipid compositional parameters can be used to model a compounding parameter like the mean ceramide carbon chain length (MCL) and potentially more complex parameters. The MCL changes when the skin is affected and has been associated with alteration in lipid organization. It is a compounding parameter of the ceramide composition and it can be expected that the shorter C34 ceramides and ultra-long EOCers have a large impact on the MCL. In chapter three it is shown that the MCL can be accurately modeled and predicted by the amount of EOCers and C34 ceramides. Exemplifying how combining parameters with modeling results in an accurate estimation of a compounding lipid parameter. Increasing the size of the dataset and including different sample types would increase the accuracy of the prediction. One could for instance combine the unbound ceramide data generated in the research described in this thesis. An example of this model is shown in **Figure 9.1**. This shows that it is possible to establish a quantitative relation predicting MCL independent of sample type.

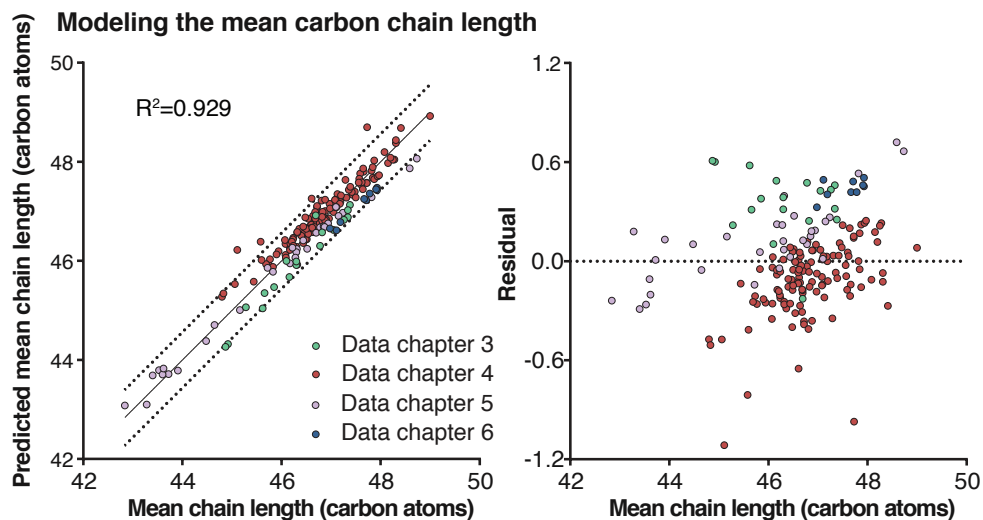


Figure 9.1: Mean chain length prediction

A general linear model to predict mean chain length was made using C34 ceramide and EOCer percentage as covariant. The unbound ceramide data used for chapters 3-6 was used as input. The predicted mean chain length is plotted against the actual mean chain length. Data for each chapter were given a separate color. The line is a linear regression with 95% predictive confidence interval indicated. The residual is also plotted per chapter.

9.2.3 Modeling the relation between lipid composition and barrier properties

The SC contains a very complex mixture of lipids and the physical chemical interactions between these lipids determine the organization of the lipid matrix and influence the barrier formed by the lipids (here called the permeability barrier). The alterations in the SC lipid composition described in 9.1 correlated to lipid organization and TEWL and each on its own could have a partial contribution to changes in the permeability barrier function. A more sophisticated predictive model can be envisioned where lipid parameters can be used to estimate lipid organization or barrier function and estimate the contribution of individual parameters. An attempt at such a model is presented in chapter three. Here a very limited number of samples was used to estimate a lipid ordering describing parameter by multiple compositional parameters. The low number of samples limited the predictive power and the accuracy of the model. More data of different sample types would be required for estimating generalized relations. A more comprehensive model including multiple changes in ceramide composition, free fatty acid composition, cholesterol amount, and bound ceramide composition, would be able to make a more genuine description. Principal component analysis (PCA) and partial least square discriminate analysis (PLS-DA) could be used to determine new parameters by comparing sample groups. Then, as explained for the MCL model, quantitative effect relations of these lipid parameters towards the lipid organization,

TEWL, or other measurements of barrier function like drug permeation could be determined. This model can test which contribution various lipid parameters have to the permeability barrier function and can distinguish the effect sizes of the different parameters.

Because of the model its complexity, the next step is to reduce the input parameters or simplify them for a more practical model. A proposed example of simplification is the following; S subclass amount has been identified as a model parameter. Yet, quantifying all S ceramides is tedious. If only one AS chain length could suffice to give the same information, it can be examined if this is a potential model parameter instead. One can envision analyzing only a limited number of ceramides which have been shown to accurately predict permeability barrier function. These ceramides could be used as clinical biomarkers to determine disease severity or in skin models to determine effects of treatment. It is, however, important to realize what these biomarkers are actually marking and how these changes relate to the processes in the epidermis. Two possible biomarkers already studied in this thesis will be discussed.

9.3 Skin barrier dysfunction biomarkers

9.3.1 C34 ceramides as signaling molecules

One ceramide group which quantity increases when the epidermis is challenged is C34 ceramides. They normally constitute only 1 molar % of all SC ceramides, but can increase significantly in diseased skin. An extensive increase of C34 ceramides can affect lipid organization because of their short chains⁴. Yet, when doubling the C34 amount one can envision the effect on lipid organization is still minimal. However, these small changes might function as powerful biomarker. C34 ceramides occur in many other tissues and showed to specifically induce the cellular processes apoptosis⁵ and autophagy⁶. Apoptosis is programmed cell death and autophagy is a process in which the cell eats itself. That both these processes are regulated by C34 ceramides begs the question if C34 ceramides have a similar role in the epidermis. Cornification is a terminal differentiation process in which almost all cellular processes are terminated and organelles are degraded. Thus, cornification has similarities to apoptosis and autophagy. It has been suggested that cornification is mediated by autophagy⁷, yet, not through regular activation⁸. That C34 ceramides increase when the epidermis requires SC generation and that they have been linked to cell death processes, suggest that C34 might play a role as inducer or signaling molecule for cornification⁹. Therefore, an increase in C34 ceramides can be interpreted as a deviation from the normal barrier formation homeorhesis and signaling for an increased epidermal differentiation.

To assess the role of C34 ceramides in differentiation, it need to be specified which pathways they interact with. A novel method of studying lipid protein interactions is by lipid based pull down assays^{10,11} combined with native MS¹². Pull down assays

based on C34 ceramides can be used on differentiating and proliferating keratinocytes to gain insight into which protein complexes they interact with and what signaling potential they have. Native MS can be used to study specific lipid protein interactions as it leaves non-covalent bonds intact.

9.3.2 Ceramide subclass ratio and lipid synthesis

The first ceramide subclass produced in ceramides biosynthesis is those with a dihydrosphingoid base (dS). These ceramides can be converted to S subclasses by dehydroceramide desaturase 1 (DES1) or to P subclasses by sphingolipid C4-hydroxylase (DES2). The enzyme producing the H-subclasses is unknown. In chapter four the correlations between subclass amount and TEWL are reported. A ratio dividing the subclasses by a negative or positive correlation to TEWL coincided with a ratio dividing the subclasses dependent on known ceramide synthesis routes: . In chapter three and four of this thesis we show that this ratio is affected when the epidermis is challenged. This ratio may thus be seen as biomarker for stress affecting DES1/DES2 and leading to a subclass constitution correlating to TEWL. If changes in subclass constitution and ratio are also causal for changes in barrier function needs to be determined. This is suggested by experiments with model lipid membranes¹³ and could be further investigated by artificially manipulating the ratio in human skin models. Furthermore, for each subclass a change in amount might affect the barrier function differently. Modeling and design of experiments could determine effect sizes for each subclass, giving a more accurate description of relation between ceramide subclass amounts than the above mentioned ratio. These new relations can be used in disease skin to determine if lipid synthesis is affected and if treatment is able to normalize the lipid synthesis. This could then be used to optimize barrier restoring and lipid replenishing formulations.

9.4 Next steps in barrier restoring formulations

In chapter three and four a VC based formulation was tested in healthy volunteers and AD patients, respectively. This formulation contained the two ceramides NS and EOS with total carbon chains of 40 66, respectively. The quantity of both was increased at the SC after application. It is difficult to establish if formulated ceramides could replenish the decreased SC ceramide amount in AD and if they were incorporated into the SC lipid matrix. Testing in *ex vivo* skin models has shown that this may occur¹⁴. When ceramides do get incorporated in the SC lipid matrix, the question arises which ceramides would have the greatest barrier enhancing effect. EOCers are reduced in AD and EOCers are essential to the lamellar organization. Therefore, EOS C66 is expected to have a beneficial effect. It would be preferred to add long chain NP ceramide to

the formulation as an additional ceramide, as these are reduced in SC of AD patients and constitute a major part of the ceramides in healthy SC. However, long chain NP ceramides are challenging to formulate and crystalizes in the VC formulation described in this thesis. Increasing NP ceramide subclass amounts have been shown to decrease permeability compared to NS ceramides¹³. Therefore, NP ceramides possibly enhances SC barrier function when formulated. Getting NP formulated into the VC would require major changes to the composition. To better understand the effects of the formulations and its composition in humans, comparison to other types of formulations like petrolatum would be essential. Linear mixed modeling can be used to compare effect sizes. To optimize the formulation's composition *ex vivo* barrier repair model testing and design of experiments should be applied.

Although there are common deviations in lipid composition, the results in chapter four indicated that AD patients can have very different lipid profiles. If we would better understand the relation between skin lipid alterations and barrier function and how to alter the lipid composition with formulations, personalized formulation could be developed. These formulations would target specific alteration observed in an AD patient. One target which is altered AD skin of which the role is not well understood are the bound lipids. In this thesis we show that the bound ceramides are closely related to the unbound EOCers due to its common precursor. Application of this precursor might function as a pro-drug and can be an interesting way of improving the SC lipid composition in diseased skin.

9.5 Conclusions

In this thesis it is shown that the analysis of SC ceramides by lipidomics can be used as a tool to study the effect of lipophilic formulations on skin barrier repair. When lipidomics is combined with *ex vivo* skin models, formulation development can become more high-throughput. Here, the *ex vivo* models function as alternative for clinical testing. Statistical models are a useful tool to determine the effect size of a formulation on diverse lipid parameters and to estimate clinical effects. Although lipophilic formulations have been extensively used in AD, it was concluded that moderate to severe AD patients require additions treatment countering inflammations. In these patients longer treatment times needs to be studied to observe the effects of the lipophilic formulation and study designs have to take into account how to counter inflammation. Nonetheless, lipidomics approaches to examine other groups of SC lipids, like bound ceramides, have shown to be able to find new targets for treatment. Using these methods, a better understanding of the role that lipids play in skin diseases was obtained.

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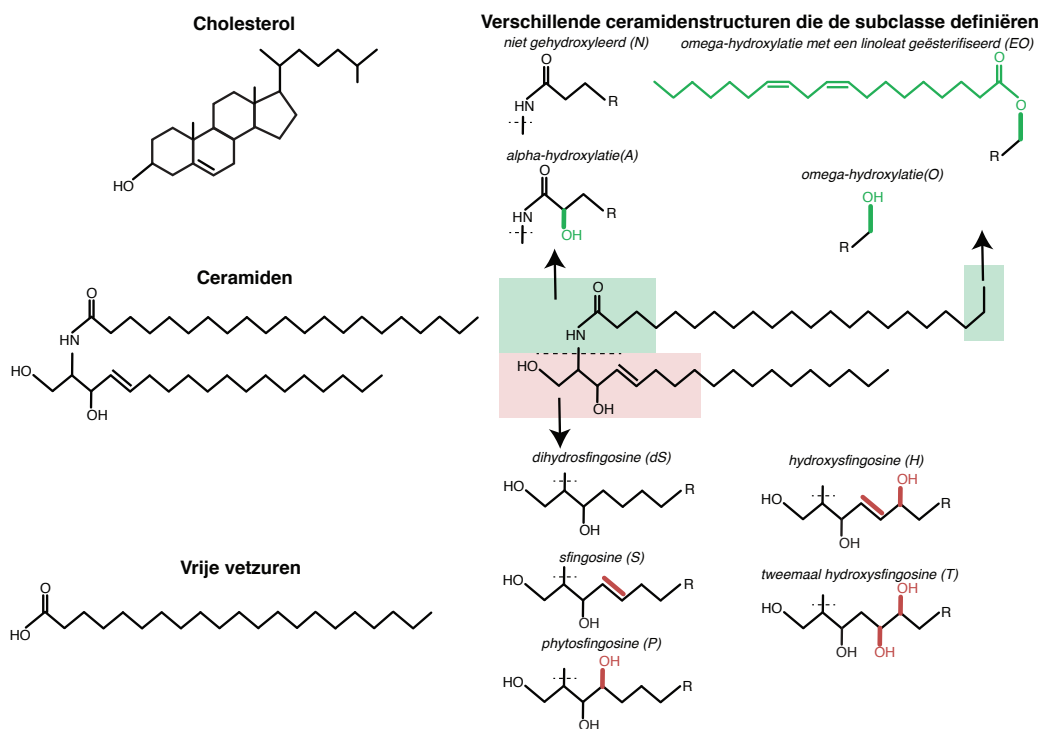
Nederlandse samenvatting

Introductie

De huid is één van de belangrijkste barrières van het menselijk lichaam. In de veel voorkomende huidziekte constitutioneel eczeem (ook wel atopisch eczeem genoemd, afgekort met AE), is deze barrière aangetast en kunnen lichaamsvreemde stoffen gemakkelijk de huid binnendringen. Dit kunnen stoffen zijn die de huid irriteren, een allergische reactie teweeg brengen, of die afkomstig zijn van ziekteverwekkers. Wanneer deze stoffen de huid binnendringen, reageert het immuunsysteem hierop en zorgt dit ervoor dat de huid op die plek gaat jeuken en rood wordt. Een ander nadeel van deze immuunreactie is dat het aanmaken van de huidbarrière, die wordt opgebouwd vanuit onderliggende huidlagen, ook wordt verstoord. Hierdoor is de nieuwgevormde huidbarrière ook verslechterd. Samen zorgen deze processen voor de kenmerkende laesies van AE. Een goed functionerende huidbarrière is essentieel om deze processen tegen te gaan.

De fysieke huidbarrière wordt gevormd door de bovenste huidlaag die het stratum corneum (SC) wordt genoemd. Deze laag bestaat uit dode huidcellen, corneocyten genoemd, en lipiden (vetten). Deze twee componenten vormen een structuur die vergelijkbaar is met een bakstenen muur. Hier zijn de corneocyten de bakstenen en de lipiden het cement. Zoals cement belangrijk is voor de stevigheid van een muur, zo zijn ook de lipiden belangrijk voor de barrierefunctie van het SC. De lipiden organiseren zich in een matrixstructuur waarbij ze dicht op elkaar gepakt zitten. Om deze structuur te vormen, is de lipidsamenstelling van groot belang. De meest voorkomende SC-lipiden zijn cholesterol, vrije vetzuren en ceramiden. Deze laatste groep is zeer divers en bestaat uit een vetzuur waaraan een sfingosinebase (een specifieke moleculaire structuur) is gekoppeld. De mogelijke chemische structuren van ceramiden die in het SC voorkomen worden weergegeven in **Figuur A.1**, samen met de structuren van de andere SC-lipiden. De naamgeving van de ceramiden is afhankelijk van de structuur van de hydrofiele kopgroep en de definities worden geven in **Figuur A.1**. Een deel van de ceramiden wordt covalent gebonden aan de buitenkant van de corneocyten. De rol van deze gebonden ceramiden voor de

huidbarrière is nog onduidelijk, maar het is wel bekend dat de ongebonden ceramiden een belangrijke functie hebben voor een correcte lipidenorganisatie en daarmee bijdragen aan de barrièrefunctie. Dit zou ook zo kunnen zijn voor de gebonden lipiden.



Figuur A.1: De chemische structuren van de lipiden die in het stratum corneum voor komen

Aan de linkerkant staan de basisstructuren van de drie verschillende hoofdgroepen van stratum corneum-lipiden. Voor de ceramiden staat aan de rechterkant de variatie in hoofdstructuur. De combinatie van vetzuur en sfingosine base bepalen ook de naamgeving. Een niet gehydroxileerde phytosfingosine heeft bijvoorbeeld als afkortingsnaam NP. Beide koolstofketens van de ceramide kunnen in lengte variëren.

In het verleden zijn er in het SC van AE-patiënten veranderingen in de samenstelling van de lipiden waargenomen en deze veranderingen correleerden met de afnamen in huidbarrière. Je kunt de huidbarrière meten door te bepalen hoeveel water er door de huid heen komt binnen een bepaalde tijd, dit proces heet trans-epidermaalwaterverlies (TEWL). Ook werd aangetoond dat de hoeveelheid ceramiden in

het SC was afgenomen in AE-patiënten. In studies van de SC ceramiden van AE-patiënten zijn daarnaast de volgende specifieke veranderingen in de ceramidenamenstelling geobserveerd:

- De hoeveelheid ceramiden met een totale koolstofketenlengte van 34 koolstofatomen is toegenomen;
- De hoeveelheid ceramiden met een sfingosinebase is toegenomen;
- De hoeveelheid ceramiden met een linoleaat veresterd aan een omega-hydroxyl ceramide (ook wel EO-ceramiden genoemd, zie **Figuur A.1**) is afgenomen.

Een gebruikelijke wijze om AE te behandelen is met vette formuleringen. Deze kunnen de huid afdekken en dienen als een extra barrière of ze kunnen vochtinbrengende bestanddelen bevatten. Ze kunnen ook als doel hebben om de lipiden in het SC aan te vullen vanuit de formulering. Dan moeten deze lipiden in de formulering zitten, wat een uitdaging kan zijn. Een natuurlijk voorkomende formulering die de SC-lipiden bevat is de vernix caseosa (VC). Dit is een laag die op huid van baby's aanwezig is voor de geboorte. In eerder onderzoek was een synthetische variant van de VC ontwikkeld. Deze VC verbeterende het herstel van de huidbarrière bij muizen met een beschadigde huid, meer nog dan standaard vette formuleringen die de huid afdekken.

Het doel van het onderzoek in dit proefschrift is om uit te vinden wat de rol van ceramiden kan zijn als therapeutisch doelwit voor het verbeteren van de huidbarrière bij ziektes waarbij deze barrière is aangetast door veranderingen in de ceramidenamenstelling. Verscheidene aspecten van de ceramidenamenstelling zijn nog onbekend. Eén daarvan is de verandering in de gebonden ceramidenamenstelling in AE. Daarnaast is de kennis over hoe de samenstelling van de gebonden en vrije ceramiden relateren aan elkaar nog gelimiteerd. Het is van belang om een goed beeld te hebben van alle veranderingen in ceramidenamenstelling om de juiste formuleringen te kunnen ontwikkelen. Daarnaast is er ook nog weinig bekend over hoe een formulering de huidlipiden beïnvloedt tijdens barrièreherstel en welke processen hieraan ten grondslag liggen. Het is van belang om deze processen te begrijpen voor het ontwikkelen van een formulering. De VC heeft een complexe samenstelling van verschillende lipiden. Het is ingewikkeld om alle componenten apart te bestuderen in klinische studies en *ex vivo*-huidmodellen (*ex vivo* betekent buiten het lichaam) zijn daarom een goed alternatief. Het is dan wel van belang dat het duidelijk is hoe deze modellen relateren aan de *in vivo* (in het lichaam) en de klinische situatie. Om de ceramiden in het SC te kunnen meten en te vergelijken tussen de *in vivo*- en *ex vivo*-huid, moest er een nieuwe methode ontwikkeld worden die alle ceramiden in beide soorten monsters kan meten. De bovengenoemde hiaten in de kennis en methodologie leiden tot de volgende onderzoeksdoelen:

1. Het ontwikkelen van een extractie en meetmethode om alle SC-ceramiden kwantitatief te meten;
2. Bepalen hoe barrièreherstel de SC-lipidensamenstelling en organisatie beïnvloedt en of *ex vivo*-huid als een model kan dienen voor klinische testen;
3. Bepalen wat de effecten van een synthetische VC-formulering zijn op huidbarrièreherstel en hoe deze formulering de SC-ceramiden beïnvloedt in gezonde huid en AE-huid;
4. Bepalen wat de gebondenceramidensamenstelling is in gezonde huid, AE-huid, en kweekhuid en wat de relatie tussen de gebonden en ongebondenceramidensamenstelling is in deze groepen.

Hoofdstuk 2: Het kwantificeren van alle SC-ceramiden

Om het eerste doel te bewerkstelligen is een methode opgezet waarbij hoge prestatie vloeistofchromatografie (HPLC) werd gecombineerd met massaspectrometrie (MS). Wanneer een methode wordt gebruikt voor het analyseren van een grote verscheidenheid aan moleculaire lipidenstructuren heet dit lipidomics. Een lipidomicsmethode voor alle ceramiden in *ex vivo*- en *in vivo*-monsters werd opgezet. *In vivo*-samples werden verkregen met tapestrippen en zijn hierdoor anders dan *ex vivo*-SC-samples. De standaard extractie werd gemodificeerd door het toevoegen van warmte en een extra lipofiele extractiestap, waardoor in plaats van 70% nu ~99% van alle ceramiden geëxtraheerd werden. Om hierna kwantitatief de hoeveelheid van alle ceramiden te bepalen, werd een model ontwikkeld om de MS-response voor elke ceramide te bepalen vanuit een gelimiteerd aantal synthetische ceramidenstandaarden. Dit model is gebaseerd op de ionisatie van de ceramide in de massaspectrometer, de isotopenverdeling en de exacte moleculaire massa van de ceramide. Om dit model te kalibreren werd de relatie tussen de MS-response, moleculaire massa en hoeveelheid in mol van de ceramidenstandaarden gebruikt. Om de toepassing aan te tonen werden monsters van gekweekte *ex vivo*-huid met direct verkregen *ex vivo*-huid vergeleken en verschillen konden worden aangetoond. Ook werd *in vivo* verkregen SC vergeleken met *ex vivo*-SC en de ceramidensamenstellingen waren vergelijkbaar. Er waren geen verschillen tussen tapestrip monsters van verschillende dieptes in het SC. Uit deze bevindingen blijkt dat de methode adequaat was om verdere vergelijkingen tussen *in vivo* en *ex vivo*-huid te kunnen doen.

Hoofdstuk 3: Het vergelijken van barrièreherstel in *ex vivo* en *in vivo* huid

Met de ontwikkelde methode kon het volgende doel worden bereikt: het bepalen of barrièreherstel *ex vivo* op dezelfde manier verloopt als het barrièreherstel *in vivo*. In deze experimenten werd de samenstelling van de SC-ceramiden vergeleken en werd de organisatie van de SC-lipiden onderzocht. Vergelijkbare veranderingen in SC-ceramidsamenstelling door barrièreherstel werden waargenomen tussen *ex vivo* gekweekte huid en *in vivo* humane huid, deze waren:

- Veranderingen in het ceramidesubklasseprofiel
 - Toegenomen sfingosine (S) subklassen;
 - Afgenomen phytosfingosine (P) subklassen;
 - Een relatief lichte afnamenvan hydroxy-sfingosine (H) subklassen
- Toegenomen percentage van ceramide met een totale koolstofketenlengte van 34 koolstofatomen, onafhankelijk van toenames in subklassen;
- De gemiddelde koolstofketenlengte van de ceramideketen nam af.

De veranderingen waren groter in *ex vivo*-huid en werden ook al waargenomen in *ex vivo*-huid die gekweekt werd maar waarbij het SC intact werd gelaten. Er werden geen veranderingen waargenomen in de lipidenorganisatie door SC-herstel, maar verschillen tussen monsters konden wel voor een deel verklaard worden door de verschillen in de ceramidsamenstelling. Daarbij waren de verschillen door SC-herstel in *ex vivo*-huid vergelijkbaar met verschillen die werden waargenomen in AE. Hieruit blijkt dat de *ex vivo*-modellen geschikt zijn om formuleringen te testen die gericht zijn op barrièreverbetering in AE. Een VC-formulering werd ontwikkeld en kon worden gebruikt voor verdere klinische studies.

Hoofdstuk 4: Therapie gericht op barrièreherstel in gezonde vrijwilligers

Omdat het nog niet bekend was wat de effecten van een barrièreherstellende formulering waren op de ceramidsamenstelling na barrièreherstel, werd de ontwikkelde VC-formulering eerst getest op gezonde vrijwilligers. Om de effecten van de formulering te testen werd het SC op een kleine plek verwijderd met behulp van tapestrippen en werd hier de formulering tweemaal daags aangebracht. Het barrièreherstel van deze plek werd vergeleken met een getapestripte plek op dezelfde persoon die niet werd behandeld. De behandeling zorgde dat de plek een mediaan herstel had dat twee dagen sneller bereikt was dan op de onbehandelde plek. Zoals

in het onderzoek beschreven in hoofdstuk 3 is aangetoond, zijn er veranderingen in de SC-ceramidensamenstelling door barrièreherstel. Wanneer na 16 dagen herstel de ceramidensamenstelling van de behandelde en onbehandelde plekken werd vergeleken waren er verschillen in de ceramidensamenstelling. De toename van zowel sfingosine subklasse als van ceramiden met een totale ketenlengte van 34 koolstofatomen was minder op de behandelde plekken dan op de niet behandelde plek. Ook correleerde de hoeveelheid van deze parameters met de barrièrefunctie gemeten als TEWL. De hoeveelheid EO-ceramiden en de gemiddelde ketenlengte van de ceramiden waren minimaal beïnvloed door de formulering, maar correleerde wel met veranderingen in de organisatie van de lipiden. Ook waren er veranderingen in het subklasseprofiel die aanduiden dat er verschillen waren in de synthese van de ceramiden tussen de behandelde en onbehandelde plekken. Uit dit onderzoek blijkt dat de VC-formulering een goede kandidaat is voor behandeling van AE-patiënten.

Hoofdstuk 5: De behandeling van atopisch eczeem-patiënten

Vanwege de bevindingen in de gezonde vrijwilligers werd er een proefonderzoek opgezet waarbij de VC-formulering werd getest in AE-patiënten. Hierbij werden patiënten gekozen die gemiddeld tot ernstig AE hadden op het moment van het onderzoek. Ze werden uitsluitend behandeld met de VC-formulering. De behandeling gaf verbeteringen van de lipidenorganisatie, maar er werden geen significante verbeteringen in de lipidensamenstelling waargenomen. De klinische score van de plekken, die de ernst van AE aangeven, verslechterde door de behandeling. De ceramidensamenstelling van plekken met laesies was verschillend van plekken die geen laesies hadden. De parameters in ceramidensamenstelling die veranderen tijdens de behandeling van plekken zonder laesies, hadden vergelijkbare verschillen in de grote van verandering (als een parameter veranderde bij een persoon veranderde een andere parameter met een vergelijkbare grootte). Dit laat zien dat veranderingen in de ceramidensamenstelling samenhangen. Over het algemeen werden er geen verbeteringen in lipidensamenstelling of TEWL gemeten. Omdat de ontstekingsreacties niet worden geremd met deze formulering, is het waarschijnlijk dat deze reacties meer effect hadden op de barrière dan de formulering. Het kan worden geconcludeerd dat bij het testen van dit soort formuleringen een combinatiebehandeling essentieel is in patiënten met gemiddeld tot ernstige AE. Behandeling met een formulering zou ook effect kunnen hebben op de gebonden ceramiden. Om dit te kunnen onderzoeken is het eerst van belang om de relatie tussen de gebonden en ongebonden ceramiden te bestuderen.

Hoofdstuk 6: Ceramiden worden selectief gebonden aan de corneocyten

Om de corneocytgebondenceramiden van menselijke *ex vivo*-huid in detail te onderzoeken, werden monsters gemeten met de ontwikkelde HPLC-MS-methode. Gebruikmakend van een specifieke fragmentatiefunctie van de MS, die moleculen in stukken breekt en meet, is er een nieuwe ceramidesubklasse ontdekt in deze monsters: een OdS-ceramide (de structuur is samen te stellen uit de gegevens in Figuur 9.1). De gebonden en specifieke ongebonden ceramiden komen voort uit dezelfde voorlopers en de samenstelling van deze beide groepen kunnen worden vergeleken. Verschillen tussen de ongebonden en gebonden ceramiden die van dezelfde voorloper afkomstig waren werden gevonden. Dit toonde aan dat er selectiviteit was in het bindingsproces, waarbij ceramiden met een extra onverzadiging en een kortere koolstofketen selectief werden gebonden. Ook werden de gebonden ceramiden gemeten van monsters die afkomstig waren van huid die geheel in kweek is gegenereerd (ook wel full thickness models, afgekort met FTMs). Een verminderde hoeveelheid gebonden ceramiden werd geobserveerd in FTMs, waarschijnlijk veroorzaakt door aanwezigheid van een oleaat in plaats van een linoleaat veresterd aan de vetzuurketen van de EO-ceramiden (een voorloper van de gebonden ceramiden). Ook waren de gebonden ceramiden voornamelijk onverzadigd en korter dan in *ex vivo*-huid. Wanneer er een lipidegevoelige receptor, de liver-x-receptor (LXR), werd geïnactiveerd, veranderde de samenstelling van de gebonden ceramiden in FTMs en werd deze meer vergelijkbaar met *ex vivo*-huid. Met deze studie is het inzicht in de relatie tussen de gebonden en ongebonden lipiden vergroot en is er aangetoond dat de gebondenceramidesamenstelling aangepast kan zijn door veranderingen in de huid. Dit doet de vraag rijzen of er niet ook veranderingen zijn in de gebondenceramidesamenstelling van AE-patiënten.

Hoofdstuk 7: Veranderingen in de gebondenceramidesamenstelling van atopisch eczeem-patiënten

Omdat de samenstelling van de ongebonden ceramiden in AE veranderde in vergelijking met gezonde huid, werd er verwacht dat de gebondenceramidesamenstelling ook veranderd zou kunnen zijn. Daarom werd een studie opgezet waarbij - met tapestrips verkregen - gebonden en ongebonden ceramiden van gezonde vrijwilligers werden vergeleken met die van AE-patiënten. Ook werd er gekeken of er op plekken met laesies aan de binnenkant van de voorarm van AE-patiënten veranderingen optreden ten opzichte van plekken zonder laesies. In AE-patiënten werden toegenomen hoeveelheden onverzadigde gebonden ceramiden waargenomen en meer ceramiden van de S-subklasse waren gebonden. Deze veranderingen waren nog sterker op de

laesionale plekken. Ook wordt er vermoed dat het binden van ceramiden met kortere ketens de hoeveelheid ongebonden EO-ceramiden beïnvloedt. De waargenomen veranderingen in AE ten opzichte van gezonde huid correleerde met de TEWL, wat aangeeft dat deze veranderingen een invloed zouden kunnen hebben op de huidbarrièrefunctie. Dit maakt de gebonden ceramiden een mogelijk doel voor nieuwe AE-therapieën.



Curriculum vitae

At the 27 of December 1985 Walter Boiten was born in Leiderdorp. He graduated his pre-university education at Rijnlands Lyceum Oegstgeest in the field of nature and technology in 2005. After one preparatory year at the conservatory, he started a bachelor in Bio-Pharmaceutical Sciences at Leiden University, which he obtained in 2010. He then continued his master in Bio -Pharmaceutical Sciences with an internship at the Division of Drug Delivery Technology. The thesis was entitled: “Multifunctional liquid chromatography mass spectrometry method for identification and analysis of the extracted lipid fraction of Stratum Corneum”. To finish his master degree an internship was performed at the pharmacology and therapeutics department of McGill University in Montreal, Canada with the thesis title: “The role of the Nerve growth factor pathway in Alzheimer disease brains form post mortem human brain samples”. Then he started his PhD at the Division of Drug Delivery Technology, now named Biotherapeutics, of the Leiden Academic Centre for Drug Research under supervision of Prof. dr. Joke Bouwstra and Dr J. van Smeden. After finishing his PhD period he continued at the same Division as a postdoc. From October 2019 he started as a postdoc at Neurochemistry Laboratory of the Department of Clinical Chemistry from the Amsterdam UMC, location VUmc.

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