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Systematic investigations into the role of ceramide subclass composition on lipid organization and skin barrier

Nadaban, A.

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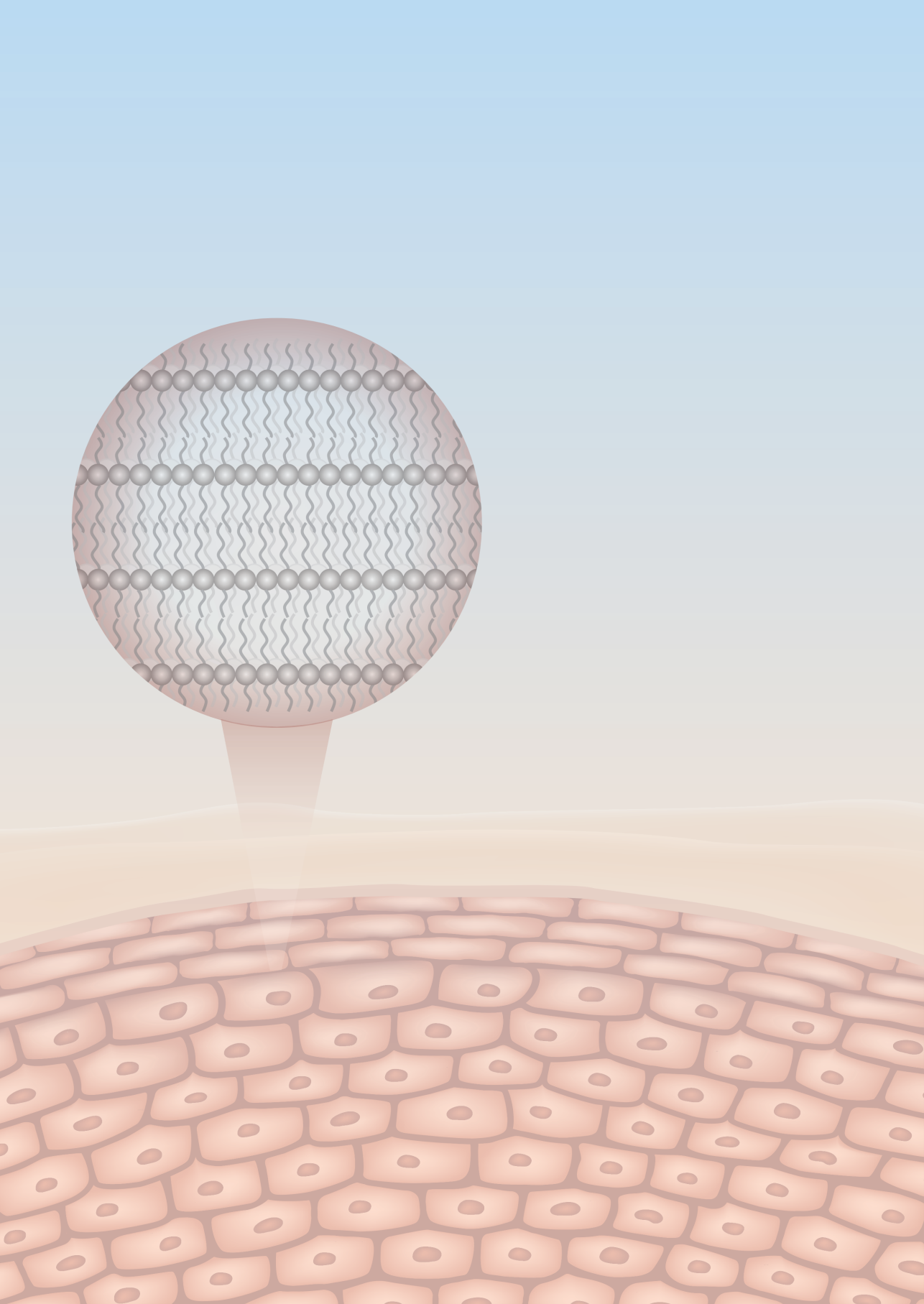

CHAPTER 1

Introduction, aim and outline of the thesis

INTRODUCTION

The structure of human skin and its barrier function

The skin, the largest organ of the body, is the main barrier against unwanted environmental factors. The essential biological functions of the skin also encompass thermoregulation, sensing external environmental stimuli, like pain and heat, synthesizing vitamin D and excreting urea and salts (1). The human skin consists of an epidermis, dermis and subcutaneous tissue (2). The epidermis, the superficial layer of the skin, continuously renews itself and it is composed of four different layers, depicted schematically in Figure 1. Keratinocytes, the most abundant cell type in the epidermis, proliferate in the stratum basale (SB) and subsequently reach the stratum spinosum (SS), where the differentiation of the keratinocytes is initiated. As the keratinocytes gradually move towards the skin surface, their function and content changes. The biosynthesis of the precursors of barrier lipids starts in the SS and is further intensified in the next layer of the epidermis, the stratum granulosum (SG). The phospholipids (precursors of free fatty acids, FFAs) and the precursors of the ceramides (CERs), the glucosylceramides and sphingomyelin, are synthesized and stored in lamellar bodies (3-6).

During the formation of the stratum corneum (SC), the final differentiation product, the keratinocytes, transform into keratin-containing corneocytes and a layer of non-polar lipids (bound lipids) is esterified to the cornified envelope, the outermost layer of these cells. Simultaneously, the formation of the intracellular SC lipid matrix is initiated. The SC lipid matrix consists of non-polar free lipids, formed by extruding lamellar bodies containing precursor lipids, together with lipid enzymes (7, 8) from the keratinocytes into the extracellular space. The monolayer of bound lipids represents a template for the free lipids to form lipid lamellae, which are oriented approximately parallel to the corneocytes (9).

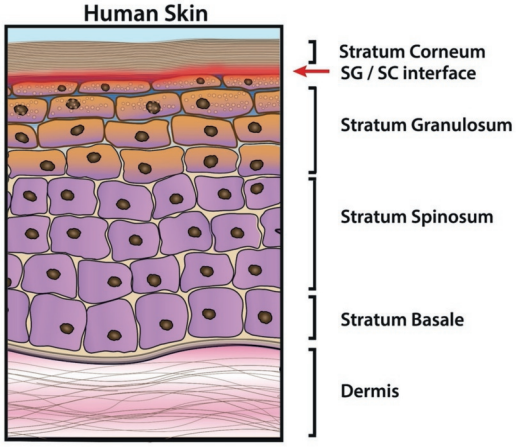

Figure 1. Schematic overview of the skin's epidermis with its four layers: stratum basale, stratum spinosum, stratum granulosum and stratum corneum (SC). Adapted from (10).

The barrier function of the skin is provided mainly by the SC, its uppermost layer (11). The only continuous pathway through the skin is the SC lipid matrix, thus any chemicals, drugs or pathogens have to pass through the intercellular lipid matrix in order to penetrate in the deeper layers. The SC has an important role to prevent unwanted environmental compounds from entering the body and prevents the excess water loss.

Lipid composition in the SC

The SC lipids consist mainly of three lipid classes: CERs, FFAs and cholesterol (CHOL), which are present approximately in an equimolar ratio in human SC (12). The CER: CHOL: FFA molar ratio varies to some extent and a comprehensive overview of the ratios obtained in previous studies is presented by Bouwstra et al. (13), also based on an earlier report of Weerheim et al. (12).

CERs are part of the sphingolipids family and besides their main function in the formation of the skin barrier, CERs are also regulators of cellular processes, such as apoptosis, proliferation and differentiation of skin cells (14, 15). CERs are composed of a sphingolipid base linked by an amide bond to an acyl chain and nowadays, there are 25 CER subclasses identified in human SC (16). In this thesis, CER subclasses are denoted according to the nomenclature introduced by Motta et al., with the first one (or two) letters representing the type of acyl chain and the last one (or two) letters indicating the type of sphingoid base (17). This is referred to as CER XY and the structures of most of the CERs subclasses present in human SC are presented in Figure 2.

A special group of CERs are the CER EO subclass (also referred to in literature as acylCERs), which have a very long ω-hydroxy acyl chain linked with an ester bond to a fatty acid chain of 18 carbon atoms (primarily a linoleate chain, but oleate or saturated chains have also been reported) (18). The CER EO subclass is very important as it is only found in the epidermis and it mostly consists of CER EOdS, CER EOS, CER EOP, CER EOH, with the exceptional structures shown in Figure 2.

Apart from the variation in the CER subclasses, in each subclass the total chain length (acyl chain + sphingoid base) varies. In this thesis, the acyl chain length of synthetic CERs is denoted as CXX. For example, CER NP C24 indicates the phytosphingosine-based CER with a non-hydroxy fatty acid chain of 24 carbon atoms. If not specified, the chain length of the sphingoid base of synthetic CERs is 18 carbon atoms in length. The total chain length of CERs is usually between 32 and 72 carbon atoms, with the variation of the acyl chain length larger than the variation in sphingoid base chain length (16, 19, 20).

The FFAs also have a large variation in the chain length (between 16 and 36 carbon atoms), with C24 and C26 representing the most abundant in human SC (21). In SC, saturated FFAs are predominantly found, with only a minor amount of monounsaturated FFAs (muFFAs) or FFAs with an extra hydroxyl group present (22).

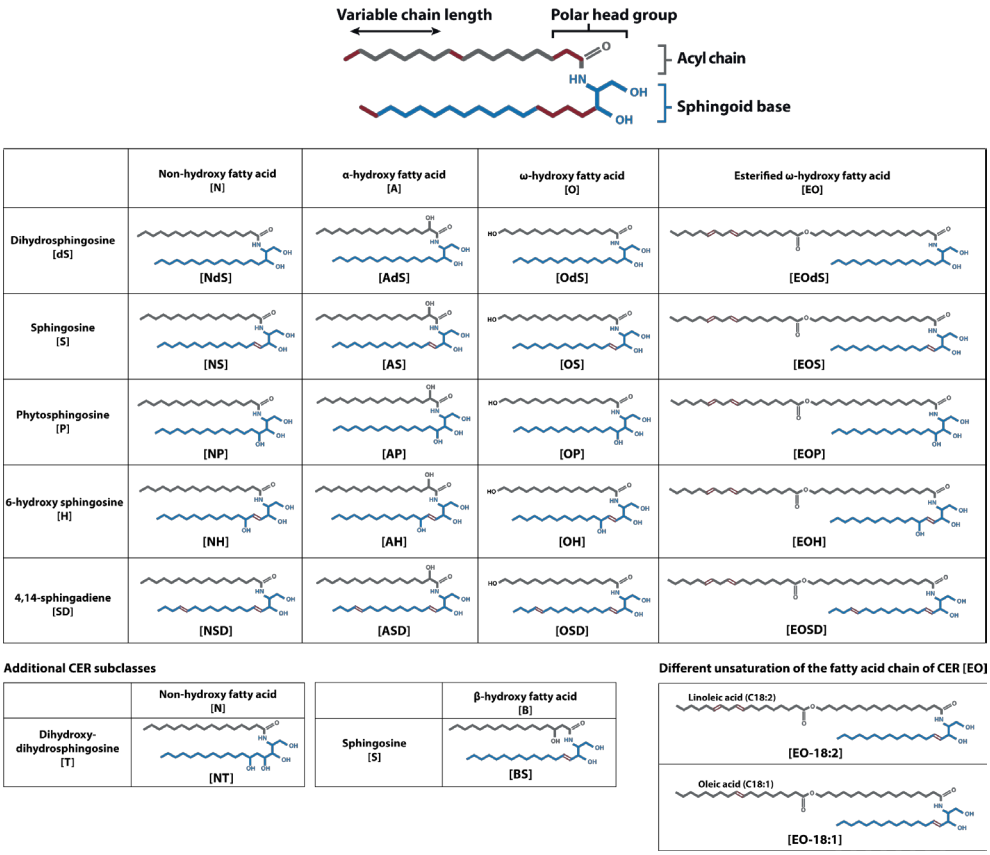

Figure 2. The structure of the SC CER subclasses and their nomenclature according to Motta et al. (17). CERs consist of an acyl chain linked with an amide bond to a sphingoid base. The CER acyl chains are non-hydroxy (N), α-hydroxy (A), ω-hydroxy (O), esterfied-ω-hydroxy (EO) or β-hydroxy acyl (B), while the sphingoid bases are dihydrosphingosine (dS), sphingosine (S), phytosphingosine (P), 6-hydroxy-sphingosine (H), 4,14-sphingadiene (SD) and dihydroxy-dihydrosphingosine (T). Figure reprinted from (13).

Lipid organization in the SC

The SC intercellular lipid matrix is organized in crystalline lamellae, arranged approximately parallel to the corneocyte surface, thus to the SC surface (9). The first studies that observed the presence of the lamellar layers in SC used freeze-fracture electron microscopy (23). Then, using ruthenium tetroxide (RuO_4) fixation of the SC the lipid lamellae were characterized by a repeating broad-narrow-broad sequence of the electron translucent bands (9, 24, 25). RuO_4 reacts with saturated SC lipid chains and the distance of this repeating pattern (referred to as the repeat distance of the unit cell) was around 12-13 nm (9). The existence of this specific repeating pattern suggests a unique molecular arrangement of the lipids in the lamellar repeating pattern. More details about the lipid organization were obtained using X-ray and neutron diffraction and Fourier

transform infrared spectroscopy (FTIR). While electron microscopy provides information in a nm-range of the field of view, X-ray diffraction and FTIR obtain average structural information.

The human SC lamellar phases were reported in 1991 using small angle X-ray diffraction, with the measurements performed at synchrotron radiation facilities. This has the advantage of a highly intense primary beam, which makes it possible to obtain structural information using a very small amount of material (26). This is important for SC as only around 15 w/w% of the material is lipids. The X-ray beam interacts with the electrons in the atoms of the lipids and the obtained data using two-dimensional detection of the X-rays can be converted to a one-dimensional plot, where equidistant peaks can be attributed to a single lamellar phase (Figure 3). X-ray studies of human SC identified two coexisting lamellar phases with repeating distances of approximately 13 nm and 6 nm, referred to as the long periodicity phase (LPP) and short periodicity phase (SPP) (26, 27). A schematic representation of these phases is presented in Figure 4.

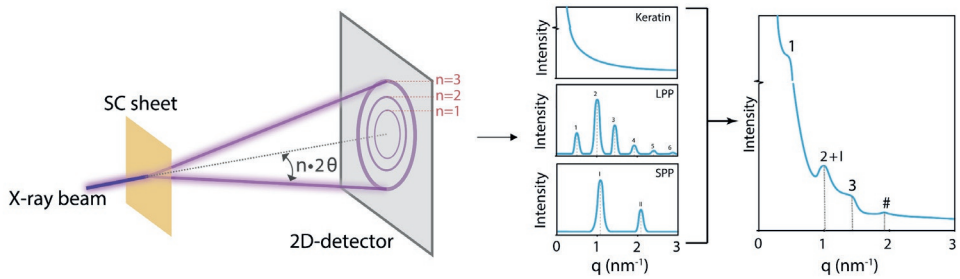

Figure 3. The lamellar organization of SC measured by X-ray diffraction. Adapted from (34).

Within the lamellar phases in the SC, the lipid chains can adopt different packing densities, referred to as the lateral organization: orthorhombic (ordered phase, very dense packing), hexagonal (an ordered phase, but the lipid chains are less densely packed) or liquid phase (highly disordered phase) (Figure 4) (28-30). In human SC, the lipids are predominantly organized in a dense crystalline orthorhombic packing, however a small fraction of lipids also adopts hexagonal packing and a minor part even a liquid phase (31, 32). The lipid packing can be analyzed using FTIR; Figure 5 provides a brief overview of the technique (13). The conformational ordering of the lipids is examined using the symmetric and asymmetric stretching frequencies of the CH_2 bond. The orthorhombic to hexagonal phase transition can be detected by measuring the FTIR spectra as a function of temperature. When the lipids adopt an orthorhombic packing, the hydrocarbon chains are very close and there is a short-range coupling of the CH_2 scissoring vibrations, resulting in two peaks at ~1463 cm^{-1} and ~1473 cm^{-1}. In case of a hexagonal packing, the hydrocarbon chains are still ordered but the distance between them causes the loss of the CH_2 frequency short-range coupling and a single peak is present in the CH_2 scissoring vibrations at ~1468 cm^{-1}. The lipid composition together with environmental factors determine the

lipid organization and thus has a great impact on the permeability of SC and skin barrier function (33, 34).

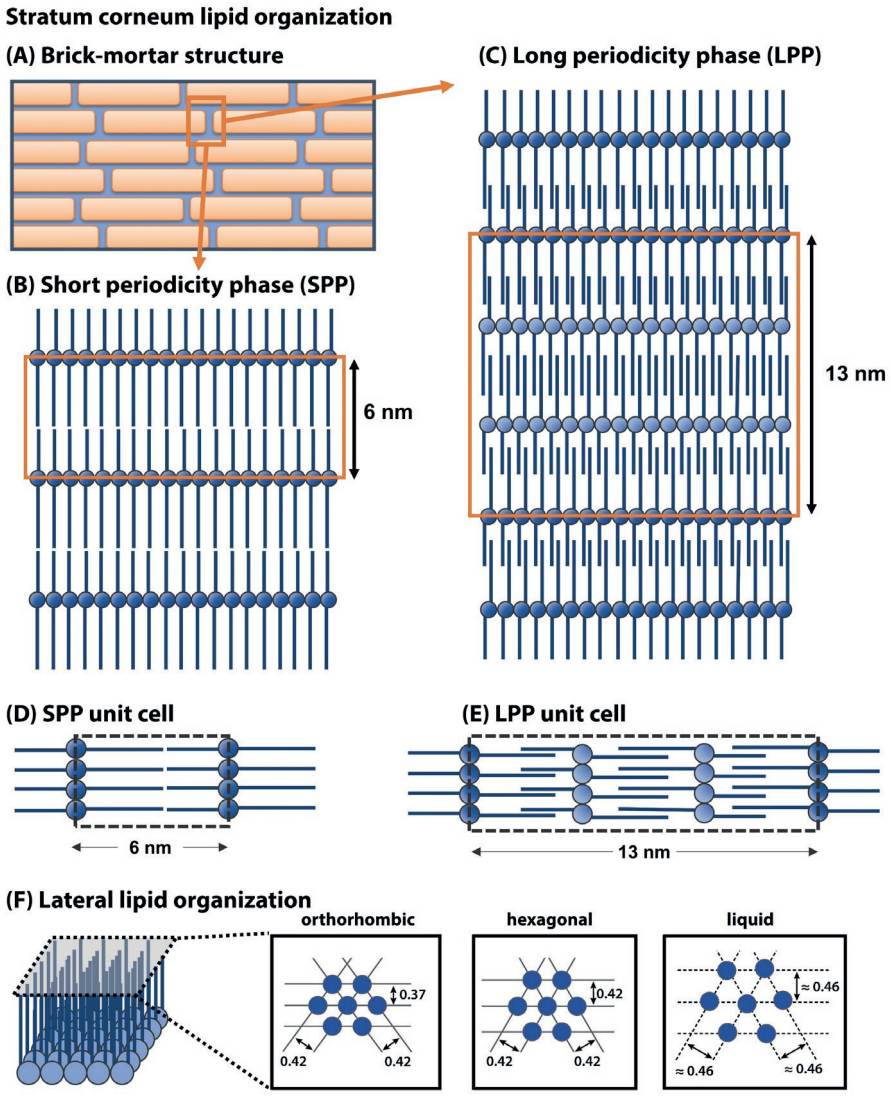

Figure 4. Schematic representation of the lamellar and lateral organization of SC lipids. The LPP and SPP with the repeat unit cells are schematically shown (B-E), along the three different packing densities of the lipids (F). Reprinted from (13).

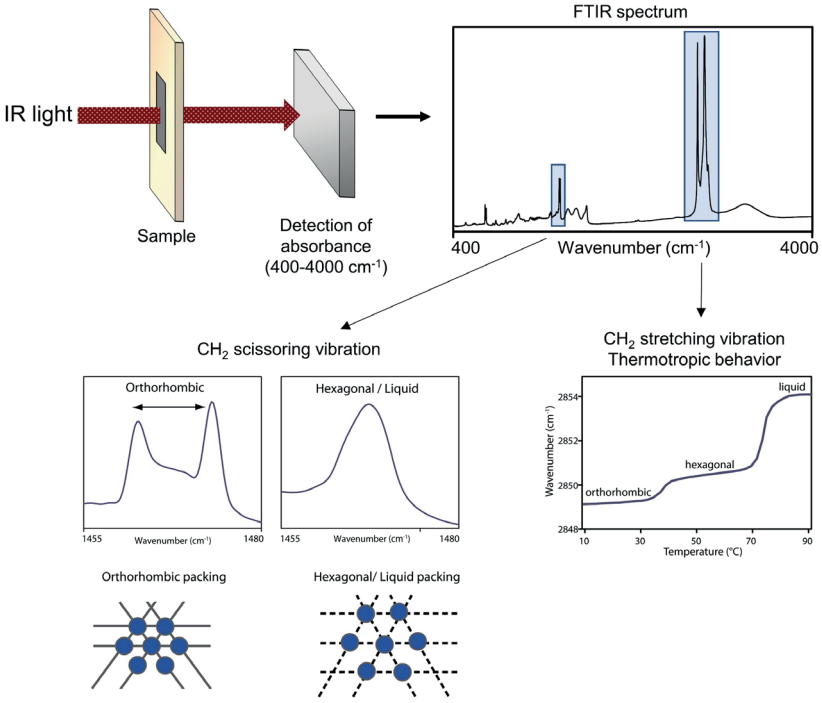

Figure 5. Brief overview of FTIR spectroscopy and the two types of vibrations mostly discussed in this thesis: the scissoring and stretching vibrations of the CH_2 and CD_2 bonds. Reprinted from (13).

Impaired barrier function and changes in barrier lipid properties in skin diseases

Inflammatory skin diseases have an important impact on our society, because of the increased patient population and the significant physical and psychological consequences of these skin disorders. Numerous patients with inflammatory skin diseases experience constant itches that cause sleep disturbances, diminished self-esteem and impacting significantly their social engagement. Due to these problems, the quality of life of the inflammatory skin disease patients is severely affected (35, 36). Psoriasis, atopic dermatitis and Netherton syndrome are some examples of inflammatory skin diseases that exhibit an impaired barrier function. Besides changes in protein composition, the SC lipid composition is also altered in these diseases. Therefore, it is important to determine whether the changes in lipid composition results in changes in lipid organization, thereby changing the skin barrier. Furthermore, it is important to determine whether there are similarities in these lipid compositional changes in the SC of these diseases.

Psoriasis is a chronic inflammatory skin disease which affects ~2% of Western countries (37). Motta et al. first reported the CER composition of lesional psoriatic SC and a reduced barrier function in lesional skin, as measured by trans epidermal water loss (TEWL) (17). A relative decrease of CER EOS, CER NP and CER AP was reported in lesional

SC, while CER NS and CER AS were increased. A clinical study by Yokose et al. reported that the ratios of CER NP and CER NH subclasses relative to CER NS and CER AS were reduced, both in lesional skin, as well as non-lesional skin (38). Moreover, the increased CER NS / CER NP molar ratio correlated positively with the TEWL values. An altered lipid composition was also observed by Uchino et al. that reported increased levels of CER NS and CER AS and reduced levels of CER NP and CER AP subclasses in psoriatic SC compared to healthy skin (39). The same study concluded that lipid composition of psoriatic SC was also characterized by shorter total chain lengths of CERs and FFAs compared to healthy SC.

Atopic dermatitis is the most frequently studied skin disease with a perturbed skin barrier. It is a chronic inflammatory skin disease that affects 2-10% of adults and over 15% of children, with an increasing prevalence in developed countries (36, 40). The barrier function of AD skin is decreased in lesional SC (34, 41-43). The major lipid compositional changes reported for atopic dermatitis SC comprise of: i) decreased total amount of CERs (44-46) and FFAs (46) in non-lesional and lesional skin, ii) reduced relative abundances of CER EOS (34, 41, 47, 48) and CER NP (47, 48), iii) increased abundance of CER NS and CER AS (45), as well as iv) short chain CER NS (34, 45). Yokose et al. showed the strong correlation of the TEWL values with the ratio of CER NS/CER NP, which was increased in lesional skin (38), in agreement with previous studies that reported separate correlations between increased TEWL values and the increased concentration of CER NS and decreased amount of CER NP in the diseased SC, respectively (34, 45, 49).

Netherton syndrome is a rare genetic skin disorder with a SPINK5 (serine protease inhibitor Kazal-type 5 gene) mutation, which is characterized by severe atopic manifestations and erythroderma (50). Similar to atopic dermatitis and psoriasis, an altered lipid composition and organization is reported in the SC of this disease. In comparison with control skin, there is a significant decrease in the FFA chain lengths and an increase in the FFAs unsaturation (51). The CER subclasses are also affected, as the concentration of CER EOS and CER NP is decreased, while the concentrations of CER NS and CER AS are elevated (51, 52). This lipid compositional change has an effect on the lipid organization as well, with a higher degree of disordering of lipid chains reported for SC of Netherton syndrome. A summary of the lipid compositional changes in atopic dermatitis, psoriasis and Netherton syndrome is presented in Table 1. As seen in the table, there are a series of lipid compositional changes occurring in all three inflammatory skin diseases.

Lipid compositional changes have an effect on the lipid organization in all three skin diseases described above: the position of the diffraction peaks (of the LPP and SPP) is shifted compared to healthy skin (34, 51, 53) and a lower fraction of lipids adopts the orthorhombic phase, as the fraction of lipids forming a less dense hexagonal organization is higher (21, 34, 51). The changes in lateral organization of the lipids in diseased SC correlated with impaired barrier function (measured by TEWL) (21, 34).

Table 1. Summary of lipid composition and organization changes observed in psoriasis, atopic dermatitis and Netherton syndrome, compared to control. Adapted from Bouwstra et al. (13).

Skin disease	Lipid composition change	Lipid organization change	References
Psoriasis	CER chain length ↓ CER EOS, EOP, EOH, NP, AP, AH ↓ CER NS, AS ↑ FFAs chain length ↓ Unsaturated FFAs ↑	Changes in lamellar phases Changes in lateral packing	(17, 38, 39, 54)
Atopic dermatitis	CER chain length ↓ CER EOS, EOP, EOH, NP, NH, AH, NdS ↓ CER NS, AS ↑ FFAs chain length ↓ Unsaturated FFAs ↑	Changes in lamellar phases Orthorhombic packing ↓ Conformational disordering of the chains ↑	(21, 34, 38, 41, 44-47)
Netherton syndrome	CER chain length ↓ CER EOS, EOP, EOH, EOdS, NP ↓ CER NS, AS ↑ FFAs chain length ↓ Unsaturated FFAs ↑	Changes in lamellar phases Conformational disordering of the chains ↑	(51, 52)

Lipid models as a tool for studying the lipid composition and organization changes

When the diseased SC is compared with healthy SC in clinical studies, the various lipid compositional changes occur simultaneously and therefore the contribution of each of the underlying factors cannot be unraveled. To understand the relationship between different lipid (sub)classes, lipid models that mimic the organization in the SC lipid matrix can be used. These models offer the possibility to systematically study the effect of compositional changes of individual components on the lipid organization and lipid barrier. In this way more detailed insights can be obtained about the main underlying factors that cause barrier impairment (55-57).

Lipid compositions

Previous studies reported that lipid model systems prepared with isolated pig (58, 59) or human CERs (60) mixed with synthetic CHOL and FFAs mimicked the unique lamellar structure observed in SC (LPP and SPP). When the various synthetic CER subclasses became available commercially, isolated pig and isolated human CERs were replaced by their synthetic counterparts. Unlike the isolated CERs, the synthetic CER mixtures have an almost uniform chain length, but this hardly influences the lamellar phase behavior: the lamellar phases of the lipid mixtures containing CERs, CHOL and FFAs, were similar to that of the mixtures prepared with isolated CER and thus also closely mimicking the native SC lipid organization (61-64). Further reducing the CER subclass composition to four, three or even two subclasses in the mixture demonstrated that selecting the proper CER

subclasses, together with CER EOS, CHOL and FFAs, it is possible to form the same lamellar phases still resembling the SC (62, 63, 65, 66). This finding is important as simple model systems with a limited number of lipid components are mandatory to obtain detailed information about the molecular arrangement of the lipids, as in such studies the protiated lipids are replaced with their deuterated counterparts.

The lipid composition of the models has an important role for the lipid organization. The presence of CERs and CHOL in lipid models is important for the formation of the lamellar phases (58, 59). Studies report the essential role of the FFAs for the lateral packing of the lipids. Short chain FFAs (C14-C18) did not influence the formation of the lamellar phases, however an additional phase was detected using X-ray diffraction. The additional phase was not formed when the FFA mix was replaced with the long-chain FFAs (with the most abundant chain lengths C22 and C24) in lipid models prepared with isolated porcine CERs (58). This was further investigated in models with synthetic CERs mimicking porcine CER composition and it was reported that short chain FFAs (C16 and C18) determine a lower conformational ordering of the lipid system, phase-separated domains, as well as a higher fraction of lipids adopting a hexagonal packing, in comparison with long-chain FFAs (C22, C24) or a FFA mix (with the most abundant chain lengths C22 and C24) (67, 68).

The presence of CER EOS in the CER composition is vital for the formation of the LPP (59, 61, 69). Increasing the CER EOS concentration from 15 mol% (mimicking the natural CER EOS concentration) to 40 mol% (of total CER fraction) results in the exclusive formation of the LPP, while the intensity distribution of the LPP diffraction orders is not changed compared to mixtures with 15 mol% CER EOS (70, 71). In the latter CER composition, the LPP and SPP are both formed. No differences in the flux of a model drug across lipid membranes of the lipid models were reported when comparing a 10 mol% and 30 mol% CER EOS fraction in the CER composition, with a significant permeability increase only present above 70 mol% CER EOS level and when no CER EOS is present (61, 72). Thus, the concentration of CER EOS can be increased or decreased to affect the fraction of lipids forming the LPP and SPP for the study of the lipid models.

Comprehensive studies were previously performed on lipid models forming only the SPP, by excluding the CER EO subclass from the lipid composition. Most frequently lipid compositions with only one or a few CER subclasses were mostly used. The most frequently investigated composition is CER NS: CHOL: FFAs. The phase behavior of these models depends on various factors (such as CER head group structure and the lipid chain length of CERs and FFAs). While CER NS C24 mixed with CHOL and FFA (C24 or the FFA_{mix} consisting primarily of C22 and C24) in equimolar ratio results in a single lamellar phase (SPP) and phase separated crystalline CHOL, using other CER subclasses in the absence of CER EOS (such as CER NP, CER AP, CER AS, CER NdS or CER NH) results in phase-separated crystalline domains, next to crystalline CHOL. This highlights the importance of the head group structure for the mixing of the lipids in the SPP (73-77). The studies using SPP

models show that CHOL improves the mixing of FFAs and CERs, and the FFAs interdigitate with the other lipid chains (78, 79).

The localization of the lipid subclasses in the LPP model

To unravel in more details the role played by the lipid (sub)classes in the lipid organization, it is important to determine the localization of the lipids in the unit cell of the lamellar phases. The unit cell represents the repeating pattern of the lamellar structure, as explained in Figure 4. Information on the lipid arrangement can be obtained using neutron diffraction, a technique that allows the identification of the exact location of the lipid chains in the unit cell. The neutron scattering of protiated and deuterated compounds is different (deuterium atoms have a higher scattering intensity than hydrogen atoms). By replacing protiated lipids by (partly) deuterated counterparts, the deuterated moiety of the lipid can be located in the structure.

The location of the water molecules can be determined by varying the D_2O/H_2O ratio in the buffer used to hydrate the samples. The water molecules are localized next to the lipid head groups, rather than the hydrophobic lipid chains. Their location indicates the four lipid head group regions in the LPP and the LPP trilayer structure comprised of a central layer with two similar outer layers (70, 80). The location of deuterated lipid chains in a lipid model can be identified by measuring the diffraction pattern of the protiated sample and its deuterated counterpart (sample with the deuterated lipid chain of interest). The scattering length density (SLD) profile of the deuterated lipid moiety is obtained by the subtraction of the SLD profiles of the protiated sample from the deuterated sample. This provides the location of the deuterated moiety in the unit cell.

The location of some lipid (sub)classes was previously examined in the LPP unit cell (Figure 6). In a lipid model with the CER subclasses mimicking the porcine CER composition, CHOL was localized in the outer layers of the LPP, with its hydroxyl group close to the inner lipid head group region (80). The position of the esterified linoleate chain of CER EOS was determined using CER EOS with a deuterated linoleate chain. The SLD profiles obtained indicate that in the LPP unit cell, the linoleate is in an isotropic phase, positioned close to the inner layer of lipid head groups (80). The proposed location of the linoleate chain of CER EOS is in agreement with previous reports of liquid droplets of esterified linoleate or oleate chains of CER EOS (81, 82). The positions of the FFA C24 and of the acyl chain of CER NS were identified in the inner layer of the LPP, with the chains interdigitating in this layer (70). Beddoes et al. determined the location of the sphingosine chain of CER NS and therefore could determine the CER NS conformation (66, 83). CER NS with the head group in the inner region of the LPP is arranged in an linear (extended) conformation: the acyl chain is mainly located in the inner layer (in agreement with the previous study of Mojumdar et al. (70)) and the sphingosine chain is positioned in the two outer layers (either side of the CER NS head group). The linear arrangement of CERs could allow a strong connection between the lipid layers and prevent the swelling

upon hydration of the LPP (81). Moreover, in this CER conformation the cross section of the polar lipid head group is reduced, allowing a higher lipid packing density (83). The localization of the two chains of CER NS is the same when comparing a complex lipid system that mimics the porcine CER composition and a lipid model with a simple composition (CER EOS, CER NS, CHOL, FFA_{mix}). Figure 6 represents a schematic arrangement of the LPP unit, according to the previously reported studies.

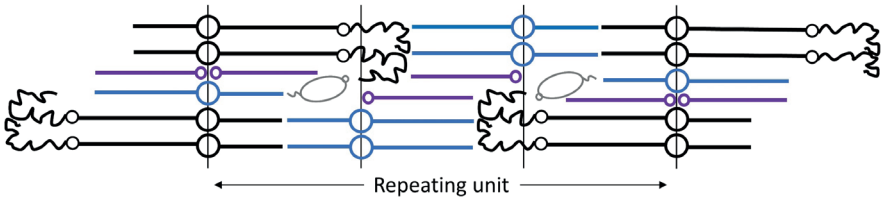

Figure 6. The lipid arrangement in the LPP unit cell (repeating unit) obtained by neutron diffraction. CER EOS is represented in black, CER NS in blue, FFAs are shown in purple and the CHOL molecules in grey. Adapted from Mojumdar et al. (80).

Lipid models for studying the lipid composition changes in skin diseases

As reported in Table 1, several changes in lipid composition have been identified in SC of inflammatory skin diseases. In order to determine the effect of each of these changes on the lipid barrier, these alterations can be systematically implemented in the lipid model systems. The influence of some lipid compositional changes on lipid barrier and phase behavior have already been studied (Table 2).

1. *Chain length of FFA and CERs*

In previous studies the effect of the lipid chain length on the conformational ordering of the lipid chains and the lateral packing was investigated. Shorter lipid chains result in a more prominent presence of lipids forming the hexagonal phase, reduce the lipid ordering and determine a difference in the lamellar organization, with a slightly altered LPP repeat distance and co-existing phases (63, 68, 84-86). This lipid compositional change also caused an impaired barrier in the lipid model membranes, as monitored by TEWL and the fluxes of model drugs, such as ethyl-p-aminobenzoic acid (E-PABA), indomethacin or theophylline.

2. *Increase in degree of unsaturation*

The degree unsaturation, that is an increase in muFFAs, influences the lateral organization resulting in a decrease of the orthorhombic packing and elevated conformational disordering of the lipid chains (87), but no change in the lamellar organization. The barrier function is also affected as monitored by TEWL and the hydrocortisone flux.

3. Variation in the fraction of CER EO subclass

Decreasing the concentration of CER EO subclass (CER EOS or a mix of CER EOS, CER EOP and CER EOdS) in lipid models has the main effect on the lamellar organization, as the LPP cannot be formed in the absence of CER EO subclass (55, 62, 64). The lateral organization is also affected, as a less dense lipid packing was predominant in the models. When the concentration of CER EO subclass in the lipid models is reduced, the lipid fraction forming the LPP is also decreased. This has a significant effect on the flux of the model drugs.

Table 2. The lipid compositional changes reported in skin diseases previously studied using lipid models.

Change in lipid composition	Barrier function	Lipid organization and molecular arrangement	References
Unsaturated FFAs ↑	TEWL and diffusion (hydrocortisone flux) ↑	Orthorhombic packing ↓ Conformational disordering ↑ No effect on lamellar phases	(87)
FFA chain length ↓	TEWL and diffusion (E-PABA, indomethacin, theophylline) ↑	Orthorhombic packing ↓	(63, 68, 84, 85)
CER chain length ↓	Diffusion (E-PABA flux) ↑	Conformational disordering ↑ No effect on lamellar phases	(63, 86)
CER EO subclass ↓	TEWL not changed Diffusion (flux of E-PABA and indomethacin) ↑	Orthorhombic packing ↓ LPP not formed when CER EO subclass is 0%	(55, 62, 64)

AIM OF THIS THESIS

The studies presented in this thesis focused on the effect of the CER subclass composition on the lipid organization, molecular arrangement and barrier function of the skin. First, the CER compositional changes were examined in human SC of seborrheic dermatitis patients by means of a clinical trial. A particular emphasis was paid to the CER NS:CER NP ratio and its correlation to the skin barrier. In the subsequent chapters lipid model systems with a simple CER subclass composition were used to study the influence of the CER NS:CER NP ratio on the barrier function, lipid organization and CER arrangement.

OUTLINE OF THIS THESIS

Chapter 2 describes a clinical study investigating the CER composition and barrier function of seborrheic dermatitis patients, an inflammatory skin disease characterized by erythema and scaling on seborrheic areas of the face and scalp. In this study, the CER composition, the severity of the disease and the skin barrier function are characterized. The CER

composition (including CER NS:CER NP ratio) was correlated with the impaired skin barrier monitored by TEWL.

In subsequent studies, lipid models with a simple lipid composition were used for a more detailed study on the influence of CER subclass composition on the lipid organization, CER arrangement and barrier function. One of the key findings regarding the CER compositional changes in SD, the CER NS:CER NP subclass ratio, was investigated using the lipid models.

Chapter 3 represents the first step in understanding the effect of changing the CER NS:CER NP molar ratio on the lipid organization, using a CER composition comprising of CER EOS, CER NS and CER NP. The location of CER NP and CER NS (ratio 1:1) in the LPP unit cell was determined.

In **Chapter 4**, the CER NS:CER NP molar ratio was changed from 1:2 (mimicking a healthy SC ratio) to 2:1 (mimicking the ratio found in severe skin conditions) in lipid models forming exclusively the LPP. The lipid organization was investigated, as well as the location of CER NS and CER NP in the LPP unit cell and the barrier function of these lipid models.

Chapter 5 continued the topic of the CER NS:CER NP molar ratio change (from 1:2 to 2:1), focusing on lipid models forming exclusively the SPP. The lipid organization of these models was investigated and compared to that of the LPP models.

In **Chapter 6**, the effect of the CER subclass composition on the arrangement of CER NP and CER NS is examined, including in a composition mimicking human CER composition. The lipid organization, molecular arrangement of CER NS and CER NP and the permeability of the lipid models with different CER head group structures were investigated.

Finally, **Chapter 7** summarizes the results of this thesis and provides an overall conclusion and some remarks on perspectives.

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