A novel formulation for skin barrier repair : from ex vivo assessment towards clinical studies

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Chapter 9

Summary and Perspectives

Summary

Introduction

The skin consists of three layers, the hypodermis, dermis, and epidermis, and protects the body from the external environment by reducing transepidermal water loss and penetration of pathogens, allergens, and irritants. The epidermis is subdivided in 4 strata, of which the stratum corneum (SC) is the outermost layer. The SC consists of terminally differentiated corneocytes embedded in a lipid matrix. The corneocytes and lipid matrix are assembled in a brick-and-mortar structure, in which the bricks represent the corneocytes and the mortar represents the lipid matrix.[1] The lipid matrix is a major penetration pathway. The lipids in the lipid matrix are excreted at the interface between the viable epidermis and the SC, after *de novo* synthesis in the keratinocytes or uptake from the systemic circulation (e.g. essential fatty acids).

Three main lipid classes in the SC are free fatty acids (FAs), ceramides (CERs), and cholesterol. A wide chain length distribution is observed for both FAs and CERs, as well as unsaturation of the carbon chain.[2-5] At least 18 subclasses of CERs are identified, based on the chemical structure of the sphingoid base and acyl chain, see Figure 1.[6-14] The SC lipids in the extracellular matrix are highly ordered in lamellae stacked on top of each other, with repeat distances of 6 nm (short periodicity phase, SPP) and 13 nm (long periodicity phase, LPP).[15-19] Within the lamellae, the lipids adopt either an orthorhombic, hexagonal, or liquid organization (Figure 2). The relative fraction of lipids adopting an orthorhombic lateral packing, the relative fraction of lipid lamellae with a repeat distance of 13 nm, and the CER composition affect the skin barrier function.[15,20-22]

In several inflammatory skin diseases, the skin barrier function is impaired. Although the underlying cause of these inflammatory diseases is different, the impaired skin barrier partly based on changes in lipid composition are very similar. These changes are e.g. increased level of CER subclass NS, decreased levels of CER NP and CER EOS, an increased fraction of unsaturated lipids, and a reduced average carbon chain length of CERs and FAs. These changes in lipid composition may contribute to a less dense lateral ordering, and a reduced lamellar repeat distance.[4,5,17,23-31] The skin barrier function is most extensively studied in atopic dermatitis (AD), and many studies focused on possible treatment strategies. However, to date treatment of AD is far from optimal: corticosteroids are used to reduce the inflammatory response, but they are associated with side effects, such as skin atrophy.[32] To reduce the absorption of pathogens, allergens, and irritants, topical application of endogenous skin barrier lipids to repair the skin barrier may be an attractive approach.[33]

Vernix caseosa (VC) is a white, cheesy substance covering and protecting a fetus in the last trimester of pregnancy and during delivery.[34-36] It consists of the barrier lipids CERs, FAs, cholesterol, as well as squalene, wax esters, sterol esters, triglycerides, and phospholipids.[36,37] Skin barrier repair in mice is enhanced after application of VC, but also after application of synthetic formulations mimicking VC.[38,39] However, the skin barrier repair response in animal skin is different from that in human skin.[40] Currently cultured human skin models (e.g. human skin equivalents) are often labor intensive and time consuming to generate. Therefore, less time consuming skin models mimicking

the *in vivo* human skin barrier response are attractive to study topical treatment, especially when focusing barrier repair of AD skin or other inflammatory skin diseases.

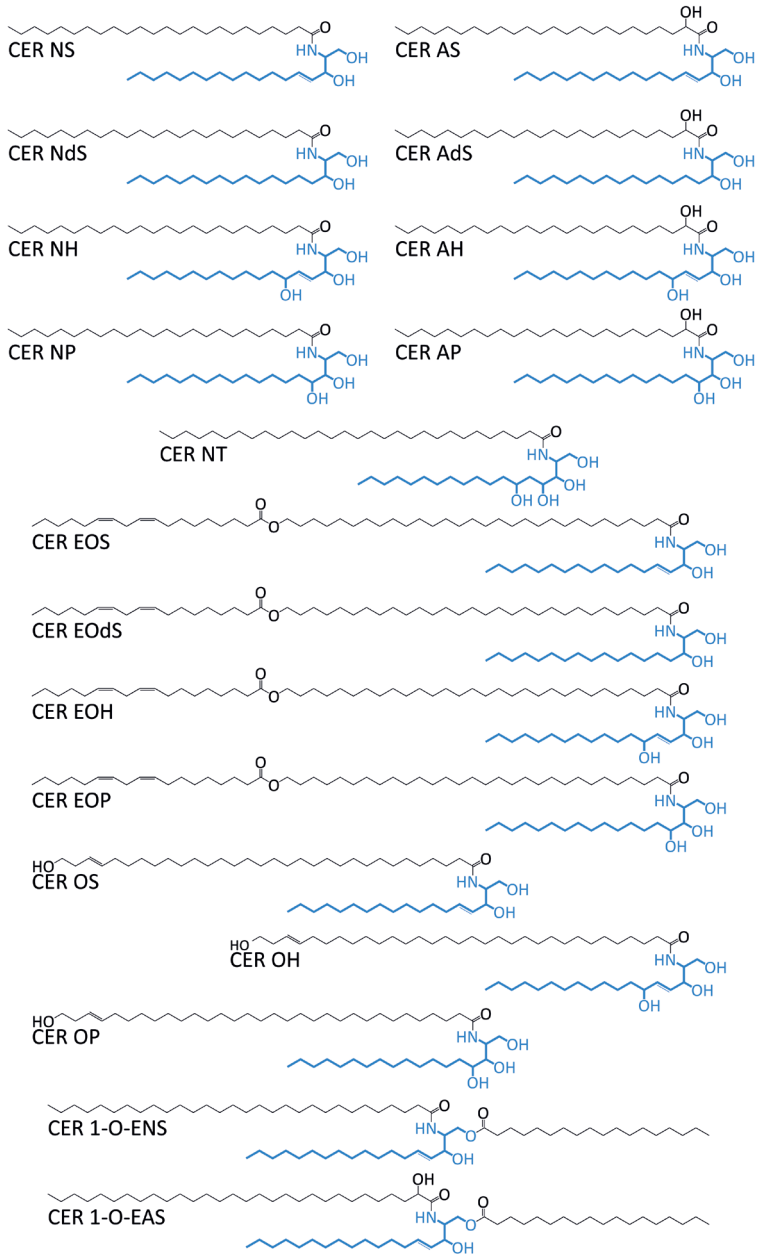

Figure 1. Ceramides subclasses in the stratum corneum lipid matrix. CERs consist of a sphingoid base coupled to a FA, which can both vary in molecular structure. CERs are named according to their molecular structure. The acyl chain can either be non-hydroxylated (N), α-hydroxylated (A), ω-hydroxylated (O), or esterified ω-hydroxylated (EO), whereas the sphingoid base is either a sphingosine (S), dihydrosphingosine (dS), phytosphingosine (P), 6-hydroxysphingosine (H), or dihydroxy dihydrosphingosine (T).

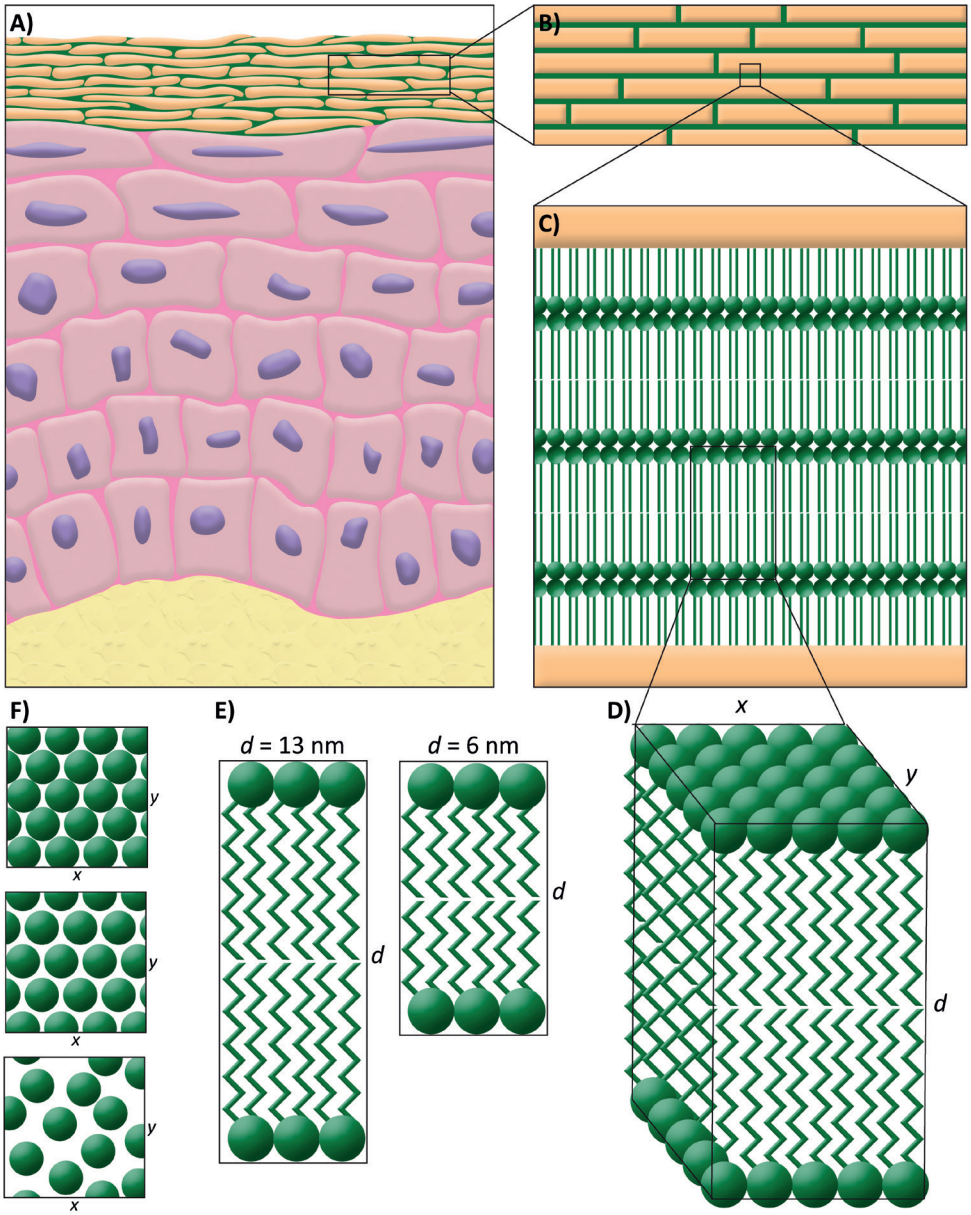

Figure 2. Organization of the lipids within the stratum corneum lipid matrix. A) Schematic overview of the epidermal morphology. **B)** The corneocytes are embedded in the lipid matrix in a brick-and-mortar structure. **C)** The lipids in the matrix are stacked in lamellae in between the corneocytes. **D)** More details of the lipid lamellae. **E)** Two lamellar phases are identified with a repeat distance (d) of either 13 nm (LPP) or 6 nm (SPP). **F)** Within the lamellae, the lipids are organized in either an orthorhombic, hexagonal, or liquid packing (from top to bottom).

Aim of the study

The aim of this thesis was to determine whether a novel VC based formulation effectively enhances skin barrier repair in AD patients and normalizes the SC lipid composition and organization. In order to achieve this goal, the following studies were performed:

1. An *ex vivo* human skin barrier repair (SkinBaR) model was developed for studying the interactions between topical applied compounds and the skin barrier. The SC lipid properties during and after skin barrier repair process were examined. The lipid composition and organization in the regenerated SC of this SkinBaR model were compared to the lipid composition and organization in regenerated SC in healthy human skin *in vivo*.
2. The effects of a selected number of barrier FAs and/or CER subclasses applied in a VC based formulation on the SkinBaR model during skin barrier repair were examined. Especially the interactions between the VC components and the SC lipid matrix were studied.
3. The effect of the VC based formulation on skin barrier repair in compromised healthy skin, and in AD skin was assessed.

Development of a skin barrier repair model

In order to study skin barrier repair *in vitro*, no suitable skin models are available. Generation of human skin equivalents is very labor intensive and therefore less attractive. In Chapter 2 a novel *ex vivo* human skin barrier repair (SkinBaR) model was developed. Cyanoacrylate was used to remove SC from the skin thereby reducing the barrier function. Epidermal morphology, differentiation, SC lipid composition and organization were examined after culturing the skin for 8 days at 37˚C and 32˚C. The results showed that the skin was actively proliferating and differentiating, which resulted in regeneration of SC. The lipids in the regenerated SC mainly adopted a hexagonal lateral lipid organization at the expense of lipids forming an orthorhombic lateral organization as observed in healthy native human SC. The altered skin barrier properties showed similarities with AD skin. Consequently, the SkinBaR model has the potential to study the skin barrier repair process and how this process may be influenced by application of topical formulations and/or by changing the environmental factors.

In Chapter 3, the reproducibility of the stripping of the SkinBaR model was examined in more detail. The influence of initial degree of barrier disruption on the lipid organization of the regenerated SC was assessed. The results showed that when 25%, 50%, and 75% of the SC was removed the SC is able to regenerate fully in a period of 8 days to a comparable number of corneocyte layers as in native SC. Major morphological differences (e.g. parakeratosis) and a change in lamellar structure were only observed when initially 75% of the SC was removed. As far as the lateral lipid organization was concerned, a gradual increase in degree of barrier disruption (percentage of SC removed) resulted in a gradually less dense packing, but the lipid ordering was only affected if at least 50% of the SC was removed. These results led to the conclusion that the degree of barrier disruption in the SkinBaR model can be controlled and that the SkinBaR model can be adjusted to match the desired lateral or

lamellar lipid organization. Therefore, the SkinBaR model offers the possibility to study the interaction of skin barrier repair formulations with the lipid matrix, in which the degree of deviation in lipid organization to that in healthy human skin can be adjusted on demand.

It is unknown how well the SkinBaR model reflects in detail the lipid properties in the regenerated SC in human skin *in vivo*. Hence, the lipid properties of the SkinBaR model were compared to those of *in vivo* skin after tape stripping. The studies are described in Chapter 4. The comparison focused on the CER composition (i.e. CER subclass, CER chain length, and degree of unsaturation), and lipid conformational ordering. Compared to control skin, in both models, levels of S subclass CERs (e.g. CER NS and CER AS, see Figure 1) were increased, whereas levels of P subclass CERs (e.g. CER NP, CER AP, and CER EOP, see Figure 1) were decreased. Furthermore, the mean ceramide chain length was decreased, and higher levels of CERs with i) a total chain length of 34 carbon atoms and/or ii) mono-unsaturation were observed. The lipid chains were less ordered in regenerated SC of the SkinBaR model, however, this was not statistically significant. Overall, changes in CER composition in the SkinBaR regenerated SC were more pronounced than changes in CER composition in the *in vivo* regenerated SC. The only pronounced difference between the models was the level of EO ceramides, which was decreased in the SkinBaR model and increased in the *in vivo* regenerated skin. Nevertheless, the lipid properties in both models mimicked quite closely the ceramide composition in AD, but also showed similarities with other inflammatory skin diseases. Therefore, the SkinBaR model and *in vivo* tape stripped healthy skin can serve as a first model to study skin barrier repair of compromised inflamed skin.

Application of VC based formulations on the skin barrier repair model

Since a VC based lipid formulation was shown to enhance skin barrier repair in mice models, this formulation was applied on the regenerating SC of the SkinBaR model. In Chapter 5 we focused on the interaction of the FA component incorporated in the VC based formulation and studied its interaction with the SC lipid matrix. FAs with a chain length of either 16, 18, or 22 carbon atoms were used in the formulation. The lipid organization and composition of the regenerated SC on which a formulation was applied immediately after stripping with cyanoacrylate were examined after regeneration. The applied formulations, especially when incorporating FA C18 and C22 resulted in an increase in the fraction of lipids adopting an orthorhombic packing. Deuterated FAs were used in order to i) distinguish between the FAs in the formulation from the endogenous FAs and ii) to examine whether FAs were partitioning in the same lattice as the SC lipids. The thermotropic behavior of the formulated deuterated FAs applied on the SC matched that of native SC, indicating that the deuterated FAs are incorporated in the SC lipid matrix. When focusing on the lateral lipid organization, the studies demonstrated that i) application of FAs with a longer chain length resulted in a higher fraction of lipids adopting an orthorhombic lateral packing, and ii) the applied deuterated FAs partitioned in one lattice with the SC lipids. Analyzing the FA composition with LC/MS showed that a fraction of the deuterated FAs with a chain length of 16 carbon atoms were elongated to mainly a chain length of 24 carbon

atoms. The studies described in this chapter demonstrate that the VC based formulation is able to improve the SC lipid properties of the *ex vivo* SkinBaR model.

Another important class of SC barrier lipids are CERs. In Chapter 6, studies are described in which CERs were used in the VC based formulation and applied on the SkinBaR model. CER subclasses NS and EOS were formulated, as well as a combination of both subclasses and FA with 16 carbon atoms. Compared to non-stripped and stripped control skin, application of the formulation with a single CER did not change the morphology of the cultured skin. A higher fraction of lipids adopted a dense orthorhombic lateral lipid packing when the formulation was applied, and CER EOS was more effective than CER NS. As CER NS was available with a deuterated acyl chain, interactions of deuterated CER NS with the SC lipid matrix were also studied, but no strong evidence could be obtained indicating that CER NS is incorporated in the lipid matrix. With regards to a formulation in which CER NS, CER EOS, and the FA were combined, interactions of these components with other lipids in the VC based formulation were examined. Interactions of CER NS with the other lipids of the formulation were observed as well as interactions of FA with the other formulation lipids. However, these studies revealed separate FA rich and CER NS rich domains. Applying the combined VC based formulation on regenerating SC resulted in participation of FA in the SC lipid matrix. When participation of CER NS from the combined formulation with the SC lipid matrix was examined, the thermotropic behavior of the formulation alone and the SC on which the formulation was applied were very similar. Therefore, changes in thermotropic behavior induced by application of CER NS (i.e. participation in the lipid matrix) could not be used to examine whether CER NS participated in the lipid matrix. As no deuterated CER EOS was available, it was not possible to determine participation of CER EOS in the SC lipid matrix. The lamellar organization of the SC lipids was not affected by application of either the formulations with a single CER or the formulation in which CER NS, CER EOS, and FA were combined. These results indicate that CERs enhance the fraction of lipids adopting a dense orthorhombic lateral packing and that the barrier lipids of the VC based formulation are, at least partly, incorporated in the SC lipid matrix. The VC based formulations might have the potential to restore a compromised skin barrier function as observed in inflammatory skin diseases.

Application of VC based formulations on human skin

As described above, tape-stripping and regeneration of healthy human skin *in vivo* resulted in i) increased levels of S subclass CERs, especially CER NS and CER AS, ii) decreased levels of P subclass CERs, especially CER NP, iii) a reduced mean chain length, iv) a higher level of CERs with a total chain length of 34 carbon atoms, and v) a higher fraction of mono-unsaturated CERs. In Chapter 7 studies are described in which a VC based formulation was applied on tape-stripped human skin *in vivo* during a regeneration period of 14 days. The formulation contained CER NS, CER EOS, and two FAs with chain lengths of 16 and 18 carbon atoms. The skin barrier repair was monitored over time for a tape-stripped treated and a tape-stripped non-treated site. For both sites and two control sites (treated and non-treated), the ceramide composition, lateral lipid ordering, and the lamellar lipid organization were examined. Accelerated

barrier repair was observed after application of the VC based formulation. Application of the VC based formulation resulted in a CER composition that was shifting toward the CER composition of the control sites. The shift was most pronounced for CERs with a chain length of C34 carbon atoms, the subclass CER AS (both reduced in relative amounts), and the average chain length (increase in chain length). Furthermore, a ratio dividing the CERs based on their synthesis route (e.g. (dS + P + H)/S) correlated with skin barrier function, with a higher ratio indicating a better barrier function. As the changes induced by tape-stripping of the skin were partially normalized by treatment with the VC based formulation indicating a positive effect of the formulation on the skin barrier, it is of interest to study its effect on the skin barrier in AD patients.

The promising results obtained after application of the VC based formulation on disrupted healthy skin justified application on naturally compromised skin of AD patients. A pilot study in which 8 moderate to severe AD patients were included is described in Chapter 8. The patients applied the formulation for 2 weeks, twice a day. The changes in disease activity, skin barrier function, ceramide composition, and lipid ordering were analyzed. After 2 weeks of treatment, the lipid ordering improved compared to control before treatment, indicating an improved skin barrier. For most patients, the transepidermal water loss decreased after applying the VC based formulation. Treatment did not affect the mean carbon chain length of the CERs, the level of CERs with 34 carbon atoms, and the level of mono-unsaturated CERs. The ratio between several CER subclasses ((dS + P + H)/S) showed a slight decrease. Overall, the lipid parameters highly correlated with transepidermal water loss and with each other. Some changes in parameters coincided for multiple patients, in that, if one was beneficial the other was as well: e.g. mean carbon chain length and level of EO CERs, CERs with 34 carbon atoms and transepidermal water loss, and level of mono-unsaturated CERs and lipid ordering. The clinical outcome, however, was negatively affected, and the severity of the AD at the start of the study was of influence on the skin barrier repair. Furthermore, the treatment affected each patient differently. Emollients could be helpful to treat AD, however, monotherapy with only a VC based formulation might not be sufficient in patients with moderate to severe AD.

Conclusions

The studies in this thesis describe the development of an *ex vivo* SkinBaR model and a novel VC based formulation to treat the skin of AD patients. We showed that the SkinBaR model is highly reproducible and the model can be used to mimic multiple degrees of skin barrier disruption. The SkinBaR model shows many parallels in lipid composition and organization with *in vivo* disrupted and regenerated skin and with that in AD skin. Application of a VC based formulation containing CERs and FAs resulted in a denser lipid organization in the SC of the SkinBaR model. Furthermore, in *in vivo* regenerated healthy human skin the VC based formulation enhanced skin barrier repair and reduced modulation in lipid composition induced by tape-stripping and SC regeneration. However, when applied as monotherapy on moderate to severe AD skin, the lipid ordering is positively affected, but limited changes in lipid composition were

observed. The overall disease activity was not influenced and the local disease severity was increased. The optimal treatment aiming to repair the skin barrier for moderate to severe AD skin needs further research.

Perspectives

Enhancement of the SkinBaR model

The SkinBaR model has been successfully developed to study the human skin barrier repair response. It is an easy to use model mimicking closely the lipid properties of AD skin. The SkinBaR model can be used to study the skin barrier repair response in *ex vivo* conditions, with a focus on the barrier lipid composition and organization. Furthermore, the effect of topical formulations on the intercellular lipid matrix can be studied. As discussed in Chapter 4, the *ex vivo* SkinBaR model shows many parallels with *in vivo* compromised skin.

Currently, the SkinBaR model reflects several aspects of AD skin. However, the ultimate goal is to have a SkinBaR model which reflects the lipid composition in healthy compromised human skin in clinical studies. However, there are several differences in the SkinBaR model compared to the compromised model *in vivo*. This is demonstrated by a substantially accelerated skin barrier repair response in the SkinBaR model compared to the *in vivo* compromised skin. This is demonstrated by a hyperproliferation indicated by a higher expression of Ki67, and more pronounced changes in the CER composition compared to *in vivo* compromised skin, especially the increase in ceramide subclass AS and NS, the level of CER with total chain length of 34 carbon atoms, and a reduction in subclass NP demonstrates an activation of the keratinocytes. In order to bring the SkinBaR model closer to the *in vivo* situation, changing the culturing conditions and environmental factors offer a suitable starting point. These are:

i) It is widely known that enzyme activity and expression are temperature dependent. In the skin, a temperature gradient exists from the core body temperature of 37˚C to the skin surface temperature of around 32˚C. This gradient might be essential for proper functioning of epidermal differentiating proteins and enzymes involved in lipid biosynthesis. A temperature gradient in the skin during culture might be obtained by maintaining the culture medium at 37˚C and reducing the environmental temperature to 32˚C, the skin surface temperature.

ii) Other factors that might influence the skin barrier repair process of the *ex vivo* cultured skin are relative humidity, UV light exposure, and physical stress. In the *in vivo* situation, the relative humidity to which the skin is exposed is much lower than in culturing conditions. Additionally, skin is exposed to a certain dose of UV light and physical stress (e.g. movement, washing, clothes) in real-life conditions. Implementing these factors during generation of the SC in the SkinBaR model might lead to a SC of the SkinBaR model mimicking the *in vivo* SC generation much closer.

iii) In the SkinBaR model common inflammatory responses that slow down the skin barrier repair in healthy compromised skin is partly lacking.[41] The inflammatory response can be established by supplementing cytokines to the medium of the SkinBaR model. For example, cytokines IL-1α and IL-6 attract immune cells to the injured site.
iv) During the culturing period, the medium composition changes because the skin uses the nutrients from the medium and releases the waste products into the medium. This may be improved by a flow through system of the medium mimicking more closely the *in vivo* situation.

Having a SkinBaR model which reflects healthy compromised human skin *in vivo*, offers a starting point to expand the use of the SkinBaR model to study detailed aspects of the skin barrier and the repair process:

i) The SkinBaR model could be used to study multiple other inflammatory skin diseases. Each inflammatory skin disease is characterized by a unique subset of changes compared to healthy human skin. These changes involve the lipid properties as well as protein expression and immunological aspects, e.g. upregulation of inflammatory cytokines.[42,43] Being able to mimic several aspects of inflammatory skin diseases in addition to the altered lipid properties induced by inflammation, may be achieved by adding pro-inflammatory cytokines to the culture medium. This may affect the morphology, enzyme expression, and lipid properties.[44] Mimicking these unique changes of each inflammatory skin disease offers the opportunity to develop disease-specific treatments tackling specific problems of the skin barrier repair response, such a disease-specific lipid composition and lipid ordering, reducing the inflammatory response, or influencing the expression and activity of specific proteins. These disease-specific treatments might involve topical formulations, but also medium supplements could be used to represent systemic treatment.
ii) Systemically circulating substances could potentially be of influence on regeneration of the SC. Therefore, adding those substances to the culture medium of the SkinBaR model allows us to examine the influence of the uptake of systemically circulating substances on the regenerated SC.

Improvement of the skin barrier repair formulation

The studies described in Chapter 5 and 6 of this thesis showed that the VC based formulation improves the lipid packing and that the applied FA with a chain length of 16 carbon atoms are elongated in the epidermis and therefore are part of the epidermal biosynthesis. Application on compromised healthy skin *in vivo* resulted in enhanced skin barrier repair. However, further improvement of the VC based formulation might be possible by changing the composition:

i) In AD skin, the level of CER subclass NS is increased and the levels of CER EOS and NP is decreased.[5,27,29,30,45,46] As described in Chapter 7, an increased CER subclass ratio ((dS+P+H)/S) correlated with an improved barrier function, indicating that increasing the relative level of CER NP in the SC might improve the skin barrier function. This novel information suggests that CER NP might

even be a better candidate to incorporate in the VC based formulation. However, due to its very stable crystal formation, CER NP is difficult to formulate. Natural VC consists of a combination of a large number of CER subclasses that are also present in human SC, including CERs NS, EOS, and NP and variations in CER chain length.[37] Perhaps changing the CER fraction of the VC based formulation by using various CER subclasses with a variation in chain length might improve the barrier repair potential of the formulation, but is not an easy task.

ii) FAs are another main lipid class in the SC lipid matrix. In healthy SC, FAs with a chain length of 24 and 26 carbon atoms are most abundant.[2-4] Unfortunately, these FAs were not available for clinical studies. Furthermore, FAs with a chain length of only 16 carbon atoms are most abundant in VC.[37] As the FA with 16 carbon atoms was elongated in the studies described in Chapter 5, this FA was used in the VC based formulation in the clinical settings. In AD skin, the average chain length of the lipids is reduced, and a higher abundance of FAs with 16 and 18 carbon atoms has been reported.[4] This might indicate that elongation of the FAs in AD skin is impaired.[47] Therefore, using FAs with longer chain lengths in the VC based formulation might be more beneficial in treating AD skin.

In the studies described in Chapter 8, the VC based formulation was applied on the skin of patients with moderate to severe AD and resulted in improvements in the lipid composition in some patients and improved lipid conformational ordering in most patients when applied on non-lesional skin. Unfortunately, the local disease severity did not improve, but this might be expected as the local disease severity is influenced by the overall disease severity and thus the degree of systemic inflammation. This might suggest that monotherapy with a VC based formulation is not sufficient. Therefore, in future it might be of interest to use this formulation in the treatment of patients with only mild AD skin or even dry skin. Another option is to investigate the use of this formulation to prevent the development of AD in those humans that have a high risk factor to develop AD.

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