Skin barrier responses to moisturizers

IZABELA BURACZEWSKA
Dissertation presented at Uppsala University to be publicly examined in Rosénsalen, Ingång 95/96, Akademiska Sjukhuset, Uppsala, Friday, October 24, 2008 at 13:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract


Moisturizers are used in various types of dry skin disorders, but also by people with healthy skin. It is not unusual that use of moisturizers is continued for weeks, months, or even years. A number of moisturizers have been shown to improve the skin barrier function, while others to deteriorate it, but the reason for observed effects remains unknown. Further understanding of the mechanism by which long-term treatment with moisturizers influences the skin barrier would have clinical implications, as barrier-deteriorating creams may enhance penetration of allergens or irritants and predispose to dry skin and eczema, while barrier-improving ones could reduce many problems.

The present research combined non-invasive techniques with analyses of skin biopsies, allowing studies of the epidermis at molecular and cellular level. Test moisturizers were examined on healthy human volunteers for their effect on the skin barrier, with regard to such factors as pH, lipid type, and presence of a humectant, as well as complexity of the product. After a 7-week treatment with the moisturizers, changes in transepidermal water loss, skin capacitance, and susceptibility to an irritant indicated a modified skin barrier function. Moreover, the mRNA expression of several genes involved in the assembly, differentiation and desquamation of the stratum corneum, as well as lipid metabolism, was altered in the skin treated with one of the moisturizers, while the other moisturizer induced fewer changes.

In conclusion, long-term use of moisturizers may strengthen the barrier function of the skin, but also deteriorate it and induce skin dryness. Moisturizers have also a significant impact on the skin biochemistry, detectable at molecular level. Since the type of influence is determined by the composition of a moisturizer, more careful selection of ingredients could help to design moisturizers generating a desired clinical effect, and to avoid ingredients with a negative impact on the skin.

Keywords: skin barrier function, moisturizers, long-term treatment, transepidermal water loss, gene expression, skin pH, lipids, urea

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ISSN 1651-6206
urn:nbn:se:uu:diva-9300 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-9300)
To my Family

'Well, in OUR country,' said Alice, still panting a little, 'you'd generally get to somewhere else—if you ran very fast for a long time, as we've been doing.'

'A slow sort of country!' said the Queen. 'Now, HERE, you see, it takes all the running YOU can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!'

Lewis Carroll, “Through the Looking-Glass”
**PAPERS INCLUDED**

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


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ABBREVIATIONS

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<th>Description</th>
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<tbody>
<tr>
<td>ACACB</td>
<td>acetyl-CoA carboxylase beta</td>
</tr>
<tr>
<td>ACSL1</td>
<td>acyl-CoA synthetase long-chain family member 1</td>
</tr>
<tr>
<td>ACTB</td>
<td>actin, beta (β-actin)</td>
</tr>
<tr>
<td>ALOX12B</td>
<td>arachidonate 12-lipoxygenase, 12R type</td>
</tr>
<tr>
<td>ALOXE3</td>
<td>epidermal arachidonate lipoxygenase 3</td>
</tr>
<tr>
<td>ARCI</td>
<td>autosomal recessive congenital ichthyosis</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>cyclin-dependent kinase inhibitor 1A</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’-6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>FASN</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FLG</td>
<td>profilaggrin</td>
</tr>
<tr>
<td>GBA</td>
<td>glucocerebrosidase, beta; acid (β-glucocerebrosidase)</td>
</tr>
<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase)</td>
</tr>
<tr>
<td>HMGCS1</td>
<td>3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMG-CoA synthase 1)</td>
</tr>
<tr>
<td>IL1A</td>
<td>interleukin-1α</td>
</tr>
<tr>
<td>IVL</td>
<td>involucrin</td>
</tr>
<tr>
<td>KLK5</td>
<td>kallikrein 5</td>
</tr>
<tr>
<td>KLK7</td>
<td>kallikrein 7</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NMF</td>
<td>natural moisturizing factor</td>
</tr>
<tr>
<td>o/w</td>
<td>oil-in-water</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>pyrrolidone carboxylic acid</td>
</tr>
<tr>
<td>PPARA</td>
<td>peroxisome proliferator-activated receptor alpha (PPAR-α)</td>
</tr>
<tr>
<td>PPARB</td>
<td>peroxisome proliferator-activated receptor beta (PPAR-β)</td>
</tr>
<tr>
<td>PPARG</td>
<td>peroxisome proliferator-activated receptor gamma (PPAR-γ)</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RXRA</td>
<td>retinoid X receptor alpha (RXR-α)</td>
</tr>
<tr>
<td>SLS</td>
<td>sodium lauryl sulfate</td>
</tr>
</tbody>
</table>
SMPD1  sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingomyelinase)
SPTLC2  serine palmitoyltransferase, long chain base subunit 2 (serine palmitoyltransferase 2)
TEWL  transepidermal water loss
TGM1  transglutaminase 1
UGCG  UDP-glucose ceramide glucosyltransferase
**INTRODUCTION**

**THE STRUCTURE AND BARRIER FUNCTION OF THE SKIN**

Terrestrial life would not be possible without protection from dehydration and harmful factors. The skin gives us such protection, but it is more than an impenetrable shield: it is a dynamic and complex tissue, mediating a multiplicity of functions. It provides a physical permeability barrier, hampering excessive water and electrolyte loss from inside-out, and protects from external chemical, microbial, and mechanical insults. The skin plays an essential role in thermoregulation, absorbance of ultraviolet radiation, sensation and sociosexual communication. Since it is our largest organ, its immunological performance as well as its ability to repair wounds and regenerate itself, is of vital importance for the entire body.\(^1,2\)

**Skin structure**

The skin consists of three distinctive layers, the subcutis, dermis, and epidermis (Figure 1). The lowest of these layers, the subcutis (hypodermis, subcutaneous fat), is built of adipose tissue, which helps to cushion and insulate the body. This layer serves as energy storage and allows for skin mobility over underlying structures.\(^1,2\)

The dermis constitutes the principle mass of the skin. Its main component, extracellular matrix (ground substance), attracts and retains water due to presence of strongly hygroscopic molecules, proteoglycans. Dermis is crossed with nerve and vascular networks and embraces epidermal appendages such as hair, sweat glands and sebaceous glands. It contains various cell types, such as fibroblasts, macrophages, mast cells, and transient circulating cells of the immune system. The dermis is tightly connected to the uppermost layer of the skin, the epidermis, by the basement membrane, which is the main part of the dermal–epidermal junction.\(^1,2\)

The epidermis, a constantly self-renewing stratified structure, is built mostly of keratinocytes, which account for at least 80% of its total cells. Therefore, the properties and functioning of keratinocytes determine the condition of the epidermis. The remaining cell types are melanocytes, Langerhans cells, Merkel cells, and various cells of the immune system. Due to the differentiation state of keratinocytes, layers of epidermis are classified into the stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Figure 2).\(^1,2\)
Figure 1 – Skin layers. From: Gilberg S., Tse D. Atlas of Ophthalmology, Edited by Richard Parrish II, David T. Tse, 2000, Current Medicine Group LLC, with permission.

Figure 2 – Layers of epidermis. From Gilberg S., Tse D. Atlas of Ophthalmology. Edited by Richard Parrish II, David T. Tse, 2000, Current Medicine Group LLC, with permission.
Stratum corneum as the skin barrier

Keratinocytes in the epidermis migrate from stratum basale with a proliferative cell type, through stratum spinosum, to the stratum granulosum, where cornification, a process of terminal differentiation, is initiated; transforming keratinocytes into flat anucleated cells called “corneocytes”. Cornification involves degradation of organelles, organization of keratin bundles inside the cell, and formation of a cornified envelope around it.1,2 This is accompanied by secretion of lamellar bodies, organelles containing a mixture of lipids, mainly polar ones: glucosylceramides, sphingomyelin, and phospholipids, and also cholesterol, as well as numerous enzymes. After secretion at the stratum granulosum/stratum corneum interface, polar lipids are enzymatically converted into non-polar products: ceramides and free fatty acids (reviewed by Feingold3). Lipids derived from lamellar bodies are assembled into lamellar structures surrounding the corneocytes.1,2

As a result, the stratum corneum consists of layers of corneocytes, which are densely packed with keratin and embedded in extracellular lipid matrix. Corneocytes are connected to each other by corneodesmosomes, and are gradually removed from the skin surface by desquamation, making place for new cells coming from underneath. Process of keratinocyte turnover in epidermis is highly organized in space and time, so that proliferation and differentiation of keratinocytes are in balance.1,2

The stratum corneum, though only 10–20 μm thick, is the most essential layer of epidermis from the perspective of its barrier properties and protection outside-in and inside-out. The structure of the stratum corneum can therefore be compared to a wall made of bricks (corneocytes) and mortar (intercellular lipids). This “brick and mortar” model was initially presented by Michales et al.4 in 1975. The skin barrier models presented later: stacked monolayer model,5 mosaic domain model,6 single gel-phase model,7 and sandwich model,8 can be regarded as extensions of the brick and mortar model (a concise summary of those models is presented by Norlén9). They accept the two-compartment structure of the stratum corneum and focus on explanation of molecular arrangements of intercellular lipids. Although the organization of the stratum corneum and its components is not completely understood, the development of new techniques, such as cryo-electron microscopy and cryo-electron tomography of vitreous skin sections (reviewed by Norlén10), gives new insight into its structure.
The function of the skin as a barrier can be regarded as protective, or even defensive, in nature, and it is localized mostly in the stratum corneum. Corneocytes and extracellular lipid matrix fulfill various protective functions, which often co-localize and/or are linked together (Table 1) (reviewed by Elias\textsuperscript{11,12}). In dermatological research, overall protective properties of the stratum corneum and epidermis are often referred to as the “skin barrier”, while their performance is known as the “skin barrier function”. However, it is important to realize that depending on the research design and the evaluation methods, the exact meaning of the term “skin barrier” may vary. For example, some studies may focus more on the permeability barrier to water from inside the skin to the outside, while others investigate selective absorption of a substance from outside into the epidermis.

<table>
<thead>
<tr>
<th>Function</th>
<th>Principal compartment</th>
<th>Biochemical basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permeability</td>
<td>Extracellular matrix of SC</td>
<td>Ceramides, cholesterol, nonessential fatty acids in proper ratio</td>
</tr>
<tr>
<td>Antimicrobial</td>
<td>Extracellular matrix of SC</td>
<td>Antimicrobial peptides, free fatty acids, sphingosine</td>
</tr>
<tr>
<td>Antioxidant</td>
<td>Extracellular matrix of SC</td>
<td>Cholesterol, free fatty acids, vitamin E, redox gradient</td>
</tr>
<tr>
<td>Cohesion (integrity) (\rightarrow) desquamation</td>
<td>Extracellular matrix of SC</td>
<td>Intercellular DSG1/DSC1 homodimers</td>
</tr>
<tr>
<td>Mechanical or rheological</td>
<td>Corneocyte</td>
<td>(\gamma)-glutamyl isopeptide bonds</td>
</tr>
<tr>
<td>Chemical (antigen exclusion)</td>
<td>Extracellular matrix of SC</td>
<td>Hydrophilic products of corneodesmosomes</td>
</tr>
<tr>
<td>Psychosensory interface</td>
<td>Extracellular matrix of SC</td>
<td>Barrier lipids</td>
</tr>
<tr>
<td>Neurosensory</td>
<td>Stratum granulosum</td>
<td>Ion channels, neurotransmitters</td>
</tr>
<tr>
<td>Hydration</td>
<td>Corneocyte</td>
<td>Filaggrin proteolytic products, glycerol</td>
</tr>
<tr>
<td>Ultraviolet light</td>
<td>Corneocyte</td>
<td>Trans-urocanic acid (histidase activity)</td>
</tr>
<tr>
<td>Initiation of inflammation (first-degree cytokine activity)</td>
<td>Corneocyte</td>
<td>Proteolytic activation of pro-interleukin-1α/β</td>
</tr>
</tbody>
</table>

*Modified from Elias\textsuperscript{12}, published with permission; SC = stratum corneum; DSG1 = desmoglein 1; DSC1 = desmocollin 1
**Intercellular lipids of the stratum corneum**

Lipids of the continuous extracellular lipid matrix of the stratum corneum are predominantly ceramides, cholesterol, cholesteryl esters (cholesterol esters with fatty acids) and free fatty acids, in an estimated molar ratio 37:32:15:16, respectively. Moreover, small quantities of cholesterol sulfate and glucosylceramides are present as well. Lipids are organized into lamellar phases, oriented approximately parallel to the surface of the corneocytes (reviewed by Bouwstra et al.). The detailed lamellar organization of intercellular lipids is still not completely known, as data vary, depending on the method used. Their lateral organization is also under investigation, since it determines the permeability of the stratum corneum: lipids are organized mostly in orthorhombic and hexagonal packing, with the first type being the least permeable arrangement, while the latter is more permeable. It has been shown that in normal skin, lipids of stratum corneum have mostly orthorhombic packing, although hexagonal packing and blends of hexagonal and orthorhombic packing are also present. By contrast, patients with atopic dermatitis and lamellar ichthyosis have been found to have predominantly hexagonal organization of intercellular lipids.

**ENZYMES AND NON-ENZYMATIC REGULATORY PROTEINS OF THE SKIN BARRIER**

The formation of components of stratum corneum, corneocytes and intercellular lipids, as well as degradation of corneodesmosomes, which results in desquamation, is regulated by several key enzymes and non-enzymatic proteins. Abnormalities in their expression, structure, or activity may lead to impairment of the skin barrier, which is often found in various skin disorders, such as atopic dermatitis, psoriasis and disorders of keratinization.

**Corneocyte formation**

Among the most important proteins in the process of cornification are transglutaminase 1, involucrin and filaggrin. Transglutaminase 1, together with transglutaminase 3, crosslinks various proteins into a mechanically and chemically resistant structure named the “cornified envelope”, formed around each corneocyte. Involucrin is one of the first proteins linked during this process and acts as a scaffold for other proteins, e.g., loricrin, trichohyalin, small proline-rich proteins, cystatin α, and elafin (reviewed by Candi et al. and Ishida-Yamamoto et al.). Reduced level of involucrin and its altered distribution in epidermis was found in patients with atopic dermatitis. Transglutaminase 1 is also probably involved in the
formation of ester bonding between involucrin, possibly also other proteins, and ω-hydroxy-derivatives, forming a lipid envelope of about 5 nm around the cornified envelope, which acts as a frame for attaching other intercellular lipids. Mutations in gene of transglutaminase 1 result in enzyme deficiency, and are found in patients with lamellar ichthyosis and congenital ichthyosiform erythroderma (nonbullous) (reviewed by Richard). Keratohyalin granules are small organelles that are visible in stratum granulosum by ordinary light microscope. They are composed primarily of profilaggrin and loricrin. Profilaggrin is cleaved into filaggrin, which aggregates keratin filaments into tight bundles and causes the collapse of filament network of keratinocytes, resulting in a flattened cell (reviewed by Candi et al.). Later, filaggrin undergoes degradation into free amino acids, pyrrolidone carboxylic acid (PCA) and urocanic acid. Amino acids and PCA are the main components of the bulk of natural moisturizing factor (NMF), helping to maintain an appropriate hydration level of the stratum corneum (reviewed by Rawlings et al.). The importance of filaggrin for the skin barrier function has been demonstrated in recent studies, which link atopic dermatitis, chronic irritant contact dermatitis, and ichthyosis vulgaris to mutations in the gene of profilaggrin. Individuals with mutation of FLG have been shown to have less NMF, which may be one of factors predisposing them to dry skin disorders. Moreover, FLG mutations are connected to a higher risk of developing asthma among patients with atopic dermatitis and severe asthma in individuals without eczema.

Another essential protein in the cell cycle is cyclin-dependent kinase inhibitor 1A, which has a broad functionality in skin biology. Together with other inhibitors of the same family, this protein is involved in cycle progression through the G1 phase into the S phase and in gene expression regulation. Differentiation of squamous epithelia, including the epidermis, is associated with increased expression of cyclin-dependent kinase inhibitor 1A (reviewed by Weinberg et al.). Increases in both its mRNA and protein levels have been found in psoriatic plaques, and also after application of irritants and tape stripping.

Desquamation

Desquamation of corneocytes from the stratum corneum surface, terminating the keratinocyte life cycle, is mediated by enzymes belonging to the group of proteases, among which kallikrein 5 and kallikrein 7 (previously known as stratum corneum tryptic enzyme (SCTE) and stratum corneum chymotryptic enzyme (SCCE), respectively) are currently assumed the most crucial. In normal skin, kallikrein 5 and 7 are found in the stratum granulosum and
corneum, as well as in the skin appendages. In transgenic mice with pathologic skin changes, such as increased epidermal thickness, hyperkeratosis, dermal inflammation, and severe pruritus, kallikrein 7 was expressed also in the suprabasal epidermal layers. Moreover, increased protein expression of kallikrein 5 and 7 was found in stratum corneum of lesional skin of psoriasis vulgaris, while non-lesional psoriasis skin had no such increase, and also in stratum corneum obtained from patients with atopic dermatitis, which had none or only very mild lichenification. These findings suggest an involvement of kallikreins in various skin disorders, including inflammatory reactions. Kallikrein 7 has also been found to be affected by the humidity of the environment: the low relative humidity decreased activity of kallikrein 7 in excised animal skin due to a diminished water concentration in the upper stratum corneum, reversible by application of a moisturizer or a humectant (glycerin).

**Formation of extracellular lipid matrix of stratum corneum**

So far, nine classes of ceramides have been identified in the human stratum corneum, and they differ from each other with regard to their head group architecture. They can contain sphingosine, phytosphingosine, or 6-hydroxysphingosine as a base. Ceramides 1, 4, and 9 are unique, as they contain linoleic acid. The ceramides, especially ceramide 1, have an important role in the organization of stratum corneum lipids (reviewed by Bouwstra et al.).

All types of ceramides are synthesized *de novo* from palmitoyl-CoA and serine, where serine palmitoyltransferase is a rate-limiting enzyme in this process. In order to be transported to stratum corneum in lamellar bodies, ceramides are converted to glucosylceramides and sphingomyelin, by enzymes UDP-glucosylceramide synthase and sphingomyelin synthase, respectively. After extrusions from lamellar bodies, enzymes β-glucocerebrosidase and serine palmitoyltransferase transform them to ceramides (reviewed by Feingold).

Deficiency in ceramides or abnormalities in enzymes involved in their formation has been found in atopic dermatitis, where a significant reduction in the quantity of ceramides has been observed in both lesional and non-lesional skin. This has been associated with a decreased activity of acid sphingomyelinase and increased activity of glucosylceramide deacetylase, but no changes were found in activity of β-glucocerebrosidase. The level of ceramides is also decreased in psoriasis, probably due to decreased levels of serine palmitoyltransferase, and the severity this disease has been reported to correlate with the level of this enzyme. In psoriasis, mRNA and protein expression of β-glucocerebrosidase has been decreased in non-lesional skin and increased in lesional skin. Except for their structural
function, ceramides are also involved in a number of cellular processes including apoptosis, the cell cycle, and cellular differentiation (reviewed by Ruvolo\textsuperscript{52}). Recently, a role for ceramides was proposed in the formation of the epidermal pH gradient, as acquiring free fatty acids from ceramides may contribute to acidification of the stratum corneum.\textsuperscript{53}

HMG-CoA synthase and reductase enzymes are involved in the synthesis of cholesterol in epidermis, with acetyl-CoA as a substrate.\textsuperscript{1} The importance of cholesterol synthesis for the barrier function and recovery has been demonstrated after acute and chronic skin barrier disruption in mice, by acetone, tape stripping and essential fatty acid-deficiency diet, which significantly increased the mRNA expression of HMG-CoA reductase and synthase.\textsuperscript{54} The same type of barrier damage increased also activity HMG-CoA reductase.\textsuperscript{55} Topical application of lovastatin, an inhibitor of HMG-CoA reductase, has been reported to impede barrier recovery.\textsuperscript{56} Cholesterol seems to be important for the organization of intercellular lipids (reviewed by Bouwstra et al.\textsuperscript{16}). Moreover, cholesterol sulfate has a significant role in desquamation (reviewed by Elias et al.\textsuperscript{57}).

Acetyl-CoA serves as a substrate for synthesis not only of cholesterol, but of fatty acids as well, involving fatty acid synthase in this process.\textsuperscript{1} The mRNA expression of fatty acid synthase was shown to increase after barrier disruption by in mice, as also does the expression of another lipid-processing enzyme, acetyl-CoA carboxylase beta.\textsuperscript{54} Interestingly, when murine skin is occluded just after tape stripping, mRNA expression of acetyl-CoA carboxylase beta, fatty acid synthase, HMG-CoA reductase and synthase is decreased, which suggests that their expression is regulated by the skin barrier function itself, not by a non-specific response to damage.\textsuperscript{54}

**Nuclear hormone receptors and lipoxygenases**

Recently, the significance of nuclear hormone receptors, namely peroxisome proliferator-activated receptors PPAR-\(\alpha\), PPAR-\(\beta\) and PPAR-\(\gamma\), and retinoid X receptor alpha (RXR-\(\alpha\)), for the normal skin barrier formation has been demonstrated. PPARs are transcription factors involved in keratinocyte differentiation and proliferation and lipid synthesis. Their activation was shown to increase expression of proteins essential in formation of cornified envelope: involucrin, loricrin, and transglutaminase 1, stimulate synthesis of epidermal lipids and formation of lamellar bodies, as well as to increase activity of lipid-processing enzymes. PPARs are also involved in anti-inflammatory processes and cutaneous carcinogenesis. Therefore, their performance is essential for the skin barrier formation and function. They
may play an important role as drug targets for such skin diseases as psoriasis and atopic dermatitis, and also in skin cancer (reviewed by Feingold, Schmuth et al., and Sertznig et al.). RXR-α is a receptor for the vitamin A metabolite, 9-cis retinoic acid, and is the most abundant retinoid X receptor in the epidermis. It heterodimerizes with PPARs, and it has been shown that these heterodimers act as signal transducers in different signaling pathways.

The exact function of RXR-α for the skin barrier formation and function remains not completely understood, but studies on mice show that mutation of RXR-α induces hyperproliferation and abnormal differentiation of epidermal keratinocytes.

The formation of the skin barrier lipids can be regulated at the transcriptional level by substances acting as ligands to the PPARs. Enzymes believed to generate endogenous ligands for these receptors have recently been described. Mutations in the coding regions of two of these enzymes, lipoxygenases arachidonate 12-lipoxygenase, 12R type and epidermal arachidonate lipoxygenase 3, were discovered in patients with autosomal recessive congenital ichthyosis (ARCI), characterized by a defect water diffusion barrier in the skin. The exact functions of those two lipoxygenases still remain unknown, but it seems that they may be connected to lipid metabolism of the lamellar granule contents or intercellular lipid layers, as well as formation of cornified envelope (reviewed by Akiyama et al.).

SKIN pH

The pH value of the skin has been investigated since the end of 19th century. The acidic nature of the skin surface was first mentioned by Heuss in 1892. In 1928, Schade and Marchionini coined the term “acid mantle” of the skin (“Säuremantel”). Since then, many studies have been carried out aiming to explain the mechanism of pH gradient formation and its importance for the skin and its barrier function, but to this day, this issue is not completely understood.

It is important to realize that the term “skin pH” is not completely correct, as pH values should refer only to diluted aqueous solutions (≤0.1 mol/kg), while the epidermis is a dense structure containing only about 20–30% of water in the stratum corneum and about 70% water in deeper layers. Moreover, various residues located on the skin surface may influence the readings. Therefore, what is actually measured is pH of the “(extractable) water-soluble constituents of skin”, and that measured pH of the skin is not the pH in a precise analytical–chemical sense. Consequently, instead of “pH of the skin”, terms such as “pH on the skin” or “apparent pH” have been proposed to be more appropriate. However, despite
the mentioned considerations, it is widely accepted to use the terms “skin pH” or “pH of the skin” and these expressions are also used in this thesis.

Several studies show that the pH value on the surface of healthy, undamaged skin of adults is slightly acidic, about 5, varying between 4 and 6. A mixture of various substances secreted on the skin surface with sweat, sebum and NMF, such as lactic acid, butyric acid, PCA, amino acids, and free fatty acids, helps to shift the surface pH towards acidic values. In addition, ingredients of exogenous origin, such as metabolites of cutaneous microflora (e.g., free fatty acids) and cosmetic products may be present. However, it seems that pH on the skin surface depends mainly on processes taking place in deeper layers of the epidermis (reviewed by Parra et al., Rippke et al., and Fluhr et al).

Below the skin surface, in the epidermis, pH increases from acidic values in the upper layers of the stratum corneum to near-neutral values of around 7.4 in viable epidermis, forming a gradient through the stratum corneum. The exact course of this gradient is still uncertain. Measurement of tape-stripped human skin with a glass electrode has shown a gradual decrease in pH towards the skin surface. Visualization of hydrogen ions in human skin biopsies demonstrated that pH decreases sharply at the stratum corneum/granulosum interface, but later slightly increases within stratum corneum, and then decreases again towards the skin surface. However, recent investigation with fluorescence lifetime imaging microscopy (FLIM) suggests that the observed pH is in fact an average from two distinct pH gradients formed by two types of acidic microdomains, each of them localized in one compartment of stratum corneum: corneocytes or extracellular lipid matrix. As the result, the average pH of the stratum corneum decreases towards the surface, due to an increase in the ratio of acidic to neutral regions.

Protons forming the pH gradient are generated most likely by several mechanisms and perhaps not all of them have been identified yet. Two endogenous mechanisms are currently believed to be the most important for acidification of the epidermis: formation of free fatty acids from phospholipids through the action of secretory phospholipase A2 (PLA2) and exchange of protons for sodium ions by non-energy-dependent sodium-proton exchangers (Na+/H+ exchanger isoform 1, NHE1) in the membranes of keratinocytes at the stratum corneum/stratum granulosum interface. The latter mechanism explains the decrease in pH at the border between the stratum corneum and the stratum granulosum, as NHE1 is expressed in the same place. Recently, a new pathway was proposed; in which epidermal ceramidase contributes to endogenous skin pH by generating free fatty acids from ceramides.
The acidic pH on the skin surface is assumed to inhibit the growth of pathogenic microorganisms and keep the skin microflora in balance (reviewed by Fluhr et al.\textsuperscript{74}). If the skin surface pH is elevated, e.g., after usage of alkaline soaps, prolonged occlusion, or in skin disorders like atopic dermatitis, the growth of pathogens increases\textsuperscript{85-88}. However, recent studies reveal another important role of skin pH. The pH gradient through the epidermis seems to be essential for several epidermal enzymes involved in the formation and function of the skin barrier, as their activity is pH-dependent, e.g., β-glucocerebrosidase has an optimum activity at pH 5.6, phospholipase A\textsubscript{2} at pH 7–8, acid sphingomyelinase at pH 4.5, and cholesterol sulfatase at pH 8 (reviewed by Redoules et al.\textsuperscript{89}). Kallikrein 5 and 7 has been shown to exhibit maximum activity at pH 8, but have a considerable activity also at pH 5.5.\textsuperscript{37} The importance of pH for activity of the epidermal enzymes, and therefore for the skin barrier, was shown in a recent study on mice. Perturbed skin barrier recovered normally when the skin was exposed to solutions buffered to an acidic pH, while initiation of the recovery was delayed when the damaged skin was exposed to neutral or alkaline pH. This delay in barrier recovery was suggested to be a consequence of a lower activity of β-glucocerebrosidases.\textsuperscript{90}

**MOISTURIZERS AND THE SKIN BARRIER**

The primary function of moisturizers is to smoothen the skin surface and to increase water content in the stratum corneum, i.e., to moisture the skin. After application, water and other volatile ingredients gradually evaporate; leaving a deposit of remaining ingredients, which may stay on the skin surface or penetrate into the epidermis and be removed from the skin surface by washing, friction and evaporation.

The increase in water content in the epidermis is achieved by water-binding properties of humectants, e.g., glycerin, and by formation of a semi-occlusive layer on the skin surface, which hampers water evaporation and increases water content in the upper epidermis.\textsuperscript{91} Moreover, an immediate increase in hydration of stratum corneum may be caused by an uptake of water from the applied product.\textsuperscript{91} The increase in water content and the simultaneous filling of the fractures on the skin surface, makes the skin more elastic, and visibly and tactilely smoother, as well as decreases itch and brings relief (reviewed by Lodén\textsuperscript{92,93})

If moisturizers are used repeatedly, as for example in the case of patients with various dry skin disorders, who require treatment over a long period, or even over a lifetime, it may be
speculated what consequences this may have for the skin and its barrier function. Recurring application of various substances of exogenous origin on the skin, followed by such physicochemical changes as increase in water content in the stratum corneum or change in skin surface pH, may influence epidermis, and therefore, the skin barrier function. Few studies about long-term treatment with moisturizers on normal and diseased human skin have shown both increases and decreases in skin barrier function, as measured by non-invasive techniques. Two, 3 or 4-week treatments of normal or atopic skin with moisturizers containing urea decreased transepidermal water loss (TEWL) and susceptibility to sodium lauryl sulfate (SLS). Treatment with a moisturizer containing another humectant, glycerin, seems to have a less pronounced impact on the skin barrier. On the other hand, in studies by Held et al., a moisturizer containing high lipid content (70%) impaired the barrier of normal skin after 5-day and 4-week treatments, measured as increased skin susceptibility to SLS, although no change in TEWL of undamaged skin was found. The same cream also increased susceptibility to nickel in nickel-allergic volunteers after 7-day treatment. In a study on patients with lamellar ichthyosis, an 8-week treatment with moisturizers containing high amount of lactic acid significantly increased TEWL, as well as dryness and scaling decreased. Moisturizers have also been shown to influence skin barrier recovery after exposure to a skin irritant. These studies demonstrate that prolonged application of moisturizers may have a substantial impact on parameters used for evaluation of the skin barrier. However, the factors responsible for the observed effects are unknown, especially since the studies were performed using moisturizers containing several ingredients.

**CHEMISTRY OF MOISTURIZERS**

Moisturizers are formulated predominantly as oil-in-water (o/w) emulsions, where oil droplets are dispersed in water and stabilized by emulsifiers. Reversed, water-in-oil (w/o) emulsions are used less frequently due to their poor spreadability and the greasier feeling they leave on the skin; however, they can offer other attributes, e.g., water resistance. Emulsions are categorized into creams or lotions, depending on their viscosity. Moisturizers may also be gels containing only hydrophilic material or ointments with only lipophilic ingredients. Other forms of moisturizers exist, but they are much less common, e.g., multiple emulsions, silicone-in-water emulsions, or suspensions. The choice of the form of a moisturizer depends on its desired effect and the ingredients that are supposed to be incorporated.
Moisturizers may either have a simple composition and contain only a few ingredients, or be a complex mixture of many substances. In the case of o/w and w/o emulsions, the simplest possible moisturizer must contain three ingredients, namely, water, a lipid (oil), and an emulsifier. Many ingredients used in moisturizers are the same as those found in the epidermis or on the skin surface: fatty acids, ceramides, vitamins, urea, lactic acid, PCA, etc. Lipids can be of vegetable, animal or mineral origin. Emulsifiers, which stabilize the lipid droplets in an emulsion, can be either low-molecular substances, e.g., PEG-100 stearate, or long-chained polymers of large size, such as acrylates/C10–30 alkyl acrylate crosspolymer. However, it is rare that emulsions contain only three ingredients, and usually they are mixtures of at least 15–20 substances. Those additional ingredients allow achieving desired properties, efficacy, and stability of the product. Moisturizers usually contain humectants, such as polyols: glycerin, propylene glycol, butylene glycol, sorbitol; alpha-hydroxy acids (AHAs) and their salts e.g., sodium lactate; low-molecular substances: urea, betaine, amino acids; and high-molecular polymers with water-binding capacity: sodium hyaluronate. To increase stability of the emulsion and to adjust its rheology to either cream or lotion form, viscosity-increasing agents must be added into the formulation, such as polymers: carbomer, acrylates copolymer, xanthan gum; and high-melting waxes: glyceryl stearate, cetearyl alcohol. Sensory properties may be modified with silicones, such as dimethicone and cyclohexasiloxane. Additionally, moisturizers may contain several other ingredients, such as antioxidants, vitamins, herbal extracts, salts, and UV filters. Depending on the composition of moisturizers, their pH is adjusted to between slightly acidic and slightly alkaline and usually ranges from 4 to 7, but in case of formulations containing stearic acid and zinc oxide, pH is slightly alkaline.

Since the majority of moisturizers contain several ingredients, identification of the parameters responsible for their effects on the skin barrier is difficult. Consequently, factors such as the concentration and type of lipids, humectants, and other ingredients, as well as pH adjustment, should be taken into account.

**Methods used for evaluation of the skin barrier function**

Methods used for evaluation of the skin and its barrier function are numerous. Skin condition may be assessed visually, e.g., for dryness, scaling and redness. There are several instruments available for non-invasive assessments of the skin, where no skin sampling is necessary, as they measure the functional changes on the skin surface or within a defined skin depth, e.g.,
TEWL, skin capacitance, skin impedance, blood flow, pH, surface topography, and elasticity. Such equipment is often portable and easy to use, and consequently, non-invasive measurements are a common tool in dermatological research. Today, assessment of TEWL is the most common method for evaluation of the skin barrier function. TEWL is increased when the skin barrier is impaired, e.g., in dry skin disorders or after damage with an irritant, but also when the skin is hydrated.

However, in order to investigate processes in the skin in greater detail, at the molecular and cellular level, skin samples are required. They may be punch or shave biopsies or samples obtained by tape stripping. Studies utilizing such invasive methods are more complicated to perform, require more resources and assessment from ethical perspective. Although they may be common in basic dermatological research, they have rarely been used in research about moisturizers and their effect on the skin barrier. It is also possible to perform studies in vivo on mice or in vitro on keratinocyte cultures or skin equivalents. However, results obtained from experiments performed using animal models or in vitro systems do not always correlate to the in vivo situation in humans. Analyses of human skin biopsies may give a lot of information about changes in epidermal structure, and gene and protein expression, as well as allowing for staining with antibodies against various proteins.

**AIM OF THE RESEARCH**

The objective of the present work was to increase the understanding of the mechanism by which long-term treatment with moisturizers influences the skin and its barrier function. The impact of formulation variables such as pH, lipid type and humectants was assessed in vivo in healthy human volunteers. Functional changes in the skin were explored using non-invasive techniques. Moreover, the impact of moisturizers on the skin barrier function was assessed also at molecular and cellular level, investigating gene and protein expression as well as histology of epidermis.
MATERIALS AND METHODS

VOLUNTEERS

All studies were performed on healthy adult human volunteers. Exclusion criteria were skin diseases, pregnancy, and allergy to ingredients used in the test preparations. Informed consent and health declarations were obtained from volunteers before commencement of the studies. The studies were approved by the Regional Ethical Review Board at Uppsala University, Uppsala, Sweden. The number and age of the volunteers participating in each study are given in Table 2.

Table 2 – Number and age of volunteers participating in each study.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of volunteers</th>
<th>Women</th>
<th>Men</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paper I</td>
<td>18</td>
<td>15</td>
<td>3</td>
<td>21–54</td>
</tr>
<tr>
<td>Paper II Treatment</td>
<td>78</td>
<td>58</td>
<td>20</td>
<td>25–60</td>
</tr>
<tr>
<td>Paper II Irritancy</td>
<td>11</td>
<td>8</td>
<td>3</td>
<td>27–46</td>
</tr>
<tr>
<td>Papers III and IV</td>
<td>20</td>
<td>15</td>
<td>5</td>
<td>23–59</td>
</tr>
</tbody>
</table>

TEST MOISTURIZERS

Altogether, five test preparations were used in the investigations: one ordinary cream, hereafter called “Complex cream”; three simplified creams emulsified with a long-chained polymer, with the hydrophobic lipid phase consisting of either the hydrocarbons isohexadecane and paraffin (“Hydrocarbon cream”) or a vegetable triglyceride oil, canola oil (“Canola cream” and “Canola/urea cream”); and one lipid-free gel consisting of the polymer used in simplified creams (“Polymer gel”). All test moisturizers, except for the Polymer gel, were oil-in-water (o/w) emulsions. Table 3 shows the detailed compositions of the test moisturizers, their pH, the number of volunteers testing each preparation, and the papers in which the results are presented.
Table 3 – Composition of the test moisturizers, their ingredients and pH, and the papers in which the results are presented.

<table>
<thead>
<tr>
<th>Test preparation</th>
<th>Lipids</th>
<th>Emulsifiers</th>
<th>Other ingredients</th>
<th>Urea</th>
<th>pH</th>
<th>Number of volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex cream</td>
<td>20% capric/caprylic triglyceride, canola oil, cetearyl alcohol, paraffin, glyceryl stearate</td>
<td>1.3% PEG-100 stearate, carboxomer, polysorbate 60</td>
<td>water, propylene glycol, glyceryl polychlorohydrate, dimethicone, sodium lactate, methylparaben, propylparaben, lactic acid, citric acid</td>
<td>5%</td>
<td>4.0</td>
<td>18</td>
</tr>
<tr>
<td>Hydrocarbon cream</td>
<td>40% isohexadecaneb (20%), paraffin e (20%)</td>
<td>0.4% acrylates/C10–30 alkyl acrylate crosspolymerd</td>
<td>water</td>
<td>0%</td>
<td>5</td>
<td>16 10</td>
</tr>
<tr>
<td>Canola cream</td>
<td>40% canola oilc</td>
<td>0.4% acrylates/C10–30 alkyl acrylate crosspolymerd</td>
<td>water</td>
<td>0%</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Canola/urea cream</td>
<td>40% canola oilc</td>
<td>0.4% acrylates/C10–30 alkyl acrylate crosspolymerd</td>
<td>water</td>
<td>5%</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Polymer gel</td>
<td>0%</td>
<td>0.4% acrylates/C10–30 alkyl acrylate crosspolymerd</td>
<td>water, methylparabenf</td>
<td>0%</td>
<td>5</td>
<td>16</td>
</tr>
</tbody>
</table>

aCanoderm® kräm 5%, ACO HUD NORDIC AB, Stockholm, Sweden; bArfamol HD, Uniqema, Gouda, The Netherlands; cMerkur White Oil Pharma, Merkur Vaseline, Hamburg, Germany; dPemulen TR-2, Noveon Inc., Cleveland, OH, USA; eAkorex L, Karlshamns AB, Karlshamn, Sweden; fNipagin M, Clariant International, Pontypridd, United Kingdom.
**EXPERIMENTAL DESIGN**

The studies were double-blinded and randomized. Test moisturizers were distributed to volunteers in white coded tubes. Volunteers were allowed to wash normally, but not to use any skin care products on the test areas (forearms and gluteal skin) at least three days before, and during, the test period.

**Paper I**

Volunteers treated each volar forearm with one of two test preparations, twice daily for 8 days. The test preparations were identical creams, except for pH, which was adjusted to either 4.0 or 7.5 (Table 3; Complex cream). Before the first application of the creams, one area of each forearm was exposed to the skin irritant SLS for 24 hours, which induced irritancy. Assessments of TEWL, blood flow, and skin capacitance, as well as visual scoring of irritancy, were performed repeatedly during the subsequent days. On day 8, the examined areas were exposed once again to SLS for 7 hours and evaluated finally on day 9.

**Paper II**

This study consisted of two parts: a long-term treatment study with the test preparations, and a test of their irritancy potential. In the long-term treatment study, volunteers treated one volar forearm twice daily for 7 weeks with one test preparation (Table 3), leaving the other forearm to serve as the untreated control. After 7 weeks, on day -1, both volar forearms, treated and control, were exposed to SLS for 24 hours. TEWL and blood flow were assessed on SLS-exposed and undamaged skin on each forearm on day 1. Skin capacitance was also measured on undamaged skin. Moreover, test preparations were assessed for their acute irritancy potential using a 24-hour patch test, evaluated by visual scoring and TEWL measurements.

**Papers III and IV**

The volunteers applied one of the test preparations (Table 3; Complex cream or Hydrocarbon cream) on one volar forearm and one buttock twice daily for 7 weeks, leaving the other forearm and buttock untreated to serve as control sites. The side of treatment was randomized. After 7 weeks, one shave and one punch biopsy were taken from each buttock, preceded by TEWL measurements of the biopsy area. The shave biopsies were used for gene expression analysis and the punch biopsies for histological and other molecular evaluations. Moreover, the skin of the forearms was patch-tested with SLS for 24 hours. Non-invasive evaluations were performed on undamaged and SLS-exposed skin.
EVALUATIONS

A list of evaluation techniques used in all presented studies is given in Table 4. The techniques are described in detail in the “Materials and methods” section of each paper.

<table>
<thead>
<tr>
<th>Analyses performed</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-invasive in vivo evaluations of the skin</td>
<td>TEWL</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Blood flow</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Skin capacitance</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Visual scoring</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular analyses of skin biopsies</td>
<td>RNA isolation and cDNA synthesis</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological evaluations</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid staining</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TEWL = transepidermal water loss; RNA = ribonucleic acid; cDNA = complementary deoxyribonucleic acid; QRT-PCR = quantitative real-time polymerase chain reaction

CALCULATIONS AND STATISTICS

The results are expressed either as percentage ratio to the corresponding values obtained from control (untreated) skin areas, which are given as 100%, or as absolute values. They are presented in graphs as box plots with the median value as a line across the box and the first quartile value at the bottom and the third at the top. The whiskers are lines that extend from the top and bottom of the box to the lowest and the highest observation within a defined region, with outliers plotted as asterisks outside this region. For results presented in a table or in the text, the median value is given, followed by the lower (Q1) and upper (Q3) quartiles in brackets.

To analyze differences between results of non-invasive measurements and molecular analyses, a Wilcoxon signed rank test on paired data was used. Possible linear relationships between two variables were analyzed using the Pearson product moment correlation
coefficient. A Kruskal-Wallis test of equality of medians was used to investigate the differences between various moisturizers.

Minitab® statistical software (Minitab Inc., State College, PA, USA), was used for calculations and plots. The level of significance was set at p < 0.05.
RESULTS

PAPER I

Treatment of surfactant-damaged skin in humans with creams of different pH values

After the initial exposure to SLS, there was no difference in the skin barrier recovery between skin treated with the pH 4.0 cream and skin treated with the pH 7.5 cream, evaluated as TEWL, blood flow, and skin capacitance, and by visual scoring. After 8 days of cream application, there was no difference between treatment groups in susceptibility to SLS at the second SLS exposure.

PAPER II

Changes in skin barrier function following long-term treatment with moisturizers, a randomized controlled trial

Treatment of normal skin for 7 weeks with Hydrocarbon cream, Canola cream, Canola/urea cream, and Polymer gel increased TEWL in comparison to the untreated areas. The opposite effect was found for Complex cream, where TEWL was reduced. Skin capacitance decreased following treatment with Hydrocarbon cream, but no difference was found for the other test preparations. Treatment with all test moisturizers did not change blood flow in comparison to control areas.

After SLS-exposure, the TEWL of skin treated with Hydrocarbon cream, Canola cream, Canola/urea cream, and Polymer gel was increased in comparison with the corresponding SLS-exposed skin on the untreated forearm, while after treatment with Complex cream, it was reduced. However, only Canola/urea cream resulted in a higher relative increase in TEWL compared with the untreated control skin after SLS exposure.

There were no differences between the simplified creams, Hydrocarbon cream, Canola cream, and Canola/urea cream, in their effects on TEWL of undamaged skin, TEWL of SLS-exposed skin, or blood flow of SLS-exposed skin. However, there was a difference in impact on skin capacitance.

The irritancy patch test showed that the five test preparations were not more irritant than water.

A summary of the effects of the test preparations on the skin barrier is presented in Table 5.
Table 5 – Summary of the effects of test moisturizers on the skin barrier after 7 weeks of exposure.

<table>
<thead>
<tr>
<th>Moisturizer</th>
<th>Undamaged skin</th>
<th>SLS-exposed skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEWL</td>
<td>Capacitance</td>
</tr>
<tr>
<td>Complex cream</td>
<td>↓</td>
<td>–</td>
</tr>
<tr>
<td>Hydrocarbon cream</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Canola cream</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Canola/urea cream</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Polymer gel</td>
<td>↑</td>
<td>–</td>
</tr>
</tbody>
</table>

↑ = increased in comparison with untreated skin; ↓ = decreased; – = no difference; SLS = sodium lauryl sulfate; TEWL = transepidermal water loss

**PAPER III**

**Long-term treatment with moisturizers affects the mRNA levels of genes involved in keratinocyte differentiation and desquamation**

After 7 weeks of twice-daily treatment of buttock and forearm skin, TEWL was increased on the Hydrocarbon cream-treated sites, but decreased on the Complex cream-treated sites, as compared with untreated skin. TEWL measured on untreated areas of gluteal skin was significantly higher than on untreated forearms: 10.8 (8.7–14.3) vs 7.3 (6.5–12.3) gm⁻²h⁻¹, respectively (p<0.001). None of the creams induced changes in superficial skin blood flow or influenced the messenger ribonucleic acid (mRNA) expression of interleukin-1α (IL1A), indicating absence of inflammation.

After SLS-exposure of forearms, a higher degree of irritation was found in skin treated with Hydrocarbon cream compared with its corresponding untreated control skin site, while skin treated with Complex cream showed less irritation than control skin. Skin capacitance decreased after exposure to Hydrocarbon cream, while no difference was found for Complex cream.

Histological analysis of sectioned skin biopsies showed no differences in the thickness of epidermis or stratum corneum after treatment with either of the creams compared with controls. In addition, the corneocyte size was not significantly influenced by any of the treatments.
Treatment with Complex cream decreased the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A), while the remaining genes were unaffected. Hydrocarbon cream significantly increased the gene expression of involucrin (IVL), transglutaminase 1 (TGM1), kallikrein 7 (KLK7), and kallikrein 5 (KLK5) compared with untreated controls, while no changes were found in the levels of profilaggrin (FLG) and cyclin-dependent kinase inhibitor 1A (CDKN1A). In an attempt to identify genes important for the normal barrier function we examined possible correlations between TEWL and mRNA expressions, thickness of the stratum corneum, and size of corneocytes of untreated buttock skin. However, no linear correlation was found between TEWL and gene expressions of analyzed genes, thickness of stratum corneum, and size of corneocytes. A summary of gene expressions analyses presented in Papers III and IV is given in Table 6.

The immunofluorescence staining did not reveal significant changes at the protein level of involucrin, transglutaminase 1, and filaggrin (Figure 3). An example of photographs used for semi-quantitative analyses of protein expression is given in Figure 4.

![Graph of protein expression](image)

Figure 3 – Protein expression of involucrin, transglutaminase 1 and filaggrin in skin treated with test moisturizers (n=10), assessed using the semi-quantitative method. For an explanation of the boxplots, see the “Calculations and statistics” section. The untreated control site is given as 100%. P-values relate to differences between treated and control areas.
Figure 4— Examples of photographs used for semi-quantitative analyses of protein expression of transglutaminase 1: untreated skin of a volunteer using Complex cream (a); treated skin of a volunteer using Complex cream (b); untreated skin of a volunteer using Hydrocarbon cream (c); and, treated skin of a volunteer using Hydrocarbon cream (d). The dashed line represents the dermal–epidermal border. Bar = 50 µm.

**PAPER IV**

*Moisturizers change the mRNA expression of enzymes synthesizing skin barrier lipids*

After a 7-week exposure of the skin to Hydrocarbon cream, an increased mRNA expression of the ceramide synthesizing enzymes β-glucocerebrosidase (GBA), serine palmitoyltransferase 2, (SPTLC2) and acid sphingomyelinase (SMPD1), but not of UDP-glucose ceramide glucosyltransferase (UGCG) was observed. Identical treatment with Complex cream did not affect the expression of any of these enzymes. Regarding the two analyzed enzymes involved in cholesterol synthesis, treatment with Hydrocarbon cream increased the expression of HMG-CoA synthase 1 (HMGCS1), but not HMG-CoA reductase (HMGCR), while Complex cream had no effect on either. None of test moisturizers significantly influenced the mRNA expression of enzymes involved in free fatty acid metabolism: acetyl-CoA carboxylase beta (ACACB), fatty acid synthase (FASN) and acyl-CoA synthetase long-chain family member 1.
(ACSL1). Treatment with Complex cream increased the mRNA expression of one nuclear receptor, PPAR-γ (PPARG), while exposure to Hydrocarbon cream decreased it. There was no effect of any of the treatments on the expression of PPAR-α (PPARA), PPAR-β (PPARB) and RXR-α (RXRA). Moreover, treatment with Hydrocarbon cream increased expression of both analyzed lipoxygenases arachidonate 12-lipoxygenase 12R type (ALOX12B) and epidermal arachidonate lipoxygenase 3 (ALOXE3), while Complex cream did not (a summary of gene expression analyzed in Papers III and IV, is presented in Table 6).

In biopsies from the untreated skin, we examined whether there was a correlation between the mRNA expression of any of the analyzed genes and TEWL (TEWL results were reported in Paper III). The mRNA expression of two of the 15 examined genes, PPARG and ACACB, exhibited an inversely linear correlation to TEWL, and high expression of these genes was associated with low TEWL.

The amount and organization of non-polar lipids, examined by Nile Red in situ staining, revealed no changes induced by either of the two treatments, in comparison with control areas.

Since treatment with the two test moisturizers altered mRNA expression of PPARG in opposite directions, the protein expression of PPAR-γ was examined in the biopsies. Two patterns of nuclear staining were observed: one was the staining of the entire viable epidermis and the other was more restricted to the rete ridges. These patterns were equally distributed between the volunteers, irrespective of the type of moisturizer used. In most cases, every volunteer exhibited the same pattern, both in treated and in control sites, and when performing semi-quantitative analysis of the staining intensity, the cream-exposed areas were no different from control areas. An example of photographs used for semi-quantitative analysis of PPAR-γ is presented in Figure 5.
Figure 5 – Examples of photographs used for semi-quantitative analyses of protein expression of PPAR-γ: untreated skin of a volunteer using Complex cream (a); treated skin of a volunteer using Complex cream (b); untreated skin of a volunteer using Hydrocarbon cream (c); and, treated skin of a volunteer using Hydrocarbon cream (d). Bar = 50 μm.
Table 6 - Summary of the gene expression analysis presented in Papers III and IV.

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene</th>
<th>Complex cream</th>
<th>Hydrocarbon cream</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins involved in keratinocyte differentiation</td>
<td>IVL</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
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<td>CDKN1A</td>
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<td>Enzymes involved in the process of desquamation</td>
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<td>KLK7</td>
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<td>Enzymes involved in ceramide synthesis</td>
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<td>Enzymes involved in cholesterol synthesis</td>
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<td>HMGCR</td>
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<td>Enzymes involved in fatty acid metabolism</td>
<td>ACACB</td>
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<td>Nuclear hormone receptors</td>
<td>PPARA</td>
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<td>Lipoxigenases</td>
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<td>ALOXE3</td>
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<td>Interleukins</td>
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↑ = increased messenger ribonucleic acid (mRNA) expression in comparison with untreated skin; ↓ = decreased mRNA expression; – = no difference; ACACB = acetyl-CoA carboxylase beta; ACSL1 = acyl-CoA synthetase long-chain family member 1; ALOX12B = arachidonate 12-lipoxygenase, 12R type; ALOXE3 = epidermal arachidonate lipoxygenase 3; CDKN1A = cyclin-dependent kinase inhibitor 1A; FASN = fatty acid synthase; FLG = profilaggrin; GBA = β-glucocerebrosidase; HMGCR = HMG-CoA reductase; HMGCS1 = HMG-CoA synthase 1; IL1A = interleukin-1α; IVL = involucrin; KLK5 = kallikrein 5; KLK7 = kallikrein 7; PPARA = PPAR-α; PPARB = PPAR-β; PPARG = PPAR-γ; RXRA = RXR-α; SMPD1 = acid sphingomyelinase; SPTLC2 = serine palmitoyltransferase 2; TGM1 = transglutaminase 1; UGCG = UDP-glucose ceramide glucosyltransferase.
DISCUSSION AND CONCLUSIONS

Moisturizers are often used as supplements to topical and/or systemic anti-inflammatory drugs in various types of skin conditions and disorders, such as contact dermatitis, atopic dermatitis, psoriasis, and ichthyosis, in order to bring relief and break a dry skin cycle (reviewed by Lodén93,105 and Proksch et al.106). Such skin conditions usually require long-lasting treatment with moisturizers, and in the case of atopic dermatitis, their use is recommended even when the eczema is cleared.107 Vehicles of many topical drugs are moisturizers as well. Use of moisturizer is also widespread among people that self-perceive their skin as dry or rough, e.g., in elderly, due to a dry climate or frequent contact with cleaning agents, and they use moisturizers to obtain relief and for smoothening of the skin. Moreover, skin protection creams (also called “barrier creams”) are widespread at various workplaces to minimize the percutaneous penetration of chemicals. Use of moisturizers is therefore common and is practiced by a significant percentage of the population. Although the importance of using moisturizers often is overlooked, and they are not perceived as an “active” treatment, they have been shown to influence skin properties and the barrier function in both healthy and diseased skin.94-98,100-103,108

Studies on the impact of moisturizers on the skin barrier have mostly focused on short-term effects, showing that moisturizers are able to increase skin hydration, decrease roughness and scaling, and improve the condition of dry skin (reviewed by Lodén et al.92,93,105,109). However, little is known about the effects of their long-term use, lasting weeks, months, or even years, which better reflects the real-life situation. The few studies that have looked into such effects, have shown that the moisturizers studied influenced the skin barrier function and recovery, as measured by non-invasive techniques, such as TEWL, skin capacitance and susceptibility to an irritant, e.g., SLS and nickel salts.94-98,100-103,108 Therefore, the aim of present research was to gain further understanding of the mechanism by which long-term treatment with moisturizers influences the skin in vivo in healthy human volunteers, using not only non-invasive techniques, but also other tools, such as quantitative real-time polymerase chain reaction (QRT-PCR), immunofluorescence, immunohistochemistry, and histological evaluations, allowing investigating the epidermis at molecular and cellular level.

Few available studies demonstrate that moisturizers may have an impact on epidermis at the molecular level, when used on normal skin. Short et al.110 showed that a moisturizer containing high amounts of glycerin and silicones increased maximum epidermal thickness,
decreased epidermal melanin content, and altered protein expressions of keratins 6, 10, and 16, as assessed with immunohistochemistry after a 4-week treatment. Moreover, all these changes were accompanied by decreased TEWL. Another study revealed that a 6-week treatment with a moisturizer containing glycerin and erythritol increased the number of keratinocytes with a well-matured cornified envelope; also, the interleukin-1 receptor agonist/interleukin-1α (IL-1ra/IL-1α) ratio in the epidermis was altered. However, it may well be individual ingredients that affect the skin at a molecular level, i.e., not the moisturizer as a whole. Linoleic acid, found in certain vegetable oils commonly used in topical preparations, is a ligand of peroxisome proliferator activated receptors (PPAR) which has been found to have effects comparable to a potent topical glucocorticoid in animal models. Another substance, nicotinamide, was shown to increase levels of ceramides and free fatty acids and to decrease TEWL, as compared with placebo-treated skin.

**CHOICE OF TEST MOISTURIZERS**

Three test moisturizers used in the present studies were formulated in such way as to minimize their complexity, in order to diminish the number of possible confounding factors. Therefore, they contained only few ingredients, in contrast to the majority of commercially available topical preparations that contain over a dozen substances. In addition, one moisturizer, Complex cream, containing several ingredients and previously shown to influence the skin barrier in normal and atopic skin, was chosen as a reference for this investigation. The following factors were examined: the impact of a humectant (urea); difference between creams of minimum complexity (Hydrocarbon, Canola and Canola/urea creams) and a cream containing more ingredients (Complex cream); and different types of lipids, which were either pure hydrophobic hydrocarbons derived from mineral oil (paraffin and isohexadecane) or a more polar vegetable oil consisting of triglycerides and sterols (canola oil). The simple creams were stabilized with a polymeric emulsifier, which was expected not penetrate and influence skin barrier function due to its large molecular size. A gel, consisting of water and this polymer, was also investigated (Polymer gel). By contrast, Complex cream contained a mixture of lipids of various origins and was emulsified with a combination of polymers and low-molecular emulsifiers. To evaluate the effect of pH on the skin barrier, Complex cream was adjusted to pH 4.0 or 7.5, as certain epidermal enzymes have optimum activity in either acidic or alkaline pH. To investigate the effect of long-term
use of moisturizers, treatment time was chosen to be 7 weeks, as the average turnover time of epidermis is reported to be 40–45 days.\textsuperscript{114,115}

**Effect of 7-Week Use of Test Preparations on the Skin Barrier**

Twice-daily treatment of normal skin for 7 weeks with all test preparations examined in this research, induced changes in the skin barrier function, as evaluated with non-invasive methods. Hydrocarbon cream, Canola cream and Canola/urea cream appear to have a negative effect on the skin barrier, as their application resulted in elevated TEWL and increased skin susceptibility to SLS. Polymer gel, consisting of polymer and water, had similar effects. Moreover, Hydrocarbon cream decreased skin capacitance. By contrast, treatment with Complex cream decreased TEWL and susceptibility to SLS. Molecular analyses of skin biopsies after use of Hydrocarbon cream and Complex cream on normal skin for 7 weeks revealed that these two creams induced different effects on the mRNA expression of genes involved in the keratinocyte differentiation, corneocyte formation and desquamation as well as lipid metabolism. Treatment with Hydrocarbon cream changed the expression of 11 out of 22 analyzed genes, while exposure to Complex cream affected expression of only two of them. At the same time, the moisturizers had no effect on protein expression of four analyzed proteins. Stratum corneum thickness, epidermal thickness, the size of corneocytes, and non-polar lipid staining, were also unaffected by the treatments.

The studies therefore revealed that moisturizers had different effects on the barrier function of normal skin and that these changes were dependent on the composition of the moisturizer. The test preparations had an impact on the normal function and/or structure of the skin. The observed changes may be caused either by the ingredients of the test preparations (e.g., lipids, humectants, emulsifiers, or water), which have a direct or indirect effect on skin barrier components, or by the physical effects of moisturizers on the skin, such as occlusion. The impact of pH of moisturizers was also investigated, and it was assessed to have no significance for their effects on the skin barrier.

**Possible Explanations of Observed Effects**

Despite the apparent relationship between gene expression and the skin barrier function, it was not possible to ascertain whether the observed functional changes, such as
increased/decreased TEWL, susceptibility to SLS or skin capacitance, were the effect of molecular changes in the epidermis, or vice versa (“hen-and-egg” situation). The first possibility is that functional changes in the skin barrier, induced by moisturizers, could trigger epidermal keratinocytes to alter their gene expression. An example of such functional change may be the delivery of exogenous lipids from the moisturizers into the intercellular lipids of the stratum corneum, resulting in altered barrier function, or the impact of emulsifiers, humectants or exposure to water.

**Lipids**

Lipids in moisturizers may remain on the skin surface or enter the skin, and more physiological lipids may penetrate into the epidermis and affect skin barrier structure and recovery. Changes in lateral packing of stratum corneum lipids were observed in patients with atopic dermatitis and psoriasis after 3-week treatment with a moisturizer containing 10% petrolatum. Therefore, in our study, the possible penetration of lipids from test moisturizers could alter skin barrier properties.

The three simplified creams investigated in our study, all of which had a negative effect on the skin barrier, contained high proportions of lipids, 40%, but the type of lipid (hydrocarbons or triglycerides) was of no importance for the effect. At the same time, Complex cream, containing 20% lipids, did not deteriorate the skin barrier. Similar effects as for simplified creams have been obtained also with a moisturizer containing 70% lipids, which made the skin more susceptible to SLS. This suggests that differences in the lipid content or uptake rate may be important for the effect of moisturizers on the skin barrier function. As exogenous lipids may change the highly organized structure of intercellular lipid layers, TEWL or the ion flux may also be altered. Such changes may be recognized as barrier impairment by epidermal keratinocytes, initiating a repair process including altered gene expression.

**Water**

Penetration of lipids, however, cannot explain the impairment of the skin barrier obtained after treatment with Polymer gel, as it contains no lipids but much more water compared with other moisturizers, 99%. After application of a moisturizer, water, which is one of the main ingredients, evaporates within a short time. The effect on the skin barrier of twice-daily exposure to water for a few weeks is unknown. It has been shown that prolonged contact with water disrupts the intercellular lipid lamellar structure in the stratum corneum, and may
contribute to dryness and increased TEWL.\textsuperscript{122} Moreover, changes in gene expression of epidermal enzymes and non-enzymatic proteins were found after exposure of skin only to water under occlusion.\textsuperscript{123} It has also been suggested that higher TEWL of the dorsal surface of hands, compared with the forearm and back, may be due to more frequent contact with water.\textsuperscript{124} Increased TEWL and dryness of the skin could also be caused by contact with irritants, but the test preparations, including Polymer gel, were shown not to be irritant, by an acute irritancy test and by blood flow measurements. Moreover, treatment with Hydrocarbon cream and Complex cream did not alter expression of IL1A, indicating lack of inflammation. Furthermore, though all simple creams and Complex cream contain similar amounts of water, around 60\%, they induced different changes in the skin barrier.

\textit{Emulsifiers and polymers}

Emulsifiers are essential in moisturizers, as they stabilize the emulsion. Emulsifiers commonly used in o/w emulsions have been shown to influence the skin barrier function in normal and SLS-exposed skin.\textsuperscript{125} Although the emulsifier used in the simple creams and Polymer gel, acrylates/C10–30 alkyl acrylate crosspolymer, was expected not to penetrate into the epidermis due to its large molecular size, its negative effect could have been caused by monomers, which may be present at low concentrations. However, the Complex cream also contained a polymeric emulsifier, carbomer, which is similar in structure to acrylates/C10–30 alkyl acrylate crosspolymer, lacking a negative impact on the skin barrier. Interestingly, it has been suggested that polymers themselves may have an effect on the skin barrier, as tested on mice, accelerating or delaying the barrier recovery.\textsuperscript{126} Although this phenomenon is not fully understood, it is possible that polymers together with their counter-ions form an electric double layer on the skin surface, influencing the skin barrier.\textsuperscript{126}

\textit{Humectants}

Humectants that are added to moisturizers, such as urea, glycerin, and propylene glycol, may penetrate into the skin.\textsuperscript{127,128} It has been suggested that the decreased TEWL and lower response to SLS after treatment with Complex cream was due to its urea content.\textsuperscript{96,97,108} Moreover, it could be expected that the addition of urea to a moisturizer would be beneficial, as it has been reported that urea replaces water in the skin, leaving the physical properties of the stratum corneum intact.\textsuperscript{129} However, not only did the addition of urea to Canola/urea cream not improve the skin barrier, but it also made the skin more susceptible to SLS in comparison to Canola cream without urea. Urea may have favoured the absorption of
potentially damaging ingredients in this formulation, or allowed for increased penetration of SLS during the 24-hour patch test exposure. It might be that in Canola/urea cream, penetration of urea was different than in case of Complex cream, due to another emulsion composition.

Urea has been reported to act as a keratolytic agent\textsuperscript{130} however, absence of a measurable thinning of the stratum corneum by the 5% urea in Complex cream in the present investigation, does not support keratolytic properties at a concentration commonly found in moisturizing creams.

Although treatment with Complex cream containing 5% urea significantly decreased TEWL and susceptibility to SLS, it had only minor effects on the mRNA expression of the analyzed genes. Therefore, the effect of urea on the barrier function may depend on the whole composition of the moisturizers, including the lipid content. Urea has also been found to diminish epidermal proliferation in psoriasis, measured as a decreased expression of involucrin and an increased expression of cytokeratins\textsuperscript{131} However, no difference in expression of proteins or the genes involved in keratinocyte differentiation and desquamation, apart from CDKN1A, was detected in the present study. Decreased expression of CDKN1A suggests influences in cell cycle progression after treatment with the urea-containing Complex cream, which could be interpreted as decrease in cell differentiation\textsuperscript{34,132} though not detectable by histological evaluations, since the thickness of the epidermis and the stratum corneum remained unchanged, as did corneocyte size.

\textbf{Occlusion}

As an alternative explanation, gene expression may be influenced by altered activity of various signaling pathways, resulting in a changed skin barrier function. However, it is still not clear what type of signal triggers changes in gene expression: the changes could be due to ingredients included in the moisturizers, or could have been indirectly induced by, e.g., changes in ion or water flux. One possible signal may be a reduction in TEWL due to occlusion by the topically applied moisturizer, hypothetically resulting in water flux changes.

We have previously shown that occlusion of the skin with a semi-permeable membrane, mimicking the occlusion effect by a moisturizer, decreased TEWL and susceptibility to an irritant, in a group wearing the membrane 23 hours a day for 3 weeks, suggesting changes in skin barrier function\textsuperscript{133} However, there is no evidence of a correlation between occlusive properties of creams and their effects on the skin barrier, determined as degree of irritation
after SLS-exposure, since Complex cream and a lipid-rich cream show similar occlusive capacity, but opposite effects on the skin barrier function. Therefore, the detected difference in the effect on the skin barrier is more likely to have causes other than difference in occlusion between the creams. It is still not known whether a prolonged occlusion per se influences mRNA expression, since no investigation of this aspect has yet been performed. It is also worth noting that the semi-occlusive layer formed by a moisturizer is usually removed from the skin within a few hours.

\textbf{pH}

The impact of the pH of topical preparations on the skin barrier is still not fully understood. The results presented in Paper I show that the pH of moisturizers seems not to be of major importance for their effects on the skin barrier. We found no difference in the impact on skin barrier recovery or susceptibility to an irritant between moisturizers of pH 4 and 7.5. The lack of difference in effect on skin barrier recovery of the moisturizers of acidic or alkaline pH values in our study disagrees with a previous study in mice, where barrier recovery was delayed after exposure to slightly alkaline pH. However, the endogenous mechanisms involved in the formation of the pH gradient in stratum corneum, as well as continuous exogenous excretion of sweat and sebum and NMF, could be expected to counteract the change in the skin surface pH induced by a topical application. It has been shown that after use of an alkaline soap, initially elevated skin surface pH decreases back towards acidic values, and therefore the same effect can be expected after a moisturizer.

Moisturizers usually contain only small quantities of buffering ingredients, which make them unable to produce persistent changes in the skin surface pH, while the mentioned skin barrier recovery study on mice was performed using strong buffer systems. Moreover, the majority of moisturizers have pH in a range of 4 to 6, which is in the range of the skin surface pH. However, it cannot be excluded that some ingredients of moisturizers may penetrate into the epidermis, and influence the pH gradient there, which would have an effect on the skin barrier function.
MOLECULAR CHANGES INDUCED BY HYDROCARBON AND COMPLEX CREAMS

Regardless of the mechanisms involved, Hydrocarbon cream and Complex cream influenced the skin barrier in a way detectable by non-invasive measurements and molecular analyses of mRNA expression: the latter, however, were not confirmed at the protein level.

Effect of Hydrocarbon cream on the skin barrier

Hydrocarbon cream appears to have a negative effect on the skin barrier, since it elevates the TEWL and makes the skin more susceptible to SLS. In the present study, Hydrocarbon cream increased the mRNA expression of genes responsible for the synthesis of ceramides and cholesterol: GBA, SPTLC2, SMPD1, and HMGCS1, but not the expression of free fatty acid metabolizing enzymes. These results can be interpreted as an epidermal response to barrier damage. Our results support such a hypothesis, since Hydrocarbon cream increased also mRNA expression of IVL and TGM1, both being key proteins in formation of cornified envelope. Other studies of skin barrier damage induced in mice by acetone, surfactant, tape stripping, or an essential fatty acid-deficient diet, also describe increased mRNA expressions or activities of lipid-processing enzymes.54,55,134,135

However, in a recent study on healthy human volunteers, two creams containing 5% urea, the same amount as in Complex cream, increased mRNA expression of IVL, FLG, loricrin, and TGM1 after a 2-week treatment, which was also accompanied by a significant decrease in TEWL.136 This gene expression profile is more similar to the effect of Hydrocarbon cream, although the effect on TEWL is like that of Complex cream. This suggests that the relationship between mRNA expression and TEWL is not so straightforward, and also that the gene expression profile may change during treatment time.

Nuclear receptors and lipoxygenases

Hydrocarbon cream decreased the mRNA expression of PPARG, as well as influenced mRNA expression of the two lipoxygenases, ALOX12B and ALOXE3. PPAR-γ and other PPARs are involved in regulation of keratinocyte differentiation and expression of several of the lipid-processing enzymes. In skin equivalent models, activation of PPAR-α receptors by synthetic ligands resulted in an increase in the mRNA expression of lipid-metabolizing enzymes, three of which were the same as in our present study (GBA, SPTLC2 and HMGCS1).137 Although Hydrocarbon cream does not contain any known PPAR agonists, the
elevated expressions of ALOX12B and ALOXE3, genes of lipoxygenases that produce derivatives acting as PPAR-α agonists, may suggest an endogenous formation of PPAR-α agonists by the Hydrocarbon cream treatment.

While Hydrocarbon cream decreased the mRNA expression of PPARG, Complex cream increased it. However, no difference in protein expression of PPAR-γ was found after use of any of the test preparations. Nevertheless, the opposite effect on the expression of PPARG and TEWL induced by Hydrocarbon and Complex cream, as well as a linear correlation between these two parameters in untreated skin, suggests an importance of PPAR-γ for the skin barrier function. Usually, PPAR-γ activation affects cell proliferation, cell differentiation, immune responses, and apoptosis in the skin. PPAR-γ signaling is triggered by various types of ligands, including linoleic acid (reviewed by Feingold, Schmuth et al., and Sertznig et al.). Although Complex cream contains a vegetable oil, which may contain a small fraction of free fatty acids, e.g., linoleic acid, our present results do not suggest any activation of PPARs.

A linear correlation between TEWL and mRNA expression in untreated skin was also found for ACACB. Enzyme encoded by this gene is involved in synthesis of fatty acids and its importance for the skin barrier has been shown after barrier disruption in mice, which increased mRNA expression of this enzyme. The regulation of ACACB in keratinocytes is unknown, but in mouse hepatocytes, it has been shown that the PPAR-α agonist WY-16,643 increases the expression of ACACB.

**Enzymes and proteins involved in keratinocyte differentiation and desquamation**

The treatment with Hydrocarbon cream increased the mRNA level of INV and TGM1, but it was not accompanied by a corresponding increase in protein expression. This may indicate that the epidermis was preparing itself for possible repair of the impaired skin barrier, or that the repair phase had already been completed, but there was still ongoing transcription. Another reason for the lack of correlation between mRNA and protein expression after treatment may be that the mRNA had not been translated into protein, or that the protein turnover had increased for unknown reasons. The inhibition of mRNA translation has been shown to be caused by micro RNA. For example, certain genes in the epidermis of psoriasis patients exhibit diminished translation due to the presence of certain micro RNAs. Furthermore, since protein and mRNA expressions were analyzed on a single occasion, after 7
weeks of treatment, it is possible that changes in protein levels occurred at another time, perhaps after a few days, but became “normalized” after a few weeks due to some adaptation mechanism. As already mentioned before, a recent study with two creams containing 5% urea, showed another mRNA expression profile of IVL, FLG and TGM1 than in our study, but analyzed after a 2-week treatment.136

It is also possible that the analytical method used was not sufficiently sensitive to detect differences in protein expression. The mRNA expression increased by about 55% in the case of IVL and 120% in the case of TGM1, which may not be enough to show in detectable differences using an immunofluorescence technique. Other techniques, e.g. western blot, may have been more powerful, but would have necessitated obtaining additional biopsies from the volunteers, which was not possible for ethical reasons. However, another study describes changes in mRNA expression of a magnitude similar to that seen in the present study, resulted in visible increased protein levels after 1–7 days after exposure to SLS.123 It suggests also that the effect of moisturizers at the protein level may also have occurred earlier.

Exposure to Hydrocarbon cream also increased the gene expression of KLK5 and KLK7, which may suggest an excessive desquamation, since these two proteases are involved in this process.36-39 However, since the thickness of the stratum corneum remained unchanged, it could be possible that treatment with Hydrocarbon cream initially induced thickening of stratum corneum, but increased performance of kallikreins counteracted it. However, the altered expression of kallikreins may also be due to other, not well known, functions in the skin barrier, since it has been shown that kallikrein 7 degrade some lipid-processing enzymes.140

The altered expression of kallikreins may also be linked in some way to decreased skin capacitance after use of Hydrocarbon cream, indicating low skin hydration levels,141 since these enzymes have been found to have increased protein expression in and psoriasis41 and atopic dermatitis.42 However, the protein expression of kallikreins was not assessed in the present study. Interestingly, decreased skin capacitance was not accompanied by any change in the mRNA expression of FLG or the protein expression of filaggrin, which degrades into free amino acids and PCA, the main components of NMF (reviewed by Rawlings et al.27,28).
CONCLUSIONS

In the present investigation, moisturizers were examined for their effect on the skin barrier after long-term treatment, with regard to such factors as pH, lipid type, and presence of a humectant, as well as complexity of the product. In conclusion, moisturizers are able to modify the skin barrier function, detected as changes in TEWL, skin capacitance, and susceptibility to an irritant, and also to change the mRNA expression of certain genes involved in the assembly, differentiation and desquamation of the stratum corneum, as well as lipid metabolism. Therefore, moisturizers should not be perceived simply as inert topical preparations, even if they do not contain any ingredients commonly perceived as “active”. However, the mechanisms behind the observed effects are still not fully understood. The observed outcome is most likely the combination of interplay and effects of several factors, rather than only one, which makes it more difficult to draw any firm conclusions. More research of this kind is needed to understand better the mechanism of action of moisturizers. Such research would aid in the treatment of various skin disorders through improved design of moisturizers, which can target specific skin problems and conditions both more efficiently and safely.
**FUTURE PERSPECTIVES**

In the present thesis, we used traditional, non-invasive methods to investigate the skin barrier function, combined with invasive techniques allowing us to study changes in the epidermis after use of moisturizers at the molecular and cellular level. Analysis of gene and protein expressions and histological evaluations lead to new hypotheses and answer more questions than non-invasive methods alone.

Our investigation showed that treatment with moisturizers might change gene expression of a number of epidermal proteins, including structural proteins and enzymes. The study was performed on selected genes known to be important for the skin barrier, and since their number was not very high, QRT-PCR was used for analyses. However, further investigations could include also analyses using cDNA microarrays, which allow for screening several thousands of genes at the same time. This would give a broader perspective over changes in gene expression in epidermis and help to identify additional genes and proteins that should be investigated more in details.

Since our research investigated the effects of moisturizers after 7 weeks of treatment, one may consider exploring also their impact on the skin barrier at earlier time points. The course of gene and protein expression and histological changes may vary between shortly after application to after few-week use. It would also be interesting to continue the investigation for some time after use of a moisturizer is terminated, following the return of the skin barrier to the state from before the treatment.

Although molecular analyses provide plenty of information about the skin barrier, non-invasive evaluations should be utilized as well, as they do not require skin sampling and provide a different type of data, i.e. skin barrier function. The new types of non-invasive methods developed during recent years help us acquire detailed facts about the skin structure and function *in vivo* and may be used in studies investigating the effects of moisturizers. For example, Raman spectroscopy helps to evaluate the moisturizing effect by giving a detailed water profile in the epidermis and assess stratum corneum and epidermal thickness, as well as to follow drug penetration (reviewed by Wartewig et al.). Multiphoton laser tomography and confocal laser scanning microscopy (reviewed by Branzan et al.), allow us to “look” inside the skin and may be exploited in various ways.
The results presented in this thesis as well as other studies demonstrate that the composition of moisturizers plays an essential role in their effects on the skin and its barrier function. Therefore, it would seem reasonable at first to systematically test various ingredients, combinations of ingredients and complete moisturizers for their effects, in order to find those giving the desired efficacy, both in healthy and in diseased skin. However, taking into consideration that over 500 ingredients classified as humectants and about 1,500 emollients are available, the number of possible combinations is endless, even if the number of ingredients were limited to only those used more frequently. Therefore, it is more realistic to continue the research about these few ingredients, which are common in many moisturizers and for which some data is already available, regarding the effect of glycerin, urea, vegetable oils, and hydrocarbons.

As the knowledge about the effect of various ingredients and their combinations on the skin barrier is scarce, testing of complete moisturizers is currently the best way to assure benefits for the consumers. However, although clinical trials are mandatory for pharmaceuticals (over-the-counter or prescription drugs) to prove their efficacy, moisturizers available as cosmetic products are rarely evaluated for their effect on the skin barrier. Therefore, in theory, some commercially available moisturizers may have a negative impact on the skin, and their use could worsen the skin condition, facilitate penetration of irritants, and even lead to eczema. Consequently, the composition of a moisturizer should be an important issue when recommending it to a patient with skin problems, since this choice may have an impact on the skin status and therefore on quality of life. Treatment with corticosteroids and other drugs improves the condition of the skin with eczema, but a relapse may only be a question of time. However, while the use of a moisturizer with the skin barrier-improving effect after corticosteroid therapy may increase an eczema-free period in comparison with no treatment, it is not known if use of a moisturizer with barrier-impairing properties causes an earlier relapse of eczema. This issue should be investigated further, as it may have a major impact on the approach to the treatment of dry skin disorders.

Improving or maintaining the skin status and quality of life may also involve more than choosing a proper moisturizer, since compliance to treatment is also required. If a moisturizer is not attractive to a patient from a cosmetic perspective, e.g., because it is too greasy and tacky, difficult to apply, has an unpleasant odour, or its package is impractical, there is a high probability that the patient will not use it according to guidelines, and as a result will require more medical attention. The same problem may exist in the case of barrier creams, which are
supposed to inhibit penetration of irritant substances. Therefore, the cosmetic properties of moisturizers must be taken into consideration during the early stages of the development, which is a challenge for researchers and formulating chemists, as various ingredients added to make a product more stable and attractive may weaken its performance or cause adverse reactions.

In conclusion, it is important to remember that moisturizers differ in their composition, which determines their impact on the skin barrier. A better understanding of the influence of moisturizers on the skin barrier, obtained by combination of non-invasive methods and molecular analyses, would facilitate designing skin care products adjusted to specific skin problems. It would also provide more efficient management of dry skin disorders, and, hopefully, help to influence the development of moisturizers so that fewer products will have negative effects on the skin.
Mjukgörande krämer (s.k. mjukgörare) används vid behandling av olika hudsjukdomar, men också av personer med frisk hud. Det är inte ovanligt att mjukgörare används kontinuerligt i veckor, månader eller till och med under flera år. Studier har visat att vissa mjukgörare förstärker hudens barriärfunktion medan andra har en negativ påverkan, av orsaker som inte är helt klarlagda. En bättre förståelse kring mekanismer hur långtidsbehandling med mjukgörande krämer påverkar hudbarriären är av stor klinisk betydelse. Krämer som försvarar hudens barriärfunktion kan leda till ökad penetration av allergener eller irriterande ämnen vilket i sin tur kan ge upphov till torr hud och eksem. Mjukgörare som stärker hudbarriären skulle dock kunna motverka många av dessa problem.

I den här avhandlingen har icke-invasiva tekniker kombinerats med analyser av hudbiopsier vilket möjliggjort studier av epidermis på celular och molekylär nivå. Olika mjukgörande formuleringar och deras effekt på hudbarriären har studerats på friska försökspersoner. Parametrar såsom mjukgörarens pH, lipidtyp, innehåll av fuktbindande ämne samt komplexiteten av formuleringar har studerats.

Efter sju veckors behandling med de olika mjukgörarna detekterades förändringar i hudens vattenavgivande förmåga, hudens kapacitans samt dess känslighet för irritation, vilket indikerar förändringar i hudens barriärfunktion. Dessutom, mRNA-uttryck av flera gener som är involverade i differentieringen och avfjällningen av stratum corneum, liksom lipidmetabolismen, var förändrad i hud behandlad med en av mjukgörarna medan en annan inducerade färre förändringar.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to:

Hans Törmä, my supervisor, for taking me under your wings and being incredibly patient and understanding, in all situations. Your guidance helped me to look at the skin from a broader perspective and ignited my enthusiasm about investigating the skin barrier at molecular level. Thank you for our vigorous discussions.

Marie Lodén, my co-supervisor, for introducing me into a field of the skin barrier and giving the opportunity to perform research in this area. Your vision and knowledge about moisturizers, countless ideas and passion for research are sincerely inspiring. I am deeply grateful for your guidance and persuading me to challenge the barriers, no matter what they are.

Berit Berne, my co-supervisor, for excellent discussions, creative brainstormings, and being very thorough in checking the manuscripts, as well as for all “clinical” help. Your inner calmness was a great counterbalance to my nervousness.

Magnus Lindberg, my co-supervisor, for sharing your knowledge and experience, teaching me to be cautious about interpretation of obtained results and to express my ideas as concise as possible (I still need to master that). I am grateful for making me to understand that stratum corneum should be treated as a ‘black box’.

Hans, Marie, Berit and Magnus–it was an honour to work with you.

Annika Grindborg, managing director of ACO HUD, for believing in me and making this research possible.

Anders Vahlquist, head of Department of Dermatology, for providing excellent work conditions.

Tove Agner, Marie Virtanen and Lars Norlén, for valuable comments and interesting discussion during my “half-time control”.

Jacek Arct, the president of the Polish Society of Cosmetic Chemists, who significantly influenced my life and showed me that cosmetics are fascinating from all perspectives.

All volunteers, for sacrificing their skin for science, although sometimes it was painful.

My parents, Janina and Krzysztof, and my brother Adam, for their love and support.
My dear Tomas, for patience and being by my side.

Ragna Lindeke-Strömqvist, Emil Schwan and Jouni Frid, for moral support, encouragement in time of despair and discussions about meaning of life.

All my colleagues at ACO HUD, for help and being enthusiastic about my research.

My colleagues at Uppsala, for all support, scientific and not only (otherwise I would have gone crazy in my dark room), and especially to Inger Pihl-Lundin, for skilful technical assistance.

Proper English AB, for help with language corrections.

This research was financially supported by ACO HUD NORDIC AB, Uppsala University and the Welander and Finsen Foundation.
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Acta Universitatis Upsaliensis

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