Evaluation of *ex vivo* human skin as model for the investigation of nanoparticle-based drug delivery systems for anti-inflammatory topical dermatotherapy

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...dedicated to an unforgotten woman.

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E. K. B. Pfannes, S. Hadam, N. Döge, S. Fimmel, U. Blume-Peytavi and A. Vogt. Mini-zone cyanoacrylate skin surface stripping: a new method for non-invasive sampling of scalp material. *Exp Dermatol* 25(7):555-556, 2016.

N. Döge, E. Thiel, G. Seewald, S. Albrecht, A. Vogt, J. Liebscher and K.-H. Schönborn. Wide-field two-photon microscopy–Transcending technology limitations. *Optik & Photonik* 10(5): 39-42, 2015.

F. Mayr, A. Schütz, N. Döge and U. Heinemann. The Lin28 cold-shock domain remodels pre-let-7 microRNA. *Nucleic Acids Res* 40(15):7492-7506, 2012.

Own oral presentations

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Abstract

Nanoparticles are promising drug delivery systems for the topical treatment of inflammatory skin diseases. Nevertheless, most useful results are based on penetration studies, which are frequently limited to model drugs such as fluorescent dyes. Investigation of penetration and efficacy would be of great value to detect potential benefits of drug-loaded nanoparticles.

The current study focused on the development of a barrier-disrupted skin model based on *ex vivo* human skin to assess preclinically the extent to which nanoparticles improve penetration and efficacy of anti-inflammatory drugs in different states of barrier disruption. Different types of skin barrier disruption were induced by physical and chemical means. Comparative studies in two short-term skin cultures systems revealed that standardization of 50-times tape-stripping and 4-hour sodium lauryl sulfate pretreatment were more reliable than abrasion and 1.5-hour sodium lauryl sulfate pretreatment: Transepidermal water loss values and interleukin-6 /-8 levels were increased reproducibly. Moreover, a wide range of inflammatory mediators was affected in cultures of skin maintained in tissue media, which preserved key skin barrier parameters and viability better than medium-free cultures. Intradermal microdialysis for up to 24 hours in one experimental setup including up to nine sets enabled comparison of intact versus barrier-disrupted skin and three dexamethasone formulations (ethyl cellulose nanocarriers, nanocrystals and a conventional cream) in parallel. Physical and chemical barrier disruption affected dexamethasone penetration differently: Penetration rates in chemically treated skin were lower than in tape-stripped skin. Nanocrystals quickly penetrated intact and disrupted skin reaching significantly higher dermal drug amounts within six hours. The benefit of encapsulation in ethyl cellulose nanocarriers was more pronounced in intact skin. High cytokine levels indicated an irritative potential of nanocrystals, while the estimation of drug-mediated efficacy over time was limited by cytokine release as a result of probe insertion. Skin barrier disruption resulted in increased penetration rates, but rare events of deeper penetration of nanoparticles were also identified in intact skin at sites of high focal particle aggregations by a newly implemented *in situ* imaging mode based on wide-field two-photon microscopy.

The current study shows an effective way for the application of *ex vivo* human skin models in advanced drug delivery studies by maximizing the read-out obtained from each donor skin sample using specifically adjusted protocols and methods. These insights into the influence of disease-related skin barrier disruptions on the penetration and efficacy of topically applied drugs provide valuable information on the benefits of each individual nanoparticle-based drug delivery system in different types of barrier alterations. The resulting more effective drug penetration has the potential to improve the topical therapy of inflammatory skin diseases.

Kurzzusammenfassung

Für die topische Therapie von entzündlichen Hautkrankheiten stellen nanopartikuläre Wirkstoffträgersysteme eine neuartige Behandlungsstrategie dar. Häufig werden aussagekräftige Ergebnisse jedoch über Penetrationsstudien geliefert, die sich auf Modellwirkstoffe wie Fluoreszenzfarbstoffe beschränken. Die Untersuchung der Penetration und Wirksamkeit wäre von großem Wert, um den potenziellen Mehrwert solcher Systeme zu ermitteln.

Im Fokus der vorliegenden Arbeit stand die Entwicklung eines barrieregestörten Hautmodells an exzidierter menschlicher Haut um präklinisch zu beurteilen, inwieweit Nanopartikel die Penetration und Wirksamkeit von entzündungshemmenden Medikamenten in verschiedenen Stadien der Hautbarrierestörung verbessern. Unterschiedliche Hautbarrierestörungen wurden durch physikalische und chemische Behandlung der Haut induziert. Untersuchungen an zwei unterschiedlichen Systemen zur Hautkultivierung zeigten, dass die 50-fache Abrissmethode mit einem Klebefilm und die 4-stündige Behandlung mit Natriumlaurylsulfat (5% w/v) zuverlässiger zu standardisieren waren im Vergleich zur 1,5-stündigen Natriumlaurylsulfat-Behandlung oder dem Abrieb mit einem Bimsstein: Sowohl die Werte des transepidermale Wasserverlusts als auch die Zytokin-Spiegel von Interleukin-6 /-8 waren reproduzierbar erhöht. Zudem waren in Hautkulturen, die mit Medium versorgt wurden, eine Vielzahl von Entzündungsmediatoren durch die induzierten Barrierestörungen erhöht. In kultivierter Haut wurde die Vitalität des Gewebes und wichtige Hautbarriereparameter besser aufrechterhalten als in Hautkulturen, die ohne Medium inkubiert wurden. Mittels intradermaler Mikrodialyse über 24 Stunden in einem Versuchsaufbau, der bis zu neun Versuchsreihen umfasste, konnte ein Vergleich von intakter und barrieregestörter Haut und drei Dexamethason-Formulierungen (Ethylcellulose-Nanocarrier, Nanokristalle und eine herkömmliche Creme-Formulierung) parallel erfolgen. Die Penetration von Dexamethason wurde durch physikalische und chemische Barrierestörung unterschiedlich beeinflusst: In chemisch behandelter Haut war die Penetration von Dexamethason geringer als in getapter Haut. Nach der topischen Applikation von Nanokristallen wurden innerhalb von sechs Stunden signifikant höhere dermale Wirkstoffmengen sowohl in intakter als auch barrieregestörter Haut gemessen. Der Mehrwert der Verkapselung in Ethylcellulose-Nanocarriern zeigte sich vor allem in intakter Haut. Hohe Zytokin-Spiegel deuten auf ein irritatives Potential von Nanokristallen hin, während die Beurteilung der wirkstoffvermittelten Wirksamkeit durch die Zytokin-Freisetzung als Folge der Einbringung von Mikrodialyse-Membranen eingeschränkt wurde. Die Störung der Hautbarriere führt zu einer erhöhten Penetration. Durch die Implementierung eines neuartigen

in situ Bildgebungsmodus, basierend auf der Zwei-Photonen-Mikroskopie, konnte jedoch auch an intakter Haut nachgewiesen werden, dass es speziell in Bereichen mit einer hohen Ansammlung von Nanopartikeln zu einer tieferen Penetration von Nanopartikeln kommt.

Die vorliegende Studie zeigt einen effektiven Weg für die Anwendung von *ex vivo* menschlichen Hautmodellen in neuartigen Studien zu Wirkstofffreisetzungsstrategien auf, wobei die aus jeder Spenderhautprobe gewonnenen Daten mit speziell angepassten Protokollen und Methoden maximiert werden. Die gewonnenen Erkenntnisse über den Einfluss krankheitsbedingter Hautbarrierestörungen auf die Penetration und Wirksamkeit von topisch applizierten Medikamenten liefern wertvolle Informationen über den Nutzen jedes einzelnen nanopartikulären Wirkstoffträgersystems in verschiedenen Zuständen der Barrierestörung. Eine resultierende effektivere Wirkstoffpenetration hat das Potenzial die topische Therapie von entzündlichen Hauterkrankungen zu verbessern.

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Abbreviations

2PM	Two-photon microscopy
AD	Atopic dermatitis
APC	Antigen-presenting cell
CLSM	Confocal laser scanning microscopy
CMS	Core-multishell
DC	Dendritic cells
GC	Glucocorticoid
HF	Hair follicle
IL	Interleukin
Ig	Immunoglobulin
LC	Langerhans cell
LC-MS/MS	Liquid-chromatography-tandem-mass spectrometry
LPS	Lipopolysaccharide
MC	Mast cell
MW	Molecular weight
NF	Nuclear factor
OECD	Organization for Economic Cooperation and Development
SC	Stratum corneum
SLS	Sodium lauryl sulfate
TEM	Transmission electron microscopy
TEWL	Transepidermal water loss
Th	T-helper
TIRFM	Total internal reflection fluorescence microscopy
TS	Tape-stripping
UV	Ultraviolet

Introduction

1 Rational for the preclinical investigation of topical nanoparticle-based drug delivery systems

Skin is a complex organ, which envelops the entire human body. Located as an interface to the external environment, skin forms an effective barrier against ultraviolet (UV) radiation, mechanical stressors, chemicals and invasion of pathogens while controlling the loss of water and the immune defense. The protection is provided by the epidermis, especially by its outermost layer, the stratum corneum (SC), which is formed by terminally differentiated corneocytes and a complex extracellular lipid matrix [1]. However, the SC is selectively permeable by passive diffusion including transepidermal and transappendageal pathways [2] (Fig. 1.1).

Topical delivery of therapeutics is the mainstay of treatment of many skin-associated problems. Many therapeutics may penetrate the skin to some extent after topical application; however, only an efficient uptake and a directed delivery to, for example, diseased cell infiltrates may lead to the desired long-term therapeutic success [3]. Increasing evidence obtained from experimental and clinical studies suggest that pathophysiological conditions affect skin barrier permeability [4] and limit topical delivery [2]. One option is to increase the dose and frequency of topical application of drugs. However, if conventional topical drug formulations are used, such as ointments or creams, problems with greasiness, stickiness, irritation, allergic reactions and uncontrolled evaporation may occur. In addition, conventional formulations often lack efficacy and pose safety concerns when used more frequently. [3].

Nanoparticle-based drug delivery systems are a promising pharmaceutical approach to overcome aforementioned limitations. The small size from 10 nm to 1000 nm, customized surfaces and improved solubility offer novel properties and functions, which differ significantly from comparable products made of identical materials. Hence, nanoparticles have the potential to revolutionize diagnosis, treatment and prevention of diseases. [5]. The central objective of applying nanoparticles in the topical treatment of inflammatory skin diseases is to increase the bioavailability of drugs by (i) a controlled transport and (ii) a release at specific sites (targeted delivery). Both facts contribute to the therapeutic efficacy and may help to reduce side effects [7]. Nevertheless, little is known about differences between intact and diseased skin on barrier function and the consequences for penetration as well as drug-mediated efficacy.

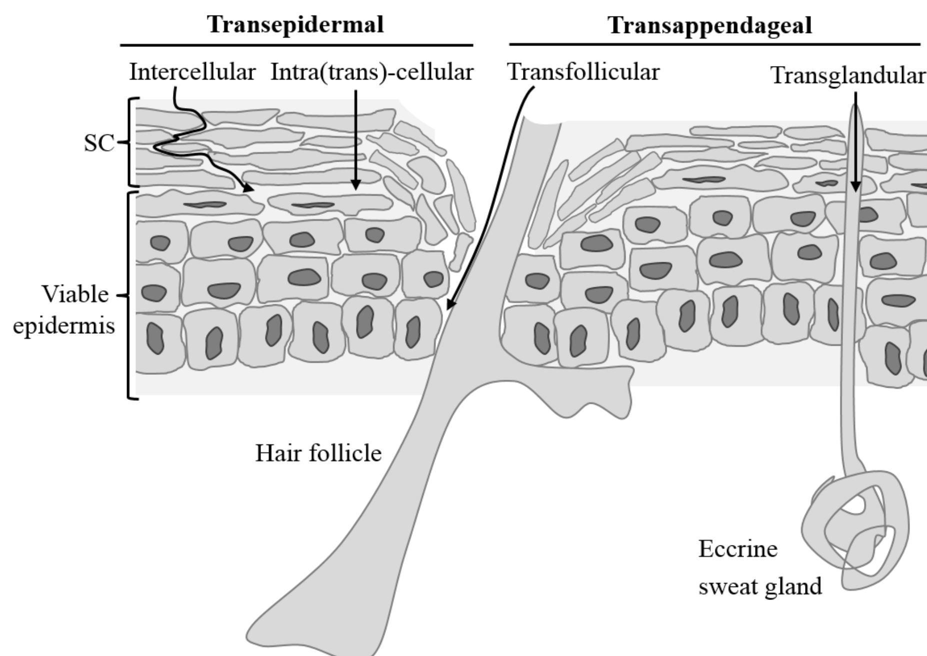

Figure 1.1: Possible routes of penetration into the SC (modified from [6])

In contrast to the generalized assumption that changes in skin permeability in diseased skin are the result of an impaired skin barrier, changes in the biochemical environment including skin surface pH, hydration, augmentation of inflammatory mediators or differential presence of enzymes [8, 9, 10] are also crucial for skin penetration, drug release and drug-mediated efficacy [4, 11]. During the initial development phase, preclinical studies are necessary to investigate the potential benefits of nanoparticle-based drug delivery systems over conventional drug formulations. Although several different preclinical skin models are available nowadays, the realization of such studies *ex vivo* is challenging. Most useful results are usually based on penetration studies, which are frequently limited to model drugs such as fluorescent dyes. Studies on drug penetration rates in intact skin and demonstration of product safety, as they are commonly applied, will not allow predictions of the future therapeutic value of drug-loaded nanoparticles. Thus, comprehensive studies on reliable and robust preclinical skin model that allow studies on penetration and efficacy in barrier-impaired skin would be of great value to decide which nanoparticle-based drug delivery strategy will be brought into clinical trials for future *in vivo* topical anti-inflammatory therapy of different skin diseases.

2 Topical glucocorticoids - Conventional therapy of cutaneous inflammation

Chronic inflammatory skin diseases represent a large group of indications for repetitive topical therapy. One of the most common chronic and relapsing inflammatory skin disease is atopic dermatitis (AD). Although it most often starts in infancy affecting 10-20% of children and tends to resolve or remarkably improve by age five, it is also highly prevalent in adults (1-3%) [12]. In the pathogenesis of AD, skin barrier dysfunction and persistent inflammation are complex reinforcing processes as schematically shown in Fig. 2.1.

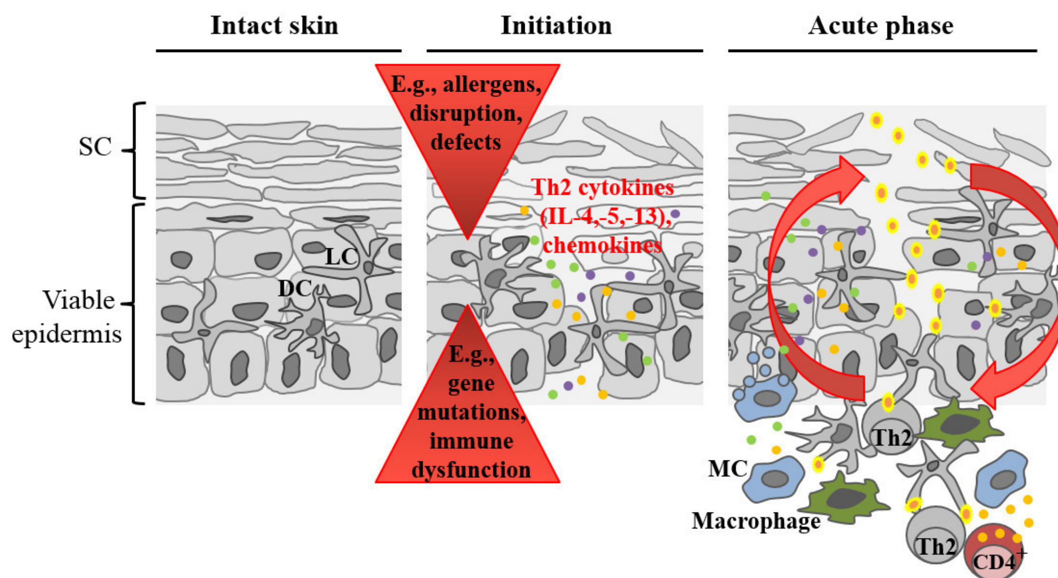

Figure 2.1: Skin barrier impairment and key pathophysiological changes involved in the progression of AD (modified from [13])

Impairment of barrier function as induced by genetic defects in skin barrier proteins, such as filaggrin and ceramides, or environmental affections activate keratinocytes to release pro-inflammatory cytokines and chemokines [8]. As a key pathogenetic role in AD, a T-helper 2 (Th2)-dominant immune response (including interleukin-4 (IL-4), IL-5, and IL-13) is mediated [14]. The release of chemoattractants causes an initial influx of inflammatory cells including antigen-presenting cells (APCs), macrophages and degranulated mast cells (MCs)

as found in the epidermis of acute AD lesions [15]. Barrier abnormalities weaken the skin barrier defense mechanism against irritant substances. APCs lead those irritants to Th cells and recruit CD4-positive T cells, which trigger non-specific inflammation. The reinforced inflammation combined with general cytokine imbalance could lead to a persistent skin barrier perturbation [16].
The presence of activated dendritic cells (DCs), the infiltration of eosinophils and macrophages as well as the rise in IL-4, -5 and -12 expression, as a part of the general overproduction of inflammatory cytokines, promote Th1 to Th2 switching. The subsequent release of Th1 and Th2 cytokines and the elevation of Immunoglobulin E (IgE) levels mark the transition from acute to chronic AD [13, 16]. Chronic AD skin lesions show an increased number of IgE-bearing LCs and inflammatory DCs in the epidermis and macrophages dominate the dermal mononuclear cell infiltrate [15]. Eosinophils and fully-granulated MCs are also increased in number. In general, the cellular infiltrate is expanded compared to acute lesions and contains both Th2 and Th1 cells [13].
AD therapy focuses on the reduction of cutaneous inflammation besides management of pruritus, infection, physiological dysfunction and identification up to elimination of triggering factors [13]. Topical agents are the mainstay of AD therapy [17]. Glucocorticoids (GCs) are frequently applied to control exacerbation of both acute and chronic cutaneous inflammation [13]. GCs mediate their anti-inflammatory effects by a cytoplasmic GC-receptor in immune cells and many other kinds of cells including keratinocytes and fibroblasts. Upon ligand binding, the GC-receptor complex translocates into the nucleus to induce two genomic effects:

- Transactivation: the GC-receptor complex binds to DNA sequences (GC-responsive elements) in the promoter regions of target genes and directly stimulates or inhibits the transactivating function of transcription factors, resulting in reduced expression of pro-inflammatory genes [18].

- Transrepression: the GC-receptor binds to various transcription factors, such as nuclear factor kappa B (NF-κB), activator protein-1, NF-IL-6, and others, via protein-protein interactions to inhibit the transcription of various pro-inflammatory genes [19].

A third mechanism, a non-genomic effect, is mediated via membrane-bound GC-receptors in T cells which form a complex with the membrane-bound GC-receptor leading to impaired T cell receptor signaling [20]. Upon topical application on a cutaneous inflammation, however, the most important mechanism is the suppression of chemokine expression. For that, topical

GCs are applied daily to treat active lesions. Once control of AD is achieved, proactive intermittent applications are frequently required to prevent relapses and maintain long-term control [17]. In a study with AD patients, fluticasone propionate (0.05% cream or 0.005% ointment) was applied once or twice daily to stabilize the inflammation. Subsequently, the continuous application of emollient with fluticasone propionate twice weekly significantly reduced the risk of an AD relapse compared to treatment with placebo base [21].

GC products are classified according to their vasoconstrictive properties from very potent (class I) to mild (class IV), the UK classification and very potent (group I) to weakest and mildest (group VII), the US classification [22]. In general, it is recommended to use ultra-high-potency GCs only for very short periods of time. Local effects including skin atrophy have been frequently reported for very potent GCs during long-term treatment or under occlusion [23], but even short-term topical treatment for three days daily with a potent GC, such as clobetasol (0.0125% - 0.05%), caused abnormalities in permeability barrier homeostasis and SC integrity [24]. Moreover, GCs should be applied only in certain body regions, because GC absorption has been demonstrated to vary not only among individuals but with respect to the anatomical location [13]. Applied on diseased skin, GC penetration is two to ten times greater than that through healthy skin [25]. Thus, delicate sites, where increased amounts of topical GCs are absorbed, tend more to local side effects from even mild-potency GCs. While significant parts of the applied GC volume are often removed from the skin surface, even small percutaneously absorbed amounts can exert systemic adverse effects, such as osteoporosis, growth retardation and glaucoma [23]. Considering these problems, research has focused on strategies to optimize dosing scheduling, to synthesize more potent and safer GCs and to adjust the effects of formulations, which decisively define the potency of topical preparations and product efficacy [26].

3 Nanoparticle-based drug delivery systems as pharmaceutical approach for topical dermatotherapy

Topical drug delivery has advantages in the treatment of skin-associated problems. It offers a lower risk of systemic side effects compared to oral or parenteral drug administration and avoids the first pass metabolism. Interestingly, several studies suggest that numerous skin barrier functions are being retained in lesional skin [4, 11]. Thus, topical drug delivery is still a challenge, especially regarding the following aspects:

- The barrier function of the SC limits efficient penetration into viable skin [27].
- An impaired barrier function significantly affects permeability, as found in AD lesions and even in uninvolved skin of AD patients compared to healthy controls [4].
- Conventional formulations often cause toxic reactions due to highly concentrated drug layers on the skin, unspecific delivery along with variable absorption rates [3].

Although skin penetration critically depends on structural integrity [4], other factors, such as an augmentation of immune reactivity as well as changes in the biochemical and metabolic environment, can have a tremendous impact on drug penetration [8, 9, 10]. For example, activated LCs have been shown to capture external allergens/ antigens by elongation of their dendrites through tight junctions [28], which might deviate in the presence of tight junction defects as recently found in AD patients [29]. Additionally, an aberrant protease activity has been associated with an inflammatory environment [8], which is known to affect drug release and drug stability [4, 11].
Manipulation of the skin barrier changes barrier integrity and is used to improve skin permeation. In addition, enhancer increase the transport across the skin barrier; however, both ways are associated with skin irritancy [6]. The choice of a correct drug or prodrug is also restricted to several limiting criteria, which numerous drugs as well as peptides, proteins, vaccines, and nucleotide fragments do not meet [27].
The main objective in topical anti-inflammatory dermatotherapy is to achieve a targeted delivery to affected sites and to reach adequate drug concentrations and simultaneously avoid high therapeutic doses and side effects. In this context, nanoparticle-based drug delivery systems have gained increasing attention during the past decades. In contrast to conventional drug formulations, particulate systems may offer high loading capacities, a protection of labile groups from degradation, a targeted drug delivery, a controlled drug release for a sustained drug activity and lower toxicity [3, 7]. A targeted delivery could be achieved by adjusting surface characteristics, such as size and shape, to the specific tissue characteristics. Such targeting can be further enhanced by the conjugation of antibodies and peptides to the nanoparticle surfaces [5].
Gupta *et al.* [3] stress the potential added value of such systems for topical treatment of skin diseases referring to work with several types of nanocarriers for skin delivery. The feasibility of using therapeutic products based on nanoparticle-based drug delivery systems in the dermatological field has been demonstrated in preclinical studies, clinical trials and, in some

cases, by the approval and marketing of new products [30, 31]. Also, a wide range of patents addresses applications of nanoparticle-based delivery systems to improve topical treatment of AD. These trends show the great interest in such delivery systems for dermatotherapy [32].

Upon application on skin, characteristics, such as size, deformability, superficial charges, formulation, and others, are determining factors for skin interaction and penetration [7]. Lipidic vesicles, such as liposomes, were first shown to be of potential value for topical therapy. Liposomes modulate mainly skin permeation with a lipid exchange between the outermost SC layers and the lipid carrier matrix. In consequence, there is a burst drug release without translocation of intact vesicles [33]. Although the burst release delivers high drug concentrations, studies indicate that systemic drug absorption is not increased, but in turn the anti-inflammatory activity of encapsulated therapeutics is improved compared to the free therapeutic when applied on inflamed skin [34]. Making those vesicles ultra-deformable, such as in the case of Transfersomes®, even penetration across an intact skin barrier by passing through pores could be achieved enabling delivery of therapeutic relevant drug amounts into the skin [35]. While vesicular particle types primarily act as penetration enhancers across the SC, the development of a broad range of nanoparticles with different structures and characteristics offers several different drug delivery strategies. For example, different generations of lipid nanoparticles, such as solid lipid nanoparticles, which combine advantages of solid particles, emulsions and liposomes, provide an improved protection of the loading and a more adaptable release. Upon application on skin, film formation properties and skin hydration [36] have been shown to lead to an improved targeting of the viable epidermis [37]. Such localizing effects have also been demonstrated by polymer-based nanoparticles, e.g. polymeric core-multishell (CMS) nanocarriers [38] and nanogels [39]. An increased penetration and prolonged skin interaction of the released drug have been shown to improve the therapeutic drug efficacy regarding erythema intensity, dermatitis severity, etc. [40]. Side effects of hydrocortisone treatment, such as elastic fiber fragmentation and fibroblast infiltration, can also be inhibited by application of polymeric drug-loaded nanoparticles compared to cream formulations which remarkably reduced drug penetation rates into the skin [41]. While the penetration of the load as well as the drug-mediated efficacy are improved, a translocation of intact nanoparticles across an intact skin barrier is generally not expected [42]. In diseased skin, the broad range of nanoparticle-based drug delivery systems may help to address various pathological conditions. For example, disease-related changes that result in limited penetration can benefit from increased penetration and exposure to sufficiently high drug doses. In contrast, a prolonged exposure to lower drug doses and wider drug distribution within the skin may

improve the therapy using high-potency drugs, which otherwise effectively penetrate the skin into systemic circulation [42]. Translation of such highly promising experimental approaches into clinical applications faces several hurdles. Beside penetration rates as determined in conventional penetration setups, skin barrier characteristics of diseased skin and biomarkers are usually less well studied, but may be of high relevance.

4 Dexamethasone as a model drug

Dexamethasone is one of the three steroids, among hydrocortisone and prednisolone, which are mainstays of GC therapy. The structures are very closely related to each other as depicted in Fig. 4.1, where the carbons in the skeleton are numbered. During the development of GCs, attempts have been made to increase their potency by chemical modification of the steroid nucleus and side groups. For example, with the introduction of a double bond between C-1 and C-2, the anti-inflammatory activity is increased, and the mineralocorticoid activity is reduced compared to hydrocortisone. Dexamethasone, 1-dehydro-9α-fluoro-16α-methylhydrocortisone, $C_{22}H_{29}FO_5$, was designed by adding a fluorine atom at C-9, which lead to an increase of the GC activity, and a methyl group at C-16 in alpha-orientation, which greatly reduces the mineralocorticoid effect [43]. It is well known that the electronegativity, size, lipophilicity, oxidation potential and electrostatic interaction of a single fluorine atom can dramatically influence chemical reactivity and biological properties of an organic compound [44]. Synthetic GCs are more potent than a natural ligand showing a higher binding affinity (K_d) 5 nM of cortisol versus 17 nM of dexamethasone [45].

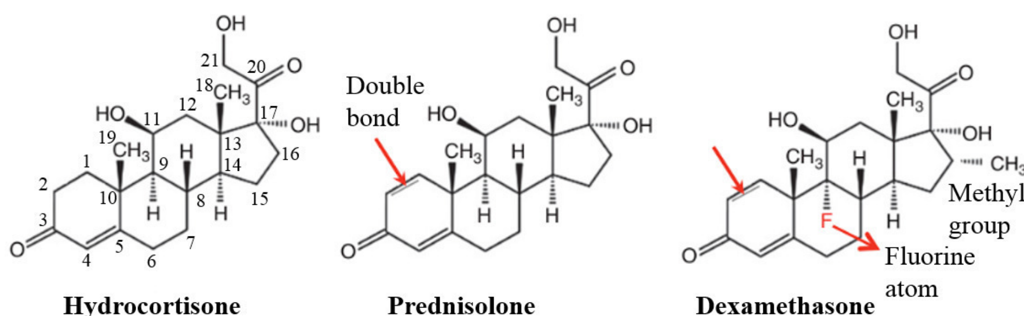

Figure 4.1: Chemical structure of hydrocortisone, prednisolone and dexamethasone [43]

In clinical dermatology, dexamethasone has been largely replaced by chemically modified structures (e.g. replacement of halogen atom and esterification). Esterification determines the solubility to a considerable extent (1-octanol/water partition coefficient of dexamethasone ≈ 1.83 [46]) as well as the duration of action. For example, dexamethasone sodium phosphate is the more soluble form of dexamethasone. Once partitioned into the skin, it undergoes ester hydrolysis, which ensures that the active form of the drug is immediately available in the skin [47]. Yet, dexamethasone remains in the light of the vast body of literature to date available a useful model molecule for cutaneous application. Fluorine is favorable for being detected without the need of labeling by means that are based on X-ray spectrometry [48]. By detection of the free drug, information about release kinetics and a correlation of penetration profiles to anatomic compartments are provided [49]. Enzyme-linked or fluorescently labeled antibodies are also available commercially for dexamethasone enabling immunohistochemical staining of the drug within tissue samples [50]. In addition, the huge amount of data available on clinical trials as well as preclinical studies regarding penetration and dexamethasone-mediated efficacy make dexamethasone an easily comprehensible candidate for the investigation of the added value of newly developed nanoparticle-based drug delivery systems compared to conventional drug formulations. For topical application, dexamethasone is classified as GC of mild potency according to the British national formulary. However, side effects result from long-term treatment combined with facilitated penetration in large skin areas [18]. Molecules with such low molecular weight (MW) as dexamethasone (MW = 392 g/mol) [27] may greatly benefit from improved selectivity and prolonged local exposure.

5 Challenges regarding preclinical assessment of nanoparticle-based drug delivery systems in human skin *ex vivo*

Nanoparticle-based drug delivery systems for topical skin application permit a more effective and sufficient delivery of new or well-known drugs with the promise of improving safety and therapeutic efficacy for patients. Although several preclinical and clinical data on the free drug, such as dexamethasone, are available, drug loading in delivery systems requires new reliable preclinical studies before clinical trials are possible. Such preclinical studies face new challenges in order to demonstrate the benefit of such delivery strategies over conventional

formulations. In the following the most important challenges will be explained in more detail based on excised human skin.

5.1 Application on ‘diseased skin‘

Most importantly, topical drug delivery systems are intended to be used on diseased skin. Preclinical investigations face new challenges since little is known about differences between intact and diseased skin including structural changes as well as altered surface pH, decreased SC hydration, augmentation of inflammatory mediators or differential presence of enzymes [8, 9, 10] on barrier function and the consequences for penetration as well as drug-mediated efficacy. While numerous preclinical data on the penetration in diseased and/ or barrier-disrupted skin are available from *in vivo* animal studies [51] or modified *in vitro* reconstructed human skin models [52], there is a lack of preclinical data on excised human skin. This can be explained by the difficulty in obtaining specimen of human diseased skin. Skin barrier disruption methods on intact human skin are applied alternatively to serve as a model for pathological alterations of skin permeability. Several means of skin barrier disruption methods have been studied so far as reviewed by Chiang *et al.* [4]. However, most studies mainly focus on the investigation of altered penetration rates associated with barrier-disrupted skin and not on induced biological effects. For example, tape-stripping (TS) is a standard skin barrier technique applied to increase cutaneous drug absorption [4] and penetration of biological macromolecules such as peptides [53] and oligonucleotides [54], which usually do not penetrate across intact skin barrier. Surfactant-treatment, e.g. with sodium lauryl sulfate (SLS), is known to enhance skin penetration as well. Both methods similarly induce increased transepidermal water loss (TEWL) values resulting in an impaired skin barrier [4] as often found in diseased skin [55]. However, simple removal of essential parts of the SC by TS could not be correlated with processes taking place in the upper skin barrier compartments after chemical treatment: SC lipids are extracted and the intercellular lamellar bilayers are disrupted by interaction with protein components converting α-keratin to β-keratin. In the epidermis, SLS has also a direct toxic effect on the keratinocytes resulting in release of pro-inflammatory cytokines, such as IL-1α [4]. This suggests that the type and intensity of the applied disruption method have different effects on the resulting pathological conditions. Whether such effects are reproducible and may also lead to a persistent inflammation in excised human skin is not

yet adequately investigated in preclinical studies. However, it is of direct relevance for the design and further development of topical drug delivery strategies.

5.2 Detection of drug efficacy

The future added value of nanoparticle-based drug delivery systems focuses mainly on the increase of therapeutic drug efficacy [3]. The therapeutic efficacy of topically applied GCs depends, besides their pharmacological potency, on their bioavailability at the site of action and the retention within the skin [18]. The assessment of skin penetration will provide information on these events; however, confirmation of increased biological efficacy is required as the presence of anti-inflammatory drugs in the skin does not necessarily correlate with significant different biological effects as demonstrated in AD [56] and psoriatic lesions [57, 58].

Especially challenging in *ex vivo* human skin is (i) the application of skin barrier disruption methods that induces skin inflammation, (ii) the conduction of drug penetration studies in an inflamed skin and (iii) the relation of penetrated drug amounts to therapeutic efficacy. *In vivo*, such experimental setups confirmed the added value of nanoparticle-mediated GC delivery as shown in a recent study on healthy versus inflamed mouse skin examining the level of penetration, clinical effectiveness and measurement of inflammatory markers [59]. Typical short-term clinical read-outs for GC bioavailability are largely based on blanching related to vasoconstriction [60] or suppression of erythema *in vivo* [59]. In excised human skin, such measurements are not applicable due to the absence of active blood flow. Additionally, the examination of drug-mediated efficacy may require prolonged culture periods as realized in human skin organ culture systems [61]. Here, the secretion of cytokines into the culture medium in response to different intensities of stimulation or inhibition can be quantified in addition to the kinetics of immune cells and the expression of certain proteins in the skin [62]. The value of such biomarkers as read-out parameter for the therapeutic efficacy of nanoparticle-based drug delivery systems has not yet been studied in detail in *ex vivo* human skin models.

5.3 Assessment of local drug penetration rates

As outlined in the previous section, the objective of nanoparticle-based drug delivery systems is to maximize the drug concentration at the site of action. Commonly, skin penetration studies are conducted for risk assessments. The Organization for Economic Cooperation and Devel-

opment (OECD) guideline 428 [63] suggests skin absorption testing in human skin mounted to Franz diffusion cells to determine systemically available amounts of a compound in contact with the skin. Such setups assess the penetrated and permeated amounts in the skin sample and the underlying receptor fluid. When standardized and pre-validated for certain skin types [64], the procedure shows predictable results for humans [65, 66]. However, assessment of flux rates may not indicate adequately local drug delivery. A more promising approach for that purpose is the quantification of drug amounts on tapes, which are harvested from the skin surface [67], or the extraction of drug amounts from epidermis and dermis sections [68]. Such studies are nevertheless relatively insensitive, time-consuming and require large pieces of tissue for e.g. the assessment of skin penetration at different time-points. Thus, a significant effort is made to apply effective and skin-sparing tools. Intradermal microdialysis, which is based on the insertion of a semipermeable membrane in the skin, is one option for this purpose. Endogenous molecules as well as topically applied or injected substances can be collected within the dermis by a passive diffusion process as shown in Fig. 5.1. The ratio between concentrations in eluate fractions and applied doses allows conclusions to be drawn about intradermal drug recovery rates [69].

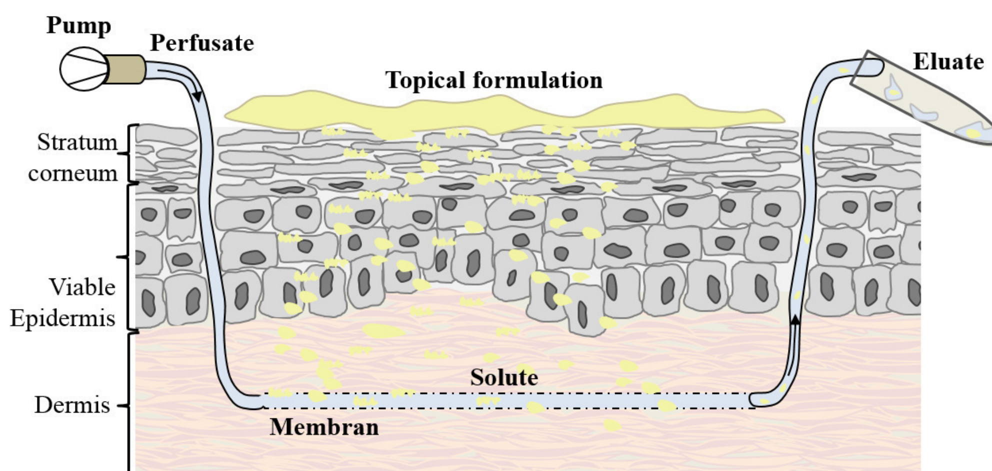

Figure 5.1: Basic principles of intradermal microdialysis

The special advantage of intradermal microdialysis studies is that chronological, real-time information about the pharmacokinetics of drugs and biological effects at the site of action at different time-points over a prolonged period can be provided. Moreover, only small skin areas or sample skin specimen are required. Consequently, microdialysis has been used in

many pharmacokinetic studies of target tissue concentrations [70] as well as for sampling of endogenous molecules in the dermis, such as histamine [71], cytokines or others [72] *in vivo* and *ex vivo*. Indeed, the method is controversially discussed in terms of its reliability and reproducibility, since relatively large inter- and intraindividual differences in the amount of recovered drug concentrations have been found [73]. However, the direct and detailed measurement of local drug concentrations or the release of inflammatory mediators in a minimally invasive manner is unique. In this way, comprehensive insights into various areas of skin research, such as the mechanisms of skin diseases, metabolism, skin penetration, etc. can be gained.

5.4 Investigation of penetration pathways and skin interactions

Drug extraction from the tissue and sampling by intradermal microdialysis enable the quantification of penetrated drug amounts. However, such studies do not focus on penetration pathways and details on skin interactions can not be given. It is important to note that there are many different mechanisms how nanoparticles interact with the skin, as described in chapter 3. Currently, ultrathin cryo-sections or harvest material of the skin surface is used for studying skin penetration. In order to investigate also small amounts of material and/ or cellular processes, high spatial resolution techniques, such as transmission electron microscopy (TEM), cryo-electron microscopy and soft X-ray spectromicroscopy, are applied in skin research. For example, TEM and stimulated Raman spectromicroscopy studies showed that nanogels induced a perturbation of the lipids and proteins resulting in increased SC hydration and deep penetration into the SC [39]. Soft X-ray spectromicroscopy was successfully applied for quantification of penetrated dexamethasone amounts and correlation of dexamethasone molecules to anatomic compartments [49]. Soft X-ray spectromicroscopy allows the label-free detection of either the drug or the nanocarrier, as demonstrated in a second study [38]. Yet, skin penetration studies on model drugs such as fluorescent dyes (e.g. Nile Red or ATTO-Oxa12 with a similar MW as several drugs of interest) are most commonly applied [74, 75]. For example, information about localization and lateral diffusion dynamics of such model drugs in the SC have been obtained by the application of total internal reflection fluorescence microscopy (TIRFM) [75]. This is also the case for most follicular penetration studies, which frequently rely on the investigation of fluorescently tagged nanoparticles or released fluorescent dyes on

ultrathin sections of HFs or harvested material from the skin surface [76, 77]. Prior to such examinations, extensive and time-consuming sample preparation is necessary. The invasiveness of such preparation steps can alter the sample quality and above all lead to the production of artifacts. In addition, the number of variables that can be examined simultaneously is considerably limited [78].

In situ imaging methods such as confocal laser scanning microscopy (CLSM) and two-photon microscopy (2PM) are promising approaches for the investigation of skin penetration. Both methods haven been successfully applied on full-thickness skin samples without the need of invasive sample preparation steps [79, 80]. Yet, limitations regarding the optical scanning depth, the field of view and tissue scattering effects must be considered, which especially restricts follicular penetration studies. Until now, in-depth scans and mosaicking of several scans are applied to reconstruct a larger field of view or 3D-scans. However, such image processing also involves the risk of producing artifacts [81]. Because both SC and HFs are important key structures for skin penetration and reservoir compartments [82, 83], insights into interfollicular and follicular penetration are essential to consider all types of delivery strategies for novel topical nanoparticle-based drug delivery systems.

6 Objectives

Nanoparticle-based drug delivery systems have gained increasing attention in topical treatment of inflammatory skin diseases, such as AD and psoriasis. Nevertheless, the impact of intact versus diseased skin on skin barrier function and the consequences for penetration as well as drug-mediated efficacy are not yet adequately investigated in preclinical studies. Most useful results are based on penetration studies, which are frequently limited to model drugs such as fluorescent dyes. The development of an *ex vivo* human skin model, in which the above-mentioned aspects can be reliably investigated, would therefore be of great value to identify the potential benefits of nanoparticle-based drug delivery systems over conventional drug formulations.

The current study was structured in the following objectives:

- Comparison of two short-term cultures of skin (skin samples maintained in tissue culture media to medium-free skin cultures incubated in humidified chambers) regarding skin barrier function and viability
- Comparison of both skin culture systems regarding structural integrity, biophysical parameters and inflammatory cytokine levels in response to different types and intensities of physical and chemical skin barrier disruption methods
- Standardization of a physical and a chemical skin barrier disruption model for the assessment of the penetration and drug-mediated efficacy in different types of skin barrier alteration
- Establishment of time- and skin-sparing protocols and methods to investigate the penetration of the anti-inflammatory drug dexamethasone parallel to cytokine levels as biological measurements of drug-mediated efficacy over time
- Comparison of intact versus barrier-disrupted skin and three different dexamethasone formulations (ethyl cellulose nanocarriers, nanocrystals and a conventional dexamethasone cream) in one experimental setup
- Investigation of nanoparticle penetration in intact skin by TIRFM and implementation of wide-field 2PM for *in situ* imaging of nanoparticles in full-thickness skin samples without invasive sample preparation

Publications and Manuscripts

Assessment of skin barrier function and biochemical changes of *ex vivo* human skin in response to physical and chemical barrier disruption

Authors: Nadine Döge, Araks Avetisyan, Sabrina Hadam, Eva Katharina Barbosa Pfannes, Fiorenza Rancan, Ulrike Blume-Peytavi, Annika Vogt

Journal: European Journal of Pharmaceutics and Biopharmaceutics 116:138-148, 2017.

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Data analysis: 100%
Interpretation of results: 70%
Writing: 90%

Ethyl cellulose nanocarriers and nanocrystals differentially deliver dexamethasone into intact, tape-stripped or sodium lauryl sulfate-exposed *ex vivo* human skin - assessment by intradermal microdialysis and extraction from the different skin layers

Authors: Nadine Döge, Stefan Hönzke, Fabian Schumacher, Benjamin Balzus, Miriam Colombo, Sabrina Hadam, Fiorenza Rancan, Ulrike Blume-Peytavi, Monika Schäfer-Korting, Anke Schindler, Eckart Rühl, Per Stahl Skov, Martin Church, Sarah Hedtrich, Burkhard Kleuser, Roland Bodmeier and Annika Vogt

Journal: Journal of Controlled Release 242:25-34, 2016.

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Data analysis: 70%
Interpretation of results: 75%
Writing: 90%

Identification of polystyrene nanoparticle penetration across intact skin barrier as rare event at sites of focal particle aggregations

Authors: Nadine Döge, Sabrina Hadam, Pierre Volz, Alexander Wolf, Karl-Heinz Schönborn, Ulrike Blume-Peytavi, Ulrike Alexiev and Annika Vogt

Journal: Journal of Biophotonics (2017). Epub ahead of print.

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Practical, experimental part: 65%
Data analysis: 65%
Interpretation of results: 70%
Writing: 85%

Discussion

7 Excised human skin in advanced drug delivery studies

Excised human skin is the preferred model and is accepted by many authorities for functional and dermal absorption studies as it has been shown that the barrier properties and structural complexity of the skin are well preserved after excision of the skin [84, 85].

Current gold standards and used as a mandatory element in preclinical toxicological and pharmacological studies of new drugs; however, are animal models. On the one hand, animal testing offers the possibility of investigating a physiological and metabolically intact system; on the other hand, it enables studies in disease models of disease mechanisms and pathogenetic stages in e. g. psoriasis or AD [85, 86]. Although such disease models have been recently used to study skin penetration and systemic distribution of innovative drug delivery systems, such as dendritic CMS nanocarriers [87, 88], obtained data cannot be extrapolated without constraints to humans. Most importantly, the application of animal skin is limited due to differences between human and animal skin histology, physiology and immunology [85, 89]. In addition, animal testing has been restricted in recent years and the replacement with alternative test systems has been increasingly introduced into practice [86, 90].

As such, mono-cellular cultures or co-cultures of different cell types in 2D mono-layers recently demonstrated the potential crosstalk of nanoparticle-based drug delivery systems with specific skin cells [91, 92] and their possible interaction with biological systems [93]. Even though important results on regulatory processes in an inflamed environment have also been provided [94], cell cultures lack organotypic properties, which are essential for the simulation of processes in a complex environment such as the skin [61].

In this context, *in vitro* models have gained considerable interest, e.g. for skin absorption testing [64, 95], for irritant [96] and phototoxic testing [97]. Although organotypic skin models composed of primary or cell-line derived keratinocytes enable *in vitro* modeling of 3D cell culture of either epidermal or full-thickness skin, the validity of obtained results in comparison to the *in vivo* situation is still limited. With the progress in tissue engineering, several different models emerged during the past years; however, experts in this field refer to general model-specific shortcomings [98, 99, 100]. A major problem is that a higher permeability of the reconstructed skin models compared to native human skin has been proven caused by differences in the skin barrier formation, the skin lipids and the skin lipid orientation [52].

Overpredictability needs to be taken into account when interpreting results regarding skin penetration [101, 102] and therefor the drug-mediated efficacy. In addition, reconstructed skin models lack structural complexity of skin, while metabolic activity comparable to that of native human skin can be demonstrated [103]. As an example, the incorporation of immune cells into the models is challenging, so that there is still a shortage of immune cells [104, 105]. This is especially crucial for pharmacological studies on the efficacy of therapies that target inflammatory cytokines, which are produced by immune cells or have immune cells as their primary target [106, 107].

Based on these considerations, human skin explants represent very relevant skin models. They are considered as a tissue of choice for regulatory dermal absorption experiments [63] as well as for studies concerning physiology and pathophysiology of human skin and epidermal appendages [108]. Excised human skin completely retains its barrier properties and contains cellular elements and appendages, such as LCs, pigment cells, nerve endings and HFs. Explants can be cultured for up to several days and weeks, which depends on culture conditions [85]. The importance of maintaining physiological tissue viability has been in the focus of several culturing strategies, since it is obvious that the metabolic capacity is diminishes over time [109], which has a significant impact on the results of experiments [103]. Indeed, the period in which *ex vivo* skin will remain viable is limited and consequently also their applicability. But due to not yet standardized culture conditions, several different supports and culture conditions have been determined for maintaining skin by the duration of the culture and/or the study purpose [85]. Such variations in culture conditions and high donor-to-donor variability; however, are disadvantageous as the comparison of different studies is difficult. At the same time, the donor-specific variability reflects the individual differences as in real life. For example, SC thickness, HF density and size have been demonstrated to differ between individuals and body regions [110, 111]. Both factors are especially important for skin penetration studies as the SC and HFs are key structures and act as long-term reservoirs [82, 83] offering translocation into the viable tissue [112]. The biological responsiveness has also been demonstrated to vary intra- and interindividual *in vivo* [113]. It is important to emphasize that only skin explants provide insights into such species-relevant and individual differences [85]. In order to simplify comparability; however, standardized tests of the skin barrier function are necessary that ensures the use of skin samples with comparable properties [114, 115]. In our own studies, we were concerned to make overpredictions based on *ex vivo* investigations and identified TEWL measurements as a non-invasive screening tool for skin barrier function in skin explants, as discussed in detail in the following section.

A point of criticism is the limited quantity and availability of skin explants. Indeed, it is necessary to use skin explants immediately after surgery or biopsies to achieve the most reliable results, in turn, researcher showed that the storage at $-20\,^{\circ}\mathrm{C}$ for up to six months does not impair skin barrier properties [116]. It could be one option to collect enough skin until the beginning of the experiments. In addition, several strategies for the application of skin-sparing protocols and methods have been developed so far [84], as discussed in detail in chapter 8.

As each model is a simplified representation of reality, it is not purposeful to exactly reproduce the *in vivo* situation, but to establish reliable and robust preclinical models for the generation of exact and predictive data. Of course, the correlation of data between *in vivo* and *ex vivo* is necessary. In terms of follicular penetration, for example, the knowledge about a general and reproducible underprediction of *ex vivo* human skin models allows for data extrapolation and a sufficient predictive value for the *in vivo* situation. Rancan *et al.* [117] reported that approximately 50-70 % of HFs are open for penetration in excised skin samples. Thus, up to ten-fold higher penetration rates *in vivo* are expected. A review of scientific literature including 30 studies dealing with the percutaneous absorption of the same compound revealed that penetration studies in human skin *ex vivo* can predict skin absorption *in vivo* [118]. Two problems were highlighted by the authors: anatomical skin sites used for testing are of great relevance for the experimental outcome and the harmonization of protocols is essential to insure a good correlation. In general, investigation of the same skin regions is recommended to ensure the interpretation and comparison of comprehensible results. Moreover, when the experiment is conducted in good accordance with validated and specified experimental conditions, the *in vivo/ in vitro* correlation can be very good [119].

It is obvious that several different skin models are available nowadays and the effort to improve this models for more advanced studies is immense. Understanding of the possibilities and limitations of each skin models for each specific purpose is very important. Just as different regulatory fields have differing requirements for the acceptance of approach criteria [84], well-designed studies with clearly defined objectives may help to find a suitable model. The remaining complexity of excised human skin and skin barrier function, albeit limited to the viability of the tissue, is the basis for multiple studies. By using efficient and skin- and time-saving protocols and methods according the research purpose, their application can be extended further.

7.1 Short-term incubation - Influence on viability, structural integrity and function

Short-term cultures of skin are a valuable research tool for studying nanoparticle-based drug delivery systems. For this purpose, it is essential to ensure that the skin model remains viable long enough for the detection of penetration but also of biological response as well as drug-mediated effects to occur. Especially skin organ cultures can be useful in this context, since skin explants can be kept in culture viable for a certain period [61, 85]. In the current study, two different skin culture systems were compared: medium-free skin cultures incubated in humidified chambers and cultures of skin maintained in tissue culture media. Since macroscopic analysis revealed that dryness and necrotic edges of medium-free skin cultures were clearly pronounced after 72 hours, the incubation time was limited to 48 hours in advance.

In accordance with previously published results [120, 121], we found that incubation for up to 48 hours led to progressive tissue degeneration, loss of cohesion in the SC and decrease of proliferative activity in both approaches over time. In the past, short-term incubation of partial-thickness skin has been used to facilitate media diffusion to the epidermis, thus in principle maximizing explant viability [61]. As a result, fluid was absorbed from the culture medium through the basal surface of the dermis causing a swelling of the specimen due to hydration. Although full-thickness skin was used for cultures of skin maintained in tissue culture media in the current study, obvious structural changes occurred including swelling of the nucleated epidermis. However, structural properties and proliferative activity of basal keratinocytes, as an indicator for viability, were better preserved than in medium-free skin cultures. The identification of such structural changes and viability as indicator for the overall condition of skin samples [122] allow for comparison between different studies on human skin explants. But nevertheless, these results did not yield detailed information on the remaining skin barrier function. Fluhr *et al.* [123] verified TEWL as a parameter for permeability barrier status *ex vivo*. Of course, the general value of TEWL measurements *ex vivo* is debatable and in many situations, this is also the case *in vivo* [124]. In the current study, TEWL measurements were applied to get an overall impression (i) as to whether it can be assessed at all, (ii) as to how far values are away from *in vivo* data in humans and most importantly (iii) find a non-invasive way to standardize skin barrier function in the setup. Indeed, many artificial factors contribute to TEWL measurements *ex vivo*, but the observation that TEWL values were reproducible and in a controllable range not far from *in vivo* data [125] suggests that it could be, among others,

one non-invasive tool for standardization of *ex vivo* experiments, as it is already applied in *ex vivo* studies [114, 126].
Although skin samples profit from the addition of culture media [85], obtained results have to be carefully interpreted considering that the composition of media is not yet standardized. Especially contained serum supplements can influence biological read-outs, which is widely discussed in the research field [120, 121]. Nevertheless, the systems in which the skin samples are maintained in tissue culture media are promising for investigating both skin penetration and biological effects. Indeed, characterization of more skin samples is needed to define an average range of the condition of excised human skin in such culture systems. For this purpose, TEWL measurements can be a simple and non-invasive screening tool to ensures standardization of sample conditions during culturing, the exclusive use of intact skin samples as well as skin samples with comparable properties [115].

7.2 Induction of an inflammatory process *ex vivo*

The application of physical and chemical skin barrier disruption methods on intact human skin explants represent valuable approaches to simulate pathological changes of the barrier-impaired skin and the associated biological changes as well as the altered permeability of diseased skin. Such models are important to investigate the added value of different nanoparticle-based drug delivery strategies for different types of skin barrier alteration. In the past, chemical and physical skin barrier disruption methods have effectively been used to reproducibly induce inflammatory processes in intact skin *ex vivo* [4]. Such skin treatments may trigger damage signals and do not really mimic diseased skin; however, the availability of explants from lesional human skin is limited and the interest in alternative models for diseased skin has constantly grown.
In the current study, abrasion and 50-times adhesive TS were applied to physically treat the skin. SLS pretreatment for 1.5- and 4-hour were used for chemical pretreatment. Baseline data obtained in intact skin revealed inconsistent IL-1α and Il-1ra levels in the skin surface directly after excision. This suggests that IL-1α and Il-1ra levels were already affected by skin surface treatment during the preparation and conduction of plastic surgery. Although the determination of skin surface cytokines including IL-1α and Il-1ra is an established method to monitor skin inflammation and barrier damage *in vivo* [127], they did not yield a robust read-out *ex vivo*. In turn, epidermal and dermal IL-6 and IL-8 levels were considerable increased

in response to skin barrier disruption. The increase was detectable 24 hours after application of the skin barrier disruption, which is consistent with *in vivo* data where increased IL-8 and IL-6 levels were found in human skin wounds within six or 24 hours, respectively [128]. It should be noted that the cytokine levels in both approaches (with and without media) were in a comparable range. This suggests that epidermal and dermal IL-6 and IL-8 levels have not been affected by the use of media. On the contrary, even more consistent results have been found in the epidermis and dermis of skin cultures maintained in tissue media. Proteomic analysis showed that several inflammatory mediators were up-regulated by 4-hour SLS incubation and TS in cultured skin samples, whereby a limited number of analytes seemed to be affected by the incubation itself.
Structural and physiological changes have also been studied for standardization of the skin barrier disruption models. The extend of observed morphological changes was in response to the applied skin barrier disruption method. The amount of removed material from the skin surface by abrasion was difficult to standardize, whereas TS led to an even and reproducible removal of SC layers. Skin physiological parameters (TEWL, surface pH, SC hydration and sebum content) are clinical standard tools to assess skin barrier properties *in vivo* [129]. In the current study, we found that surface pH, SC hydration and sebum content are not suitable parameter for the *ex vivo* situation. For example, directly after excision the pH changed compared to the physiological skin pH of about 5.5 [125] to a slightly alkaline pH, which decrease during further incubation. However, the pH did not change after application of skin barrier disruption. The influence of artificially induced skin barrier disruptions on the surface pH is controversially discussed: *In vivo* studies in human skin revealed that in response to skin barrier disruption there is a short decrease and thereafter a clear and significant increase in the pH [130]. In contrast, Fluhr *et al.* [131] found only minor pH changes after chemical or physical treatment. Interestingly, it has been demonstrated that skin samples showed a significantly higher pH than human skin *in vivo* [122], as indicated by our own results. This could be due to the fact that the skin is affected by (i) extensive disinfection during surgery, (ii) contamination of the skin surface with blood, serum, fat tissue during transport and (iii) incubation in a cell culture incubator with 37 °C, 5% CO_2, 100% humidity.

Although surface pH, sebum content and SC hydration values did not change in response to the applied skin barrier disruption method, TEWL values indicated differences between intact and barrier-disrupted skin being reproducibly increased after skin barrier disruption. Netzlaff *et al.* [126] showed that TEWL was not suitable to detect slight barrier changes *ex vivo*, but clearly increased after TS. In accordance, Dabboue *et al.* [132] showed a strong cor-

relation between the number of TS and TEWL. In another study researcher applied 15-times TS and found no correlation between TEWL and skin permeability [133]. These results reveal that above all the intensity of applied barrier disruption plays a significant role for a relation between applied skin barrier disruption method and TEWL values *ex vivo*. Admittedly, the complexity of changes in diseased skin cannot be simulated by the application of skin barrier disruption methods. This has to be considered as a limitation of the *ex vivo* skin models compared to the *in vivo* situation. But several of the mediators are involved in skin diseases and are within the target of anti-inflammatory active compounds. For example, proteases have been shown to play a key role in main skin functions such, as homeostasis and chemotaxis, but also in skin inflammation [8]. Increased TEWL values are also well-known to be associated with various skin diseases, including AD and psoriasis [55, 134]. Indeed, *ex vivo* skin models lack an active influx of other immune cells and biochemicals; however, we found that local cytokine levels increased in response to skin barrier disruption within 48 hours of short-term incubation. This demonstrates the general possibility to make use of such markers for biological read-outs. TEWL values were reproducible increased with the intensity of the applied skin barrier disruption method. This suggests that TEWL measurements might be useful as non-invasive screening tool to control and standardize such experimental setups. Although surface pH measurements did not yield a robust read-out for skin changes, it should be noted that skin surface pH is considered as a critical parameter of skin well-being [135] and integrity [136]. For the interpretation of obtained results, increased pH values have to be considered. Although there are model-specific shortcomings, screening experiments on tape-stripped and 4-hour SLS-treated skin models may provide important insights into the influence of different types of skin barrier alterations on skin penetration and biological efficacy. The gained knowledge can be used to develop more selective and effective topical drug delivery strategies and finally to improve the topical treatment of various inflammatory skin diseases.

8 Investigation of nanoparticle-based drug delivery systems

The investigations performed with intradermal microdialysis on differently treated skin samples with three different types of dexamethasone formulations provided a complex data set that yield valuable information on skin penetration rates and biological effects *ex vivo*. In animal models such data are easily obtained, since drugs can be repetitively applied over time in diseased skin models allowing for drug penetration analysis in line with clinical and molecular

efficacy read-outs [59]. To perform such studies *ex vivo*, the evaluation of time- and skin-saving protocols and methods was necessary. The application of an experimental setup with up to nine test series allows investigations on chemically and physically barrier disrupted skin compared to intact skin as well as comparison of three different drug formulations. In addition, intradermal microdialysis permits the simultaneous sampling of penetrated drug amounts and cytokine levels in the dermis, enabling conclusions to be drawn about the biological efficacy. Consequently, it is an effective screening tool to study skin penetration and biological effects *ex vivo*.

8.1 Influence of barrier disruption on skin penetration

Striking differences between the penetration of dexamethasone in tape-stripped skin compared to 4-hour SLS-treated skin indicate that the penetration rates correspond to the intensity of the applied skin barrier disruption. Even though both approaches end up with a disruption of the skin barrier, penetration rates were not equally increased. Such conclusions can only be drawn from complex experimental setups, which are often rare as detailed information on skin penetration is usually restricted to comparison of one barrier disruption model and intact skin.

In current setup, protocols and methods were adapted in such a way that they were not only time- and sample-saving, but also enabled a maximum read-out in a very short time. Sampling at three different time-points within 24 hours by intradermal microdialysis and quantification of drug amounts in tissue media, skin surface, epidermis and dermis provided detailed information on dexamethasone penetration rates. In addition, we used liquid-chromatography-tandem-mass spectrometry (LC-MS/MS) as highly sensitive and specific detection method to measure even least amounts of dexamethasone with high accuracy. Considering future dose-sparing drug delivery strategies [137], such highly sensitive detection techniques are of great interest in skin research.

According to previously published results [4], increased penetration rates were found in barrier-disrupted skin compared to intact skin. Interestingly, even though morphological changes were most pronounced in chemically treated skin, penetration rates were lower compared to tape-stripped skin. *In vivo* intradermal microdialysis studies revealed increased penetration through both tape-stripped and SLS-treated skin, which correlated significantly with a TEWL increase [138]. However, the skin was longer exposed to SLS so that it can be assumed that induced skin barrier changes were stronger. Although TEWL values and cytokine

levels were similarly increased, penetration rates may not necessarily have to correlate with those events. For example, the remaining SC in SLS-treated skin may impede high flux rates unlike the removal of significant parts of the SC by TS. Several studies indicate that many of the characteristics of the skin barrier function remain preserved even in chronic inflammatory skin diseases [139]. Smits *et al.* [140] showed an increased accumulation of protoporphyrin IX following topical aminolevulinic acid application in psoriatic and actinic keratosis lesions compared to non-lesional skin. The increase was more pronounced in psoriatic lesions [140]. This suggests that structural integrity alone is not the major determinant of skin penetration. The effort to understand the complexity of the specificities of the skin barrier in diseased skin and its relevance for penetration processes has recently been demonstrated by the development of SC models similar to those observed in psoriatic lesions [141]. Even though the same observations obtained *ex vivo* need to be carefully interpreted, our findings revealed that the applied barrier disruption models *ex vivo* are suitable for simulation of different types of skin alterations. In addition, it was possible to differentiate between different penetration rates, depending on the intensity and applied method of the skin barrier disruption. It can be assumes that especially in advanced drug delivery studies it is of considerable advantage to detect differences in the penetration of intact and barrier-disrupted skin with the focus on a higher selectivity and therefore improved therapy. The examination of skin models with different changes in skin barrier allows conclusions to be drawn about the optimal drug delivery strategy for future application in different types of barrier alterations.

8.2 Distinction between specific characteristics of drug formulations

Ex vivo intradermal microdialysis studies combined with culture media analysis provides an effective method to assess dexamethasone penetration rates of three different formulations at therapeutic doses. Understanding of the specific properties of each individual formulations and delivery strategy is of crucial importance in order to recommend a certain formulation for a specific pathological alteration.

During the selection process, we applied two different delivery systems in order to increase the probability of measuring differences in the penetration of the loaded drug, as shown in previous studies [142]. For the interpretation of the influence of the specific characteristics of drug formulations, general effects were considered including increased penetration rates through

occlusion or ingredients of the cream in comparison to condensation of active ingredients on the skin surface by water evaporation of an aqueous solution [143]. We selected dexamethasone nanocrystals and dexamethasone loaded in ethyl cellulose nanocarriers as two nanoparticle formulations with very different drug release kinetics, as demonstrated in previous *in vitro* studies. Differences in dexamethasone penetration by ethyl cellulose nanocarriers and nanocrystals compared to the cream formulation were found according to the specific characteristics of the drug formulations: The assessment at different time-points revealed much faster penetration through intact and barrier-disrupted skin when applied as nanocrystals. The differences were already visible within 6 hours after topical application. In contrast, encapsulation in ethyl cellulose nanocarriers led to a reduced amount of penetrated dexamethasone, which is in line with the significant slower release kinetics compared to nanocrystals. All obtained results were in line with penetration rates obtained in *ex vivo* Franz diffusion cell studies.

Although, several studies in the field focus on the enhancement of skin drug delivery [144, 145], a delayed release and resulting lower skin penetration rates relative to commercial dexamethasone cream makes ethyl cellulose nanocarriers a favorable delivery approach for e.g. GC therapy. In detail, drugs with high therapeutic potency that effectively penetrate skin may benefit from prolonged exposure to lower doses and possible delayed drug distribution as well as increased local intradermal availability within the skin, as it has recently been demonstrated [59]. In fact, high application frequencies can be avoided and an risk of systemic blood levels can be reduced. By contrast, penetration enhancement and tissue exposure to sufficiently high drug doses is relevant as well. For this purpose, nanocrystals have gained increasing attention for dermal application [146]. Nanocrystals might be interesting for drugs with poorer solubility, limited penetration or weaker biological effects. In mice, it has been shown that the use of nanocrystals has led to improved biological efficacy. This was accompanied by an improved skin deposition of diclofenactic acid in damaged skin *ex vivo* [147]. In the current study, we found evidence that application of nanocrystals stimulated the release of pro-inflammatory cytokines, which may point toward an irritative potential, as discussed in detail in the following section. This reinforces our assumption that the use of nanocrystals is useful especially for drugs with poor skin penetration.

Current penetration analyses on human skin explants largely rely on Franz diffusion cell setups, according to the OECD guidelines [63]. However, TEWL values have been shown to correlate poorly with percutaneous absorption in such setups due to changes in the hydration state of the skin [115]. However, TEWL measurements are particularly important in order

to assess the skin barrier function. Especially after the application of skin barrier disruption methods, which have not proved to be easy to perform in Franz diffusion cell setups [126], the read-out is of great interest. As it is recommended by the guideline, we further applied drug extraction from epidermis and dermis. Cyanoacrylate skin surface stripping was applied to recover drug amounts from the upper approximately 30% of the SC and from HF openings [148]. All these methods were rather time consuming and the production of artifacts during drug extraction has to be considered [78]. Moreover, the analysis at different time-points will require large pieces of skin from one donor. This can limit the number of variables that can be examined in a set of experiments. In contrast, the analyses of microdialysis eluates at different time-points and sampling of culture media after 24 hours allowed conclusions to be drawn about altered penetration rates as consequence of the applied formulation or skin barrier status. Considering the need for tissue-saving preclinical examinations, such complex studies without the need of harvesting tissue is very attractive in skin research [78]. Thus, the current experimental setup is an effective screening tool to compare different drug delivery strategies and differently treated skin samples in parallel. This yields a complex data set, which has a high prevalence for advanced drug delivery studies.

8.3 Cytokine sampling

The current experimental setup allows not only complex penetration studies, but also investigations on biological effects based on cytokine levels as indicator for therapeutic efficacy. These investigations are crucial because higher penetration alone does not necessarily correlate with significantly higher biological effects [56, 58], which could motivate further development towards a product [78]. In *ex vivo* short-term culture models, the absence of an active blood flow restricts numerous typical short-term clinical read-outs for GC bioavailability [60, 149]. Moreover, the viability of the excised skin is a crucial limiting factor. In the current study, structural deteriorations over time restrict the use of skin explants to 48 hours. In fact, the experiments must be completed within 48 hours after removal of the skin. Indeed, effective regulation studies require longer time periods to observe a correlation between penetrated drug amounts and cytokine levels [150, 151]. From *in vivo* studies it is well-known that not all clinical markers with anti-inflammatory effects are equally suitable to demonstrate the added value of a topical drug formulation [147]. From this it can be concluded that such investigations in *ex vivo* models, where even more limiting factors exist, are far more chal-

lenging and largely rely on molecular read-outs [85].
In current setup, the application of intradermal microdialysis provided both analysis of drug penetration rates and simultaneous sampling of local cytokine levels. Highly sensitive detection by Luminex® multiplex technology allowed for detection of IL-6 and IL-8 levels in small sample volumes. According to *in vivo* intradermal microdialysis studies in humans [152], high cytokine levels after probe insertion revealed that a trauma was also induced *ex vivo*. This effects affected the biological read-outs associated with skin barrier disruption or drug application. Nevertheless, higher cytokine levels in response to the application of nanocrystals were clearly visible. This suggests that although significantly higher levels of the anti-inflammatory drug penetrated the skin, irritant reactions were triggered in the skin when dexamethasone was applied in a highly condensed form as in the case of nanocrystals. Such local side effects upon administration of topical GCs are well known [23]; however, nanocrystal-based delivery strategies are most promising for drug candidates with poor skin penetration and low biological effects, as mentioned in the previous section.

The aim of this study was to find out whether topically applied dexamethasone can reverse or inhibit cytokine levels in barrier-disrupted skin models. The basis for these investigation is that dexamethasone is a GC with well-known anti-inflammatory activities on local cytokine levels. For example, *in vivo* mice studies revealed that subcutaneously injected dexamethasone significantly reduced pro-inflammatory cytokine levels in skin wounds compared to cytokine levels observed in phosphate buffered saline-treated skin wounds [153]. The effect was also demonstrated in *ex vivo* skin models: Frušić-Zlotkin *et al.* [154] observed that the secretion of cytokines including IL-6 and IL-8 was significantly reduced within 48 hours after GC-treatment of psoriatic human skin culture models. Companjen *et al.* [62] reported the same effect in Lipopolysaccharide (LPS) and IL-1β stimulated *ex vivo* skin organ culture systems even within 24 hours. In both studies, cytokines were collected exclusively in the medium without providing data on local cytokine levels. In the current experimental setup based on intradermal microdialysis, local cytokine levels could be investigated. Moreover, within 48 hours the down-regulation of cytokines induced after skin barrier disruption and not only the inhibition of pro-inflammatory mediators was focused. Conclusion on such drug-mediated effects based on cytokine levels were difficult due to the trauma induced by probe insertion. In future, the application of an appropriate equilibration period, so that there is enough time to return to near baseline values after innate insertion, is one option. In addition, increasing the residence time of the probes in the skin and a constant collection over time could be another possibility to detect fluctuations in local cytokine levels more sensitively [70, 152].

Although a drug-mediated down-regulation of cytokine levels could not be investigated in detail, the general possibility of using such markers *ex vivo* as indicators of therapeutic efficacy was demonstrated by observation of the irritative potential of high drug doses. Thus, the simultaneous sampling by intradermal microdialysis of local cytokine levels over time provides additional insights into the benefits of nanocrystals and ethyl cellulose nanocarriers compared to conventional drug formulations, as obtained in penetration studies, in order to better decide which strategy is most appropriate for which disease-related skin condition.

9 Do nanoparticles penetrate skin?

By detecting the penetration of nanoparticles *in situ* without invasive sample preparation using a newly implemented 2PM setup, new insights into penetration pathways, skin barrier architecture and function of intact skin were gained. This knowledge is of crucial importance for the design and further development of topical drug delivery strategies based on nanoparticles [155]. Immunization studies must be emphasized in particular, which suggest that even exposure to small amounts of vaccine can result in immunological responses in intact skin [156, 157]. Otherwise, it has been highly debated in terms of toxicological evaluation of nanomaterials [158].

In the current study, we implemented a wide-field 2PM to study interfollicular and follicular penetration of fluorescently tagged nanoparticles in full-thickness skin samples. By adaptation of sample preparation, considerations regarding the production of artifacts could be reduced to a minimum. Thus, nanoparticle penetration could be investigated with high accuracy and even smallest amounts of penetrated material were identified. This is of high relevance as current *ex vivo* penetration analyses largely rely on layer-wise extraction, microscopy of cryosections and Franz diffusion cell experiments. Techniques that reach their limits when it comes to the detailed assessment of insignificant amounts of penetrated material and the dissection of penetration pathways. Moreover, the results obtained from such studies need to be carefully differentiated from artifacts due to technical limitations or tissue processing [78]. In accordance with the fact that intact skin provides effective barrier [27, 143], the vast majority of topically applied nanoparticles remained in the upper SC layers. Close to regions with high focal particle aggregations, such as in skin furrows and HF openings; however, we observed a further distribution into deeper layers of the SC and HF infundibulum, respectively. Single molecule high resolution microscopy and single particle tracking by TIRFM was applied to investigate these events in more detail as previously reported for detection of fluorescent dye

molecules [75] or fluorescently labeled drug molecules [159]. By calculating the lateral diffusion dynamics and confinement areas of the nanoparticles in lipid regions within the SC, between or close to corneocytes, TIRFM measurements confirmed results obtained by 2PM detecting nanoparticles directly beneath the SC within the viable tissue with high accuracy.

In general, multi-photon microscopy offers considerable advantages for studying skin, such as no out-of-focus effects and low scattering effects [160], which leads to increased scan depth in samples with minimal photobleaching and phototoxic effects [161]. Still, the scan area of the focus of a standard high magnification 2PM setup is limited by the field of view of the objective lens [162]. Until now, image processing is applied to reconstruct larger scan areas, which also bear risks on producing artifacts [81]. Using a wide-field 2PM [162] and laterally positioned skin sample, horizontal scanning in XY-plane with a principle width of 10 mm x 10 mm both interfollicular and follicular penetration could be investigated in histomorphological correlation. In contrast, CLSM studies, as one example for common *in situ* investigations, are limited by the strong scattering of biological samples and the mechanical scanning depth (smaller than 60 μm) [163]. Thus, most often follicular penetration could not be addressed. Alvarez-Román *et al.* [164] investigated both interfollicular and follicular penetration by CLSM. Beyond the reservoir function of HFs, further insights into follicular penetration could not be obtained. The current 2PM setup provides a more promising approach to study also follicular penetration in histomorphological correlation, a common view into the skin.

It has to be noted that although the fluorescence signals reliably belong to the intact nanoparticles and not to the free dye of the applied model nanoparticle, the brightness of the spots does not allow for quantification. This is also the case for other *in situ* imaging technquies. 2PM provides nevertheless a promising approach for this purpose: the concomitant detection of emission spectra of 2P-sensitive delivery systems loaded with drugs that act as fluorophores in the wavelength dependent detection mode [165] could be one option. For example, Stracke *et al.* [80] demonstrated that multiphoton microscopy could also be used to study the release mechanism of nanoscale carrier particles using nanoparticles covalently labeled with fluorescein and a dye as drug-model. By quantitative spectral analysis of the concentration of the load, the release process in different skin compartments could be investigated. Indeed, label-based investigations are limited as it is not clear if the same results could be obtained by label-free approaches. For that purpose, Raman approaches, X-ray spectromicroscopy and TEM have gained increasing attention [78]. Nevertheless, these findings on fluorescently labeled model nanoparticles provide important baseline data for further investigations of more

advanced nanoparticle-based delivery systems, such as CMS nanocarriers [38] or nanogels [39]. When comparing such different nanoparticles, it has to be considered that also certain characteristics of the nanoparticles affect skin penetration [7]. For example, it is well-known that the dimension of nanoparticles is one of the most important parameters regarding follicular penetration [166]. Studies on nanoparticles with diameter ranging between 7 and 20 nm showed exclusive distribution in the HF infundibulum and below [167, 168, 169]. The same was shown for 200 nm particles that preferably accumulate in follicle openings [164]. In the pig ear-skin model, it could be shown that especially 643 nm particles penetrated deepest into the HFs [83, 170]. We compared both 20 and 200 nm particle penetration in vellus and intermediate-type HFs; however, significant differences in penetration depth were not observed, but much more work is required to draw conclusions about the impact of nanoparticle size on penetration. Nevertheless, a deep penetration into HFs of nanoparticles is of crucial importance as it has been postulated that nanoparticles are able to permeate through the follicular epithelium. For example, Baroli *et al.* [171] reported that topical exposure of metallic nanoparticle dispersions (smaller than 10 nm) on excised human skin led to passive penetration through SC lipid matrix (deepest deposit 100 μm below SC) and follicular orifices (around 170 μm) reaching the viable epidermis. Vogt *et al.* [76] showed that 40 nm particles were internalized by APCs surrounding the HF infundibulum which reveals that the particles were able to penetrate the perifollicular dermis through the HF. Deposition into viable tissue also depends on the softness of nanoparticles [172]. In contrast to rigid particles, a high elasticity allows for penetration through channel-like structures into the SC [173]. Consequently, it has been demonstrated that elastic particles of 100-130 nm size passed the SC and reach the SC-viable epidermis junction *in vivo*, whereas rigid particles were recovered only in superficial SC layers [174], still suggesting that the penetrated amount is minimal [175]. The deformability of nanoparticles is a key determinant of penetration [176] and may offer an interesting alternative to conventional cream formulations, as there is the possibility of triggering the release of active ingredients [177].

In summary, scanning by wide-field 2PM of full-thickness human skin samples provided a non-invasive and preparation-sparing method for the *in situ* imaging of nanoparticle penetration. The combination with single-molecule, high-resolution microscopy and single-particle tracking using TIRFM enabled selective detection of individual as well as clusters of nanoparticles with high accuracy. Technical refinements can provide insights into the penetration of nanoparticles with other physicochemical properties, such as higher elasticity and deformability, which are known to increase the penetration rate. In conclusion, our proof-of-concept

study represents a decisive step towards the answer of the question whether and to which extend nanoparticles penetrate the skin.

10 Outlook

In the future, established *ex vivo* human skin models maintained in short term cultures could be used to improve our understanding of the impact of a complex altered environment as found in inflamed skin on skin penetration. In addition to the structural integrity, biochemical changes (e.g. inflammation mediators, altered protease activity and inflammatory infiltrate), which contribute to a changed skin barrier function and drug penetration, should be evaluated in the current skin model. If these changes refer to changes in drug release and stability and thus to penetration and therapeutic efficacy [178, 179], important conclusions can be drawn from the investigation of different nanoparticles regarding their *in vivo* behavior in lesional skin. Furthermore, effects such as a triggered release due to changes in the pH value or enzymatic cleavage of enzyme-sensitive elements in adopted nanocarriers can be investigated regarding their effect on the release of active substances [180]. For these studies, the induction of considerably more complex or targeted inflammatory processes would be helpful. The use of more specific biochemical stimuli (e.g. trypsin- and plasmin-like proteases, LPS, Tumor necrosis factor-α should be tested to determine whether it is possible to regulate inflammatory processes [62, 181].

Additionally, the measurement of biological effects, which was shown to be feasible in this work on cytokine levels, should be further optimized. Especially regarding drug-mediated efficacy read-outs, the use of other biomarkers should be further emphasized. One option is to predict anti-inflammatory action of GCs on other mechanisms such as the gene expression of pro-inflammatory cytokines [182] or the presence of pro-inflammatory transcription factors [183, 184]. The anti-inflammatory efficacy read-outs of GCs could be at both transcriptional and post-transcriptional levels [185, 186]. GCs are also known to have a profound effect on several immune cell subpopulations [187]. Membrane protein expression, kinetics and morphology of immune cell subsets can be an indicator for the activation status in response to topical GCs as previously reported [62, 187]. While *in vivo* studies point to the fact that not all clinical markers of anti-inflammatory effects are equally suited to demonstrate biological efficacy of topical drug formulations [147, 154], the suitability of other biomarkers within the applied setup is a subject for further investigations.

The investigations of the advantages of nanocarriers should not only be extended to standard active substances, but also to new innovative active substances that are not yet approved for topical dermatotherapy, e. g. the JAK-inhibitor tofacitinib and the protein-based biological etanercept [188]. Hence, it is important for the broad application of the skin model that it is adapted for penetration and efficacy studies of more innovative active ingredients. For investigations of drugs, which are known to mainly act at targets such as the level of T cells or other immunocompetent cells in the skin [189], new biological read-outs should be established based on e.g. mRNA expression of target genes, release of relevant cytokines as well as immune cells, which allow for preclinical screening of drug delivery and biological effects.

The subject of further investigations chould also be the dissection of the role of HFs as penetration pathway. Even though not all HFs are accessible for penetration [190], the effect of HFs on the overall penetration rates could be investigated in intradermal microdialysis setups by applying an established method of closing the HF orifices [191]. Hence, conclusion can be drawn about the prominent role of HFs as reservoirs and penetration pathways as well as the potential of nanoparticle-based drug delivery systems for the treatment of inflammatory infiltrates along the HF [192], which could also be effectively addressed also by new drug candidates [193].

2PM investigations should be carried out on innovative nanoparticle-based delivery systems in order to gain insights into the penetration of nanoparticles and the penetration of the released cargo. It has already been shown that such examinations with the 2PM setup are possible and enable studies on the release mechanism of nanoscaled carrier particles using nanoparticles covalently labeled with fluorescein and a dye as drug-model [80]. Our 2PM setup provides a promising approach, since resulting emission spectra could be individually detected in the wavelength dependent detection mode. In addition to penetration studies, the question of how deep the particles have to penetrate for a possible cellular uptake by e.g. LCs, which was impressively demonstrated by Kubo *et al.* [28] in mice, could be answered in further investigations. Staining protocols, which were previously applied as read-out on epidermis sheets [148], could be applied on whole tissue samples for multi-imaging of stained cells and applied particles.

Summary

The current study focused on the development of an *ex vivo* human skin model to investigate the added value of nanoparticle-based drug delivery systems on skin penetration and drug-mediated efficacy compared to conventional drug formulations for the topical therapy of inflammatory skin diseases. In order to induce different types and intensities of skin barrier disruption, physical and chemical means were applied on human skin explants obtained from plastic surgery. Comparative studies on structural integrity, biophysical parameters and cytokine levels were performed three times within 48 hours in two culture systems of skin. Cultures of skin maintained in tissue media preserved key skin barrier parameters and viability better than medium-free cultures in humidified chambers. The standardization of 50-times tape stripping and 4-hour sodium lauryl sulfate (5% w/v) pretreatment was reliable: transepidermal water loss values and interleukin-6 /-8 levels as examples of a wide range of affected inflammatory mediators were increased reproducibly compared to intact skin. Different structural and biological changes were induced, which are usually associated with pathological changes in diseased skin. Transepidermal water loss measurements provide a noninvasive screening tool to control and standardize skin barrier disruption *ex vivo*.

Based on these results, time- and tissue-sparing protocols and methods for further investigations were adapted to a short-term skin culture system. Intradermal microdialysis in one experimental setup including up to nine sets for up to 24 hours enabled comparison of intact versus physically and chemically barrier-disrupted skin on the skin of one donor as well as three dexamethasone formulations (ethyl cellulose nanocarriers, nanocrystals and a conventional cream) in parallel. The application of highly sensitive detection methods, such as liquid-chromatography-tandem-mass spectrometry and Luminex® multiplex technology, provided a complex set of data on skin penetration and biological effects. The model successfully demonstrated that skin barrier disruption and the characteristic properties of the dexamethasone formulations affected drug penetration differently. Penetration rates in chemically treated skin were lower compared to tape-stripped skin. Nanocrystals quickly and effectively penetrated intact and barrier-disrupted skin. Thus, significantly higher dermal drug concentrations were achieved within six hours compared to the other formulations. The benefit of encapsulation in ethyl cellulose nanocarriers was more pronounced in intact skin. The results were largely in line with the prediction made based on *in vitro* release kinetics and Franz diffusion cell studies. High local cytokine levels indicated a trauma induced by probe insertion,

which restricted the estimation of drug-mediated efficacy over time. Nevertheless, evidence was found that the application of nanocrystals was associated with high dermal cytokine levels, although significantly higher amounts of the anti-inflammatory drug penetrated the skin. This could point toward an irritative potential of the nanocrystals. In summary, conclusion can be drawn about drug penetration and local biological effects in intact versus barrier-disrupted skin after the application of up to three different drug formulations. Such a complex screening tool can help to characterize the added value of each individual nanoparticle-based drug delivery systems compared to conventional formulations for different pathological skin changes in order to determine the most appropriate for a particular clinical indication.

To answer the questions as to whether and to which extent nanoparticles are capable of translocating across an intact skin barrier, an *in situ* imaging mode based on wide-field two-photon microscopy was implemented. The analysis of full-thickness samples allowed sample preparation steps to be reduced to a minimum, which mainly avoids the production of artifacts. Moreover, both interfollicular and follicular penetration were investigated in histomorphological correlation. Proof-of-concept work on fluorescently tagged nanoparticles revealed that the vast majority of nanoparticles remained in the upper Stratum corneum. However, rare events of deeper penetration were also observed in intact skin close to regions with high focal nanoparticle aggregations in the Stratum corneum and in the infundibulum of hair follicles. Total internal reflection fluorescence microscopy confirmed barrier crossing with high sensitivity. Individual nanoparticles as well as clusters of nanoparticles were detected in the Stratum corneum and within the epidermal layer directly beneath the Stratum corneum. The combination of both technologies provides highly important insights into penetration processes and pathways as well as into the barrier function of the skin, which are crucial for the development of nanoparticle-based drug delivery systems for improved topical application of drugs for inflammatory skin diseases.

Zusammenfassung

In der vorliegenden Studie wurden Hautmodelle an exzidierter menschlicher Haut entwickelt, um den potenziellen Nutzen von nanopartikulären Wirkstoffträgersystemen gegenüber herkömmlichen Arzneimittelformulierungen hinsichtlich der Penetration und der Wirksamkeit von Medikamenten für die topische Therapie entzündlicher Hauterkrankungen zu ermitteln. Zur Induktion von unterschiedliche Prozesse und Intensitäten der Hautbarrierestörung wurden an menschlichen Hautexplantaten verschiedene physikalische und chemische Barrierestörungsmethoden durchgeführt. Vergleichende Studien bzgl. der strukturellen Integrität, physiologischer Parameter und lokaler Zytokinspiegel wurden dreimal innerhalb von 48 Stunden in zwei unterschiedlichen Hautkultursystemen durchgeführt. Die Kultivierung von Haut in Gewebemedium erwies sich im Vergleich zur Inkubation in einer feuchten Kammer als effektiver, da es zu einer verbesserten Aufrechterhaltung von verschiedensten charakteristischen Hauteigenschaften bzw. der Vitalität der Haut kam. Zudem konnte in diesem Modell gezeigt werden, dass sowohl der 50-fache Abriss mit einem Klebefilm als auch die Inkubation mit Natriumlaurylsulfat (5% w/v) effektiv und einfach zu standardisieren waren. Nach Applikation dieser Barrierestörungen wurden sowohl die transepidermale Wasserverlust-Werte als auch die Zytokin-Spiegel von IL-6 und IL-8 als Beispiele von einem breiten Spektrum an betroffenen Entzündungsmediatoren reproduzierbar erhöht, wobei diese in Korrelation mit der aufgewendeten Intensität der Barrierestörung standen. Durch die Behandlung intakter Haut mit physikalischen und chemischen Barrierestörungsmoethoden wurden unterschiedliche strukturelle als auch biologische Veränderungen der Haut hervorgerufen, die sonst in Folge einer entzündlichen Hauterkrankung auftreten. Messungen des transepidermalen Wasserverlusts erwiesen sich als vielversprechend, wobei dieser Parameter als Screening-Tool für eine nicht-invasive Kontrolle und Standardisierung von Hautbarrierestörungen auch am *ex vivo* Hautmodell dienen kann.
Basierend auf diesen Daten wurden in nachfolgenden Untersuchungen effektive und zeitsparende Methoden und Protokolle auf das Hautmodell angepasst. Die Anwendung der intradermalen Mikrodialyse über einen Zeitraum von 24 Stunden in einem Vesuchsaufbau, der bis zu neun Versuchsreihen an der Haut eines Spenders umfasste, ermöglichte hierbei sowohl Untersuchungen bzgl. der Auswirkungen von unterschiedlichen Hautbarrierestörungen als auch verschiedener nanopartikulärer Trägersysteme (Ethylcellulose-Nanocarrier und Nanokristalle) im Vergleich zur Creme-Formulierung von Dexamethason. Zudem lieferte die Anwendung

hochempfindlicher Detektionsmethoden, wie LC-MS/MS und Luminex® Multiplex Technology, einen komplexen Datensatz über Hautpenetrationsraten und biologische Effekte *ex vivo*. Dadurch konnte im Modell erfolgreich gezeigt werden, dass sowohl die induzierte Barrierestörung als auch die Applikationsform von Dexamethason einen wesentlichen Einfluss auf das Penetrationsverhalten hatten. Die Penetrationsraten in chemisch behandelter Haut waren insgesamt niedriger als in physikalisch behandelter Haut. Dexamethason-Nanokristalle penetrierten schnell und effektiv sowohl in intakte als auch barrieregestörte Haut, wodurch bereits innerhalb von sechs Stunden signifikant höhere Dexamethason-Konzentrationen in der Dermis gemessen wurden. Der Mehrwert einer verzögerten Wirkstoff-Freisetzung mittels Ethylcellulose-Nanopartikeln war im intakten Hautmodell am deutlichsten ersichtlich. Mittels intradermaler Mikrodialyse und der Analyse des Kultivierungsmediums konnte gezeigt werden, dass die Penetrationsraten von Dexamethason mit den *in vitro* Freisetzungsstudien und mit den Ergebnissen der Franz-Diffusionszell-Untersuchungen an intakter Haut übereinstimmten. Zudem deuteten hohe lokale Zytokinspiegel auf ein Trauma des Gewebes hin, das durch die Einführung der Mikrodialyse-Membran induziert wurde. Diesem Trauma waren biologische Effekte, die durch Barrierestörungen oder durch die Penetration des Wirkstoffes verursacht wurden, untergeordnet. Dennoch wurden Hinweise darauf gefunden, dass die topische Anwendung von Dexamethason-Nanokristallen mit höheren Zytokinwerten assoziiert war, obwohl signifikant höhere Mengen des entzündungshemmenden Wirkstoffes in die Haut penetrierten. Folglich konnte in dieser Serie von Untersuchungen sowohl der Einfluss einer Veränderung der Haut in Folge unterschiedlicher Barrierestörungen untersucht werden, als auch unterschiedliche Eigenschaften von Wirkstoffformulierungen auf die Penetration von Dexamethason und biologische Effekte gegenübergestellt werden. Ein derart komplexes Screening-Tool kann dazu beitragen, den Mehrwert jedes einzelnen nanopartikel-basierten Wirkstoffträgersystems im Vergleich zu herkömmlichen Formulierungen für verschiedene pathologische Hautveränderungen zu charakterisieren, um das für eine bestimmte klinische Indikation am besten geeignetste zu bestimmen.

Um die Frage zu beantworten, ob und inwieweit Nanopartikel in der Lage sind in intakte Haut zu penetrieren, wurde ein nicht-invasives *in situ* Bildgebungsverfahren basierend auf der Zwei-Photonen Mikroskopie für Penetrationsstudien von Nanopartikeln implementiert. Durch die Analyse von Vollhautproben konnten die Probenvorbereitungsschritte auf ein Minimum reduziert werden, was vor allem die Produktion von Artefakten vermeidet. Zudem gelang es erstmals sowohl Einblicke in die interfollikuläre als auch follikuläre Penetration in histomorphologischer Korrelation zu geben. In ersten Ergebnissen an fluoreszierenden

Modell-Nanopartikeln unterschiedlicher Größe konnte gezeigt werden, dass die überwiegende Mehrheit der Nanopartikel im oberen Stratum corneum verblieben ist. Jedoch konnte auch eine tiefere Penetration in untere Stratum corneum- Ebenen und in das Infundibulum von Haarfollikeln nachgewiesen werden, vor allem in Bereichen, in denen es zu einer hohen Ansammlung von Nanopartikeln kam. Die Ergebnisse der Zwei-Photonen Mikroskopie-Studien konnten durch interne Totalreflexionsfluoreszenzmikroskopie mit hoher Sensitivität bestätigt werden. Dabei konnten einzelne Nanopartikel sowie Ansammlungen von Nanopartikeln im Stratum corneum und innerhalb der epidermalen Schicht direkt unter dem Stratum corneum detektiert werden. Durch die Kombination dieser Technologien können Einblicke in Penetrationsprozesse, -wege und in die Barrierefunktion der Haut gewonnen werden, die für die Entwicklung nanopartikel-basierter Wirkstoffträgersysteme für die topische Therapie entzündlicher Hauterkrankungen von entscheidender Bedeutung sind.

Bibliography

[1] P. M. Elias. Epidermal lipids, barrier function, and desquamation. *J Invest Dermatol*, 80, 1983.

[2] J. Hadgraft. Skin, the final frontier, 2001.

[3] M. Gupta, U. Agrawal, and S. P. Vyas. Nanocarrier-based topical drug delivery for the treatment of skin diseases. *Expert Opin Drug Deliv*, 9(7):783–804, 2012.

[4] A. Chiang, E. Tudela, and H. I. Maibach. Percutaneous absorption in diseased skin: an overview. *J Appl Toxicol*, 32(8):537–563, 2012.

[5] R. Singh and J. W. Lillard. Nanoparticle-based targeted drug delivery. *Exp Mol Pathol*, 86(3):215–223, 2009.

[6] B. W. Barry. Novel mechanisms and devices to enable successful transdermal drug delivery. *Eur J Pharm Sci*, 14(2):101–114, 2001.

[7] T. W. Prow, J. E. Grice, L. L. Lin, R. Faye, M. Butler, W. Becker, E. M. T. Wurm, C. Yoong, T. A. Robertson, H. P. Soyer, and M. S. Roberts. Nanoparticles and microparticles for skin drug delivery. *Adv Drug Delivery Rev*, 63(6):470–491, 2011.

[8] P. M. Elias and M. Schmuth. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Curr Allergy Asthma Rep*, 9(4):265–272, 2009.

[9] T. S. Kupper. Immune and inflammatory processes in cutaneous tissues. Mechanisms and speculations. *J Clin Invest*, 86(6):1783, 1990.

[10] L. Korkina. Metabolic and redox barriers in the skin exposed to drugs and xenobiotics. *Expert Opin Drug Metab & Toxicol*, 12(4):377–388, 2016.

[11] S. Gattu and H. I. Maibach. Modest but increased penetration through damaged skin: an overview of the *in vivo* human model. *Skin Pharmacol Physiol*, 24(1):2–9, 2010.

[12] N. Katoh. Future perspectives in the treatment of atopic dermatitis. *J Dermatol*, 36(7):367–376, 2009.

[13] D. Y. Leung. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J Allergy Clin Immunol*, 105(5):860–876, 2000.

[14] M. Grewe, C. A. Bruijnzeel-Koomen, E. Schöpf, T. Thepen, A. G. Langeveld-Wildschut, T. Ruzicka, and J. Krutmann. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today*, 19(8):359–361, 1998.

[15] M. K. Oyoshi, R. He, L. Kumar, J. Yoon, and R. S. Geha. Cellular and molecular mechanisms in atopic dermatitis. *Adv Immunol*, 102:135–226, 2009.

[16] M. J. Cork, D. A. Robinson, Y. Vasilopoulos, A. Ferguson, M. Moustafa, A. MacGowan, G. W. Duff, S. J. Ward, and R. Tazi-Ahnini. New perspectives on epidermal barrier dysfunction in atopic dermatitis: gene–environment interactions. *J Allergy Clin Immunol*, 118(1):3–21, 2006.

[17] L. F. Eichenfield, W. L. Tom, T. G. Berger, A. Krol, A. S. Paller, K. Schwarzenberger, J. N. Bergman, S. L. Chamlin, D. E. Cohen, K. D. Cooper, others, and K. M. Cordoro. Guidelines of care for the management of atopic dermatitis: section 2. Management and treatment of atopic dermatitis with topical therapies. *J Am Acad Dermatol*, 71(1):116–132, 2014.

[18] S. Wiedersberg, C. S. Leopold, and R. H. Guy. Bioavailability and bioequivalence of topical glucocorticoids. *Eur J Pharm Biopharm*, 68(3):453–466, 2008.

[19] T. Rhen and J. A. Cidlowski. Anti-inflammatory action of glucocorticoids: new mechanisms for old drugs. *N Engl J Med*, 353(16):1711–1723, 2005.

[20] M. Löwenberg, C. Stahn, D. W. Hommes, and F. Buttgereit. Novel insights into mechanisms of glucocorticoid action and the development of new glucocorticoid receptor ligands. *Steroids*, 73(9):1025–1029, 2008.

[21] J. Berth-Jones, R. J. Damstra, S. Golsch, J. K. Livden, O. Van Hooteghem, F. Allegra, and C. A. Parker. Twice weekly fluticasone propionate added to emollient maintenance treatment to reduce risk of relapse in atopic dermatitis: randomised, double blind, parallel group study. *BMJ*, 326(7403):1367, 2003.

[22] S. E. Jacob and T. Steele. Corticosteroid classes: a quick reference guide including patch test substances and cross-reactivity. *J Am Acad Dermatol*, 54(4):723–727, 2006.

[23] U. R. Hengge, T. Ruzicka, R. A. Schwartz, and M. J. Cork. Adverse effects of topical glucocorticosteroids. *J Am Acad Dermatol*, 54(1):1–15, 2006.

[24] J. S. Kao, J. W. Fluhr, M.-Q. Man, A. J. Fowler, J.-P. Hachem, S. K. Crumrine, D.and Ahn, B. E. Brown, P. M. Elias, and K. R. Feingold. Short-term glucocorticoid treatment compromises both permeability barrier homeostasis and stratum corneum integrity: inhibition of epidermal lipid synthesis accounts for functional abnormalities. *J Invest Dermatol*, 120(3):456–464, 2003.

[25] M. Turpeinen, E. Lehtokoski-Lehtiniemi, S. Leisti, and O. P. Salo. Percutaneous absorption of hydrocortisone during and after the acute phase of dermatitis in children. *Pediatr Dermatol*, 5(4):276–279, 1988.

[26] T. Senyigit and O. Ozer. Corticosteroids for skin delivery: challenges and new formulation opportunities. In *Glucocorticoids-New Recognition of Our Familiar Friend*. InTech, 2012.

[27] J. D. Bos and M. M. H. M. Meinardi. The 500 dalton rule for the skin penetration of chemical compounds and drugs. *Exp Dermatol*, 9(3):165–169, 2000.

[28] A. Kubo, K. Nagao, M. Yokouchi, H. Sasaki, and M. Amagai. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. *J Exp Med*, 206(13):2937–2946, 2009.

[29] K. Yoshida, A. Kubo, H. Fujita, M. Yokouchi, K. Ishii, H. Kawasaki, T. Nomura, H. Shimizu, K. Kouyama, T. Ebihara, K. Nagao, and M. Amagai. Distinct behavior of human Langerhans cells and inflammatory dendritic epidermal cells at tight junctions in patients with atopic dermatitis. *J Allergy Clin Immunol*, 134(4):856–864, 2014.

[30] E. Desmet, M. Van Gele, and J. Lambert. Topically applied lipid-and surfactant-based nanoparticles in the treatment of skin disorders. *Expert Opin Drug Deliv*, 14(1):109–122, 2017.

[31] M. Singhal, M. Lapteva, and Y. N. Kalia. Formulation challenges for 21st century topical and transdermal delivery systems, 2017.

[32] N. Akhtar, A. Verma, and K. Pathak. Exploring preclinical and clinical effectiveness of nanoformulations in the treatment of atopic dermatitis: Safety aspects and patent reviews. *Bull Fac Pharm, Cairo Univ*, 2016.

[33] H. Schreier and J. A. Bouwstra. Liposomes and niosomes as topical drug carriers: dermal and transdermal drug delivery. *J Controlled Release*, 30(1):1–15, 1994.

[34] M. Manconia, J. Pendás, N. Ledón, T. Moreira, C. Sinico, L. Saso, and A. M. Fadda. Phycocyanin liposomes for topical anti-inflammatory activity: *in-vitro in-vivo* studies. *J Pharm Pharmacol*, 61(4):423–430, 2009.

[35] G. Cevc, D. Gebauer, J. Stieber, A. Schätzlein, and G. Blume. Ultraflexible vesicles, Transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. *Biochim Biophys Acta - Biomembranes*, 1368(2):201–215, 1998.

[36] R. H. Müller, M. Radtke, and S. A. Wissing. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Adv Drug Delivery Rev*, 54:S131–S155, 2002.

[37] C. S. Maia, W. Mehnert, and M. Schäfer-Korting. Solid lipid nanoparticles as drug carriers for topical glucocorticoids. *Int J Pharm*, 196(2):165–167, 2000.

[38] K. Yamamoto, A. Klossek, R. Flesch, T. Ohigashi, E. Fleige, F. Rancan, J. Frombach, A. Vogt, U. Blume-Peytavi, P. Schrade, S. Bachmann, R. Haag, S. Hedtrich, M. Schäfer-Korting, N. Kosugi, and E. Rühl. Core-multishell nanocarriers: Transport and release of dexamethasone probed by soft x-ray spectromicroscopy. *J Controlled Release*, 242:64–70, 2016.

[39] M. Giulbudagian, F. Rancan, A. Klossek, K. Yamamoto, J. Jurisch, V. C. Neto, P. Schrade, S. Bachmann, E. Rühl, U. Blume-Peytavi, A. Vogt, and M. Calderón. Correlation between the chemical composition of thermoresponsive nanogels and their interaction with the skin barrier. *J Controlled Release*, 243:323–332, 2016.

[40] Z. Hussain, H. Katas, M. C. I. M. Amin, E. Kumolosasi, F. Buang, and S. Sahudin. Self-assembled polymeric nanoparticles for percutaneous co-delivery of hydrocortisone/hydroxytyrosol: an *ex vivo* and *in vivo* study using an NC/Nga mouse model. *Int J Pharm*, 444(1):109–119, 2013.

[41] Z. Hussain, H. Katas, M. C. I. M. Amin, E. Kumolosasi, and S. Sahudin. Downregulation of immunological mediators in 2,4-dinitrofluorobenzene-induced atopic dermatitis-

like skin lesions by hydrocortisone-loaded chitosan nanoparticles. *Int J Nanomed*, 9:5143, 2014.

[42] F. Rancan, U. Blume-Peytavi, and A. Vogt. Utilization of biodegradable polymeric materials as delivery agents in dermatology. *Clin Cosmet Investig Dermatol*, 7:23–34, 2014.

[43] P. C. Hindmarsh and K. Geertsma. *Congenital Adrenal Hyperplasia: A Comprehensive Guide*. Academic Press, p. 212, 2017.

[44] J. Wang, M. Sanchez-Rosello, J. L. Acenna, C. del Pozo, A. E. Sorochinsky, S. Fustero, V. A. Soloshonok, and H. Liu. Fluorine in pharmaceutical industry: fluorine-containing drugs introduced to the market in the last decade (2001–2011). *Chem Rev*, 114(4):2432–2506, 2013.

[45] P. Mulatero, M. Panarelli, D. Schiavone, A. Rossi, G. Mengozzi, C. J. Kenyon, L. Chiandussi, and F. Veglio. Impaired cortisol binding to glucocorticoid receptors in hypertensive patients. *Hypertension*, 30(5):1274–1278, 1997.

[46] F. A. A. Núñez and S. H. Yalkowsky. Correlation between logP and ClogP for some steroids. *J Pharm Sci*, 86(10):1187–1189, 1997.

[47] J. Paturi, H. D. Kim, B. Chakraborty, P. M. Friden, and A. K. Banga. Transdermal and intradermal iontophoretic delivery of dexamethasone sodium phosphate: quantification of the drug localized in skin. *J Drug Targeting*, 18(2):134–140, 2010.

[48] G. Tarsoly, M. Ovari, and G. Zaray. Determination of fluorine by total reflection x-ray fluorescence spectrometry. *Spectrochim Acta, Part B*, 65(4):287–290, 2010.

[49] K. Yamamoto, R. Flesch, T. Ohigashi, S. Hedtrich, A. Klossek, P. Patoka, G. Ulrich, S. Ahlberg, F. Rancan, A. Vogt, U. Blume-Peytavi, P. Schrade, S. Bachmann, M. Schäfer-Korting, N. Kosugi, and E. Rühl. Selective probing of the penetration of dexamethasone into human skin by soft X-ray spectromicroscopy. *Anal Chem*, 87(12):6173–6179, 2015.

[50] A. S. Grewal, J. M. Nedzelski, J. M. Chen, and V. Y. W. Lin. Dexamethasone uptake in the murine organ of corti with transtympanic versus systemic administration. *Indian J Otolaryngol Head Neck Sur*, 42(1):19, 2013.

[51] I. Jakasa and S. Kezic. Evaluation of *in vivo* animal and *in vitro* models for prediction of dermal absorption in man. *Hum Exp Toxicol*, 27(4):281–288, 2008.

[52] S. Küchler, K. Strüver, and W. Friess. Reconstructed skin models as emerging tools for drug absorption studies. *Expert Opin Drug Metab & Toxicol*, 9(10):1255–1263, 2013.

[53] M. J. Choi, H. Zhai, H. Löffler, F. Dreher, and H. I. Maibach. Effect of tape stripping on percutaneous penetration and topical vaccination. *Exog Dermatol*, 2(5):262–269, 2004.

[54] V. Regnier, A. Tahiri, N. Andre, M. Lemaıtre, T. Le Doan, and V. Preat. Electroporation-mediated delivery of 3-protected phosphodiester oligodeoxynucleotides to the skin. *J Controlled Release*, 67(2):337–346, 2000.

[55] Y. Werner and M. Lindberg. Transepidermal water loss in dry and clinically normal skin in patients with atopic dermatitis. *Acta Derm Venereol*, 65(2):102–5, 1985.

[56] N. Inagaki, N. Shiraishi, K. Igeta, T. Itoh, T. Chikumoto, M. Nagao, J. F. Kim, and H. Nagai. Inhibition of scratching behavior associated with allergic dermatitis in mice by tacrolimus, but not by dexamethasone. *Eur J Pharmacol*, 546(1):189–196, 2006.

[57] A. V. Powles, B. S. Baker, J. McFadden, A. J. Rutman, C. E. M. Griffiths, L. Fry, and H. Valdimarsson. Intralesional injection of cyclosporin in psoriasis. *The Lancet*, 331(8584):537, 1988.

[58] H.-J. Schulze, G. Mahrle, and G. K. Steigleder. Topical cyclosporin A in psoriasis. *Br J Dermatol*, 122(1):113–114, 1990.

[59] M. M. A. Abdel-Mottaleb, B. Moulari, A. Beduneau, Y. Pellequer, and A. Lamprecht. Nanoparticles enhance therapeutic outcome in inflamed skin therapy. *Eur J Pharm Biopharm*, 82(1):151–157, 2012.

[60] S. Wiedersberg, A. Naik, C. S. Leopold, and R. H. Guy. Pharmacodynamics and dermatopharmacokinetics of betamethasone 17-valerate: assessment of topical bioavailability. *Br J Dermatol*, 160(3):676–686, 2009.

[61] R. Tammi and H. I. Maibach. Skin organ culture: why? *Int J Dermatol*, 26(3):150–160, 1987.

[62] A. R. Companjen, L. I. van der Wel, L. Wei, J. D. Laman, and E. P. Prens. A modified *ex vivo* skin organ culture system for functional studies. *Arch Dermatol Res*, 293(4):184–190, 2001.

[63] *OECD, OECD guideline for the testing of chemicals. Skin absorption: in vitro method No. 428, Adopted 13 April 2004, pp. 1 to 8. 2004*.

[64] M. Schäfer-Korting, U. Bock, W. Diembeck, H. J. Düsing, A. Gamer, E. Haltner-Ukomadu, C. Hoffmann, M. Kaca, H. Kamp, others, and M. Weimer. The use of reconstructed human epidermis for skin absorption testing: Results of the validation study. *Altern Lab Anim - ATLA*, 36(2):161–187, 2008.

[65] C. Herkenne, A. Naik, Y. N. Kalia, J. Hadgraft, and R. H. Guy. Dermatopharmacokinetic prediction of topical drug bioavailability *in vivo*. *J Invest Dermatol*, 127(4):887–894, 2007.

[66] H. Wagner, C.-M. Lehr, U. F. Schaefer, and K.-H. Kostka. Human skin penetration of flufenamic acid: *in vivo/in vitro* correlation (deeper skin layers) for skin samples from the same subject. *J Invest Dermatol*, 118(3):540–544, 2002.

[67] J. Lademann, U. Jacobi, C. Surber, H.-J. Weigmann, and J. W. Fluhr. The tape stripping procedure–evaluation of some critical parameters. *Eur J Pharm Biopharm*, 72(2):317–323, 2009.

[68] A. Gysler, B. Kleuser, W. Sippl, K. Lange, H. C. Korting, H.-D. Höltje, and M. Schäfer-Korting. Skin penetration and metabolism of topical glucocorticoids in reconstructed epidermis and in excised human skin. *Pharm Res*, 16(9):1386–1391, 1999.

[69] N. Leveque, P. Muret, S. Makki, S. Mac-Mary, J. P. Kantelip, and P. Humbert. *Ex vivo* cutaneous absorption assessmentof a stabilized ascorbic acid formulation using a microdialysis system. *Skin Pharmacol Physiol*, 17(6):298–303, 2004.

[70] R. Holmgaard, E. Benfeldt, N. Bangsgaard, J. A. Sorensen, K. Brosen, F. Nielsen, and J. B. Nielsen. Probe depth matters in dermal microdialysis sampling of benzoic acid after topical application: an *ex vivo* study in human skin. *Skin Pharmacol Physiol*, 25(1):9–16, 2011.

[71] L. J. Petersen, M. Brasso, K.and Pryds, and P. S. Skov. Histamine release in intact human skin by monocyte chemoattractant factor-1, RANTES, macrophage inflammatory protein-1α, stem cell factor, anti-IgE, and codeine as determined by an *ex vivo* skin microdialysis technique. *J Allergy Clin Immunol*, 98(4):790–796, 1996.

[72] M. K. Church, S. P. Skinner, L. J. Burrows, and A. P. Bewley. Microdialysis in human skin. *Clin Exp Allergy*, 25(11):1027–1029, 1995.

[73] M. Kreilgaard. Dermal pharmacokinetics of microemulsion formulations determined by *in vivo* microdialysis. *Pharm Res*, 18(3):367–373, 2001.

[74] A. Boreham, M. Pfaff, E. Fleige, R. Haag, and U. Alexiev. Nanodynamics of dendritic core–multishell nanocarriers. *Langmuir*, 30(6):1686–1695, 2014.

[75] P. Volz, A. Boreham, A. Wolf, T.-Y. Kim, J. Balke, J. Frombach, S. Hadam, Z. Afraz, F. Rancan, U. Blume-Peytavi, A. Vogt, and U. Alexiev. Application of single molecule fluorescence microscopy to characterize the penetration of a large amphiphilic molecule in the stratum corneum of human skin. *Int J Mol Sci*, 16(4):6960–6977, 2015.

[76] A. Vogt, B. Combadiere, S. Hadam, K. M. Stieler, J. Lademann, H. Schaefer, B. Autran, W. Sterry, and U. Blume-Peytavi. 40 nm, but not 750 or 1,500 nm, nanoparticles enter epidermal CD1a+ cells after transcutaneous application on human skin. *J Invest Dermatol*, 126(6):1316–1322, 2006.

[77] J. Lademann, N. Otberg, H. Richter, H.-J. Weigmann, U. Lindemann, H. Schaefer, and W. Sterry. Investigation of follicular penetration of topically applied substances. *Skin Pharmacol Physiol*, 14(Suppl. 1):17–22, 2001.

[78] A. Vogt, C. Wischke, A. T. Neffe, N. Ma, U. Alexiev, and A. Lendlein. Nanocarriers for drug delivery into and through the skin: Do existing technologies match clinical challenges? *J Controlled Release*, 242:3–15, 2016.

[79] R. Alvarez-Román, A. Naik, Y. N. Kalia, H. Fessi, and R. H. Guy. Visualization of skin penetration using confocal laser scanning microscopy. *Eur J Pharm Biopharm*, 58(2):301–316, 2004.

[80] F. Stracke, B. Weiss, C.-M. Lehr, K. König, U. F. Schaefer, and M. Schneider. Multiphoton microscopy for the investigation of dermal penetration of nanoparticle-borne drugs. *J Invest Dermatol*, 126(10):2224–2233, 2006.

[81] E. Decencière, E. Tancrède-Bohin, P. Dokládal, S. Koudoro, A.-M. Pena, and T. Baldeweck. Automatic 3D segmentation of multiphoton images: a key step for the quantification of human skin. *Skin Res Technol*, 19(2):115–124, 2013.

[82] U. Blume-Peytavi and A. Vogt. Human hair follicle: reservoir function and selective targeting. *Br J Dermatol*, 165(s2):13–17, 2011.

[83] J. Lademann, H. Richter, S. Schanzer, F. Knorr, M. Meinke, W. Sterry, and A. Patzelt. Penetration and storage of particles in human skin: perspectives and safety aspects. *Eur J Pharm Biopharm*, 77(3):465–468, 2011.

[84] S. Gordon, M. Daneshian, J. Bouwstra, F. Caloni, S. Constant, D. E. Davies, G. Dandekar, T. Hartung, M. Leist, and C. M. Lehr. Non-animal models of epithelial barriers (skin, intestine and lung) in research, industrial applications and regulatory toxicology. *Altern Anim Ex - ALTEX*, 32(4):327–378, 2015.

[85] N. Lebonvallet, C. Jeanmaire, L. Danoux, P. Sibille, G. Pauly, and L. Misery. The evolution and use of skin explants: potential and limitations for dermatological research. *Eur J Dermatol*, 20(6):671–684, 2010.

[86] I. Parvova, N. Danchev, and E. Hristov. Animal models of human diseases and their significance for clinical studies of new drugs. *J Clin Med*, 4(1):19–29, 2011.

[87] H. Pischon, M. Radbruch, A. Ostrowski, P. Volz, C. Gerecke, M. Unbehauen, S. Hönzke, S. Hedtrich, J. W. Fluhr, others, and L. Mundhenk. Stratum corneum targeting by dendritic core-multishell-nanocarriers in a mouse model of psoriasis. *Nanomed Nanotechnol Biol Med*, 13(1):317–327, 2017.

[88] M. Radbruch, H. Pischon, A. Ostrowski, P. Volz, R. Brodwolf, F. Neumann, M. Unbehauen, B. Kleuser, R. Haag, others, and A. D. Gruber. Dendritic core-multishell nanocarriers in murine models of healthy and atopic skin. *Nanoscale Res Lett*, 12(1):64, 2017.

[89] K. Shortman and Y.-J. Liu. Mouse and human dendritic cell subtypes. *Nat Rev Immunol*, 2(3):151–161, 2002.

[90] C. Auxenfans, J. Fradette, C. Lequeux, B. Germain, L.and Kinikoglu, N. Bechetoille, F. Braye, F. A. Auger, and O. Damour. Evolution of three dimensional skin equivalent

models reconstructed *in vitro* by tissue engineering. *Eur J Dermatol*, 19(2):107–113, 2009.

[91] A. Edlich, C. Gerecke, M. Giulbudagian, F. Neumann, S. Hedtrich, M. Schäfer-Korting, N. Ma, M. Calderón, and B. Kleuser. Specific uptake mechanisms of well-tolerated thermoresponsive polyglycerol-based nanogels in antigen-presenting cells of the skin. *Eur J Pharm Biopharm*, 116:155–163, 2017.

[92] C. Gerecke, A. Edlich, M. Giulbudagian, F. Schumacher, N. Zhang, A. Said, G. Yealland, S. B. Lohan, F. Neumann, others, and B. Kleuser. Biocompatibility and characterization of polyglycerol-based thermoresponsive nanogels designed as novel drug-delivery systems and their intracellular localization in keratinocytes. *Nanotoxicology*, 11(2):267–277, 2017.

[93] K. Obst, G. Yealland, B. Balzus, E. Miceli, M. Dimde, C. Weise, M. Eravci, R. Bodmeier, R. Haag, M. Calderón, N. Charbaji, and S. Hedtrich. Protein corona formation on colloidal polymeric nanoparticles and polymeric nanogels: impact on cellular uptake, toxicity, immunogenicity and drug release properties. *Biomacromolecules*, 2017.

[94] R. Arnold, M. Seifert, K. Asadullah, and H. D. Volk. Crosstalk between keratinocytes and T lymphocytes via Fas/Fas ligand interaction: modulation by cytokines. *J Immunol*, 162(12):7140–7147, 1999.

[95] M. Schäfer-Korting, U. Bock, A. Gamer, A. Haberland, E. Haltner-Ukomadu, M. Kaca, H. Kamp, M. Kietzmann, H. C. Korting, others, and A. Vuia. Reconstructed human epidermis for skin absorption testing: results of the german prevalidation study. *Altern Lab Anim - ATLA*, 34(3):283–94, 2006.

[96] *OECD, OECD guideline for the testing of chemicals. In vitro skin irritation: Reconstructed human epidermis test method No. 439, Adopted 26 July 2013, pp. 1 to 8. 2014.*

[97] *OECD, OECD guideline for the testing of chemicals. In vitro 3T3 NRU phototoxicity test No. 432, Adopted 13 April 2004, pp. 1 to 10. 2004.*

[98] M. Schäfer-Korting, A. Mahmoud, S. L. Borgia, B. Brüggener, B. Kleuser, S. Schreiber, and W. Mehnert. Reconstructed epidermis and full-thickness skin for absorption testing: influence of the vehicles used on steroid permeation. *Altern Lab Anim - ATLA*, 4(36):441–52, 2008.

[99] F. Netzlaff, C. M. Lehr, P. W. Wertz, and U. F. Schaefer. The human epidermis models EpiSkin®, SkinEthic® and EpiDerm®: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. *Eur J Pharm Biopharm*, 60(2):167–178, 2005.

[100] M. Van Gele, B. Geusens, L. Brochez, R. Speeckaert, and J. Lambert. Three-dimensional skin models as tools for transdermal drug delivery: challenges and limitations. *Expert Opin Drug Deliv*, 8(6):705–720, 2011.

[101] N. Alnasif, C. Zoschke, E. Fleige, R. Brodwolf, A. Boreham, E. Rühl, K.-M. Eckl, H.-F. Merk, H. C. Hennies, U. Alexiev, R. Haag, S. Küchler, and M. Schäfer-Korting. Penetration of normal, damaged and diseased skin: an *in vitro* study on dendritic core multishell nanotransporters. *J Controlled Release*, 185:45–50, 2014.

[102] M. Witting, M. Molina, K. Obst, R. Plank, K. M. Eckl, H. C. Hennies, M. Calderón, W. Frieß, and S. Hedtrich. Thermosensitive dendritic polyglycerol-based nanogels for cutaneous delivery of biomacromolecules. *Nanomed Nanotechnol Biol Med*, 11(5):1179–1187, 2015.

[103] F. M. Bätz, W. Klipper, H. C. Korting, F. Henkler, R. Landsiedel, A. Luch, U. von Fritschen, G. Weindl, and M. Schäfer-Korting. Esterase activity in excised and reconstructed human skin–biotransformation of prednicarbate and the model dye fluorescein diacetate. *Eur J Pharm Biopharm*, 84(2):374–385, 2013.

[104] E. H. Van Den Bogaard, G. S. Tjabringa, I. Joosten, M. Vonk-Bergers, E. Van Rijssen, H. J. Tijssen, M. Erkens, J. Schalkwijk, and H. J. Koenen. Crosstalk between keratinocytes and T cells in a 3D microenvironment: a model to study inflammatory skin diseases. *J Invest Dermatol*, 134(3):719–727, 2014.

[105] D. Y. S. Chau, C. Johnson, S. MacNeil, J. W. Haycock, and A. M. Ghaemmaghami. The development of a 3D immunocompetent model of human skin. *Biofab*, 5(3):035011, 2013.

[106] Y. Cai, C. Fleming, and J. Yan. New insights of T cells in the pathogenesis of psoriasis. *Cell Mol Immunol*, 9(4):302–309, 2012.

[107] P. Blanco, A. K. Palucka, V. Pascual, and J. Banchereau. Dendritic cells and cytokines in human inflammatory and autoimmune diseases. *Cytokine Growth Factor Rev*, 19(1):41–52, 2008.

[108] B. De Wever, S. Kurdykowski, and P. Descargues. Human skin models for research applications in pharmacology and toxicology: Introducing NativeSkin®, the "missing link"bridging cell culture and/or reconstructed skin models and human clinical testing. *Appl In Vitro Toxicol*, 1(1):26–32, 2015.

[109] R. C. Wester, J. Christoffel, T. Hartway, N. Poblete, H. I. Maibach, and J. Forsell. Human cadaver skin viability for *in vitro* percutaneous absorption: storage and detrimental effects of heat-separation and freezing. *Pharm Res*, 15(1):82–84, 1998.

[110] J. Sandby-Moller, T. Poulsen, and H. C. Wulf. Epidermal thickness at different body sites: relationship to age, gender, pigmentation, blood content, skin type and smoking habits. *Acta Dermato Venereologica*, 83(6):410–413, 2003.

[111] N. Otberg, H. Richter, H. Schaefer, U. Blume-Peytavi, W. Sterry, and J. Lademann. Variations of hair follicle size and distribution in different body sites. *J Invest Dermatol*, 122(1):14–19, 2004.

[112] B. Mahe, A. Vogt, C. Liard, D. Duffy, V. Abadie, O. Bonduelle, A. Boissonnas, W. Sterry, B. Verrier, U. Blume-Peytavi, and B. Combadiere. Nanoparticle-based targeting of vaccine compounds to skin antigen-presenting cells by hair follicles and their transport in mice. *J Invest Dermatol*, 129(5):1156–1164, 2009.

[113] C. M. Jongh, I. Jakasa, M. M. Verberk, and S. Kezic. Variation in barrier impairment and inflammation of human skin as determined by sodium lauryl sulphate penetration rate. *Br J Dermatol*, 154(4):651–657, 2006.

[114] A. Nangia, B. Berner, and H. I. Maibach. Transepidermal water loss measurements for assessing skin barrier functions during *in vitro* percutaneous absorption studies. In *Percutaneous Absorption: Drugs, Cosmetics, Mechanisms, Methods*, pages 497–507. CRC Press, 2005.

[115] K. Guth, M. Schäfer-Korting, E. Fabian, R. Landsiedel, and B. Van Ravenzwaay. Suitability of skin integrity tests for dermal absorption studies *in vitro*. *Toxicol in Vitro*, 29(1):113–123, 2015.

[116] S. Schreiber, A. Mahmoud, A. Vuia, M. K. Rübbelke, E. Schmidt, M. Schaller, H. Kandarova, A. Haberland, U. F. Schäfer, U. Bock, H. C. Korting, M. Liebsch, and M. Schäfer-Korting. Reconstructed epidermis versus human and animal skin in skin absorption studies. *Toxicol In vitro*, 19(6):813–822, 2005.

[117] F. Rancan, D. Papakostas, S. Hadam, S. Hackbarth, T. Delair, C. Primard, B. Verrier, W. Sterry, U. Blume-Peytavi, and A. Vogt. Investigation of polylactic acid (PLA) nanoparticles as drug delivery systems for local dermatotherapy. *Pharm Res*, 26(8):2027–2036, 2009.

[118] P. A. Lehman, S. G. Raney, and T. J. Franz. Percutaneous absorption in man: *in vitro-in vivo* correlation. *Skin Pharmacol Physiol*, 24(4):224–230, 2011.

[119] F. M. Williams. *In vitro* studies - how good are they at replacing *in vivo* studies for measurement of skin absorption? *Environ Toxicol Pharmacol*, 21(2):199–203, 2006.

[120] Z. Lu, S. Hasse, E. Bodo, C. Rose, W. Funk, and R. Paus. Towards the development of a simplified long-term organ culture method for human scalp skin and its appendages under serum-free conditions. *Exp Dermatol*, 16(1):37–44, 2007.

[121] K. Kleszczyński and T. W. Fischer. Development of a short-term human full-thickness skin organ culture model *in vitro* under serum-free conditions. *Arch Dermatol Res*, 304(7):579–587, 2012.

[122] S. Messager, A. C. Hann, P. A. Goddard, P. W. Dettmar, and J.-Y. Maillard. Assessment of skin viability: is it necessary to use different methodologies? *Skin Res Technol*, 9(4):321–330, 2003.

[123] J. W. Fluhr, K. R. Feingold, and P. M. Elias. Transepidermal water loss reflects permeability barrier status: validation in human and rodent *in vivo* and *ex vivo* models. *Exp Dermatol*, 15(7):483–492, 2006.

[124] J. Kottner, A. Lichterfeld, and U. Blume-Peytavi. Transepidermal water loss in young and aged healthy humans: a systematic review and meta-analysis. *Arch Dermatol Res*, 305(4):315–323, 2013.

[125] S. Luebberding, N. Krueger, and M. Kerscher. Skin physiology in men and women: *in vivo* evaluation of 300 people including TEWL, SC hydration, sebum content and skin surface pH. *Int J Cosmet Sci*, 35(5):477–483, 2013.

[126] F. Netzlaff, K.-H. Kostka, C.-M. Lehr, and U. F. Schaefer. TEWL measurements as a routine method for evaluating the integrity of epidermis sheets in static Franz type diffusion cells *in vitro*. Limitations shown by transport data testing. *Eur J Pharm Biopharm*, 63(1):44–50, 2006.

[127] N. Garcia Bartels, L. Lünnemann, A. Stroux, J. Kottner, J. Serrano, and U. Blume-Peytavi. Effect of diaper cream and wet wipes on skin barrier properties in infants: a prospective randomized controlled trial. *Pediatr Dermatol*, 31(6):683–691, 2014.

[128] W. Grellner, T. Georg, and J. Wilske. Quantitative analysis of proinflammatory cytokines (IL-1β, IL-6, TNF-α) in human skin wounds. *Forensic Sci Int*, 113(1):251–264, 2000.

[129] R. Darlenski, S. Sassning, N. Tsankov, and J. W. Fluhr. Non-invasive *in vivo* methods for investigation of the skin barrier physical properties. *Eur J Pharm Biopharm*, 72(2):295–303, 2009.

[130] K.-P. Wilhelm and H. I. Maibach. Susceptibility to irritant dermatitis induced by sodium lauryl sulfate. *J Am Acad Dermatol*, 23(1):122–124, 1990.

[131] J. W. Fluhr, H. Dickel, O. Kuss, I. Weyher, T. L. Diepgen, and E. Berardesca. Impact of anatomical location on barrier recovery, surface pH and stratum corneum hydration after acute barrier disruption. *Br J Dermatol*, 146(5):770–776, 2002.

[132] H. Dabboue, N. Builles, É. Frouin, D. Scott, J. Ramos, and G. Marti-Mestres. Assessing the impact of mechanical damage on full-thickness porcine and human skin using an *in vitro* approach. *Biomed Res Int*, 2015, 2015.

[133] R. P. Chilcott, C. H. Dalton, A. J. Emmanuel, C. E. Allen, and S. T. Bradley. Transepidermal water loss does not correlate with skin barrier function *in vitro*. *J Invest Dermatol*, 118(5):871–875, 2002.

[134] K. Grice, H. Sattar, H. Baker, and M. Sharratt. The relationship of transepidermal water loss to skin temperature in psoriasis and eczema. *J Invest Dermatol*, 64(5):313–315, 1975.

[135] T. Abe, J. Mayuzumi, N. Kikuchi, and S. Arai. Seasonal variations in skin temperature, skin pH, evaporative water loss and skin surface lipid values on human skin. *Chem Pharm Bull (Tokyo)*, 28(2):387–392, 1980.

[136] J.-P. Hachem, D. Crumrine, J. W. Fluhr, B. E. Brown, K. R. Feingold, and P. M. Elias. pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. *J Invest Dermatol*, 121(2):345–353, 2003.

[137] N. Brunacci, C. Wischke, T. Naolou, and A. Neffe, A. T.and Lendlein. Influence of surfactants on depsipeptide submicron particle formation. *Eur J Pharm Biopharm*, 116:61–65, 2017.

[138] E. Benfeldt, J. Serup, and T. Menne. Effect of barrier perturbation on cutaneous salicylic acid penetration in human skin: *in vivo* pharmacokinetics using microdialysis and non-invasive quantification of barrier function. *Br J Dermatol*, 140:739–748, 1999.

[139] N. R. Blickenstaff, G. Coman, C. M. Blattner, R. Andersen, and H. I. Maibach. Biology of percutaneous penetration. *Rev Environ Health*, 29(3):145–155, 2014.

[140] T. Smits, C. A. Robles, P. E. J. van Erp, P. C. M. van de Kerkhof, and M.-J. P. Gerritsen. Correlation between macroscopic fluorescence and protoporphyrin IX content in psoriasis and actinic keratosis following application of aminolevulinic acid. *J Invest Dermatol*, 125(4):833–839, 2005.

[141] L. H. Basse, D. Groen, and J. A. Bouwstra. Permeability and lipid organization of a novel psoriasis stratum corneum substitute. *Int J Pharm*, 457(1):275–282, 2013.

[142] C.-F. Hung, W.-Y. Chen, C.-Y. Hsu, I. A. Aljuffali, H.-C. Shih, and J.-Y. Fang. Cutaneous penetration of soft nanoparticles via photodamaged skin: Lipid-based and polymer-based nanocarriers for drug delivery. *Eur J Pharm Biopharm*, 94:94–105, 2015.

[143] H. Trommer and R. H. H. Neubert. Overcoming the stratum corneum: the modulation of skin penetration. *Skin Pharmacol Physiol*, 19(2):106–121, 2006.

[144] H. A. E. Benson. Transdermal drug delivery: penetration enhancement techniques. *Curr Drug Deliv*, 2(1):23–33, 2005.

[145] S. Küchler, M. R. Radowski, T. Blaschke, M. Dathe, J. Plendl, R. Haag, M. Schäfer-Korting, and K. D. Kramer. Nanoparticles for skin penetration enhancement–a comparison of a dendritic core-multishell-nanotransporter and solid lipid nanoparticles. *Eur J Pharm Biopharm*, 71(2):243–250, 2009.

[146] R. H. Müller, S. Gohla, and C. M. Keck. State of the art of nanocrystals–special features, production, nanotoxicology aspects and intracellular delivery. *Eur J Pharm Biopharm*, 78(1):1–9, 2011.

[147] R. Pireddu, C. Caddeo, D. Valenti, F. Marongiu, A. Scano, G. Ennas, F. Lai, A. M. Fadda, and C. Sinico. Diclofenac acid nanocrystals as an effective strategy to reduce *in vivo* skin inflammation by improving dermal drug bioavailability. *Colloids Surf , B*, 143:64–70, 2016.

[148] A. Vogt, S. Hadam, I. Deckert, J. Schmidt, A. Stroux, Z. Afraz, F. Rancan, J. Lademann, B. Combadiere, and U. Blume-Peytavi. Hair follicle targeting, penetration enhancement and Langerhans cell activation make cyanoacrylate skin surface stripping a promising delivery technique for transcutaneous immunization with large molecules and particle-based vaccines. *Exp Dermatol*, 24(1):73–75, 2015.

[149] J. Reuter, A. Jocher, J. Stump, B. Grossjohann, G. Franke, and C. M. Schempp. Investigation of the anti-inflammatory potential of aloe vera gel (97.5%) in the ultraviolet erythema test. *Skin Pharmacol Physiol*, 21(2):106–110, 2008.

[150] S. Zöller, N. N.and Kippenberger, D. Thaçi, K. Mewes, M. Spiegel, A. Sättler, M. Schultz, J. Bereiter-Hahn, R. Kaufmann, and A. Bernd. Evaluation of beneficial and adverse effects of glucocorticoids on a newly developed full-thickness skin model. *Toxicol in Vitro*, 22(3):747–759, 2008.

[151] G. D. Keeler, J. M. Durdik, and J. A. Stenken. Effects of delayed delivery of dexamethasone-21-phosphate via subcutaneous microdialysis implants on macrophage activation in rats. *Acta Biomater*, 23:27–37, 2015.

[152] F. Sjögren, M. Davidsson, K.and Sjöström, and C. D. Anderson. Cutaneous microdialysis: cytokine evidence for altered innate reactivity in the skin of psoriasis patients? *The AAPS journal*, 14(2):187–195, 2012.

[153] G. Hübner, M. Brauchle, H. Smola, M. Madlener, R. Fässler, and S. Werner. Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. *Cytokine*, 8(7):548–556, 1996.

[154] M. Frušić-Zlotkin, Y. Soroka, R. Tivony, L. Larush, L. Verkhovsky, F. M. Brégégère, R. Neuman, S. Magdassi, and Y. Milner. Penetration and biological effects of topically

applied cyclosporin A nanoparticles in a human skin organ culture inflammatory model. *Exp Dermatol*, 21(12):938–943, 2012.

[155] S. Gupta, R. Bansal, S. Gupta, N. Jindal, and A. Jindal. Nanocarriers and nanoparticles for skin care and dermatological treatments. *Indian Dermatol Online J*, 4(4):267, 2013.

[156] C. Scheicher, M. Mehlig, H.-P. Dienes, and K. Reske. Uptake of bead-adsorbed versus soluble antigen by bone marrow derived dendritic cells triggers their activation and increases their antigen presentation capacity. In *Dendritic Cells in Fundamental and Clinical Immunology*, pages 253–255. Springer, 1995.

[157] A. F. Charest, J. McDougall, and M. B. Goldstein. A randomized comparison of intradermal and intramuscular vaccination against hepatitis B virus in incident chronic hemodialysis patients. *Am J Kidney Dis*, 36(5):976–982, 2000.

[158] P. H. M. Hoet, I. Brüske-Hohlfeld, and O. V. Salata. Nanoparticles–known and unknown health risks. *J Nanobiotechnology*, 2(1):12, 2004.

[159] G. Zoubari, S. Staufenbiel, P. Volz, U. Alexiev, and R. Bodmeier. Effect of drug solubility and lipid carrier on drug release from lipid nanoparticles for dermal delivery. *Eur J Pharm Biopharm*, 110:39–46, 2017.

[160] K. König and I. Riemann. High-resolution multiphoton tomography of human skin with subcellular spatial resolution and picosecond time resolution. *J Biomed Opt*, 8(3):432–440, 2003.

[161] W. R. Zipfel, R. M. Williams, and W. W. Webb. Nonlinear magic: multiphoton microscopy in the biosciences. *Nat Biotechnol*, 21(11):1369, 2003.

[162] N. Döge, E. Thiel, G. Seewald, S. Albrecht, A. Vogt, J. Liebscher, and K.-H. Schönborn. Wide-field two photon microscopy - transcending technology limitations. *Optik & Photonik*, 10(5):39–42, 2015.

[163] C. S. J. Campbell, L. R. Contreras-Rojas, M. B. Delgado-Charro, and R. H. Guy. Objective assessment of nanoparticle disposition in mammalian skin after topical exposure. *J Controlled Release*, 162(1):201–207, 2012.

[164] R. Alvarez-Román, A. Naik, Y. N. Kalia, R. H. Guy, and H. Fessi. Skin penetration and distribution of polymeric nanoparticles. *J Controlled Release*, 99(1):53–62, 2004.

[165] N. S. H. Motlagh, P. Parvin, F. Ghasemi, and F. Atyabi. Fluorescence properties of several chemotherapy drugs: doxorubicin, paclitaxel and bleomycin. *Biomed Opt Express*, 7(6):2400–2406, 2016.

[166] J. Lademann, A. Richter, H.and Teichmann, N. Otberg, U. Blume-Peytavi, J. Luengo, B. Weiss, U. F. Schaefer, C.-M. Lehr, R. Wepf, and W. Sterry. Nanoparticles–an efficient carrier for drug delivery into the hair follicles. *Eur J Pharm Biopharm*, 66(2):159–164, 2007.

[167] A. C. Lauer, C. Ramachandran, L. M. Lieb, S. Niemiec, and N. D. Weiner. Targeted delivery to the pilosebaceous unit via liposomes. *Advanced drug delivery reviews*, 18(3):311–324, 1996.

[168] R. Toll, U. Jacobi, H. Richter, J. Lademann, H. Schaefer, and U. Blume-Peytavi. Penetration profile of microspheres in follicular targeting of terminal hair follicles. *J Invest Dermatol*, 123(1):168–176, 2004.

[169] V. M. Meidan, M. C. Bonner, and B. B. Michniak. Transfollicular drug delivery-is it a reality? *Int J Pharm*, 306(1):1–14, 2005.

[170] A. Patzelt, H. Richter, F. Knorr, U. Schäfer, L. Lehr, C.-M.and Dähne, W. Sterry, and J. Lademann. Selective follicular targeting by modification of the particle sizes. *J Controlled Release*, 150(1):45–48, 2011.

[171] B. Baroli, M. G. Ennas, F. Loffredo, M. Isola, R. Pinna, and M. A. López-Quintela. Penetration of metallic nanoparticles in human full-thickness skin. *J Invest Dermatol*, 127(7):1701–1712, 2007.

[172] F. Rancan, M. Asadian-Birjand, S. Dogan, C. Graf, L. Cuellar, S. Lommatzsch, U. Blume-Peytavi, M. Calderón, and A. Vogt. Effects of thermoresponsivity and softness on skin penetration and cellular uptake of polyglycerol-based nanogels. *J Controlled Release*, 228:159–169, 2016.

[173] B. A. van den Bergh, J. Vroom, H. Gerritsen, H. E. Junginger, and J. A. Bouwstra. Interactions of elastic and rigid vesicles with human skin *in vitro*: electron microscopy and two-photon excitation microscopy. *Biochim Biophys Acta - Biomembranes*, 1461(1):155–173, 1999.

[174] P. L. Honeywell-Nguyen, G. S. Gooris, and J. A. Bouwstra. Quantitative assessment of the transport of elastic and rigid vesicle components and a model drug from these vesicle formulations into human skin *in vivo*. *J Invest Dermatol*, 123(5):902–910, 2004.

[175] G. Cevc, A. Schätzlein, and H. Richardsen. Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements. *Biochim Biophys Acta - Biomembranes*, 1564(1):21–30, 2002.

[176] M. J. Morilla and E. L. Romero. Carrier deformability in drug delivery. *Curr Pharm Des*, 22(9):1118–1134, 2016.

[177] M. Asadian-Birjand, J. Bergueiro, F. Rancan, J. C. Cuggino, R.-C. Mutihac, K. Achazi, J. Dernedde, U. Blume-Peytayi, A. Vogt, and M. Calderón. Engineering thermoresponsive polyether-based nanogels for temperature dependent skin penetration. *Polym Chem*, 6(32):5827–5831, 2015.

[178] K. Rizi, R. J. Green, M. X. Donaldson, and A. C. Williams. Using pH abnormalities in diseased skin to trigger and target topical therapy. *Pharm Res*, 28(10):2589–2598, 2011.

[179] A. Abtin, L. Eckhart, M. Mildner, M. Ghannadan, J. Harder, J.-M. Schröder, and E. Tschachler. Degradation by stratum corneum proteases prevents endogenous RNase inhibitor from blocking anti-microbial activities of RNase 5 and RNase 7. *J Invest Dermatol*, 129(9):2193–2201, 2009.

[180] E. Fleige, M. A. Quadir, and R. Haag. Stimuli-responsive polymeric nanocarriers for the controlled transport of active compounds: concepts and applications. *Adv Drug Delivery Rev*, 64(9):866–884, 2012.

[181] S. Hönzke, C. Gerecke, A. Elpelt, N. Zhang, M. Unbehauen, V. Kral, E. Fleige, F. Paulus, R. Haag, M. Schäfer-Korting, B. Kleuser, and S. Hedtrich. Tailored dendritic core-multishell nanocarriers for efficient dermal drug delivery: a systematic top-down approach from synthesis to preclinical testing. *J Controlled Release*, 242:50–63, 2016.

[182] A. Ahluwalia. Topical glucocorticoids and the skin-mechanisms of action: an update. *Mediators Inflamm*, 7(3):183–193, 1998.

[183] W. V. Berghe, E. Francesconi, K. De Bosscher, M. Resche-Rigon, and G. Haegeman. Dissociated glucocorticoids with anti-inflammatory potential repress interleukin-6 gene expression by a nuclear factor-κb-dependent mechanism. *Mol Pharmacol*, 56(4):797–806, 1999.

[184] K. De Bosscher, W. V. Berghe, and G. Haegeman. Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. *J Neuroimmunol*, 109(1):16–22, 2000.

[185] M. Schäfer-Korting, B. Kleuser, M. Ahmed, H.-D. Höltje, and H. C. Korting. Glucocorticoids for human skin: new aspects of the mechanism of action. *Skin Pharmacol Physiol*, 18(3):103–114, 2005.

[186] K. De Bosscher, W. V. Berghe, and G. Haegeman. The interplay between the glucocorticoid receptor and nuclear factor-κb or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev*, 24(4):488–522, 2003.

[187] M. Furue and S. I. Katz. Direct effects of glucocorticosteroids on epidermal Langerhans cells. *J Invest Dermatol*, 92(3):342–347, 1989.

[188] M. Giulbudagian, G. Yealland, S. Hönzke, A. Edlich, B. Geisendörfer, B. Kleuser, S. Hedtrich, and M. Calderón. Breaking the barrier-potent anti-inflammatory activity following efficient topical delivery of etanercept using thermoresponsive nanogels. *Theranostics*, 8(2):450, 2018.

[189] O. Čulić, V. Eraković, and M. J. Parnham. Anti-inflammatory effects of macrolide antibiotics. *Eur J Pharmacol*, 429(1):209–229, 2001.

[190] A. Patzelt, H. Richter, R. Buettemeyer, H. J. Huber, U. Blume-Peytavi, W. Sterry, and J. Lademann. Differential stripping demonstrates a significant reduction of the hair follicle reservoir *in vitro* compared to *in vivo*. *Eur J Pharm Biopharm*, 70(1):234–238, 2008.

[191] A. Teichmann, M. Ossadnik, H. Richter, W. Sterry, and J. Lademann. Semiquantitative determination of the penetration of a fluorescent hydrogel formulation into the hair follicle with and without follicular closure by microparticles by means of differential stripping. *Skin Pharmacol Physiol*, 19(2):101–105, 2006.

[192] A. Vogt, N. Mandt, J. Lademann, H. Schaefer, and U. Blume-Peytavi. Follicular targeting–a promising tool in selective dermatotherapy. In *J Investig Dermatol Symp Proc*, volume 10, pages 252–255. Elsevier, 2005.

[193] U. Blume-Peytavi and A. Vogt. Translational positioning of janus kinase (JAK) inhibitors in alopecia areata. *EBioMedicine*, 2(4):282–283, 2015.