Electron paramagnetic resonance spectroscopy as method for investigating the redox status in inflammatory skin

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List of abbreviations

AD	atopic dermatitis
CCR	carbon-centered radicals
DMPO	5,5-dimethyl-1-pyrroline-*N*-oxide
DNA	deoxyribonucleic acid
DUOX	dual oxidase
EPR	electron paramagnetic resonance
FLG	filaggrin
GSH	glutathione
GSSG	glutathione disulfide
H_2DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
IFN	interferon
IL	interleukin
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
NIR	near-infrared
NMR	nuclear magnetic resonance
NOX	NADPH oxidase
PCA	3-(carboxy)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy
RNS	reactive nitrogen species
ROS	reactive oxygen species
SC	stratum corneum
SOD	superoxide dismutase
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TNF	tumor necrosis factor
UV	ultraviolet
VIS	visible

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1 Introduction

Oxidative stress describes the imbalance between oxidants and antioxidants and is involved in a variety of diseases like cancer, cardiovascular and neurodegenerative diseases [1]. To determine the oxidative stress or redox status in tissues and cells, oxidants and antioxidants have often been investigated individually [2–4]. However, these methods are very time-consuming and cost-intensive due to preparation of tissue or cell homogenates and the need for numerous assays [5]. In addition, the results usually only reflect the endpoint of the investigation and give only few information about kinetics. A fast and easy method to get an overview of the redox status in real time would be advantageous, e.g. to detect potential differences in pathological conditions. The direct detection of free radicals is a major challenge, as they only occur in low concentrations in biological material due to their short lifetime in the micro- and nanosecond range [6–9]. In order to detect radicals in real time, electron paramagnetic resonance (EPR) spectroscopy represents a possible method where various information about the redox status can be obtained [7]. The skin is a suitable target organ to investigate the redox status, since it is continuously exposed to an oxidative environment [6]. In some chronic inflammatory skin diseases, e.g. atopic dermatitis (AD), the role of the redox status has not yet been fully elucidated [10]. Here, EPR spectroscopy might be a powerful tool to get further insight into the redox status of this tissue, showing the potential of EPR in dermatological research.

1.1 Skin

1.1.1 Structure and function of human skin

The skin of a human adult has an area of about 2 m^2 and accounts for about 16 % of total body weight [11]. It serves as a barrier between the organism and its external environment and thus has several important functions. The skin protects against physical influences like solar radiation, chemical influences like xenobiotics and biological influences like microorganisms [12,13]. Furthermore, it is greatly involved in thermoregulation and sense of touch and is responsible for vitamin D synthesis. Structurally, skin is divided into the three layers epidermis, dermis and hypodermis, into which skin appendages such as hair follicles, nails, sweat glands and sebaceous glands are incorporated (Figure 1) [14,15].

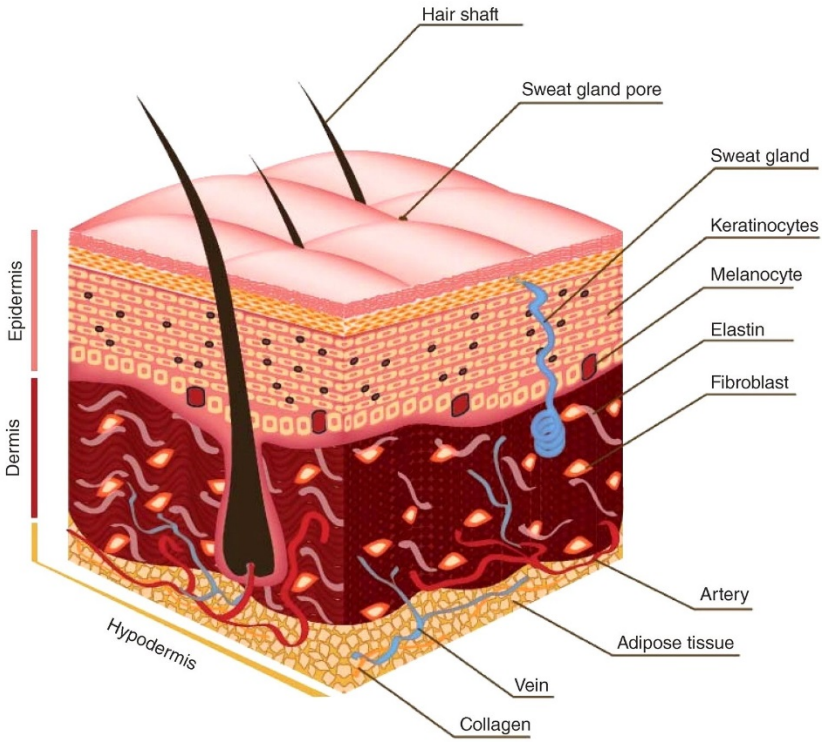


Figure 1: Structure of the skin. Skin is composed of the three layers epidermis, dermis and hypodermis. Reprinted with the permission from [14].

The outermost skin layer, the epidermis, is a stratified squamous epithelium including four or five layers where the maturation and differentiation of keratinocytes take place. The epidermis consists primarily of keratinocytes, which make up approximately 90 % of its bulk. Keratinocytes proliferate in the innermost epidermal layer, the *stratum basale*. The newly formed cells move upwards through the *stratum spinosum* and *stratum granulosum* during differentiation. The *stratum lucidum* is located above the *stratum granulosum* but is only present in thick skin areas such as on the palm of the hands and soles of the feet [15]. The final layer of the keratinized epidermis is the *stratum corneum* (SC) with a varying thickness from 10 to 20 µm [16,17], that depends on body regions, age and gender [17,18]. The human SC consists of 15 to 25 layers of flattened, anucleated corneocytes containing keratin filaments, enzymes and water. The corneocytes are enveloped by a densely cross-linked protein network known as the cornified envelope, which includes filaggrin (filament-aggregating protein, *FLG*), loricrin and involucrin. A monolayer of nonpolar lipids is bound to this protein layer. This lipid envelope serves as a template for the formation of the intercellular lipid matrix surrounding the corneocytes [19,20]. The main lipid classes of the intercellular lipid matrix in the human SC are ceramides, cholesterol and free fatty acids with an equal molar ratio. Additionally, the lipid matrix also consists of small amounts of cholesterol sulphate and other lipids [21]. The synthesis of the skin lipids takes place within the keratinocytes. Firstly, the polar precursors sphingomyelins and glucosylceramides (for

ceramides), cholesterol sulphate (for cholesterol) and phospholipids (for free fatty acids) are produced and stored with catabolic enzymes in lamellar granules. At the interface between the *stratum granulosum* and the SC, the granules migrate to the surface of the keratinocytes, where they fuse with the plasma membrane and the lipid precursors and enzymes are released into the intercellular space of the SC. The active enzymes convert the precursors into lipids, which assemble into multilamellar structures in the human SC and fill the intercellular space between the corneocytes [20]. The compactness of the intercellular lipid matrix contributes significantly to the barrier function of the skin, which on the one hand protects the body from excessive water loss and on the other hand prevents the penetration of foreign microorganisms and substances from the environment [1]. Corneodesmosomes are also responsible for the stability and cohesion of the barrier, connecting the corneocytes primarily via glycoproteins including corneodesmosin [22]. The SC has a low water content of only 15 % to 30 %, where the intercellular lipid matrix contributes to [23]. The SC surface is acidic and has a pH between 4.1 and 5.8, which is important to the maintenance of a healthy skin microbiome. Furthermore, a low pH also plays an important role in the ceramide synthesis, since the responsible enzymes such as β-glucocerebrosidase and acid sphingomyelinase are most active at low pH [24].

The epidermis undergoes constant shedding and renewal, where the turn-over of intact epidermis takes approximately 26 to 28 days [25]. Furthermore, melanocytes are located in the *stratum basale* of the epidermis that produce the pigment melanin, which protects against damage from ultraviolet (UV) radiation and is responsible for skin pigmentation. Further cell types in the epidermis are Merkel cells, which are responsible for the sense of touch and Langerhans cells, which are involved in the immune response to defend against invading microorganisms and foreign substances [15]. The epidermis forms an extensive interface with the underlying skin layer, the dermis, which is described as the epidermal-dermal junction zone. Between these two skin layers there is the basement membrane including the *lamina lucida*, *lamina densa* and *lamina fibroreticularis*. The basement membrane is connected to the *stratum basale* of the epidermis by anchoring filaments and hemidesmosomes and to the dermis by anchoring fibrils, dermal microfibril bundles and collagen fibers [26,27]. The epidermal-dermal junction is characterized by downward projections of the epidermis (rete ridges) and upward projections of the dermis (dermal papillae) [25]. This zone serves on the one hand for the mechanical connection between epidermis and dermis and on the other hand for the exchange of signals and molecules [26,27].

The dermis consists of the two layers: papillary and reticular dermis. The main cell type are fibroblasts that are embedded in a gel-like matrix composed of fibrous proteins such as fibroblast derived collagen and elastin as well as proteoglycans and hyaluronic acid, which is called extracellular matrix. This matrix is responsible for the stability and elasticity of connective tissue. The upper papillary dermis is thinner, has a higher cell density and fine collagen bundles and is more vascularized and innervated. In contrast, the reticular dermis is thicker, shows a lower cell density and well-organized collagen bundles. Furthermore, the dermis also includes macrophages, mast cells, neutrophils, lymphocytes as well as the vascular and lymphatic systems. The hypodermis is located under the dermis and represents the upper part of the subcutaneous tissue. It consists mainly of adipocytes and connective tissue and serves as energy storage, thermal insulation and protection against mechanical influences [25].

1.1.2 Skin models in dermatological research

Due to ethical and practical reasons, clinical studies are not always feasible. For this reason, different skin models are used in the investigation of dermatologically relevant aspects in current basic and preclinical research. The aim of the skin models used is to reflect the structural and functional properties of *in vivo* skin in the best possible way. Excised human skin, which is mostly obtained from plastic surgery, represents the best surrogate for *in vivo* skin. However, availability is limited and ethical approval is required to use *ex vivo* human skin. In addition, different body regions show differences in e.g. SC thickness, hydration and lipid composition [11].

A further skin model frequently used in dermatological research is *ex vivo* porcine skin. As a waste product of slaughterers or animal experiments, porcine skin is easy to obtain. This is used, among other things, for the investigation of transdermal drug testing, melanoma and psoriasis research, wound healing and therapies and skin aging [11,25]. Porcine skin shows strong similarities in anatomy and physiology to human skin [28–30]. The cell composition, epidermal and dermal thickness and epidermal-dermal junction zone between the two skin models are very similar. Furthermore, they show similarities in SC lipid composition, collagen and elastin content in the dermis as well as in immune cell composition and melanocyte distribution. In addition, the hair-follicle density in porcine skin is similarly low as in humans in contrast to murine skin. However, in porcine skin the fat tissue is thicker and the SC lipid packing less dense than in human skin [11,25].

Mice are also often used in dermatological research and are considered the most widely used animal models for pathophysiological studies. Thus, they are used for the research of immunological diseases, cancer, skin repair, genetic diseases and hair disorders [25]. Hence, mice are the most commonly used skin models to study AD. Since mice cannot

develop AD spontaneously, atopic lesions can be induced e.g. genetically or by topical application of haptens like oxazolone [31]. Advantages of mice are that they are easy to obtain and handle as well as they have a short generation time compared to e.g. pigs, making them well suited for genetic modifications. Furthermore, in contrast to other skin models, systematic effects in mice can be investigated. However, due to structural, physiological and molecular differences in murine skin compared to human skin, it is often difficult to transfer the data obtained from murine skin to human skin [25]. The differences include a smaller thickness of all three skin layers epidermis, dermis and hypodermis [25,32,33], a three times faster epidermal renewal (8-10 days), a lack in the epidermal rete ridges as well as different melanocyte distribution to human skin. Furthermore, murine skin shows genetic differences to human skin [25]. Nevertheless, ethical approval is also required for working with *in vivo* mice.

To reduce the use of animal models, tissue-engineered human skin equivalents have been developed that mimic the main structural and functional properties of human skin *in vitro*. Reconstructed skin equivalents are divided into reconstructed epidermis equivalents and full-thickness skin equivalents. Epidermal equivalents only consist of human keratinocytes building a sheet of stratified epithelia and represents the first generated tool *in vitro*. In order to improve the histological quality of these models, three-dimensional skin equivalents were developed. Primary keratinocytes are seeded on a dermal substrate containing dermal primary fibroblasts. Dermal substrates can be of natural origin (collagen, fibrin, hyaluronic acid) or synthetic hydrogels. After 24 h, the skin construct is moved to the air-medium interface to promote epidermal differentiation and stratification. Advantages of skin equivalents are that they consist of human skin cells, show a comparable morphology and therefore no differences in species occur [25]. In addition, they are commercially available, which results in high availability and no ethical approval is required for commercially obtained skin equivalents. A further advantage of skin equivalents is that they can emulate certain characteristics of skin diseases *in vitro*. Skin equivalents of various skin diseases already exist, such as non-melanoma skin cancer [34], atopic dermatitis [35] or psoriasis [36]. Therefore, skin equivalents are mainly used to investigate cellular and molecular mechanisms of cutaneous pathophysiological conditions. In addition, skin equivalents are used for safety analysis of cosmetics and toxicity testing of drugs [37]. Nevertheless, skin equivalents lack of immune cells and the blood vessel system found in human skin and have an altered lipid composition in the SC, giving them clearly higher permeability compared to other skin models [25]. However, in the last generation of skin equivalents different cell types, like adipocytes or T cells and skin appendages, like hair follicle, were incorporated into the skin equivalents [25,31].

1.1.3 Solar radiation as exogenous stress factor

As interface between body and environment, the skin is permanently exposed to physical, chemical and biological environmental influences. These include xenobiotics, exhaust gases, tobacco smoke, microbes and as main noxious agent solar radiation [12,13]. Solar radiation composed of the following wavelength ranges: 5 % UV from 200 to 400 nm, 50 % visible (VIS) from 400 to 740 nm and 45 % infrared (IR) radiation from 740 nm to 1 mm. UV radiation is divided into UVC (200-280 nm), UVB (280-320 nm) and UVA (320-400 nm), whereby UVC is absorbed by the ozone layer and does not reach the surface of the earth [38,39]. IR radiation is also further divided into IRA (740-1400 nm), IRB (1400-3000 nm) and IRC (3000 nm-1 mm), where IRA is also known as near-infrared (NIR) radiation [38,40]. The penetration depth of the radiation into the skin depends on its wavelength. Accordingly, the short-wave and therefore high-energy UVB radiation reaches the basal cell layer of the viable epidermis, where UVA radiation penetrates deeper into the dermis. VIS and NIR radiation can even penetrate into the hypodermis [38].

UV exposure has positive effects, such as vitamin D synthesis in the skin, stimulation of hormones that regulate the circadian rhythm and mood as well as the use in photodynamic therapy. However, excessive UV radiation can lead to skin damage, which can be divided into acute and chronic damage. Acute damages include sunburn, inflammation, tanning and local as well as systemic immunosuppression. Chronic damages also include immunosuppression and furthermore photoaging and photocarcinogenesis [41]. UV radiation can interact directly or indirectly with biomolecules in tissue. In the direct interaction, molecules absorb UV radiation and undergo chemical modification. The most common direct interaction is the reaction of UVB radiation with deoxyribonucleic acid (DNA) which can lead to the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) photoproducts representing crosslinks between pyrimidine bases. In the indirect interaction endogenous photosensitizers of the skin, such as melanin, nicotinamide adenine dinucleotide (NADH) or flavins, absorb UV radiation and change to an excited state. The excited species transfer the energy to molecular oxygen (O_2) when returning to the ground state which leads to the formation of free radicals, especially singlet oxygen ($^1O_2$) or superoxide anion ($\cdot O_2^-$). Furthermore, UV radiation stimulates inflammatory processes which also lead to the accumulation of free radicals [41,42]. Although most attention is focused on UV radiation in the case of solar radiation, it has been shown that actually up to 49 % of the radicals are produced by the VIS and NIR radiation of solar radiation [43–45]. This illustrates that the whole range of solar radiation should be considered for investigations of sun-induced skin effects.

1.2 Redox status

1.2.1 Oxidants and antioxidants

Solar radiation and other exogenous stress factors either act as oxidants or induce their formation [12]. The most important oxidants include free radicals and related species, which are divided into the main groups reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are generated from molecular oxygen and includes radicals, e.g. superoxide anion ($\cdot O_2^-$) and hydroxyl radical ($\cdot OH$) as well as non-radicals like hydrogen peroxide (H_2O_2). RNS are derived from nitrogen and include e.g. the radical nitric oxide ($NO\cdot$) and the non-radical peroxynitrite ($ONOO^-$) [1,46]. Reactive lipid species (RLS) like carbon-centered radicals (CCR) ($\cdot R$) and alkoxyl radicals ($\cdot RO$) represent a further group of reactive species, which are produced by the reaction of ROS with lipid molecules of cell membranes [46,47]. In addition to environmental factors, ROS and RNS are constantly produced during physiological processes, e.g. the mitochondrial electron transport chain, immune response of phagocytes and enzymatic reactions [48–50]. In moderate levels, reactive species are involved in numerous cellular processes and act as important regulators in signal transduction, e.g. in cell proliferation and differentiation, apoptosis and immune-inflammatory responses to internal and external factors [47,49,51].
Although free radicals are necessary for several physiological functions, they can become harmful with excessive production. Since free radicals are unstable and highly reactive due to their unpaired electrons, they attack biomolecules, such as proteins, lipids and DNA, to achieve a stable state. In this process, the biomolecules are oxidized by the radicals taking electrons from them. This turns the biomolecules themselves into radicals, which in turn can attack other molecules and continue the chain reaction. Due to the excessive radical formation, the balance between oxidants and antioxidants, known as redox status, shifts towards oxidants, creating an imbalance which is described as oxidative stress. Oxidative stress leads to protein oxidation, lipid peroxidation and DNA mutation, which can lead to defective gene expression and protein formation. As a result, the functions of molecules can be reduced or lost, causing the development of pathological conditions [1,48,51]. These radical chain reactions continue till it is stopped by anaerobic conditions, substrate depletion or by antioxidants [52].

The body includes a complex antioxidative protection system consisting of endogenous and exogenous components which physiologically rapidly neutralizes ROS and RNS to protect the body against oxidative attacks and keep the balance. Endogenous antioxidants are produced by the cells themselves and include enzymatic antioxidants, e.g. catalase, superoxide dismutase (SOD) and non-enzymatic antioxidants, e.g. glutathione (GSH) [53]. In contrast, exogenous antioxidants cannot be produced by cells and have to be taken up

by food, especially through fruits and vegetables. Examples are carotenoids, ascorbic acid (vitamin C) and α-tocopherol (vitamin E) [52]. Antioxidants stop the described chain reaction by also undergoing oxidation by radicals, without becoming radicals themselves. The oxidized form can be then reduced and recycled to its initial form by e.g. other antioxidants [1,54]. For example, GSH is oxidized to glutathione disulfide (GSSG), a non-radical molecule, which is then recycled to GSH by glutathione reductase [55].

1.2.2 Redox status in skin

Due to their structure, the skin layers are exposed to environmental influences of varying intensity and have different protective systems according to their composition. The outermost layer of the epidermis, the SC, is exposed most to the oxidative environment. The SC of healthy human skin shows mainly lipophilic non-enzymatic antioxidants due to its high lipid content in the intercellular lipid matrix. These include the main antioxidant α-tocopherol, which mainly protects against lipid peroxidation and stabilize the lipid bilayers in the SC. It is distributed in a gradient with decreasing concentrations from the innermost to the outermost layer of the SC [53]. Further antioxidants in the SC are ubiquinol (coenzyme Q10), squalene and sterols which are taken up by food or produced by sebum glands. In the viable epidermis, α-tocopherol is also the predominant lipophilic antioxidant in addition to enzymatic antioxidants such as catalases, SOD, GSH peroxidases and peroxiredoxins. Furthermore, hydrophilic non-enzymatic antioxidants such as ascorbic acid, uric acid and GSH also occur in the epidermis. The extracellular matrix of the dermis contains high amounts of hydrophilic antioxidants, e.g. ascorbic acid, uric acid and GSH. Additionally, endogenous melatonin and its metabolites were detected in keratinocytes, melanocytes and fibroblasts. Due to their scavenging properties for the hydroxyl radicals (·OH), they are the most potent protectors against UV radiation-induced oxidative stress [13,47]. The diversity of antioxidants in the skin can be explained by the need for protection against the high amount of endogenously and exogenously produced radicals [13]. In Figure 2, the redox status in skin cells is shown. It represents the interaction between the radicals caused by environmental factors (solar radiation and xenobiotics), such as superoxide anion ($\cdot O_2^-$) and singlet oxygen ($^1O_2$) and the endogenous antioxidants, such as SOD, GSH system, catalase and nitric oxide synthase [12].

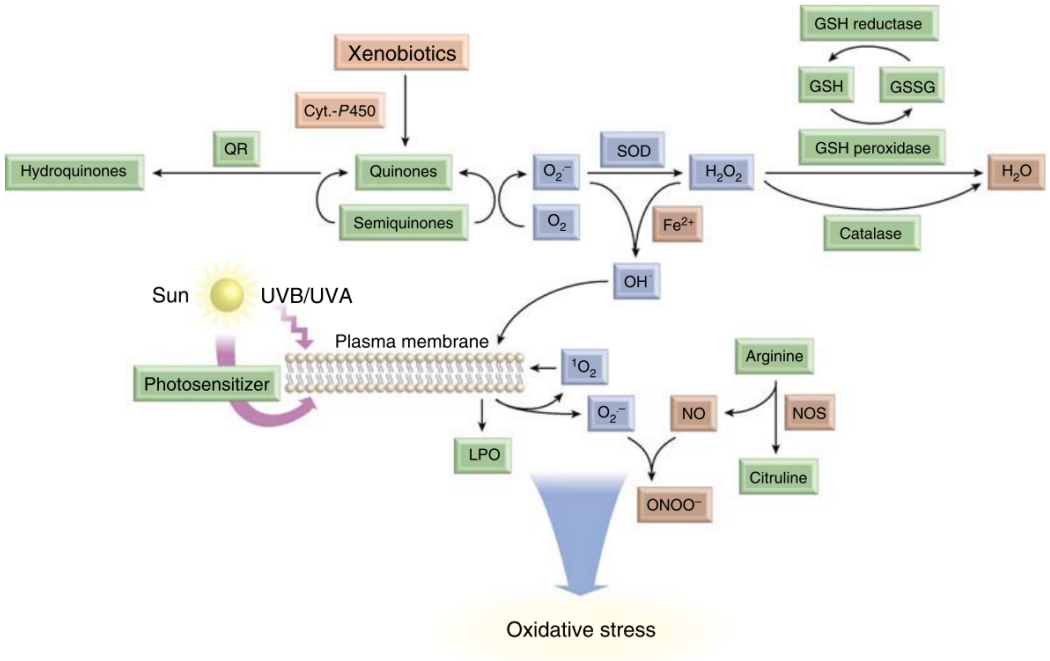


Figure 2: Overview of the redox status in skin cells. Exogenous stress factors such as solar radiation and xenobiotics lead to the production of ROS and RNS. Solar radiation and the presence of photosensitizers lead to the production of singlet oxygen ($^1O_2$) and superoxide anion ($\cdot O_2^-$). Superoxide anion can on the one hand react with nitric oxide (NO·), which is produced during the conversion of arginine into citrulline by nitric oxide synthase (NOS), to form peroxynitrite ($ONOO^-$). On the other hand, superoxide anion is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), which in turn is reduced to water (H_2O) by catalase or the GSH system. Hydrogen peroxide and superoxide anion can also react to the hydroxyl radical (·OH) in an iron-dependent reaction. This in turn can attack cell membranes and lead to lipid peroxidation (LPO). Xenobiotics lead by means of cytochrome P450 enzymes to the production of redox-sensitive quinones, which can be reversibly reduced to semiquinones, generating superoxide anions. Superoxide anions are neutralized as already described. Quinones can be also reduced into less toxic hydroquinones by quinone reductase (QR). Reprinted with the permission from [12].

1.2.3 Role of redox status in the inflammatory skin disease atopic dermatitis

The skin, as the first line of defense against environmental influences, is particularly susceptible for oxidative stress and the various associated diseases. It is already known that oxidative stress is involved in the development of skin cancer [51,56] and skin aging [57]. Additionally, it is also known that oxidative stress is associated with tissue inflammation because ROS/RNS induce and upregulate the expression of proinflammatory cytokine genes such as interleukin- (IL-) 1, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) through the activation of the transcription factor nuclear factor kappa B (NF-κB) [10,58]. These inflammatory reactions lead to the activation of immune cells, in particular polymorphonuclear leukocytes and macrophages, which then infiltrate into the inflammatory tissue [59]. These immune cells also release ROS for immune defense in form of superoxide anions ($\cdot O_2^-$), which are the result of the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$ by NADPH oxidases (NOX) known as phagocytosis [60].

Therefore, oxidative stress can induce inflammatory reactions, but can also be a result of inflammation.

For some inflammatory skin diseases like psoriasis, there is evidence that oxidative stress can contribute to the development of the disease [51,61,62]. However, in other inflammatory skin diseases like atopic dermatitis (AD) the pathogenesis and thus the role of oxidative stress is still not fully understood. AD is a multifactorial disease combining genetic, immunological and environmental aspects and is one of the most common chronic skin diseases worldwide with 15-20 % affected children and 1-3 % affected adults [63,64]. According to current knowledge, up to 50 % of AD patients have a loss-of-function mutation in the *FLG* gene, which might contribute to the impaired epidermal differentiation and skin barrier function [65]. Furthermore, in atopic skin the SC lipid composition is changed and the lipids are less ordered, likely correlating with a disturbed barrier function [19,66]. It is assumed that the disturbed skin barrier increases the penetration of allergens and pathogens into the skin, which activates the immune system. Since the immune response in AD is also dysregulated due to T helper type 2 (Th2) cell dominance and elevated immunoglobulin E (IgE) levels, leading to increased release of proinflammatory cytokines especially IL-4 and IL-13, the development of inflammation is further intensified. Inflammatory reactions in turn also contribute to the disturbed skin barrier [63,67]. However, it is not yet clear whether the disturbed skin barrier or the inflammation is the starting point for AD. AD also often occurs simultaneously with further allergic diseases such as asthma and allergic rhinitis known as atopic march [68]. Histologically, atopic skin is characterized by a thickened epidermis with parakeratosis which describes the retention of nuclei in the SC and edema with spongiosis which means fluid accumulation. The dermis is also infiltrated by many types of inflammatory cells including mast cells, eosinophils as well as B and T lymphocytes [10]. These skin changes lead to the typical main symptoms of atopic skin patches characterized by dryness, redness, itching as well as cracking or scaling [64]. The current standard therapy for symptomatic treatment includes moisturizing lotions and creams, the topical application of anti-inflammatory corticosteroids, e.g. dexamethasone as well as calcineurin inhibitors, e.g. tacrolimus. In severe cases, the oral administration of corticosteroids or cyclosporine is necessary [10]. Additionally, therapies with monoclonal antibodies like dupilumab, that is an IL-4 receptor antagonist inhibiting the cytokine responses of IL-4 and IL-13, are innovative approaches [10,69].

Since AD is characterized by inflammatory processes, ROS could be involved, but it is yet unknown, whether they contribute to the induction of inflammation or whether they are induced by inflammation. Several studies with AD patients indicate an increased oxidative stress level in blood and urine samples. In detail, the blood of 25 AD patients was examined in an *in vivo* study and it was shown that in the blood of AD patients the levels of

malondialdehyde, a marker for lipid peroxidation, were increased and the levels of enzymatic and non-enzymatic antioxidants were decreased compared to the control group [70]. Furthermore, Tsuboi *et al.* [71] investigated the urine of AD patients and detected an increase in the oxidative marker for DNA damage (8-hydroxy-2`-deoxyguanosine), which also indicated an increased oxidative level. These results are supported by two further studies where the urine of 1.5 to 15 years old children with AD also showed elevated oxidative markers, such as 8-hydroxy-2`-deoxyguanosine [72,73]. However, elevated oxidative stress levels in the blood do not necessarily corresponds to the situation in other organs like the skin, as it has been shown for muscle cells [74]. Unfortunately, studies on redox status in atopic skin are limited and human *in vivo* data are especially rare [75]. On the one hand, few studies showed an increased oxidative stress level in atopic skin like in blood and urine [76–78]. In contrast, in one study more antioxidants were detected in atopic skin compared to control skin [79].

1.3 Electron paramagnetic resonance spectroscopy

1.3.1 Physical principles and functionality

The electron paramagnetic resonance (EPR) or electron spin resonance spectroscopy was discovered by the Russian physicist Yevgeny Zavoisky in 1944 [80,81] and is now a widely-used method in different scientific fields [82]. It has also become well established in dermatology and is used to study the skin redox status, drug penetration and controlled release in the skin as well as melanin in the skin, skin tumors and skin adnexa [83].

In general, EPR spectroscopy is based on the absorption of microwave radiation by unpaired electrons in an external magnetic field. Accordingly, the physical principle is similar to nuclear magnetic resonance (NMR) spectroscopy, except that EPR measures the spin of unpaired electrons, while NMR measures the spin of nuclei. Therefore, EPR spectroscopy can be used for the detection of paramagnetic substances, e.g. free radicals, transition metal ions or photochemical intermediates, which are characterized by one or more unpaired electrons [8,82,84,85]. The basis of this method is that unpaired electrons have an intrinsic angular momentum, which is known as spin and is described by the spin quantum number m_s. Here, unpaired electrons can take the following two spin orientations to the magnetic field: parallel ($m_s = -½$) or antiparallel ($m_s = +½$). Due to the spin, unpaired electrons have a magnetic moment. In the absence of an external magnetic field, the energies of the two spin orientations are equal, i.e. they are degenerate. By applying a magnetic field, the degeneracy is eliminated and the electrons take either parallel or antiparallel orientation according to their spin. Since both spin orientations have different energies, a splitting of the energy states in the magnetic field occurs, which is called

Zeeman effect and is shown in the left part of Figure 3. The energy difference ΔE between the parallel and antiparallel state is calculated according to equation 1 and is directly dependent on the magnetic field B_0, since g and μ_B are constants. The Landé g-factor or g-value describes the position of the EPR spectrum lines in the magnetic field and reflects the polarity of the microenvironment. The Bohr magneton μ_B is $9.27 \cdot 10^{-24}$ JT^{-1}. The energy difference can also be described as the product of Planck's constant h and the microwave frequency v, because electromagnetic radiation, especially microwave radiation, is used to induce the transition from the parallel to the antiparallel energy states [8,85,86].

$$\Delta E = g \cdot \mu_B \cdot B_0 = h \cdot v \qquad (1)$$

A klystron is used as a microwave source from where the microwaves are radiated into the resonator, where the sample is located between two electromagnets in a magnetic field. If the energy of the constantly irradiated microwave frequency corresponds to the energy difference between the two spin orientations at a certain magnetic field, resonance absorption of the microwave radiation by the sample occurs. The reflected microwave radiation from the sample is detected and then recorded [84,85]. An absorption spectrum is obtained and the first derivative is used for facilitated spectrum evaluation. One peak in the EPR spectrum is typical for a free unpaired electron in the magnetic field, since only the Zeeman interaction is involved [8].

In addition to the interaction with the external magnetic field (Electron Zeemann interaction), unpaired electrons also interact with the nuclei of neighboring atoms in molecules, because they also have their own magnetic field. This has an influence on the EPR spectra, as it leads to a further splitting of the energy states, known as hyperfine splitting [82,83]. The energy states are split into the number of lines calculated according to equation 2, where I describes the nucleus spin number and n the number of such nuclei [9].

$$\text{number of lines} = 2 \cdot I \cdot n + 1 \qquad (2)$$

In the case of nitroxide radicals, which are often used as spin probes in EPR, the unpaired electron interacts with the nucleus of the nitrogen atom (^{14}N). Since nitrogen has a nucleus spin number I of 1 (I=1) and is present once in nitroxide radicals (n=1), the parallel and antiparallel state is split in three further energy states (m_I = +1, 0, -1). This results in three transition lines and thus three peaks (low, middle, high field) are visible in the EPR spectrum of nitroxide radicals. The distance between the transition lines is described as hyperfine splitting constant A (Figure 3) [82–84].

Depending on the microwave frequency, different EPR spectrometers are distinguished: W-band (95 GHz), Q-band (35 GHz), X-band (9.5 GHz), S-band (3.0 GHz) and L-band (1.3 GHz) [85,87]. With decreasing frequency, the resolution and sensitivity of the EPR

spectra decreases, but the penetration depth of the radiation into the sample increases [85]. For this reason, the different EPR spectrometers can be used for various applications. The W-band is used for the measurement of solutions [88]. The X-band enables the detection of paramagnetic substances in *ex vivo* and *in vitro* biological samples [89,90] and the L-band can be used for *in vivo* investigations as it ensures adequate penetration of radiation into the body and avoids overheating of aqueous biological samples [8,91,92].

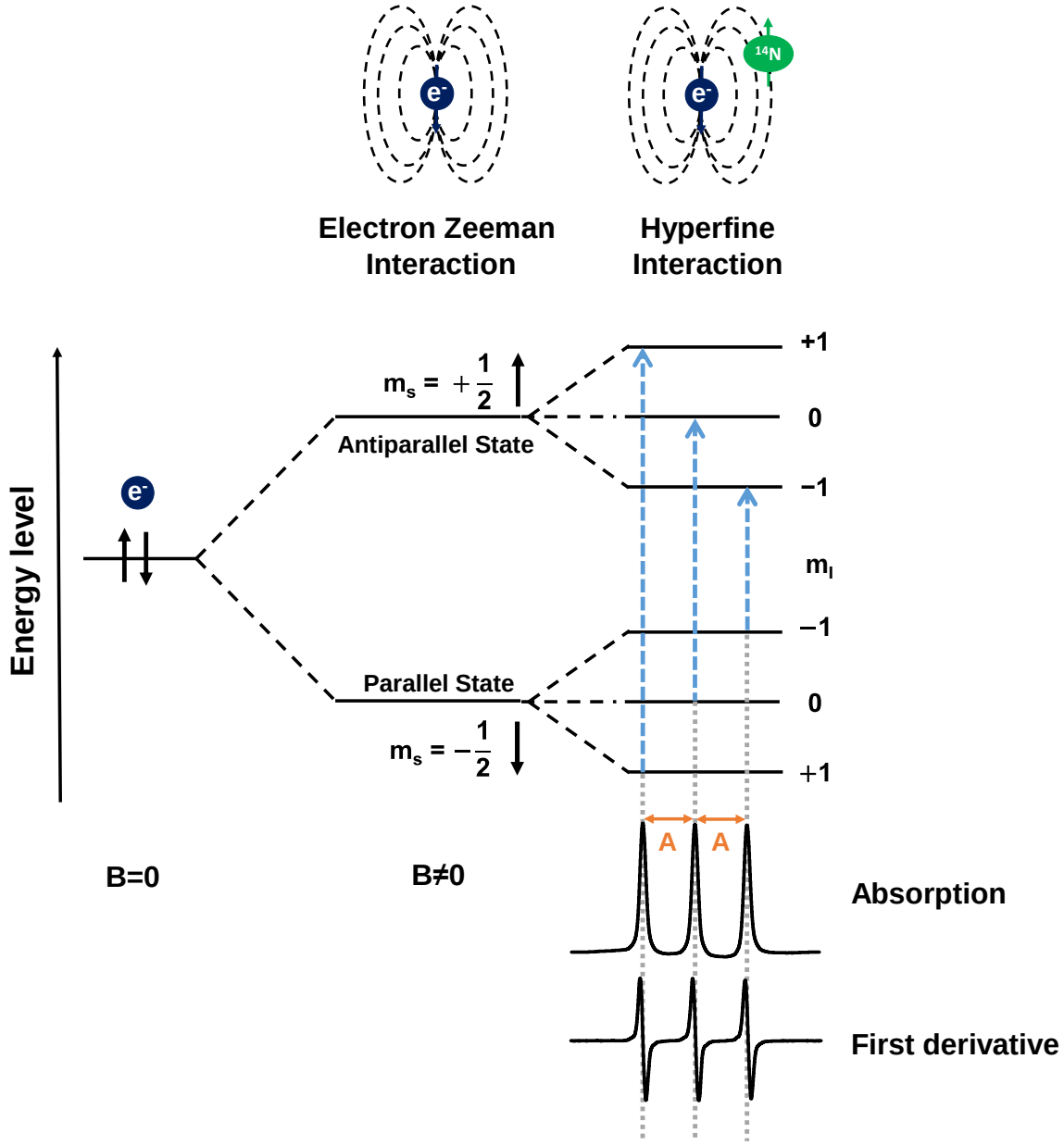


Figure 3: Energy level diagram and resulting EPR spectrum for nitrogen atom (^{14}N) as a function of applied magnetic field B. Hyperfine levels and transition for nitroxide nitrogen nucleus; spin system with spin s=½ and a nucleus spin number of I=1; The hyperfine splitting constant A describes the distance between the transition lines (according to [82–84,93]).

1.3.2 Spin probes

1.3.2.1 Redox status

Spin probes or spin labels in EPR spectroscopy are stable exogenous radicals that include a chemical group with an unpaired electron and thus they can be detected by EPR and show a specific EPR spectrum [83]. They are used to monitor physiological processes and microenvironmental properties. Common used spin probes in biological systems are nitroxide radicals, also called aminoxyl radicals according to IUPAC nomenclature [93,94]. They are composed of a nitroxide group with an unpaired electron (N-O·) and are of low toxicity. Nitroxide radicals can be used as an indicator for the redox status as they participate in redox reactions in cells and tissues. For redox status investigations, in particular cyclic nitroxide radicals with a five- (pyrrolidine, pyrroline, oxazolidine) or six- (piperidine) membered ring are used in EPR. In this case the nitroxide group is stabilized by methyl groups at α-positions. These nitroxide radicals can be reduced to the corresponding hydroxylamine by antioxidants or oxidized to the corresponding oxoammonium cation by radicals, whereby in both cases the products are EPR-silent and cannot be detected by EPR (Figure 4) [94–96].

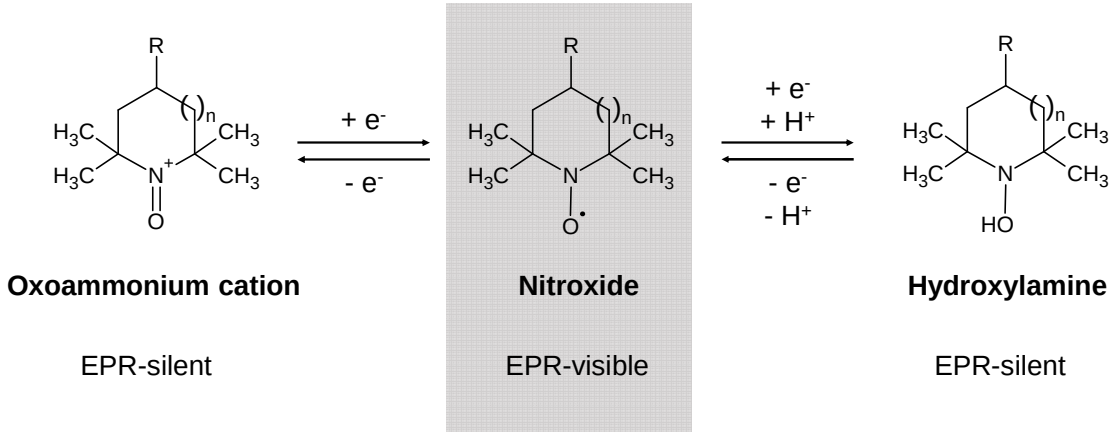
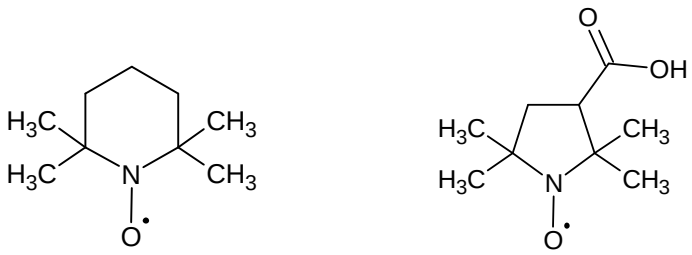


Figure 4: Possible redox reactions of cyclic nitroxide radicals (pyrrolidine n=0; piperidine n=1). Cyclic nitroxide radicals can be reduced to hydroxylamine by antioxidants and oxidized to oxoammonium cation by oxidants. Both products are EPR-silent and cannot be detected by EPR (according to [96]).

The reaction of spin probes with antioxidants or radicals to EPR-silent products leads to a decay of the EPR signal over time. The decay rate can be used to get information about the redox status. In biological systems, the reduction of nitroxide radicals by antioxidants occurs mainly within cells and therefore nitroxide radicals have to pass the cell membrane. The cell uptake of the nitroxide radicals is mainly dependent on their lipophilicity and charge. Consequently, hydrophilic and charged compounds have a very low penetration rate into the cells. Within the cell, the reduction rate of the nitroxide radicals depends on their stability which differs strongly between the various nitroxide radicals. Piperidine (six-membered ring)

nitroxide radicals are reduced faster than pyrrolidine (five-membered ring) nitroxide radicals. Ring substituents influence the reduction rate as well, but to a lesser extent. Accordingly, cationic nitroxide radicals are reduced faster than neutral ones which are in turn reduced faster than anionic nitroxide radicals [94,95]. Commonly used nitroxide radicals are the spin probes 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) [97] and 3-(carboxy)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PCA) [98]. The structure of both nitroxide radicals are presented in Figure 5. TEMPO is an amphiphilic piperidine (six-membered ring) nitroxide radical with a log*P* value of 1.8 [99,100]. It penetrates into cells and skin very fast and shows a fast reduction by antioxidants, mainly caused by ascorbate and thiol-dependent antioxidants, as shown in cultured human skin and skin cells by Fuchs *et al.* [97]. However, TEMPO can also act as an antioxidant and react with radicals [101], although the reaction with antioxidants in the skin is preferred. In contrast to TEMPO, PCA reacts mainly with radicals in the skin [98,102], because it is very stable against reduction as pyrrolidine (five-membered ring) nitroxide radical [94]. Furthermore, the penetration of PCA into cells is very low due to its hydrophilicity (log*P*=-1.7) [103] and the charged carboxy group at pH 7, but it can reflect intra- and extracellular radical production [95,104].



TEMPO **PCA**

Figure 5: Structure of TEMPO and PCA. TEMPO is an amphiphilic six-membered ring nitroxide radical, which penetrates fast into the cells and can be reduced by antioxidants. PCA is a hydrophilic five-membered ring nitroxide radical whose penetration into the cells is very low and is stable against reduction due to its five-membered ring (according to [85]).

1.3.2.2 Microenvironmental polarity

Nitroxide radicals can also provide information on the characteristics of their microenvironment, such as polarity, viscosity and pH [95]. The distinction of the polarity in the microenvironment is based on different hyperfine splitting constants. In the hydrophilic microenvironment the nitroxide group is present as a zwitterion and the unpaired electron is located at the nitrogen atom (Figure 6). This increases the electron spin density at the nucleus of the nitrogen atom, leading to a higher hyperfine splitting. In contrast, in the lipophilic microenvironment, the nitroxide group is neutral and the unpaired electron is located at the oxygen atom and thus delocalized from the nitrogen nucleus. The influence

of the unpaired electron on the nitrogen atom becomes lower, resulting in lower hyperfine splitting compared to the hydrophilic microenvironment [93,95]. Both mesomeric structures of TEMPO as representative nitroxide radical are illustrated in Figure 6 [93]. TEMPO is often used as an indicator for differences in the microenvironmental polarity, since its log*P* value allows the distribution into hydrophilic and lipophilic microenvironments [92,105].

In addition to the hyperfine splitting constant, the g-value also depends on the polarity of the microenvironment, which results in a shift of the EPR spectrum on the x-axis. With increasing polarity, the hyperfine splitting constant increases and the g-value decreases (right shift). With decreasing polarity, the hyperfine splitting constant decreases and the g-value increases (left shift). However, the influence of the g-value on the EPR spectrum depends on the sensitivity of the EPR spectrometer. In the W-band, which has a high sensitivity due to the frequency of 95 GHz, the influence of the g-value is greatest, leading to visible lipophilic and hydrophilic peaks in the low, middle and high field peak of the EPR spectrum due to clearly left shift in the lipophilic microenvironment. However, in the X-band with a 10-times lower frequency (9.5 GHz), the influence of the g-factor is smaller than in the W-band and thus the shift to the left on the x-axis is smaller in the lipophilic microenvironment. Therefore, only the lipophilic and hydrophilic peak at the high field peak can be resolved in X-band and the low and middle field peak are broadened (Figure 6) [85].

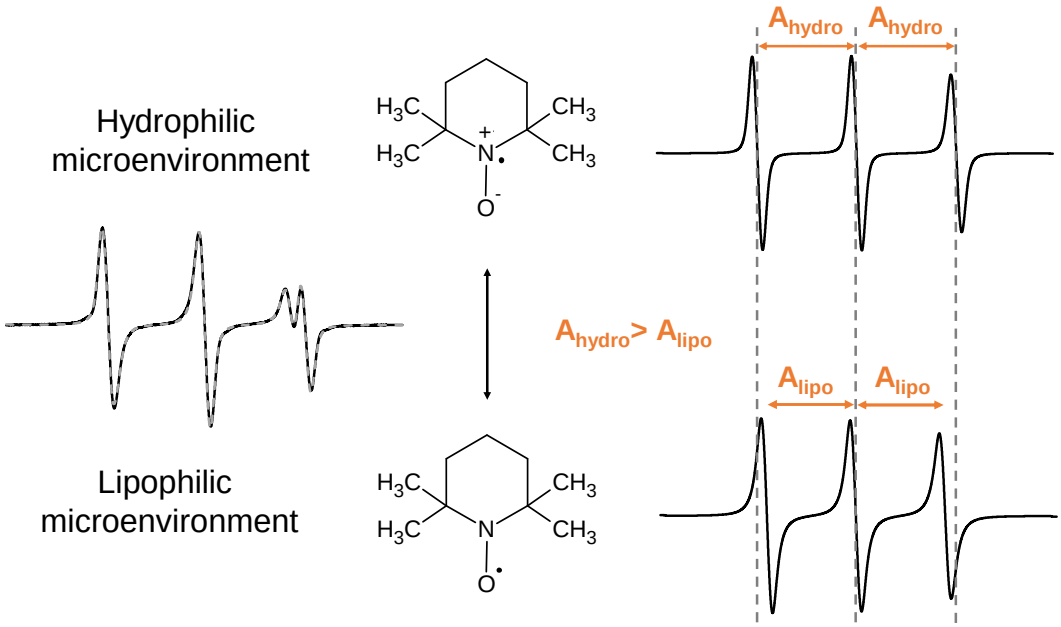


Figure 6: TEMPO in different microenvironmental polarities. Representative X-band EPR spectrum of TEMPO in human excised skin (experimental – solid line; simulated – dashed line); mesomeric structure and representative simulated EPR spectrum of TEMPO in the hydrophilic and lipophilic microenvironment of human skin. In the hydrophilic microenvironment the unpaired electron is located on the nitrogen atom and the hyperfine splitting constant (A_{hydro}) is higher than in the lipophilic microenvironment (A_{lipo}) where unpaired electron is located at the oxygen atom (mesomeric structures according to [93]).

The investigation of microenvironmental polarity of spin probes is mainly applied for studying the structure and dynamics of biological and artificial membranes at cellular level [105,106]. Furthermore, it is used to analyze the distribution and localization of spin-labeled drugs within lipid bilayers [107] and nanoparticular carrier systems [88]. The distribution of spin probes themselves (TEMPO) in hydrophilic and lipophilic compartments within carrier systems has also been investigated [108]. The microenvironmental polarity has also been used in skin experiments to investigate the localization of TEMPO within invasomes and nanostructured lipid carriers during skin penetration [92,109].

1.3.3 Spin traps

Spin traps are nitrones and nitroso compounds which have no radical character themselves in contrast to spin probes and therefore they are not detectable by EPR. They are used to stabilize and detect short-lived radicals by reacting with them and forming stable spin adducts. The stable products are nitroxide radicals, which can be detected by EPR. The nitrones are divided into linear and cyclic nitrones and the most common spin trap is the cyclic nitrone 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) [110,111]. It is a hydrophilic molecule with a log*P* value of -1.0 [112] and highly cell permeable [113]. DMPO reacts with oxygen-centered radials, like hydroxyl radicals (·OH) and superoxide anion ($\cdot O_2^-$) and also with carbon-, nitrogen- and sulphur-centered radicals. Each DMPO-radical adduct has a specific pattern in the EPR spectrum, which allows DMPO to distinguish between different types of radicals. Figure 7 shows the reaction of DMPO with ·OH and $\cdot CH_3$ radicals and the resulting EPR spectra [110,111].

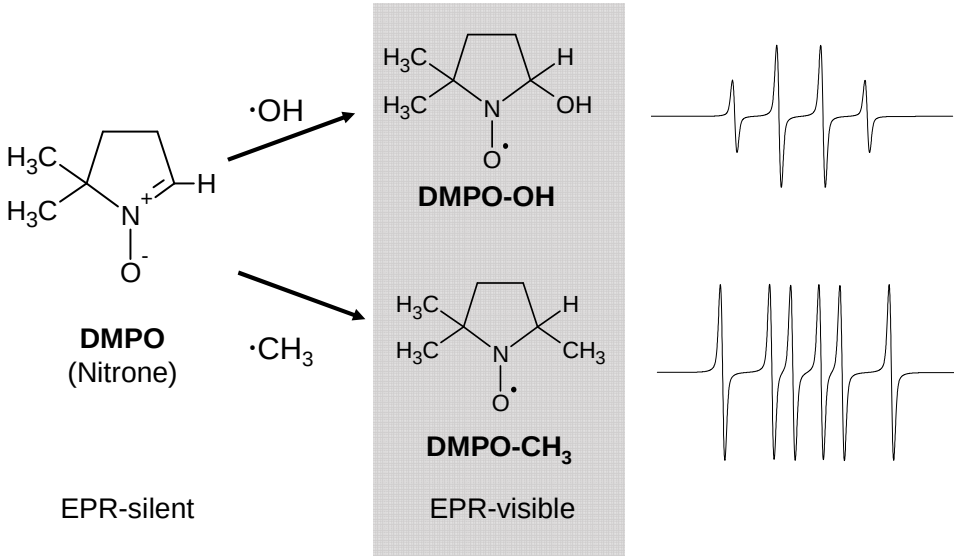


Figure 7: Principle of spin trapping. Reaction of the spin trap DMPO with ·OH and $\cdot CH_3$ radicals to stabilized spin adducts DMPO-OH or DMPO-CH_3, which can be detected by EPR. DMPO-OH shows four characteristic peaks and DMPO-CH_3 shows six characteristic peaks in the EPR spectrum (according to [111]); DMPO spectra were taken from Bruker software Xepr 2.6b.143.

1.4 Aim

The pathogenesis of the chronic inflammatory skin disease AD is not yet fully understood, especially with regard to the role of oxidative stress. Since inflammatory reactions are associated with oxidative stress and the skin is permanently exposed to environmental factors, oxidative stress may play an important role in AD. Previous data on the redox status in atopic skin are limited, therefore this study aims to investigate the redox status in atopic skin to deepen the understanding. For this purpose, EPR spectroscopy is used, as it offers the possibility to obtain different information on the redox status in the skin in real time by using spin probes and spin traps. The following points will be addressed in particular:

- *Establishment of an EPR method for investigating the redox status in skin*

  Since the redox status always represents an interaction between oxidants and antioxidants, it is essential to consider both the oxidative and the antioxidative status in order to obtain a comprehensive overview of the redox status. For this purpose, different EPR-based approaches with the spin probes PCA and TEMPO and the spin trap DMPO are established on different *ex vivo* and *in vitro* skin models which are commonly used in dermatological research.

  a) Oxidative status

     The spin probe PCA and spin trap DMPO are used to quantify and characterize the radical production in the skin. Furthermore, the effect of UV-NIR irradiation on the radical production is investigated to analyze the resistance of skin models against this external stress factor.

  b) Antioxidative status

     To determine the antioxidative status, the decay of the spin probe TEMPO is investigated. Since TEMPO can be distributed in different microenvironmental polarities, it is investigated whether TEMPO can also provide spatially resolved information on the antioxidative status in the skin.

- *Investigation of the redox status in atopic skin*

  In order to gain a deeper insight into the redox status of atopic skin by EPR, normal and inflammatory *in vitro* skin equivalents emulating certain characteristics of AD were used and analyzed comprehensively concerning redox status using the established EPR method.

2 Results

2.1 Quantification and characterization of radical production in human, animal and 3D skin models during sun irradiation measured by EPR spectroscopy

The manuscript has been published in Free Radical Biology and Medicine:

S. Albrecht*, A. Elpelt*, C. Kasim, C. Reble, L. Mundhenk, H. Pischon, S. Hedtrich, C. Witzel, J. Lademann, L. Zastrow, I. Beckers, M.C. Meinke, Quantification and characterization of radical production in human, animal and 3D skin models during sun irradiation measured by EPR spectroscopy, Free Radic. Biol. Med. 131 (2019) 299–308.

* Both authors contributed equally

DOI: 10.1016/j.freeradbiomed.2018.12.022

https://doi.org/10.1016/j.freeradbiomed.2018.12.022

Personal contribution:

- Experimental design
- Acquisition of foreskin and murine skin
- Isolation and cultivation of human primary skin cells
- Generation of skin equivalents
- Investigation of skin equivalents together with S. Albrecht
- Drafting of the manuscript together with S. Albrecht
- Revision of the entire manuscript in cooperation with all co-authors

2.2 Investigation of TEMPO partitioning in different skin models as measured by EPR spectroscopy – Insight into the stratum corneum

The manuscript has been published in Journal of Magnetic Resonance:

A. Elpelt, D. Ivanov, A. Nováčková, A. Kováčik, M. Sochorová, S. Saeidpour, C. Teutloff, S.B. Lohan, J. Lademann, K. Vávrová, S. Hedtrich, M.C. Meinke, Investigation of TEMPO partitioning in different skin models as measured by EPR spectroscopy – Insight into the stratum corneum, J. Magn. Reson. 310 (2020) 106637.

DOI: 10.1016/j.jmr.2019.106637

https://doi.org/10.1016/j.jmr.2019.106637

Personal contribution:

- Experimental design
- Acquisition of foreskin and porcine abdominal skin
- Isolation and cultivation of human primary skin cells
- Generation of skin equivalents
- Determination of the log*P* value of TEMPO together with S. Saeidpour
- Preparing of skin samples for cryosectioning
- Preparing of *stratum corneum* samples for lipid analysis
- Conduction of EPR and tape stripping experiments
- Analysis of EPR spectra
- Interpretation of the entire data set in cooperation with the co-authors
- Drafting and revision of the manuscript

2.3 Insight into the redox status of inflammatory skin equivalents as determined by EPR spectroscopy

The manuscript has been published in Chemico-Biological Interactions:

A. Elpelt*, S. Albrecht*, C. Teutloff, M. Hüging, S. Saeidpour, S.B. Lohan, S. Hedtrich, M.C. Meinke, Insight into the redox status of inflammatory skin equivalents as determined by EPR spectroscopy, *Chem. Biol. Interact*. 310 (2019) 108752.

* Both authors contributed equally

DOI: 10.1016/j.cbi.2019.108752

https://doi.org/10.1016/j.cbi.2019.108752

Personal contribution:

- Experimental design
- Acquisition of foreskin
- Isolation and cultivation of human primary skin cells
- Generation of skin equivalents
- Conduction of viability assays and GSH assays
- Conduction of EPR experiments using TEMPO
- Analysis of EPR spectra of TEMPO
- Interpretation of the entire data set in cooperation with the co-authors
- Drafting and revision of the manuscript

3 Discussion

3.1 Establishment of an EPR method for investigating the redox status in skin

Oxidative stress is involved in numerous diseases [1], therefore the investigation of the redox status is essential to contribute to the understanding of disease pathogenesis. In AD, especially in atopic skin, the role of oxidative stress is not fully elucidated so far. To get a comprehensive overview of the redox status in atopic skin, EPR spectroscopy represents a suitable method, because different information can be obtained by using spin probes and spin traps. For this purpose, it is first necessary to establish an EPR method, which can be used to determine the oxidative as well as the antioxidative status. Therefore, in this thesis different EPR-based approaches with the spin probes PCA and TEMPO as well as the spin trap DMPO were established. To investigate the radical production and thus the oxidative status in skin, the decay of the spin probe PCA was used. Herrling *et al.* [98] showed that PCA is a stable spin probe in skin, because it is resistant to reduction by antioxidants due to its five-membered ring structure (pyrrolidine) [94,95]. Additionally, they showed that PCA is decreased in skin by ROS generated by UV radiation [98]. These results are in accordance with those of Albrecht *et al.* [102], who showed that PCA has a comparable relative radical production as the spin trap N-tert-Butyl-α-phenylnitrone (PBN), which reacts exclusively with radicals.

To successfully establish this method using PCA, the radiation-induced radical production as a measure of stress resistance was investigated and quantified in different *ex vivo* and *in vitro* skin models using X-band EPR spectroscopy. Differences in the radiation-induced radical production between the skin models were found. *Ex vivo* human skin showed a significant lower radical production and thus higher stress resistance compared to *ex vivo* murine skin and *ex vivo* porcine skin, whereas the radical production of murine and porcine skin was very similar. In previous investigations, Shindo *et al.* investigated a set of enzymatic and non-enzymatic antioxidants individually in human [2] and murine skin [3]. Clear differences could be shown between the individual antioxidants in human and murine skin, with some antioxidants elevated in human skin and others in murine skin. GSH represents one of the most important endogenous antioxidants and is an obligate co-substrate for GSH peroxidase which catalyzes the degradation of hydrogen peroxide (H_2O_2) to oxygen and water. In this reaction GSH is oxidized to GSSG which is then recycled by the GSH reductase [55]. The percentage of oxidized GSH is a suitable marker for oxidative stress and was found to be 5.0 ± 0.86 % in human epidermis and 10.5 ± 2.7 % in human dermis [2], whereby murine skin showed a higher percentage of oxidized GSH with 5.5 ± 1.6 % in epidermis and 21.7 ± 4.2 % in dermis [3]. Consequently, murine skin has a

higher oxidative level and thus a lower antioxidant capacity which is in agreement with the lower stress resistance in our investigations. A further endogenous antioxidant is SOD that is responsible for the conversion of superoxide anion ($\cdot O_2^-$) to hydrogen peroxide (H_2O_2), which in turn is detoxified by GSH peroxidase or catalase [12]. Human epidermis showed a SOD activity of 17.8 ± 1.0 U/mg protein [2], whereas murine epidermis showed a SOD activity of 11.7 ± 1.4 U/mg protein [3] which also reflects the lower antioxidant activity of murine skin compared to human skin that is consistent with our results of stress resistance determined by EPR. In addition, the melanin in human skin which protects against radiation, as well as carotenoids which are taken up by food and also have photo-protective properties contribute to the higher stress resistance and thus higher antioxidant capacity in human skin compared to murine and porcine skin. In contrast, murine and porcine skin contain only little or no melanin and carotenoids [114,115]. Haag *et al.* [115] also showed that the radical production after a 9-minute UV irradiation was significantly lower in *ex vivo* human skin than in *ex vivo* porcine skin which is in accordance with our results. However, the catalase activity in the SC of human and porcine skin was very similar [115]. All in all, although human and porcine skin are structurally very similar [28,29], they show clear differences in the redox status.

Compared to the *ex vivo* skin models, normal *in vitro* skin equivalents showed a significant higher radiation-induced radical production. On the one hand, this can be explained that *in vitro* skin equivalents only contain an endogenous antioxidant system of the keratinocytes and fibroblasts and contain no exogenous antioxidants or melanin. On the other side, skin equivalents are living, metabolic active and produce and consume oxygen in contrast to the *ex vivo* skin models allowing the formation of ROS [44,116]. In accordance with the *in vitro* skin equivalents, *in vivo* investigations also showed a higher radiation-induced radical production compared to *ex vivo* skin models by using L-band EPR spectroscopy [116].

To characterize the produced radicals after irradiation, EPR measurements with the most commonly used spin trap DMPO were established. DMPO reacts with radicals and form spin-adducts which have a specific pattern in the EPR spectrum [110]. In order to detect radicals, the skin models were irradiated to simultaneously detect the produced radicals during irradiation. In *ex vivo* human skin, porcine skin and *in vitro* skin equivalents ·OH radicals, which are produced first, as well as, CCR produced in the second step of radical cascade, could be detected. In *ex vivo* human and porcine skin a similar ratio between ·OH and CCR were detected whereby *in vitro* skin equivalents showed more ·OH radicals, which can be explained by a better supply of oxygen leading to higher production of adenosine triphosphate (ATP) during mitochondrial electron transport chain and thus to higher ROS production. During the mitochondrial electron transport chain, 85 % of the molecular oxygen is reduced initially to superoxide anion ($\cdot O_2^-$), which is further reduced to hydrogen peroxide

(H_2O_2), then hydroxyl radicals (·OH) and lastly water [46,51]. These results show that the established EPR approaches using PCA and DMPO reflect the oxidative status in the different skin models and showed that the *in vitro* skin equivalents are more comparable to *in vivo* and are suitable for further redox status measurements.

For the investigation of the antioxidative status the spin probe TEMPO was used. It has already been used to analyze the radical scavenging capacity or total antioxidant capacity in human skin biopsies, keratinocytes and fibroblasts [97,98] as well as murine skin homogenates [99]. In this context, Fuchs *et al.* [97] showed that a decrease of cytosolic ascorbate and thiol-dependent antioxidants, like GSH, inhibits the TEMPO decay, which indicates that TEMPO reacts mainly with these intracellular antioxidants. Additionally, TEMPO has the potential to partition into different microenvironmental polarities of the skin due to its *log*P value of 1.8 [99,100,117]. For this reason, TEMPO can also provide additional spatially resolved information on the antioxidant capacity in different skin layers. There are no comparable studies on the microenvironment in the skin to date, however this methodological approach has already been applied to *in vivo* mice. Yamato *et al.* [118] investigated the free radical production in the hydrophilic and lipophilic microenvironment of *in vivo* mice by using spin probe methoxycarbonyl-PROXYL and a L-band (1.1 GHz) EPR spectrometer with a loop-gap resonator. In their study they found out, that in transient MCAO mice the decay of the spin probe in the lipophilic microenvironment is increased and thus more lipid-derived radicals are available than in the sham-operated MCAO mice.

In order to define the skin compartments of different polarities, the TEMPO partitioning into hydrophilic and lipophilic microenvironment in different *ex vivo* and *in vitro* skin models was investigated. In this thesis, it was shown that the relative TEMPO amount in the lipophilic microenvironment correlates with the SC thickness and thus the lipophilic microenvironment corresponds to SC and the hydrophilic microenvironment to viable skin (viable epidermis and dermis). The SC thicknesses determined were consistent with literature data [33,119,120] and showed species and body region dependent differences [17,18,119,121]. This supports the validity of this method. We assume that TEMPO is distributed in the whole skin sample including the SC, viable epidermis and a part of the dermis based on existing studies [117,122]. Additionally, TEMPO most likely penetrates the SC through the intercellular pathway which consists of lipid matrix including ceramides, cholesterol and free fatty acids [21]. Thus, with increasing SC thickness more TEMPO could be localized in the SC and thus in the lipophilic microenvironment. This knowledge about the distribution of TEMPO in SC and viable skin can be used to obtain spatially resolved information on the antioxidant capacity in the skin. Consequently, the spin probe TEMPO can not only be used to determine the antioxidant capacity in the whole skin sample, but also in the SC (lipophilic) and the viable skin (hydrophilic) individually and thus provide spatially resolved information.

3.2 Investigation of the redox status in diseased skin

3.2.1 Atopic dermatitis

Since the role of oxidative stress in atopic skin is not completely known yet, the investigations are aimed to contribute to the understanding of this topic. Following the successful establishment of the different EPR-based approaches, they were used to get a comprehensive overview of the redox status in normal and atopic skin. *In vitro* skin equivalents were used as skin model for the investigations, since *in vitro* skin equivalents showed comparable stress resistance to *in vivo* compared to *ex vivo* skin models (chapter 3.1), are independent of external stress factors, are easily reproducible and have the potential to emulate certain characteristics of diseases such as AD. Hönzke *et al.* [35] established inflammatory *in vitro* skin equivalents that emulate specific characteristics of AD, in particular a disturbed skin barrier and a defect T helper type 2 (Th2) cell immune response. The inflammatory skin equivalents showed thickening of the epidermis, parakeratosis and spongiosis as well as an increase in the surface pH and in the expression of the thymic stromal lymphopoietin (TSLP) and ß-defensin-2 (hBD-2) representing typical histological and physiological properties of atopic skin [35].

The spin probe TEMPO was used to investigate the antioxidant capacity in the whole skin sample as well as in the SC (lipophilic) and viable skin (hydrophilic). Between normal and inflammatory skin equivalents, no differences in the antioxidant capacity, neither in the SC nor in the viable skin, could be observed, although the relative TEMPO amount in the SC was lower in the inflammatory skin equivalents than in the normal skin equivalents. The lower TEMPO amount originates from the smaller SC thickness in the inflammatory skin equivalents which is consistent with measurements of SC thickness in AD patients [123]. The similar antioxidant capacity can be explained by the fact that the TEMPO decay depicts a sum of several antioxidative components. Since skin equivalents contain no ascorbate, it is assumed that here, the TEMPO decay is caused by thiol-dependent antioxidants. These include GSH and thioredoxin, among others. The antioxidant capacity of these antioxidants may vary individually between the skin equivalents, however TEMPO only detects the total change of the antioxidant capacity. Therefore, the concentration of the essential antioxidant GSH was investigated individually by a fluorescence-based assay and the inflammatory skin equivalents exhibited a decreased GSH concentration in the epidermis compared to the normal skin equivalents. In agreement with this, a higher metabolic radical production was detected in inflammatory skin equivalents using the spin probe PCA which indicates a higher oxidative stress level under inflammatory conditions. To induce inflammatory conditions, the proinflammatory cytokines IL-4 and IL-13, which are upregulated in the skin [124,125] and blood [126] of AD patients, were supplemented to the culture medium of the

skin equivalents. It is already known that IL-4 and IL-13 increase the ROS generation as already shown in microglia [127,128] and tracheobronchial epithelial cells [129]. Hirakawa *et al.* [130] also showed that the supplementation of IL-4 and IL-13 increases the ROS production in human primary keratinocytes by increased expression of the enzyme dual oxidase 1 (DUOX1). The possible mechanism is illustrated in Figure 8 and is based on the fact that the binding of IL-4 or IL-13 to the respective IL-13 receptor activates the JAK/STAT6 signaling pathway [131]. The transcription factor STAT6 induces the expression of DUOX1, increasing its expression on the gene and protein level. DUOX1 belong to the NOX/DUOX family which represents major producers of hydrogen peroxide (H_2O_2) in cells, because they oxidize NADPH to $NADP^+$ and thereby the electrons are transferred to molecular oxygen and hydrogen peroxide (H_2O_2) is produced [130,132]. Therefore, increased presence of DUOX1 increases extracellular hydrogen peroxide (H_2O_2) and consequently intracellular ROS in keratinocytes [130], which could explain the increased metabolic radical production in the inflammatory skin equivalents. The increased intracellular radical production can lead to an increased oxidation of GSH to GSSG, which could explain the lower epidermal GSH concentration in the inflammatory skin equivalents. Furthermore, ROS could induce the NF-κB signaling pathway which activates further proinflammatory processes [12]. In addition, DUOX1 inhibits the protein tyrosine phosphatase 1B (PTP1B) by oxidizing the cysteine residues in its active site, which no longer control DUOX1 expression and radical production in keratinocytes [130]. This signaling pathway could also contribute to an increased radical production in atopic skin.

Due to the higher metabolic radical production in the inflammatory skin equivalents, more antioxidants such as GSH are consumed, which explains the lower GSH concentration in the inflammatory skin equivalents. However, the lower GSH concentration was only detected in the epidermis of the inflammatory skin equivalents, whereas there were no differences in the dermis. A possible explanation for this could be that DUOX1 is mostly expressed in epithelial cells like keratinocytes [129,130,133–135] and additionally that fibroblasts contain fewer IL-13 receptors than keratinocytes as shown by Akaiwa *et al.* [136]. Thus, the proinflammatory interleukins and DUOX1 might play a minor role in fibroblasts and do not induce additional radicals, which support the comparable GSH concentration in the dermis of both skin equivalents. Furthermore, there are clearly fewer fibroblasts in the dermis than keratinocytes in the epidermis of the skin equivalents, which makes it more difficult to detect differences in the dermal GSH concentration.

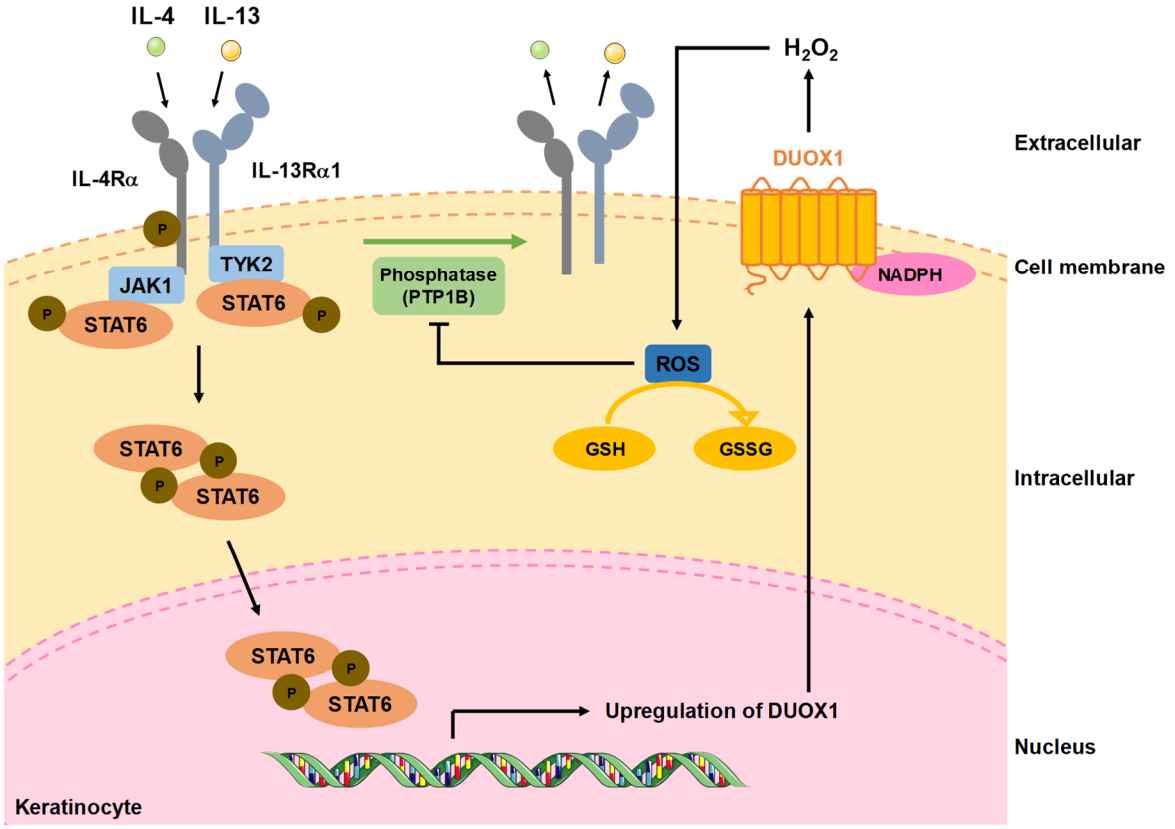


Figure 8: Proposed signal transduction pathway in keratinocytes induced by IL-4/IL-13 supplementation. The binding of the supplemented proinflammatory cytokines IL-4 and IL-13 to the IL-13 receptor consisting of IL-4Rα chain and IL-13Rα1 chain triggers the JAK/STAT6 signal transduction pathway. The receptor is phosphorylated by the janus kinases JAK1 and tyrosine kinase 2 (TYK2). As a result, the transcription factor STAT6 binds to the receptor, which is also phosphorylated. Dimerization and translocation of STAT6 into the nucleus leads to increased expression of dual oxidase 1 (DUOX1). DUOX1 is membrane-bound and catalyzes the reduction of oxygen to hydrogen peroxide (H_2O_2) by using NADPH. Consequently, H_2O_2 is released into the extracellular compartment, from where it also enters the intracellular compartment. Intracellular ROS can oxidize GSH to GSSG and also inhibit the phosphatase PTP1B by oxidation. This prevents dephosphorylation of the IL-13 receptor and continues the JAK/STAT6 signaling cascade (according to [130,131,137]). Figure uses illustrations from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License.

The number of studies on redox status in atopic skin is limited, nevertheless our *in vitro* results are mostly comparable to the existing results. Sapuntsova *et al.* [76], detected increased free radical oxidation products and inhibited antioxidative system in skin biopsies of AD patients resulting in higher oxidative stress level which is in accordance to our results. The study of Niwa *et al.* [77] is also consistent with this, as it shows an increase in protein carbonyl, a marker for protein oxidation, in the SC of skin biopsies of Japanese AD patients. Furthermore, Eto *et al.* [78] investigated the redox status of AD mouse model *in vivo* which was generated by repeated topical application of mite antigen in NC/Nga mice. They detected a faster nitroxide decay in acute and chronic AD mice compared to healthy mice using a non-invasive method also indicating an excessive oxidative stress. In addition, an *in vivo* dermal microdialysis study in AD patients showed that the levels of oxidative stress

markers in lesional skin were increased compared to non-lesional skin, although no control group was included [138]. In contrast, Antille *et al.* [79] showed that the SC of AD patients had a significantly lower oxidative status indicated by a lower concentration of lipid peroxides and a higher concentration of α-tocopherol compared to control group. This difference could have been caused by the fact that only the SC and not the whole skin biopsy was investigated and that only non-lesional skin was investigated, where the used inflammatory skin equivalents most likely emulate lesional atopic skin.

To obtain further information on the redox status, the effect of external factors can be investigated. In this thesis, UVB-NIR irradiation was used as an external stress factor to investigate the oxidative status, especially the radiation-induced radical production and produced radical types in skin equivalents. Neither the radical production determined by PCA nor the radical types measured by DMPO after UVB-NIR radiation showed differences between the normal and inflammatory skin equivalents, which is consistent with the similar antioxidant capacity detected by TEMPO decay. This result is in agreement that no adverse effects of solar radiation have been observed in AD patients. On the contrary, solar radiation can even improve eczema of atopic skin [139,140].

The application of radiation represents one possibility to evaluate the redox status in skin which was already applied to study the influence of dietary carotenoids [141] and hyperforin-rich cream [142] as well as to compare different *ex vivo* skin models [115] and *in vivo* [116]. A further possibility to evaluate the redox status is the administration of exogenous antioxidants. It has been shown in several human *in vivo* studies that the oral administration of vitamin C [143] or curly kale extracts containing carotenoids [141] leads to an improved redox status in the skin. The effect of exogenous antioxidants has also been studied in other species. For example, the supplementation of vitamin C showed an improvement in total antioxidant capacity in the blood of dogs with AD representing a suitable model due to spontaneously developed AD [31,144]. Furthermore, the supplementation of extracts of red cabbage and grape skin exhibited an increase in the antioxidant capacity in the blood of pigs. However, it was also shown that hydrogen peroxide (H_2O_2) is increased in the blood of pigs [145], which in turn shows that it is important to study both the oxidative and antioxidative status to get a comprehensive overview.

Consequently, differences in the redox status between normal and inflammatory *in vitro* skin equivalents concerning epidermal GSH concentration and metabolic radical production were detected and thus indicating that oxidative stress could play a role in atopic skin. However, the results showed that the redox status is not consistent and can vary depending on the investigated skin layer and the type of investigation, since the total antioxidant capacity and stress resistance were similar in normal and inflammatory skin equivalents.

3.2.2 Other skin diseases

The redox status, in particular oxidative stress, also plays a key role in other skin diseases. Psoriasis is also a chronic inflammatory skin disease like AD and is characterized by keratinocyte hyperproliferation and impaired cell differentiation. In addition, unlike AD, psoriasis has a T helper type 1 (Th1) cell dominance, which upregulates interferon-gamma (IFN-γ) [13,49]. It has already been reported that oxidative stress plays a role in the pathogenesis of psoriasis. Therefore, increased lipid peroxidation and decreased antioxidant levels have been detected in the blood [146] and skin [147] of psoriasis patients [49,62]. This is caused by the increased infiltration of leukocytes, which lead to increased ROS release at psoriatic inflammatory lesions. ROS in turn activates the redox-sensitive transcription factor NF-κB, which is also upregulated in psoriasis. Since NF-κB regulates the expression of proinflammatory cytokines, there is an upregulation of mainly TNF-α and IL-8, which in turn can activate NF-κB and thus represent a positive cytokine loop [49,58]. The inhibition of NF-κB in psoriasis showed a reduction of the inflammatory component, which reflects its central role in the inflammatory process [148,149]. In addition, the upregulated cytokines TNF-α, IL-8 and IFN-γ are triggers for the inducible nitric oxide synthase (iNOS), which produces nitric oxide (NO·) and is expressed in the keratinocytes of psoriatic skin lesions [58]. In atopic skin, NF-κB might also play an important role, as it could also be activated by the increased ROS production and trigger further inflammatory processes. Increased radical production could be caused in atopic skin on the one hand by the infiltrated leukocytes like mast cells in the dermis [10] and on the other hand by the described DUOX1 signaling pathway in the epidermis (chapter 3.2.1). There are already studies showing that NF-κB is elevated in the blood of AD patients [150] and that inhibition of NF-κB led to an improvement of AD in mouse models [151,152].

Skin cancer is another skin disease with oxidative stress as a major factor. In this context, UV radiation is the main trigger, which leads to increased radical production and is associated with an increased mutation rate driving the tumor development [42,51]. Apart from causing mutations, ROS also causes an impairment of the immune system, which also contributes to the development of skin cancer. This impairment is caused by damage of the DNA of skin leukocytes through ROS leading to the death or an impaired function of these leukocytes. In addition, ROS also induces the upregulation and release of proinflammatory cytokines like IL-6 and TNF-α by the keratinocytes in skin cancer [51,58]. This demonstrates the impact of oxidative stress for the immune system of the skin and further strengthens the argument of the involvement of oxidative stress in the pathogenesis of AD.

3.3 Redox status in different skin compartments

Differences in redox status have already been shown between physiological and pathological conditions. In this thesis, the investigation of normal and inflammatory skin equivalents showed that the redox status also varies in different skin compartments and thus is not homogenous within the skin. In the SC (lipophilic) of the skin equivalents a slightly lower antioxidant capacity was detected than in the viable skin (hydrophilic). One possible reason could be that less antioxidants are available in the SC compared to the viable epidermis and dermis which is in accordance to a previous study with murine skin [3,153]. Furthermore, in the epidermis of skin equivalents a higher GSH concentration was detected compared to the dermis. Regional differences in the redox status, especially between epidermis and dermis, were already extensively investigated in human skin by Shindo *et al.* [2]. They detected a higher concentration of each investigated enzymatic and non-enzymatic antioxidant in the epidermis than in the dermis. However, this pattern could not be observed in murine skin, which shows the species dependency [3]. A further study of human primary keratinocytes and fibroblasts showed a higher concentration of reduced GSH in human primary keratinocytes compared to fibroblasts, which is also in accordance to our results [4]. However, this study also showed that keratinocytes have a higher oxidative stress level due to the accumulation of superoxide anions ($\cdot O_2^-$) caused by lower activity of mitochondrial Mn^{2+}-SOD.

Another reason for the differences in redox status between the epidermis and dermis may be differences between the intracellular and extracellular compartment [154–156]. In the dermis, the extracellular compartment plays a more dominant role. Here, the protein rich extracellular matrix provides an oxidizing environment, since thiol groups of the cysteine residues are prone to oxidation, influencing the activity and function of the proteins [157]. However, the extracellular and intracellular compartments are constantly interacting to maintain the physiological redox status. For example, extracellular SOD as well as intracellular Cu^{2+}/Zn^{2+}-SOD (cytoplasm) and mitochondrial Mn^{2+}-SOD are responsible for the dismutation of superoxide anions ($\cdot O_2^-$) produced by membrane-bound NOX [154–156].

The redox status also varies intracellularly between different cell compartments and each cell compartment has its individual redox potential known as redox compartmentalization. Here, mitochondria and nucleus belong to the compartments with more reducing redox potential and lysosomes and peroxisomes represent compartments with more oxidizing redox potential [155,158–160]. In a study with HaCaT cells, it was shown that oxidative stress induced by the supplementation of epidermal growth factor only led to a significant

oxidation of cytosolic thioredoxin-1. Nuclear thioredoxin-1, mitochondrial thioredoxin-2 and cellular GSH remained unchanged [161].

This knowledge illustrates that the redox status is a complex system of many individual components at the tissue, cellular and subcellular level. Methodologically, redox sensors can be used to investigate the redox status in different compartments without homogenizing the tissues or cells [155]. For this purpose, EPR spectroscopy with specific spin probes can be used. In a study, mito-TEMPO was used as a probe for mitochondria, methoxy-TEMPO for the intracellular compartment and carboxy-PROXYL for the extracellular compartment to investigate the proliferation rates in different cells [162].

3.4 Methods for investigating the redox status in skin

In this thesis, the total redox status in the skin was investigated using EPR to obtain a comprehensive overview. However, there are also other methods to investigate the redox status in the skin. There are numerous spectrophotometric or fluorescence-based methods to investigate the redox status in tissue samples and skin cells. The concentration of individual non-enzymatic antioxidants, ROS, oxidation damage marker, enzyme activities, but also the total oxidant and antioxidant capacity can be determined using various detection reagents [4,163,164]. Examples are the ABTS and FRAP assay for the determination of the total antioxidant capacity [7,164]. An established and common assay for the determination of intracellular ROS production of cells is the H_2DCFDA assay. 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) is hydrolysed by intracellular esterases and then oxidized by radicals to the fluorescent 2′,7′-dichlorofluorescein. However, this assay shows unspecific reactions of H_2DCFDA with medium components, such as serum, as well as heme, heme proteins like cytochrome C or metalloporphyrins which increases the fluorescence signal and limits its application [165,166]. The investigation of the redox status using spectrophotometric or fluorescence-based methods requires the removal of skin biopsies as well as tissue or cell homogenization in most cases, which is time-consuming and can cause additional oxidative stress [5,155]. These methods can be used for investigating tissue samples and cells, but it is not possible to apply the methods to the skin *in vivo*.

A method for determining the redox status in skin that can be used *in vivo* is based on electrochemical detection. Kohen *et al.* (1999) [5] and Brainina *et al.* (2013) [167] published a non-invasive *in vivo* method for the determination of the oxidative and antioxidative status in the skin. In 2019, an innovative method known as PAOT (Pouvoir AntiOxydant Total) Skin Score® was published, which can also be used to measure the total oxidative and antioxidative status in the skin [168]. Here, a patch containing both oxidized and reduced iron complex forms (mediator) is applied to skin areas and connected by microelectrodes.

The electrochemical potential of the oxidized/reduced mediator is measured by microelectrodes over a time period of 10 min and its change is used to determine the oxidative and antioxidative status. This method is non-invasive, inexpensive, fast and easy to use. However, it is not possible to obtain spatially resolved information about the redox status and it is not described in which skin depth the redox status is measured with this method.

EPR spectroscopy also offers the possibility to measure the redox status in skin *in vivo* using L-band (1.3 GHz) in addition to the application of *in vitro* and *ex vitro* skin. *In vivo* EPR by L-band allows the detection of applied nitroxide radicals up to a skin depth of 2 cm and has also the potential to investigate the redox status in different microenvironmental polarities [85,92]. Furthermore, EPR also offers the possibility to visualize the redox status by EPR imaging, providing additional information on the spatial distribution of the spin probe along the skin depth. EPR imaging has already been applied to *ex vivo* skin [122] as well as *in vivo* [95,169] to investigate e.g. the pharmacokinetics of nitroxide radicals. However, EPR imaging requires additional equipment and qualified staff for implementation and EPR spectra analysis. For the investigation and visualization of the redox status *in vivo* a further method combining NMR and EPR, the dynamic nuclear polarization magnetic resonance imaging, has also been established using AD mouse [78]. Here, the signal from NMR spectroscopy is enhanced by transferring the polarization of unpaired electrons to the surrounding nuclei using microwave irradiation [170]. All in all, there are a variety of methods for the investigation of the redox status in skin. While the investigation of individual oxidative and antioxidative markers by spectrophotometric or fluorescence-based methods can give detailed analysis of differences in the redox status, EPR spectroscopy is an established method to offer a broad range of information to investigate comprehensively the redox status in *ex vivo*, *in vitro* and *in vivo* skin.

3.5 Outlook and further perspectives

We investigated the redox status in normal and inflammatory *in vitro* skin equivalents by EPR and detected a clearly increased metabolic radical production in inflammatory skin equivalents. Since the immune system plays a crucial role in AD, immune cells such as $CD4^+$ T cells could be added to the skin equivalents as described in Wallmeyer *et al.* [171] and thus the effects of the immune cells on the redox status could be investigated by EPR. Furthermore, these *in vitro* skin equivalents could be used to further elucidate the molecular mechanism between ROS and AD, which is not yet known. Especially, the role of DUOX1 and NF-κB could be investigated more in detail. In addition, further spin probes such as mito-TEMPO could be used to obtain more information on redox status in individual compartments, like mitochondria [162,172].

Since human *in vivo* studies on the redox status in atopic skin are very rare and results of *in vivo* AD mouse are hard to transfer to humans [75,78], an *in vivo* study with AD patients is planned in the future. The ethics proposal for this study was prepared by me and has already been accepted by the ethics committee of Charité – Universitätsmedizin Berlin. In this *in vivo* study, the spin probes PCA and TEMPO will be used to gain an overview of the oxidative and antioxidative status in normal and atopic skin by L-band EPR spectroscopy. PCA [45,142,173] and TEMPO [141,143] have already been used in several *in vivo* studies. The use of the spin trap DMPO for radical characterization could also be a possibility in this study although no *in vivo* studies on humans with DMPO have been published so far. When using PCA, TEMPO and DMPO *in vivo*, the toxic and irritant effects of these spin probes and trap have to be taken into consideration. Previous *in vitro* or *in vivo* data showed no adverse effects for PCA, TEMPO [174] and DMPO [175,176] on the skin [90]. Furthermore, the UVB-NIR radiation used in the experiments has to be controlled and should not exceed one minimal erythema dose to prevent adverse effects in the volunteers. Since *in vivo* EPR uses a L-band EPR spectrometer with a surface coil resonator allowing measurements on volunteers [109,142], a splitting of the peaks into the hydrophilic and lipophilic microenvironment cannot be measured directly. However using simulation, the EPR spectra could be deconvoluted into their hydrophilic and lipophilic spectra to also gain insight into the redox status of the different layers [92]. Since L-band spectrometer can detect spin probes till a depth of 2 cm, the detection of TEMPO in the subcutaneous tissue could increase the lipophilic peak that should be considered. Furthermore, in the *in vivo* study with AD patients, carotenoids in the skin will be measured non-invasively by resonance Raman spectroscopy [116] and the GSH concentration as well as the catalase activity in the SC will be determined [115]. To compare the redox status in the skin and in the blood of the AD patients, oxidative and antioxidative markers in the blood will also be determined.

If the *in vivo* study shows that AD patients have an elevated oxidative stress level, antioxidants could be administered orally or topically to the patients in further *in vivo* studies to observe a potential improvement of the redox status. Promising results have already been shown with vitamin C, vitamin E [177,178], SOD [179] as well as goat milk fermented with antioxidative probiotic in adult patients with mild to moderate AD [163]. Furthermore, antioxidants could be also supplemented to the culture medium or applied topically to the *in vitro* skin equivalents. This might open the possibility to develop new therapeutic approaches for AD based on antioxidants in the future.

3.6 Conclusion

This work highlights the potential of EPR spectroscopy for the investigation of the redox status in *ex vivo* and *in vitro* skin models. Different EPR-based approaches were used successfully to determine the oxidative status including radical quantification and characterization as well as stress resistance using the spin probe PCA and the spin trap DMPO. The antioxidative status in the whole skin sample as well as in SC and viable skin individually was also analyzed using the spin probe TEMPO. Investigation of the redox status of inflammatory *in vitro* skin equivalents emulating characteristics of AD showed an increased metabolic radical production and decreased epidermal GSH concentration compared to the normal skin equivalents. It was also observed that the redox status varies between different skin layers and the type of investigation used. The results of this work indicate that the redox status under inflammatory conditions is shifted towards oxidants and that oxidative stress could play a role in atopic skin which could offer new therapeutic approaches. In conclusion, it has been shown that EPR is a powerful method for determining the redox status in real time in *ex vivo* and *in vitro* skin models, as well as in different microenvironments. It can complement or partially replace commonly used spectrophotometric and fluorescence-based methods to save time and offers the possibility to visualize the redox status by EPR imaging.

4 Summary

4.1 Summary

The redox status describes the balance between oxidants and antioxidants and is essential for maintaining physiological processes. However, excessive radical production can lead to an imbalance, known as oxidative stress, which is involved in a variety of pathological changes associated with cancer or chronic inflammatory skin diseases. However, the role of oxidative stress in atopic dermatitis (AD) has not been elucidated so far and studies on the redox status in atopic skin are very limited. Since inflammatory reactions are associated with oxidative stress and the skin is constantly exposed to environmental factors, oxidative stress could play a role in atopic skin. Here, electron paramagnetic resonance (EPR) spectroscopy offers the opportunity to get a comprehensive overview of the redox status in atopic skin and a deeper understanding of this topic.

Therefore, different EPR-based approaches using redox active spin probes and a spin trap were established in different *ex vivo* and *in vitro* skin models to assess both the oxidative and antioxidative status in the skin. In the investigation of the oxidative status, the spin probe PCA was used to quantify the radical production and the spin trap DMPO was used to characterize the radical types. In addition, UVB-NIR radiation was used as an exogenous stress factor to investigate radiation-induced radical production in the skin and thus gaining information about the stress resistance. Furthermore, the spin probe TEMPO was used to investigate the antioxidative status, which gives information on the antioxidant capacity in the skin. The established EPR measurements showed differences in the redox status between the different skin models (*ex vivo* human, porcine, murine skin, *in vitro* skin equivalent), especially in the amount and type of radicals after UVB-NIR irradiation.

Apart from the antioxidant capacity, TEMPO can also be used to obtain spatially resolved information of skin, since it can be distributed in both hydrophilic and lipophilic microenvironments. TEMPO showed a correlation of the distribution into the lipophilic microenvironment with the thickness of the *stratum corneum* (SC) in different *ex vivo* and *in vitro* skin models. Thus, the TEMPO amount in the lipophilic microenvironment directly corresponds to the SC, demonstrating the use of TEMPO to not only provide information on the antioxidant capacity in the whole skin, but also in the lipophilic SC and hydrophilic viable skin.

The EPR-based approaches have been successfully established and therefore used to comprehensively investigate the redox status in atopic skin. For this purpose, normal and inflammatory *in vitro* skin equivalents emulating characteristics of AD were used, which are not influenced by exogenous stress factors and showed a comparable stress resistance to

in vivo. The investigations showed a significantly increased metabolic radical production in the inflammatory skin equivalents, which could be due to an increased expression of dual oxidase 1 (DUOX1) in the keratinocytes by stimulation of interleukin- (IL-) 4 and IL-13. In agreement, the glutathione (GSH) concentration in the epidermis of the inflammatory skin equivalents was reduced, which indicates an increased consumption of antioxidants due to increased radical production. In contrast, the antioxidant capacity in both hydrophilic (viable skin) and lipophilic (SC) microenvironment showed no differences between normal and inflammatory skin equivalents. Furthermore, the radical production after UVB-NIR irradiation as well as the radical types produced also showed no differences between the skin equivalents, which represents a similar stress resistance. These results illustrate that the redox status can differ depending on whether the total status or individual components are investigated.

In summary, EPR spectroscopy can be used to determine the oxidative and antioxidative status in the skin by using the spin probes PCA and TEMPO and the spin trap DMPO, which illustrates the potential of EPR spectroscopy in the investigation of oxidative stress in dermatological research. Furthermore, the EPR results showed that an increased metabolic radical production and associated oxidative stress could play a role in atopic skin, thus human *in vivo* studies in particular should be performed in the future in order to develop potential therapeutic approaches.

4.2 Zusammenfassung

Der Redoxstatus beschreibt das Gleichgewicht zwischen Oxidantien und Antioxidantien und ist essentiell für die Erhaltung physiologischer Prozesse. Bei einer übermäßigen Radikalproduktion kann jedoch ein Ungleichgewicht entstehen, das als oxidativer Stress bezeichnet wird und bei einer Vielzahl von pathologischen Veränderungen wie Krebs oder chronisch entzündlichen Hauterkrankungen beteiligt ist. Die Rolle von oxidativem Stress in atopischer Dermatitis (AD) ist jedoch bisher nicht aufgeklärt und Studien zum Redoxstatus in atopischer Haut sind sehr limitiert. Da Entzündungsreaktionen mit oxidativem Stress assoziiert sind und die Haut ständig Umweltfaktoren ausgesetzt ist, könnte oxidativer Stress in atopischer Haut eine Rolle spielen. Hierbei bietet die Elektronen-Spin-Resonanz- (ESR-) Spektroskopie die Möglichkeit, einen umfassenden Überblick über den Redoxstatus in atopischer Haut sowie ein tieferes Verständnis zur Thematik zu erhalten.

Daher wurden verschiedene ESR-basierte Ansätze mittels redoxaktiven Spinsonden und einer Spinfalle an verschiedenen *ex vivo* und *in vitro* Hautmodellen etabliert, um sowohl den oxidativen als auch antioxidativen Status in der Haut zu erfassen. Zur Untersuchung des oxidativen Status wurde die Spinsonde PCA zur Quantifizierung der Radikalproduktion als auch die Spinfalle DMPO zur Charakterisierung der Radikaltypen verwendet. Zusätzlich wurde UVB-NIR Strahlung als exogener Stressfaktor verwendet, um strahlungsinduzierte Radikalproduktion in der Haut zu untersuchen und somit Aussagen zur Stressresistenz zu erhalten. Weiterhin wurde die Spinsonde TEMPO zur Untersuchung des antioxidativen Status verwendet, womit Aussagen zur antioxidativen Kapazität in der Haut getroffen werden können. Die Etablierungsmessungen zeigten Unterschiede im Redoxstatus zwischen den verschiedenen Hautmodellen (*ex vivo* Human-, Schwein-, Maushaut, *in vitro* Hautäquivalent) auf, insbesondere in der Radikalmenge und -art nach UVB-NIR Bestrahlung.

Zusätzlich zur antioxidativen Kapazität kann TEMPO ebenfalls genutzt werden, um ortsauflösende Informationen in der Haut zu erhalten, da es sich sowohl in hydrophile als auch lipophile Mikroumgebungen verteilen kann. An verschiedenen *ex vivo* und *in vitro* Hautmodellen konnte gezeigt werden, dass die TEMPO Verteilung in der lipophilen Mikroumgebung mit der SC Dicke korreliert. Somit entspricht die TEMPO Menge in der lipophile Mikroumgebung direkt dem SC, weshalb TEMPO nicht nur Informationen zur antioxidativen Kapazität in der gesamten Haut, sondern ebenfalls im lipophilen SC und der hydrophilen viablen Haut liefern kann.

Die ESR-basierten Ansätze wurden erfolgreich etabliert und wurden daher verwendet, um den Redoxstatus in atopischer Haut umfassend zu untersuchen. Hierfür wurden normale und entzündliche *in vitro* Hautäquivalente verwendet, die Charakteristika von AD imitieren,

durch keine exogenen Stressfaktoren beeinflusst werden sowie eine vergleichsweise ähnliche Stressresistenz zu *in vivo* aufweisen. Die Untersuchungen zeigten in den entzündlichen Hautäquivalenten eine deutlich erhöhte metabolische Radikalproduktion, das auf eine erhöhte Expression von der Dualoxidase 1 (DUOX1) in den Keratinozyten durch die Stimulation von Interleukin- (IL-) 4 und IL-13 zurückzuführen sein könnte. In Übereinstimmung dazu war die Glutathion- (GSH-) Konzentration in der Epidermis der entzündlichen Hautäquivalente reduziert, das auf einen erhöhten Verbrauch von Antioxidantien aufgrund der erhöhten Radikalproduktion hinweisen lässt. Im Gegensatz dazu zeigte die antioxidative Kapazität sowohl in hydrophiler (viabler Haut) als auch in lipophiler (SC) Mikroumgebung keine Unterschiede zwischen normalen und entzündlichen Hautäquivalenten. Zudem zeigten die Radikalproduktion nach UVB-NIR Bestrahlung als auch die entstandenen Radikaltypen ebenfalls keine Unterschiede zwischen den Hautäquivalenten, das eine ähnliche Stressresistenz darstellt. Diese Ergebnisse verdeutlichen, dass sich der Redoxstatus in Abhängigkeit davon unterscheiden kann, ob der totale Status oder einzelne Komponenten untersucht werden.

Zusammenfassend kann die ESR-Spektroskopie unter Verwendung der Spinsonden PCA und TEMPO sowie der Spinfalle DMPO verwendet werden, um den oxidativen und antioxidativen Status in der Haut zu bestimmen, dass das Potential der ESR-Spektroskopie zur Untersuchung von oxidativem Stress in der dermatologischen Forschung verdeutlicht. Zudem zeigten die ESR-Ergebnisse, dass eine erhöhte metabolische Radikalproduktion und damit verbundener oxidativer Stress eine Rolle in atopischer Haut spielen könnte, weshalb zukünftig vor allem humane *in vivo* Studien durchgeführt werden sollten, um potenzielle Therapiemöglichkeiten entwickeln zu können.

5 References

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List of publications

A. Elpelt, S.B. Lohan, M.E. Darvin, J. Lademann, M.C. Meinke, Carotenoids - Effective Radical Scavengers for Healthy and Beautiful Skin, Heal. Educ. Public Heal. 2 (2019) 227–231. doi:10.31488 /heph.129.*

A. Elpelt, D. Ivanov, A. Nováčková, A. Kováčik, M. Sochorová, S. Saeidpour, C. Teutloff, S.B. Lohan, J. Lademann, K. Vávrová, S. Hedtrich, M.C. Meinke, Investigation of TEMPO partitioning in different skin models as measured by EPR spectroscopy – Insight into the stratum corneum, J. Magn. Reson. 310 (2020) 106637. doi:10.1016/j.jmr.2019.106637.

A. Elpelt*, S. Albrecht*, C. Teutloff, M. Hüging, S. Saeidpour, S.B. Lohan, S. Hedtrich, M.C. Meinke, Insight into the redox status of inflammatory skin equivalents as determined by EPR spectroscopy, *Chem. Biol. Interact*. 310 (2019) 108752. doi:10.1016/j.cbi.2019.108752 (* both authors contributed equally).

S. Albrecht*, **A. Elpelt***, C. Kasim, C. Reble, L. Mundhenk, H. Pischon, S. Hedtrich, C. Witzel, J. Lademann, L. Zastrow, I. Beckers, M.C. Meinke, Quantification and characterization of radical production in human, animal and 3D skin models during sun irradiation measured by EPR spectroscopy, Free Radic. Biol. Med. 131 (2019) 299–308. doi:10.1016/j.freeradbiomed.2018.12.022 (* both authors contributed equally).

*not part of this dissertation

Oral presentations

Conference

The 52nd Annual International Meeting of the ESR Spectroscopy Group of the Royal Society of Chemistry, Glasgow, United Kingdom, April 2019: *EPR Spectroscopy for Characterization of Skin Barrier*.

20th International Conference on Oxidative Stress Reduction, Redox Homeostasis and Antioxidants, Paris, France, June 2018: *Investigation of redox status in different skin models.*

Intern

Research Seminar at the Department of Dermatology, Venerology and Allergology, Charité – Universitätsmedizin Berlin, April 2019: *Is EPR spectroscopy a suitable method for assessment of skin barrier function?*

Research Seminar at the Department of Dermatology, Venerology and Allergology, Charité – Universitätsmedizin Berlin, May 2018: *Investigation of environment and redox status in skin models using nitroxide radical TEMPO*

Disciplinary Forum Area B and C of SFB1112, Freie Universität Berlin, September 2017: *Investigation of redox status in different skin models by EPR*

Disciplinary Forum Area B of SFB1112, Freie Universität Berlin, February 2017: *Investigation of redox status by EPR spectroscopy*

Disciplinary Forum Area B of SFB1112, Freie Universität Berlin, July 2016: *Investigation of redox status in skin models and influence of nanotransporters and other exogenous stress factors*

Poster presentation

Albrecht S, Kasim C, **Elpelt A**, Hedtrich S, Gruber A, Witzel C, Lademann J, Zastrow L, Beckers I, Meinke MC: Quantification and Characterization of cutaneous irradiation induced radical formation by EPR spectroscopy in different skin models, *2nd Sino-German Symposium*. Berlin, May 30 - June 1, 2017.

Scholarships and Grants

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Statement of Authorship (Eigenständigkeitserklärung)

Hiermit versichere ich, Anja Elpelt, die vorliegende Arbeit selbstständig verfasst zu haben. Alle verwendeten Hilfsmittel und Hilfen habe ich angegeben. Die Arbeit wurde weder in einem früheren Promotionsverfahren angenommen noch als ungenügend beurteilt.

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Anja Elpelt