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Atopic eczema and its relation to the skin barrier

Atopic eczema (AE) is a multifactorial, chronic relapsing, pruritic, inflammatory skin disease. The disease is characterized by eczematous lesions as well as a broad spectrum of clinical manifestations, like xerosis (dry skin), erythema (red skin), and pruritus (itch) (Figure 1). These symptoms affect the quality of life substantially, and patients often show difficulties in their social life. The incidence of AE in developed countries is rapidly increasing over the last two decennia to a current prevalence of 5-10% in adults and around 20% in children, making it one of the most common skin diseases. The diagnosis of AE is based on a constellation of clinical findings, as there is no pathognomonic biomarker for diagnosis. Formerly, AE was considered as a solely inflammatory disease and therefore often referred to as atopic dermatitis (but also neurodermatitis and endogenous eczema). Patients show an increased reaction to antigens, thereby inducing a dominant T-cell response that upregulates the production of cytokines. Although the disease is called ‘atopic’, up

Figure 1: An example of a child with AE. In general, patients show the presence of lesional areas (affected, presence of erythema) as well as non-lesional sites (unaffected, which appear healthy).
to 60% of patients with the clinical phenotype of AE do not have elevation of total or allergen-specific IgE levels in serum. As only a subpopulation show increased levels of IgE antibodies and eosinophils (white blood cells associated with allergy), there is still a controversy in terminology.

However, a strong association between AE and loss-of-function mutations in the filaggrin gene (FLG) was observed in 2006. Filaggrin is not directly related to the primary inflammatory response in AE, but is crucial for a proper formation of the outermost layer of the skin, the stratum corneum (SC), and SC hydration. The SC functions as the primary barrier of the skin. Often referred to as a ‘brick and mortar’ structure, both the corneocytes (bricks) and lipids (mortar) are essential for the skin barrier. This finding created a paradigm shift in the understanding of the disease away from the inflammatory aspects towards AE as an epidermal barrier disorder. One of the current hypotheses is that defects in the barrier function of the SC may facilitate the transport of pathogens, allergens, and irritants across the skin, thereby provoking dysfunctional innate and adaptive immune responses (Figure 2). This inflammation exacerbates the barrier defects, which allows successive pathogens to penetrate the skin, initiating a vicious circle. Recent studies corroborate this theory that a skin barrier dysfunction is causative for the risk of developing AE.

**Human epidermis**

Mammalian skin is divided into two layers, the dermis and epidermis (Figure 2). The former consists of predominantly collagen and elastin, but also contains fibroblasts and...
the lymph vessels crucial for lymphocyte distribution in the dermis. Lymphocytes act as a second line of defense, and may induce a T-cell response when arrest of exogenous compounds occurs. In AE, lymphocyte activation leads to an inflammatory response resulting in skin lesions. The fact that exogenous compounds can reach the second line of defense means that it has already surpassed the epidermis.

The human epidermis can be divided into 4 layers (strata). The stratum basale is the deepest located layer that contains the epidermal stem cells. In this layer, proliferation of keratinocytes the major cell type takes place. Keratinocytes migrate upwards, and differentiate during this migration. This differentiation is a sequential process crucial for formation of the barrier function of the skin. First, keratinocytes are migrating to the stratum spinosum where the formation of lamellar bodies (LBs) is initiated. LBs are secretory organelles that contain the precursors of the lipids essential for a proper skin barrier as well as many enzymes necessary to convert the lipids into their final structure in human SC. The corneocytes are gradually differentiating and migrating towards the stratum granulosum, where numerous processes take place in a very short time period: Keratin filaments aggregate after interaction with filaggrin, and enzymes will start to degrade cell components like the nucleus and cell organelles. In addition, desmosomes that keep the keratinocytes together are transformed into corneodesmosomes, and a cornified envelope is formed around the plasma membrane of the keratinocytes. Lipids and enzymes of the LBs are extruded at the interface between the stratum granulosum and the uppermost layer of the skin, the SC.

The stratum corneum as the primary skin barrier

The SC acts as the primary barrier against penetration of pathogens, allergens and other exogenous substances into the lower layers of the skin (the so-called outside-in barrier), and also prevent excessive transepidermal water loss (TEWL, Figure 2), the so-called inside-out barrier. The SC has a 'brick-and-mortar' like structure. The 'bricks' are corneocytes: flattened, terminally differentiated keratinocytes. The SC contains around 20 layers of corneocytes and is around 15-20 μm thick. The corneocytes are embedded in a lipid matrix that is the ‘mortar’ of the SC barrier. The mortar is formed at the interface of the stratum granulosum and SC: The lipid disks stored in the LBs are extruded into the intercellular space between the stratum granulosum and SC. These lipid disks fuse together and create the highly ordered lipid lamellae. Metabolism, transport and extrusion of the SC lipids will be discussed below.

The SC is constantly renewed. In human skin this occurs in approximately 2 to 4 weeks. At the surface, continuous shedding of the SC takes place at a rate of approximately 5·10^8
cells/day, a process called desquamation\(^{44}\). It is an active process that depends on pH, enzyme activity, and also on SC water levels in which kallikreins (KLKS) and cathepsins degrade the strong cohesive links between the corneocytes, the corneodesmosomes\(^{45-49}\). Friction or sheer stress with the environment enhances the shedding of corneocytes at the skin surface.

The major penetration pathway of most molecules through the SC is along the intercellular pathway (i.e. penetration via the extracellular lipid matrix)\(^{50,51}\). This emphasizes the importance of the SC lipids as the primary barrier components of the skin.

**Extracellular lipid matrix in human stratum corneum**

The lipids located in the extracellular matrix of the SC consist primarily of three lipid classes: ceramides (CERS), free fatty acids (FFAS), and cholesterol (CHOL), in approximately equimolar amounts\(^{52-55}\). The former two consist of carbon chains that are exceptionally heterogeneous in their molecular structure and are therefore classified into subclasses. Under healthy conditions, human SC FFAs are predominantly saturated, but mono-unsaturated FFAs (MUFA\(S\)) and trace amounts of poly-unsaturated FFAs (PUFAs) are present as well\(^{53,56,57}\). FFAs with an additional hydroxyl-group (OH-FFAs) are occasionally reported too\(^{57}\). Moreover, FFAs show an extensive variation in their carbon chain length. The most predominant FFAs have a chain length around 24 carbon atoms, but the full chain length distribution varies between 16 and 36 carbon atoms\(^{56,57}\). The second lipid class, CERS, show even more structural diversity: They consist of a sphingoid base linked via an amide bond to a fatty acid (acyl) chain and are classified according to the different functional groups they have in both chains (Figure 3)\(^{58}\): The sphingoid moiety can either be a sphingosine [S], dihydrosphingosine [dS], phytosphingosine [P] or 6-hydroxysphingosine [H]. The acyl chain is either non-hydroxylated [N], α-hydroxylated [A], or can even be ω-hydroxylated and successively linked to another fatty acid resulting in an esterified ω-hydroxylated [EO] acyl chain. The latter [EO] subclass (also named acyl-CER subclass) has an exceptionally long carbon chain which is unique for SC lipids. Different combinations in both carbon chains lead to the possibility of 12 different CER subclasses which have all been observed in human SC except for CER [EODS]\(^{59}\). In addition to their variation in subclasses, CERS show a wide distribution in their total chain length (that is, the chain length of both the sphingosine base and the acyl chain together). CER chain lengths between 34 carbon atoms and 72 carbon atoms have been reported in healthy human subjects\(^{59,60}\).

The SC lipid matrix is not only distinctive in its composition, but also shows a unique three-dimensional ordering (Figure 4)\(^{61}\). Lipids are organized in stacked layers, lamellae\(^{62,63}\).
The structures formed by these layers have a repeat distance \((d)\) of approximately 6 nm or 13 nm, referred to as the short periodicity phase (SPP) and long periodicity phase (LPP), respectively\(^{64-66}\). In particular the LPP is considered to be important for the barrier function of the skin, as is demonstrated from in vitro studies using lipid membranes\(^{67-69}\).

Approximately perpendicular to the lamellar organization, lipids are packed with certain density. This lateral lipid packing is also of high importance for a proper SC barrier function\(^{70}\). At physiological temperature, human SC lipids are mainly present in a very dense orthorhombic organization. However, some lipid domains may be arranged in a less dense, hexagonal organization or even liquid organization, making the SC more permeable\(^{71-75}\).

**Epidermal lipid metabolism**

SC lipids (viz. FFAs, CERS and CHOL) are either generated in viable keratinocytes by several enzymatic reactions (de novo synthesis) or taken up by keratinocytes from extracutaneous...
sources, such as the dietary lipids (e.g. essential FFAs) or lipids synthesized in other organs like the liver\textsuperscript{76}. Several enzymatic reactions are required for lipids synthesis and the subsequent lipid transport from the keratinocytes into the extracellular matrix of the SC, which will be discussed briefly (Figure 5).

Fatty acid synthase synthesizes FFAs to a chain length of 16 carbon atoms (palmitic acid) using acetyl-CoA and malonyl-CoA. Successively, FFAs can be elongated over 16 carbon atoms by a series of 7 elongases (ELOVLs)\textsuperscript{77-79}. This results in a wide chain
length distribution of FFAs. In addition to elongation, FFAs can be converted to mono-unsaturated FFAs (MUFAs) and poly-unsaturated FFAs (PUFAS) catalyzed by stearoyl-CoA desaturases (SCDs). Subsequently, the synthesized FFAs can either be used for synthesis of CERS (described below) or transformed to phospholipids and stored into LBs.

CER synthesis occurs in the endoplasmic reticulum. The first step involves the enzyme serine palmitoyl transferase that catalyzes the condensation of serine and palmitoyl-CoA to form 3-keto-dihydrosphingosine, which is successively reduced to form dihydrosphingosine. The next enzymatic step acylates a FFA to dihydrosphingosine. In this step, 6 CER synthases are involved. Each of the different CER synthases are more specific to certain FFA chain length and degree of unsaturation. The final step involves the conversion of the dihydrosphingosine into one of the several sphingoid subclasses.
catalyzed by dihydroceramide desaturase (DES) enzymes. These subclasses of sphingoid bases are either sphingosine [S], dihydrosphingosine [ds], phytosphingosine [P], or 6-hydroxysphingosine [H]. The different CERS are then transported to the Golgi complex and converted to glucosyl-CERS and sphingomyelin, before transported into LBs.

CHOL is the third main component of the SC lipid matrix and needs no conversion for storage into the LBs. It can also be converted to oxysterol or CHOL-sulfate, which stimulates keratinocyte differentiation and plays a crucial role in the desquamation process by inhibiting the activity of proteases that promote the degradation of the desmosomes. Moreover, CHOL-sulfate is highly amphiphilic and can therefore cross the keratinocyte membrane and directly enter the stratum corneum where it will be partially converted back to CHOL by cholesterol sulfatase to be incorporated into the lipid lamellae.

The LBs generated in the stratum spinosum and stratum granulosum contain both the lipid precursors (CHOL, phospholipids, glucocylceramides and sphingomyelin) as well as the enzymes that convert the precursor-lipids into the final extracellular barrier lipids. At the interface of the stratum granulosum and SC, secretion of the lipids from the LBs into the stratum corneum occurs. This process is called lamellar body extrusion, and is triggered by changes in the local calcium concentration. The lipids are then converted to their final substituents by enzymes that are cosecreted in the LBs: The phospholipids are converted back to FFAs (and glycerol) by phospholipases, whereas β-glucocerebrosidase and acidic sphingomyelinase convert respectively glucosylceramides and sphingomyelin back into CERS.

Crucial for an optimal lipid synthesis and successive LB formation is a proper differentiation of keratinocytes. Important regulators of keratinocyte proliferation and differentiation are the nuclear hormone receptors like peroxisome proliferator-activated receptors (PPARS) and liver X receptor (LXR). These transcription factors are designated as ‘liposensors’, as they can sense cellular lipid levels and adjust gene expression accordingly. PPARs are primarily activated by FFAs and their metabolic products, while LXR is activated by oxysterol metabolites. Activation of PPARs and LXR stimulate expression of keratinocyte differentiation markers, such as loricrin, involucrin and filaggrin. Consequently, as the amount of FFAs and CHOL necessary for LB formation increases, PPAR and LXR activation is also increased which stimulate corneocyte formation. In addition to their role in the formation of the SC ‘bricks’, PPARs and LXR also act directly on the SC lipids: They stimulate lipid synthesis, facilitates both LB formation and LB secretion, and promote extracellular lipid processing.
Stratum corneum hydration

The permeability, flexibility, and enzymatic activity of the SC is highly dependent on its water content. Proper SC hydration is regulated by (derivatives of) amino acids and specific salts, commonly referred to as the ‘natural moisturizing factor’ (NMF)\textsuperscript{111,112}. Reduced NMF levels in the SC may negatively affect the hydration level, but is also suggested to increase the local pH, as NMF are derived from amino acids. Sub-optimal SC hydration levels or pH can have an effect on hydrolytic enzymes and the aforementioned permeability and flexibility. This may thereby induce a negative effect on many processes like desquamation, keratinocyte differentiation, lipid synthesis, cornified envelope formation, and the SC barrier function\textsuperscript{111,113-119}. Most of the amino acid derived NMF are breakdown products of filaggrin. FLG mutations lead to reduced NMF levels which is associated with dry skin\textsuperscript{120,121}. This may explain the relation between AE, dry skin and FLG mutations\textsuperscript{122}. Although loss-of-function mutations in the FLG gene are the foremost genetic risk factor for developing AE, a relatively large percentage of AE patients around 50-80% do not carry such a mutation\textsuperscript{123,124}. Besides, there is no convincing data that shows a clear relation between FLG mutations and a reduced skin barrier function in AE patients as measured by transepidermal water loss. Therefore, the role of filaggrin for an impaired SC barrier function remains indistinct, suggesting that other components of the epidermis and the skin barrier are likely to be involved as well\textsuperscript{125-127}.

The cutaneous immune response in AE

The primary cause for inflammation in AE is related to penetration of antigens through the disrupted SC barrier. This leads to an acute Th2-driven inflammatory response in the early stage of AE, but can develop in a Th1-driven response when the disease becomes chronic\textsuperscript{11,20,128,129}. In addition, lesional epidermis (affected skin sites, Figure 1) in AE is characterized by infiltration of dermal dendritic cells, memory T cells, eosinophils, mast cells, lymphocytes and (IgE coated) macrophages\textsuperscript{11,130-134}. The Th2-response leads to increased expression of cytokines which play a crucial role in the cutaneous immune response. Among these are the pro-inflammatory interleukins (ILs), like IL-4, IL-5, IL-13, IL-17, IL-22 as well as IL-31, the initiator of the itch-response. These cytokines reduce the expression of filaggrin, thereby exaggerating AE\textsuperscript{11,135,136}. The production of these cytokines occurs in the T-cells, except for thymic stromal lymphopoietin (TSLP), which is produced by the keratinocytes. TSLP enhances the production of Th-2 cytokines by mast cells and mediates the dendritic cells towards a Th2-response\textsuperscript{137,138}.

The hydration of the skin is also of importance for the immune response. It is known that an increase in local pH induces the activity of serine proteases, leading to
the generation of primary cytokines, interleukin (IL)-1α and IL-1β. These are considered to be important for triggering the cytokine cascade and are related to the skin barrier dysfunction.

Studies on the SC barrier lipids in AE

The specific role of the lipid composition and organization with respect to AE has yet to be elucidated, though several studies show the importance of these aspects regarding AE. Concerning the lipid composition, there is conflicting information reported: some studies notice no change in the CER composition, whereas others demonstrate a decrease in total CER level as well as a decrease of CER subclasses [EOS], [EOH] and/or [NP], and increase in CER [AS]. A study by Ishikawa et al. tended to show differences in the chain length of some CER subclass in lesional skin only. In addition, they show that the levels of individual CER subclasses were altered even in non-lesional AE skin. With respect to FFAs, little is known on the changes in SC of AE compared to that in SC of control subjects. Results on the FFA lipid class in AE are scarce, but two studies report a decrease in SC FFAs longer than 24 or 26 carbon atoms in AE patients. With respect to the lipid organization in AE patients, almost no information is available. Pilgram et al. performed a limited study in 3 AE subjects in which they observe a significant increase in hexagonally ordered lipids compared to controls, as studied by electron diffraction. Farhat et al. show that LB extrusion is delayed in AE, resulting in diminished delivery of the lipids into the intercellular regions. The metabolic enzymes involved in lipid synthesis have been studied as well, since these are involved in the underlying causes for possible changes in the SC lipids of AE patients. However, the data are scarce. For example: two studies report no changes in enzyme activity of sphingomyelinase and β-glucocerebrosidase in skin of AE patients compared to healthy controls. However, this is contradictory to a study published by Jensen et al., who reported a reduced activity of sphingomyelinase in both lesional and non-lesional skin of AE patients. Hara et al. report that ceramide deficiency is related to another enzyme, sphingomyelin deacylase, which converts sphingomyelin into sphingosylphosphorylcholine and FFAs instead of CERS. Another example of important SC lipid modulators which are modified in AE are the PPARs. Lesional skin of AE patients showed an increased expression of PPARβ/δ, while the expression of PPARα and PPARγ was decreased. As discussed before, PPARs stimulate keratinocyte expression and have a direct effect on lipid synthesis, LB formation and its secretion, and promote extracellular lipid processing. Changes in these nuclear receptor proteins are therefore suggested to affect the SC lipid barrier. However, the changes in differentiation in relation to lipid biosynthesis and lipid composition are not fully established. One of the important
piec in the puzzle is a comprehensive analysis of the barrier lipids, in which not only the lipid classes, but also the chain length distribution of the lipids is studied.

**Analysis of the SC lipid composition**

Analyzing the three main SC lipid classes (CHOL, FFA, CERS) can be challenging, as these lipids are very non-polar and show a large diversity. FFAs are most commonly analyzed by gas chromatography (GC) or gas liquid chromatography (GLC). Although the lipid composition can be examined in detail, the main drawback of these methods is related to the labor intensive derivatization of the sample prior to analysis. Despite all technological improvements, identification and quantification of SC FFAs has only been reported twice by Ansari and Norlen in healthy human skin.

The structural variation is the main challenge for analysis of CERS. Usually, thin layer chromatography (TLC) is used to separate some of the CER subclasses, and quantification or additional structural information can be obtained by successive densitometry or nuclear magnetic resonance (NMR) spectroscopy. Main drawbacks are that TLC is usually cumbersome, cannot separate all subclasses at once, and has a low linear dynamic range in terms of quantification. Better separation of lipids can usually be achieved by GC, but CERS are non-volatile and unstable in the gas-phase, making GC only compatible for analysis of CERS when derivatized. Liquid chromatography (LC) in combination with light-scattering detection (LSD) has proven its potential for proper SC CER separation and analysis, but the inability for quantification is a major disadvantage. Mass spectrometry (MS) is currently the most sensitive and powerful tool for identification of CERS, and although quantification is relatively difficult, technological developments over the last decennia have led to improvements on this major issue. In combination with LC, it allows for analysis of all CER subclasses and can distinguish between different chain lengths as well. Whereas TLC in combination with NMR demonstrates the presence of 9 CER subclasses, the introduction of LC/MS has led to the discovery and identification of 2 additional subclasses. MS can be a powerful tool as it gives information on the mass of a compound, a unique feature very useful for identification. However, the aforementioned quantification issue is a major drawback. Proper quantification is usually difficult as MS needs extensive validation, multiple internal standards per sample and quality controls. Nevertheless, reported data on SC CERS by LC/MS has proven its potential and its high sensitivity makes LC/MS the preferred method when small quantities of material are used.

The analysis of all lipid classes at once is currently limited to TLC only. This method has led to enormous advancements in the understanding of lipids in the SC, and is still...
frequently used for SC lipid analysis. However, the aforementioned disadvantages makes this method not appropriate for detailed analysis, especially when focusing on the lipid chain length distribution or when using small lipid amounts. There is currently no method for detailed analysis of all SC lipids, in which chain lengths of individual FFAs and CERS as well as all their subclasses can be studied at once. One of the primary challenges that will be addressed in this thesis is regarding development of a robust and high-throughput method, using straightforward sample preparation that enables detailed analysis of all main SC lipid classes in a single setup using very limited sample amounts. LC in combination with MS seems most promising, as it can both separate lipid classes based on polarity (like TLC), and in addition on a second dimension: molecular mass.

The analytical methods reported in literature have also been used to study the SC lipids in AE, although the results remain inconclusive. For example, two (relatively recently) developed methods report contradictory information regarding AE. Farwanah et al. developed a high performance TLC method to compare the CER composition in non-lesional skin of 7 AE patients with 7 healthy control subjects. However, they observed no differences in any of the CER subclasses between the two groups. In contrast, Masukawa et al. developed an LC/MS method to study 8 AE patients and 7 control subjects. In contrast to Farwanah et al., they observed significant changes in some of the CER subclasses of both lesional skin and non-lesional skin. They suggest that besides CER subclasses, CER chain length may be of importance for a proper SC lipid composition. These contradictory results show that more information is required to fully elucidate the lipid composition in these patients. To achieve this, there is a need for proper analytical methods enabling the analysis of all lipid classes in SC as well as the lipid chain length.

Analyzing the SC lipid organization

The SC lipids are organized in a highly ordered 3D-structure (Figure 4). The lamellar lipid organization can be studied by means of small angle X-ray diffraction (SAXD). The principle of SAXD is that X-rays are scattered by a sample (i.e. SC sheets). The scattered X-rays are recorded as a function of its scattering vector \( q \), defined as \( q = \frac{2\pi \cdot \sin \theta}{\lambda} \), in which \( \lambda \) is the wavelength of the X-rays and \( \theta \) the angle of the scattered X-rays (Figure 6a). As the lamellar lipid organization is characterized by repeating lipid layers (periodicity phase), a typical SAXD profile of human SC (see Figure 6b) shows sequential maxima from which the repeat distance \( d \) of the LPP and SPP can be determined, according to the equation \( d = n \cdot \frac{2\pi}{q_n} \) \((n = \text{order of diffraction peak})\).

The lateral lipid organization can be studied by Fourier transform infrared spectroscopy (FTIR). An infrared beam is emitted on a sample (i.e. SC sheet), and the amount of IR
radiation that is absorbed due to resonating atom bond vibrations is recorded. Analyzing specific vibrations in different wavelength regions provide information on the 3-dimensional SC lipid organization. These are e.g. CH$_2$ symmetric stretching vibrations (2848-2053 cm$^{-1}$) and the CH$_2$ scissoring vibrations (1460-1480 cm$^{-1}$; Figure 7$^{71-75}$. The former provides information on the conformational ordering of the lipids, and peak positions at a lower frequency (~2848 cm$^{-1}$) contribute to a higher degree of conformational ordering than peak positions located at a higher wavenumber (2853 cm$^{-1}$). The bandwidth of the CH$_2$ scissoring vibrations is indicative for the amount of orthorhombic domains

![Figure 6: Principle of SAXD. An X-ray beam is scattered at a certain angle (θ) by the SC sheet, resulting in a 2D-SAXD image, which can be transformed into a typical SAXD plot shown in b): A representative SAXD profile of human SC. The scattering intensity is plotted as a function of q, which is defined by q = $\frac{2\pi\sin \theta}{\lambda}$. The X-ray diffraction graph of human SC is characterized by a high intensity at low q values due to keratin in the corneocytes and a series of peaks. The peaks indicated by I (weak peak), II (strong peak) and III (weak peak) are attributed to the LPP. Peak II is also attributed to the SPP. The peak indicated by # is due to CHOL.](image-url)

![Figure 7: The lateral organization can be measured by FTIR. a) CH$_2$ stretching vibrations: At a lower frequency (~2848 cm$^{-1}$), CH$_2$ stretching vibrations indicate a high degree of conformational ordering of the lipids, whereas a high wavenumber (2853 cm$^{-1}$) is indicative for a liquid organization (low degree of conformational ordering). b) CH$_2$ scissoring vibrations (1460-1480 cm$^{-1}$): An orthorhombic organization results in a splitting of the scissoring vibrations, while a hexagonal packing results in a single vibration.](image-url)
present in human SC. A small, single peak with a limited bandwidth is indicative for the presence of a hexagonal organization, whereas an increased bandwidth of the scissoring vibrations is indicative for an increased fraction of lipids present as orthorhombic lipid domains.

**This thesis**

The rapid increase in prevalence of AE urges for novel treatments, also with respect to SC barrier repair. However, the exact role of the SC lipids for the impaired skin barrier function in AE is inconclusive. This lack of knowledge hampers the development for restoring the SC barrier by e.g. topical treatments. The studies described in this thesis aim in providing detailed information on the SC lipid composition, the lipid organization, and the role of SC lipids for the skin barrier function. In other words: we want to study the role of the lipids in the impaired skin barrier in AE. To study these three basal parameters in a combined study is unique, but needs several scientific barriers to be breached before this can be realized. The next chapter will describe the objectives and aims that facilitate in realizing the main goal of the study.
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