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Review

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Defects of corneocyte structural proteins and epidermal barrier in atopic dermatitis

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Abstract: The main function of the epidermis is to establish a vital multifunctional barrier between the body and its external environment. A defective epidermal barrier is one of the key features of atopic dermatitis (AD), a chronic and relapsing inflammatory skin disorder that affects up to 20% of children and 2–3% of adults and often precedes the development of allergic rhinitis and asthma. This review summarizes recent discoveries on the origin of the skin barrier alterations in AD at the structural protein level, including hereditary and acquired components. The consequences of the epidermal barrier alteration on our current understanding of the pathogenesis of AD, and its possible implications on the treatment of patients, are discussed here.

Keywords: atopic dermatitis; corneocytes; eczema; epidermal barrier; filaggrin; keratinocytes; skin inflammation.

Introduction: epidermal barrier functions and proteins involved

The skin not only forms a physical barrier between the inside and the outside of the body, but is also a biochemical and immunological barrier. Most of these functions are provided by the epidermis, a multilayered, squamous and cornified epithelium, mainly composed of keratinocytes and studded with Langerhans cells, which act as sentries. Throughout their migration from the basal layer of the epidermis toward the external surface of the skin, keratinocytes differentiate in an oriented process and sequentially express a large number of specific proteins, such as keratins K1/K10, involucrin, loricrin, corneodesmosin, filaggrin and other S100-fused type proteins (SFTPs). In parallel, they undergo a series of structural changes and transform into spinous then granular keratinocytes that form the *stratum spinosum* and *stratum granulosum*, respectively (Fuchs and Raghavan, 2002; Harding, 2004; Candi et al., 2005). The cytoplasm of granular keratinocytes contains numerous granules, known as the keratohyalin granules, and tubulo-vesicular structures related to lysosomes, derived from the Golgi apparatus and named lamellar bodies. The keratinocytes are linked together by desmosomes, the components of which include proteins of the desmosomal-cadherin family and change during differentiation. At the level of the granular layer, tight junction proteins also form focal attachment structures that may delineate extracellular compartments and define apical-basal polarization of the upper cells. The ultimate step, known as cornification or keratinization, is a genuine process of programmed cell death that results in the formation of corneocytes. These ‘dead’, large (with a diameter of 30–40 μm), flattened, anucleated cells form the outermost layer of the epidermis called the stratum corneum, also known as the horny or cornified layer. In direct contact with the external environment, the stratum corneum allows the epidermis to perform its vital function of multiple barrier thanks to its high mechanical strength,

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its ability to quench some of the reactive oxygen species, to absorb UV-B radiation, and to prevent the infiltration of allergens and microorganisms, and its capacity to limit water and electrolyte loss from the body (Elias, 2008; Proksch et al., 2008; Jonca et al., 2011). The impermeability is not total, however, and a slight evaporation of water from the epidermis through the stratum corneum can be measured and this is called the transepidermal water loss (TEWL). As the water is in the form of vapor, ions probably remain trapped under the stratum corneum. To maintain the thickness of the stratum corneum constant, corneocytes detach from the epidermis surface during the desquamation process and are constantly replaced by newly differentiated cells (Caubet et al., 2004).

In addition to the rapid disappearance of the nucleus and other organelles, cornification is characterized by sudden biochemical and morphological changes: i) the replacement of the cellular plasma membrane by a 15-nm-thick, resistant, highly insoluble, lipid and protein structure, a real shell, called the cornified cell envelope (Candi et al., 2005); ii) the transformation of desmosomes into corneodesmosomes, intercellular rivet-like structures that tightly connect adjacent corneocytes (Jonca et al., 2011); iii) the formation of a dense intracellular fibrous matrix; preceded by iv) the release of lamellar body content into the extracellular interstices (Fartasch, 2004). Cornified cell envelopes result from the formation of covalent ϵ -(γ -glutamyl)-lysine isopeptide bonds between various cytoplasmic precursors, including involucrin and loricrin. These cross-links are catalyzed by calcium-dependent enzymes called transglutaminases (EC 2.3.2.13; TGases), i.e. TGases 1, 3 and 5. Then, a monolayer of Ω -hydroxyceramides is linked to the external surface of the cornified cell envelopes by esterification, also promoted by TGase1. The ultrastructural morphology of desmosomes changes dramatically during cornification with the densification of their extracellular core, in parallel to the addition of an adhesive protein named corneodesmosin, and the incorporation of their intracellular plaque components within the cornified cell envelope. Proteins resulting from the dispersion of keratohyalin granules and aggregates of keratin intermediate filaments fill the interior of corneocytes. The ‘corneocyte matrix-cornified cell envelope-corneodesmosomes’ super structure provides the structural scaffold for mechanical resistance and cohesion of the stratum corneum. Finally, intercellular spaces are filled by nonpolar lipids (essentially ceramides, free fatty acids and cholesterol) organized into lamellae parallel to the corneocyte surface and derived from the secreted lipid content of lamellar bodies, after their processing by various concomitantly secreted

lipid-modification enzymes, including beta-glucocerebrosidases, acid sphingomyelinase, secreted phospholipases and steroid sulfatase. These lipids are essential for the permeability barrier (Feingold and Elias, 2014). During lamellar body secretion, antimicrobial peptides (cathelicidin, β -defensins, etc.), proteases (kallikreins, cathepsins and others) and protease inhibitors (cystatins and Kazal-type inhibitors) are also delivered to the extracellular spaces. The antimicrobial peptides are part of the innate immune system that controls skin infection, whereas proteases and protease inhibitors finely regulate desquamation through the orderly digestion of corneodesmosomes. To carry out its barrier functions effectively, the stratum corneum remains hydrated regardless of ambient moisture, thanks to the natural moisturizing factor (NMF) (Rawlings and Harding, 2004). This is formed by the combination of hygroscopic molecules – in particular, amino acids and pyrrolidone-5-carboxylic acid, a derivative of glutamine (Harding et al., 2000; Rawlings and Harding, 2004). The NMF also contributes to skin photoprotection because of its high *trans*-urocanic acid content (Barresi et al., 2011) produced from histidine by the activity of the stratum corneum histidine ammonia-lyase (histidase; EC 4.3.1.3.) (Gibbs et al., 2008; Barresi et al., 2011). These amino acids and derivatives mainly come from the total degradation of the histidine-rich protein filaggrin (Harding et al., 2000), but also from the filaggrin-related protein filaggrin-2 (Hsu et al., 2011).

Although corneocytes are no longer able to synthesize new proteins, several dynamic processes occur in the horny layer because of the presence of numerous enzymes and their substrates produced before cornification occurs. The extent of the biochemical reactions and their speed are regulated under physiological conditions. In particular, the environment has some influence (Rogers et al., 1996; Fluhr et al., 2010; Singh and Maibach, 2013), as indicated below for filaggrin degradation.

Two key proteins, filaggrin and filaggrin-2

The epidermal differentiation complex, a 2-Mb region located on human chromosome 1 in 1q21, contains 60 genes expressed in differentiated keratinocytes, many of them encoding structural and regulatory proteins that are of crucial importance for the stratum corneum functions. Besides the genes encoding the proteins already mentioned, loricrin and involucrin, there are four groups of genes: two of them encoding other components of the

cornified cell envelope, namely the small proline-rich proteins (SPRRs) and the late cornified envelope proteins; one group encoding small calcium-binding S100A proteins; and one group coding for the seven members of the SFTP family, including two proteins essential for the epidermal barrier functions, filaggrin and filaggrin-2 (Henry et al., 2012).

Filaggrin is synthesized by granular keratinocytes, as a large (400 kDa) precursor called profilaggrin (Figure 1). Profilaggrin is highly phosphorylated on serine residues and stored in the cytoplasmic keratohyalin granules. It consists of 10–12 repeats of filaggrin, joined by short hydrophobic linker peptides (FLYQVST), flanked by two truncated repeats, an NH₂-terminal domain homologous to the S100A proteins and a peculiar COOH-terminal domain (Dale et al., 1985, 1994; Gan et al., 1990; Sandilands et al., 2009). The amino-terminus contains two active EF-hand calcium-binding sites, however the effect of calcium-binding is unknown. The carboxy-terminus may be necessary for profilaggrin to filaggrin processing. At the *stratum granulosum-stratum corneum* transition, concomitantly to keratohyalin granule dissolution, profilaggrin is dephosphorylated. In particular, dephosphorylation of phosphoserines located just upstream of the

linker peptides is suspected to be required for further profilaggrin processing (Resing et al., 1985, 1989). The implicated phosphatase(s) is(are) not formerly known, but protein phosphatase 2A has been suspected (Kam et al., 1997). Profilaggrin is then proteolytically processed to give highly cationic filaggrin monomers (324 amino acids long; 37 kDa) via at least two proteolysis steps performed by both endo- and exo-peptidases (Resing et al., 1993; Thulin and Walsh, 1995). Calpain 1 could be one of the calcium-activated proteases involved, at least in rat epidermis (Resing et al., 1993). The released amino-terminal domain of profilaggrin is translocated to the nucleus, where it plays a role that is, as yet, undefined (Pearson et al., 2002), and the filaggrin monomers associate with and promote the aggregation of intermediate filaments (Steinert et al., 1981). Then, filaggrin monomers are deiminated by peptidylarginine deiminases 1 and 3 (PAD1 and PAD3). This post-translational modification (transformation of arginyl residues into citrullyl) induces the detachment of filaggrin monomers from the corneocyte filamentous matrix (Méchin et al., 2005, 2007; Nachat et al., 2005) and their subsequent degradation into free amino acids, mainly histidine (10.7% of the filaggrin monomer constituting total amino acids), serine (25.3%), glycine (13.6%) and

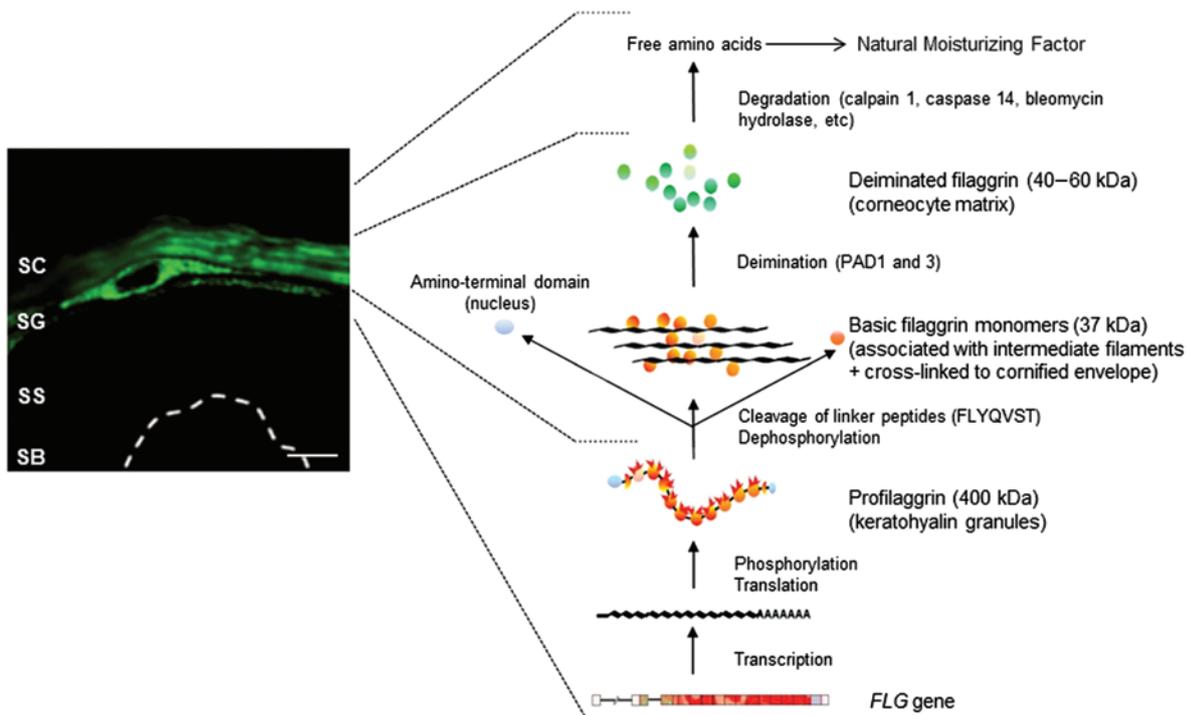


Figure 1: Immunofluorescence detection of profilaggrin and filaggrin (left, green) and schematic representation of filaggrin metabolism (right) in human epidermis.

The four epidermal layers shown are *stratum basale* (SB), *stratum spinosum* (SS), *stratum granulosum* (SG) and *stratum corneum* (SC). Scale bar = 20 μ m.

glutamine (9.6%). Several enzymes are involved in this proteolysis step, including caspase 14, calpain 1 and bleomycin hydrolase (Denecker et al., 2007; Kamata et al., 2009; Hoste et al., 2011). Subsequently, glutamine and histidine are modified to form pyrrolidone-5-carboxylic acid and *trans*-urocanic acid respectively, two main components of the NMF. The former is involved in hydration of the stratum corneum (Rawlings and Harding, 2004) and Barresi and colleagues provided robust evidence that *trans*-urocanic acid is involved in keratinocyte photoprotection (Barresi et al., 2011), as discussed by Gibbs and Norval (Gibbs and Norval, 2011). In addition, some filaggrin monomers are cross-linked to cornified cell envelopes (Steinert and Marekov, 1995; Simon et al., 1996). This may contribute to the mechanical properties of these structures and, beyond, of corneocytes. The amino acid sequence of filaggrin monomers in a single individual or between two different individuals displays up to 40% variation (Gan et al., 1990). This strongly suggests that a precisely defined succession of amino acids is not necessarily important for the functions of filaggrin, but rather its global composition. This is easy to understand as its ultimate destiny is to form a pool of free amino acids. UV-B radiation down-regulates filaggrin expression (Del Bino et al., 2004). Environmental humidity appears to trigger filaggrin degradation, at least in rodents (Scott and Harding, 1986; Katagiri et al., 2003).

The importance of filaggrin in skin homeostasis has recently been demonstrated using both experimental and

animal models (Table 1). In three-dimensional epidermis reconstructed on a polycarbonate membrane with human keratinocytes treated with shRNA targeting filaggrin expression, marked downregulation of the protein ($\geq 90\%$) leads to an absence of keratohyalin granules, an impaired keratinocyte differentiation, at both the mRNA and protein levels, a disturbed corneocyte matrix ultrastructure, reduced amounts of pyrrolidone-5-carboxylic and urocanic acids associated with an increase in UV-B sensitivity, and increased outside-in stratum corneum permeability (Pendaries et al., 2014). In an organotypic human skin model, when filaggrin has been effectively down-regulated with siRNAs, similar hypogranulosis, increased dye penetration and reduced urocanic acid concentration associated with increased UV-B-induced apoptosis has been observed. However, in this model, keratinocyte differentiation and keratin aggregation appear normal whereas lamellar body formation is impaired (Mildner et al., 2010). In a similar model, filaggrin knockdown has been shown to also induce the accumulation of free fatty acids in the stratum corneum interstices, leading to less ordered lipid lamellae and higher permeability, associated with production of interleukin (IL)6 and IL8 (Küchler et al., 2011; Vavrova et al., 2014). Lesser effects of filaggrin silencing were observed by the team of J Bouwstra, but in that case the N/TERT cell line was used instead of human primary keratinocytes to produce human skin equivalents (van Drongelen

Table 1: Comparison of biochemical and ultrastructural features in filaggrin and filaggrin-2 knockdown reconstructed human epidermis, *Flg* knockout mouse epidermis, and the epidermis of ichthyosis vulgaris (IV) and atopic dermatitis patients.

	Filaggrin knockdown ^{a-d}	Filaggrin-2 knockdown ^e	<i>Flg</i> knockout mice ^f	IV ^g	AD
pH of the SC surface	= ^{a,b}	↗	=	↗	↗
SC hydration	NA	NA	=	↘	↘
Parakeratosis	- ^{a-d}	+	-	-	+/-
Altered corneocyte matrix	+ ^b / ^{-b}	-	+	-	+
Vesicles inside corneocytes	- ^{a-d}	+	-	-	+
Amounts of urocanic and pyrroxylic acids	↘ ^{a-c}	↘	↘	↘	↘
Alterations in stratum corneum lipids	+ ^a / ^{-d}	unknown	unknown	+	+
Outside-in SC permeability	↗ ^{a-d}	=	↗	↗	↗
Transepidermal water loss	Unknown	unknown	=	↗	↗
Hypogranulosis	+ ^{a-d}	-	+	+	+
Filaggrin-2 and hornerin monomers	↘ ^a	↘	unknown	unknown	↘
Filaggrin monomers	↘ ^{a-d}	↘	↘	↘	↘
Activation of caspase-14	↘ ^a	↘	unknown	unknown	↘
Amount of bleomycin hydrolase	↘ ^a	↘	unknown	unknown	↘
Expression of loricrin	↘ ^{a,b=c,d}	↘	=	=	↘
Cytokine production by keratinocytes	+ ^{a,d}	Unknown	unknown	-	+

^aKüchler et al. (2011); Vavrova et al. (2014); ^bPendaries et al. (2014); ^cMildner et al. (2010); ^dvan Drongelen et al. (2013); ^ePendaries et al. (2015); ^fKawasaki et al. (2012); ^gGruber et al. (2011); Thyssen et al. (2013). SC, stratum corneum; NA, not applicable.

et al., 2013). Newborn *Flg*^{-/-} mice exhibit dry scaly skin, associated with hypogranulosis and a marked decrease in the free amino acid content of the stratum corneum. Absence of filaggrin induces stratum corneum fragility, as shown by increased desquamation under mechanical stress, probably related to the abnormal keratin filament aggregation observed. The knockout mice display a normal TEWL, associated with an apparently normal tight junction-related epidermal barrier, but increased outside-in stratum corneum permeability, leading to enhanced penetration of both hapten and protein antigens followed by exaggerated immune responses (Kawasaki et al., 2012; Yokouchi et al., 2015). Loss-of-function mutations in the gene encoding filaggrin (*FLG*) cause ichthyosis vulgaris, the most commonly inherited skin disease (Smith et al., 2006). Some patients with homozygous or compound heterozygous mutations and largely devoid of clinical signs of cutaneous inflammation completely lack filaggrin monomers, the N-terminal domain of profilaggrin being still present but with an abnormal distribution (Smith et al., 2006; Gruber et al., 2011; Thyssen et al., 2013). Characterization of their epidermis also gave some important information about the role of filaggrin (Table 1): abnormal keratin filaments, decreased tight junction protein expression, decreased lamellar body content, corneocyte defects, and abnormalities in epidermal barrier functions and stratum corneum properties have been observed (Gruber et al., 2011; Thyssen et al., 2013).

In agreement with our current knowledge of filaggrin metabolism, caspase-14-deficient mice display abnormal processing of filaggrin monomers with a strong accumulation of filaggrin-derived large peptides in the epidermis. In parallel, there is a substantial reduction in the amount of urocanic acid and increased sensitivity to UV-B radiation. These mice have a reduced level of stratum corneum hydration and increased TEWL, as well as weakened cornified cell envelopes (Denecker et al., 2007; Demerjian et al., 2008). Also, bleomycin hydrolase knockout newborn mice present mild ichthyosis, generalized scaling and sloughing of the skin. At 7–10 days, the scaling resolves except on the tail. The tail dermatitis includes thickening of the epidermis with parakeratotic hyperkeratosis and acanthosis. Mutant mice are more prone to develop the tail dermatitis when kept in a low-humidity atmosphere (Schwartz et al., 1999). However, filaggrin processing in these mice remains to be analyzed in detail.

One of the SFTPs most closely related to filaggrin, i.e. filaggrin-2, has recently been characterized from a biochemical and functional point of view (Toulza et al., 2007; Wu et al., 2009; Hsu et al., 2011; Henry et al., 2012). Filaggrin-2 and filaggrin are very similar in terms of protein

structure, amino acid composition and pattern of expression. Filaggrin-2 is synthesized by granular keratinocytes as a large precursor (profilaggrin-2; 250 kDa) consisting of an S100-homologous NH₂-terminal domain, a spacer domain, a large repetitive central domain and a unique COOH-tail (Figure 2). The central domain contains two types of 75–77 amino acid long repeats: nine A-type repeats and fourteen B-type repeats related to filaggrin monomers (28–39% identity). Profilaggrin-2 is located in the keratohyalin granules. It is then proteolytically processed to fragments of 130 kDa and smaller. One of the proteases involved seems to be calpain 1. Two peptides derived from the spacer domains have been identified by mass spectrometry in purified cornified cell envelopes (Vermeij et al., 2011). The role of A-type repeats is not known, but they have been suggested to be also cross-linked to cornified cell envelopes (Wu et al., 2009; Henry et al., 2012). Filaggrin-2 B-type repeats are detected in the intracorneocyte fibrous matrix of the lower corneocytes where they are colocalized with filaggrin. Both proteins disappear concomitantly in the upper cornified layer. To investigate the role of filaggrin-2 in the epidermis, its expression has been down-regulated using lentivirus-mediated shRNA interference in reconstructed human epidermis (Pendaries et al., 2015). This resulted in premature cornification with a reduction in the thickness of the spinous/granular layers, retention of vesicles in the corneocyte matrix and parakeratosis. These morphological changes are paralleled by a decrease in the degradation of corneodesmosin and in the level of filaggrin monomers, a reduction in the amount of urocanic acid, an increase in photosensitivity and a higher pH at the stratum corneum surface. These data demonstrate the importance of filaggrin-2 for a proper cornification process and a fully functional stratum corneum (Figure 3). In addition, a recombinant fragment of mouse filaggrin-2 has been shown to interact with and bundle keratin filaments *in vitro* (Presland et al., 2006) so filaggrin-2 may also contribute to the aggregation of intermediate filaments to form the intracellular corneocyte matrix.

Epidermal barrier impairment in atopic dermatitis: reasons and consequences

Atopic dermatitis (AD or atopic eczema) is a chronic skin disease. It is very common in children (almost 20% in industrialized countries) and frequent in adults (up to

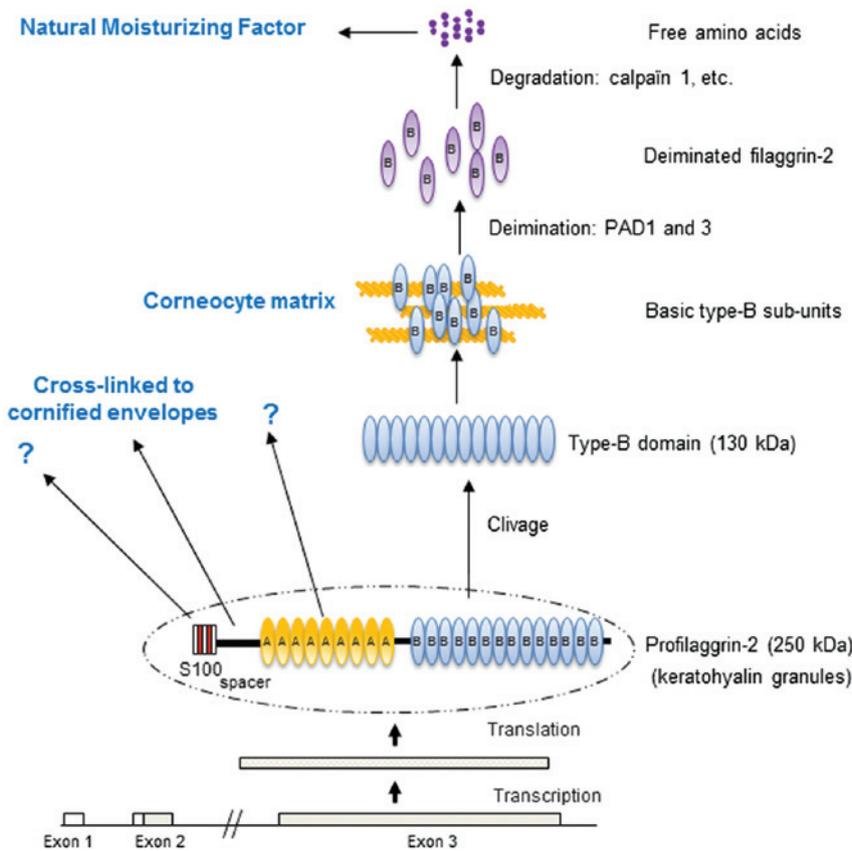


Figure 2: Schematic representation of filaggrin-2 metabolism in the epidermis.

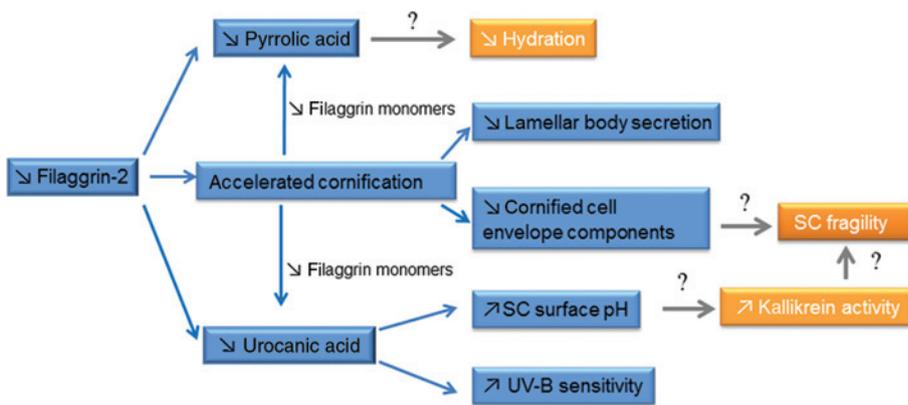


Figure 3: Experimentally observed (blue boxes) and hypothesized (orange boxes) downstream consequences on the stratum corneum (SC) of filaggrin-2 silencing in reconstructed human epidermis.

3%). AD is a highly pruritic inflammatory skin condition. The affected body areas depend on the age of the patient: infants under 1 year of age usually have widely distributed lesions, the cheeks often being the first site to be affected; adults have more localized eczema lesions predominantly occurring in the skin flexures, the extremities and the face. Acute lesions are characterized by erythematous patches

with edema, vesicles and oozing/crusting. Chronic lesions are characterized by skin thickening (lichenification). Pruritus is a hallmark of AD. Scratching can disrupt the patient’s sleep, and ability to concentrate at work or school. It can make skin bleed, and contribute to secondary staphylococcal infections, which are common. AD is often associated with IgE-mediated sensitizations and

other atopic conditions such as asthma, allergic rhinitis and food allergy. AD is related with complex hereditary factors and the increase in its prevalence since the 1950s highlights the influence of environmental factors (Oyoshi et al., 2009; Bieber, 2012). There is currently no cure for AD, and exploration of the pathophysiological mechanisms is therefore of major medical interest.

An increase in the permeability of the stratum corneum, assessed by measuring the TEWL, is a characteristic clinical feature of the skin of AD patients. In addition, the magnitude of the increase is proportional to the severity of the disease. This anomaly, obvious at the level of the AD lesions, is also observed in the apparently healthy skin (Taieb, 1999; Jensen et al., 2004; Elias and Wakefield, 2014; and references therein). Although more scarce, several works studying the epidermal barrier properties by measuring percutaneous penetration also indicate an outside-in defect of involved and non-involved skin. Skin penetration of both hydrophilic and lipophilic dyes, of sodium lauryl sulfate and of polyethylene glycol is higher in the skin of AD patients than in healthy subjects (Hata et al., 2002; Jakasa et al., 2006; Jensen et al., 2009). Increased penetration of hydrophilic dye is significantly correlated with disease severity (Hata et al., 2002). Structural abnormalities of the stratum corneum that may impact the efficacy of epidermal barrier functions have also been described in lesional skin – decreased compaction/cohesion of the layer, reduced secretion of lamellar bodies (Elias and Wakefield, 2014), and altered aspects of the corneocyte intracellular matrix and cornified cell envelopes (Guttman-Yassky et al., 2009); as have biochemical impairments – a reduction in the amounts of intercorneocyte lipids and defects in their nature, including a reduction of the ceramide side chain length, and an increase in the proportion of cholesterol (Janssens et al., 2012; van Smeden et al., 2014), an increase of surface pH (Schmid-Wendtner and Korting, 2006) and reduction of hydration. These abnormalities in lipids and biophysical properties have been observed in lesional as well as non-lesional skin. Furthermore, the atopic skin is prone to bacterial (*Staphylococcus aureus*) and viral (*Herpes simplex*) infections, reflecting an abnormal skin immune response to microbes (Cork et al., 2006; Oyoshi et al., 2009; Guttman-Yassky et al., 2011; Tauber et al., submitted).

These abnormalities of lesional and also non-lesional atopic skin are the result of multiple factors: mutations in genes coding for proteins essential to the functions/properties of the stratum corneum; effect of the proinflammatory cytokines that reduce the mRNA levels of various cornification-specific proteins, including filaggrin, and inhibit the over-expression of antimicrobial peptides

normally induced by alterations of the stratum corneum; changes in the program of keratinocyte differentiation; and proteolytic activity secreted by *S. aureus*.

Genetic factors involved in stratum corneum barrier defects

Recent genetic analyses (pan-genomic association studies or candidate-gene approaches) have helped to identify many loci/genes for susceptibility to AD, on several chromosomes. These genes relate to the immune response, both innate and adaptive, and to the epidermal barrier (Barnes, 2010). The genes that code factors of the immune system are not the subject of this review, and interested readers can refer to previously published articles (Oyoshi et al., 2009; Barnes, 2010; Guttman-Yassky et al., 2011). A breakthrough in the genetics of AD was achieved in 2006 with the identification of loss-of-function mutations in the *FLG* gene, encoding profilaggrin. The two variants (R501X and 2282del4) initially identified as the cause of ichthyosis vulgaris (Smith et al., 2006), a common monogenic non-inflammatory keratinization disorder, were subsequently demonstrated to be also strong risk factors for AD in Irish and Scottish populations (Palmer et al., 2006). This association has since been confirmed in several family studies or in case-control analyses including populations from various ethnic backgrounds. More than 40 different mutations have so far been identified in patients of Caucasian and Asian origin. All of them are non-sense mutations, four (R501X, 2282del4, R2447X and S3247X) being predominant in the populations of northern and central Europe (for reviews, see Irvine et al., 2011; Brown and McLean, 2012; Kezic et al., 2014). All these mutations induce a decrease or absence of filaggrin in the epidermis. Two meta-analyses of the data have shown that the presence of a mutation of at least one allele of *FLG* increases the risk of developing the disease by a factor of between 3 and 5 (Baurecht et al., 2007; Rodriguez et al., 2009). This strong association between *FLG* mutations and AD is one of the most robust genotype-phenotype links observed in complex human genetic disorders. In addition, *FLG* mutations appear to define a subgroup of patients with early-onset, longer duration and more severe course of disease, a higher prevalence of IgE-mediated sensitizations and a higher risk of developing asthma, allergic rhinitis, and nickel and peanuts allergies (Weidinger et al., 2006; van den Oord and Sheikh, 2009; Brown et al., 2011; McAleer and Irvine, 2013). The risk of asthma with AD was higher in patients with two mutated alleles of *FLG* (Marenholz et al.,

2006; Brown et al., 2008). The relationship between *FLG* mutations and sensitization to other foods is controversial (Thyssen et al., 2015). Consistent with the importance of *FLG* loss-of-function mutations in AD are the similarities between clinical features of the patient skin and the phenotype of epidermis/skin substitutes where filaggrin is knocked down, and the phenotype of *Flg*^{-/-} mice (Table 1). In particular, hypogranulosis, decreased amounts of urocanic and pyrrolidone-5-carboxylic acids, and enhancement in the stratum corneum outside-in permeability are characteristics of both the disease and the experimental models.

These genetic data showing that a primary defect in a structural epidermal protein underlies AD has led to a profound change in the understanding of the disease. However, *FLG* mutations are neither necessary nor sufficient to develop AD. Only a fraction of patients (between a few and 50% depending on the populations studied) carry *FLG* mutations, and 60% of *FLG* mutation carriers do not develop AD (Weidinger et al., 2008). Therefore additional genetic and environmental factors are needed. Interestingly, one publication reports that individuals who are homozygous for filaggrin gene null mutations (one in nine adults and one in three children) do not always develop dermatitis (Thyssen et al., 2012). In addition, *FLG* non-sense mutations have not been detected in Ethiopian, Tunisian and South African populations (Winge et al., 2011; Thawer-Esmail et al., 2014; Thyssen et al., 2014), and are not associated with AD in African-American patients (Margolis et al., 2012; Garrett et al., 2013). One can assume that other inherited factors affecting the epidermal barrier function could explain the AD cases where *FLG* mutations are not present.

In fact, *FLG* non-sense mutations are detected in about 10% of normal Europeans and 5% of normal Asians. A puzzling question is why these mutations have been selected in the normal population during evolution? An intriguing recent observation may give the answer: a decreasing North-South gradient exists in the mutation prevalence in Europe and is positively correlated to a parallel gradient of blood vitamin D3 concentration. As most of the vitamin D needed by the human body is formed in the skin through the action of UV radiation, and as loss of filaggrin results in reduced production of trans-urocanic acid, a major UV-B filter in low-pigmented individuals, non-sense *FLG* mutations would favor the cutaneous generation of the vitamin (Thyssen et al., 2014).

A genome-wide search analysis has revealed an association between AD and a genetic variant, distinct from *FLG* but also located on chromosome 1q21 in the epidermal differentiation complex (Esparza-Gordillo et al., 2009).

Recently, mutations in the *FLG2* gene, in particular a non-sense mutation, were shown to be associated with persistent AD in a cohort of 60 US patients of African ancestry (Margolis et al., 2014). Considering the close proximity between filaggrin and filaggrin-2 as described above, and the similarities between filaggrin-2 knockdown reconstructed epidermis and the epidermis of atopic patients (Table 1), the relationship between *FLG2* mutations and AD deserves to be further investigated. In 2011, an insertion of 24 base pairs in the coding sequence of the *SPRR3* gene, encoding another component of the cornified envelope, was recognized as another genetic risk factor for AD. The association has been replicated in a large cohort of cases (1314) with early onset dermatitis from central Europe and has led to an odds ratio of 1.3 (Marenholz et al., 2011). The *SPRR3* protein variant carries an insertion of eight amino acids (CTKVPEPG) in its central repetitive proline-rich domain. Whether and how this could alter the epidermal barrier function can only be speculated but the amounts of *SPRR* incorporated in the envelopes are suspected to confer various mechanical properties on the structure. Finally, a single nucleotide polymorphism (rs877776; C/G substitution) located 7 kb downstream of the hornerin gene has been reported as an AD susceptibility variant (Esparza-Gordillo et al., 2009), although this association was not replicated in another study (O'Regan et al., 2010). Hornerin, one of the SFTPs, is another component of cornified cell envelopes (Henry et al., 2012). Whether and how this polymorphism could contribute to barrier defects in AD patients remains unknown.

Variants in genes located outside 1q21 and encoding other proteins involved in cornification have also been associated with AD, especially polymorphisms of the gene *SPINK5* (Walley et al., 2001) that encodes the lympho-epithelial Kazal-type inhibitor (LEKTI) of serine proteases, and of the kallikrein 7 gene (Vasilopoulos et al., 2011). These two proteins are essential participants in the degradation of corneodesmosomes and in the control of desquamation. The identification of *SPINK5* as the defective gene in Netherton syndrome, a severe recessive genodermatose with a profound epidermal barrier defect and multiple allergic manifestations (Furio and Hovnanian, 2014), is a strong argument in favor of a deficient stratum corneum as underlying AD. In addition, in patients with Netherton syndrome, excess active kallikrein levels induce, through activation of PAR2 receptor, the production of thymic stromal lymphopoietin, a pro-Th2 cytokine highly expressed in atopic skin, which is known to be involved in AD pathogenesis and probably links skin defects to other manifestations of atopy (Liu et al., 2007). As already mentioned, LEKTI and kallikrein

7 are secreted by granular keratinocytes via the lamellar bodies (Ishida-Yamamoto et al., 2005). Less convincing in the actual stage of our knowledge but of potential interest, a missense mutation in the *TMEM79/MATT* gene, rs6694514, has a small but significant negative association with AD (OR, 0.91; 95% CI, 0.82–0.96; $p=0.001$), as shown by a meta-analysis of more than 4000 patients and 10 000 controls (Saunders et al., 2013). *TMEM79* encodes matrin, a transmembrane component of the trans-Golgi network mainly expressed in the granular keratinocytes. Mice deficient in matrin display a reduced secretion of lamellar body cargo and a fragile stratum corneum with significantly increased TEWL. They spontaneously develop marked dermatitis at the age of 1 week with subsequent scratching behavior and high amounts of serum IgE (Sasaki et al., 2013; Saunders et al., 2013). In line with the abnormal presence of vesicles within the corneocyte matrix, this convincingly shows that variants in genes encoding lamellar body components, either membranous or transported proteins, may be involved in stratum corneum and epidermal barrier abnormalities. Finally, the importance of a variant of claudin-1, a component of tight junctions, has been highlighted in atopic North American populations of African and European descent (De Benedetto et al., 2012). A reduction in the expression of claudin-1 has been reported in the patients. In the meantime, claudin-1 and tight junctions have been observed in the lower stratum corneum (Haftak et al., 2011; Igawa et al., 2011), where they are supposed to be involved in controlling the access of desquamation-related proteases to corneodesmosomes. These observations, in line with the fatal, postnatal permeability barrier defect of claudin-1 null mice (Furuse et al., 2002), suggest that non-functional tight junctions in the granular and/or cornified layers of the epidermis of AD patients could participate in the process of epidermal barrier alteration.

As a whole, the recent genetic/functional data above described strongly support the view that a combination of inherited defects in proteins necessary for the epidermis to fulfil its barrier functions can predispose to AD. *FLG* loss-of-function mutations were the first to be identified but one can speculate that many others will be discovered in the future.

Effects of cytokines on the stratum corneum barrier

A downregulation of filaggrin monomers is observed in the epidermis of adult AD patients, regardless of *FLG*

mutation status (Pellerin et al., 2013). This may be due to the effect of Th2 proinflammatory cytokines, some of the predominant cytokines in AD lesions. In fact, treatments of normal keratinocytes with IL4, IL13 and pro-Th2 cytokines including IL25 and IL-33 strongly reduces the filaggrin mRNA levels (Boniface et al., 2005; Nograles et al., 2008; Howell et al., 2009; Hvid et al., 2011; Pellerin et al., 2013; Von Hesler et al., 2015). Th2-cytokines affect the expression not only of filaggrin but also of other structural components of corneocytes, e.g. filaggrin-2, involucrin, loricrin, hornerin and probably others (Kim et al., 2008; Pellerin et al., 2013, 2014; Omori-Miyake et al., 2014). In agreement with these findings, high throughput gene expression analyses have identified broad defects in the terminal differentiation of keratinocytes in AD, both in the lesional as well as non-involved skin. In particular, a reduction in the expression of numerous cornified envelope components, including loricrin, involucrin, LCE1, LCE2, cornifelin and sciellin has been confirmed (Guttman-Yassky et al., 2009; Suárez-Fariñas et al., 2011). When the analysis was performed after separation of the epidermis from the dermis using laser capture microdissection, some proteins of the tight junctions, i.e. claudin-8 and -23, were also shown to be down-regulated in the lesional skin (Esaki et al., 2015). Other evidence points to a role of IL4 and IL13: mice that overexpress these cytokines in their skin (Chan et al., 2001; Lee and Flavell, 2004) or display constitutive activation of signal transducer and transcription activator 6 (Stat6) in T cells (Sehra et al., 2008), a downstream protein in IL4/13 signaling, develop dermatitis-like lesions with a marked down-regulation of filaggrin; and polymorphisms in IL4, IL13 and IL4 receptor have been linked to AD susceptibility (Barnes, 2010).

Caspase-14 expression and/or activation and bleomycin hydrolase expression are reduced in the patient's skin (Broccardo et al., 2011; Kamata et al., 2011; Suárez-Fariñas et al., 2011; Pellerin et al., 2014), and also in reconstructed human epidermis models where either filaggrin or filaggrin-2 is silenced (Pendaries et al., 2014, 2015). IL4 and IL13 have also been shown to downregulate the expression of bleomycin hydrolase in keratinocytes at the mRNA and protein levels (Pellerin et al., 2014). In addition, the profilaggrin/filaggrin ratio is increased in patients (Tan et al., 2012; Pellerin et al., 2013, 2014). Therefore, acquired defects in enzymes involved in the processing of profilaggrin to filaggrin monomers and in filaggrin degradation could alter NMF metabolism and contribute to AD pathophysiology. This hypothesis warrants further investigation.

Environmental factors involved in stratum corneum barrier defects

Many studies have indicated a role of environmental factors such as microorganisms and aeroallergens in exacerbations of eczema. In some cases, this may occur through a direct effect on the epidermal barrier.

Atopic dermatitis lesions are commonly infected by *S. aureus* and herpes simplex virus, and these agents are implicated in lesion worsening. Staphylococcal density for example appears to be a significant independent predictor of disease course severity (Tauber et al., submitted for publication). Th2 cytokines are thought to be involved in the bacterial colonization as they reduced the production of antimicrobial peptides by keratinocytes and the expression of epidermal barrier protein, thus providing a permissive environment for bacterial growth (Travers, 2014). In addition, both profilaggrin downregulation and Th2 cytokines appear to reduce the expression/secretion of acid sphingomyelinase (Brauweiler et al., 2013, 2014). This in turn could not only affect the lipid organization in the stratum corneum interstices but also increase the *S. aureus* alpha-toxin toxicity as sphingomyelin is the main binding site for the toxin. Conversely, exfoliative toxin-negative *Staphylococcus* strains target tight junctions during infection (Ohnemus et al., 2008). Proteases secreted by *S. aureus*, including exfoliative toxin and V8 protease, could also exacerbate the impairment of the epidermal barrier and the lesions through degradation of corneodesmosomes and subsequent loss of corneocyte cohesion (Hirasawa et al., 2010; Takai and Ikeda, 2011). Common aeroallergens, such as house dust mites and pollens, also produce and release proteases that are frequently allergens themselves, e.g. Der p 1 and Der f 1 of *Dermatophagoides*, and that are able to degrade stratum corneum structural proteins and to break down the epidermal barrier, as well as the tracheal and bronchial epithelial barrier (Takai and Ikeda, 2011). As a whole, environmental proteases may well contribute to disease exacerbation through their action on proteins important for the epidermal barrier.

The rise in AD incidence since the early 1950s in industrialized countries and its current increase in developing countries point to the significant importance of environmental conditions (Langan and Irvine, 2013; Flohr and Mann, 2014). Among the number of potential triggers, indoor and outdoor air pollutants have been suspected. Air pollutants include particulate matter, volatile organic compounds, such as formaldehyde and benzene, and sulfur and nitrogen oxides. They originate from engine

vehicle emissions, tobacco smoke, furniture and electronic devices. Because a variety of confounders may false the results, a definitive conclusion about the effects of air pollutants on the development and/or aggravation of AD and about the exact nature of the responsible pollutants could not be obtained from the current studies. However, many data back up this hypothesis, as discussed in a recent review (Ahn, 2014). The involved biological mechanisms remain unclear. They may include a Th2-immune response, oxidative stress damages to the stratum corneum and an impairment of the epidermal barrier (Ahn, 2014). For example, exposure of AD patients to volatile organic compounds for 4 h lead to increased TEWL 48 h later (Huss-Marp et al., 2006). Climate is also suspected to influence AD activity (Silverberg et al., 2013). High sun exposure, warm temperatures and high humidity seem to protect against AD development. In contrast, low UV exposure, low outdoor temperatures and indoor heating may increase the risk. However, increased sun exposure and higher temperatures have also been associated with a poorly controlled disease in some patients (Sargen et al., 2014). The underlying mechanism(s) for improvement of AD symptoms in some patients in summer is (are) ill-known. They might include changes in keratinocyte metabolism and immune regulation. The climate could act also partly through filaggrin expression/degradation that is regulated by external humidity and UV radiation, as mentioned above.

We can suspect that scratching also can reduce the expression of filaggrin and of other stratum corneum proteins, through disruption of the epidermal calcium gradient, which is known to be involved in keratinocyte differentiation. Similarly, epidermal barrier acute disruption by tape stripping induced a downregulation of structural proteins including filaggrin (de Koning et al., 2012). Exposure of the skin to detergents and soaps is associated with skin dryness and AD flare-ups. These environmental

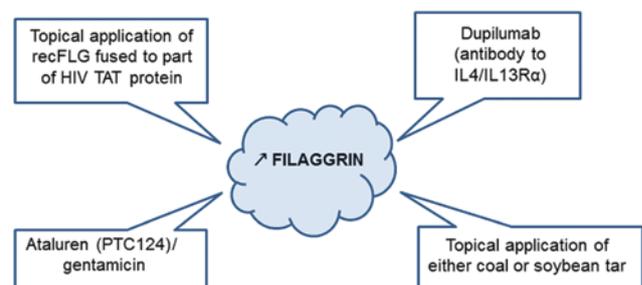


Figure 4: Therapeutic strategies based on filaggrin level induction, previously used or envisaged in atopic dermatitis, ichthyosis vulgaris and dry skin.

factors probably act by promoting lipid and NMF component extraction from the superficial layers of the stratum corneum, and therefore participate in the alterations to the epidermal barrier (Cork et al., 2006; Flohr and Mann, 2014).

Pathophysiology of AD: a new paradigm and clinical implications

In the proposed current pathophysiological model for AD, defect in the epidermal barrier function is emerging as an important point. The discovery of the importance of filaggrin defects in AD development and severity has put gene/environment interactions and how environmental factors impact on epidermal barrier under the spotlight (Thyssen and Kezic, 2014). In healthy skin under steady-state conditions, the penetration of allergens and microorganisms is prevented by the stratum corneum. However, because mutations in the genes encoding proteins essential to cornification, including filaggrin, lead to a reduced effectiveness of the epidermal barrier, the penetration is allowed in the skin of atopic patients. Keratinocytes and immune cells are then activated and produce proinflammatory cytokines, including IL4 and IL13, that in turn reduce the expression of late proteins of

keratinocyte differentiation. The penetration of allergens is again increased and the inflammation becomes chronic in a ‘leaky’ vicious cycle (Pellerin et al., 2014). In strong agreement with this model, impairment of the epidermal barrier at birth and at 2 months of age, as measured by TEWL, precedes the clinical signs of AD. Moreover, the degree of barrier impairment predicts the occurrence of the disease at 1 year (Kelleher et al., 2015).

This model reflects the marked and rapid therapeutic efficacy of dupilumab (clinicaltrials.gov, number NCT01859988; Beck et al., 2014), a fully human monoclonal antibody directed against the common subunit of IL4 and IL13 receptors (IL-4R α). It also explains the efficacy of longstanding treatments, the topical application of coal or soybean tar extracts (McLean and Irvine, 2013). In AD patients, coal tar can completely restore the expression of major stratum corneum proteins, including filaggrin, loricrin and hornerin. This is achieved through activation of the aryl hydrocarbon receptor that interferes with the STAT6-mediated Th2 cytokine signaling (van den Bogaard et al., 2013). Because coal tar is toxic, its usage is however not allowed in many countries. Soybean tar Glyteer has been shown to act in a similar way (Takei et al., 2015). Other strategies to improve AD patients through upregulation of filaggrin have been proposed (Figure 4). This includes the use of a candidate drug that promotes filaggrin expression

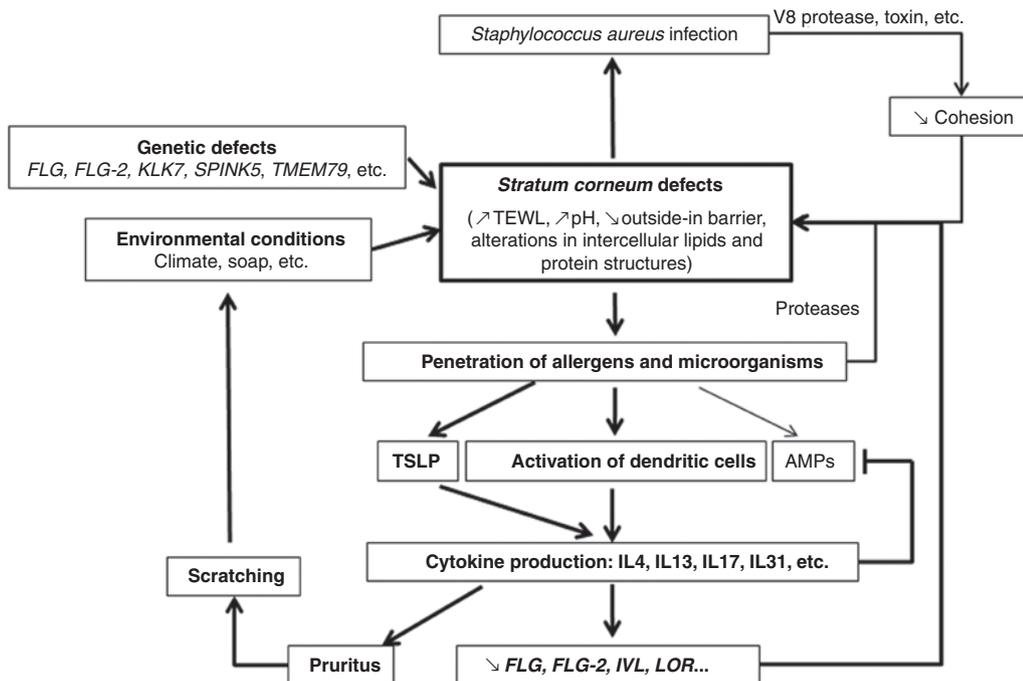


Figure 5: Schematic representation of the current pathophysiological model of atopic dermatitis, with several vicious feedback loops. TSLP, thymic stromal lymphopoietin.

Table 2: Key points.

The upper part of the epidermis, the stratum corneum, is a vital barrier (physical, chemical and immunological) between the body and its external environment.

Filaggrin and filaggrin-2 are key proteins for the epidermal barrier. They are involved in the control of keratinocyte differentiation, and in the hydration, mechanical resistance, photo-protective and permeability properties of the stratum corneum.

The epidermal barrier is altered in AD, and non-sense mutations of *FLG* – encoding filaggrin – are a major risk factor for the disease.

FLG mutations define a subgroup of patients more susceptible to subsequent allergies, the ‘atopic march’.

Mutations in other genes that also compromise the epidermal barrier predispose to AD.

Filaggrin and filaggrin-2 expression are reduced in the atopic skin of adult patients regardless of *FLG* mutations, probably due to the downregulation effects of proinflammatory cytokines.

A new pathophysiological model of AD has been proposed, with important consequences for treatment strategies.

AD, atopic dermatitis; *FLG*, filaggrin gene.

in vitro and in a mouse model of the disease, NC/Nga mice (Otsuka et al., 2014). A topically applied recombinant filaggrin monomer fused to a cell-penetration-promoting peptide has been shown to penetrate the epidermis, be internalized in the granular keratinocytes and restore the epidermal barrier function, in flaky-tail mice (mice with both *Flg* loss-of-function and *matt* mutations). This could also be a therapeutic approach for patients with AD (Stout et al., 2014). The use of a drug such as gentamycin or ataluren that allows the ribosomes to read through premature stop-codons has been proposed but not tested yet (McElroy et al., 2013). Finally, restoration of a functional lipid epidermal barrier could also be effective (Elias and Wakefield, 2011).

Conclusions

Two AD pathophysiological paradigms have been proposed. Historically, it was thought that the primary defect resided in the immune system, leading to excessive inflammation and to a subsequent local epidermal barrier disruption (the inside-outside model). The discovery that loss-of-function mutations in the filaggrin gene were the strongest risk factor for the disease led to the proposal of an alternative view detailed in this review (the outside-inside model). These two models are not exclusive. On the contrary, they help to explain the onset and chronicity, respectively, of atopic lesions. Intrinsic defects in proteins important for the epidermal barrier, aggravated by scratching, the use of detergents and by *S. aureus* colonization, allow the penetration of allergens into the skin and the following activation of immunity. Some inflammatory cytokines could further alter the epidermal barrier and perpetuate the lesions through signal transduction cascades affecting keratinocyte differentiation, in potentially negative feedback loops (Figure 5 and Table 2). Recent

studies similarly suggest that disruption of the epithelial barrier in the lung is a feature of airway inflammation in asthmatic patients (Georas and Rezaee, 2014) and that, in the gut, it is central in the pathogenesis of inflammatory bowel disease (Pastorelli et al., 2013; Watson, 2015).

In conclusion, it is recommended that AD patients avoid frequent washing with products aggressive for the skin. The treatments should aim to reduce the negative effects of proinflammatory cytokines on the expression of stratum corneum proteins, and to ‘fix’ the epidermal barrier.

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