Aus dem Universitätsklinikum Münster
Klinik und Poliklinik für Hautkrankheiten
- Allgemeine Dermatologie und Venerologie -
der Westfälischen Wilhelms-Universität Münster

- Direktor: Univ.-Prof. Dr. med. T. Luger -

Untersuchungen der Protease-Aktivität und Expression bei Filaggrin Mangel

INAUGURAL-DISSERTATION

zur

Erlangung des doctor medicinae
der Medizinischen Fakultät
der Westfälischen Wilhelms-Universität Münster

vorgelegt von:

Irina Dreizler, geb. Zaraeva
aus Uraj
2018

Gedruckt mit Genehmigung der Medizinischen Fakultät
der Westfälischen Wilhelms-Universität Münster

Dekan: Univ.-Prof. Dr. med. Mathias Herrmann

1. Berichterstatter: Priv.-Doz. Dr. med. V. Oji

2. Berichterstatter: Univ.-Prof. Dr. med. D. Föll

Tag der mündlichen Prüfung: 21. Februar 2018

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ZUSAMMENFASSUNG

Untersuchungen der Protease-Aktivität und Expression bei Filaggrin Mangel

Dreizler, Irina

Ichthyosis vulgaris (IV) ist die häufigste genetisch bedingte Hauterkrankung aus der Gruppe hereditärer Ichthyosen. Ursächlich für die Erkrankung sind die Mutationen im Profilaggrin-Gene, die zu einem Mangel von Filaggrin (FLG) in der Epidermis führen. Gleichzeitig stellen die FLG Mutationen den wichtigsten genetischen Risikofaktor für die atopische Dermatitis (AD) und andere allergische Erkrankungen dar. Bei Patienten mit IV wurde ein pH-Anstieg im Stratum corneum festgestellt, der seinerseits zu einer erhöhten Aktivität der Kallikrein-verwandten Peptidasen (KLK) führen kann.

Ziel dieser Arbeit ist daher die Analyse der Proteinexpression und Aktivität der KLK in der Epidermis sowie die Untersuchung des Multidomänen-Serinproteinase-Inhibitors LEKTI. Hierfür wurden zunächst die organotypischen dreidimensionalen (3D) Hautäquivalente mit Hilfe von epidermalen und dermalen Zellen von Patienten mit IV mit oder ohne AD etabliert. Dadurch konnten die umweltbedingten und immunologischen Einflüsse ausgeschlossen werden.

Die Ergebnisse dieser Arbeit zeigten Unterschiede der Expression und Aktivität der KLK bei IV, wobei aufgrund der kleinen Anzahl der Probanden keine signifikanten Unterschiede nachweisbar waren. Die Proteinexpression von KLK5 und KLK7 wurde mittels Immunofluoreszenzmikroskopie und ergänzend Immunoblotting analysiert. Die Expression von LEKTI wurde mittels Immunoblotting bestimmt. Die Aktivität von KLK5 und KLK7 wurde mittels Fluoreszenzspektroskopie mit spezifischen Substraten gemessen. Hierbei zeigte die Expression von KLK7 eine schwache Erhöhung in 3D Hautäquivalenten bei Patienten mit IV. Demgegenüber blieb die Expression und Aktivität von KLK5 und LEKTI unverändert. Interessanterweise zeigte die Aktivität von KLK7 eine geringfügige Erhöhung in $IV_{non\text{-}AD}$. Ergänzende Messungen der Aktivität von KLK5 und KLK7 wurden mittels D-Squames von Patienten mit IV (n = 11) und Kontrollen (n = 8) durchgeführt. KLK5 Aktivität korrelierte mit Ergebnissen *in vitro, w*ährend die KLK7 Aktivität in IV_{AD} signifikant erhöht war (p=0,004). Wir schließen daraus, dass ein deutliches Muster mit Dysbalancen unter epidermalen Serinproteasen bei Patienten mit IV eher zu beobachten war, wobei eine erhöhte Aktivität von KLK7 eine Rolle in der Entwicklung einer Atopie bei Patienten mit IV spielen könnte.

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SUMMARY

Investigation of protease activity and expression in filaggrin deficiency

Dreizler, Irina

Ichthyosis vulgaris (IV) is a frequent inherited skin disease caused by loss-of-function mutations in the profilaggrin gene resulting in filaggrin deficiency and being strongly associated with atopic dermatitis (AD). Lack of filaggrin and its acidic degradation products may increase the skin surface pH and activity of kallikrein-related proteases (KLK) accordingly.

Therefore, we studied the expression and activity of serine proteases of the epidermis in filaggrin deficient skin as well as the presence of the multidomain serine protease inhibitor LEKTI. To exclude environmental as well as immunologic influences, we have established a skin organotypic (3D) coculture model using epidermal and dermal cells of patients with IV with or without additional AD.

Our results show that subtle differences of the protease protein expression and activity are present in filaggrin deficient skin. However, probably due to the small number of subjects no significant differences could be confirmed. The distribution of KLK5 and KLK7 was analysed by immunofluorescence microscopy, additionally by immunoblotting, while the protein expression of the serine protease inhibitor LEKTI was determined by immunoblotting. KLK5 and KLK7 activities were measured by fluorescence spectrometry using specific substrates. In the filaggrin deficient 3D models KLK7 expression showed a slight increase, but expression and activity of KLK5 and LEKTI was unchanged. Interestingly, KLK7 activity was slightly higher in the $IV_{non\text{-}AD}$ subgroup. To correlate the results obtained from the skin models, we examined the epidermal protease activity *in vivo* measuring activity of KLK5 and KLK7 in ichthyosis vulgaris (n=11) and healthy subjects (n=8) using D-Squames. KLK5 activity correlated with that from the skin models, whereas KLK7 activity was significantly increased in the IV_{AD} (p=0,004). We conclude that there is a probably distinct pattern of dysbalances among epidermal serine proteases in FLG deficiency and that a higher KLK7 activity may act as a modifier for to the risk of developing atopic disorders in ichthyosis vulgaris.

Tag der mündlichen Prüfung: 21. Februar 2018

ERKLÄRUNG

Ich gebe hiermit die Erklärung ab, dass ich die Dissertation mit dem Titel:

Untersuchungen der Protease-Aktivität und Expression bei Filaggrin-Mangel

in der/im (Klinik, Institut, Krankenanstalt):

Universitätsklinikum Münster, Klinik und Poliklinik für Hautkrankheiten

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Meinen Eltern,
meinem Mann und
meinem Sohn
in großer Dankbarkeit
gewidmet

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ABBREVIATIONS

3D	three dimensional
α-1AT	Alpha 1 antitrypsin
AD	Atopic dermatitis
AMC	Amino-Methyl-Coumarin
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BPE	Bovine pituitary extract
°C	Grad in Celsius
CD	Cluster of differentiation
CDSN	Corneodesmosin
CE	Cornified envelope
CTRL	Healthy controls
D1-D15	Inhibitory domains of LEKTI
DMEM	Dulbecco's Modified Eagle's medium
DSG1,2	Desmoglein 1,2
DSC1	Desmocollin 1
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
f	female
FBS	Fetal bovine serum
FCS	Fetal calf serum
FLG	Filaggrin
g	Gram
GAG	Glycosaminoglycane
GEE	Generalized Estimating Equations
HCl	Hydrogen chloride
ICE	Interleukin 1b-converting enzyme
ID	Identity document
IFG	Interferon gamma
IgE	Immunglobuline E
IL	Interleukin
IV	Ichthyosis vulgaris
$IV_{non\text{-}AD}$	Ichthyosis vulgaris without atopy
IV_{AD}	Ichthyosis vulgaris with atopy
hCAP18	Human 18 kDa cationic antimicrobial protein
kb	Kilobyte

kDa	Kilodalton
kg	Kilogram
KH	Keratohyalin
KHG	Keratohyalin granula
KLKs	Kallikrein-related Peptidases
KLK5	Humane kallikrein 5, SCTE
KLK7	Humane kallikrein 7, SCCE
KSFM	Serum-free culture medium
L	Liter
LB	Lamellar bodies
LEKTI	Lympho-epithelial-kazal-type-related inhibitor
m	male
M	Mol
MeDOC	Mendelian disorders of cornification
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimol
µg	Microgram
ng	Nanogram
NMF	Natural moisturizing factor
nm	Nanometer
PAR2	Proteinase-activated receptor 2
PEG	Polyethylenglykol
PBS	Phosphate buffered saline
PCA	Pyrrolidone-5-carboxylic acid
pH	Decimal logarithm of the reciprocal of the hydrogen ion activity, aH+, in a solution
q	Long arm of a chromosome
resp.	Respectively
rpm	Rounds per minute
RT	Room temperature
SB	Stratum basale
SC	Stratum corneum
SCCE	Stratum corneum chymotryptic enzyme
SCCP	Stratum corneum cysteine protease
SCTE	Stratum corneum tryptic enzyme
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec	Second
SG	Stratum granulosum
SKALP	Skin-derived antileukoproteinase
SLPI	Secretory leukocyte protease inhibitor
SP	Serine proteases
SPI	Serine protease inhibitor
SPINK5	Serine Protease Inhibitor Kazal type 5
SPR	Small proline-rich protein
SS	Stratum spinosum
TEWL	Transepidermal water loss
TGF	Transforming growth factor
TGM	Transglutaminase
Th	T helper
TLR	Toll-like receptor
t-UCA	*trans*-urocanic acid
U	Unit
WB	Western blot

1. INTRODUCTION

1.1. Structure and function of the skin

The skin represents the outer body surface and a protective interface of the organism towards the environment. It belongs to the largest human organ with the surface of about 2m² (Jung *et* al., 1995; Garidel 2003). The skin functions are very varied. Its many tasks include the protection against physical, chemical and biological effects, also against the dehydration of organism, the radiation protection, the control of water loss and body temperature (Proksch *et al.,* 2008). The skin is also a sense organ and has various receptors and namely cold, heat, pressure receptors and nociceptors (Leonhardt, 1990). Three layers are distinguished from the outside to the inside: epidermis, dermis and subcutis (Geneser, 1990; Montagna, 1992).

Epidermis

Based on the body location, the age and sex, the thickness of epidermis can be varied between 30 µm on the head and 1,5 mm on the palms and soles of the feet (Kittler *et al.*, 2009). The epidermis is a dynamic skin layer that develops within four weeks (Marks, *et al.,* 2006). The epidermis represents a complex multilayered squamous epithelium of ectodermal origin that is further divided from basal to apical (Figure 1): stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (Fritsch, 1998; Fuchs, 1990; Watt, 1989). The basal cells form the lowest layer of the epidermis - the stratum basale (SB). It consists of cylindrical constructed cells forming single layer that is linked to an underlying basement membrane by hemidesmosomes, anchoring filaments and fibrils (epidermal junction zone) (Richter und Linss, 1998). Here, the regeneration of keratinocytes begins from the native cells via mitosis (Fritsch, 1998). The keratinocytes form the main population with nearly 90% of epidermal cells and are a subject to a continuous differentiation process – keratinization (Houben, *et al.,* 2007). The stratum spinosum (SS) is characterized by two to five layers of large polygonal

keratinocytes firmly linked together by desmosomes (Leonhardt, 1990; Garrod *et al.,* 2002b). The cell contact serves the exchange of substances using the „gap junctions“ (Fritsch, 1998, Kerl *et al*., 2003). The expression of membranous transglutaminase and coating protein involucrin also begins in SS. The stratum granulosum (SG) consists of two to three cell layers. The cells become impoverished of cell organelles, lose their cell nucleus resulting in the cell death. The keratinocytes begin to produce the keratohyalin (KH) in the SG tightly packed in the keratohyalin granules that represent the precursor of keratin in the stratum corneum. The stratum lucidum is only represented on the appointed body location (hands and feet) and includes strong refractive cells. The stratum corneum (SC) forms the outermost skin layer and is the end product of the epidermal cell differentiation (Fritsch, 1998). Most commonly, the horny layer consists of 10 - 20 cell layers of keratinized corneocytes that are about 0,5 - 3 µm thick and have a diameter of 30 - 40 µm (Young B *et al.,* 2000). Those are the largest cells in the human organism. The cells are predominantly constructed from keratin filaments and are enveloped with the closely networked outer stable layer of proteins and lipids - the cornified cell envelope (Figure 1).

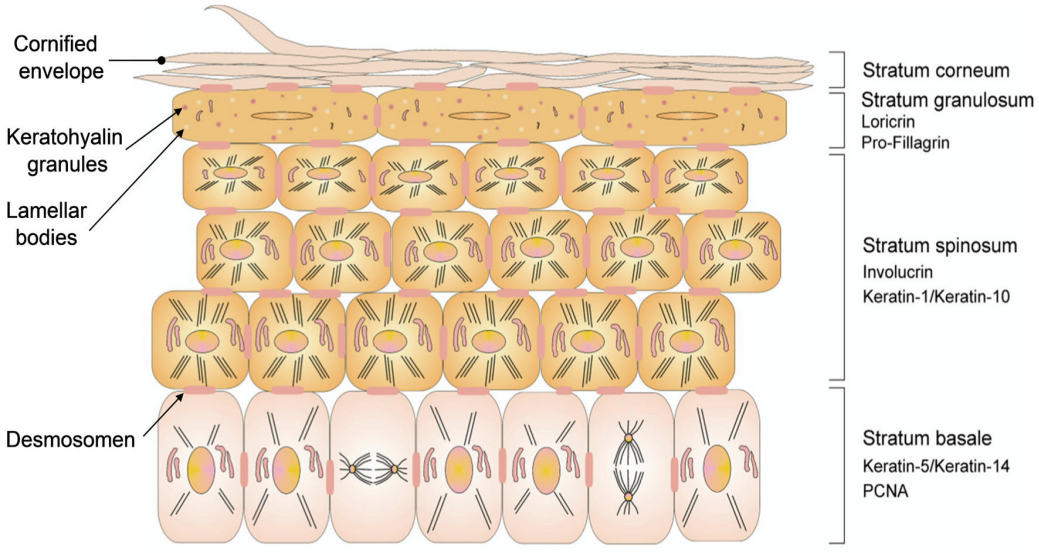

Figure 1. Structure of the epidermis (modified according to Alonso and Fuchs, 2003). Picture shows the main four layers of the epidermis: stratum basale, stratum spinosum, stratum granulosum, stratum corneum. The following structures can be identified: cornified envelope, desmosomes, lamellar bodies and keratohyalin granules.

Dermis and subcutis

The dermis is located between the epidermis and subcutis and is separated from the epidermis by the basement membrane. The dermis is about 0,6-3 mm thick composed of collagenous and elastic fibers, fibroblasts, histiocytes, mast cells, lymphocytes and extravascular leucocytes. The nutrition and fixation of the epidermis are the most important functions of the dermis (Myllyharju J, Kivirikko KI, 2004; Schultz GS, Wysocki A, 2009). The outmost reactive vascular network is located in the papillary layer on the border with epidermis that plays not only a role in the nutrition of the epidermis but also is involved in the skin inflammatory processes.

The subcutis is a loose connective tissue composed of botryoidal ordered fat cells. The hypodermic adipose tissue serves to cryoprotection, water and energy reservoir. However, this layer is differently thick on the various body location depending on nutrition, hereditary, and hormone balance.

1.2. Synthesis of cornified cell envelope

The cornified cell envelope (CE) is a chemical and proteases resistant structure essentially contributing to the physical and chemical resistance of the horny layer. The corneocytes are predominantly constructed from keratin filaments and are enveloped with the closely networked outer stable layer of proteins and lipids, the cornified cell envelope (Steinert *et al.,* 1981; Lonsdale- Eccles *et al.,* 1984; Magnaldo *et al.,* 1990; Hohl *et al.,* 1991a; Hohl *et al.,* 1991b). In the stratum granulosum the proteins are deposited on the inside cell membrane of the keratinocytes. During terminal differentiation, they are cross-linked to the cornified envelope membrane by a calcium-dependent membrane-bound transglutaminase (TGM). The proteins are covalently connected on the outside of the envelope with the ceramide-rich lipid layer (lipid envelope) (Figure 2). This envelope plays a crucial role for the epidermal barrier and protection of the transepidermal water loss (Fritsch, 2003; Madison, 2003). The mature

CE formed in the differentiation process consists of 10 nm thick protein envelope and 5 nm thick lipid envelope. Both structures are crucial together with the intercellular lipid lamellae for the permeability skin barrier (Kalinin *et al.*, 2002).

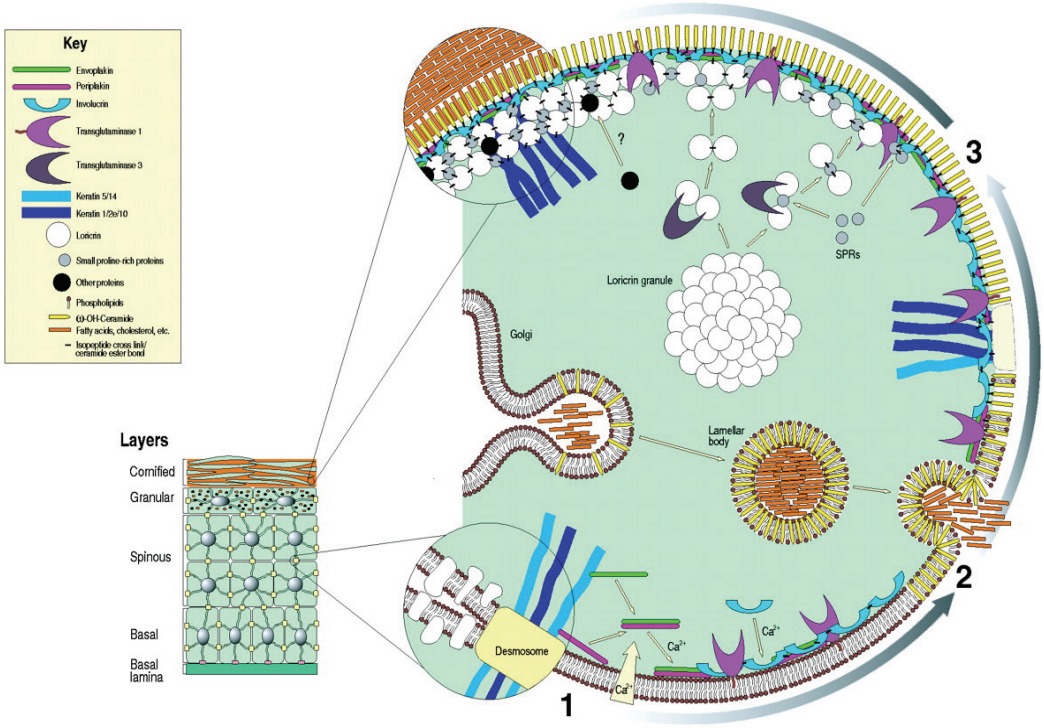

Figure 2. The cornified cell envelope (Kalinin *et al.,* 2002). The first step is the initiation occurred in the SS and included the synthesis of the cornified envelope structural proteins. The CE-formation begins with the expression of periplakin und envoplakin. An increase of the local calcium concentration leads to the interconnection between involukrin-monomers and involukrin with envolukrin catalyzed by TGM1 (Elias *et al.*, 2005; Kalinin *et al.*, 2002). The second step is the synthesis of the lipid envelope and the extrusion of lipids into the intercellular space of SC. The epidermal lipid synthesis occurs in the lamellar bodies. They are released into the intercellular space of SC composed an intercellular lipid bilayer. The next step is the reinforcing phase occurred in the SG. CE consists of 80% of the loricrin-SPR-oligomers. Loricrin is linked intracellular with the small proline-rich protein (SPR) to the loricrin-SPR-oligomers by TGM1 and 3 (Candi *et al.*, 1999). In this way, the networking occurs with the existing protein scaffold. Meanwhile, the most junction proteins are broken down. Keratin filaments (keratin 1, keratin 2e, keratin 10) and filaggrin are cross-linked with the proteins of CE. At the same time occurs the formation of the keratin filament-filaggrin-complex (Dale *et al.*, 1997).

1.3. Synthesis of filaggrin

Beverly Dale discovered a high-molecular phosphorylated histidine-rich and strong basic protein, so-called profilaggrin in 1977. Profilaggrin (400 kDa) is a molecular precursor of filaggrin. Filaggrin (35 kDa) is an intermediated filament-associated protein. The

profilaggrin- or *filaggrin*-gene is located on the chromosome 1q21.3 and consists of three exons (Figure 3). The exon 3 is very long (12-14 kb) and contains a variable number of 10 to 12 repeat structures (Presland *et al.*, 1992).

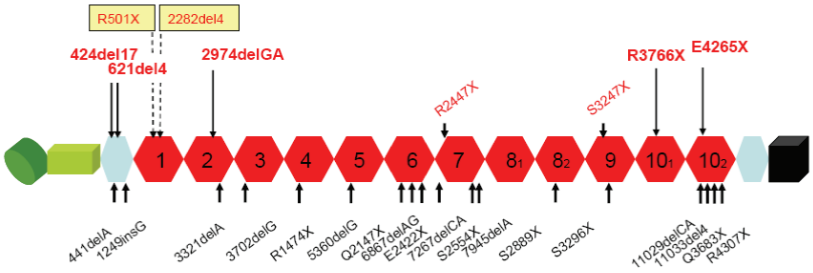

Figure 3. Overview of *FLG* gene with presently known mutations. The above named mutations were found in the German cohort (Oji *et al.*, 2009).

1.4. Filaggrin and the Natural Moisturizing Factors (NMF)

Profilaggrin is synthesized in the outmost stratum spinosum and forms with keratin filaments the clumpy aggregates, so-called keratohyalin granules. Profilaggrin is phosphorylated in the stratum granulosum and transformed to stable FLG-monomeres by endopeptidases resulting in the aggregation of keratin filaments and in the networking via disulfide bonds (Presland *et al.*, 1992, Fritsch, 2003). The formation of keratin is then completed. Filaggrin is further cleaved. The degradation products of FLG are various amino acids, namely arginine, glutamine and histidine or its derived products citrulline, pyrrolidone-5-carboxylic acid (PCA) and *trans*-urocanic acid (*t*-UCA) that are of great importance for water binding in the stratum corneum. The healthy skin has about 40% hydroscopic active amino acids in the stratum corneum, filaggrin filaments, that together with lactate and urea form the Natural moisturizing factors (Rawlings and Matts, 2005). The filaggrin deficiency thus results in skin dehydration.

Natural moisturizing factors (NMF) are derived from the different substances and namely from the sweat, sebum and degradation products of natural skin cornification. Their function is to connect the water between and within the keratinocytes in the stratum

corneum (Figure 4). In particular, NMF consists of various acids and especially amino acids, lactic acid, hyaluronic acid, urea and mineral acids.

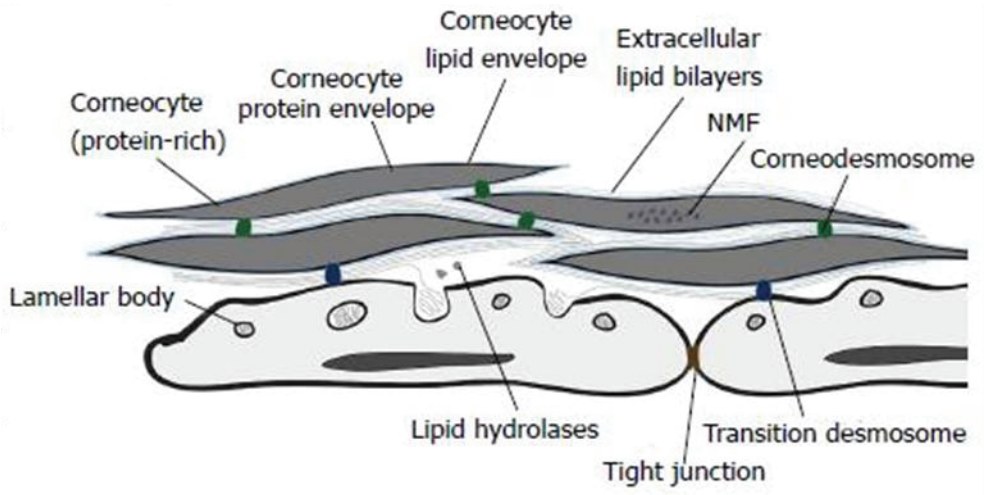

Figure 4. Skin moisturizing (Gillespie and Brown, 2015). The proteins in the stratum granulosum are covalently connected on the outside of the envelope with the ceramide-rich lipid layer (lipid envelope). This envelope plays a crucial role for the epidermal barrier and protection of the transepidermal water loss. The corneocytes are predominantly constructed from keratin filaments and are enveloped with the closely networked outer stable layer of proteins and lipids, the cornified cell envelope. Natural moisturizing factors have the function the water between and within the keratinocytes in the SC.

Amino acids are the protein building blocks that are a large part of NMF. They are derived from the protein filaggrin that is present in all mammals and is responsible for the structural connection of corneocytes. Urea is a degradation product of amino acids. It has a very strong hygroscopic effect. The sweat delivers it to the skin, where proteins and water are bound to it. It is a part of the NMF. Hyaluronic acid belongs to the so-called glycosaminoglycanes (GAGs) that are important building blocks of connective tissue. They pull large amounts of water. Glycerol is also located in the stratum corneum in lower level. It forms the basic building block for all fats, the so-called triglycerides, consisting of connection from three fat acids with glycerol. It is released during hydrolysis of the main lipids. Lactic acid is a chemical compound playing an important role as an intermediate product in the whole metabolism and is found in the blood, sweat and various inner organs.

1.5. Epidermal barrier

The stratum corneum with its lipid-rich intercellular substance represents the effective permeability skin barrier. The lipids of SC are essential to protect the skin against dehydration (Candi *et al.,* 2005; Wertz, 2000). The transepidermal water loss without lipids would increase by a factor of 2500 (Landmann, 1988). The SC is impermeable for water and externally supplied hydrophilic substances. Furthermore, the stratum corneum protects against penetration of harmful substances like allergens, microorganisms and irritants and serves as a barrier for water and small molecules from inside to outside (Elias *et* al., 2005; Elias P *et* al., 2007). These manifold functions require a highly complex organized dynamic 10-15 µm thin horny layer composed of arranged dead keratinized corneocytes.

The traditional model of the SC is the so-called “brick and mortar model” (Elias, 1983). It does not fully explain the complexity of the SC functioning. Bouwstra proposed a "sandwich model". According to this model, the lipid layer is represented as a fluid phase (narrow) within the hexagonal or orthorhombic ordered lipid structures (wide) (Bouwstra *et al.*, 2000). Forslind described the lipid matrix of the SC as a discontinuous system in his "domains-mosaic-model". The crystalline domains are kept together over lipids of the fluid-crystalline phases (Forslind, 1994). Norlen suggested a modified variation of this model - „membrane folding model “. In this model the lipids are ordered into a single-phase coherent lamellar gel (Norlen, 2001).

The corneodesmosomes consist out trans-membrane proteins interlocked in the intercellular space and "rivet" the corneocytes with each other (Table 1). This connection, that is not mechanically the strongest element, contributes to the cohesion of the corneocytes. There are about 400 bis 600 corneodesmosomes per one horny cell and side. The result is a complex and dense network that together with hook- and clamp-like horny cell structures forms a stable structural framework ensuring the necessary mechanical stability.

Table 1: Transmembrane proteins in the stratum corneum

Structures in the stratum corneum	Transmembrane glycoproteins
Desmosomes	Desmoglein 1-3 Desmocollin 1-3
Corneodesmosomes	Corneodesmosin
Tight junctions	Occludin Claudin 1-15 ZO (1, 2, 3)

The tight junctions serve as the last condition for the mechanic stability. The tight junctions consist of a network of transmembrane proteins, especially of claudin and occludin surrounding belt-like the entire cell scope. This way, they form a connected diffusion barrier in the SC. Tight junctions serve the mechanic stability of epidermis by interconnecting the cytoskeleton of the participating cells and involving in this way all cells in the tissue statics. The intercellular space is sealed by tight junctions. This prevents the paracellular seepage of the molecules and ions into the epidermis. Tight junctions also provide protection against invading microorganisms. Furthermore, tight junctions have a so-called "fence-function“. Tight junctions hold the cell polarity by prevention of the free movement of the cell membrane components. They are subdivided into the apical and basal cell pole. These are separated from each other by the tight junctions that is a necessary prerequisite for directed substance transport.

The reason for the disturbed barrier are the changes in the lipid metabolism, epidermal proliferation and differentiation.

1.6. Transepidermal water loss

The transepidermal water loss of the skin (TEWL) is limited by an intact barrier. The water content of the epidermis declines from inside to outside (Tagami *et al.,* 1980). Thus, it is falling from 70-80% (m/m) in the stratum basale and stratum spinosum to a maximum of 20% (m/m) in the stratum corneum (Warner *et al.* 1988). Measurement of the TEWL is clinically used for the assessment of the epidermal barrier function (Pinnagoda *et al.,* 1990; Rogiers, 2001). An impaired permeability barrier is a

characteristic feature of atopic eczema in the lesional and nonlesional skin (Chikakane K and Takahashi H, 1995) and in most disorders of cornification like the ichthyoses (Oji *et* al., 2010).

1.7. The pH gradient

The pH gradient is a key characteristic for the epidermal barrier function. The lipid secretion is controlled by Ca2+ and K+ (Imokawa *et al*., 1991). Changes of hydrolase function and expression lead to a disturbed structure and function of SC. In the first 4 weeks of life, the skin surface-pH gradient changes from pH 7.0 to pH 5.0 (Behrendt H and Green M, 1958; Giusti F *et al.,* 2001; Yosipovitch *et al.,* 2000; Hoeger PH and Enzmann CC, 2002; Afsar, 2009). Later in life, the skin surface-pH gradient is stable from infancy to adulthood and ranges at pH 5.0-5.4 (Braun-Falco O, Korting HC, 1986, Zlotogorski A, 1987). In contrast, the skin surface-pH gradient at the age of 70 - 80 years of life increases up to pH 6.0 (Thune *et al*., 1988; Wilhelm *et al*., 1991; Man *et al*., 2009).

Within the stratum granulosum a “physiological” pH of around 7,5 is present. Thus there is a gradient from the stratum granulosum to the stratum corneum. It is of note that the more acidic pH of the stratum corneum allows that the inhibitory complex formed by KLK5 with LEKTI can dissociate and free KLK5 can be activated by matriptase finally resulting in KLK5 mediated degradation of corneodesmosomes (Deraison *et al*., 2007).

2. KALLIKREIN – RELATED PEPTIDASES

2.1. Serine proteases

Proteases are a major group of enzymes in mammals. Based on the amino acid residue presented in the active site, serine proteases can further be subdivided into four major classes: serine proteases, cysteine proteases, aspartic proteases and metalloproteases (Laskowski and Kato, 1980; Brattsand and Egelrud, 1999; Hansson *et al.,* 1994; Bernard *et al.,* 2003; Horikoshi *et al.,* 1998).

Almost one third of all proteases are serine proteases divided into two groups: plasmatic kallikreins (KLK) and tissue kallikreins. The first member of the tissue KLK family was identified in 1930 as the most abundant protease in pancreas and hence was named tissue "kallikrein", for pancreas (kallikreas) in Greek (Werle, 1934; Kraut *et* al., 1930). In the past few years, serine proteases have been intensively investigated and are the best characterized enzyme family today consisting of 15 different trypsin- and chymotrypsin-like KLKs (Yousef *et al*., 2005). Only three KLKs, namely KLK3, KLK7 and KLK9 have chymotrypsin-like activity in the human organism (Skytt *et al*., 1995). KLK1, KLK2, KLK4 - 6, KLK8, KLK10 - 15 belong to the trypsin-like serine proteases.

Due to identical tertiary structure the serine proteases are subdivided into two superfamilies, the chymotrypsin and subtilisin family. The serine proteases have an identical catalytic mechanism, which differentiates them from other protease groups. The first step involves a formation of an acyl enzyme, the so-called acylation. In the second step, a hydrolysis of ester takes place (Hedstrom, 2002; Fersht *et al.,* 1999; Berg *et al.,* 2002; Page *et al.,* 2008). The majority of enzymes only show their catalytic activity in a narrow pH range. Trypsin und chymotrypsin are most active at the pH of 7.0 to 8.0 (Brattsand *et al.,* 2005; Caubet *et al.,* 2004; Ekholm *et al.,* 1999; Borgono *et al.,* 2007).

KLKs characteristically have a reactive serine residue in the active enzyme center - hence the name of this enzyme group. In connection with two additional amino acids, serine forms the so-called catalytic triad composed of serine-histidine-aspartate. The

active form of this serine residue could catalytically hydrolyze the peptide bonds. As each protease only splits identified places in the protein, there is a need for the common influence of several KLKs in the process of proteolysis. According to the amino acid sequence of catalytic residues, KLKs are further differentiated into trypsin- and chymotrypsin-like groups. The first group prefers the peptide bonds followed by alkaline amino acids (lysine (K) oder arginine (R)). They include an aspartate or glutamate residue in the substrate binding domain that could enter into a strong electrostatic bond with the alkaline amino acids. Chymotrypsin-like KLKs have a nonpolar substrate binding domain and prefer an aromatic and nonpolar amino acids as substrate (tryptophane (W), tyrosine (Y), phenynalaline (F) or leucine (L)) (Bond *et* al., 1989).

Most serine proteases are secreted as zymogens (inactive pro-forms) subsequently processed extracellularly to their active form by limited proteolyses (Neurath and Dixon, 1957). The activation of the zymogens in their active form is a precise by controlled process. However, this activation is irreversible. Therefore, there are many endogenous serine protease inhibitors in the human organism that are responsible to prevent uncontrolled proteolysis.

2.2. Nomenclature

The accepted nomenclature for kallikrein-related peptidases was until recently by gene (KLK 1, KLK 2, etc.) and enzyme/protein (K 1, K 2, etc.). To characterize the species, a prefix has been used, for example h (*human*) for the human species and m (*mouse*) for the species of rodents.

The new nomenclature refers proteins without proven kininogenase activity as kallikrein-related peptidases. Except for KLK 1 that has kininogenase activity and therefore still is known kallikrein 1, the new names of KLK 2 - 15 are kallikrein-related peptidases followed by the number of gene. To differentiate between protein and gene, KLK is written in standard capital letters for the protein, *KLK* in italics is written for the gene names (Table 2) (Lundwall *et al.,* 2006).

Table 2: Gene and protein names of human tissue kallikreins 1-15 (modified according to Yousef und Diamandis, 2003).

Gene names	Protein names	Other proteins names
KLK 1	KLK 1	Tissue kallikrein, kidney/pancreas/salivary gland kallikrein, hPRK
KLK 2	KLK 2	Kallikrein-related peptidase 2, glandular kallikrein 1, hGK-1
KLK 3	KLK 3	Kallikrein-related peptidase 3, prostate specific antigen, PSA
KLK 4	KLK 4	Kallikrein-related peptidase 4, PRSS17, kallikrein-like protein 1, KLK-L1, Enamel Matrix Serin Protease (EMSP1), Androgen regulated message 1 (ARM1), Prostase
KLK 5	KLK 5	Kallikrein-related peptidase 5, kallikrein-like protein 2, KLK-L2, stratum corneum trypsin-like enzyme (SCTE)
KLK 6	KLK 6	Kallikrein-related peptidase 6, PRSS9, protease M, zyme, neurosin, myelencephalon specific protease (MSP)
KLK 7	KLK 7	Kallikrein-related peptidase 7, PRSS6, stratum corneum chymotrypsin-like enzyme (SCCE)
KLK 8	KLK 8	Kallikrein-related peptidase 8, PRSS19, neuropsin, tumor-associated differentially expressed gene 14 (TADG14), ovasin, brain serine protease 1(BSP1)
KLK 9	KLK 9	Kallikrein-related peptidase 9, kallikrein-like protein 3, KLK-L3
KLK 10	KLK 10	Kallikrein-related peptidase 10, normal epithelial specific-1 (NES1 Protein)
KLK 11	KLK 11	Kallikrein-related peptidase 11, PRSS20, like serine protease (TLSP), hippostatin
KLK 12	KLK 12	Kallikrein-related peptidase 12, kallikrein-like protein 5 (KLK-L5)
KLK 13	KLK 13	Kallikrein-related peptidase 13, kallikrein-like protein 4 (KLK-L4)
KLK 14	KLK 14	Kallikrein-related peptidase 14, kallikrein-like protein 6 (KLK-L6)
KLK 15	KLK 15	Kallikrein-related peptidase 15, prostin (ogen), ACO protease

2.3. Gene locus and structure

The human KLK genes are located on the long arm of chromosome 19q13.3-q13.4 and constitute the largest contiguous serine protease cluster in the human genome (Yousef *et al*., 2000; Gan *et al*., 2000; Harvey *et al*., 2000). Three classical kallikreins (*KLK 1 - 3)* and *KLK 15* are only located in the 60 kb large region, followed by the 11 remaining kallikrein-related peptidases. The genomic structure of kallikreins is highly conserved. The coding regions are fundamentally divided among the five exons with similar and even identical length. All genes code probably secreted serine proteases with a typical catalytic triad consisting of histidine-aspartate-serine in the exon 2, 3 and 5 (Yousef and Diamandis, 2001, Yousef *et al.,* 2005) and except for *KLK2* and *KLK3,* which are transcribed in the direction of telomere to the centromere (Figure 5).

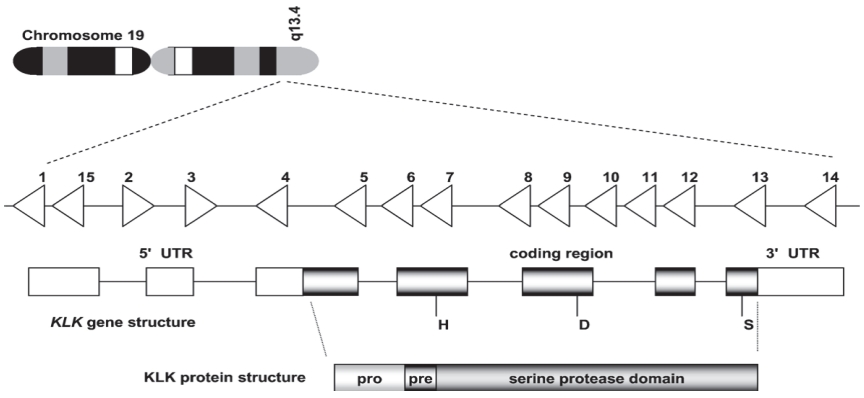

Figure 5. Gene cluster of human tissue kallikreins (Pampalakis and Sotiropoulou, 2007). The human KLK genes are located on the long arm of chromosome 19q13.3-q13.4. The coding regions are fundamentally divided among five exons with similar and even identical length. All genes code probably secreted serine proteases with a typical catalytic triad consisting of histidine-aspartate-serine in the exon 2, 3 and 5, which are transcribed in the direction of telomere to the centromere.

All KLKs include five exons and in between four introns that are located in a 5-10 kb large region. The coding exons are even sized and receive the identical sequences to 36-80% (Yousef *et* al., 2001; Lundwall *et al*., 2008). Moreover, the KLK gene locus encompasses the pseudogenes. However, it is not yet known, how many pseudogenes there are and what role they play (Clements *et al*., 2001). Every protein is synthesized

as proenzyme. The catalytic triad consists of His 57, Asp 102 and Ser 195 (Hedstrom *et* al., 2002). The arrow shows the place of amino acids in the substrate binding domain determining whether the late protein has trypsin- or chymotrypsin-cleaving skills.

The relative short signal peptide consists of 16-33 amino acids, the propeptide – of 4-9 amino acids. An exception is *KLK 5*, which propeptide includes 37 amino acids. The mature enzymatic active enzyme composes of 227-252 amino acids. The 10-12 preserved cysteine residues form five (KLK 1 - 3, KLK 13) or six (residual KLKs) disulphide bridges.

2.4. Kallikrein-related peptidases in the skin desquamation

Kallikreins are responsible for the coordination of various physiological skin functions. They regulate many biological processes and are involved in the important physiological processes via activation of different substrates and receptors.

The skin desquamation is a physiological process in the epidermis characterized by the shedding of the upper layers of SC. This process requires the proteolysis of the corneodesmosomal adhesion molecules followed by serine proteases. Many proteases are involved in this process, but the most important are KLK5 and KLK7 (Egelrud and Lundstrom, 1991; Suzuki *et al.,* 1993;1994). Desquamation normally occurs invisibly and is referred as desquamatio insensibilis.

The desquamation of the stratum corneum is a serine protease-dependent process regulated not only by serine proteases but also by serine protease inhibitors (Suzuki *et* al., 1994; Suzuki *et al*., 1996). Many kallikreins are expressed in the epidermis and skin appendages as inactive pro-forms (pro-KLK), transformed in the active form by various mechanisms. Recently it has been reported that eight kallikreins (KLK 5 - 8, 10, 11, 13 and 14) are expressed in healthy skin, of which KLK5, KLK7, KLK8, and KLK14 seem to be the most important (Lundwall and Brattsand, 2008; Komatsu *et* al., 2003). So far the role of different kallikreins can not be assessed with precision.

KLK5, KLK7 and KLK14 are mainly essential for the skin desquamation (Suzuki *et* al., 1994, 1996). KLK5 is also known as stratum corneum tryptic enzyme (SCTE) and KLK7 - as stratum corneum chimotryptic enzyme (SCCE) (Brattsand and Egelrud, 1999; Egelrud, 1993. Yousef *et* al., 1999; Hansson *et* al., 1994). Both enzymes are secreted as zymogens by lamellar granules of upper keratinocytes in the stratum granulosum, then transported to the stratum corneum and are subsequently released into the extracellular interstate (Sondell *et al*., 1995). KLK5 and KLK7 are located in the upper layers of SG and in lower layers of SC. The actual knowledge about proteolytic cascade of the human KLKs in the skin is represented in the Figure 6.

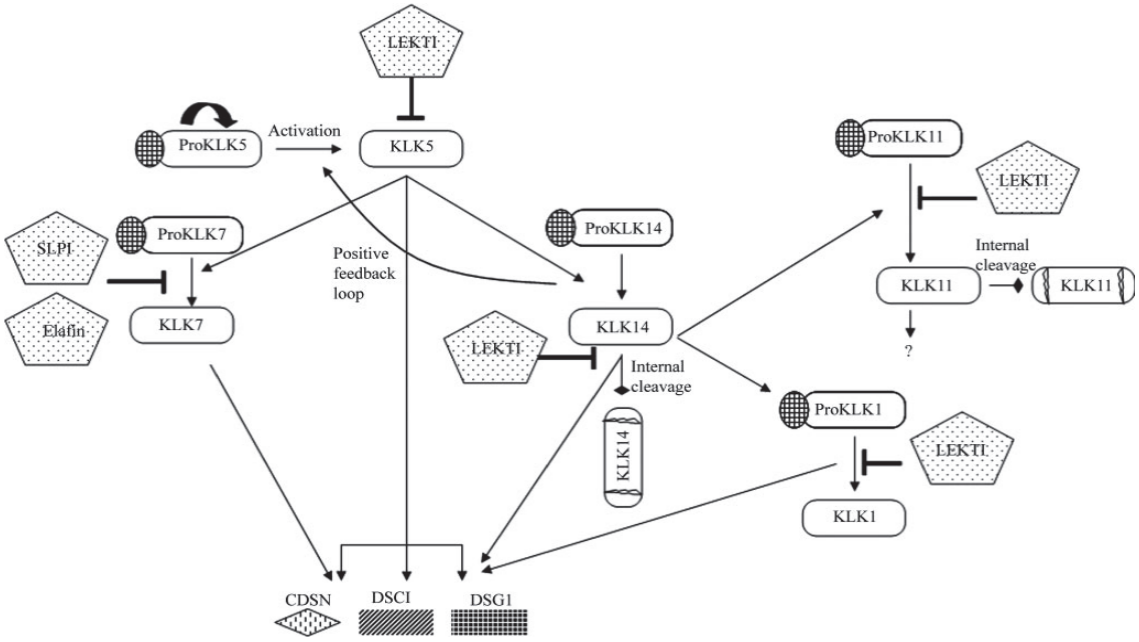

Figure 6. Scheme of KLK cascade in the skin (Emami and Diamandis, 2008). KLK5 is activated by itself and activates pro-KLK14 and pro-KLK7. Activated KLK5, 7, 11 and 14 split the corneodesmosome proteins (DSG1, DSC1 and CDSN). Desquamation is regulated by different serine protease inhibitors like SLPI, elafin and LEKTI.

KLK7 consists of 253 amino acids and has a secretion signal prepeptide (22 amino acids), followed by an activation propeptide (7 amino acids) and the mature chain (224 amino acids) (Ying Dong *et al*., 2009). Because of 7 amino acids on the N-terminus the constructed intermediate product fails to be active and is saved in the lamellar bodies. The intermediate protein is released into the extracellular interstate and is activated by

KLK5 during the keratinocyte differentiation. KLK5 has 227 amino acids, while the zymogen consists of 293 amino acids. Both, KLK5 and KLK7 are transformed to the active form by several interstages. KLK5 is activated by itself and by KLK1 (Yousef and Diamandis, 2001; Yousef *et* al., 2005; Emani and Diamandis, 2007).

However, proteolytic activity of kallikreins is strongly regulated by pH dependent matter, hydration and serine protease inhibitors (Deraison *et* al., 2007). Serine proteases are responsible for integrity and cohesion of the SC. Human SC has a pH gradient ranged from pH 7.5 at the inner SC layers to pH 5.4 at the SC surface. (Hanson *et* al., 2002; Ohman *et* al., 1994). This creates a steep pH gradient of 2 units between the thickness of 10-15 μm (Ohman and Vahlquist A, 1994). The decrease of pH gradient leads to the activation of serine proteases. Deraison et al. suggested 2007 a model for desquamation regulation (Figure 7).

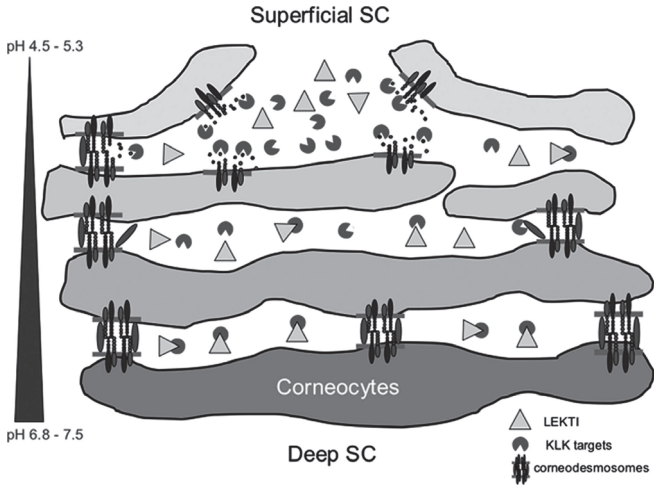

Figure 7. Desquamation model (Deraison *et al*., 2007). The decrease of pH gradient from pH 7.5 to 5.4 being more acidic at the SC surface leads to the activation of KLKs resulting in the complete degradation of corneodesmosomal components and the exfoliation process.

KLK5 cleaves the corneodesmosome proteins: corneodesmosin (CDSN), desmocolin1 (DSC1), desmoglobin1 (DSG1), desmoglobin2 (DSG2) and plactoglobin. It was established that KLK5 does not cleave DSG1 and DSG2 at pH 7.2 (Simon *et al.*, 2001; Caubet *et al.,* 2004). Nevertheless, at pH 5.6 both these proteins were degraded by KLK5 (Caubet *et* al., 2004), i.e., that the induced degradation of corneodesmosome

proteins DSG1 and DSG2 by KLK5 could be only effective in the acid pH. Thus, the cell junctions are destructed and degraded followed by the scaling of corneocytes (Caubet *et al*., 2004). KLK5 has a trypsin-like activity to 33%, and like KLK14 - to 22%. KLK7 cleaves CDSN, DSC1 and plactoglobin, but does not cleave DSG1 and DSG2 independent from pH gradient (Simon *et* al., 2001; Caubet *et* al., 2004). KLK7 is responsible for 76% chymotripsin-like activity. The increase of TEWL does not lead to elevated activity of KLK7.

KLK5 and KLK14 play an important role in the skin inflammatory processes by activation of protease-activated receptor 2 (PAR2). Activated KLK7 can also play a role in the inflammation, because it is able to activate a precursor of cytokine interleukin-1b (Nylander Lundqvist *et al*., 1997).

Further KLKs can influence the desquamation process. KLK1, KLK6 and KLK14 are able to cleave DSG1. Although KLK1 has a less importance, KLK14 is an efficient enzyme in cleaving DSG1 (Borgoño *et* al., 2007).

KLK7 is associated with the terminal differentiation of keratinocytes and cornification. It is a constant process in the skin during the life. In contrast to KLK7, the concentration of KLK5 is less in those aged 30 - 70 years. Based on this, KLK5 may play a role in skin aging.

Interestingly, KLK5, KLK7, LEKTI (Lympho-epithelial-kazal-type-related inhibitor) and CDSN are transported in the different lamellar bodies (LB). This process is exactly regulated in the LB, when and where the molecules should be transported to. LEKTI is expressed in the upper layers of SS and transported in LB to the upper layers of SG. KLK5 and KLK7 are released extracellularly in the SC. This physiological distribution of proteases, substrates and serine protease inhibitors prevents premature proteolysis. The pH gradient regulates the KLK activity by interaction of LEKTI and other serine protease inhibitors (SPI). In the deep layers of SC with a neutral pH gradient LEKTI inhibits KLK activity by forming inhibitory complexes. Thus, the proteolysis of corneodesmosome proteins is prevented. As opposed to this, the more acidic pH gradient in the superficial layers of SC instigates the KLK activity and the shelling of corneocytes. A lack of LEKTI

causes a genetic disease, Netherton Syndrome, characterized by hyperactivity of KLK5, KLK7 and abnormal proteolysis of corneodesmosomes.

2.5. KLK in immunological and inflammation skin processes

The KLKs are involved in a variety of inflammatory and immunological processes in the skin (Table 4) (Richard, 2005; Ramachandran and Hollenberg, 2008).

Kallikreins, especially trypsin-like proteases like KLK5 and KLK14, induce the proinflammatory processes in the epidermis by PAR2. (Steinhoff *et al*., 2000). KLK7 fails to active PAR2, but it is also involved in the skin inflammation by other mechanisms. PAR2 is a G-protein-coupled receptor expressed in the superficial layers of epidermis.

Cathelicidin is a cationic antibacterial protein of 18 kDa (hCAP18) in the epidermis converted into the active form LL-37 by KLK5 (Sørensen *et al*., 2001; Yamasaki *et al*., 2006). LL-37 is cleaved into the small antimicrobial peptides KS-29, KS-30, and KS-22 that are active against various pathogens like Escherichia coli, Staphylococcus aureus (Zaiou *et al*., 2003) and several stems of Streptococcus (Dorschner *et al*., 2001). This process is also regulated by KLK5. KLK7 cleaves LL-37 in the antimicrobial peptides RK-31 and KR-20 that are not particularly stable. Based on it, KLK7 rather plays a role as inactivator of LL-37. Furthermore, KLKs are relevant to antimicrobial defense system (Yamasaki *et al*., 2006). Among the inflammatory diseases, for example, rosacea appears to have an increased expression of cathelicidin or of its isoform LL-37.

Thus, KLK7 plays a role in rosacea, psoriasis and atopic dermatitis activating the proinflammatory cytokines. In this context interleukin 1b (IL-1b) is relevant. IL-1b is a cytokine produced by macrophages, endothelial cells and keratinocytes (Kupper *et al*., 1986; Ansel *et al*., 1988; Janeway *et al*., 1999). It affects the receptors CD121a (IL-1RI) and CD121b (IL-1RII). Thus, the proinflammatory effects are activated in the epidermis. IL-1b (31kDa) is expressed as molecular precursor of IL-1b and transformed to an active form (18kDa). This process is regulated by KLK7 and 1b-converting enzyme (ICE).

Based on the pH shift to an alkaline range, the increase of KLK activity is assumed in patients with atopic dermatitis (AD). According to several studies, it is known that the expression of KLK7 is increased and strongly diffused in the epidermis by psoriasis patients versus controls. Values of KLK6, 8, 10 and 13 are elevated in the serum by patients with psoriasis and significantly correlated with PASI score. The table 4 provides an overview about the role of KLKs in the skin inflammation.

Table 3: Kallikrein-related peptidases in inflammatory skin diseases (Magdolen *et al.*, 2013).

Skin disease	Pathogenesis	KLK levels	KLK activity
Atopic dermatitis	chronic inflammatory disease with multiple factors in the pathogenesis (immunological, endocrine, metabolic, infectious)	Increased expression of KLK	pH shift in SC leads to the increased KLK-activity that causes the abnormal desquamation, epidermal barrier dysfunction, inflammation, pruritus and manifestation of allergy
Psoriasis vulgaris	chronic inflammatory disease characterized by erythematous plaques and hyperproliferation of keratinocytes	Increased expression of KLK; diffused in the deep layers of SC	pH shift in the epidermis leads to increased of KLK. Hyperactivity of KLK leads to the abnormal desquamation, activation of IL-1b, inflammation and manifestation of allergy
Netherton syndrome	rare autosomal recessive genodermatosis caused by mutations in the *SPINK5* gene on the chromosome 5q32 and failure of epidermal serine protease inhibitor LEKTI	Hyperexpression of KLK; diffused deeper in the SS/SG to the periphery of keratinocytes	Hyperactivity of KLK leads to the abnormal desquamation, inflammation and manifestation of allergy. Abnormal expression of cathelicidin is caused by hyperactivity of KLK
Rosacea	chronic inflammatory skin disease characterized by erythema, papules, papulopustules and telangiectasis in the face	Increased expression of KLK; diffused deeper in the epidermis	Abnormal expression of hCAM18, cathelicidin and LL-37 leads to the inflammation, pruritus and epidermal barrier dysfunction via KLK-hyperactivity

3. SERINE PROTEASE INHIBITORS

The protease inhibitors are necessary to prevent an uncontrolled proteolysis and a damage of the functional cells and tissues (Bode and Huber, 1991). The serine protease inhibitors (SPI) are characterized by the serine residue in the active center. They regulate the serine protease activity and thus control various of the biological processes in the organism. The coagulation system, complement activation and proteolytic cascades are regulated by serine proteases and likewise by serine protease inhibitors. The endogenous inhibitors in the skin include α-1 antitrypsin, skin-derived antileukoproteinase (Elafin), secretory leukocyte protease inhibitor (SLPI) and lymph-epithelial Kazal-Typ inhibitor (LEKTI).

3.1. Alpha-1 Antitrypsin

Alpha 1 antitrypsin (α-1AT) consists of 350-500 amino acids. A-1AT was first detected in a complex with KLK7 in the plantar skin. This inhibitor plays an important role in lungs and liver function. Genetic diseases with α-1AT deficiency cause pulmonary emphysema and hepatic cirrhosis.

3.2. Skin-derived Antileukoproteinase (SKALP)

Skin-derived Antileukoproteinase (SKALP) is better known as elafin. SKALP is an elastase specific inhibitor that cannot be demonstrated in the healthy skin (Pol *et al*., 2003). It is located in the buccal mucosa, esophagus and vagina (Pfundt *et al*., 1996). Firstly, elafin was detected suprabasally in patients with psoriasis. Also, elafin was shown to be present the inflammatory diseases (Schalkwijk *et* al., 1990; Wiedow *et al*., 1990; Schalkwijk *et al*., 1991). Up to now, the expression of elafin remains controversial in the healthy epidermis.

Elafin is synthesized as precursor with 117 amino acid residues including a hydrophobic signal peptide of 22 residues. The presence of signal peptide indicates that elafin is secreted (Schalkwijk *et al*., 1999).

However, it is not clear yet, if SKALP is an effective serine protease inhibitor. SKALP and SLPI share several homologous amino acids, but SLPI is more effective inhibitor of KLK7 as SKALP.

3.3. Secretory Leucocyte Protease Inhibitor (SLPI)

Secretory leukocyte protease inhibitor (antileucoprotease (ALP)), human seminal inhibitor-1 (HUSI-1)) inhibits various of enzymes like elastase, trypsin, chymotrypsin and cathepsin. The molecular weight is 11.7 kDa (Thompson *et al*., 1986; Eisenberg *et al*., 1990; Seemüller *et al*., 1986). SLPI is synthesized in the inflammatory skin. SLPI was found in the SG of the psoriatic skin like SKALP. Franzke et al. reported in 1996 that SLPI is the most important inhibitor for KLK7 in the epidermis. Indeed, it was established that SLPI is able to inhibit the desquamation of keratinocytes in the plantar skin. At that time, LEKTI was not yet detected. In the meantime it has been established that SLPI inhibits KLK7 less markedly than LEKTI.

Except for the inhibitory effect, SLPI also possesses antimicrobial activity (Hiemstra *et al*., 1996). SLPI is effective against several bacteria and fungi like Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, and Candida albicans (Wiedow *et al*., 1998). SLPI also appears to have antiretroviral activity and inhibits human immunodeficiency virus 1-activity (McNeely *et al*., 1995).

3.4. Lympho-epithelial Kazal-type inhibitor (LEKTI)

LEKTI belongs to a family of Kazal-type serine protease inhibitors consisting of 1064 amino acids and comprising of 15 inhibitory domains (D1-D15). LEKTI is located on the chromosome 5q32 and is encoded by gene *SPINK5* (serine protease inhibitor Kazal-Typ 5). Domains 2 and 15 (D2 und D15) almost exactly match Kazal-type pattern with specific

bonds of six cysteine residues linked with each other by disulfide bondage. However, the other 13 domains only exhibit four instead of six cysteine residues with 1-4/2-3 disulfide bondage and have a high homology with this inhibitor family (Mägert *et al*., 1999). The domains 8 - 11 of LEKTI have the biggest inhibitory activity. Domain 6 of LEKTI is responsible for the inhibition of KLK5 and KLK7 (Egelrud *et al*., 2005), whereas D15 does not exhibit any effects for these two KLKs. Furthermore, KLK5 is inhibited by domains 6-9 (D6-D9) and 9-12 (D9-D12) of LEKTI (Jayakumar *et al*., 2004; Schechter *et al*., 2005). In contrast to KLK5, KLK7 is only inhibited by LEKTI domains 6-9 (D6-D9) (Jayakumar *et al*., 2004; Schechter *et al*., 2005). According to Deraison et al., KLK5 is the most important objective for LEKTI.

All known *SPINK5*-mutations cause a genetic disease namely Netherton-Syndrome, and result in a premature stop codon in the LEKTI transcript (Raghunath *et al*., 2004; Chavanas *et al*., 2000). Thus, it is believed that truncated LEKTI forms are formed having a lack of several inhibitory domains. Most mutation reports have not investigated the protein by detailed immunostaining approaches. In most studies LEKTI is absent in the skin irrespective of where the mutation in the gene is located. Thus, it is conceivable that due to mRNA mediated decay the entire LEKTI protein is abolished. Due to the absence of this inhibition there is an up opposed increased serine proteases activity, especially the hyperactivity of KLK5 and KLK7. Thus, an abnormal proteolysis of corneodesmosomes and intensified scaling occurs in the epidermis. This in turn results in the skin barrier dysfunction (Yang *et al*., 2004). LEKTI can be considered as a key regulator of epidermal protease activity. In this context it is important to note the epidermal proteolytic cascade and the key role KLK5 plays in this cascade (Furio *et* al., 2015). Thus inhibition of KLK5 by LEKTI reduces overall epidermal proteolytic activity in particular of downstream target proteases such as KLK7, KLK14 and ELA2 (Furio *et* al., 2015). The overview of proteases and protease inhibitors is given in Table 3.

Table 4: Proteases and protease inhibitors in the epidermis

Protease	Substrate	Protease inhibitors
Serine proteases		
KLK5	DSG1, DSG2, DSC1, CDSN	LEKTI
KLK7	DSC1, CDSN	SKALP, SLPI, LEKTI
Cysteine protease		
SCCP	DSC1, CDSN	Cystatin M/E, Cystatin-a, SLPI
Aspartate protease		
Cathepsin G	DSC 1, CDSN	SLPI

The inhibition of serine proteases is a pH dependent process. LEKTI is active by pH 7.0 in the superficial layers of SG or in the deep layers of SC. In the upper layers of SC with pH 4.5 LEKTI loses its inhibitory activity as it dissociates e.g. from KLK5 and cannot longer inhibit KLK5. In this manner is it possible that serine proteases can become active. This results in degradation of corneodesmosome and in physiological skin desquamation.

3.5. SERPINs

SERPINs (**Ser**ine **Pr**oteinase **In**hibitors) comprise a superfamily of proteins that have various of functions in the human organism. Interestingly, they recognize about ten percent of plasma proteins. Antitrypsin, antitrombin, ovalbumin, plasminogen-aktivator-inhibitor and neuroserpin belong to the most important SERPINs. They are involved in intracellular as well in extracellular processes. Among other functions, they regulate blood coagulation, fibrinolysis, cell differentiation, tissue development, apoptosis and tumor growth. SERPINs are involved in the regulation of proteolytic processes in the different biological systems. The originally identified SERPINs are able to inhibit serine proteases. Certainly, the terms SERPINs and serine protease inhibitors are not interchangeable, because the first group inhibit not only serine proteases but also other

proteases. Furthermore, the serine proteases are inhibited by SERPINs as well by other protease inhibitors.

SERPINs structurally have three β-sheets A, B, C and nine α-Helices A-I forming an inter tension standing structure with a reactive center. This can result in a loss of inhibitory properties by proteolytic cleavage of this structure. The inhibition is also irreversible. Inhibitory SERPINs bind to proteases close to catalytic domains. Initially, the inhibitory effect is obtained by steric hindrance. In addition, SERPINs are cut open by the proteases. The released tension in SERPINs leads to profound conformation changes and finally to degradation of proteases.

It has been reported that many SERPINs are able to inhibit KLKs in the skin (Goettig *et al.*, 2010). For instance, KLK5 is inactivated by SERPINA5 (proteinase C inhibitor) and SERPINF2 (alpha2-antiplasmin). SERPINA, SERPINA3, SERPINA4, SERPINA5 and SERPINF2 have been shown to be potent inhibitors of KLK7 in the skin.

4. SKIN DISEASES

4.1. Mendelian disorders of cornification (MeDOC)

Ichthyoses are genetically determined Mendelian disorders of cornification (MeDOC) that are characterized by universal scaling. Today a distinction is made between non-syndromic and syndromic forms (Traupe *et al*., 2014). Ichthyosis vulgaris (IV; OMIM 146700) is the most frequent form of MeDOC having a prevalence in Central Europe of 1:400 (Traupe, 1989; Wells *et al*., 1965; Oji *et al*., 2010). Clinically it presents with mild generalized fine scaly skin with flexural sparing, palmar hyperlinearity being a particular hallmark (Wells and Jennings, 1967; Wells and Kerr, 1965) (Figure 8).

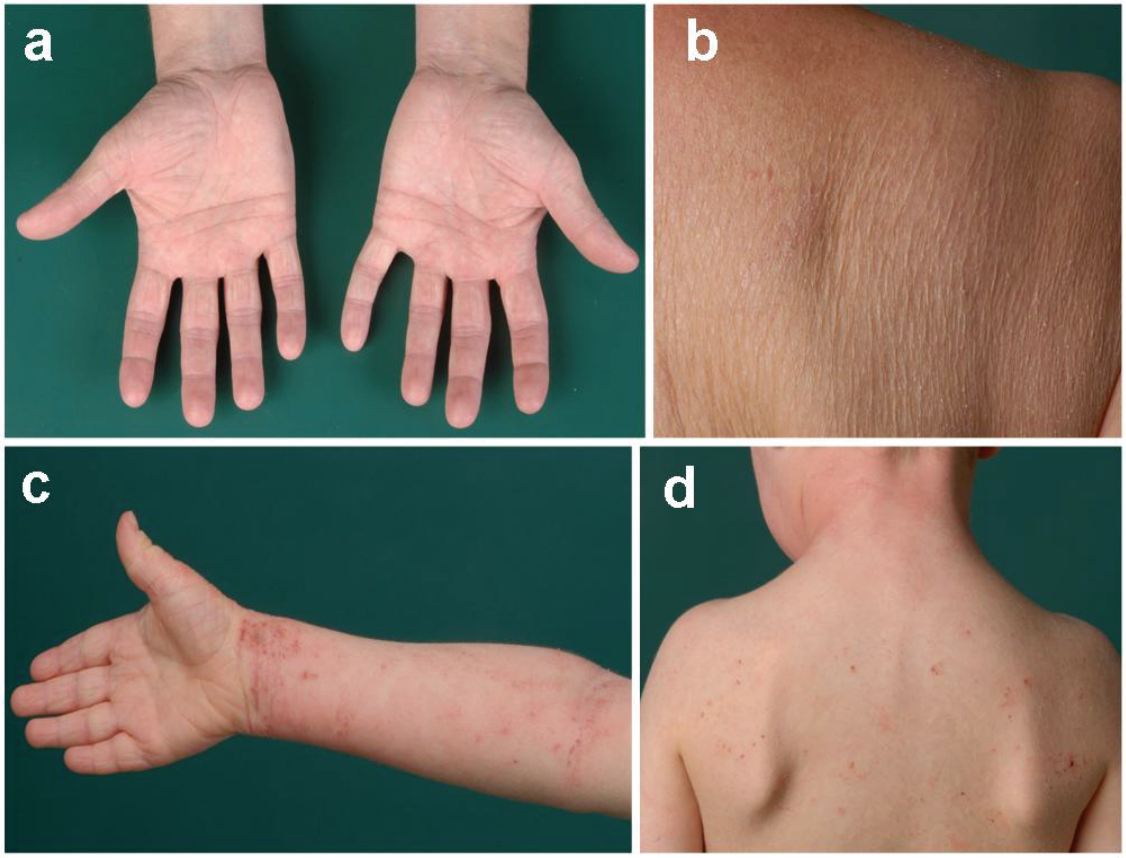

Figure 8. Clinical presentation of ichthyosis vulgaris due to compound heterozygous mutations in the *FLG* gene: hyperlinearity of palms in a 37 years old young man (ID 7, a), moderate fine, light grey scaling on the trunk (b). Clinical features of ichthyosis vulgaris with atopic dermatitis in a 4 years old boy (ID 11): hyperlinearity of palms, erythematous and papular patches on the forearm (c) and trunk (d), redness on the neck (d) (Photos from Department of Dermatology, University of Münster).

Atopic manifestations are a frequent finding in up 50-70% of the patients (Brown *et al*., 2008; Brown *et al*., 2008; Akiyama *et* al., 2010; Oji *et al*., 2009).

The condition is inherited in a semidominant pattern, i.e. homozygous and compound heterozygous patients develop a more severe form of the disease. Ichthyosis vulgaris is caused by nonsense mutations in the profilaggrin gene (*FLG*) (Smith *et al*., 2006; Sandilands *et al*., 2007), which at the same time represent the strongest genetic predisposing factor for atopic dermatitis and asthma (Palmer *et al*., 2006; Sandilands *et al*., 2006; Weidinger *et al*., 2006). More than 45 nonsense mutations in the *FLG* gene have been found in IV and/or atopic dermatitis worldwide (Irvine *et al*., 2011). In Europe, R501X and 2282del4 represent the most common mutations accounting for over 80% of the spectrum (Brown *et al*., 2012). Considering the function of FLG in the epidermis its deficiency may critically affect the barrier function (≈ TEWL). Indeed, a lack of NMFs has been shown together with an increase of the skin surface pH, which has been observed in two studies of IV (Ohman *et al*., 1994; Gruber *et al*., 2011), but not in a study from our department in which only a trend towards a more alkaline pH was noted (Perusquia-Ortiz *et* al., 2013).

Tippelt.H reported that the increased pH gradient is present in lesional as well as in nonlesional skin areas in patients with ichthyosis (Tippelt, 1969). Based on the reduced sweating in these patients, the armpit area is characterized by a lower skin surface pH. Recently it was shown that in two out of three studies patients which ichthyosis vulgaris have an increased skin surface pH. The deficiency of filaggrin and filaggrin fragments or amino acids is believed to play an essential role. In the broadest sense, the pH shift leads to the increased protease activity. As most proteases have an alkaline pH optimum. On the other hand activation of the proteolytic cascade by KLK5 requires an acidic pH (Decraison *et* al., 2007). In contrast to IV, X-linked recessive ichthyosis is characterized by a reduced skin surface pH (Ohman H and Vahlquist, 1995).

Histologically, IV is characterized by reduction or absence of stratum granulosum, orthohyperkeratosis and deep follicular hyperkeratosis. Electron microscopy shows a

typical defect of keratohyaline granules, which is characterized by abnormally small or crumbly granules (Figure 9).

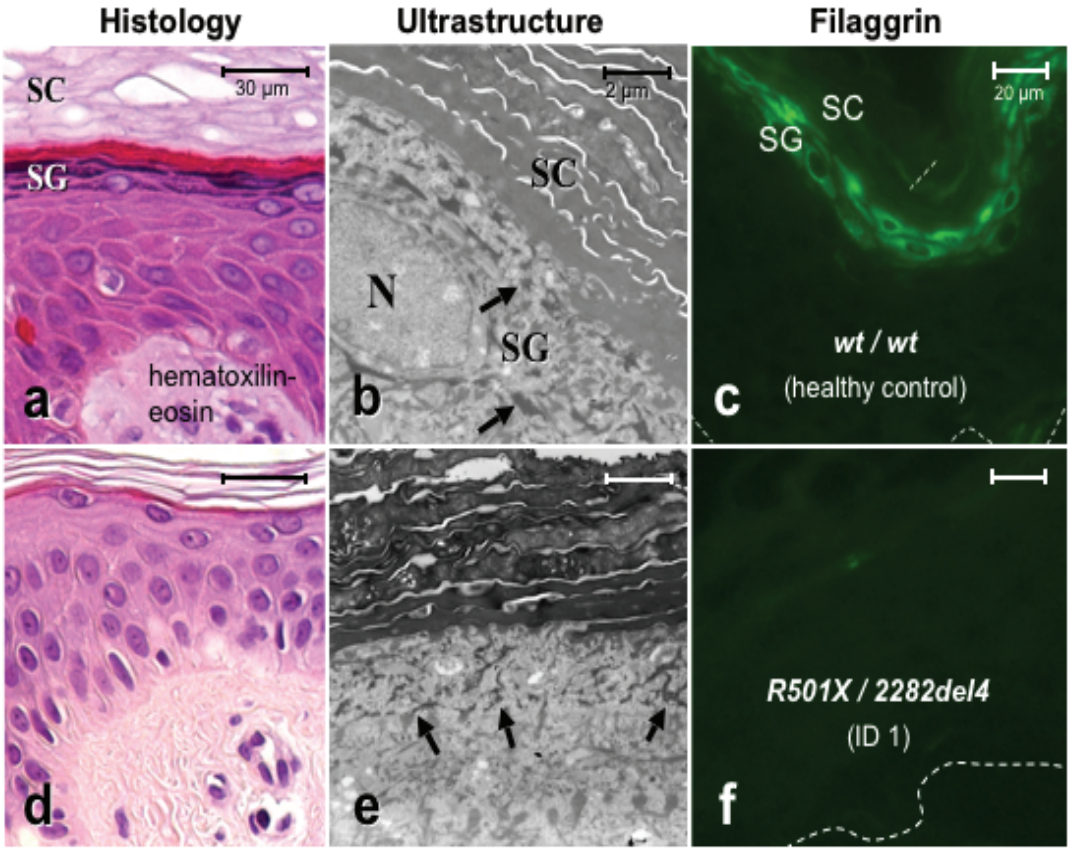

Figure 9. Histology, ultrastructure and filaggrin antigen mapping of healthy skin and ichthyosis vulgaris due to compound heterozygous mutations in *FLG* gene (Oji *et al.,* 2008). Healthy control showed a SG with marked filaggrin staining (a,c). KH can be identified by electron microscopy in a healthy control (b). The IV skin with heterozygous mutation *R501X/2282del4* showed a lack of SG (d, e). Compared to normal skin, filaggrin antigen mapping was completely negative (f).

4.2. Atopic dermatitis

Atopic dermatitis (AD) is a very complex multifactorial chronic inflammatory disease. The prevalence of AD has greatly increased in the last 20 years (Leung *et al*., 2003). This disease is more frequent in infancy and adolescence than in adulthood. In children, about 70% of cases manifest the disease before age 5 years (Fritsch, 2003). About one-third of the patients are free from symptoms in adulthood. Furthermore, about one-third of the patients have clinical features in the adulthood. One third of patients have disease onset in adulthood.

The disease is defined by the criteria of Hanifin and Rajka (1980). Various aspects include microbial superinfection such Staphylococcus aureus infection of the skin. Important dermatological symptoms like frequent palmar hyperlinearity refer to those of ichthyosis vulgaris.

Etiology and pathogenesis

Considering the clinical association of AD and IV it is not surprising that mutations of FLG have been shown to be strongly associated with AD (Palmer *et* al., 2006). Various genetic studies confirm FLG as the most important genetic susceptibility factor for AD (Rodríguez *et* al., 2009). Of course there multiple other genetic loci associated with disease. They mostly refer to the immunological part of the pathogenesis. For instance, it is recognized that there are gene polymorphisms that are especially prevalent in atopic patients. This also concerns a common loci with psoriasis vulgaris on chromosome 1q21 (PSOR 4) and 17q25 (PSOR 2). Furthermore, the association of atopic dermatitis with cytokine- and cytokine receptor gene has been established, especially TGF-beta 1, SPINK-5, the toll-like-receptors TLR2 and TLR4. It is not yet known, how these gene polymorphisms trigger and regulate atopic dermatitis (Folster-Holst R *et* al., 2005; Kuo IH *et* al., 2013).

The initial phase of an atopic immune response is determined by Th2-type that results in acute inflammation. T-lymphocytes secrete a large series of cytokines. IgE-synthesis is induced by IL-4 and IL-13. IL-5 is responsible for the proliferation and maturation of the eosinophilic granulocytes, whereas IL-10 is considered to have a role in the inhibition of Th1-cells.

However, the immunological skin profile is changing during the chronic phase of the disease. Instead of Th2-cytokines, Th1 cytokines dominate like IL-12 and interferon gamma (IFG). The last cytokine inhibits the IgE-synthesis and the Th2-proliferation but supports the growth of Th1 cells (Böhm and Bauer 1997; Leung 2000). The lichenification of atopic lesions is a particular feature of the chronic phase of AD characterized by manifesting tissue remodeling. Thus cytokines like IL-11 and IL-17 appear to be very

important mediators of this event (Howell *et* al., 2009; Cornelissen *et* al., 2012; Deleuran *et* al., 2012; Gutowska-Owsiak *et* al., 2011, 2012; Hvid *et* al., 2011; Kim *et* al., 2011).

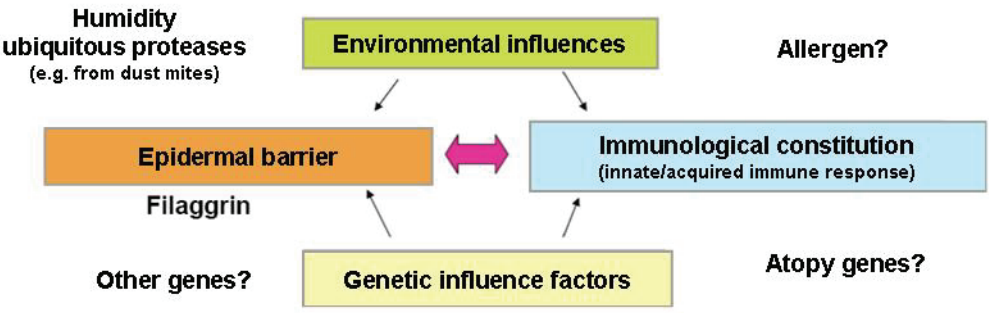

Figure 10. Pathogenetic model of epidermal barrier and atopy. AD is a complex multifactorial chronic inflammatory disease genetically determined and triggered by environmental factors, having alterations in barrier function and in immune system.

A major problem of AD is a disturbed skin barrier function that can be based on the mutations in the coding gene for filaggrin peptides (De Benedetto *et al*., 2012). They are essential for an intact skin barrier that is lost due to mutations in the *FLG*-gene. Moreover, the impaired skin barrier function has been recognized as an important key factor in the pathogenesis of AD (Sugarman *et al*., 2003; Seidenari and Giusti, 1995; Proksch *et al*., 2006; Chamlin *et al*., 2002). The skin barrier of atopic patients is more susceptible to the penetration of environmental allergens into the skin (house dust mites, epithelial tissues of cats, dogs and grass pollen).

Other causative factors for atopic dermatitis are bacteria like *Staphylococcus aureus* that are colonizing over 90% of atopic skin lesions (Rippke *et al*., 2004; Johansson *et al*., 2003). The particular vulnerability of atopic patients for infections with Staphylococcus aureus may be due to the reduced cutaneous production of antimicrobial peptides, especially defensins und cathelicidins (Rippke *et al*., 2004; Ong *et al*., 2002).

The reduced concentration of ceramides is also present in the atopic skin. This results in the dry skin, increased TEWL and alkaline pH-value (Novak, Bieber, 2000).

5. AIM OF STUDY

We wondered why 65% of patients with ichthyosis vulgaris develop atopic dermatitis, whereas approximately 35% patients remain free from atopy (Oji *et al*., 2008). The terminal differentiation of the stratum corneum is a dynamic process regulated by a complex network of serine proteases and serine protease inhibitors. Filaggrin deficiency is associated with NMF reduction (Kezic *et al*., 2011). Slight changes of the skin surface pH in ichthyosis vulgaris have been shown by the group in Innsbruck (Gruber *et al*., 2011) and in a study from Münster at least a trend toward an increased skin surface pH was found (Perusquía-Ortiz *et al*., 2013). Hence, it is tempting to speculate that there may be an imbalance of serine proteases and serine protease inhibitors in the skin of filaggrin deficiency and that such imbalances play a role in determining proneness for atopy.

Specific aims of this study are as follows:

I. Clinical characterization of patients with ichthyosis vulgaris with and without atopic dermatitis

II. Culture of keratinocytes and fibroblasts from skin biopsies of the patients

III. Establishing skin equivalents (three dimensional skin models) of patients with ichthyosis vulgaris

IV. Studying the kallikrein expression and serine protease activity from skin equivalents (*in vitro)* and from tape strips (*in vivo*)

V. Investigating LEKTI as an important regulator of kallikrein activity in three dimensional skin models of filaggrin deficiency

6. METHODS

6.1. Patients

IV was clinically diagnosed by two experienced dermatologists (H.T., V.O.) in our specialized outpatient clinic of the department of dermatology of the University Hospital Münster. The ichthyosis vulgaris severity score provided a valuable clinical tool for evaluation of *FLG* status: Mean values of > 7.8 indicate the presence of two *FLG* mutations (Oji *et al*., 2009). For the 3D model studies, individuals were categorized into healthy controls (n=3), patients with IV without atopic dermatitis ($IV_{non\text{-}AD}$ [n=3]), or IV with additional AD (IV_{AD} [n=3]). Electron microscopy and/or *FLG* restriction enzyme analyses have been performed as published recently (Oji *et al*., 2009). Punch biopsies (4-5 mm) were taken from the upper arms of three healthy volunteers and from six patients with filaggrin deficiency (43.5 ± 14.1 years).

For *in vivo* material, tape strips from forearms of eight healthy volunteers and eleven patients with IV were collected. Three groups studied were: healthy controls (age 33.5 + 7.5 years, n = 3), $IV_{non\text{-}AD}$ (age 28.2 + 7.5 years, n = 3), and IV_{AD} (age 25.5 + 7.2 years, n = 3). Nonlesional skin was used in $IV_{AD.}$ All studies performed were done within the framework of activities of the Network for ichthyoses and keratinization disorders (NIRK) and informed consent of the probands and institutional ethical approval had been obtained in accordance with the Declaration of Helsinki Guidelines.

6.2. Isolation and culture of cells from normal skin and ichthyosis vulgaris skin

Primary keratinocytes and fibroblasts were obtained by enzymatic digestion from 3-5 mm punch biopsies according to a standard protocol described previously (Rheinwald *et al., 1975*; Aufenvenne *et al*., 2012). Briefly, biopsies were incubated over night at 4°C in 0.5 mg/ml protease X (Sigma-Aldrich, Taufkirchen, Germany). Epidermal sheets were peeled off the dermis and incubated in 0.25% trypsin/0.02% EDTA (PAA, Pasching, Austria) for 15 min at 37°C in order to achieve keratinocyte suspension. Trypsin activity

was stopped with FCS containing medium. The suspensions were centrifuged at 1000xg for 5 min, and cells were resuspended in serum-free culture medium (KSFM) supplemented with 10 ng/ml epidermal growth factor (EGF), 50 mg/ml bovine pituitary extract (BPE) (all from Life Technologies, Darmstadt, Germany), 2 mM glutamine and 100 U/ml penicillin, and 100 mg/ml streptomycin (PAA, Pasching, Austria).

To obtain fibroblasts, dermis was incubated for 2-4 h in 0.5 mg/ml collagenase IA (Sigma-Aldrich, Taufkirchen, Germany). After centrifugation cells were resuspended in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all three from PAA, Pasching, Austria). Medium was renewed every 2-3 days and cells were usually passaged at 80% confluence.

6.3. Organotypic (3D) coculture of cells from normal and IV skin

The 3D cocultures were grown in 3 µm filter inserts placed in six well plates (BD Biosciences, NJ, USA) according to published protocols (Stark *et al*., 1999; Mildner *et al*., 2010; Eckl *et al*., 2011) with slight modifications (A.2.1). In brief, per well $0.1x10^6$ fibroblasts, suspended in FCS were added to ice cold bovine collagen I (PureCol, San Diego, CA) solutions and after neutralising (ca. pH 7) incubated for 2 h at 37°C at 5% CO_2. The resulting collagen gels were equilibrated with KSFM containing 10 ng/ml EGF, 0.49 mM calcium chloride, 4 mM glutamine, 1% penicillin/streptomycin, 0.4 µg/ml hydrocortisone and 48 µg/ml ascorbic acid (Sigma-Aldrich, Taufkirchen, Germany). After 1.5 hours two ml of keratinocyte suspensions ($1.5x10^6$ per ml supplemented KGM) were added to each collagen-gel and cocultures were incubated at 37°C at 5% CO_2. After 48 hours, when the culture surface was exposed to the air-liquid interface and medium was replaced by DMEM, supplemented with 21.5% Ham's F-12, 2% FBS (all from Life Technologies, Darmstadt, Germany), 0.4 µg/ml hydrocortisone, 50 µg/ml ascorbic acid, 10^{-10} M cholera toxin, 10 ng/ml EGF, 5 µg/ml insulin, 5 µg/ml transferrin, 5 g/ml sodium selenite, 0,18 mM adenine, 6.1 µg/ml ethanolamine, 14.1 µg/ml phosphorylethanolamine, and 4 mM glutamine (all from Sigma Aldrich, Taufkirchen,

Germany). The feeding medium was changed by 50% daily. Keratinocytes were uniformly used at passage three and for individual cocultures cells of the same donor were used.

6.4. Sample preparation from 3D cocultures

On day 9 of 3D coculture epidermis (diameter of tissue ~ 2.5 x 2.5 cm²) was separated from the dermis, immediately transferred into 200 µl ice-cold lysis buffer (82 mM Tris/HCl [pH 8.0], 0.01% Triton X-100, 1 µM pepstatin, 1 mM 1,10-phenantroline), and incubated on ice for 8 min. Samples were homogenized by repeated sonication (2 min), aliquoted and immediately frozen in liquid nitrogen. The samples have been preserved for further treatment in a freezer at temperatures of ca. -80°C.

6.5. Immunohistochemical analysis

6.5.1. Preparation of frozen sections

Sections of three µm were prepared from cryoconserved tissue samples in the cryostat at a block temperature of -30°C. The sections were cut and placed on the superfrost plus slides (Thermoscientific, US) that were kept in the freezer at -80°C up to the further treatment or were directly used.

The immunohistochemical analyse of FLG, KLK5 and KLK7 expression were carried out on frozen sections to study protein localization in the tissue sections with dye-labeled antibodies according to established protocols. The staining was carried out in a dark moist chamber to avoid the drying of the sections. All primary and secondary antibodies are commercially available. Positive and negative controls were performed to test for antibody specificity and quality. For this, a section of the normal skin in KLK5 and KLK7 reaction was only coated with the antibody dilute buffer instead with the primary antibody. In the FLG reaction, the antibody dilute buffer instead the primary antibody was

dispensed onto the section of the normal skin. As a further negative control, the antibody dilute buffer instead of the polyclonal antibody was replaced in the FLG reaction.

6.5.2. Presence of FLG, KLK5 and KLK7 in the epidermis

The frozen sections of normal and affected skin were air-dried for 10 minutes at room temperature, labeled and marked with DAKO-Pen. A blockage was then followed by a incubation in 10% goat normal serum for 30 minutes at room temperature to unblock unspecific binding sites on the sections (A.2.2, A.2.3). Excessive serum on the sections was aspirated prudently with a water jet pump. After that the incubation with fresh diluted primary antibodies in measured unit quantities (rabbit anti-KLK5 (1:50, H-55), and rabbit anti-KLK7 (1:25, H-50, all from Santa Cruz Biotechnology, USA) followed overnight at room temperature. Filaggrin was detected with mouse anti-filaggrin (1:50, Novocastra Laboratories Ltd., UK) for 45 min at room temperature (Table 7). After the wash steps with PBS three times for 5 minutes the secondary antibody was applied. After the incubation the sections were washed again with PBS three times for 5 minutes.

The sections were covered subsequently in Mowiol and fixed with coverslips. Images were taken with an Axioscope 2, using an Axiocam HR video camera and Axiovision 3.0 software (all Carl Zeiss, Jena, Germany). The preparations were kept at – 20°C.

Table 5. Primary antibodies used in immunohistochemical analysis.

Antigen	Species	Immunogen	Antibody	Company/reference
FLG	Mouse	Synthetic peptide	147204 35806	Novocastra Laboratories Ltd., UK
KLK5 (H-55)	Rabbit	Synthetic peptide	sc: 20623	Santa Cruz Biotechnology, Inc.,USA
KLK7 (H-50)	Rabbit	KLK7	sc: 20625	Santa Cruz Biotechnology, Inc.,USA

6.6. Electrophoresis and Immunoblotting

For immunoblotting of KLK5, KLK7 and LEKTI, equal protein amounts (90 µg) were separated on 15% SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by electroblotting at 80A for 75 min (A.2.4). For LEKTI immunoblotting a 7.5% SDS-PAGE was used. Protein concentrations of each sample were determined by BCA protein assay kit (Pierce, USA). Membranes were blocked with 5% BSA in TBS and incubated with primary antibodies: rabbit anti-KLK5 (1:1,000), rabbit anti-KLK7 (1:200) and mouse anti-LEKTI (1:1,000) (Table 8).

Table 6. Primary antibodies used in immunoblotting.

Antigen	Species	Immunogen	Antibody	Company/reference
KLK5 (H-55)	Rabbit	Synthetic peptide	sc: 20623	Santa Cruz Biotechnology, Inc.,USA
KLK7 (H-50)	Rabbit	KLK7	sc: 20625	Santa Cruz Biotechnology, Inc.,USA

Proteins were visualized on X-ray film using the chemiluminescence detection reagent Rotilumin (Roth, Karlsruhe, Germany). Band densities were captured by the ImageJ Software (http://imagej.en.softonic.com/), normalizing to *β*-actin.

6.7. Enzyme activity in 3D cocultures

The protease activity of patients with ichthyosis vulgaris were assayed using a test for KLK5 and KLK7 function. In the literature, the test for the KLK5 function had been done in similar manner with samples of stratum corneum of Netherton patients (Komatsu *et al*., 2002) and patients with ichthyosis vulgaris (Suzuki *et al*., 1996). The samples derived from 3D skin equivalents were solved in buffer containing 0.005% Triton X-100, 2.3% µl N,N-dimethylformamide, 82 mM Tris-HCl, pH 8.0 (A.2.5, A.2.6). Trypsin-like and chymotrypsin-like activities were assayed using the fluorogenic protease substrates Boc-

Phe-Ser-Arg-AMC (10 µM) (B6388, Sigma, USA) and Suc-Ala-Ala-Pro-Phe-AMC (20 µM) (230914, Calbiochem, Darmstadt, Germany), respectively (Table 9).

Table 7: Using protease substrates and buffers in fluorescence spectrometry.

Protease	Sample buffer	Substrate	Final concentration
KLK5	200 mM Tris/HCl, pH 8,0	Boc-Phe-Ser-Arg-AMC	10 µM
KLK7	N,N-Dimethylformamide	Suc-Ala-Ala-Pro-Phe-AMC	20 µM

Reaction was initiated by adding specific substrates to the samples in a final volume of 500 µl. The fluorescent measurements were performed for 30 min at 37°C on a fluorescence spectrometer (LS 55, Perkin Elmer, USA) with excitation and emission wavelengths of 380 and 460 nm, respectively. Enzyme activity was adjusted to protein concentrations running all experiments in triplicates. An adequate number of samples was measured twice or more on different daytimes to control the consistency and reproducibility of the assay.

6.8. Enzyme activity in scales from normal and diseased skin

Before the tape stripping procedure, the skin was cleaned by distilled water and allowed to dry. D-Squame standard sampling discs with a diameter of 22 mm (CuDerm Corporation, Dallas, USA) were applied on the forearm of patients and human volunteers. The skin sites were marked with a surgical marker to ensure that the tapes were consistently applied to the same area. After removal, the tape strips were sealed into a 1.5 mL Eppendorf tube, immediately frozen in liquid nitrogen, and stored at -80°C until use. Tapes 5-10 were incubated in buffer containing 0,5% Triton X-100, 82 mM Tris/HCl (pH 8.0) and 2.3% N,N-dimethylformamide for 1 hour on ice. Protease assays were performed as described above and activities were normalized to total protein contents as measured by BCA assay.

6.9. Statistical analyses

Statistical analyses were performed using the GEE methods (Generalized Estimating Equations) calculating P-values for differences between samples, which were considered significant at $P<0.05$ (GenMod Procedure in SAS/ver. 9.2 for Windows; SAS Institute Inc., Cary, NC).

The calculation of the different subgroup, i.e. atopic versus nonatopic IV etc, we used the data of three patients per group, two skin models per patient and technical triplicates.

7. RESULTS

7.1. Clinical data and filaggrin status of IV patients

An overview on the clinical characteristics of the patients with IV (n=13) is given in Table 6. Six of those patients were biopsied for the generation of 3D coculture models, five of them showing complete lack and one significant reduction of filaggrin. This was confirmed by electron microscopy as well as sequencing of the *FLG* gene, e.g. patient 3 showed two novel and rare *FLG* mutation showing minimal crumbly KH by electron microscopy. Moreover, mean values > 7.8 of the IV severity score indicated the presence of two *FLG* mutations in this patient (Oji *et al*., 2009). Three patients had a history of atopic dermatitis (IV_{AD}), the other three suffered only from IV without additional atopic dermatitis ($IV_{non\text{-}AD}$).

Table 8. Clinical data and filaggrin status in patients with ichthyosis vulgaris.

Groups	ID	Age (years)/sex	IV score	Electron microscopy	Filaggrin antigen mapping	*FLG* mutations
$IV_{non\text{-}AD}$	1^{3D}	60/f	8	no KHG	strongly reduced	*R501X/2282del4*
	2^{3D}	54/f	7	no KHG	negative	*2282del4/2282del4*
	3^{3D}	21/m	9	minimal crumbly KHG	n.d.	*R3409X/new*
	4	8/f	10	n.d.	n.d.	*R501X/2282del4*
	5*	31/f	9	minimal crumbly KHG	strongly reduced	*2282del4/424del17*
	6	27/f	7	minimal crumbly KHG	n.d.	*R501X/2282del4*
IV_{AD}	7^{3D}	37/m	9	no KHG	strongly reduced	*R501X/2282del4*
	8^{3D}	39/f	8	no KHG	negative	*R501X/G1253X*
	9^{3D}	50/m	5	crumbly KHG	reduced	*2282del4/wt*
	10	31/f	10	n.d.	n.d.	*R501X/2282del4*
	11	4/m	9	minimal crumbly KHG	strongly reduced	n.d.
	12*	18/f	9	n.d.	n.d.	*R501X/2282del4*
	13*	11/f	9	n.d.	n.d.	R2447X/2282del4

^{3D} = skin models; * = patients were published in (Oji *et al*., 2009); KHG = keratohyalin granules; n.d. = not done

7.2. Characterization of 3D skin model

Macroscopically, three-dimensional skin models derived from patients with IV (with and without AD) did not show differences from those of healthy volunteers, but light microscopy revealed main phenotypic characteristics of IV, i.e. compact hyperkeratosis and lack of SG (Figure 12a-c). Antigen mapping of filaggrin confirmed a complete lack of the protein in IV (Figure 12d-f). To further characterize the model, we investigated markers of the dermal-epidermal junction, i.e. collagen IV, CD49f, integrin α6 and β1, and epidermal differentiation markers such as filaggrin and LEKTI, and monitored transglutaminase-1 activity *in situ* (Table 10) (Oji *et al*., 2006). There were no substantial differences between IV and normal cell cocultures, though a slightly increased expression of KLK7 may be noted in the $IV_{non\text{-}AD}$ subgroup.

Table 9. Immunohistochemical analysis of markers of the dermal-epidermal junction, epidermal differentiation markers and proteases in 3D skin models of IV and normal skin.

Marker/ ID	CTRL (n=3)	$IV_{non\text{-}AD}$			IV_{AD}		
		1	2	3	7	8	9
TG1 activity	normal	normal	normal	normal	normal	normal	normal
Collagen IV	+	+	+	+	+	+	+
Integrin α6	+	+	+	+	+	+	+
Integrin β1	+	+	+	+	+	+	+
LEKTI	+	+	+	+	+	+	+
Filaggrin	+	negative	negative	strongly reduced	strongly reduced	negative	reduced
KLK5	++	+++	+++	++	+++	+++	++
KLK7	+/++	+++	+++	+++	++	++	++

CTRL = healthy controls

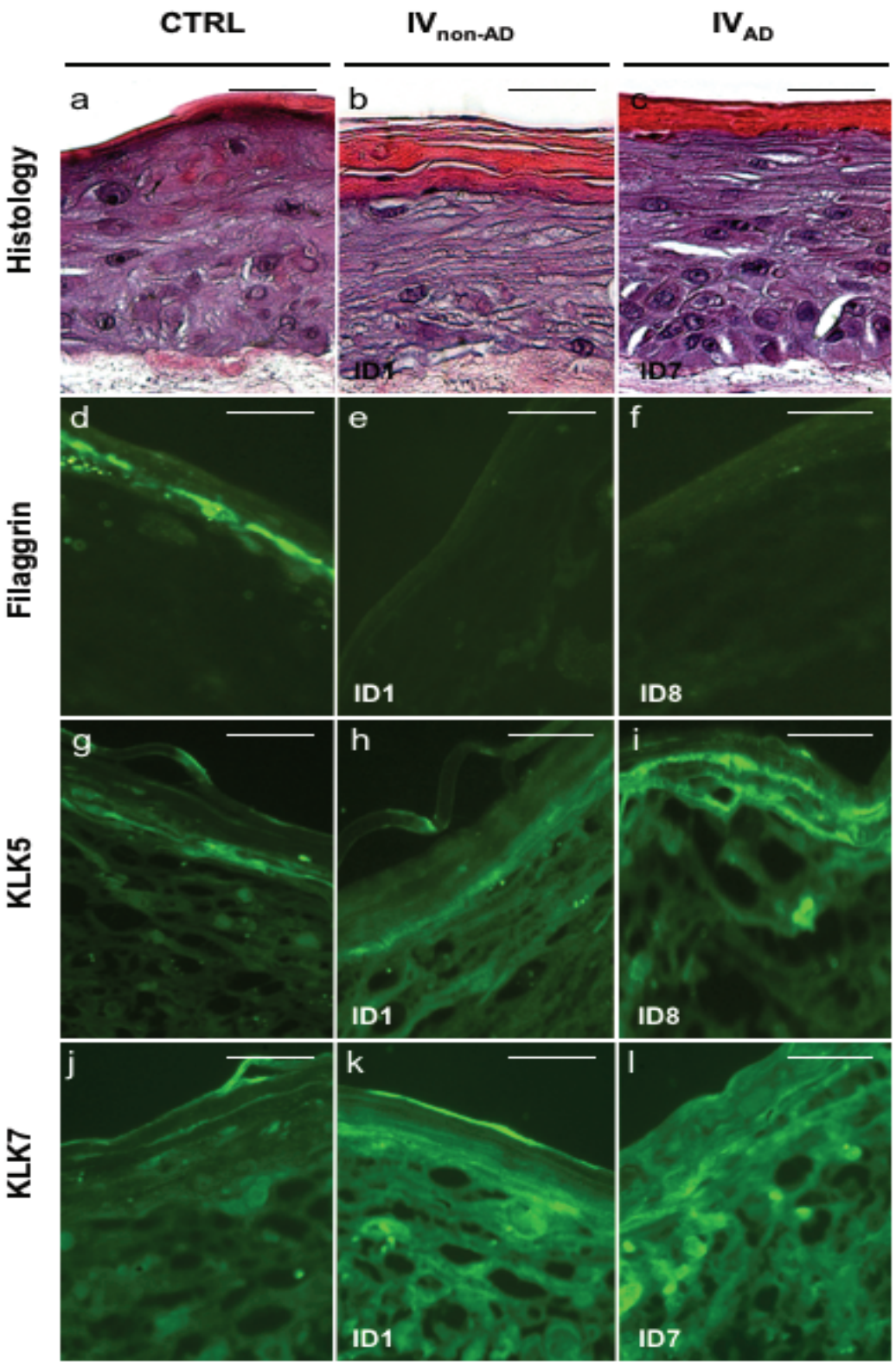

Figure 11. Histological characterization of three-dimensional skin models with antigen mapping of FLG, KLK5 and KLK7. Healthy controls (CTRL) showed a SG with marked filaggrin staining (a, d). Skin models of IV showed a lack of SG (b, c) and filaggrin antigen mapping was negative or strongly reduced (e, f). KLK5 and KLK7 showed a stronger signal in the upper epidermal layers including the SC in IV_{AD} as well as $IV_{non\text{-}AD}$ subgroup (h, i, k, l) when compared to the normal skin model (g, j).

7.3. Disease related protein changes in the 3D model

Regarding proteases and other disease relevant proteins, we localized KLK5, KLK7 as well as the inhibitor LEKTI in the 3D cocultures. According to fluorescence, all these components (except LEKTI) were increased in both types of IV cocultures with filaggrin deficiency. In particular, for KLK5 (Figure 11 g-i), the signal was most pronounced at the SG-SC interface and in the deeper layers of the SC. A more even distribution was seen for KLK7 and LEKTI. Changes in the protein levels were analyzed by western blots, showing for KLK5 and KLK7 single bands at 39 and 48 kDa, respectively (Figure 12a and 13a). A densitometric evaluation of the respective bands was done to obtain values for protein expression that can be compared.

While slight increasing levels of KLK5 were found in the $IV_{non\text{-}AD}$ when compared to controls, this was rather variable in IV_{AD} samples, in comparison to normal controls (Figure 12b). On the other hand, protein expression levels of KLK7 were slightly elevated in both $IV_{non\text{-}AD}$ and IV_{AD} subgroups (Figure 13b). However, these results should not be interpreted as statistically significant. Expression of the serine protease inhibitor LEKTI was also confirmed by western blots, which did not show any significant differences between the three coculture types (Figure 14b).

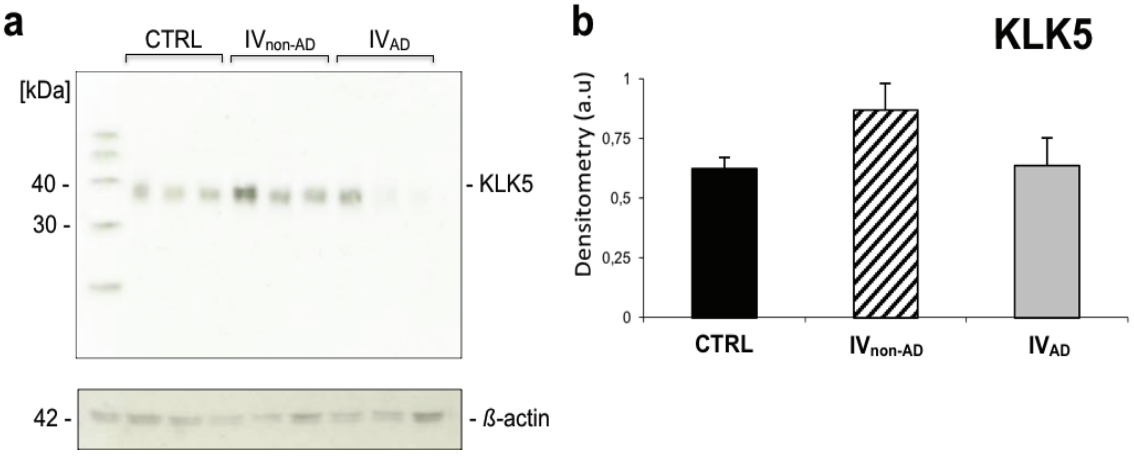

Figure 12. Protein expression of KLK5 in three-dimensional skin models. (a) Western blot analysis of KLK5 protein expression. KLK5 shows a single band at 39 kDa. Protein loading was normalized by *ß*-actin. (b) Densitometric analysis of KLK5 protein expression in skin models of normal and IV skin. Expression has been adjusted for *β*-actin expression in WB. Data presented as mean values ± SEM. CTRL = healthy controls.

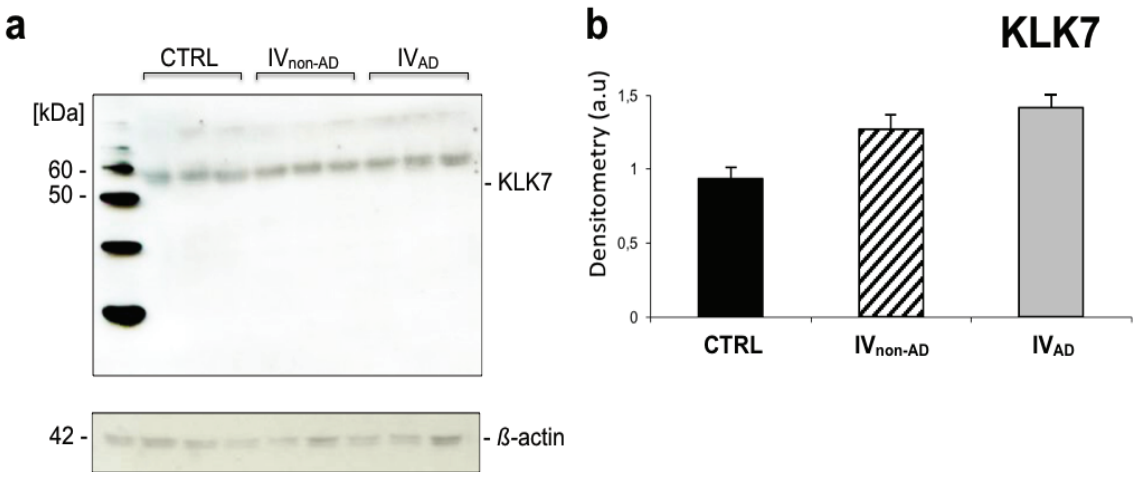

Figure 13. Protein expression of KLK7 in three-dimensional skin models. (a) Western blot analysis of KLK7 protein expression. KLK7 shows a single band at 48 kDa. Protein loading was normalized by *ß*-actin. (b) Densitometric analysis of KLK7 protein expression in skin models of normal and IV skin. Expression has been adjusted for *β*-actin expression in WB. Data presented as mean values ± SEM. CTRL = healthy controls.

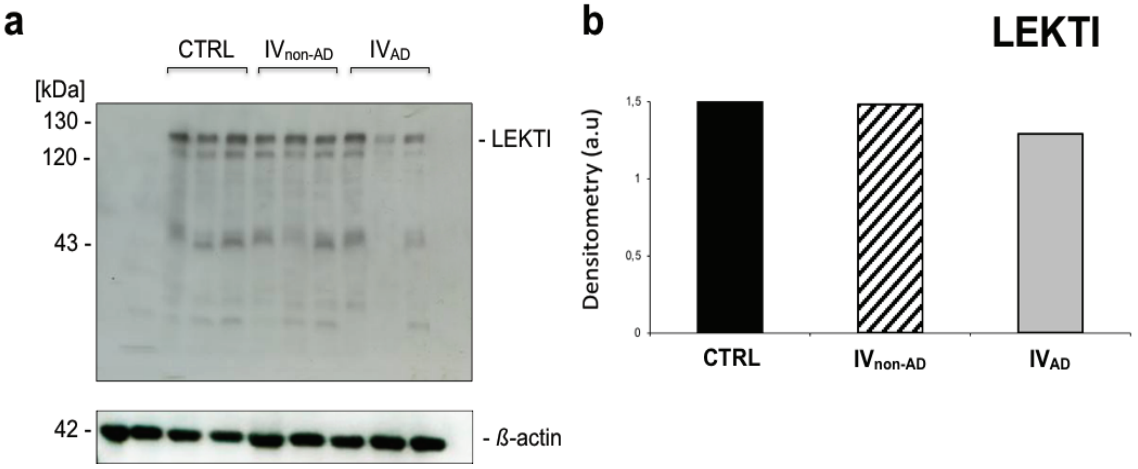

Figure 14. Protein expression of LEKTI in three-dimensional skin models. (a) Western blot analysis of LEKTI protein expression. Protein loading was normalized by *ß*-actin. (b) Densitometric analysis of LEKTI protein expression in skin models of normal and IV skin. There was no difference for LEKTI expression among the groups. Expression has been adjusted for *β*-actin expression in WB. Data presented as mean values ± SEM. CTRL = healthy controls.

7.4. Epidermal serine protease activity in 3D coculture

Protease activities of KLK5 and KLK7 were carried out in cocultures from healthy (n=3), $IV_{non\text{-}AD}$ (n=3), and IV_{AD} skin (n=3) by cleavage of enzyme specific substrates [Boc-Phe-Ser-Arg-AMC and Suc-Ala-Ala-Pro-Phe-AMC]. KLK5 activity of $IV_{non\text{-}AD}$ as well as IV_{AD} did not differ from that of healthy controls (Figure 15a). However, mean value of KLK7 activity shows a slight tendency of increase in $IV_{non\text{-}AD}$ when compared to controls (Figure 15b), which may correlate with its subtle increase of expression as seen by immunostaining (Table 10).

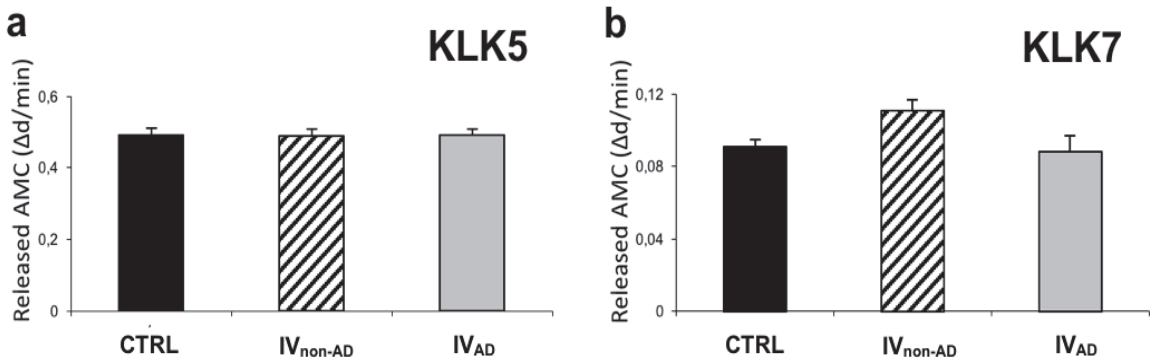

Figure 15. Epidermal protease activities of KLK5 and KLK7 in 3D skin model of IV patients and healthy subjects. There was no significant difference of the mean values of KLK5. KLK7 activity showed a slight increasing tendency in skin models of $IV_{non\text{-}AD}$ patients (a). Activity has been adjusted for total protein contents as measured by BCA assay. Data presented as mean values ± SEM. CTRL = healthy controls.

7.5. Enzymatic activity in scales from healthy and IV skin

As an attempt to corroborate our data from the 3D model and to test the assay reliability on authentic samples, we analyzed the epidermal protease activity in scales of patients with IV (n=11) and healthy subjects (n=8), obtained by standard tape stripping (D Squames). Interestingly, KLK5 activity of scales correlated with the findings in the 3D model showing a slight tendency of increased KLK5 activity (Figure 16a). But KLK7 activity was significantly increased in IV especially in the AD subgroup (p=0.004) (Figure 16b).

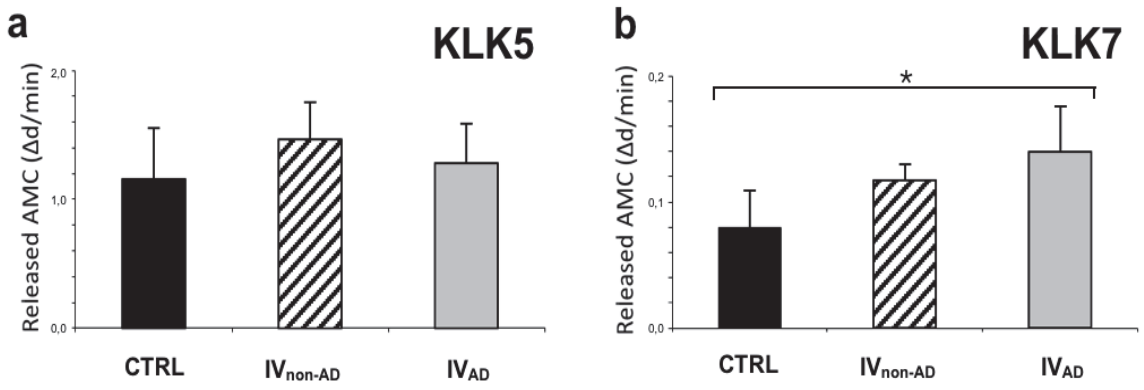

Figure 16. Epidermal protease activities of KLK5 and KLK7 in scales of IV patients and healthy subjects. There was no difference for KLK5 activity in scales (a). KLK7 activity in scales was significantly increased in $IV_{non\text{-}AD}$ patients (n = 5) as well as in IV_{AD} patients (n = 6) compared with healthy controls (n = 8) (p=0,004) (b). Activity has been adjusted for total protein contents as measured by BCA assay. Data presented as mean values ± SEM. CTRL = healthy controls. * P < 0.05.

8. DISCUSSION

Loss-of-function mutations in the *FLG* gene underly ichthyosis vulgaris and are the major risk factor for atopic dermatitis (Smith *et al*., 2006; Sandilands *et al*., 2007; Palmer *et al*., 2006; Sandilands *et al*., 2006; Weidinger *et al*., 2006). Herein we have primarily focused on patients suffering from ichthyosis vulgaris with a confirmed deficiency of filaggrin and investigated whether the balance of proteases and protease inhibitors is impaired in the epidermal compartment of 3D cocultures of skin cells from those individuals. In this context a question of particular interest is, why there are patients with severe IV, who never suffered from any additional atopic manifestations (around 30-50%), despite the strong association with *FLG* loss-of-function mutations (Akiyama *et al*., 2010; Oji *et al*., 2009; Irvine *et al*., 2011).

It has been suggested that a lack of profilaggrin and its acidic breakdown products may lead to a constitutional elevation of the skin pH that results in an increase of the activity of the SC serine proteases (Elias *et al*., 2009).

Data that could support this model are inconsistent (Figure 17). In an early study Suzuki *et al.* 1996 observed decreased levels of serine protease activity in ichthyosis vulgaris (Suzuki *et al*., 1996), whereas other investigators have reported increased levels and activities of serine protease in the SC of atopic dermatitis. For instance, Hansson *et al*. 2002 observed increased expression of KLK7 in chronic lesions (Hansson *et al*. 2002). Komatsu *et al.* 2007 found that elevation of KLK7 was predominant compared with other trypsin-like KLKs (KLK5, KLK8 and KLK11), while according to another study trypsin- and chymotrypsin-like activities did not differ significantly (Komatsu *et al.,* 2007). Except for KLK11, they confirmed elevated protein expression levels of KLKs in the atopic lesions. At the same time, however, the activity of KLKs was not increased. More recently, Voegeli *et al*. 2009 found increased trypsin- as well as chymotrypsin-like expression correlating with the KLK enzyme activities in the lesional skin of AD versus nonlesional skin (Voegeli *et al*., 2009).

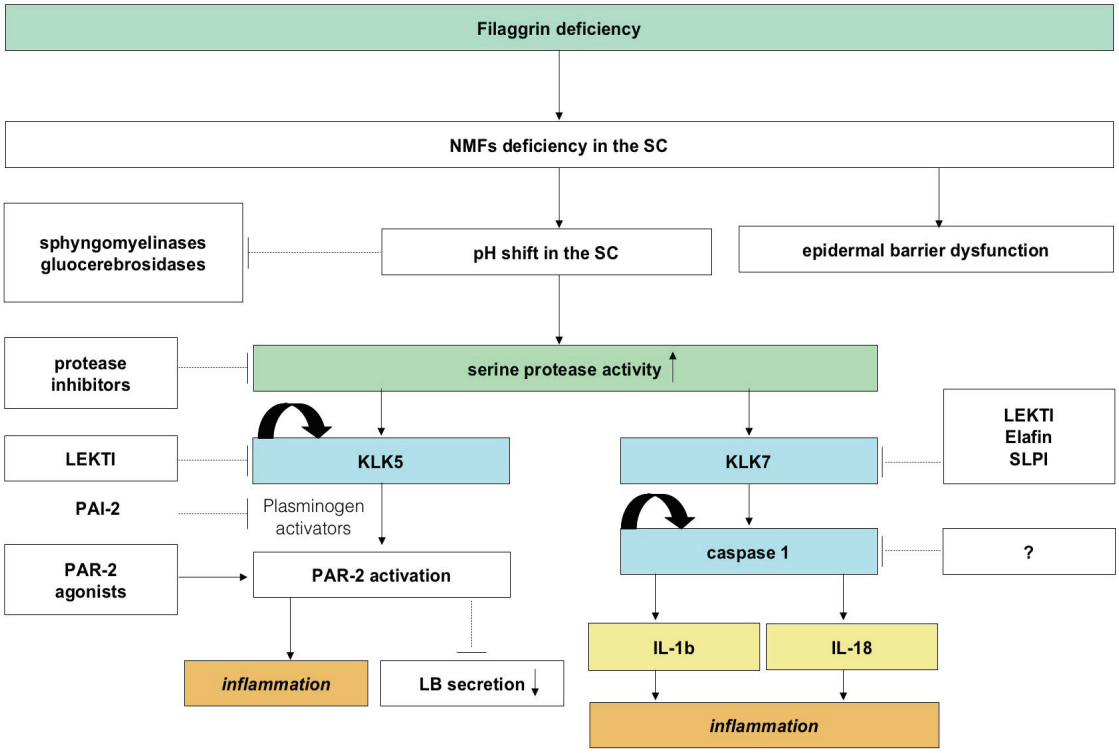

Figure 17. Relationship between increased pH value, protease activity and epidermal differentiation (modified according to Hachem *et al.*, 2006, Pampalakis *et al.*, 2007; Kim *et al.*, 2013; Choi *et al.*, 2014). Filaggrin deficiency results in a lack of NMFs in the stratum corneum consequences being in epidermal pH shift, impaired skin barrier and overall increased serine protease activities. KLK5 activates KLK7 and itself contributing to an inflammation via PAR2 pathway. KLK7 can activate caspase 1 that acts on proinflammatory cytokines with the consequence of elevated IL-1b.

Likewise, increased activity of serine proteases was found in subjects with dry skin (Simon *et al.*, 2008). Considering possible variables due to patient conditions and heterogeneity of *in vivo* generated samples, herein we primarily focused on a 3D model using cells from patients suffering from IV with confirmed filaggrin deficiency to investigate whether the balance of proteases and protease inhibitors is impaired in the epidermal compartment.

The use of patients' epidermal keratinocytes and dermal fibroblasts in this skin-organotypic coculture model certainly rules out confounding environmental effects on epidermal barrier and influences of the individual adaptive immune system. Regular differentiation in 3D cocultures of cells from healthy skin (n=3) was confirmed by histology and immunofluorescence (IF), while those from IV skin (n=6) completely lacked

the SG and more or less detectable filaggrin signals. We have recently demonstrated that the skin ultrastructure strongly correlates with the *FLG* mutation status (Oji *et al*., 2009). Electron microscopy and IF of IV patients with two loss-of-function mutations show at best minimal and crumbly keratohyalin and no detectable filaggrin staining, respectively. Biopsies of the patients with IV showed complete filaggrin deficiency in five cases and partial filaggrin deficiency in one. Patient 11 without any classical *FLG* mutation showed minimal crumbly KH by electron microscopy. Based on the clear-cut correlation between *FLG* genotype and KH morphology, we assume that this patient most likely has rare mutations in *FLG* gene that we could not detect by our allele-specific DNA analysis. Considering this aspect, we have assembled organotypic cocultures with keratinocytes and fibroblasts from (i) IV patients without AD ($IV_{non\text{-}AD}$ [n=3]) and (ii) IV patients suffering from additional AD (IV_{AD} [n=3]). Using the criteria of Hanifin and Rajka (Hanifin and Rajka, 1980) history of atopic dermatitis was positive in three patients with IV. SCORAD or EASI have not been assessed, as patients did not suffer from acute eczema when skin biopsies were taken.

First, we examined the protein expression of serine proteases and found a slight tendency of increased levels of KLK5 in $IV_{non\text{-}AD}$. However, KLK7 protein expression was slightly elevated in both subgroups when compared with normal skin (Figure 13). Interestingly, slight increased protein levels of KLK5 did not result in a higher activity of this enzyme (Figure 15). These results are in line with those of Suzuki *et al.* 1996, who used the same fluorescent substrate for KLK5 (Boc-Phe-Ser-Arg-AMC) and actually observed decreased levels of KLK5 activity of the SC of IV patients (Suzuki *et al*., 1996). In the current study, using a different specific substrate for KLK7 (Suc-Ala-Ala-Pro-Phe-AMC), we found a slight increasing level of KLK7 activity in the $IV_{non\text{-}AD}$ subgroup compared with normal skin.

Given these observations, we investigated the expression of the multidomain serine protease inhibitor LEKTI, which selectively inhibits KLK5, KLK7, and KLK14 and has been implicated in the regulation of epidermal desquamation (Figure 17) (Deraison *et al*., 2007). Recently, it has been reported that the expression of LEKTI was significantly

decreased in AD (Roedl *et al*., 2009). *SPINK5*, the gene encoding LEKTI and mutations of which cause Netherton syndrome (NTS) (Chavanas *et al*., 2000a; Chavanas *et al*., 2000b), was considered a candidate gene for AD by some studies (Walley *et al*., 2001; Kato *et al*., 2003; Nishio *et al*., 2003). It has been reported that patients with different loss-of-function in *SPINK5* gene may show different levels of KLK7 (Komatsu *et al*., 2008). In this study, we did not observe any significant difference of LEKTI expression; and consequently no correlation between the levels of serine proteases activity and LEKTI expression in our IV model. These observations in $IV_{non\text{-}AD}$ and IV_{AD} do not suggest a primary role of LEKTI for the development of the atopic phenotype in IV. However, our results do not rule out the involvement of other epidermal serine protease inhibitors.

Finally, we examined the epidermal protease activity *in vivo* to correlate the results obtained from the skin models. Therefore, we measured the SC activity of KLK5 and KLK7 in patients with filaggrin deficiency (n=11) and healthy subjects (n=8), using D-Squames (Voegeli *et al*., 2009). KLK5 activity correlated with that from the skin models, whereas KLK7 activity was significantly increased in the IV_{AD} (p=0,004). Hence, in contrast to the cell culture data there was a higher KLK7 activity in atopic filaggrin deficiency (IV_{AD}).

In summary, statistical results of our investigations *in vitro* are limited by the small number of samples (n = 9) and did not show significant differences. However, the *in vitro* studies on 3D epidermal models of filaggrin deficiency propose that alterations of activity of epidermal proteases are present and may reflect an early event, which may increase the risk for the development of atopic dermatitis. This susceptibility may be independent from the immunology of eczema. Imbalances of expression and activity of epidermal proteases in the context of normal and stable expression of LEKTI suggests that there may be other compensatory mechanisms and/or inhibitors.

Finally, our study opens the question why patients with severe forms of IV do not suffer from AD. Further investigations are necessary to provide an answer to this enigma. Possibly the enhanced KLK7 activity found in scales from IV_{AD} patients may act as a risk

factor by influence on the caspase 1 activity in filaggrin deficiency and may result in inflammation mediated for example by IL-1b.

Vasilopoulos *et* al. 2004 suggested that an AACC insertion in the KLK7 gene was significantly associated with atopic dermatitis. One could speculate that this mutation or other activating mutations could act as a modifier in FLG deficiency being a KLK7 sensitive disorder.

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10. LIST OF FIGURES

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A. APPENDUM

A.1. Table 10: Clinicogenetic classification of inherited ichthyosis: nonsyndromic forms (Oji *et al.,* 2010).

Disease	Inheritance	Gene(s)
Common ichthyosis		
Ichthyosis vulgaris (IV)	autosomal-semidominant	*FLG*
Recessive X-linked ichthyosis (XRI) nonsyndromic presentation	XR	*STS*
Autosomal recessive congenital ichthyosis (ARCI), major types		
Harlequin ichthyosis (HI)	AR	*ABCA12*
Lamellar ichthyosis (LI)	AR	*TGM1/NIPAL4/ALOX 12B/ABCA12/locus on 12p11-q13*
Congenital ichthyosiform erythroderma (CIE)	AR	*ALOXE3/ALOX12B/A BCA12/CYP4F22/ NIPAL4/TGM1/locus on 12p11-q13*
ARCI, minor variants		
Self-improving congenital ichthyosis (SICI)	AR	*TGM1/ALOX12B/ALO XE3*
Acral LI	AR	*TGM1*
Bathing-suit-ichthyosis (BSI)	AR	*TGM1*
Keratinopathic ichthyosis (KPI), major types		
Epidermolytic ichthyosis (EI)	AD	*KRT1/KRT10*
Superficial epidermolytic ichthyosis (SEI)	AD	*KRT2*
KPI, minor variants		
Annular epidermolytic ichthyosis	AD	*KRT1/KRT10*
Ichthyosis hystrix of Curth-Macklin	AD	*KRT1*
Autosomal recessive epidermolytic ichthyosis (AREI)	AR	*KRT10*

APPENDUM

Disease	Inheritance	Gene(s)
Epidermolytic nevi	somatic mutations	*KRT1/KRT10*
Congenital reticular ichthyosiform erythroderma (CRIE)	AD	*KRT1/KRT10*
Other nonsyndromic ichthyoses		
Loricrin keratoderma	AD	*LOR*
Erythrokeratodermia variabilis	AD	*GJB3/GJB4*
Peeling-skin-disease	AR	*CDSN*
Keratosis-linearis-ichthyosis-congenital-keratoderma (KLICK)	AR	*POMP*

XR, X-linked recessive; AD, autosomal dominant; AR, autosomal recessive.

A.2. EXPERIMENTAL PROTOCOL

A.2.1. Cell culture protocol

Prepare cell growth mediums

KSFM 1

KSFM 100%

Epidermal Growth Factor Kit

Bovine Pituitary Extract Kit

Penicillin/Streptomycin 1 X

KSFM 2

KSFM 100%

Epidermal Growth Factor Kit

CaCl2 0,49 mM

Glutamine 4 mM

Hydrocortisone 0,4 µg/ml

Ascorbic acid 48 µg/ml

Penicillin/Streptomycin 1 X

DMEM

DMEM 100%

Fetal bovine serum 10%

L-Glutamine 4 mM

Na-Pyruvate 4 mM

Penicillin/Streptomycin 1 X

SuperMedium 1

DMEM 64,5%

Ham’s F-12 21,5%

Fetal bovine serum 2%

Glutamine 4 mM

Hydrocortisone 0,4 µg/ml

Ascorbic acid 50 µg/ml

Epidermal Growth Factor 10 ng/ml

Cholera toxin 10−10M

Insulin 5 µg/ml

Transferrin 5 µg/ml

Sodium selenite 5 ng/ml

Adenine 0,00018 M

Ethanolamine 6,1 µg/ml

Phosphorus ethanolamine 14,1 µg/ml

SuperMedium 2

DMEM 64,5%

Ham’s F-12 21,5%

Fetal bovine serum 4%

Glutamine 4 mM

Hydrocortisone 0,4 µg/ml

Epidermal Growth Factor 10 ng/ml

Cholera toxin 10−10M

Insulin 5 µg/ml

Transferrin 5 µg/ml

Sodium selenite 5 ng/ml

Adenine 0,00018 M

Ethanolamine 6,1 µg/ml

Phosphorus ethanolamine 14,1 µg/ml

On day 1:

- Prepare and inscribe the plates (patient's names and inserts from a-f)
- ➢ Provide the plate with cell culture inserts
- Prepare human fibroblasts for collagen gel (for 3 wells = 1/2 plate)
- Trypsinate human fibroblasts in 75cm² bottle:
- ➢ Incubate 3,5 ml 1xPBS for 5 min at 37°C; aspirate
- ➢ Incubate 2,5 ml Trypsin for 5 min at 37°C
- ➢ Stop trypsin reaction mit 5 ml DMEM (resuspend well cell structures) and transfer all into a falcon
- ➢ Wash well the bottle mit 5 ml 1 x PBS and transfer into a falcon
- ➢ Centrifuge for 10 min at 7°C with 1.000 rpm; aspirate supernatant
- ➢ Resuspend cells in 1 ml DMEM + 2 ml 1xPBS and count

16µl cell suspension +16µl Trypan blue = mix well and give ca. 20µl into a counting chamber

Count cells in two square fields [e.g. (30+30)* 3ml cell suspension*10000 = 1.800.000 total number]

The glass must be in correct position (violet stripes)

- ➢ Resuspend 900.000 Fibroblasten in 0,9 ml FCS and keep on the ice

(100.000 cells pro 1 ml gel / with something surplus = 1.000.000 human fibroblasts in 1 ml FCS)

- Prepare collagen gel for 3 wells
- ➢ 7,2 ml PureCol (Collagen) = 8 parts (incubate ca. 1 h at RT)
- ➢ 0,9 ml 10x HBSS = 1 part

▪ Titrate with 2 M NaOH to weak pink color

- ➢ 0,9 ml human fibroblasts suspension in FCS = 1 part

▪ Mix/pan very well and pipette free of air bubbles 2,5 ml pro insert

- ➢ Incubate in a humid environment for 2 h at 37°C / 5% CO2/
- Equilibrate collagen gel with KSFM2

- Incubate 2 ml (insert) + 12 ml (well) in a humid environment for 2 h at 37°C / 5% CO2/

- Prepare human keratinocytes

- Incubate 7 ml 1 x PBS for 5 min at 37,2°C; aspirate
- Incubate 5 ml 1 x PBS for 5 min at 37,2°C; aspirate
- Incubate 2,5 ml Trypsin for 5-6 min at 37,2°C
- Stop the trypsin reaction with 2,5 ml trypsin inhibitor (resuspend well cell structures) and transfer all into a falcon
- Wash well the bottle mit 5 ml KSFM1 and transfer into a falcon
- Centrifuge for 10 min at 7°C with 1.000 rpm; aspirate supernatant
- Resuspend cells in 4-6 ml KSFM2 and count

16µl cell suspension +16µl Trypan blue = mix well and give ca. 20µl into a counting chamber

- 1.500.000 keratinocytes/ml
- evacuate medium and give 2 ml cell suspension pro well in the middle free of air bubbles

On day 2:

- Leave to rest

On day 3-6: at the air-liquid interface

- Treatment with fresh SuperMedium1
- total volume 10 ml
  - 5,2 ml old medium (transfer into a falcon)
  - 5,2 ml SuperMedium1 (transfer into a falcon and mix well)

On day 7-10:

- Treatment with fresh SuperMedium2
- total volume 10 ml

- 5,2 ml old medium (transfer into a falcon)
- 5,2 ml SuperMedium2 (transfer into a falcon and mix well)

On day 11:

- Harvest

A.2.2. Evidence of KLK5 and KLK7 in the epidermis

Specific antibodies: **KLK5 – AB**

KLK7 – AB

Skin type: 3D skin equivalent

Preparation: Cryo (Cryo- eibedding compound/ Mikrom Laborgeräte GmbH/ 350 100)

Cutting thickness: ca. 3µm

1. Frozen sections allow to dry for 10 minutes

2. Fixation: none

3. Marked the sections with DAKO-Pen / allow to dry

4. Blocking

AB/ Serum: buffer:	Firma:	Lot.: Code.:	Protein concentration	*Dilution:*	*Volume:*	*Intubation:*
Goat normal serum	Jackson Immuno Research	56599 005-000-121		*10%*	*100µl/ section*	*30 min.at RT*

5. Serum was aspirated with a wash jet pump

6. Primary antibodies

KLK5 Rabbit AK KLK7 Rabbit AK	Santa Cruz Santa Cruz	922- 420 306 14243- V		***1:50*** *in antibody dilute buffer* ***1:25*** *in antibody dilute buffer*	*50µl/ section*	*overnight at RT*

7. Washing procedure:

PBS	PAA	H00210-0198 H-31-002		*1x*	*ca. 100ml (cuvette)*	*3x5 min at RT*

8. Marking with secondary anybody

Goat Anti-rabbit (FITC)	Jackson ImmunoResearch Inc	58627 111-096-006	1.5 mg/ml	***1:100*** *in antibody dilute buffer*	*50µl/section*	*30 min. at RT*

9. Washing procedure:

PBS	PAA	H00210-0198 H-31-002		*1x*	*ca. 100ml (cuvette)*	*3x5 min at RT*

10. Embedding: coverslips + Mowiol

11. Storage: dark at -20°C

A.2.3. Evidence of FLG in the epidermis

Specific antibody: **Filaggrin - AB**

Skin type: 3D skin equivalent

Preparation: Cryo (Cryo- embedding compound/ Mikrotom Laborgeräte GmbH/ 350 100)

Cutting thickness: ca. 3µm

1. Frozen sections allow to dry for 10 minutes

2. Fixation: none

3. Marked the sections with DAKO-Pen / allow to dry

4. Blocking

AK/ Serum: Puffer:	Firma:	Lot.: Code.:	Protein concentration	*Dilution:*	*Volume:*	*Intubation:*
Goat normal serum	Jackson ImmunoResearch	56599 005- 000- 121		*10%*	*100µl/ section*	*30 min at RT*

5. Serum was aspirated with a wash jet pump

6. Primary antibody

Filaggrin Mouse AB	Novocastra	147204 35806		***1:50** in antibody dilute buffer*	*50µl/ section*	*45 min at RT*

7. Washing procedure:

PBS	PAA	H00210-0198 H-31-002		*1x*	*ca. 100ml (cuvette)*	*1 x short 1 x 3 min.*

8. Marking with polyclonal anybody

Polyclonal Goat Anti-Maus Immunoglobulins/Biotinylated	DAKOCytomation	E 0433		***1:100** in antibody dilute buffer*	*50µl/ section*	*15 min. at RT*

9. Washing procedure:

PBS	PAA	H00210-0198 H-31-002		*1x*	*ca. 100ml (cuvette)*	*1 x short* *1 x 3 min.*

10. Marking with secondary anybody

Fluorescein (DTAF)-conjugated Streptavidin	Jackson ImmunoResearch Inc	71126 116-010-0842	1.5 mg/ml	***1:100*** *antibody dilute buffer*	*50µl/section*	*30 min. RT*

11. Washing procedure:

PBS	PAA	H00210-0198 H-31-002		*1x*	*ca. 100ml (cuvette)*	*1 x short* *1 x 3 min.*

12. Embedding: coverslips + Mowiol

11. Storage: dark at -20°C

A.2.4. Immunoblotting

Protein separation by gel electrophoresis

- Protein concentrations of each sample were determined by BCA protein assay kit (Pierce, USA).
- Load equal amounts of protein (90 µg) into the wells of a SDS- PAGE gel, along with molecular weight markers.
  - For KLK5 and KLK7 was used a 15% SDS-PAGE gel
  - For LEKTI was used a 7,5% SDS-PAGE gel
- Run the gel for 5 min at 50 V and increase the voltage to 100–150 V to finish the run in about 1 hour.

Transferring the protein from the gel to the nitrocellulose membrane

- Transfer the proteins onto the nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) for 75 min at a constant current of 80 mA.
- Blocking the membrane overnight in 5% BSA/TBS solution at + 8°C with shaking.

Antibody incubation

- Incubate the membrane with primary antibody for 1 hour.
  - The primary antibodies were diluted in the blocking buffer
  - rabbit anti-KLK5 (1:1,000), rabbit anti-KLK7 (1:200) and mouse anti-LEKTI (1:1,000)
- Wash the membrane three times for 5 min in TBS buffer.
- Incubate the membrane with anti-rabbit antibody coupled to horseradish-peroxidase for 45 min at room temperature.
  - the antibody was diluted using 10% skim milk in TBS (1:10,000)
- Rinse the membrane three times for 5 min in TBS buffer.

Imaging and data analysis

- Apply the chemiluminescent detection reagent Rotilumin (Roth, Karlsruhe, Germany) to the blot for 3 min according to the manufacturer's recommendation.
- Acquire image using darkroom development techniques on X-ray film.
- Capture the band densities by the ImageJ Software (http://ImageJ.en.softonic.com/(, normalizing to β-actin.

A.2.5. Measurement of KLK5 activity

Aim: Measurement of KLK5 activity by fitting of Boc-Phe-Ser-Arg-AMC.

Lysis buffer

Tris/HCl, pH 8.0	1528 µl
1% Triton X-100	16 µl
Pepstatin A	16 µl (dilution 1:100 in Tris/HCl)
1,10-Phenantroline	40 µl

Sample preparation

Cell lysates (after 9. day of cell differentiation)

- BCA-test: concentration of cell lysates ca. 400-600 µg/ml (1:10), i.m. the samples have overall 4-6 mg/ml.
- Final concentration of total protein into the solution is 40-60 µg/ml (dilution factor 1:100), aliquots evenly 10 µl

All protein sample were aliquots (cell lysates ca.10 µl), immediately frozen in liquid nitrogen and kept at -80°C.

Trypsin buffer:

N,N-Dimethylformamide	20 µl
0,01% Triton X-100 (wt/wt)	480 µl
200 mM Tris–HCl, pH 8.0	350 µl

Substrate preparation

Boc-Phe-Ser-Arg-AMC	5 mg
DMSO	250 µl

- stock solution 20 mg/ml (30 mM)
- aliquot evenly 5 µl

- Proteins were added fresh on measurement day.
- We need for assay 5 µl 1 mM (final concentration 10 µM) Boc-Phe-Ser-Arg-AMC.

➢ 20 mg/ml Boc-Phe-Ser-Arg-AMC equates to 30 mmol/l

Boc-Phe-Ser-Arg-AMC	1 µl (20 mg/ml)
DMSO	29 µl
	30 µl (1 mmol/l)

➢ final concentration of substrate 10 µM

Important: protect the assay buffer from light, i.m. pack into an aluminum foil

Provision

Sample buffer	490 µl
Boc-Phe-Ser-Arg-AMC 10 µM	5 µl
Sample	5 µl
	500 µl

Preparation of serine protease inhibitors

AEBSF

➢ final concentration 2 mM

➢ volume 10 µl

Provision

Sample buffer	480 µl
Boc-Phe-Ser-Arg-AMC 10 µM(f.c.)	5 µl
AEBSF	10 µl
Sample	5 µl
	500µl

- Prepare the corresponding volume of sample buffer in a glass bottle (100-200 ml), solve carefully protein using a magnetic stirrer
- Temper the sample buffer into the hot block for ca. 5-10 min at 37°C
- Check the microcuvette for potential contaminations
  - water (aqua dest) → ethanol → water (aqua dest)
- Cuvette into the sample compartment shall be heated for ca. 5-10 min
- Thaw samples on ice and leave during the whole measuring time on ice
- Pipette the substrate and then the sample into the sample buffer, pipette then without bubbles into the cuvette and put it into the sample compartment
- Start the measurement immediately for 30 min

After the measurement

- Rinse the cuvette properly: water → ethanol → water → 10 mM HCl overnight

Controls

- Positive control: sample buffer + AMC + sample
- Negative control: (sample buffer + AEBSF + sample, incubate for 10 min) + AMC
- Blank sample: sample buffer + AMC

Provision of activity measurement

- The fluorescent spectrometer should be set with following parameters:
  - Excitation wavelength 380 nm
  - Emission wavelength 460 nm
  - Band filter 5 nm
  - Program FL WinLab, TimeDrive Modul
- Set water bath and hot block to the desired temperature
- Register computer

- Start fluorimeter
- Start program FL WinLab
- Application – Time Drive – Setup parameters: set measuring parameters
  - Wavelength:
    - Excitation: 380 nm
    - Emission: 460 nm
  - Band filter
    - Excitation: 5.0 nm
    - Emission: 5.0 nm
  - Time of measurement: 30 min

After the measurements, all cuvettes and used glass equipment were washed with distillate water and then stored.

A.2.6. Measurement of KLK7 activity

Aim: measurement of KLK7 activity by fitting of Suc-Ala-Ala-Pro-Phe-AMC (Suc-AAPP-AMC).

Lysis buffer

Tris/HCl, pH 8.0	1528 µl
1% Triton X-100	16 µl
Pepstatin A	16 µl (dilution 1:100 in Tris/HCl)
1,10-Phenantroline	40 µl

Sample preparation

Cell lysates (after 9. day of cell differentiation)

- BCA-test: concentration of cell lysates ca. 400-600 µg/ml (1:10), i.m. The samples have overall 4-6 mg/ml.
- Final concentration of total protein into the solution is 40-60 µg/ml (dilution factor 1:100), aliquots evenly 10 µl

All protein sample were aliquots (cell lysates ca.10 µl), immediately frozen in liquid nitrogen and kept at -80°C.

Chymotrypsin buffer:

N,N-Dimethylformamide	20 µl
0,01% Triton X-100 (wt/wt)	480 µl
200 mM Tris–HCl, pH 8.0	350 µl

Substrate preparation

Suc-Ala-Ala-Pro-Phe-AMC	25 mg
DMSO	1895 µl

- stock solution 13,2 mg/ml
- aliquot evenly 5 µl

- Proteins were added fresh on measurement day.
- We need for assay 5 µl 1 mM (final concentration 20 µM) Suc-Ala-Ala-Pro-Phe-AMC.

Suc-Ala-Ala-Pro-Phe-AMC	5 µl (13,2 mg/ml)
DMSO	25 µl
	30 µl (2 mmol/l)

Important: protect the assay buffer from light, i.m. pack into an aluminum foil

Provision

Sample buffer		490 µl
Suc-AAPP-AMC	(f.c. 20 µM)	5 µl
Sample		5 µl
		500 µl

Preparation of serine protease inhibitors

AEBSF

- final concentration 2 mM
- volume 10 µl

Provision

Sample buffer		480 µl
Suc-AAPP-AMC	(f.c. 20 µM)	5 µl
AEBSF		10 µl
Sample		5 µl
		500 µl

- Prepare the corresponding volume of sample buffer in a glass bottle (100-200 ml), solve carefully protein using a magnetic stirrer
- Temper the sample buffer into the hot block for ca. 5-10 min at 37°C

- Check the microcuvette for potential contaminations
  - water (aqua dest) → ethanol → water (aqua dest)
- Cuvette into the sample compartment shall be heated for ca. 5-10 min
- Thaw samples on ice and leave during the whole measuring time on ice
- Pipette the substrate and then the sample into the sample buffer, pipette then without bubbles into the cuvette and put it into the sample compartment
- Start the measurement immediately for 30 min

After the measurement

- Rinse the cuvette properly: water → ethanol → water → 10 mM HCl overnight

Controls

- Positive control: sample buffer + AMC + sample
- Negative control: (sample buffer + AEBSF + sample, incubate for 10 min) + AMC
- Blank sample: sample buffer + AMC

Provision of activity measurement

- The fluorescent spectrometer should be set with following parameters:
  - Excitation wavelength 380 nm
  - Emission wavelength 460 nm
  - Band filter 5 nm
  - Program FL WinLab, TimeDrive Modul
- Set water bath and hot block to the desired temperature
- Register computer
- Start fluorimeter
- Start program FL WinLab
- Application – Time Drive – Setup parameters: set measuring parameters
  - Wavelength:

- Excitation: 380 nm
- Emission: 460 nm

▪ Band filter

- Excitation: 5.0 nm
- Emission: 5.0 nm

▪ Time of measurement: 30 min

After the measurements, all cuvettes and used glass equipment were washed with distillate water and then stored.

A.3. MATERIALS

A.3.1. Enzymes, antibodies and other proteins

1, 10-Phenantroline monohydrate	Sigma-Aldrich, Darmstadt, Germany
AEBSF- Hydrochlorid	AppliChem, Darmstadt, Germany
Antibody dilution buffer	DCS Innovative Diagnostik-Systeme, Hamburg, Germany
Boc-Phe-Ser-Arg-MCA	Sigma, USA
DAKO Pen	Firma DAKO GmbH, Denmark
E64	Sigma-Aldrich, Darmstadt, Germany
Fluorescein (FITC)-conjugated AffiniPure F (ab')2 fragment goat anti-rabbit IgG (H+L)	Firma ImmunoReseach, USA
Fluorescein (DTAF)-conjugated Streptevidin	Firma ImmunoReseach, USA
Goat normal serum	Firma Jackson Immunoresearch, USA
Insulin (bovine pancreas)	Sigma Aldrich, Darmstadt, Germany
KLK5 (H-55)	Santa Cruz Biotechnology, Inc., USA
KLK7 (H-50)	Santa Cruz Biotechnology, Inc., USA
Mowiol 4-88, Polyvinylalkohol	Firms Hoechst, Frankfurt, Germany
NCL-filaggrin mouse monoclonal	Firma Novocastra, UK
Pepstatin A	Sigma-Aldrich, Darmstadt, Germany
Phosphate buffered saline (PBS)	Firma PAA, Austria
Polyclonal goat anti-mouse immunoglobulins/Biotinylated	Firma DakoCytomation, Denmark
Rabbit polyclonal antibody (WB)	
Suc-Ala-Ala-Pro-Phe-MCA	Calbiochem, Darmstadt, Germany
Trypsin-EDTA-Lösung	PAA, Austria
Trypsin Inhibitor, Soybean	Invitrogen, USA

A.3.2. Chemicals and „Kits“

Acrylamide 30%,	CarlRothGmbh, Karlsruhe, Germany
0.8% Bisacrylamide Rotiphorese Gel 30	
Adenine (free bases)	Sigma Aldrich, Darmstadt, Germany
Albumin, from bovine serum ich	Sigma, USA
Ammonium persulfate (APS)	Sigma, USA
Ammonium sulfate	Fluka AG, Buchs, Switzerland
BCA Protein Assay Kit	Pierce, USA
BCA Protein Assay Reagent A, 1x500ml	
BCA Protein Assay Reagebt B, 1x25 ml	
Albumin Standard, 10x1 ampules	
Bromphenol blue	Fluka AG, Buchs, Switzerland
Blotte, Non-Fat Dry Milk	Santa Cruz Biotechnology, Inc., USA
Calcium chloride dihydrate	Merck, Darmstadt, Germany
Cholera Toxin	Sigma Aldrich, Darmstadt, Germany
Dimethylformamide (DMF)	Serva Elektrophoresis, Heidelberg
Dimethyl sulfoxide (DMSO)	Sigma, USA
1,4-Dithio-DL-threit (DTT)	AppliChem, Darmstadt, Germany
DMEM	Invitrogen, USA
Epidermal Growth Factor	Sigma Aldrich, Darmstadt, Germany
Ethanol 70% technical	Applichem, Darmstadt, Germany
FBS (inaktiviert)	Gibco, USA
Glycine	CarlRothGmbh, Karlsruhe, Germany
Ham’s F-12	Invitrogen, USA
HBSS (w/o Ca, Mg, phenol red)	Gibco, USA
Hellmanex (R) III	Hellma, Mühlheim, Germany
HEPES 1 M	Invitrogen, USA
Hydrocortisone	Sigma Aldrich, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany

KSFM + EGF u. BSP	Gibco, USA
L-Glutamine	PAA, Austria
MagicMark™ XP (WB)	Thermo Fisher Scientific, USA
Methanol for analysis	Sigma-Aldrich, Darmstadt, Germany
Natrium EDTA	Merck, Darmstadt, Germany
Ortho-phosphoric acid	Fluka AG, Buchs, Switzerland
Tween 20	AppliChem, Darmstadt, Germany
Roti-Lumin	CarlRothGmbh, Karlsruhe, Germany
120 ml Roti-Lumin 1	
120 ml Roti-Lumin 2	
Hydrochloric acid concentrated (HCl)	
dPBS ohne Ca^{2+}/Mg^{2+}	Gibco, USA
Pen/Strep	PAA, Austria
ProFreeze-CDM NAO	Lonza, Switzerland
SeeBlue Plus2 Per-stained standard	Invitrogen, USA
Sodium chloride	Serva Elektrophoresis, Heidelberg
Sodium dodecyl sulphate (SDS)	Sigma, USA
Sterile water (cell culture pure)	Invitrogen, USA
TEMED	AppliChem,Darmstadt, Germany
Tris (hydroxymethyl)-aminomethane	Sigma-Aldrich, Darmstadt, Germany
Triton X-100	Sigma, USA
Trichloroacetic acid (TCA)	AppliChem, Darmstadt, Germany

A.3.3. Solutions and buffers

Lysis buffer

200 mM Tris/HCl

v/v Triton X-100

Pepstatin A

Phenantrolyne

The pH gradient was adjusted to 8.0 with concentrated HCl.

Sample buffer

200 mM Tris/HCl

0.01% v/v Triton X-100

Dimethylformamide (DMF)

The pH gradient was adjusted to 8.0 with concentrated HCl.

The mix was prepared into a glass bottle.

200 mM Tris-HCl, pH 8,0

12.11 g Tris(hydroxymethyl)aminomethane (MW 121,14), 100 ml A. bidest.

The pH gradient was adjusted to 8.0 with concentrated HCl.

Separating gel buffer

1.5 M Tris/HCl (MW 121,14)

0.4% SDS

The pH gradient was adjusted to 8.0 with concentrated HCl.

Sodium dodecyl sulfate

1.5 M Tris/HCl (MW 121,14)

0.4% SDS

The pH gradient was adjusted to 6.8 with concentrated HCl.

Electrodes buffer

25 mM Tris

192 mM Glycine

0.075% SDS

The pH gradient was adjusted to 8.3 with concentrated HCl.

TEMED

10% (v/v) TEMED in dH2O, 10 ml

APS

10% (v/v) APS in dH2O, 10 ml

3 x sample buffer

2.28g Tris/HCl

9g SDS

30 ml Glycerin

2 drops Bromphenol blue

ad 85 ml dH2O

For using: 850 µl 3x sample buffer + 150 µl 0,5M DTT

The pH gradient was adjusted to 6.8 with concentrated HCl.

Separating gel, 10%

2.6 ml dH2O

1.6 ml separating gel buffer

2.1 ml acrylamide stock solution

50 µl TEMED, 10%

50 µl APS, 10%

Sodium dodecyl sulfate

1.55 ml dH2O

625 µl SDS buffer

325 µl acrylamide stock solution

25 µl TEMED, 10%

25 µl APS, 10%

TCA solution

12% (w/v) Trichloroacetic acid (TCA) in dH2O

Stock solution 1

5% Coomassie Brilliant Blue G 250-solution (CBBG250) in dH2O

Stock solution 2

2% (w/v) phosphoric acid

10% ammonium sulphate

Stock solution 3

0.1% (w/v) CBBG250 (complies 1:50 diluted stock solution 1) + stock solution 2

Coomassie staining solution

20 ml stock solution 3

5 ml methanol abs.

Cathode buffer

25 mM Tris/HCl

40 mM glycine

20% (v/v) methanol

The pH gradient was adjusted to 8.0 with concentrated HCl.

Anode buffer

300 mM Tris/HCl

20% (v/v) methanol

The pH gradient was adjusted to 10.4 with concentrated HCl.

TBS buffer, pH 7.5

20 mM Tris/HCl

137 mM NaCl

The pH gradient was adjusted to 7.5 with concentrated HCl.

TBS Tween/Triton (TBST+T)

20 mM Tris/HCl

500 mM NaCl

0.05% (v/v) Tween 20

0.2% (v/v) Triton X-100

The pH gradient was adjusted to 7.5 with concentrated HCl.

Skimmed-milk powder solution (MMP)

1, 5 bzw. 10% (w/v) skimmed-milk powder in TBS buffer, pH 7.5

3 bzw. 5 % BSA solution

3 bzw. 5% (w/v) bovine serum albumin (BSA) in TBS buffer, pH 7.5

Anti-mouse antibody coupled to horseradish-peroxidase (secondary antibody)

(Amersham Pharmacia Biotech, Freiburg)

Dilution 1:10000 in 10% skimmed-milk powder solution

Anti-rabbit antibody coupled to horseradish-peroxidase (secondary antibody)

(Sigma, USA)

Dilution 1:10000 in 10% skimmed-milk powder solution

A.3.4. Equipment und other materials

6-Well-Plates	BD Biosciences, Belgium

Adaptor, 4 mm, PowerPac ™	Bio-Rad Laboratories, USA
Assay plate, 96 Well (for BCA assay)	Becron Dickinson Labware, USA
Automatic pipettes	*edp* Rapid Charger / 250 µl - Rainin, USA
Balance	BP 3100S Sartorius, Göttingen, Germany
Biopsy punch 4 mm	PFM Medical, Köln, Germany
Bottle sterile 25 ml, 150 ml	Corning Incorporated, Mexico
Cell culture bottle, 75 m², 175 m²	Sarstedt, Nümbrecht, Germany
Cell safe biopsy inserts	Science Servies, München, Germany
Cell safe biopsy capsules	LEICA, Wetzlar, Germany
Cell stainer, cell sieve 70 µl, 100 µl	BD Biosciences, Belgium
Centrifuges and accessories:	Eppendorf, Hamburg, Germany
Comb, Mini Protean® System	Bio-Rad Laboratories, USA
Complete filtration system 150 ml	VWR (Nunc), US
Coverslips for microscopy	Glaswarenfabrik Karl Hecht, Sondheim/Rhön, Germany
D-Squame	CuDerm Corporation, Dallas, USA
Deep-Well-Plates	VWR (BD), Hannover, Germany
Electrophoresis equipment	Bio-Rad Laboratories, USA
Fluorospectrometer	PerkinElmer, USA
Glass plates and accessories for WB	Bio-Rad Laboratories, USA
Hot plate IKAMAG RET-G	Kika-Labortechnik, Staufen, Germany
Horizontal electrophoresis chamber Typ	FEBIKON Labortechnik Gmbh, Köln
Immobilon P,	Millipore, Eschborn, Germany
Inserts for 6-Well-Plates	VWR (BD), Hannover, Germany
Magnetic stirrer	RCT basic - IKA Labortechnik, Staufen
Metal block termostat DB-2A	Techne, UK
Microscope Axioscope 2	Carl Zeiss, Jena, Germany
Microliter Syringes	HAMILTON Bonaduz AG, Switzerland
Microscopic Slides (superfrost plus)	Thermo Fisher Scientific, USA

Microtome Blades	PFM Medical, Köln, Germany
Nitrocellulose membrane	Schleicher & Schuell, Dassel, Germany
Nunc Cryo Tube vials	Sigma-Aldrich, Darmstadt, Germany
Pasteur Pipettes 145 mm, 225 mm	Omnilab, Bremen, Germany
pH meter	InoLab, Weilheim, Germany
Pipettes P 10, P 20, P 200, P 1000	Eppendorf, Hamburg, Germany
Pipetus battery	Hirschmann Laborgeräte, Eberstadt
Quartz glass cuvette	PerkinElmer, USA
Shaker Polymax	Heidolph, Kelheim, Germany
Software Axiovision 3.0	Carl Zeiss, Jena, Germany
Standard Power Pack P25	Biometra, Göttingen, Germany
Tubes 15 ml, 50 ml	Corning Incorporated, Mexico
Tubes Falcon 50 ml	BD Biosciences, Belgium
Ultrasound	Bandelin electronic, Berlin, Germany
UV-translucent gel carrier with seals	FEBIKON Labortechnik Gmbh, Köln
Video camera Axiocam HR	Carl Zeiss, Jena, Germany
Vortex IKA-VIBRO-FIX VF2	Kika-Labortechnik, Staufen, Germany
Whatman paper	Maidstone, UK
X-ray films Fuji Super RX	Stamford, CT

ACKNOWLEDGEMENTS

This work was supported by the Medical Faculty (OJI120817) of the University of Münster and the German Federal Ministry for Education and Research as part of the Network for Rare Diseases (NIRK, GFGM01143901).

I would like to express my very profound gratitude to PD. Dr. med. Vinzenz. Oji for his dedicated support and expert discussions during my research. In particular, I would like to thank him for his trust and helpfulness.

I am very grateful to Prof. Heiko Traupe as the second reader of this thesis for his friendly support and constructive cooperation during my investigation.

I would like to thank, too, Dr. rer. nat. Karin Aufenvenne (Leibniz Research Institute for Environmental Medicine, Düsseldorf) for her very valuable suggestions and comments on this thesis.

I also owe an enormous debt of gratitude to Mrs. Tatjana Tarinski for numerous types of assistance for the cell culture, preparation of cryosection and support in the laboratory.

I am grateful to all patients and volunteers who took part in this research.

The excellent technical assistance of Christa Post and Androniki Kolovou is gratefully acknowledged. A special thanks go to Mrs. Brigitte Willis and Mr. Frederic Valentin.

I would like to express my gratitude to my parents, my husband and my son who have supported me so well during my research. They gave me so much power and energy and helped me in this matter to bring my investigation to a conclusion.

In closing, my thanks also go out to German Academic Exchange Service (DAAD) for financial support.