Junctional epidermolysis bullosa

Wing Yan Yuen, 2012

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List of abbreviations

α	cronbach alpha
α6	integrin α6
α6β4	integrin α6β4
α3β1	integrin α3β1
AL	apical lateral
ANOVA	analysis of variance
β4	integrin β4
BMP-1	human bone morphogenetic protein-1
BMT	bone marrow transplantation
BMZ	basement membrane zone
BP	bodily pain
BPAG1-e	bullous pemphigoid antigen 1-e
Col7	type VII collagen
Col17	type XVII collagen
CS	connecting segment
DDEB	dominant dystrophic epidermolysis bullosa
DEB	dystrophic epidermolysis bullosa
DEBR	Dutch Epidermolysis Bullosa Registry
DLQI	Dermatology Life Quality Index
EB	epidermolysis bullosa
EB-PA	epidermolysis bullosa with pyloric atresia
EBS	epidermolysis bullosa simplex
EBS-AR	epidermolysis bullosa simplex autosomal recessive
EBS-DM	epidermolysis bullosa Dowling Meara
EBS-gen	epidermolysis bullosa simplex generalized non-Dowling Meara
EBS-loc	epidermolysis bullosa simplex localized
EBS-migr	epidermolysis bullosa simplex migratory circinate
EBS-MP	epidermolysis bullosa simplex with mottled pigmentation
EBS-PA	epidermolysis bullosa with pyloric atresia
EGF	epidermal growth factor
EM	electron microscopy
FNIII	type III fibronectin repeats

GH	general health
HB-EGF	heparin-binding epidermal growth factor-like growth factor
HCP	health care professional
HRQOL	health related quality of life
IF	immunofluorescence antigen staining
IH	immunohistochemistry staining
JEB	junctional epidermolysis bullosa
JEB-H	junctional epidermolysis bullosa, type Herlitz
JEB-lo	junctional epidermolysis bullosa of late onset
JEB-nH	junctional epidermolysis bullosa, type non-Herlitz
JEB-nH gen	junctional epidermolysis bullosa, type non-Herlitz generalized
JEB-nH loc	junctional epidermolysis bullosa, type non-Herlitz localized
JEB-PA	junctional epidermolysis bullosa with pyloric atresia
LABD97	linear IgA bullous disease antigen of 97 kDa
LAD-1	120 kDA linear IgA disease antigen
LM-332	laminin-332
LOC	laryncho-onycho-cutaneous
mAb	monoclonal antibody
MAP	mitogen activated protein
MH	mental health
MMP	matrix metalloproteineases
NC	non-collagenous
NEBR	National Epidermolysis Bullosa Registry
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3 kinase
PF	physical functioning
PTC	premature termination codon
QOLEB	quality of life in epidermolysis bullosa
ρ_s	Spearman's rho correlation coefficient
RDEB	recessive dystrophic epidermolysis bullosa
RE	role emotional
RP	role physical
SCC	squamous cell carcinoma

SD	standard deviation
SF	social functioning
SF-36	Short Form-36
TEM	transmission electron microscopy
VT	vitality

1

Introduction

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The skin and its structure

The skin, together with its appendages, the hair, nails, sweat glands, and sebaceous glands, form the integumentary system, which is human's largest organ, as it covers the whole body with an average surface of 1.5-2.0 m² and weighing around 5 kg.[1,2] One of the most important functions of the skin is to protect the body. It does so by forming a protective barrier that prevents loss of fluids and electrolytes, and, at the same time, preventing penetration of toxic materials and UV radiation.[3,4] The skin also protects against external blunt trauma, and against infections through an intricate system of innate and adaptive immunity.[5,6] Other functions of the skin are sensory perception, excretion of waste, regulating body temperature, synthesizing vitamin D, storage of water, fat, and glucose, and as a way of sociosexual communication.[7-10] The skin consists of two distinct compartments: the epidermis and the underlying dermis. At the basement membrane zone (BMZ) these two compartments are connected.[4] This thesis focuses on genetic diseases that are characterized by blistering at the BMZ.

Epidermis

The epidermis is a stratified squamous epithelium originating from the embryonic ectoderm, with a thickness varying from 0.05 mm on the eyelids to 1.5 mm on the palms and soles.[4,11] The epidermis is a cellular network harboring four different cell types: keratinocytes, melanocytes, Langerhans' cells, and Merkel cells.[4] The most common cell type is the keratinocyte, accounting for 95% of the epidermis. Keratinocytes are continuously renewed through cell division of stem cells that are located in the basal interfollicular epidermis, bulge area of hair follicles, and upper isthmus located between the bulge and sebaceous glands.[12] This causes keratinocytes to migrate upwards, forming four distinct differentiation layers in the epidermis: successively the basal layer, the spinous layer, the granular layer, and the corneal layer (Figure 1). On the palms and soles a fifth layer, called the lucid layer, is situated between the granular and corneal layer.[4] Migration from the basal layer to eventually desquamation from the corneal layer takes approximately one month.[13] The structural integrity of keratinocytes is provided by keratin intermediate filaments (~7-10 nm), actin microfilaments (~7nm), and microtubules (~20-25 nm).[4,14-16] Desmosomes are the major adhesion complexes for keratinocytes, anchoring keratin intermediate filaments to the cell membrane and bridging adjacent keratinocytes.[17] The attachment of actin microfilaments is provided by adherens

junctions and tight junctions. Tight junctions also regulate the para-cellular flux of water-soluble molecules between adjacent epidermal cells.[17] The cytoplasm of adjacent keratinocytes are connected by gap junctions, that permit sharing of low molecular mass metabolites and ion exchange.[18,19] Characteristic for keratinocyte differentiation is the switch in expression of keratin intermediate filaments 5 and 14 in the basal layer, to expression of keratin intermediate filaments 1 and 10 in the spinous layer.[20] In the spinous and granular layer the flattened keratinocytes transform from dividing cells, to metabolically active cells. For example, the keratinocytes in the granular layer synthesize keratohyaline granules containing glutamine and lysine rich proteins like involucrin and loricrin. In the corneal layer, keratinocytes, now called corneocytes, undergo programmed cell death and lose their nucleus and cytoplasmic organelles. Corneocytes develop a protective barrier by forming a highly insoluble cornified envelope by formation of transglutaminase-catalysed glutamyl-lysyl-isodipeptide bonds between envelope proteins, like involucrin and loricrin.[21]

The dendritic melanocytes reside in the basal layer of the epidermis, where they produce the photo-protective pigment melanine and accumulate it in the specialized organelles called melanosomes. Melanosomes are transferred to neighboring keratinocytes where they are located over the nucleus to protect DNA from harmful UV radiation.[22]

Langerhans' cells play an important role in the adaptive immunity as antigen presenting cells. They are dendritic cells located in the spinous layer of the epidermis, that catch, phagocytose, process, and present antigens to T-lymphocytes in regional lymph nodes.[23]

Located in the basal layer, Merkel cells comprise 0.2-5% of the epidermal cells. They are closely associated with the ends of nerve fibres and act as skin mechanoreceptors that are sensitive for touch, pressure, texture, and low frequency vibrations.[24]

Dermis

The ectoderm-derived dermis provides nutritional and structural support to the epidermis. The major constituent of the dermis is the extracellular matrix connective tissue; other components are nerves, vasculature, and cells, such as fibroblasts, macrophages, mast cells, and dendritic cells. The fibroblast is the major cell type in the dermis and it is responsible for the synthesis and maintenance of the four extracellular matrix

components: 1) collagens, providing strength and resistance to the skin (75-80%), 2) elastic fibres, providing elasticity and resilience to the skin (4%), 3) proteoglycan/glycosaminoglycan macromolecules, important for hydration and binding a diversity of molecules (0.1-0.3%), and 4) non-collagenous glycoproteins, such as fibronectins, fibulins and transmembrane integrins that organize the extracellular matrix and facilitate cell adhesion and cell motility.[4,25,26] The extracellular matrix consists of a complex interconnected network of these macromolecules, resulting in a gel-like structure, acting as a scaffold through which cells can move.[4]

Basement membrane zone

The epidermal BMZ is a specialized structure located between the epidermis and the dermis. It has functions in tissue repair, cell migration, cell differentiation, and cell adhesion.[27,28] The BMZ is subdivided in the plasma membrane of the basal keratinocyte, lamina lucida, lamina densa and sublamina densa (Figure 1). The adhesion of the epidermis to the dermis is provided by focal adhesions that connect the actin and tubulin microtubule cytoskeleton to the dermis, and by hemidesmosomes that connect the keratin intermediate filament cytoskeleton to the lamina densa (Figure 1).[4]

The hemidesmosome

Hemidesmosomes are specialized multi-protein complexes, that mediate the adhesion of epithelial cells to the underlying basement membrane.[29,30] Hemidesmosomes also facilitate signal transduction regulating cell growth, differentiation, proliferation, migration, and apoptosis, making them important factors in wound healing and tumor invasion.[31,32] Hemidesmosomes in the skin contain at least five proteins: bullous pemphigoid antigen 1-e (BPAG1-e, also known as BP230), plectin, integrin α6β4 (α6β4), type XVII collagen (Col17), and CD151 (Figure 2).[33-37]

In the skin, hemidesmosomes appear ultrastructurally as small (<0.5 μm) regularly spaced electron dense plaques on the dermal surface of basal keratinocytes.[32] The electron dense plaque can be further subdivided from inside out in three compartments: 1) the inner plaque, consisting of the proteins BPAG1-e and plectin, that act as cytolinkers of keratin intermediate filaments to the hemidesmosome, 2) the outer plaque overlying the plasma membrane, containing the cytoplasmic domains of the transmembrane proteins Col17 and α6β4, and 3) an extracellular subbasal dense plate running parallel to the plasma membrane, consisting of tetraspanin CD151, and the extracellular domains of Col17 and α6β4. Adhesion of the hemidesmosome to the

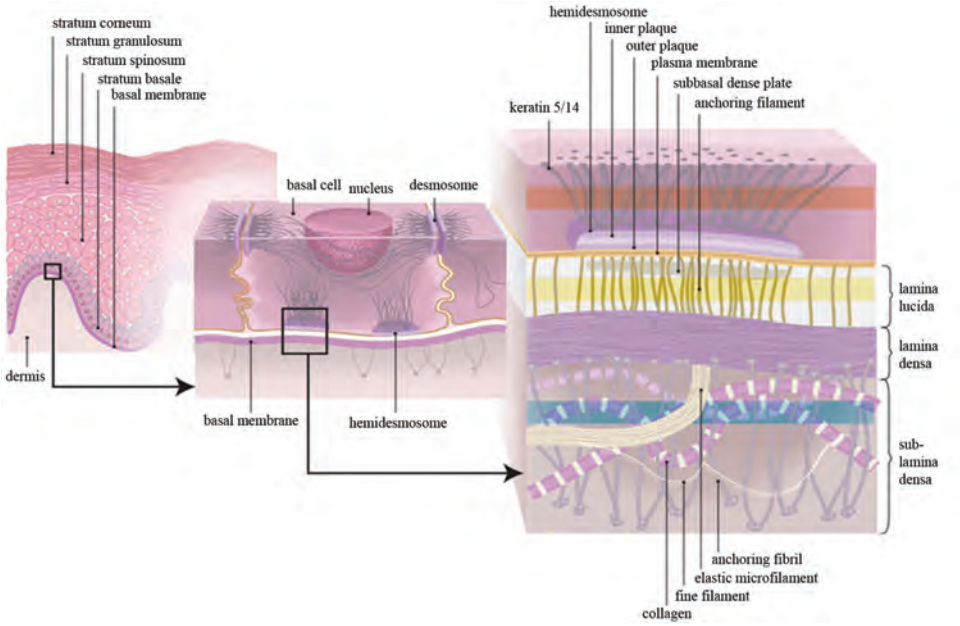

Figure 1. On the left an illustration of the skin comprising the five layers of the epidermis with the underlying dermis. In the middle the epidermal-dermal junction is shown with on the right a magnification of the basement membrane zone. Courtesy of M.F. Jonkman

underlying extracellular matrix is facilitated by a structure called the hemidesmosome-stable adhesion complex, consisting of anchoring filaments, anchoring fibrils and the hemidesmosome (Figure 2).[27,32] The extracellular domain of α6β4 and Col17 connect to laminin-332 (LM-332) in thin thread-like anchoring filaments, which cross the lamina lucida into the lamina densa. The lamina densa is a complex meshwork in which type IV collagen and different laminins are crosslinked via nidogen, fibulin, and other glycoproteins.[27,38] Here, the anchoring filaments connect to semicircular anchoring fibrils, consisting of type VII collagen (Col7). The anchoring fibrils extend into the dermis and loop back into the lamina densa.[39-41]

The assembly of hemidesmosomes can be driven entirely from within the cell, and starts with a crucial interaction between integrin β4 (β4) with plectin.[42-44] Then β4 interacts with Col17, after which Col17 recruits BPAG1-e in hemidesmosomes.[31] When LM-332 is not bound to β4, plectin clusters α6β4 into tight complexes. The interaction of β4 with LM-332 restricts the degree of clustering, allowing the formation of

hemidesmosomes.[45] It is hypothesized that the disassembly of hemidesmosomes results from phosphorylization of certain sites in the β4 cytoplasmic domain that are important in the interaction with plectin.[46-48]

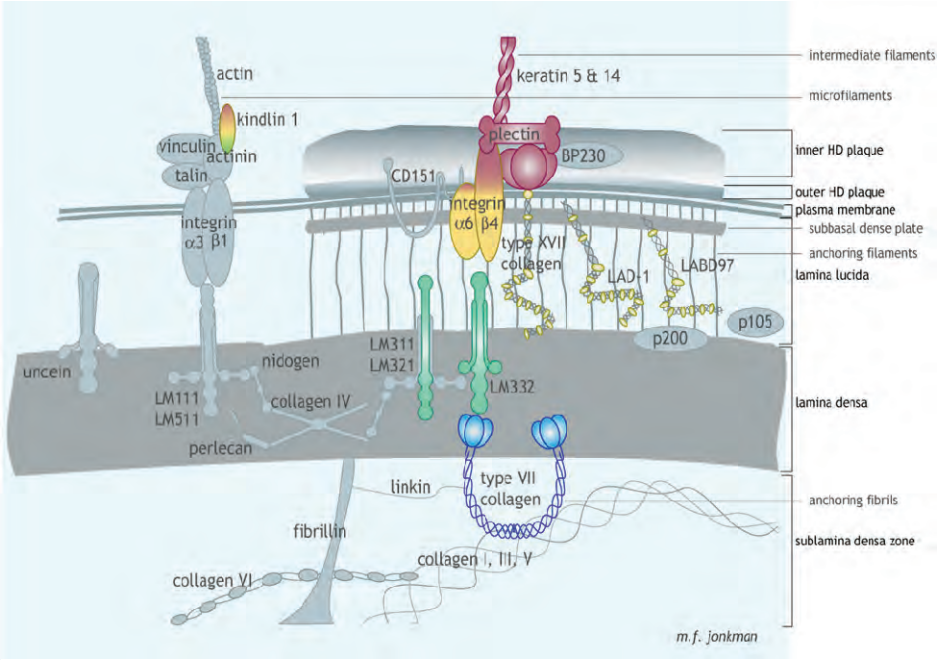

Figure 2. Overview of the hemidesmosome-stable adhesion complex (right) and focal adhesion (left). The proteins associated with epidermolysis bullosa are in colour. Courtesy of M.F. Jonkman

There are two types of hemidesmosomes: type II hemidesmosomes are found in simple epithelia and consists only of α6β4 and plectin;[37,49,50] type I hemidesmosomes contain all five hemidesmosomal proteins and are present in stratified, transitional, and pseudo-stratified squamous epithelium, such as the skin, cornea, parts of the gastrointestinal- and respiratory tract, and the amnion.[31,32] In the skin, both type I as type II hemidesmosomes can be seen, however it is thought that the occurrence of type II hemidesmosomes reflects the assembly and disassembly of type I hemidesmosomes.[51]

BPAG1-e

BPAG1-e (gene: *DST*) is one of the seven family members of the cytoskeletal linker proteins called plakins. Other family members are plectin, desmoplakin, envoplakin, microtubule-actin crosslinking factor, epiplakin and periplakin. Plakins preserve the tissue integrity of the cell by crosslinking cytoskeletal intermediate filaments and attaching them to their membrane attachment site.[52,53] Plakins are characterized by the presence of numerous repeat motifs that form complex tertiary structures with highly versatile binding properties.[53] Alternative splicing and alternative initiation sites in *DST* result in multiple tissue isoforms with various expression in the skin, neurons, muscles, and the central nervous system: BPAG1-a is located in the nervous system and BPAG1-b in the heart and skeletal muscles.[54,55] The major epidermal isoform BPAG1-e, is expressed in hemidesmosomes in basal epithelial cells.[53,54]

BPAG1-e contains a central coiled-coil rod domain, mediating dimerization of the protein. The N-terminal globular head domain is known as the plakin domain. It consists of six α-helical segments that are organized into anti-parallel α-helical bundles, and facilitates the interaction of BPAG1-e with Col17 and β4.[51] The C-terminus contains two plakin repeat domains that interact with various intermediate filaments. Plakin repeat domains consist of four-and-a-half, 38 residues long, plakin repeat motifs, that are organized in tandem arrays. Of the three known plakin repeat domain classes (A, B, and C), BPAG1-e has two (B, and C).[53]

BPAG1-e has been identified as an auto-antigen in the auto-immune blistering disease bullous pemphigoid.[56,57] In mice, mutations in *DST* lead to the hereditary disease dystonia musculorum, characterized by progressive degeneration of sensory neurons.[58-60] *DST* knockout mice show skin blistering upon trauma, after which they die after 4-5 weeks due to dystonia musculorum. Ultrastructurally, the hemidesmosomes miss the inner plaque and they do not associate with intermediate filaments. However, the rest of the hemidesmosomal component remained normal and the attachment of the hemidesmosome to the underlying BMZ was not altered.[59] In humans, three cases of *DST* abnormalities have been reported to lead to disease. A 6;15 translocation of the isoforms BPAG1-a and BPAG1-b has led to encephalopathy, severe motor and mental retardation, and delayed visual maturation without skin involvement.[61] The homozygous nonsense mutation p.Gln1124X located in the coiled-coil rod domain of BPAG1-e was shown to be pathogenic in autosomal recessive epidermolysis bullosa simplex (EBS).[62] Recently, a second case of recessive EBS was linked with a homozygous nonsense mutation (p.R1249X) in the coiled-coil rod domain of BPAG1-e.[63]

Plectin

Plectin (gene: *PLEC1*) is the most versatile of the plakins, as it can associate with intermediate filaments, actin, and microtubules.[53,64] Alternative splicing of *PLEC1* at the 5'end creates many tissue specific isoforms, and, except in some neurons, plectin is seen in all mammalian cells, especially in skin, skeletal muscle, heart, and nerve tissue.[64-67] In the skin, plectin provides the linkage of the cytoskeleton to adjacent keratinocytes with desmosomes, to the lamina densa with hemidesmosomes and focal adhesions, and to cell organelles, such as the nucleus and mitochondria.[53] Furthermore, plectin may also have an impact on signaling processes that regulate cell migration.[55]

Plectin is a large polypeptide of over 500 kDa, composed of a central coiled-coil rod domain that is flanked by a globular N-terminal head domain and a C-terminal tail domain. The N-terminal head domain contains a plakin domain, and an actin-binding domain composed of two calponin-homology domains.[53] The actin-binding domain and the first two repeats of the plakin domain are essential in the interaction with β4.[68] Furthermore, Col17 binds to the plakin domain.[51] The C-terminus consists of six plakin repeat domains, a linker subdomain located between the fifth and sixth plakin repeat domains, and a glycine-serine-arginine domain. Intermediate filaments interact with the plakin repeat domain 5 and 6, near the linker subdomain,[66] and the glycine-serine-arginine domain binds microtubules.[69] The plakin repeat domain also interacts with β4, although this interaction is less crucial in hemidesmosome formation.[68]

Plectin-knockout mice die postnatal after 2-3 days with extensive skin fragility, and muscular and cardiac abnormalities. Ultrastructurally, the hemidesmosomes appeared unaffected, although they were reduced in number.[70] Mutations in *PLEC1* in humans have been associated with EBS-Ogna,[71] EBS with pyloric atresia,[72] and EBS with muscular dystrophy.[73]

CD151

CD151 (gene: *CD151*) is a member of the tetraspan superfamily, that are cell surface proteins that intersect the plasma membrane four times and form multimolecular complexes with each other and other membrane proteins, such as integrins.[74]

CD151 is expressed in a variety of epithelial and mesenchymal cells, like the skin, vascular endothelium, Schwann cells, and muscle cells.[75] In the skin, CD151 is localized in basal keratinocytes, both intracellular as in hemidesmosomes,[37] where it functions in cell adhesion, cell motility, transport of integrins, signal transduction, and linking the cytoskeleton.[76-80]

CD151 contains four transmembrane passes that are interconnected by a small and a large extracellular loop, and a short intracellular N-and C-terminal domain.[74,81] The large extracellular loop of CD151 is involved in the binding of the α-subunit of heterodimeric integrins.[77] In hemidesmosomes, CD151 interacts with α6β4. CD151 is not required for the formation of hemidesmosomes, and its role is still unclear. A hypothesis is that CD151 forms complexes with integrin α3β1 to provide platforms on which hemidesmosomal components can be localized and organized.[37,74,82]

CD151-knockout mice are normal, healthy and fertile, with no structural abnormality of the skin and hemidesmosomes.[74] In humans, two siblings with a homozygous *CD151* nonsense mutation, resulting in truncated or absence of CD151 protein, showed pretibial epidermolysis bullosa with hereditary nephritis, sensorineural deafness, and β-thalassemia minor.[83] This discrepancy in mouse and human, has led to the hypothesis that truncated CD151 is more deleterious than no CD151 at all, or that mice have other tetraspanins to compensate for CD151 loss, that are not available in human.[74,82]

Type XVII collagen

Col17 (gene: *COL17A1*) belongs to the collagenous type II transmembrane proteins, a versatile group that functions as cell-surface receptors and extracellular matrix molecules with structural and regulatory roles.[84] Col17 is expressed in skin, cornea, teeth, mucous membranes, brain, placenta and the umbilical cord.[85-88] In the skin, Col17 is found in basal keratinocytes as a component of hemidesmosomes.[34]

Col17 is a homotrimer of α1-chains of 1497 residues and comprises an intracellular N-terminus (466 residues), followed by a transmembrane protein (23 residues) and an extracellular C-terminus (1008 residues). The C-terminus consists of a linear rod domain that crosses the lamina lucida and a flexible tail domain localized in the lamina densa.[51,89,90] It contains 15 collagenous (COL1-COL15) domains that are intersected by 16 non-collagenous (NC1-16) domains. The juxtamembraneous non-collagenous linker region NC16A (residues 490-566) is involved in the triple-helix folding of Col17,[91] and in this region (residues 528-547) the ectodomain of Col17 is also shed from the cell surface yielding the soluble 120-kDA linear IgA disease antigen 1 (LAD-1).[92-94] The shedding is activated by furin and executed by the metalloproteinases ADAM-9, ADAM-10, and ADAM-17. LAD-1 is further cleaved at the C-terminus, resulting in the linear IgA bullous disease antigen of 97 kDa (LABD97) with possible cleavage sites ranging from amino acids 1209-1310.[95,96] The function of ectodomain shedding is

not yet fully understood. Hypotheses are that it releases the cell to form other binding partners and allows it to migrate, or that these molecules settle into the BMZ where they strengthen the epidermal-dermal adhesion.[97,98]

The binding partner of the extracellular domain of Col17 is LM-332. The intracellular domain binding partners are β4, plectin, and BPAG1-e.[51,98-100]

Auto-antibodies against Col17 are found in the auto-immune blistering diseases bullous pemphigoid, pemphigoid gestiationes, cicatricial pemphigoid, lichen planus pemphigoides, linear IgA disease, and mucous membrane pemphigoid.[101] The NC16A domain is the major site of epitopes, and it has been proposed that ectodomain shedding at this domain creates neo-epitopes.[102] Mutations in *COL17A1* are associated with junctional epidermolysis bullosa (JEB), and in some cases with EBS.[103-105]

Integrin α6β4

Integrin α6β4 belongs to the integrin superfamily of transmembrane cell adhesion receptors that recognize mainly extracellular matrix ligands, cell surface ligands and soluble ligands. They function as links of extracellular ligands to the cytoskeleton, and as two-way signaling proteins that influences many aspects of cell behavior, like proliferation, survival/apoptosis, shape, polarity, motility, gene expression and differentiation.[106] Due to its signaling and migratory properties, α6β4 contributes to wound healing, but also in stimulating carcinoma migration and invasion.[107,108] Integrins are non-covalently bound heterodimers consisting of an α and β subunit. To date, 18 α and 8 β subunits are known, that form 24 distinct integrins, all with their own binding specificity and signaling properties.[109] Integrin α6β4 binds with both LM-332 and laminin-511, and is expressed in epithelial tissues, endothelia and peripheral nerves.[110,111] In the skin, α6β4 is located in hemidesmosomes.[36]

Integrin α6 (α6) is encoded by *ITGA6* and consists of 1050 amino acids, subdivided in a large N-terminal ectodomain (991 residues), a transmembrane protein (23 residues), and a short C-terminal endodomain (36 residues).[112,113] An alternative transcript with a larger endodomain of 54 residues, termed integrin α6B, exists, but is not seen in the epidermis.[112,114] The α6 ectodomain folds into a seven-bladed β-propellor connected to a thigh and two calf domains, that interacts with integrin β4 or β1.[108,110,115,116]

Integrin β4 (gene: *ITGB4*) consists of a signal peptide (residues 1-27), followed by an extracellular domain (residues 28-710), a transmembrane protein (residues 711-733), and an intracellular domain (residues 734-1752).[117-119] The N-terminal extracellular

domain of β4 binds to the α6 subunit, and acts as a receptor for LM-332 and laminin-511.[120,121] The β4 intracellular domain contains a calx-β domain (residues 991-1054),[122] and two pairs of type III fibronectin (FNIII) repeats (residues FNIII-1 1128-1215; FNIII-2 1220-1313; FNIII-3 1458-1548; FNIII-4 1571-1664) which are separated by a connecting segment (CS) (residues 1314-1457).[113,118,123] The FNIII-1,2 domains and the first 35 N-terminal residues of the connecting segment binds with the actin-binding domain of plectin.[42,43] This interaction is required for the stability of hemidesmosomes, and without it hemidesmosomes will not be formed.[42,43] Additional binding sites of β4 with plectin are in the end of the CS and in the FNIII-4 and C-tail region.[124] The C-terminal of the CS and FNIII-3,4 interacts with BPAG1-e, and the FNIII-3 repeat interacts with Col17.[43,51,125]

Mutations in *ITGB4* and *ITGA6* can cause epidermolysis bullosa with pyloric atresia, JEB type non-Herlitz, and EBS.[126,127,128,129]

Laminin-332

LM-332 is located in the lower lamina lucida and lamina densa and is part of the laminin family, which are glycoproteins associated with adhesion of cells, binding with cell surface receptors, activating intracellular signaling cascades, and interaction with components of the extracellular matrix to form scaffolds for migrating cells.

All laminins are heterotrimers composed of an α, β, and γ chain. To date 5 α, 3 β, and 3 γ chains, forming 16 different laminins, have been described.[130] LM-332 is composed of an α3 chain (consisting of three (I-III) domains), a β3 chain (consisting of six (I-VI) domains), and a γ2 chain (consisting of five (I-V) domains). In the skin, LM-332 is produced by basal keratinocytes where it forms a cross-shaped trimer. The C-terminal rod-like coiled-coil long arm of the cross consists of domain I and II. At the base of the long arm, the α3 chain contains five globular LG1-5 domains; LG3 binds integrins (α3β1, α6β1, and α6β4), and LG4-5 binds heparin and syndecan, that is necessary for matrix assembly.[131-133] These LG domains are also important in efficient chain assembly and secretion.[134] The three short arms of the cross are formed by the N-terminal parts of the individual subunits that consist of LE domains with laminin EGF-like motifs arranged in rod-like structures. The N-terminus of the β3 chain (domain III-VI) contains a globular LN-domain, that interacts with LM-332, laminin-311, and Col7.[135,136] At the N-terminus of the γ2 chain (domain III-V), a globular L4 site provides the interaction with nidogen and fibulin, that facilitates the adherence of LM-332 to Col7.[137]

The genes encoding the α3, β3, and γ2 chain are *LAMA3*, *LAMB3*, and *LAMC2*, respectively. Through alternative initiation sites, *LAMA3* (76 exons) transcribes the

shorter α3A subunit, encoded by exon 39-76 (1714 residues), and the longer α3B subunit, encoded by exon 1-38 and exon 40-76, with a skip of exon 39 (3333 residues). Furthermore, the α3B subunit can also skip exon 10 (3289 residues).[138,139] LM-332 containing the α3A subunit is detected in stratified squamous epithelia, such as the skin and hair follicles, whereas the α3B is found in transitional epithelium of the lung, salivary glands and intestine.[140-142]

The assembly of LM-332 in keratinocytes starts with the formation of a stable, disulfide-linked dimer of the β3 (140 kDa) and γ2 chain (155 kDa), after which the α3A chain (190 kDa) is incorporated and cross-linked by disulfide bridges in the rough endoplasmic reticulum.[143,144] LM-332 is secreted in the lamina densa as a heterotrimer. Only the γ2 chain can be secreted as a monomer by basal keratinocytes.[145] Once secreted, the short arms of LM-332 are orientated towards the lamina densa and the long arm crosses the lamina lucida.[146] Here, LM-332 undergoes extracellular proteolytic processing influencing its biological functions. A necessary step for interaction with hemidesmosomes is that the hinge between the LG3 and LG4 domain of the α3 chain is cleaved by plasmin, which converses LM-332 from a motility to an adhesion factor.[147,148] Furthermore, the γ2 chain is processed at the N-terminus by metalloproteinases, although the relevance of this remains unclear.[149] Due to its migratory and signalling properties, LM-332 is also involved in wound healing and carcinogenesis.[150-156]

Auto-antigens against LM-332 are found in patients with anti-LM-332 mucous membrane pemphigoid and cicatricial pemphigoid.[101,157] Mutations in the genes coding for LM-332 lead to JEB, type Herlitz and non-Herlitz.[158-160] Specific mutations in the *LAMA3* chain lead to laryncho-onycho-cutaneous (LOC) syndrome.

Epidermolysis bullosa

Epidermolysis bullosa (EB) comprises a heterogenous group of inherited diseases characterized by trauma-induced blistering. The International classification of inherited EB distinguishes four major EB subtypes based upon the ultrastructural level of blistering: EBS with an intra-epidermal cleavage, JEB with a junctional cleavage, dystrophic epidermolysis bullosa (DEB) with a dermal cleavage, and Kindler syndrome with a mixed cleavage (Figure 3).[161] The level of intra-epidermal blistering in EBS, further subdivides it in suprabasal and basal EBS, with cleavage planes above and in the basal

keratinocytes, respectively.[161] The major EB subtypes include a total of 29 minor subtypes, caused by 15 distinct genes.[62,161,162]

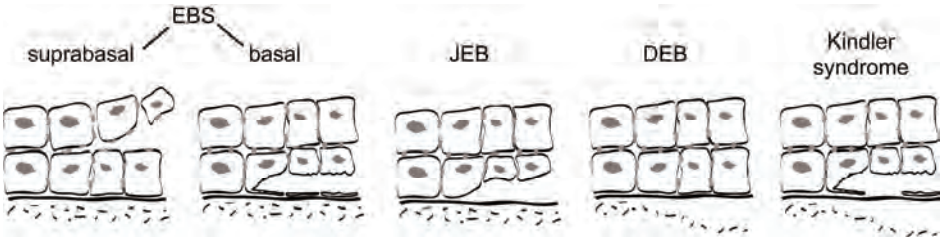

Figure 3. Illustration of the blister levels of the four major EB subtypes. Courtesy of M.F. Jonkman

Three diagnostic tools exist to achieve a correct diagnosis and classification of EB. In immunofluorescence (IF) antigen staining, antibodies are used to stain against specific proteins associated with EB, to see if their expression is normal, reduced or absent. Furthermore, with IF antigen mapping, the level of blistering can be determined by investigating the location of different proteins in the dermal-epidermal junction in a fresh blister.[163] The level of blistering can also be distinguished by transmission electron microscopy (TEM). Moreover, TEM can show ultrastructural abnormalities in keratin filaments and hemidesmosome-adhesion complexes, that are typical for some EB subtypes.[164] When IF antigen mapping and TEM have established an EB subtype and a candidate gene, molecular analysis can be performed to identify the pathogenic mutation, which is important for the confirmation of the diagnosis and for prenatal diagnosis.[165]

In the Netherlands, the incidence of EB has been estimated at 100 new cases per one million live births, with a prevalence of 45 patients per one million inhabitants. Of these patients 40% suffers from EBS, 25% of JEB, and 35% of DEB.[166] DEB can be inherited in an autosomal dominant fashion, resulting in the milder dominant DEB (DDEB), or in an autosomal recessive fashion, resulting in the generally more severe recessive DEB (RDEB). All DEB cases are caused by mutations in the gene coding for type VII collagen (*COL7A1*), resulting in absent or truncated anchoring fibrils.[161] Kindler syndrome is an autosomal recessive subtype caused by mutations in *FERMT1*, coding for the actin cytoskeleton-associated protein fermitin family homologue I, located in focal adhesions.[167] As this thesis focuses mainly on JEB and also on basal EBS, they will be discussed in more detail.

Basal epidermolysis bullosa simplex

Studies in the Netherlands and Scotland have revealed that 70-75% of basal EBS cases are caused by mutations in the genes encoding keratin 5 (*KRT5)* and keratin 14 (*KRT14).*[168-171] Mutations in specific regions in *KRT5* and/or *KRT14* give rise to three common major EBS subtypes ranging from mild to severe: 1) EBS localized (EBS-loc), 2) EBS generalized non-Dowling Meara (EBS-gen), and 3) EBS Dowling Meara (EBS-DM), and to three less common minor subtypes: 1) EBS with mottled pigmentation (EBS-MP), 2) EBS migratory circinate (EBS-migr), and 3) EBS autosomal recessive (EBS-AR) (for details see Table 1).[168,169,172-174] All these subtypes, except for EBS-AR, inherit in an autosomal dominant fashion, in which missense mutations or small deletions/insertions result in aberrant keratin 5 or 14, that is incorporated into the keratin intermediate filament cytoskeleton, where they compromise the structure and the function of the cytoskeleton, exerting a dominant negative effect. EBS-AR inherits in an autosomal recessive manner, caused by loss-of-function nonsense or missense mutations in *KRT14*. Recently, a new gene was found to be associated with EBS-AR; *DST* nonsense mutations located in the coiled-coil rod domain of the hemidesmosomal BPAG1-e were shown to be pathogenic.[62,63]

Plectin has been associated with the minor EBS subtypes EBS-Ogna, EBS with muscular dystrophy (EBS-MD), and EBS with pyloric atresia (EBS-PA). EBS-Ogna is an autosomal dominant variant caused by missense mutations in the rod domain or plakin repeat domains of *PLEC1*.[71,175,176] EBS-MD and EBS-PA are both rare autosomal recessive subtypes.[72,73] The phenotype-genotype correlation is related to the presence of an alternative spliced RNA transcript, that lacks exon 31 encoding the central rod domain of plectin.[177] In EBS-MD, nonsense or frameshift mutations located within exon 31, or in-frame mutations elsewhere in *PLEC1*, are found. For the nonsense or frameshift mutations, alternate splicing of exon 31 may restore the *PLEC1* open reading frame. A possibility that is not available in EBS-PA, which is caused by nonsense or frameshift mutations located outside exon 31, leading to a more severe and lethal subtype.[178]

Other proteins involved in EBS are β4 and Col17. Both have been associated with single cases of EBS-AR with mild skin blistering and a pseudo-junctional blister level.[103,105,129] Furthermore, β4 has been associated with EBS-PA.

Table 1. Clinical and diagnostic features of basal epidermolysis bullosa simplex subtypes

Basal EBS subtype	Onset	Clinical features	Gene	Inheri-tance	IF	TEM
EBS-localized	Early childhood	Blistering hands and feet; focal PPK	*KRT14* / *KRT5*	AD		Basal split; basal cell lysis
EBS-generalized	Birth	Generalized blistering; focal PPK	*KRT14* / *KRT5*	AD		Basal split; basal cell lysis
EBS Dowling Meara	Birth	Severe generalized herpetiform blistering; mucosa and nail involvement; diffuse PPK	*KRT14* / *KRT5*	AD		Basal split; KIF clumping
EBS mottled pigmentation	Birth	Mottled pigmentation trunk and limbs; mild generalized blistering	*KRT 5* / sporadic *KRT14*	AD		Basal split; abnormal clustering of melanosomes
EBS migratory circinate	Birth	Generalized migratory circinate erythema with multiple vesicles on the border; postinflammatory hyperpigmentation	*KRT 5*	AD	K5 reduced	Basal split; reduced KIF
EBS autosomal recessive	Birth	Generalized blistering with variable severity; focal PPK; mucosal and nail involvement	*KRT 14* / *DST*	AR	K14 absent or reduced / BPAG1-e absent	Basal split; absent or reduced KIF in basal keratinocytes; inner plaque HD poorly formed or absent
EBS Ogna	Birth	Acral blistering; blood blebs	*PLEC1*	AD	Plectin reduced	Low basal split; normal or aberrant HD; impaired insertion KIF in HD
EBS pyloric atresia	Birth	Generalized blistering; pyloric atresia; can be lethal in chidlhood; possible aplasia cutis; mucosal involvement	*PLEC1* / *ITGB4*	AR	Plectin absent or reduced / integrin β4 absent or reduced	Low basal split; small HD with abnormal subbasal dense plate
EBS muscular dystrophy	Birth	Generalized blistering; muscular dystrophy from infancy-childhood	*PLEC1*	AR	Plectin absent or reduced	Low basal split; small HD plaque

EBS epidermolysis bullosa simplex; PPK palmoplantar keratoderma; AR autosomal recessive; AD autosomal dominant; KIF keratin intermediate filament; HD hemidesmosome; IF immunofluorescence antigen staining; TEM transmission electron microscopy

Junctional epidermolysis bullosa

JEB inherits in an autosomal recessive manner and is characterized by blistering through the lamina lucida.[161] The most common subtype is junctional epidermolysis bullosa, type non-Herlitz (JEB-nH), caused by mutations in the genes coding for LM-332, Col17, and less frequently in β4.[104,128] JEB-nH is divided in a localized subtype with blistering mainly limited to the hands and feet, and a generalized subtype with widespread blistering.[179-184]

A total absence of LM-332, caused by nonsense mutations or out-of-frame deletions or insertions in *LAMB3*, *LAMC2* or *LAMA3*, leads to the severe subtype JEB, type Herlitz (JEB-H).[158-160] The extensive and persistent blistering of the skin and mucous membranes results in extracutanous complications such as failure to thrive, dyspnea, anemia, and susceptibility to infections.[185-187] The severity of these symptoms and complications are so overwhelming, that they lead to death in early childhood.[188,189]

Another potential lethal variant is JEB with pyloric atresia (JEB-PA), which is caused by mutations in the genes coding for α6β4, and is associated with generalized blistering, pyloric atresia, aplasia cutis, and urinary tract stenosis.[126,127,190] In most cases JEB-PA has a fatal outcome despite surgical correction of the pyloric atresia, however some milder non-lethal cases have been described.[190-194]

Other rare subtypes are JEB of late onset (JEB-lo), JEB inversa, and LOC syndrome. In JEB-lo blistering starts in childhood or young adulthood and is primarily located on the hands and feet.[195-199] In chapter 9 we reveal the pathogenesis of JEB-lo.

In JEB inversa blistering is predominantly located in the intertriginous areas and the presumed pathogenesis are mutations in the genes coding for LM-332.[161,199]

The potentially lethal LOC (laryncho-onycho-cutaneous) syndrome is characterized by chronic granulation tissue in the mucosa, larynx, and eyes.[200-204] The pathogenesis lies in a specific out-of-frame insertion in exon 39 of *LAMA3* (the first and exclusive exon for the laminin-α3A subunit), after which an alternative initiation codon in exon 45 is used to produce a laminin α3A chain lacking 226 amino acids at the N-terminus.[202]

Aim of this thesis

The Center for Blistering Diseases, located at the University Medical Center Groningen, is the single national referral center for EB in the Netherlands. We see patients from all over the country and also from other countries, such as Belgium and Germany. Since

1988 over 400 patients have visited our center and each of these patients have been carefully examined, diagnosed, treated, and followed-up. These data have been documented in the Dutch EB Registry. This information gives us great insight into the spectrum of EB in the Netherlands, such as the epidemiology, clinical features, diagnostic features, mutational profile, disease progression, and therapeutic options. The aim of this thesis was to distill and summarize this information for JEB. Our goal is that this knowledge will function as a guide for health care professionals to base future decisions to enhance the care for EB patients.

In **chapter 2 and 3** we focus on the lethal JEB-H subtype. We discuss all 22 patients who have visited our center for the past 23 years between 1988 and 2011. In **chapter 2** we discuss the mutational profile and genotype-phenotype correlation of these patients. Furthermore, we have calculated the incidence and carrier frequency of JEB-H in the Netherlands. In **chapter 3** we report the long-term follow-up of these 22 JEB-H patients and, among others, the following topics are discussed: clinical symptoms, complications, end-of-life, treatment, and predictors of lifespan.

Losing a child suffering from EB has a great impact on parents. Health care professionals should attend to their needs, and in **chapter 4** we have identified these needs by conducting in-depth semi-structured interviews with parents who have lost their child to a lethal EB subtype.

Chapter 5 focuses on the genetic basis of the Dutch JEB-nH cohort. As expected most pathogenic mutations were found in the genes coding for Col17 and LM-332. However, we have found pathogenic *ITGB4* mutations in a substantial percentage of our JEB-nH cohort, whereas in the literature only a sporadic case has been described. In this chapter we describe these patients.

In **chapter 6** the occurrence of squamous cell carcinoma (SCC) in JEB patients is discussed. We show that the risk of developing an SCC differs for the subtypes JEB-H and JEB-nH, and that a discrepancy is seen with the frequencies observed in the United States National EB Registry (NEBR). In **chapter 7** we propose that the limited diagnostic validity of the NEBR is the cause of this discrepancy.

In **chapter 8** we present our experiences with the relatively simple method of punch grafting to treat small, persistent ulcers that adversely affect the quality of life of JEB-nH patients.

JEB is characterized by an autosomal recessive inheritance pattern. However, some carriers of *COL17A1* mutations have been associated with mini-symptoms, such

as teeth abnormalities. In **chapter 9** we describe the first carriers of a *LAMA3* null mutation to show teeth abnormalities.

JEB-lo is a rare subtype with only 22 reported cases, and with an unknown pathogenesis. In **chapter 10** we show that JEB-lo is caused by a specific missense mutation in the NC4 domain of *COL17A1*.

IF antigen staining is crucial in diagnosing JEB and in appointing a candidate gene for molecular analysis. Therefore, it is important that antibodies remain available for IF analysis. We noticed an imminent extinction of antibodies against the Col17 endodomain. In **chapter 11** we describe the development of five new versatile antibodies against the Col17 endodomain that are functional in IF antigen staining, immunohistochemistry staining and western blotting. Furthermore, we discuss the biological turnover of Col17 in the BMZ.

In **chapter 12** we focus on the Dutch basal EBS cohort. Previous work in our group has shown that *KRT5*, *KRT14*, and *PLEC1* mutations underlie 83% of all Dutch basal EBS cases. The pathogenesis of the remaining 17% of the cases remains unknown. In these cases we have analyzed all other known genes that are associated with basal EBS (*DST, ITGB4*, and *COL17A1*).

In **chapter 13** we have developed and validated a Dutch quality of life measurement tool specifically for EB patients. This questionnaire was derived from the English variant developed in Australia that was launched in 2009. Furthermore, in this chapter we assess the quality of life in Dutch EB patients.

Finally, in **chapter 14** we discuss our work and give ideas for future studies.

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2

Herlitz junctional epidermolysis bullosa: diagnostic features, mutational profile, incidence, and population carrier frequency in the Netherlands

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Abstract

Background Junctional epidermolysis bullosa, type Herlitz (JEB-H) is a lethal, autosomal recessive blistering disease caused by null mutations in the genes coding for the lamina lucida/densa adhesion protein laminin-332 (*LAMB3, LAMA3* and *LAMC2*).

Objective To present the diagnostic features and molecular analyses of all 22 JEB-H patients in the Dutch Epidermolysis Bullosa Registry between 1988-2011, and to calculate the disease incidence and carrier frequency in the Netherlands.

Methods All patients were analyzed with immunofluorescence antigen mapping (IF), electron microscopy (EM), and molecular analysis.

Results The average lifespan of our JEB-H patients was 5.8 months (range 0.5-32.6 months). IF showed absent (90.9%) or strongly reduced (9.1%) staining for laminin-332 with monoclonal antibody GB3. In EM the hemidesmosomes and subbasal dense plates were hypoplastic or absent. We identified mutations in all 22 patients: in 86.4% we found *LAMB3* mutations, in 9.1% *LAMA3* mutations, and in 4.5% *LAMC2* mutations. We found three novel splice site mutations in *LAMB3*: (1) c.29-2A>G resulting in an out-of-frame skip of exon 3 and a premature termination codon (PTC); (2) c.1289-2_1296del10 leading to an out-of-frame skip of exon 12 and a PTC; (3) c.3228+1G>T leading to an exon 21 skip.

Conclusions All diagnostic tools should be evaluated to clarify the diagnosis JEB-H. We have identified eleven different mutations in 22 JEB-H patients, three of them novel. In the Netherlands the incidence rate of JEB-H is 4.0 per one million live births. The carrier frequency of a JEB-H mutation in the Dutch population is one in 249.

Introduction

Junctional epidermolysis bullosa (JEB) is an autosomal recessive subtype of the hereditary heterogeneous mechanobullous disease epidermolysis bullosa, and is characterized by a blister level through the lamina lucida. JEB can be roughly subdivided into two major subtypes: (1) JEB, type non-Herlitz (JEB-nH), in which patients have a normal lifespan, and (2) JEB, type Herlitz (JEB-H), in which patients do not survive childhood.[1,2] In 80% JEB-H is caused by null mutations in *LAMB3* encoding for the laminin β3 chain, in the remaining cases null mutations have been detected in *LAMC2* coding for the laminin γ2 chain, or *LAMA3* coding for the laminin α3 chain.[3-6] To date, 101 mutations have been associated with JEB-H, 52 of them located in *LAMB3*, 25 in *LAMA3*, and 24 in *LAMC2*.[6-10] These three genes encode the laminin chains that together compose the glycoprotein laminin-332 (LM-332). It is secreted by keratinocytes as a cross-shaped heterotrimer and provides epidermal-dermal adhesion by linking hemidesmosomal proteins, such as integrin α6β4 and type XVII collagen to type VII collagen, which forms anchoring fibrils in the dermis.[6] In JEB-H, LM-332 is either absent or strongly reduced.[11,12] In the absence of LM-332 no hemidesmosomes can be formed, leading to extensive blistering of the skin and mucous membranes after minor trauma.[6,13-15] LM-332 is also involved in regulating the motility and proliferation of keratinocytes, and its absence leads to impaired wound healing with chronic erosions and formation of granulation tissue as a result. The absence of LM-332 in extracutanous tissues leads to nail anomalies, dental hypoplasia, eye involvement, anemia, gastrointestinal involvement resulting in failure to thrive, and laryngeal involvement with hoarseness, dyspnea and stridor as a result.[2,16,17] The complications are so overwhelming and therapy resistant that all JEB-H patients die within the first few years of their life.[2,18] JEB-H is a rare disease, and only a few incidence rates and carrier risks of JEB-H have been reported worldwide. In the USA the incidence rate is estimated at <0.41 new cases per million births, and in Italy it is estimated at 0.68 per million births.[7,19] The carrier frequency is the risk of an individual in a given population carrying a JEB-H mutation, and it is helpful in calculating recurrence risks for the disease. In the USA the carrier frequency has been estimated at 1/781 and in Italy at 1/375.[7,20]

We describe the diagnostic features and mutational profile of Dutch JEB-H patients, and have estimated the incidence and carrier frequency of JEB-H in the Dutch population.

Materials and Methods

Patients

JEB-H patients referred to the Centre for Blistering Diseases in Groningen between 1988 and 2011 were included in the Dutch Epidermolysis Bullosa Registry. As the single national referral center for EB, we are almost certain that all JEB-H patients born in the Netherlands have been included in this study. The patients were referred by their dermatologist or pediatrician. The diagnosis of JEB-H was established on the basis of immunofluorescence antigen mapping (IF), electron microscopy (EM) and mutation analysis.

Immunofluorescence antigen mapping

For IF 4 mm punch biopsies of perilesional skin and non-lesional skin on the inner upper arm were taken and prepared for IF microscopy as described before.[21] The monoclonal antibody (mAb) GB3 was used to stain for LM-332, BM165 for the laminin α3 chain, K140 for the laminin β3 chain, and D4B5 for the laminin γ2 chain.[21] As a secondary step against these primary mouse mAb, we used Alexa488-conjugated goat anti-mouse IgG.[22] The slides were examined with a Leica DMRA fluorescence microscope (Leica, Solms, Germany). Staining intensity was compared to controls and scored as normal (+++), slightly reduced (++), reduced (+), strongly reduced (±), or absent (-).

Electron microscopy

For EM 2 mm punch biopsies were taken at the same locations as for IF and prepared as described previously.[23] The biopsies were analyzed with a Philips CM100 transmission electron microscope (Philips, Eindhoven, the Netherlands) for the ultrastructural level of the blister split, and for any abnormalities in hemidesmosomes, subbasal dense plates, tonofilaments, anchoring filaments and anchoring fibrils.

Mutation analysis

Genomic DNA was extracted from peripheral blood. The 22 coding exons of the *LAMB3* gene (GenBank accession number NM_001127641.1), the 23 coding exons of the *LAMC2* gene (GenBank accession number NM_005562.2), and the 38 coding exons of the *LAMA3* gene (GenBank accession number NM_000227.3), were amplified with polymerase chain reaction (PCR) together with their flanking introns. For the amplicons

in which mutations were identified, the primer sequences are shown in Table 1. PCR amplification was performed with 1.5 µl sense primer and 1.5 µl antisense primer in a concentration of 0.5 pmol/µl, 2 µl genomic DNA in a concentration of 40 ng/µl, and 5 µl Amplitaq gold® Master Mix (Applied Biosystems, Foster City, CA, USA). PCR was performed on a Perkin-Elmer Geneamp 9700 (Applied Biosystems) using the following PCR program: an initial denaturation at 94°C for 1 minute, followed by 5 cycles of denaturation at 94°C for 5 seconds, annealing starting at 65°C for 30 seconds with a step down of 1°C every cycle, and elongation at 72°C for 1 minute, followed by 20 cycles of denaturation at 94°C for 5 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 1 minute, followed by 15 cycles of denaturation at 94°C for 5 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute with a final step at 72°C for 5 minutes, after which the samples are cooled down to 20°C. Afterwards the PCR products were purified with 4 µl Exo-SAP IT (Fermentas, Vilnius, Lithuania) by incubating at 37°C for 15 minutes, and inactivated at 80°C for 15 minutes. All amplicons were sequenced directly on an automated DNA sequencer (ABI 3730 DNA analyzer, Applied Biosystems). Verification of mutations was performed by repeating the PCR amplification and sequence analysis of the relevant exons and the inheritance from both parents was verified.

Table 1. Overview of the primer sequences used to identify the mutations in our JEB-H cohort

Gene	Exon	Forward primer	Reverse primer
LAMB3	3	5'- GCGAGTGGAAGCTTGAGTGAG-3'	5'- TACACAGGGCTTGGCCTAC-3'
	7	5'-GCCTCATAGCCATGGTCTATC-3'	5'- GCAAGCAGGGCAAGTATC-3'
	8	5'-AGGATGGCCCACGGTATCAC-3'	5'-GCCCTTCCTGCTTTACAGGAG-3'
	10	5'-TCCCTGCCTTGATCATCTTG-3'	5'-TGCCCAGGAACATGTACTC-3'
	11	5'-TGAGGCACAGAGGGATTAAG-3'	5'-TACCCTGCAGTGGGATTTC-3'
	12	5'-GCCCTGCCCAGTTCTTTTC-3'	5'-AAGACGCCCAGTCTGACAG-3'
	14	5'-AGCCTCAGGTCAGACCTC-3'	5'-ACCAGCATGCCCGGTACT-3'
	15	5'-CCCTTAGCCTGTGGATTCTCTG-3'	5'-GCGAGAAGCATGAGGAATGG-3'
	21	5'-GTGAGAGGTGGGCAATTGTG-3'	5'-TGACTCTCAAGCCTCTTCTG-3'
LAMC2	8	5'-TTAGCTGTTCCCGTATCCTC-3'	5'-TGGTGCTGGGTGCTGACATC-3'
LAMA3	30	5'-CCTATTGTGCTGCCAAAC-3'	5'-CCCAATATCTCCCACAAG-3'

Disease incidence and carrier frequency calculation

The incidence of JEB-H patients born in the Netherlands was calculated from data in the Dutch Epidermolysis Bullosa Registry. The number of births per year in the Netherlands was collected from Statistics Netherlands (www.cbs.nl). The Dutch carrier frequency was calculated using the Hardy-Weinberg law and the equation $p^2+2pq+q^2 = 1$, in which 2pq is the heterozygote carrier frequency.

Results and Discussion

Demographics

We saw 22 JEB-H patients (13 males, 9 females) in our centre between 1988 and 2011, of whom 18 patients were born in the Netherlands, two patients were Caucasian twin sisters born in Germany, and two patients were Caucasians born in Belgium (Table 2). Of the 18 Dutch patients, 13 were Caucasian, two originated from Africa, two from Central Asia, and one from the Middle East (Table 2). One Dutch Caucasian patient and the three patients originating from Central Asia and the Middle-East had consanguineous parents. All patients died before the age of three years (average age 5.8 months, range 0.5–32.6 months) (Table 2).

Diagnostic features

For the diagnosis of JEB, IF analysis is the main important diagnostic tool. It yields a candidate gene for molecular diagnosis, and is important in differentiating between JEB-H (death in early childhood) and JEB-nH (survival to adulthood). In all our patients the cleavage plane of blisters was located through the lamina lucida (Table 3). As expected from the literature, immunofluorescence staining for LM-332 with mAb GB3 was negative in 20 patients (90.9%), and strongly reduced in two patients (9.1%) (Table 3).[11,12] In our opinion, absence of LM-332 staining is consistent with a diagnosis of JEB-H. The biggest pitfall in IF is when GB3 staining is strongly reduced, which can be seen in cases of JEB-H as well as JEB-nH.[12,24] Staining with mAb BM165 for the laminin α3 chain was performed in 11 patients: two patients had normal, three patients slightly reduced, five patients reduced, and one patient had absent staining (Table 3). Molecular analysis revealed that none of these patients had mutations in *LAMA3*. Staining for the laminin γ2 chain with mAb D4B5 was reduced in eight patients, strongly reduced in one patient, and absent in two patients (Table 3). One of the two patients with absent staining for the

laminin γ2 chain did indeed have *LAMC2* mutations. Staining for the laminin β3 chain with mAb K140 was reduced, strongly reduced, or absent in patients with *LAMB3* mutations. However, in two patients with *LAMA3* and *LAMC2* mutations, staining with mAb K140 was also reduced or absent (Table 3). Our results indicate that staining of separate LM-332 chains does not assist in defining a candidate gene in JEB-H patients. This is consistent with the results of McMillan *et al.*[25]

The results of EM analysis in our patients are listed in Table 3 and indicate that: (1) in almost all patients the hemidesmosomes are reduced in number and hypoplastic; in only one patient the hemidesmosomes were present in a normal number, (2) in all patients the subbasal dense plates were absent, (3) the tonofilaments were reduced in number and/or had an inadequate projection into the hemidesmosomes in all except one patient, who had normal tonofilaments. Although in JEB-H the hemidesmosomes and subbasal dense plates are absent or attenuated, whereas in JEB-nH they are normal or attenuated, EM analysis is often not sufficient to differentiate between JEB-H and JEB-nH without further testing.[26,27]

Molecular diagnosis allows further differentiation between JEB-H and JEB-nH. In patients with JEB-H we expect to find two null mutations caused by nonsense or frameshift mutations, leading to downstream premature termination codons (PTC), while in JEB-nH we expect missense mutations or in-frame deletions, whether or not in combination with a null mutation.[9] However, cases of patients carrying two null mutations, who had a milder phenotype due to genetic escape mechanisms, such as illegitimate in-frame/exon skipping restoring the open reading frame, have also been reported.[28,29]

Table 2. Overview of our 22 JEB-H patients: patient characteristics and molecular analysis

	Patient	Sex	Age (months)	Ethnic background	Genetic status	Gene	Nucleotide	Amino acid	Exon	Conseque-nce	Reference
1	007-01	F	4.0 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5
2	008-01	M	14.1 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5
3	021-01	M	1.7 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5
4	061-01	M	1.5 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5
5	081-01	M	3.6 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5
6	091-01	F	2.5 †	Caucasian	Homozygous	*LAMB3*	c.786delG	p.Lys262AsnfsX134	8	Frameshift	35
7	107-01	F	32.6 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5
8	124-01	F	15.1 †	Caucasian	Compound heterozygous	*LAMB3*	c.1903C>T / **c.3228+1G>T**	p.Arg635X / Splice site	14 / *21	PTC / Splice site	This article, 5
9	126-01	F	0.6 †	Caucasian	Compound heterozygous	*LAMB3*	c.1903C>T / c.565-2A>G	p.Arg635X / Splice site	14 / *6	PTC / Splice site	20, 35
10	142-01	M	5.0 †	Middle East (Kurdistan)	Homozygous	*LAMB3*	c.1978C>T	p.Arg660X	15	PTC	33
11	176-01	M	9.4 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5
12	187-01	M	5.7 †	Africa (Nigeria/Sudan)	Compound heterozygous	*LAMB3*	**c.29-2A>G** / c.957ins77	Splice site / p.Asn345MetfsX77	*2 / 10	Splice site / Frameshift	This article, 34
13	188-01	F	0.5 †	Central Asia (Afghanistan)	Homozygous	*LAMB3*	**c.1289-2_1296del10**	Splice site	*11/12	Splice site	This article
14	200-01	F	0.9 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5
15	206-01	M	0.8 †	Africa (Burundi)	Homozygous	*LAMB3*	c.957ins77	p.Asn345MetfsX77	10	Frameshift	34
16	212-01	M	5.0 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5

Table 2 continued

	Patient	Sex	Age (months)	Ethnic background	Genetic status	Gene	Nucleotide	Amino acid	Exon	Conseque-nce	Reference
17	231-01	M	3.5 †	Central Asia (Pakistan)	Homozygous	*LAMC2*	c.1045C>T	p.Arg349X	8	PTC	37
18	238-01	M	2.5 †	Caucasian	Homozygous	*LAMB3*	c.1903C>T	p.Arg635X	14	PTC	5
19	155-01	F	6.1 †	Caucasian (Germany)	Homozygous	*LAMA3*	c.3928A>T	p.Lys1310X	30	PTC	9
20	155-02	F	6.0 †	Caucasian (Germany)	Homozygous	*LAMA3*	c.3928A>T	p.Lys1310X	30	PTC	9
21	263-01	M	3.8 †	Caucasian (Belgium)	Compound heterozygous	*LAMB3*	c.1903C>T / c.1978C>T	p.Arg635X / p.Arg660X	14 / 15	PTC / PTC	5, 33
22	269-01	M	2.0 †	Caucasian (Belgium)	Compound heterozygous	*LAMB3*	c.31insC / c.1903C>T	p.Leu11ProfsX43 / p.Arg635X	3 / 14	Frameshift/ PTC	5, 33

M male; F female; † age at death; NA not available; * intron; PTC premature termination codon; novel mutations are in bold

Table 3. Immunofluorescence antigen mapping and electron microscopy in our JEB-H cohort (n=22)

	Patient	IF: Blister mapping	IF: GB3	IF: BM165 (α3)	IF: K140 (β3)	IF: D4B5 (γ2)	EM: Split level	EM: Hemidesmosomes	EM: Subbasal dense plate	EM: Tonofilaments
1	007-01	Junctional	-	++	+/-	+	Junctional	reduced number; hypoplastic	absent	NA
2	008-01	Junctional	-	NA	+	NA	Junctional	reduced number; hypoplastic	absent	inadequate adhesion to HD
3	021-01	NA	-	++	+/-	+	Junctional	reduced number; hypoplastic	absent	inadequate adhesion to HD
4	061-01	Junctional	-	NA	+	NA	NA	hypoplastic	absent	reduced number
5	081-01	Junctional	-	NA	+	NA	Junctional	reduced number	NA	reduced number
6	091-01	Junctional	-	NA	NA	NA	Junctional	hypoplastic	absent	inadequate adhesion to HD
7	107-01	Junctional	-	NA	+	-	Junctional	reduced number; hypoplastic	absent	reduced in number
8	124-01	Junctional	+/-	NA	NA	NA	Junctional	reduced number; hypoplastic	absent	Inadequate adhesion to HD
9	126-01	Junctional	-	NA	NA	NA	Junctional	NA	NA	NA
10	142-01	Junctional	-	NA	+/-	NA	Junctional	reduced number; hypoplastic	absent	NA
11	176-01	Junctional	-	NA	NA	NA	Junctional	hypoplastic	absent	NA
12	187-01	Junctional	-	+	-	+	Junctional	reduced number; hypoplastic	absent	reduced number; inadequate adhesion to HD
13	188-01	Junctional	-	+	+	+	Junctional	reduced number; hypoplastic	absent	reduced number; inadequate adhesion to HD
14	200-01	Junctional	-	+	-	+	Junctional	reduced number; hypoplastic	absent	NA
15	206-01	Junctional	-	-	-	+/-	Junctional	reduced number; hypoplastic	absent	NA
16	212-01	Junctional	-	+	+	+	Junctional	reduced number; hypoplastic	absent	NA
17	231-01	Junctional	-	+	-	-	Junctional	reduced number; hypoplastic	absent	normal
18	238-01	Junctional	-	+++	+	NA	Junctional	reduced number; hypoplastic	absent	NA
19	155-01	Junctional	+/-	NA	+	NA	NA	NA	NA	NA
20	155-02	Junctional	-	NA	NA	NA	NA	NA	NA	NA

Table 3 continued

	Patient	IF: Blister mapping	IF: GB3	IF: BM165 (α3)	IF: K140 (β3)	IF: D4B5 (γ2)	EM: Split level	EM: Hemidesmosomes	EM: Subbasal dense plate	EM: Tonofilaments
21	263-01	Junctional	-	+++	+/-	+	NA	normal number; hypoplastic	absent	NA
22	269-01	Junctional	-	++	-	+	NA	NA	NA	NA

IF immunofluorescence antigen mapping; GB3 monoclonal antibody (mAb) staining for laminin-332; BM165 mAb staining for laminin α3 chain; K140 mAb staining for laminin β3 chain; D4B5 mAb staining for γ2 chain; - absent; +/- strongly reduced; + reduced; ++ slightly reduced; +++ normal; NA not available; EM electron microscopy; HD hemidesmosome

Molecular analysis

We identified mutations in all 22 patients. Nineteen patients (86.4%) were explained by mutations in *LAMB3* (Table 2). Of these, 14 showed homozygosity for a single mutation, and five appeared to be compound heterozygous. Two patients (9.1%) were homozygous for a *LAMA3* null mutation. One patient (4.5%) was homozygous for a *LAMC2* null mutation. All the mutations were confirmed in the parents (i.e. we detected no *de novo* mutations in our patients).

We identified three novel mutations in *LAMB3*: (1) in patient 187-01 we saw heterozygosity of the novel mutation c.29-2A>G on the maternal allele, together with the known mutation c.957ins77 on the paternal allele. The patient was from African descent with a Nigerian mother and a Sudanese father. The mutation c.29-2A>G is located in the highly conserved consensus AG acceptor splice site sequence of intron two (Fig.1a). Several splice site prediction programs predict the loss of the acceptor splice site at the 5' end of exon 3, most likely resulting in an out-of-frame exon-skipping of exon 3, leading to a PTC in exon 4.[30,31] (2) We found the homozygous novel mutation c.1289-2_1296del10 in the Pakistani patient 188-01, which deletes the acceptor splice site sequence at the 5' end of intron 11 (Fig.1b). This most likely results in an out-of-frame skipping of exon 12 and a PTC. (3) We detected the heterozygous donor splice site mutation c.3228+1G>T on the paternal allele in the Caucasian patient 124-01, together with the known maternal mutation p.Arg635X. The highly conserved consensus GT donor splice site sequence in intron 21 is abolished by the mutation c.3228+1G>T, which most likely results in the skipping of exon 21 (Fig.1c).[30,31] The loss of exon 21 does not lead to an out-of-frame deletion, which may account for the strongly reduced staining of LM-332 with mAb GB3 in patient 124-01. Diagnosis of the Herlitz phenotype was therefore uncertain until the patient died at the age of 15.3 months. In the literature, two very similar mutations abolishing the same donor splice site showed two different phenotypes.[20,32] A Hispanic patient carrying the compound heterozygous mutation c.3228+1G>A, together with p.Q868X, had the milder non-Herlitz phenotype,[32] whereas a patient carrying the mutation c.3228+2T>A, together with an unknown mutation, was affected with the Herlitz phenotype.[20]

The other mutations found in *LAMB3, LAMA3* and *LAMC2* in our JEB-H cohort have been described previously, and included nonsense mutations, frameshift mutations, and a splice site mutation.[5,9,20,33-35] All these mutations lead to a PTC, resulting most likely in mRNA decay.[9]

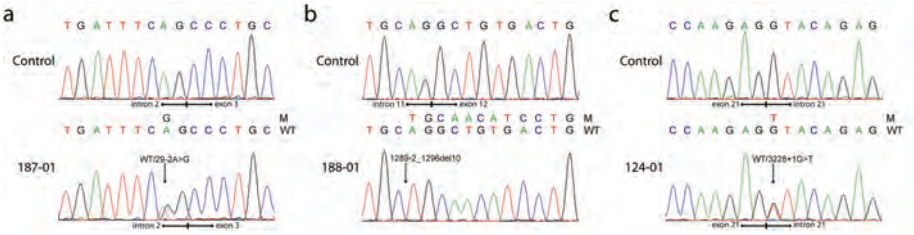

Figure 1. Novel splice site mutations in *LAMB3* (GenBank NM_001127641.1) associated with junctional epidermolysis bullosa, type Herlitz. (a) Molecular analysis revealed a heterozygous c.29-2A>G mutation in patient 187-01, (b) a homozygous deletion of 10 base pairs designated c.1289-2_1296del10 in patient 188-01, and (c) a heterozygous c.3228+1G>T mutation in patient 124-01.

In *LAMB3* the most frequent mutation we found was p.Arg635X; it accounts for 24 alleles (63.2%) in 14 patients (73.7%). Ten patients carried the p.Arg635X mutation homozygously, while four patients were compound heterozygous for p.Arg635X, combined with either the nonsense mutation p.Arg660X, the insertion c.31insC, or one of the splice site mutations c.3288+1G>T or c.565-2A>G. Although all these patients were Caucasians born in the Netherlands and Belgium, p.Arg635X represents a hotspot mutation rather than propagation of a common ancestral allele.[33,36] In the literature, the frequency of p.Arg635X in all mutated *LAMB3* alleles in JEB-H is estimated at 45.4%.

The mutation c.957insC in *LAMB3* was seen in two of our patients of African descent. Patient 206-01 has parents originating from Burundi and was homozygous for the c.957insC mutation. Patient 187-01 is discussed above and carried the mutation heterozygously with the novel splice site mutation c.29-2A>G. In the literature, the mutation c.957insC is highly prevalent, especially in patients of African origin.[20,35]

In two patients we detected the recurrent mutation p.Arg660X in *LAMB3*: homozygously in a Dutch patient (142-01) of Kurdish descent, and heterozygously in a Caucasian Belgian patient along with p.Arg635X (263-01).

In the Caucasian Belgian patient 269-01, the insertion c.31insC was seen heterozygously with p.Arg635X. The insertion c.31insC is a recurrent mutation first described by Kivirikko *et al*. and it leads to a frameshift resulting in a PTC.[10,33]

We did not detect the hotspot mutation p.Arg42X in any of our patients.[33]

In the Caucasian patient 091-01 the deletion c.786delG in *LAMB3* was present homozygously. The mutation was described by Varki *et al*.[35] and it leads to a frameshift with a PTC 134 amino acids downstream.

We found *LAMA3* mutations in Caucasian monozygotic twin sisters originating from Germany (155-01 and 155-02). They both showed homozygosity for the nonsense mutation c.3896A>T (p.Lys1310X). These patients were originally seen in Germany, and were referred to us at age 5.8 months. These patients were probably included in reports by Mühle *et al*.[9]

A patient of Pakistani origin (231-01) was the only patient in our cohort to carry a *LAMC2* mutation. He showed homozygosity for the nonsense mutation c.1045C>T (p.Arg349X).[37]

Genotype-phenotype correlations

Apart from the fact that JEB-H is caused by null mutations in the genes encoding for LM-332, little is known about the genotype-phenotype correlation in JEB-H patients. In 12 patients analyzed by Mühle *et al*. there were no clear-cut genotype-phenotype correlations.[9] In our cohort 73% of the patients died before they reached the age of six months. Only six patients became six months or older, but they also died in early childhood before reaching the age of three years. Of the six patients who survived to six months, three patients were homozygous for the *LAMB3* mutation p.Arg635X, one patient carried the *LAMB3* mutation p.Arg635X heterozygously with c.3228+1G>T, and two patients were homozygous for the *LAMA3* mutation p.Lys1310X. The mutation p.Arg635X in *LAMB3* is most frequently associated with a longer survival, however, one of our patients who had one of the shortest recorded lifespan of 0.9 months, also appeared to be homozygous for this mutation. It seems that no apparent genotype-phenotype correlations can be made in our cohort.

Disease incidence and carrier frequency

Of the 22 JEB-H patients seen in our center between 1988 and 2011, 18 were born in the Netherlands (Table 2, #1-18). This means that an average of 0.78 JEB-H patients is born in the Netherlands per year. With an average of 193,305 annual live births per year in the Netherlands between 1988 and 2011, we calculated the incidence of JEB-H at 4.0 new cases per million live births (Table 4), i.e. one case of JEB-H per 247,524 births. The Dutch incidence is more than five times higher than that reported for the USA and Italy, with incidence rates of <0.41 and 0.68 per million live births, respectively.[7,19] Among the 18 JEB-H patients born in the Netherlands since 1988, 94% (n=17) had mutations in *LAMB3*, 6% (n=1) had mutations in *LAMC2*, and none had mutations in *LAMA3*. This allowed us to estimate the JEB-H population carrier frequency (Table 4).

The risk of an individual in the Dutch population carrying a JEB-H mutation is 1/249; the risk of carrying a JEB-H mutation in *LAMB3* is 1/266, and in *LAMC2* 1/1056 (Table 4). The carrier risk of a JEB-H mutation in the USA has been established at 1/781,[19,20] and in Italy 1/375.[7] Since mutations were identified in *LAMB3, LAMC2*, or *LAMA3* in all 16 Italian JEB-H patients, the carrier frequency of the Italian cohort for the separate LM-332 chains could be calculated at 1/917 for *LAMB3*, 1/858 for LAMC2, and 1/2427 for *LAMA3*.[7] The Dutch risk of carrying *LAMB3* mutations is higher than in the Italian population, whereas *LAMC2* and *LAMA3* mutations are more common in the Italian population.

Table 4. Dutch incidence rate and carrier frequency for JEB-H

	Proportion	Incidence per 10^6 births	Carrier frequency
LAMA3 mutations	0/18 (0%)	-	-
LAMB3 mutations	17/18 (94.4%)	3.82	1/266
LAMC2 mutations	1/18 (5.6%)	0.22	1/1056
Total	18/18 (100%)	4.04	1/249

It is remarkable that the incidence and the carrier risk for JEB-H in the Netherlands is markedly higher than in the United States and Italy. This discrepancy might be explained by a different genetic background, or by the small number of patients studied, which could lead to distortion of the results. However, the most likely cause of this discrepancy is an under-reporting of cases in the United States and Italy, leading to an underestimated incidence and carrier risk in these countries. As we are almost certain that all JEB-H cases born in the Netherlands for the past 23 years have been included in this study, we believe that the calculated Dutch incidence and carrier risk is quite accurate. However, the risk of under-reporting in our study is not excluded, which would mean that the actual incidence and carrier risk are even higher. It would be interesting if incidence rates were calculated in other countries, to see if regional and/or global trends can be discovered. With more accurate information about the incidence and carrier frequency in the Netherlands, we will be able to provide better genetic counseling. Although the carrier frequency is higher than seen in other countries, there is still only a small recurrence risk in the offspring of healthy siblings and other family members. Molecular prenatal diagnosis[38] and preimplantation genetic diagnosis[39,40] do therefore not seem to be indicated. However, for the parents of an affected child, a 25% recurrence risk clearly justifies molecular diagnosis in any further pregnancies.[41]

Conclusion

We have presented the diagnostic features of 22 JEB-H patients who visited our EB referral center between 1988-2011. Their average lifespan was 5.8 months (range 0.5-32.6 months). We identified mutations in all 22 patients, with a total of 11 different mutations in *LAMB3*, *LAMC2* and *LAMA3*. Three of the mutations we found in *LAMB3* had not been reported previously. In the Netherlands, the incidence of JEB-H is 4.0 new cases per million live births and the carrier frequency of a JEB-H mutation in the Dutch population is 1/249.

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3

Long-term follow-up of patients with Herlitz type junctional epidermolysis bullosa

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Abstract

Background Junctional epidermolysis bullosa, type Herlitz (JEB-H) is a rare, autosomal recessive disease caused by absence of the epidermal basement membrane adhesion protein laminin-332. It is characterized by extensive and devastating blistering of the skin and mucous membranes, leading to death in early childhood.

Objective Here we present the long-term follow-up of a cohort of JEB-H patients, and we give guidelines for prognosis, treatment and care.

Methods All JEB-H patients included in the Dutch Epidermolysis Bullosa (EB) Registry between 1988 and 2011 were followed longitudinally by our EB team. Diagnosis was established by immunofluorescence antigen mapping, electron microscopy and DNA analysis.

Results In total, we included 22 JEB-H patients over a 23-year period. Their average age at death was 5.8 months (range 0.5-32.6 months). The causes of death were, in order of frequency, failure to thrive, respiratory failure, pneumonia, dehydration, anemia, sepsis, and euthanasia. The pattern of initial weight gain is a predictor of lifespan in these patients. Invasive treatments to extend life did not promote survival in our patients.

Conclusions It is important to diagnose JEB-H as soon as possible after birth, so that the management can be shifted from life saving to comfort care. The palliative end-of-life care can take place in hospital, but is also safe in the home setting. Suffering in JEB-H patients can become so unbearable, that in some patients, who do not respond to adequate analgesic and sedative treatment, newborn euthanasia, performed according to the Groningen protocol, is legally permitted in the Netherlands.

Introduction

Inherited epidermolysis bullosa (EB) is a diverse group of genodermatoses characterized by blistering of the skin and mucous membranes after trivial trauma.[1,2] Junctional epidermolysis bullosa (JEB) is an autosomal recessive disorder characterized by a blister level through the lamina lucida. It is roughly subdivided into JEB, type non-Herlitz (JEB-nH) in which patients survive through to adulthood, and the rarer and more severe JEB, type Herlitz (JEB-H). The incidence of JEB-H is 4.0 per million live births based on the Dutch EB Registry.[3] JEB-H is caused by null mutations in the genes encoding an adhesion protein in the epidermal basement membrane: laminin-332.[4-8] In the absence of laminin-332 in JEB-H, the skin is prone to generalized painful blistering, with persistent erosions and granulation tissue formation. Other symptoms are nail anomalies, corneal erosions, enamel hypoplasia, hoarseness, stridor, dyspnea, anemia, reflux, dysphagia, constipation and failure to thrive.[1,6,9-11]

Patients suffering from JEB-H die in the first few years of life,[6,12,13] with the most important causes of death being failure to thrive, sepsis, pneumonia, and respiratory failure.[12,14] There is no cure for JEB-H patients. Treatment is aimed at comfort care, although there is no consensus on the extent of invasive medical interventions that should be performed, since it seems that these interventions do not promote survival.[1,6,12,13]

This study aims to describe the long-term follow-up of all JEB-H patients in the Netherlands. We discuss our results and provide some guidelines for the prognosis, medical treatment, and care of JEB-H patients.

Materials and Methods

Patients

All JEB-H patients from the Dutch EB Registry in the period 1988-2011 were included. We are almost certain that all JEB-H patients born in the Netherlands in this period have been included in the study, as the Center for Blistering Diseases in Groningen is the single national referral center for EB. Patients were inhabitants of the Netherlands, Belgium or Germany and were referred by their dermatologist or pediatrician. They were examined by one professional (MJ), and followed longitudinally by the EB team in our center. Diagnosis of JEB-H was established on the basis of clinical findings and

immunofluorescence antigen staining. Confirmation of the diagnosis was obtained by electron microscopy and mutation analysis. The results of these studies and the diagnostic criteria of JEB-H were described in a separate study.[3] Weight was converted to standard scores, which is the number of standard deviations the patient's weight is above or below the mean of gender- and age-matched weight according to WHO reference values.

Statistics

Statistical analyses were performed with SPSS 16.0 (IBM, Chicago, Ill, USA) using the Mann-Whitney test, Kruskal-Wallis test, and Spearman's rho test. Significance was classified as $p<0.05$.

Results

Patients

The Dutch EB Registry contained 22 JEB-H patients from 21 unrelated families collected between 1988 and 2011 (Table 1). Thirteen patients (59.1%) were male, and nine (40.9%) were female. All patients were inhabitants of the Netherlands, Belgium or Germany. Seventeen patients were Caucasian (77.3%). The other patients were born from parents who had migrated from Africa (9.1%), Central Asia (9.1%) and the Middle East (4.5%). Four patients had consanguineous parents. The average time between birth and the first consultation in the EB center was 36 days (range 2-179 days). Immunofluorescence antigen staining for laminin-332 with monoclonal antibody GB3 was absent in 20 patients, and strongly reduced in the other two patients (Table 1).[3]

Clinical symptoms

Blistering was present at birth in fourteen patients (63.6%); in the other eight patients blisters were noticed after an average of 3.4 days (range 0.5-10 days). Blistering and erosions gradually progressed during their life in 19 patients (86.4%), remained mild in the first four months in patients 13 and 18, and remained mild throughout her whole life in patient 7. Hypergranulation was present at birth in six patients, and developed later in ten patients. Cutis aplasia was present at birth in five patients (22.7%). Nail deformities were present at birth in all patients (100%), consisting of periungual crusts (72.7%),

Table 1. Demographics of our 22 JEB-H patients

Nr	EB-nr	Sex	Year of birth	Ethnicity	IF (GB3)	Age (months)	Cause of death	Standard score at birth	Standard score at death	Place of death	Treatment/ diagnostics at end of life*
1	007-01	F	1988	Caucasian	-	4.0 †	Failure to thrive	1.7	- 4.8	Hospital	
2	008-01	M	1988	Caucasian	-	14.1 †	Failure to thrive	- 0.5	- 12.0	Home	-
3	021-01	M	1993	Caucasian	-	1.7 †	Pneumonia	- 4.7	- 7.2	Hospital	-
4	061-01	M	1997	Caucasian	-	1.5 †	Failure to thrive	- 1.3	- 10.2	Hospital	-
5	081-01	M	1999	Caucasian	-	3.6 †	Failure to thrive	2.4	- 4.2	Home	-
6	091-01	F	2000	Caucasian	-	2.5 †	Failure to thrive	- 0.8	- 2.3	Home	-
7	107-01	F	2001	Caucasian	-	32.6 †	Respiratory failure	1.4	- 1.7	Home	Nebulizing, oral dexamethasone
8	124-01	F	2001	Caucasian	+/-	15.1 †	Respiratory failure/ larynx strictures	0.4	- 1.6	Acute hospitalization	Nebulizing, oral dexamethasone, oxygen
9	126-01	F	2002	Caucasian	-	0.6 †	Respiratory failure/ morphine elevation	0.9	0.2	Hospital	-
10	142-01	M	2003	Middle East (Kurdistan)	-	5.0 †	Dehydration / gastroenteritis	- 0.3	- 4.7	Acute hospital-ization in Iraq	-
11	155-01	F	2003	Caucasian (Germany)	+/-	6.1 †	Failure to thrive	- 1.9	- 5.8	Hospital	-
12	155-02	F	2003	Caucasian (Germany)	-	6.0 †	Failure to thrive	- 4.3	- 7.4	Hospital	-
13	176-01	M	2005	Caucasian	-	9.4 †	Respiratory failure	0.3	- 1.1	Home	-
14	187-01	M	2006	Africa (Nigeria/Sudan)	-	5.7 †	Failure to thrive	1.9	- 8.2	Home	-

Table 1 continued

Nr	EB-nr	Sex	Year of birth	Ethnicity	IF (GB3)	Age (months)	Cause of death	Standard score at birth	Standard score at death	Place of death	Treatment/ diagnostics at end of life*
15	188-01	F	2006	South-East Asia (Afghanistan)	-	0.5 †	Failure to thrive	0.4	NA	Hospital	-
16	200-01	F	2007	Caucasian	-	0.9 †	Pneumonia	- 0.9	- 2.4	Acute hospitalization	Chest X-ray, bowel X-ray, ballooning
17	206-01	M	2007	Africa (Burundi)	-	0.8 †	Anemia	- 1.9	NA	Hospital	Antibiotics
18	212-01	M	2008	Caucasian	-	5.0 †	Dehydration	- 2.8	- 5.4	Home	-
19	231-01	M	2009	South-East Asia (Pakistan)	-	3.5 †	Respiratory failure	- 2.2	- 5.6	Acute hospitalization	Chest X-ray, antibiotics, oral dexamethasone
20	238-01	M	2009	Caucasian	-	2.5 †	Euthanasia	0.1	- 6.7	Hospital	-
21	263-01	M	2009	Caucasian (Belgium)	-	3.8 †	Sepsis	- 0.6	- 2.1	Home	-
22	269-01	M	2010	Caucasian (Belgium)	-	2.0 †	Respiratory failure	1.0	- 2.3	Hospital	

M male; F female; IF immunofluorescence antigen mapping; - absent; +/- strongly reduced; Standard score is the number of standard deviations the weight was above or below the mean of gender- and age- matched weights according to WHO reference values; NA not available; Acute hospitalization occurred at end-of-life; * except for pain relief medication

raised nails (68.2%), absent nails (68.2%), distal onycholysis (54.5%), abnormally long nails (54.5%), paronychia (50%), dystrophic nails (27.3%), and subungual blisters (18.2%). The mucous membranes were affected in all patients. Hoarseness was present in 59.1% of the patients at birth, increasing to 86.4% of the patients later in life. Five patients developed teeth and all had enamel hypoplasia.

Complications

The complications encountered were failure to thrive (91.0%), anemia (54.5%) (Figure 1), dyspnea/stridor (36.4%), gastroenteritis (18.2%), edema (18.2%), fever of unknown origin (13.6%), pneumonia (13.6%), constipation (13.6%), sepsis (13.6%), vomiting (9.1%), dehydration (9.1%), conjunctivitis (9.1%), cornea erosions (9.1%), pyodermia (4.5%), bradycardia (4.5%), convulsions (4.5%), and pneumothorax (4.5%).

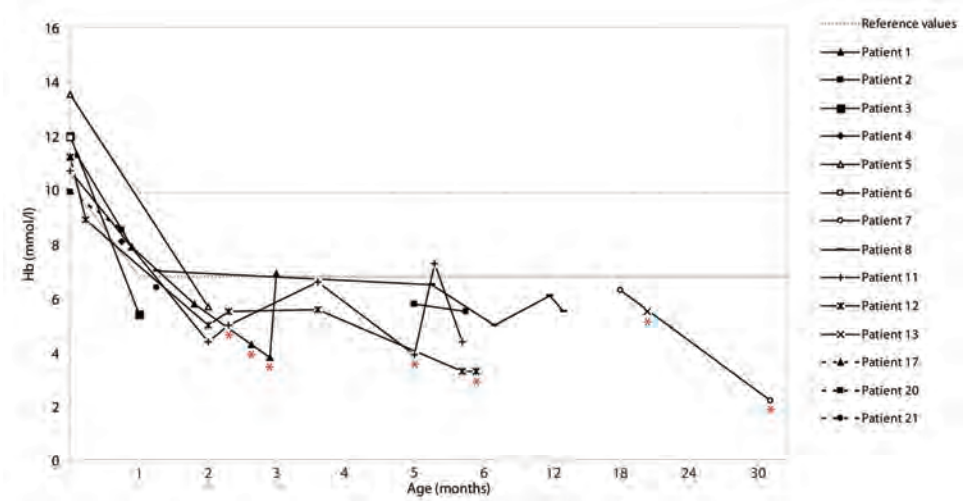

Figure 1. Hemoglobin levels in the course of life of JEB-H patients. Anemia was seen in twelve patients (54.5%). Five patients received one or more blood transfusions, indicated by a red asterisk (*). Blood transfusions in patients 1 and 11 did not result in long-term elevation of Hb values.

End-of-life

All of the 22 patients died before the age of three years. The average age at death was 5.8 months, with a range of 0.5-32.6 months (Table 1). The causes of death were failure to thrive (40.9%), respiratory failure (27.3%), pneumonia (9.1%), dehydration (9.1%), anemia (4.5%), sepsis (4.5%), and euthanasia (4.5%) (Table 1). The cause of respiratory failure was unknown in six patients, in two patients it was caused by laryngeal strictures, and in another patient by elevated morphine gifts for comfort. Patient 20 suffered from

uncontrollable discomfort and weakening, and newborn euthanasia was performed according to the Groningen protocol.[15] Fourteen of our patients died in hospital and eight patients died at home. Four of the 14 patients who died in hospital, were brought there in an acute condition by ambulance (Table 1). In three patients this was a result of worsening of dyspnea and in one patient as a result of dehydration and convulsions. No patients were hospitalized acutely due to uncontrollable pain.

Growth

The average birth weight was 3.2 kilograms (range 1.7-4.7 kg), with a mean standard score of -0.54 (Table 1). For 20 patients, a weight-for-age growth diagram could be made (Figure 2). Three types of growth can be seen: (1) patients with no weight gain. The average lifespan of these patients was 2.2 months (range 0.6-5.8), (2) patients with an insufficient initial weight gain. The average lifespan of these patients was 4.7 months (range 1.7-14.3), and (3) patients with a sufficient initial weight gain. The average lifespan of these patients was 12.0 months (range 3.9-32.6).

In the terminal phase, body weight followed two patterns: (1) patients continued their pattern of (minimal) weight gain or loss and then died, or (2) patients experienced a period of extra weight loss before they died. The latter was only seen in patients with a lifespan longer than two months. The average period of extra weight loss was 2.8 months (range 0.6-10 months). All patients had a lower standard score on death compared to their birth weight. The mean standard score at death was -4.8 (range -12.0 to 0.6) (Table 1). A total of seven patients (nos 1, 2, 4, 5, 9, 14, 20) died at a lower weight than their birth weight, after an average of 4.6 months (range 1.5-14.3), with an average loss of 0.6 kg (range 0.2-1.4 kg).

Treatment

We can subdivide the treatment given to our patients into three periods: (1) prior to diagnosis, (2) after diagnosis, and (3) at the end of life (Figure 3a). Treatment for constipation and topical antibiotics to treat skin infections, were given as required to all patients. Prior to the diagnosis, oral antibiotics were the most frequent medication given, mainly due to a suspected infectious disease. Use of antibiotics declined after the correct diagnosis was made. Anemia was treated with oral iron supplementation. In five patients one or more blood transfusions were given (Figure 1). Patients with insufficient growth were treated with nutritional supplements, such as specialized formula feeds. Nasogastric tube and/or parenteral feeding were installed in seven patients in total (in

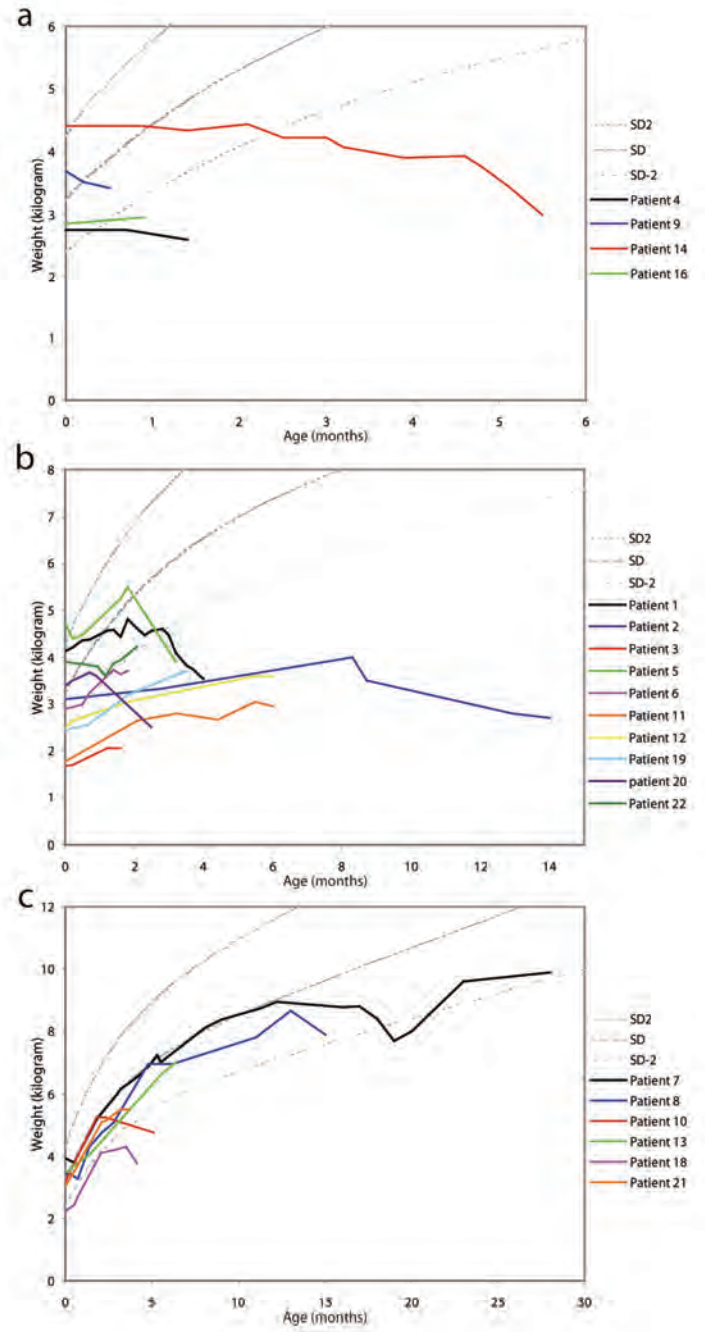

Figure 2. The weight-for-age diagram in patients with (a) no initial weight gain, (b) insufficient initial weight gain, and (c) sufficient weight gain.

two patients prior to the diagnosis, in four patients after the diagnosis was clarified, and one patient had both). None of the patients received extra feeding at the end of life. Dyspnea was treated with oral dexamethasone and/or nebulizers containing oxygen, budesonide, salbutamol and/or adrenaline. In one patient a pneumothorax was treated with a chest tube. One patient was intubated before the diagnosis was made, and was extubated when the diagnosis with its poor prognosis became clear. Two patients (nos 11, 12) were intubated in Germany after the diagnosis was made. Mask ventilation was given to three patients, in one before diagnosis, one after diagnosis, and one at the end of life. One patient, in whom the diagnosis was clear, suffered from bradycardia and received heart massage, which she survived. Comfort medication given to patients consisted of paracetamol, NSAIDs, morphine, and benzodiazepines (Figure 3b). In the period prior to the diagnosis being clarified, nine patients were given paracetamol, and three of them were given it in combination with morphine. After the diagnosis was made, all except one patient received several types of comfort medication, and at the end of life, 95.5% of the patients received comfort medication, mostly morphine and benzodiazepines. There was no significant difference in the amount or type of pain medication given to patients who died at home or in hospital (Table 2).

Table 2. Pain medication given to our JEB-H cohort in relation to the place of death

Place of death	No. of patients	Paracetamol	NSAIDs	Tramadol	Morphine
Home	8	25.0% (n=2)	12.5%, (n=1)	12.5%, (n=1)	75.0% (n=6)
Hospital	14	35.7% (n=5)	7.1% (n=1)	7.1% (n=1)	85.7% (n=12)
p value*		p=0.60	p=0.67	p=0.67	p=0.53

'*' significant difference at $p<0.05$

Predictors of lifespan

Twelve patients received one or more invasive treatments in their life, such as heart massage, mask ventilation, (par)enteral feeding, blood transfusion, and intubation. These patients survived on average for 6.0 months. The average lifespan (5.5 months) of the ten patients who did not receive any of these invasive treatments was not significantly different (p=0.81). The 20 patients with an absence of GB3 staining had an average lifespan of 5.3 months, whereas the two patients with a strongly reduced GB3 staining had an average lifespan of 10.6 months. The difference in these two groups was not significant (p=0.08). Also no significant correlation between the lifespan and birth weight of the patients was seen (p=0.49) (Figure 4). However, we did find a significant

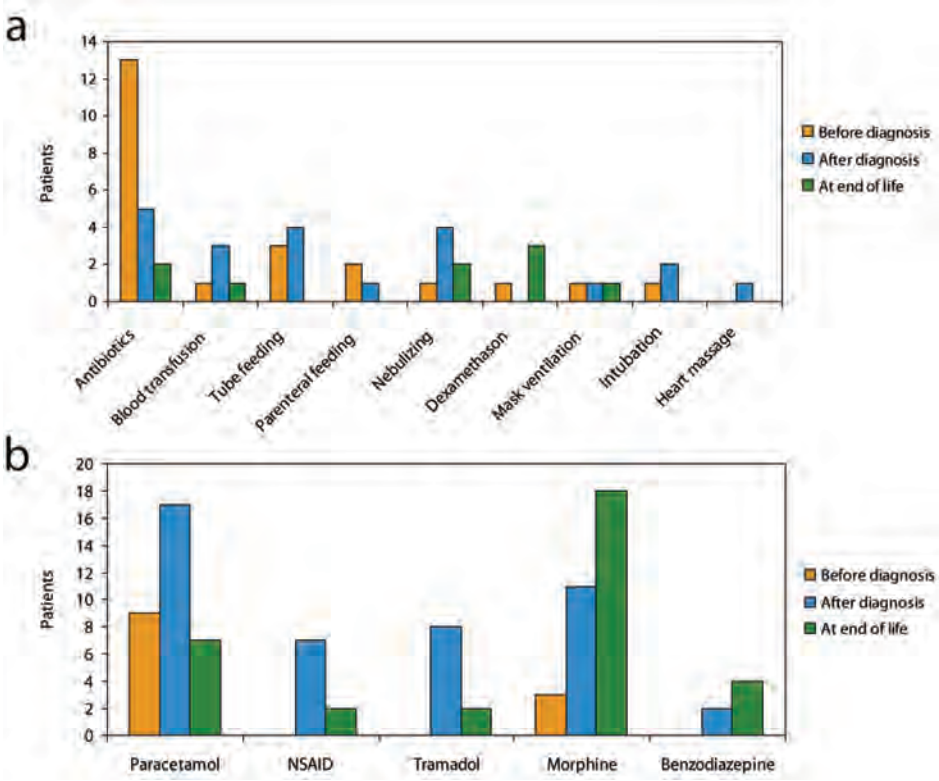

Figure 3. Summary of (a) treatments, and (b) comfort medication received by patients, subdivided into the categories: prior to diagnosis, after diagnosis was clarified, and at the end of life.

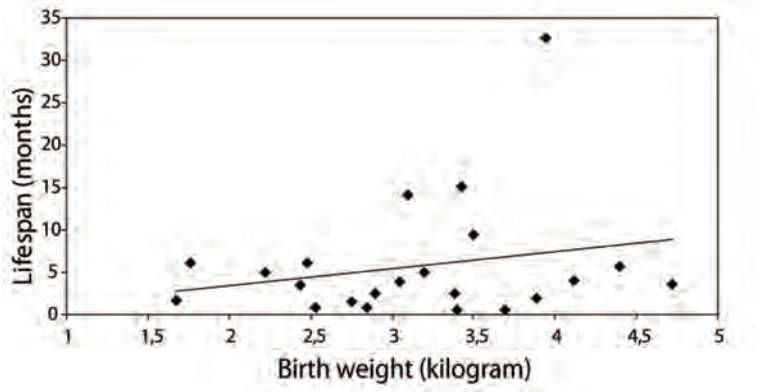

Figure 4. No significant correlation between the lifespan and the birth weight of JEB-H patients was seen (p=0.49)

difference in lifespan between the patients belonging to the three different growth groups discussed above (p=0.02) (Figure 2).

Discussion

To our knowledge this is the largest cohort of JEB-H patients (n=22) in which the long-term follow-up is described. Early diagnosis of this lethal EB subtype is crucial to parents. In JEB, the most important differentiation that should be made is that between JEB-H and JEB-nH. The first direction in the diagnosis could be present in the clinical symptoms that present at birth, like nail abnormalities (100%) and hoarseness (59.1%). However, although these symptoms may correlate with disease severity, they are not specific and may also be present in JEB-nH.[16,17] The area of skin blistering at birth is also not helpful in diagnosing a patient, since 36.4% of the patients lack blisters at birth. Hypergranulation tissue is not common at birth; it seems to develop later in life, with 72.7% of the patients affected, and the most common site being periungual. It is remarkable that hypergranulation around the mouth, often seen as a pathognomic feature,[1,6] is rare in JEB-H patients; only one patient was affected in our cohort. From our own experience, patients classified with JEB-nH are also affected with perioral hypergranulation tissue (unpublished data). Therefore the pathognomicity of hypergranulation in JEB-H patients is not clear. Aplasia cutis is not helpful in differentiating EB subtypes, as it has been associated with all the major subtypes (simplex, junctional and dystrophic).[18-22] So it seems that it is not possible to distinguish between different EB subtypes using the clinical symptoms at birth.

To differentiate between JEB-H and JEB-nH, immunofluorescence antigen staining using monoclonal antibody GB3 and molecular analysis are most valuable.[3]

The general consensus is that JEB-H patients do not survive to adulthood.[13] None of our 22 patients survived longer than 33 months. JEB-H cohorts in Australia (n=11) and in Austria (n=6) also showed a 100% mortality in childhood, with patients not surviving past 13 months.[6,12] It is remarkable that in 41 JEB-H patients from the United States National EB Registry, 34% (n=14) of the patients survived to the age of 15 years.[14] It is believed that these patients were incorrectly classified due to a limited diagnostic validity.[6] Similar to our results, JEB-H patients in Australia and the USA died due to failure to thrive, sepsis, respiratory failure, or pneumonia.[12,14]

As all patients with JEB-H will eventually succumb to the complications associated with the disease, our opinion is that medical procedures intended to extend life, such as blood transfusion, tracheostomy, nasogastric feeding tubes, gastrostomy, parenteral feeding, intubation, mask ventilation, and heart massage, may compromise the comfort of the JEB-H child.[12,13,23] Furthermore, in our cohort, these invasive treatments did not prolong life significantly (p=0.81), and a similar average lifespan was seen in the patients who were treated, compared to those who were not treated. We also question the long-term effectiveness of some invasive treatments. Blood transfusions did not resulted in long-term increased Hb values (Figure 1). Nor did we see an improved weight gain in the six patients receiving nutritional support by nasogastric tube and parenteral feeding. A study by Kho *et al*. showed similar results, with no apparent contribution to long-term survival in JEB-H patients receiving tracheostomy or gastrostomy.[12]

Although we decided to give some less aggressive medical treatments, such as nasogastric tube feeding and blood transfusion, to some patients, in an attempt to enhance their comfort, the harm and possible ineffectiveness of invasive medical treatment should be discussed as early as possible with parents and caregivers to prevent unnecessary treatments being carried out.

Our restraint also applies to invasive diagnostic procedures. This could have led to underreporting of complications in our cohort, and it could also account for some complications that are described in the literature, but that were not seen in our patients, such as esophageal stenoses, urethral meatal stenosis, bladder hypertrophy, hydronephrosis, and osteopenia.[1,6,9,24-26]

The mainstay of treatment should be comfort care, such as pain medication, dressing changes, laxatives and nebulizers.[13,23] Early diagnosis allows the treatment to be switched from aggressive life-extending treatments to comfort care. In our cohort, the average age at diagnosis was 36 days. The diagnosis can mostly be confirmed within two days after consultation.

When parents are confronted with the diagnosis of JEB-H in their child, it is important to discuss the prognosis with them. Although parents can be told that their child will not survive through childhood, there have been no prognostic indicators of their lifespan available. Patients with a strongly reduced immunofluorescence antigen staining with GB3 for laminin-332 have a 2-fold higher average life expectancy, compared to patients who have absent GB3 staining. This difference is not significant, however, we believe that this is due to the small amount of patients included in the strongly reduced GB3 staining group (n=2). A higher birth weight did not predict a longer lifespan (Figure

4). However, patients with a sufficient initial weight gain did live significantly longer than patients with an insufficient or no initial weight gain. Some conclusions from our patient cohort concerning growth, body weight, and death can be made: (1) patients who do not have an initial weight gain will most likely die in the first six months of their life; (2) if patients do have an initial (sufficient) weight gain, they may survive beyond six months of age. These patients can nonetheless die with or without an extra period of weight loss. In the former, they will die on average two months after they started losing weight.

In our JEB-H cohort, the setting in which patients died was either in hospital or at home. Comfort care, measured by the amount and type of pain medication given to patients, was equally available to those in hospital or at home. The number of medical treatments given was also comparable. The group that seemed to be the most overtreated and overdiagnosed at the end of life were the four patients who were transferred in an acute condition from their home to hospital, due to dyspnea and dehydration. However, it appears adequate to follow a palliative end-of-life care program in the home setting, instead of in the hospital, if parents, home care givers, and the family doctor are well informed about what they should expect, how to realize comfort care, and how to act if there are complications. In the latter case, they should follow an agreement written specifically to cover terminal diseases. This will reduce the number of acute transfers to hospital and, as a result, also the burdensome diagnostics and treatments given to patients at the end of their life.

Newborn euthanasia according to the Groningen protocol was performed in one of our patients.[15,27] The lifelong tendency to develop blisters and erosions means JEB-H patients live in pain and distress their entire life. Bathing, changing dressings, feeding, and even cuddling, can cause excruciating pain (see link to movie 1 in the Supplementary Appendix).[13] Furthermore, complications such as anemia, dyspnea, and failure to thrive also contribute to suffering in patients. Taking this into account, along with the fatal prognosis and the absence of a cure, newborn euthanasia is legally permitted in the Netherlands in some patients with JEB-H. Patient 20 could not be treated sufficiently for his suffering, and the parents requested for euthanasia. However, we realize that euthanasia in newborns with a fatal prognosis and hopeless, unbearable suffering is not acceptable in many countries.[28] In the future, (mesenchymal) stem cell transplantation may possibly provide a form of treatment for this fatal and devastating condition.[29,30]

Conclusions

The symptoms and complications of JEB-H are so severe that they are lethal in early childhood, with failure to thrive and respiratory failure as the most common causes of death. The pattern of initial weight gain is a predictor of lifespan in these patients. As life saving treatments do not promote survival in JEB-H, the management of these patients should be shifted from invasive medical treatments to comfort care as soon as the diagnosis with its lethal prognosis is known. The palliative end-of-life care can take place in hospital, but is also safe in the home setting. Suffering in JEB-H patients can become so unbearable, that in some patients, who do not respond to adequate analgesic and sedative treatment, newborn euthanasia, performed according to the Groningen protocol, is legally permitted in the Netherlands.

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4

The needs of parents with children suffering from lethal epidermolysis bullosa

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Abstract

Background Some subtypes of the heterogeneous genetic blistering disease epidermolysis bullosa (EB) lead to lethality in childhood. The severity and extent of blistering leaves these patients living in excruciating pain and distress their entire lives. Parents of these patients experience some specific problems, such as the unfamiliarity of EB amongst health care professionals and the suffering and loss of their child.
Objective To identify the needs of parents who have lost their child to lethal EB.
Methods A qualitative study was performed, comprising of semi-structured in-depth interviews with 16 parents. The transcripts were analyzed and common themes were identified.
Results Parents indicated that they have the need (1) for a fast and correct referral to a specialized EB clinic, (2) to be informed as honestly as possible about the diagnosis and lethal prognosis, (3) to have a structured network of care givers in the palliative care, (4) to be involved in the care and the medical decisions involving their child, (5) to be informed about the end-of-life and to discuss euthanasia, (6) for guidance and to have remembrances of their child, and (7) for genetic counseling.
Conclusions Our job as health care professionals is not only to provide the best care for the children suffering from lethal EB, but also for their parents. In this study, parents have provided us with some guidelines to care for them. However, it is important to keep in mind that every parent is different, and that the guidance should be tailored to their individual needs.

Introduction

Epidermolysis bullosa (EB) is a group of genetic diseases characterized by blistering of skin and mucous membranes.[1] EB is further classified in many subtypes with different degrees of severity, ranging from an occasional palmoplantar blister to lethality in early childhood.[2] The lethal EB subtypes comprise Herlitz type junctional EB (JEB-H) due to absence of laminin-332, EB with pyloric atresia (EB-PA) due to absence of plectin or integrin $\alpha 6\beta 4$, and lethal acantholytic EB due to deficiency of desmoplakin or plakoglobin. Lethal EB is rare (the incidence for JEB-H is 4 per million life births).[2-4] The severity and the extent of blistering leaves these patients living in pain and distress their entire, albeit, short life, in which normal activities such as dressing changes, bathing, feeding and even cuddling can cause excruciating pain.[5,6] Even with invasive treatments, these patients eventually succumb to the complications of the disease mostly within the first two years of life. Therefore the treatment of these children should be palliative, focusing on comfort care: reducing pain and discomfort.[5,6]

The process that parents of a child with lethal EB go through is a painful and difficult path: the expectance of a healthy child, the uncertainty before the diagnosis is made, the lethal prognosis, the child's suffering from pain, the loss of the child, and the mourning period. Our job as health care professionals (HCP) is not only to provide the best care for the child, but also to guide the parents in this difficult time. To improve this, it is important to know what parents want, expect, and appreciate from HCP.

Research performed in this field has mainly focused on parents that have lost their child to a malignancy.[7-9] Although much can be extracted and learned from this research, parents of children suffering from lethal EB encounter some specific issues. Previous research in children with non-lethal EB has shown that parents worry about their child being in pain and being different. These parents have feelings of uncertainty, anger, fear, and guilt. They also experience difficulties in organizing the care, restrictions in employment and leisure time, and ignorance and lack of skills of HCP.[10,11] In addition to this, the loss of a child is a life changing event. The way parents continue their lives is influenced by the care for them during and after their child's death.[12,13] The aim of this study is to identify the needs of parents with children suffering from lethal EB, and to give guidelines for their guidance so the care for future parents can be improved. To do this we have performed a qualitative study by interviewing parents who have lost their child to lethal EB.

Materials and Methods

This study was performed at the Center for Blistering Diseases in Groningen. All EB patients visiting this single national referral center for EB in the Netherlands from 1988-2011 were included in the Dutch Epidermolysis Bullosa Registry (DEBR). We are almost certain that all patients with lethal EB born in the Netherlands for the past 23 years have been included in the DEBR. For this study all parents who had lost their child to lethal EB one year prior to the start of the study were selected from the DEBR. A total of 25 parents were eligible for the study, and they were contacted with a letter informing them about the nature and the purpose of the study. One week after receiving the letter, the parents were called by the primary investigator (W.Y). Further information was given orally and the parents were given the opportunity to ask questions. Parents were asked if they wanted to participate and it was emphasized that cooperation was entirely voluntary. Five parents did not want to participate and four parents were untraceable. Sixteen independent parents were willing to cooperate and an appointment was made to conduct a telephone interview. In six cases only the mother was interviewed, in five cases only the father, and in five cases both parents were interviewed. The interviews were conducted by the primary investigator and lasted from 30 to 150 minutes. Of the participants, fourteen parents had lost their child to JEB-H, of which one couple had lost their twin. Two parents lost their child to EB-PA. All parents resided in the Netherlands, Belgium or Germany. Two couples did not speak Dutch and a certified interpreter was arranged to translate. The interviews were semi-structured and open-ended, in which the investigator invited parents to narrate their experiences and thoughts about their child's illness, with a focus on the period before the diagnosis was made, the delivery of the diagnosis, the palliative care, the end-of-life of their child, and their grieving process. The interviews were recorded on tape and transcribed ad verbatim by the primary investigator. The transcripts were analyzed by multiple line by line readings. Significant statements, phrases, and sentences were identified and were used to distil common themes. Seven themes emerged from the interviews and they are separately presented in the results and discussed. Parents quotes (Q) can be found in Table 1, and are referred to in the text.

Results and Discussion

1. The need for a fast and correct referral to a specialized EB clinic

Children with lethal EB are born with blisters or develop them in the first days of their life.[3,6] The time until referral to the specialized EB clinic in Groningen lasted on average 35 days (range 1-179 days). For the Dutch patients the average time until referral was 28 days between 1988-1999 and 14 days between 2000-2011. In this pre-diagnostic period parents experienced insecurity, stress, loneliness, and anger.

The initial HCP involved in the care for children with lethal EB are mostly family doctors, pediatricians, and dermatologists. Parents were of opinion that these HCP were not competent in caring for their child, lacking knowhow, and resulting in injuries and suffering to their child by inadequate pain medication and incorrect wound care (Q1). Some parents experienced that a lack of awareness of EB has led to an incorrect or late referral to the specialized EB clinic (Q2), and that a faster and correct referral could have or has prevented suffering and injuries inflicted to their child (Q3). One mother indicated that she would have spent her time differently with her child, if she had known the diagnosis sooner (Q4). Our survey shows that awareness of EB among HCP is not optimal and should be improved; this could be achieved by education and publicity.

2. The need to be informed as honestly as possible about the diagnosis and lethal prognosis

After the first consultation in our specialized EB clinic, the diagnosis was made within one or two days, or a few weeks in the early days. The diagnosis with its lethal prognosis was discussed honestly with parents. Although parents thought it was hard to hear the news, they were all glad that they were informed honestly (Q5). Parents indicated that important factors in the conversation in which the news was delivered were empathy, compassion, intimacy, room for emotions and questions, visual aids, and written brochures. Some parents indicate that they approached their child differently knowing the lethal prognosis (Q6).

In the literature, it is shown that HCP find it stressful and difficult to give a poor prognosis to patients and parents.[14,15] In addition, many HCP think it is cruel to take away hope.[16,17] As a result, HCP withhold the truth about the prognosis or they avoid the discussion.[18,19] Consequently, patients and parents have an overestimation of the prognosis and more treatments are given that are not effective and that lead to more

adverse effects.[18,20] However, consistent with our results, research has shown that parents want to be informed about the prognosis as much as possible, although they find it upsetting.[21,22] Parents evaluate the quality of care higher if clear information is given, and they feel more distress when they are not informed.[21-23]

3. The need to have a structured network of caregivers in the palliative care

After the diagnosis of lethal EB was made, parents choose to situate the palliative care at home (n=9) or at the hospital (n=8). None of the children were brought to a hospice. In hindsight all parents were satisfied with their choice, and parents should be free to choose the location of palliative care after being provided with information about the pros and cons of both options.

Reasons for parents to choose for hospital-based palliative care were, among others, that they thought their children would receive better care and more attention in the hospital. Important for these parents was the possibility to transfer their child from the EB clinic to a nearby hospital. All parents were satisfied with the provided care of the hospital and its consultations with the EB clinic.

Home-based palliative care can be perceived as very burdensome by parents (Q7). In most cases the regular and acute pediatric care was facilitated by a pediatrician affiliated to a nearby hospital, who consulted the EB clinic in case of problems. For parents accessibility of the nearby hospital was one of the most important factors. In general, parents were very satisfied with the care provided. In some cases the family doctor was also actively involved in the care, usually with a more coordinating role. It is important that parents visit their own nearby hospital or family doctor, as the latter have become familiar with the diagnosis, the prognosis, and the treatment of lethal EB. When other HCP are consulted, this can lead to difficult situations (Q8).

Almost all parents that choose for home-based palliative care received home care, and the overall opinion was that this was a great support. However, one of the most heard complaints is the large number of home care nurses involved in the care for their child, that all needed instruction (Q9).

Parents indicated that the EB clinic played a supportive role in the palliative care. Much appreciated was the provided custom made handbook on caring for a child with lethal EB, and the personal education on wound care given to parents and/or the home care nurses. Some parents indicated that education made them the expert of the disease, and this empowered them in the care for their child and enabled them to take charge of the situation (Q10). Medical care provided by the EB clinic could go through

email (Q11), or personally in the multidisciplinary EB carousel in which patients are seen by all relevant specialists on one day (Q12).[24] Parents indicated that accessible contact with EB nurses through email or telephone supported them (Q13).

4. The need to be involved in the care and in medical decisions involving their child

Parents indicated the importance to be actively involved in all phases of care for their child. Most parents want to keep participating in the daily care of their child, including the wound care. In the literature it is also reported that parents appreciate this.[12,21] The participation in the care of a child facilitates the adjustment to the death of their child, it increases the parents sense of control, and it decreases the feelings of helplessness.[8] However, some parents appreciated that (home care) nurses took over the wound care, so their child would not associate the parents with negative and painful experiences.

After the diagnosis had been made and the lethal prognosis was known, most parents wanted to minimize the examinations and investigations performed in their child (Q14). Also most parents were reluctant to invasive treatments, as they did no want to elongate their child's life of suffering (Q15). In the parents' view the main therapy for their child should be pain medication (Q16). Although pain medication was given, children still suffered, especially during painful events such as dressing changes (Q17).

5. The need to be informed about the end-of-life and to discuss euthanasia

On average, participating parents lost their child at 5.7 months, with a range of 0.1 to 32.6 months. Twelve of the children died in hospital, and five at home. The literature regarding the preference of parents for the location of death (LOD) is not conclusive, with studies showing a preference for home death or with an equally divided preference.[25,26] It has been thought that the LOD can have implications for the bereavement of families, as home deaths are associated with a more adequate family adjustment and less psychological distress of the family, compared to when a child dies at the hospital.[27,28] However, a recent study of Dussel *et al*. shows that the opportunity to plan the LOD is more important than the actual LOD.[29] In our cohort all parents were satisfied with the LOD.

Some parents did not expect their child to suffer as they did at the end of their life, and they wanted to be more prepared for this (Q18). Three children received elevated morphine gifts to minimize the suffering at their end of life. As a result, this could have hastened their death (Q19). In the Netherlands most physicians think it is permissible to

give pain medication and sedatives to alleviate the suffering in dying children, even if it shortens their life. It is estimated that in most children, palliative medicine shortens the life with less than one week.[30-32]

Newborn euthanasia is a delicate subject, and unacceptable in most countries. In the Netherlands newborn euthanasia performed according to the Groningen protocol is legally permitted in some children with hopeless, unbearable suffering who do not respond to adequate analgesic and sedative treatment.[33] There are no studies regarding parent's perspectives on newborn euthanasia. In our survey many parents brought up the subject euthanasia during the interview, and indicated that they had the need to talk about it. One couple was against euthanasia on principle. All other parents were open minded on euthanasia, with the reason that they did not want their child to suffer. These parents were more satisfied if they could discuss euthanasia with their doctors, although most of them never requested it (Q20), since the suffering of their child could be adequately treated with palliative medicine. In one child, the hopeless and unbearable suffering could not be adequately treated with analgesic or sedative treatment, and newborn euthanasia according to the Groningen protocol[33] was performed on request of the parents (Q21). Now, two years after the death of their child, the parents do not feel any regrets and still stand behind their decision.

6. The need for guidance and to have remembrances of their child

An important factor in the guidance of parents is how they and their child are treated by HCP. Parents want to be treated compassionately. They also want to be heard and taken seriously. As regard to their child, it is important that HCP do not only see a baby with EB, but also as a baby with other needs. One mother told us that she appreciated that HCP also had eye for the development of her child by arranging music in the hospital room and a mobile above the bed.

The need for guidance of professional social workers during the life of their child and in the grieving process is variable in parents. There were three opinions on it. Firstly, the opinion that there was no need for it, as parents found support with each other, their family, their friends, their surroundings, their church, or by contact with fellow sufferers. Secondly, the opinion that guidance from social workers was valued. And thirdly, parents that missed the contact with social workers, as this was not offered to them. Parents indicate that it is important that social workers know what lethal EB entails and that they are aware of the wound care and the pain of their child, so that they can focus on what that specifically does with them as parents (Q22). Although not all parents needed the

guidance and care of a social worker, it is a necessity to offer it to all parents during their child's' life and after their death.

Not only social workers, but also family doctors can be an important factor in the guidance of parents (Q23), and some parents missed the support and involvement from the family doctor.

In our survey, parents indicated that they had misjudged the impact of losing a sibling to lethal EB on their older child. Some siblings developed separation anxiety (Q24). HCP should anticipate on this and provide the needed guidance. Literature reveals that the loss of a sibling can lead to emotional or behavioral problems.[34] Also, parents can feel alone in parenthood after the death of a child and they could need support and guidance from others.[35]

For the grieving process parents indicated that it is important to be with their deceased child (Q25), and to have tangible memories of them, such as photos, movies, plaster casts of their hand or foot, a lock of the hair, and diaries kept by parents and/or nurses. Having these keepsakes is an important step in the grieving process, as it can help parents hold on to their relationship with their child.[36,37] Parents appreciated it, if HCP urged them to take keepsakes, as they did not think about it at that moment (Q26), or because the sight of severe blistering made them reluctant to do this.

7. The need for genetic counseling

An issue much broached by parents is the genetic testing and counseling. The desire for another child can be very great, so for most parents the genetic results are very important. Most parents received the results rapidly, and were glad with that. However, some parents had to wait a long time for the DNA results, or received little information about the hereditary of the disease (Q27). Parents should be referred to a clinical geneticist, and DNA analysis should be started as soon as possible.

Table 1. Selection of parents' quotes

Q1	They used the wrong adhesive plasters, and when they took the adhesive plasters off, all of his skin came off. It never healed again. That was really a mistake.
Q2	The doctors did not know what it was. That is okay, but then refer me! It took too long. Only after making a fuss they referred me.
Q3	He had so much pain (…) Because we were referred to the EB clinic so late; you have the feeling that you have caused your child so much pain for the past two months (…) If we were in the EB clinic within two weeks, we would know how to care for his wounds (…) it would have prevented a lot of suffering for him.
Q4	Because of his blisters, I often left him lying in his bed (...) I just thought that [holding him] would come later, but we did not have that time.
Q5	He could not make it better then it was. It was very hard to hear it, but on the other side, he couldn't have told it in a different way. I wouldn't want that.
Q6	If you're not honest to people, then they keep hope (…) That will give problems, as you will give them more [treatments]. That should not happen.
Q7	We were busy day and night; we didn't have a social life anymore. It was survival.
Q8	Of course we wanted to go to our own hospital, but instead they took us to the nearest hospital. There, they wanted to directly intubate her and measure her saturation. And then I had to hang over her and I said: "You stay away from my child" (…) Those HCP just saw a baby with clothing and some bandages underneath. And we told them that she has EB, but they didn't know what that was. They wanted to treat her. We thought: "we have to get out of here." I became very angry.
Q9	I constantly had to instruct the nurses. They were thrown in the deep end, they had never seen it [EB] before, and they all thought it was very scary. Every day a new nurse came. At one moment I said: "please give me two or three permanent nurses." At one point the sixth came, and I was like: "do I have to teach all those six nurses everything what I have learnt?" I will succumb to it. It was more of a load then a relief.
Q10	The people from the EB clinic told me: "you have to monitor what HCP do with him, and if they do something that is not allowed you have to tell them that." We did that. We were on top of it. And every time we went to the hospital, we told them. For us this was a positive experience. They listened to us.
Q11	It was so nice that we could send photo's or email to the EB nurse. We could fall back on a lot of people. That is an enormous support (…) just to know that what you are doing is right.
Q12	The journey to the EB clinic was not possible any more for John. In the state he was in, I couldn't put him in the car anymore. It was just too painful for him. And eventually it's useless, just to let the EB team see how much he had deteriorated. For us it wasn't interesting enough anymore to drag him to Groningen every time.
Q13	Just to get things off your chest (...) the feeling that you are understood. Because in your surroundings no one knows what the disease entails.
Q14	He always had to be undressed to be weighed, but I think that if you know that a child is terminal, you can omit that. There is no point in it.
Q15	Why would you want to prolong the life of your son in such a miserable state (…) you know that you can not be selfish.
Q16	The most important thing for us is that he did not have pain. He absolutely should not have pain. That was our only agreement, the most important agreement that we have made.

Table 1 continued

Q17	It's horrible to hurt your own child. But you don't have another option. When your child has EB you can choose between a bad option and an even worse option. There isn't a good solution.
Q18	He got very intense breathing problems and we were not prepared for that. The struggle that he had to go trough the last hours was very painful for us (…) For us these are very difficult moments, because you hope that your child will pass away peacefully and that he does not have to struggle as he did.
Q19	At one moment he was very uncomfortable. And then the doctor said: "if we give him morphine again, it could become fatal." Then we were like: "what are we going to do, what are we going to do?" It was a matter of hours. It could have lasted for hours, but there was no need that he would be in so much pain. And then I gave him a little bit morphine on a teaspoon, and then he flew away peacefully.
Q20	Of course, you would prefer that it all just ends, that it stops. You are caught in a nightmare. On one hand you want her to die very soon so she does not have to suffer anymore, but on the other hand you are very proud that she is so strong (…) We talked about euthanasia after she died. But that is a very difficult question. My husband would say yes, and I say that I would not know. Maybe it's selfish, but I'm glad we had the time that she was with us.
Q21	If you keep him in sleep the whole day, he has to wake up to drink. And then the pain broke through. And for him drinking was not pleasant anymore. He drank, but he had horrific pain. That was the point that we said: "we don't want this." (…) Especially on one day, when it went really bad with him. They told us to stay with him, because it could be his last day. So we stayed with him and in the morning it went really bad, but he recovered. He woke up and looked around. That was a turning point; I thought he is not going to give up. How far should you go? That's when we decided it was enough. You can't do this to a child.
Q22	In the beginning I was a little bit cool towards Jane and I was also a little bit scared. Then she [psychologist] told me: "now you can hold her, later it will not be possible anymore." Then I thought she is right. I opened myself up and it went better.
Q23	The family doctor called me regularly, to see how we were doing (...) That had a lot of value for me. There were moments that it was mentally very difficult for us. And if he called me (…) it was very nice that I could get things of my chest. I really had some sleepless nights, my wife too. So sometimes he prescribed us a sleeping pill. And he also supported us mentally.
Q24	In the beginning it was really difficult for him, he asked me: "mama, are you going to get rid of me too?" He slept in our bedroom for a year and a half; he did not dare to let us go. For him the world became very unsafe (…) Of course, you expect a sister and then your sister is gone in two days. Your mother is constantly crying and your father is upset. And then you have to stay there and then there. That is very difficult.
Q25	[After she died] we bathed her at our ease, and then we groomed here and dressed here. I thought that was fantastic. It was great, my baby lying in her cradle and she doesn't have any pain anymore.
Q26	We made very few photos. I regret that.
Q27	The pediatrician shortly told us about the heredity (…) Later, we got more children and they are all very healthy, but statistically I think that could have been different.

Limitations

There are several potential limitations to this study. This study is performed retrospectively. To make the research population as large and as representative as possible, we have chosen to include all parents of children with lethal EB included in the DEBR, with the resulting disadvantage that we had to rely on the accurate recollection of events that took place ranging from 1 to 23 years prior to the interview. However, as this period was so intensely experienced by parents and the unlikelihood that they have forgotten about the death of their child, we believe that their recollections do represent their needs.

Another possible limitation of this study is the potential selection bias. Parents who choose to co-operate could have different ideas about the care and guidance, compared to parents that did not want to co-operate. Nevertheless, with a participation rate of 76.2% (16 out of 21 traceable parents), we believe that the presented results are reliable.

Conclusion

In this survey, parents of children with lethal EB have provided HCP with some guidelines to care for them. The implementation of these guidelines will help to improve the quality of care in lethal EB. However, it is important to keep in mind that every parent is different, and that the guidance should be tailored to their individual needs.

Acknowledgements

We are grateful to all families who have participated in this study. We are thankful to Bouwe Molenbuur for his contribution to the study. We thank *Vlinderkind* (Dutch Butterfly Child Foundation) and the J.P. Nater Foundation for their financial support of the study.

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5

ITGB4-associated junctional epidermolysis bullosa, type non-Herlitz: report of two new cases carrying two novel *ITGB4* mutations

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Under revision

To the editor

Epidermolysis bullosa (EB) is a genetic, trauma-induced, blistering disease consisting of 29 subtypes.[1] Mutations in *ITGB4,* the gene coding for the hemidesmosomal protein integrin β4 (β4), cause the rare subtype EB with pyloric atresia (EB-PA), that can be lethal in childhood.[2,3] *ITGB4* mutations have also been associated to a case of junctional EB, type non-Herlitz (JEB-nH),[4] that is usually caused by mutations in the genes coding for laminin-332 (*LAMA3, LAMB3, LAMC2*) or for type XVII collagen (*COL17A1*). The other *ITGB4* mutated non-pyloric EB case was previously published by us and classified as EB simplex due to a low intrabasal split level.[5] We found two more JEB-nH cases carrying pathogenic *ITGB4* mutations, corresponding with an incidence of 6.5% in the Dutch JEB-nH cohort (two *ITGB4*-associated JEB-nH families out of a total of 31 families). Here we report these two new JEB-nH cases carrying two novel *ITGB4* mutations.

Patient 1 (EB112-01)

Patient 1 was an 81-year-old man, who has suffered from generalized blistering since his birth. The blisters healed with post-inflammatory hyperpigmentation (Fig.1a). He had male pattern baldness and hypotrichosis of eyelashes and eyebrows. Secondary hair in his axillae and pubis was absent, although it was present in the beard region (Fig. 1b). Other clinical symptoms were mild distal onycholysis of the nails, and enamel defects that led to extraction of the whole dentition. He did not contract any atresias or stenoses.

With electron microscopy (EM) of lesional skin we found blister formation through the lamina lucida. In non-lesional skin, duplications of the lamina densa with an irregular thickness were seen (supplementary appendix 1). The hemidesmosomes were hypoplastic with a loss of the sub-basal dense plate.

With immunofluorescence (IF) antigen staining of lesional skin, we found a *high* junctional split: pankeratin and plectin exclusively stained the roof of the blister, whereas the endodomain of type XVII collagen (Col17), and the extracellular domains of integrin α6 (α6) and β4 stained both roof and floor of the blister, and laminin-332 (LM-332) was mostly present in the floor of the blister. In non-lesional skin, staining for LM-332 and type VII collagen was normal. Staining for plectin, Col17 endodomain, and Col17 ectodomain was slightly reduced. The extracellular domain of α6 stained normal, while the intracellular domain showed reduced staining compared to control. Staining of the intracellular and extracellular domains of β4 was strongly reduced (Fig. 2).

Mutation analysis of *ITGB4* showed the previously described mutation c.600delC (p.F201SfX9), that results in a frameshift and a premature termination codon (PTC).[6] The mRNA from this allele will likely be targeted for nonsense-mediated mRNA decay. On the other allele, we found the novel mutation c.3040C>T in exon 26. This mutation predicts a substitution of an arginine to a tryptophan at codon 1014 (p.R1014W). R1014 is highly conserved in many species and is located in the intracellular domain of β4.

Patient 2 (EB113-01)

Patient 2 is a 64-year-old woman suffering from localized blistering since birth. The blistering is mainly localized on the hands, feet, lower legs, forearms and genital area, and sporadically on the oral and nasal mucosa (Fig. 1c). The blisters heal without scarring and are aggravated by sunlight. The fingernails show pachyonychia and onychogryphosis; the toenails are absent (Fig. 1c). Primary hair is normal, whereas secondary hair of the axillae and pubis is absent (Fig. 1d). Enamel defects required extraction of the total dentition. Her medical history lists multiple squamous cell carcinomas of the skin and rheumatoid arthritis. She has no history of any atresias or stenoses.

EM of lesional skin showed a split through the lamina lucida. In non-lesional skin hypoplastic hemidesmosomes were seen with duplications of the lamina densa (supplementary appendix 1).

IF of lesional skin showed a split *high* in the lamina lucida: pankeratin and plectin stained exclusively the blister roof, while the ecto- and endodomain of Col17, LM-332 and type VII collagen stained exclusively the blister floor. IF of non-lesional skin showed normal staining of pankeratin, Col17 endodomain, Col17 ectodomain, LM-332, and type VII collagen, compared to control. The staining of the extracellular domain and intracellular domain of β4 was slightly reduced in intensity, and interruptions in the basement membrane were seen, especially in the extracellular domain of β4 (Fig. 2).

Mutation analysis of *ITGB4* showed the novel mutation c.4975G>T in exon 37 homozygously. This mutation results in a PTC at position 1659 (p.E1659X). As IF staining of β4 remained visible, this mutation predicts the production of a truncated β4, missing 93 amino acids at the C-terminus.

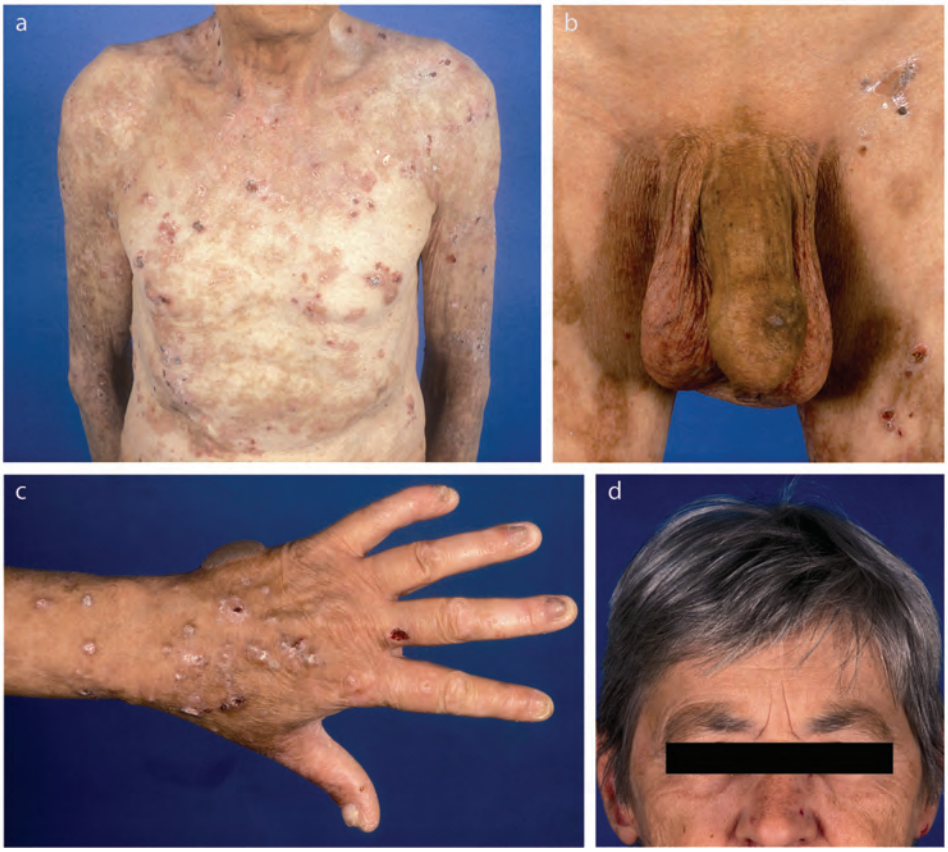

Figure 1. Junctional epidermolysis bullosa, type non-Herlitz caused by *ITGB4* mutations. (a) Bullae, erosions and crustae that have healed with hyperpigmentation on the trunk and arms of patient 1. (b) A nearly total absence of secondary hair in the genital area of patient 1, a single hair is visible in the pubic area. (c) Blisters and crustae together with multiple prurigo papules on the back of patient 2's hand. The nails show pachyonychia and onychogryphosis. (d) Normal primary hair on the scalp and eyebrows of patient 2.

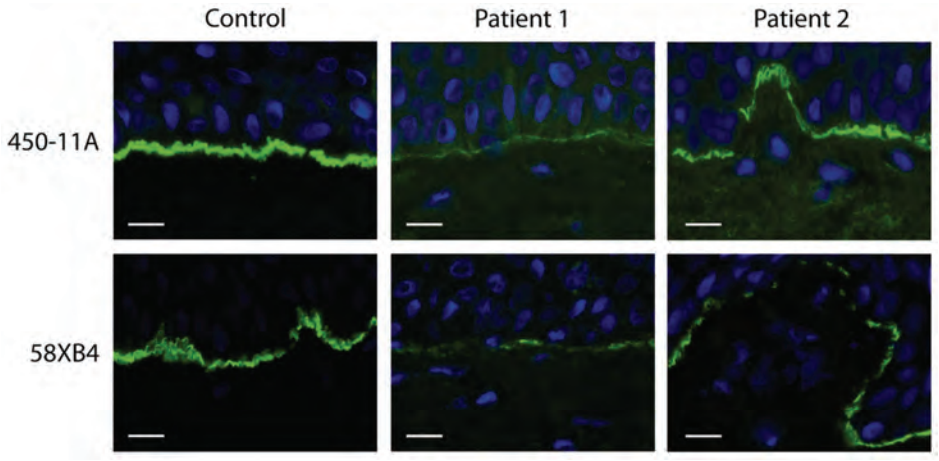

Figure 2. Immunofluorescence staining with antigen 450-11A for the intracellular domain of integrin β4 and with antigen 58XB4 staining for the extracellular domain of integrin β4 showed a strongly reduced staining in patient 1 and a slightly reduced staining in patient 2. Interruptions in the basement membrane are seen in patient 2, especially in the staining with 58XB4. Scale bar = 10 μm.

Conclusion

ITGB4-associated EB may be without pyloric atresia and cause a generalized or a localized pattern of blistering of either JEB-nH or EB simplex. IF antigen mapping can be helpful in appointing *ITGB4* as the first candidate gene.

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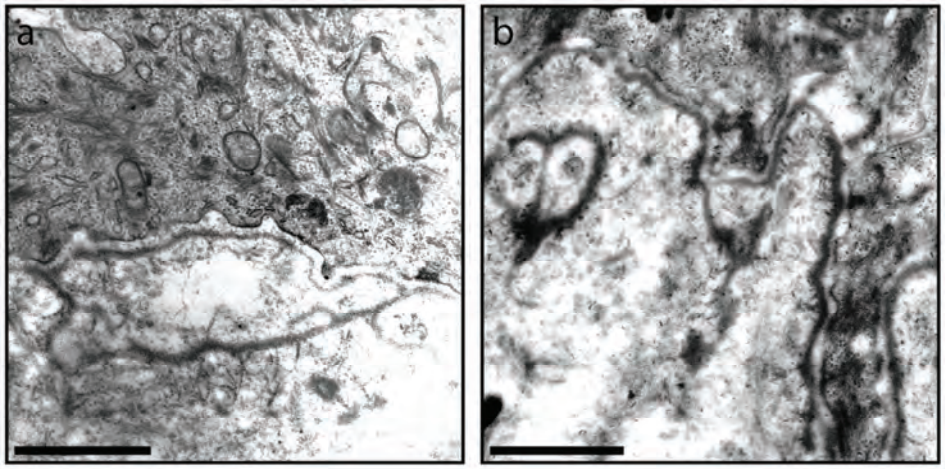
Supplementary Appendix



Supplement 1. Electron microscopy of non-lesional skin shows duplications in the lamina densa of (a) patient 1 and (b) patient 2. Scale bar = 1 µm.

6

Risk of squamous cell carcinoma in junctional epidermolysis bullosa, non-Herlitz type: report of seven cases and a review of the literature

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Abstract

Background Squamous cell carcinoma (SCC) is the most severe complication and most common cause of death in patients with recessive dystrophic epidermolysis bullosa. The risk of developing SCC among junctional epidermolysis bullosa (JEB) patients is unclear from the literature, however, in our center we noticed an unexpected number of SCCs among adult patients with JEB.

Objective To review all documented patients with JEB who developed an SCC, both from our EB center and those reported in the literature.

Methods A search in our EB registry documenting all JEB patients visiting our EB referral center from 1990 through 2010 revealed seven JEB patients who developed one or more SCCs. A systematic literature search revealed eight relevant articles documenting a total of seven patients who developed an SCC.

Results In our EB registry we found seven cases of JEB who developed an SCC; these were all adults classified with non-Herlitz type of JEB. The frequency of developing an SCC among adult JEB patients (n=28) in our center was therefore 25%. In the literature, we found seven case reports of JEB complicated by SCC (also classified as JEB, non-Herlitz type), bringing the total number of documented cases to 14. The first SCC in JEB patients developed at an average age of 50 years (median 52 yrs, range 28-70 yrs). In 9 out of 14 cases, multiple primary SCCs occurred, with a total of 45 SCCs. The SCCs are most often located on the lower extremities, in areas of chronic blistering, long-standing erosions or atrophic scarring. Three (21%) patients developed metastases and died on average 8.9 years after diagnosis of the initial SCC.

Limitations This study was retrospective and the statistical analyses were based on a small number of patients.

Conclusions From their third decade, adult JEB patients have an increased risk (1:4) of developing SCC on their lower extremities. The SCCs have a high recurrence rate and follow an aggressive course that results in death in one out of five patients. We recommend annual checks of all JEB patients for SCC from age 25.

Introduction

Epidermolysis bullosa (EB) is a heterogeneous group of diseases characterized by trauma-induced blistering of the skin and mucous membranes.[1] Four major EB types can be distinguished based on the ultra-structural level in which blisters develop.[2] One of these is junctional epidermolysis bullosa (JEB), an autosomal recessive inherited subtype characterized by cleavage through the lamina lucida. JEB is caused by mutations in one of the genes encoding for the lamina lucida/densa adhesion protein laminin-332 (*LAMA3*, *LAMB3*, *LAMC2)*, or the hemidesmosomal proteins type XVII collagen (*COL17A1*) and integrin α6β4 (*ITGA6*, *ITGB4*).[2-4] JEB can be roughly subdivided into two types: JEB, Herlitz type (JEB-H), which leads to death within the first three years of life, and JEB, non-Herlitz type (JEB-nH) with a life expectancy extending to adulthood.[1,2,4] The clinical features of JEB-nH are generalized or localized trauma-induced blisters that often heal with scarring, erosions, crustae, alopecia, nail dystrophy or loss, enamel hypoplasia, dental caries, corneal scarring and keratitis. JEB-H is complicated by systemic involvement, leading to mortality in infancy caused by a failure-to-thrive, sepsis, pneumonia and tracheolaryngeal obstruction.[1,4]

Another of the four major types of EB is dystrophic EB, which is characterized by cleavage through the sub-lamina densa. The development of squamous cell carcinoma (SCC) is well known in dystrophic EB, especially in patients with severe generalized recessive dystrophic epidermolysis bullosa (RDEB). The frequency of SCC in severe RDEB is 23% and the estimated cumulative risk of developing a primary SCC is 90.1% by age 55.[5] This is much higher than the lifetime risk of developing an SCC in the American-Caucasian population, which is estimated to be 7-11%, with an incidence rate of 0.08-1.36 per 1,000 person-years.[6,7] The lifetime risk of developing an SCC in the Netherlands is lower, with an estimate between 3-6%.[8] Although SCCs in RDEB patients are mostly well differentiated with respect to their histopathology, they have an aggressive course with a tendency to metastasize.[1] Metastatic SCC is the most common cause of death in severe RDEB patients, with a cumulative risk of death of 87.3% at age 45.[5]

In the literature, the risk of developing SCC for JEB patients is unclear. It is assumed that patients with JEB-nH do not have an increased risk of developing SCCs since there have been only a few case reports of SCC in JEB-nH patients. Furthermore, in a prevalence study by Fine *et al*., the frequency of developing an SCC among JEB-nH

patients was estimated at 0.5%.[5] However, in our EB referral center, we noticed a high rate of SCCs among JEB-nH patients which we did not observe among JEB-H patients, whereas Fine *et al*. saw a higher frequency (4.4%) of SCCs among JEB-H patients.[5]

Here we report and review all the JEB patients who developed an SCC, including patients in our center and those reported in the literature. We then discuss the clinical consequences of our findings.

Materials and Methods

EB registry

Analysis of our EB registry database containing 362 EB patients visiting our EB referral center between 1990 through 2010 produced seven patients with JEB complicated by one or more SCCs. These cases were followed longitudinally. Patients EB 117-01[4], EB 078-01[9] and EB 029-01[9] have been described previously. Diagnosis of the EB subtype was confirmed by transmission electron microscopy, immunofluorescence and, in some cases, mutation analysis. Details of biopsy sites[10], electron microscopy[11], source of antibodies[9,10], immunofluorescence procedures[10], and DNA and RNA isolation and analysis[4,9] have been reported elsewhere. The diagnosis of SCC was confirmed by biopsy specimens or excision specimens of suspected skin lesions and these were studied using histopathological methods. Statistical analysis was performed with SPSS (IBM, Chicago, Ill, USA) using Kaplan-Meier type estimates.

Literature review

A systematic literature search was performed in the MEDLINE and Embase databases using the terms '*junctional epidermolysis bullosa*' and '*squamous cell carcinoma'*. Eleven results were found in MEDLINE and 39 results in Embase, including eight relevant articles documenting a total of seven JEB patients who developed SCC.

Results

Case reports

Analysis of our EB registry revealed seven patients with JEB who had developed one or more SCCs. The patients are described briefly below (see tables 1 and 2 for details).

Patient 1 (EB 117-01)

A 42-year-old woman suffering from generalized JEB-nH with a complete loss of type XVII collagen presented with three ulcers in a giant plaque of scarred skin covering the pretibial aspect of the lower right leg (figure 1a). These ulcers had developed years ago in an area with frequent and extensive blistering and they had each grown to a size of 4 cm in diameter. Biopsy of the three tumors showed poorly, moderately, and well-differentiated SCCs. Cytology of small lymph nodes that were palpable in the right groin was negative for malignancy. The patient refused amputation of the right leg. The tumors were excised and the wound closed with an autologous split skin graft. Although histopathology confirmed tumor-free margins, the most distal tumor was still macroscopically visible and was excised with Mohs micrographic surgery and left open for healing by second intention. One month later she developed a 3x3 cm indurated hyperkeratotic lesion on her lateral right knee, which was diagnosed as a well-differentiated SCC and excised with tumor-free margins. Nine months later the lymph nodes in her right groin had grown to 2 cm and cytology confirmed metastasis of SCC. She also developed lymph node metastasis on her right upper leg. The patient refused possible curative lymph node dissection, and when the lymph node metastases on her leg and groin had grown to 20 and 10 cm in diameter, respectively, a palliative resection was performed. The patient died due to metastasis, 21 months after the diagnosis of the first SCC, at age 43.

Patient 2 (EB 029-01)

Patient 2 is a 67-year-old man suffering from generalized JEB-nH with reduced laminin-332 (LM-332). The patient was involved in a motorcycle accident at age 16, which resulted in a skin defect on the left lower leg that has never closed. At age 48, the defect had grown for a year and showed more hypergranulation and papillomatosis. Histopathology showed a moderately to well-differentiated SCC and after two excisions, tumor-free margins were obtained. These were followed by multiple, failed autologous split skin grafts and a blister-roof graft, but at age 54 the patient still had an ulcer that covered 50% of his left lower leg. Multiple biopsies were taken and showed a moderately differentiated SCC. The ulcer was excised twice before tumor free margins were obtained and the wound was closed with an autologous skin transplant. A year later he experienced intolerable pain in his left leg with diffuse osteomyelitis. His left leg was amputated and histopathology showed massive growth of SCC with invasion of the dermis, subcutis, muscle, tendon and cortical bone. Extensive tumor staging

investigations showed no signs of metastases in the abdomen, thorax or skeleton. The patient has been in follow-up for 11 years without recurrence or developing another SCC.

Patient 3 (EB 119-01)

Patient 3 is an 81-year-old woman with generalized JEB-nH with reduced LM-332 since birth. She presented at age 61 with a hyperkeratotic plaque on her left lateral malleolus. Biopsy showed a verrucous carcinoma and the lesion was excised. Twelve years later, two hyperkeratotic crusted tumors developed at the excision site (figure 1b), which were diagnosed as well-differentiated SCCs. The lesions were excised by Mohs microscopic surgery and left open for healing by second intention. She has not developed any new primary tumors or metastases in 8 years of follow-up.

Patient 4 (EB 078-01)

Patient 4 was a 47-year-old man suffering from generalized JEB-nH with reduced LM-332. The pretibial area of his right leg had been erosive for several years. At age 28 he presented with two verrucous tumors within the pretibial erosions (figure 1c). Biopsy showed well-differentiated SCCs and the tumors were excised. In the following years he developed another six primary SCCs, all on his right lower leg. At age 46 his right leg was amputated after histopathology of a tumor showed an invasive SCC with a pseudosarcomatous growth pattern. A year later two lymph node metastases were found in his right groin, as well as two suspected areas on his lungs. He died two months later at age 47.

Patient 5 (EB 050-01)

Patient 5 is a man born in 1925 with localized JEB-nH with reduced LM-332. At age 71, he experienced more pain in his right lower leg with a pretibial 10x15 cm infiltrated ulcerative lesion. Biopsy showed an ulcerative SCC and the lesion was excised with tumor-free margins and closed with an autologous split skin graft. There were no lymph nodes palpable and a CT scan of his brain showed no signs of metastases. The patient was lost to follow-up.

Patient 6 (EB 113-01)

Patient 6 is a 72-year-old woman suffering from generalized JEB-nH. At age 49 she presented with a perianal tumor that she had had for a few months; it had reached a size

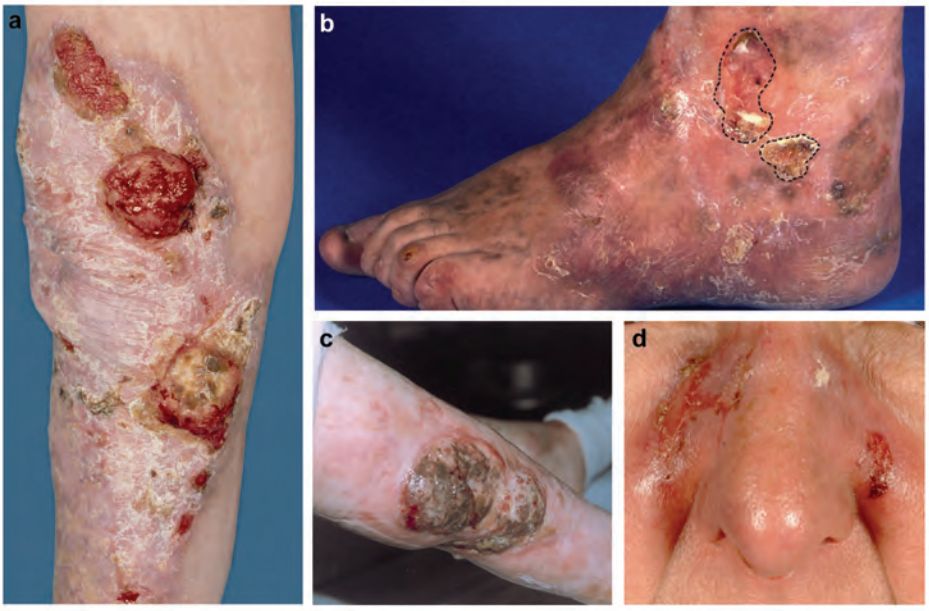

Figure 1. Squamous cell carcinoma in JEB patients. (a) Three erosive tumors in an erythemato-squamous plaque of scarring on and below the right knee of patient 1. (b) Two hyperkeratotic crusted plaques located at the excision site of a previous squamous cell carcinoma on the left lateral malleolus in patient 3. (c) Two papillomatous tumors on the right lateral lower leg of patient 4. (d) Radiodermatitis on the nose after radiotherapy of a squamous cell carcinoma on the inner left ala of the anthrum nasi in patient 7.

of 8x15 mm. Histopathology showed a well-differentiated SCC and the lesion was excised and closed primarily. No lymph nodes were palpable. Eleven years later she presented with an elevated tumor, 2x1.5 cm, on her left lower leg. Histopathology showed a well-differentiated SCC with a differential diagnosis of a pseudocarcinomatous hyperplasia. The lesion was excised and closed with an autologous split skin graft. Eight years later, at age 68, she presented with hyperkeratosis on her right lower arm. Biopsy showed a well-differentiated SCC, but after excision the prevalent histopathological diagnosis of the excised tumor was pseudoepitheliomatous hyperplasia. The patient has not developed any more SCC in 4 years of follow-up.

Patient 7 (EB 043-01)

Patient 7 is a 62-year-old man diagnosed with localized JEB-nH. At age 55 he presented with rhinorrhea and a cutaneous erosion with a raised border in the left ala of the

vestibulum nasi. He had had erosions in his nasal cavity for the previous three years. Biopsy showed an SCC, but further extensive tumor staging showed no metastases or local invasion of the septum or bone. The patient was treated with radiotherapy, which resulted in remission and a slow-healing radiodermatitis with erythematous erosions on the nose (figure 1d). In 7 years follow-up, he has not developed any further SCCs.

Occurrence and risk of SCC in JEB patients in our EB registry

Our EB registry contains 67 live-born JEB patients, of which 40 are classified as JEB-nH, 22 as JEB-H, and five as JEB with pyloric atresia. Of these 67 patients, 28 had reached the adult age of 18 in 2010; these were all patients with JEB-nH. Seven of these adult patients had a history of one or more SCCs, which corresponds with a frequency of 25%. The incidence rate of a primary SCC is 7.6 per 1000 person-years for adult JEB patients (with a total of seven cases and 916 adult follow-up years). If JEB patients of all ages (n=67) are included, we see a frequency of 10.4%, with an incidence rate for a first SCC of 4.6 per 1000 person-years (seven cases and 1522 follow-up years). The cumulative risk of occurrence of a first SCC in all JEB patients is 3.6% by age 30, increasing to 22.7% at age 50, 31.8% at age 60, 47.2% at age 70, and 70% at age 75. The cumulative risk of death from metastatic SCC in all JEB patients is 10.9% by age 50. If only those JEB patients with a history of SCC are considered, a cumulative risk of death from metastatic SCC is 33.6% by age 50.

Review of the literature

A literature review revealed seven JEB patients who developed one or more SCCs (see tables 1 and 2).[12-19] Together with the seven patients from our EB registry, we had a total of 14 cases to study (10 males (71%) and 4 females (29%)). All the patients were classified as JEB-nH. Nine of them (64%) had reduced or absent LM-332, there was loss of type XVII collagen in two patients (14%), and in three patients the cause was not documented. Of the eight mutations known, most (n=6) are located on the *LAMB3* gene encoding for the β3 chain of LM-332. The other two mutations involve *COL17A1*, which causes complete loss of type XVII collagen. None of the 14 patients had the localized JEB-nH phenotype associated with residual type XVII collagen expression.[4] The average age of developing a first SCC was 50 years, with a median of 52 years (range 28-70 years).

Of the 14 patients, 64% developed multiple primary SCCs. Together they had at least 45 SCCs, with a median of 2 SCCs per patient. Patients 4 and 9 had the most

tumors, (respectively 9 and more than 8). The second SCC was most often seen in the surrounding area or at the excision site of the first SCC, while in almost all the patients, SCCs arose in areas of chronic blistering, long-standing erosions/ulcers, or atrophic scarring. Figure 2 shows the approximate distribution of all 45 SCCs, with most located on the lower extremities. The appearance of SCCs in JEB patients is described by adjectives such as indurated,[12] verrucous,[12,19] hyperkeratotic,[12,14] exophytic,[17,18] or fungating[12] plaques or tumors. Other adjectives were ulcerative, erosive lesions[12,15] and hypergranulation. In a few cases, pain and visible change of the lesion were noted, and one case reported rhinorrhea. The therapy chosen for 13 patients consisted of surgical excision, either conventional or with Mohs microscopic surgery. Three of the patients needed an amputation. In one patient curative radiation therapy for a primary SCC was achieved, but this resulted in a slow-healing radiodermatitis. Although most SCCs were histopathologically well differentiated, three (21%) patients developed metastases and died, on average 8.9 years after the primary SCC.

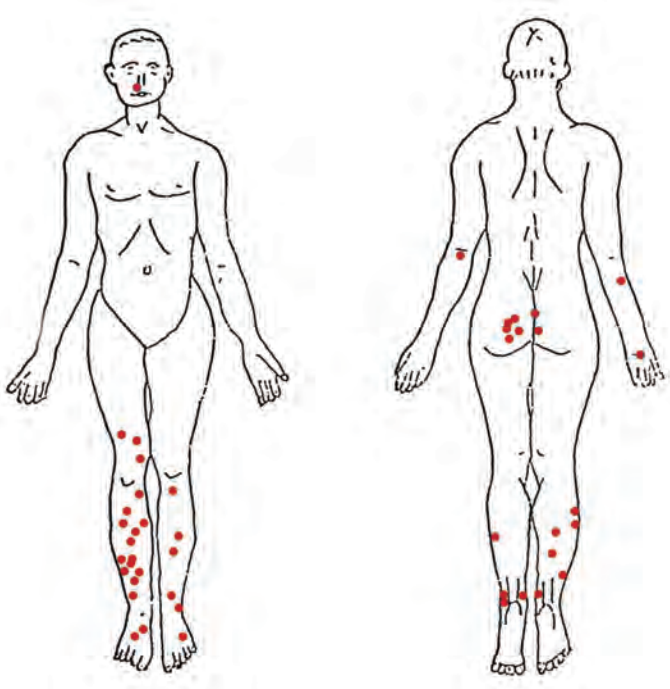

Figure 2. The approximate locations of 45 squamous cell carcinomas in 14 JEB patients.

Table 1. Reported cases of squamous cell carcinoma in patients with junctional epidermolysis bullosa: patient characteristics

Patient	Age of onset	Sex	subtype	IF	Mutation	Consequences	Reference
1 (EB 117-01)	Birth	F	JEB-nH gen	Col17 negative	*COL17A1* c.[3236delC]+[3236delC]	p.[Ser1079CysfsX26]+[Ser1079CysfsX26]	This article
2 (EB 029-01)	Birth	M	JEB-nH gen	LM-332* reduced	*LAMB3* c.[628G>A]+[628G>A]	p.[Glu210Lys]+[Glu210Lys]	This article
3 (EB 119-01)	Birth	F	JEB-nH gen	LM-332* reduced	*LAMB3* c.[29insC]+[628G>A]	p.[Leu11ProfsX43]+[Glu210Lys]	This article
4 (EB 078-01)	Birth	M	JEB-nH gen	LM-332* reduced	*LAMB3* c.[628G>A]+[1903C>T]	p.[Glu210Lys]+[Arg635X]	This article
5 (EB 050-01)	Birth	M	JEB-nH loc	LM-332* reduced			This article
6 (EB 113-01)	Birth	F	JEB-nH gen				This article
7 (EB 043-01)	Birth	M	JEB-nH loc				This article
8	Birth	M	JEB-nH gen	LM-332 negative	*LAMB3* c.[29insC]+[2500C>T]	p.[Leu11ProfsX43]+[Gln834X]	14, 18, 47
9	Birth	M	JEB-nH gen	LM-332 negative	*LAMB3* c.[29insC]+[2500C>T]	p.[Leu11ProfsX43]+[Gln834X]	14, 18, 47
10		M	JEB-nH				17
11	Birth	F	JEB-nH gen	LM-332 reduced	*LAMB3* c.[1903C>T]+[1048A>C]	p.[Arg635X]+[Thr350Pro]	12, 48
12	Teens	M	JEB-nH loc	LM-332 reduced			12
13	Seven	M	JEB-nH loc	LM-332 reduced			12, 13, 15, 16
14	Birth	M	JEB-nH gen	Col17 negative	*COL17A1* c.[4003delTC]+[4003delTC]		19

M male; F female; JEB-nH junctional epidermolysis bullosa, type non-Herlitz; gen generalized; loc localized; IF immunofluorescence antigen staining; * IF staining with GB3 monoclonal antibody; LM-332 laminin-332; Col17 type XVII collagen

Table 2. Reported cases of squamous cell carcinoma in patients with non-Herlitz junctional epidermolysis bullosa: tumor characteristics

Patient	Age of first SCC	No. of SCC	Localisa-tion	Arisen in a site with	Clinical features SCC	Histopathological differentiation of SCC	Therapy	Metas-tasis	Outcome (follow-up after diagnosis of last SCC)	Refere-nce
1 (EB 117-01)	42	4	Lower extremity	Chronic blistering; atrophic scarring	Erosive; hyperkeratotic	Well, moderately, poorly	Excision, MMS, lymph node dissection	Yes	Died at age 43	This article
2 (EB 029-01)	48	2	Lower extremity	Chronic ulcer	Hypergranulation; papillomatosis; ulcerative; pain	Well, moderately	Excision, amputation	No	Alive (follow-up 11 years)	This article
3 (EB 119-01)	61	2	Lower extremity	Atrophic scarring	Hyperkeratotic	Well	Excision, MMS	No	Alive (follow-up 8 years)	This article
4 (EB 078-01)	28	9	Lower extremity	Chronic ulcer	Verrucous	Well, poorly	Excision, amputation	Yes	Died at age 47	This article
5 (EB 050-01)	71	1	Lower extremity		Ulcerative; pain		Excision	No	Unknown (lost to follow-up)	This article
6 (EB 113-01)	49	3	Perianal; lower and upper extremities	Blistering	Hyperkeratotic	Well	Excision	No	Alive (follow-up 4 years)	This article
7 (EB 043-01)	55	1	Nasal cavity	Chronic erosion	Erosive; rhinorrhea		Radiotherapy	No	Alive (follow-up 7 years)	This article
8	39	>8	Lower extremities		Hyperkeratotic; exophytic	Well	Excision, MMS	No	Alive (follow-up 1 year)	14, 18

Table 2 continued

Patient	Age of first SCC	No. of SCC	Localisation	Arisen in a site with	Clinical features SCC	Histopathological differentiation of SCC	Therapy	Metastasis	Outcome (follow-up after diagnosis of last SCC)	Reference
9	32	>4	Lower extremities	Atrophic scarring		Well	MMS	Yes	Died at age 39	14, 18
10	54	1	Upper extremity	Atrophic scarring	Non-healing friable exophytic plaque		MMS	No	Alive (follow-up 2,5 years)	17
11	70	>6	Sacrum; buttock	Chronic erosion	Warty; fleshy; nodules; indurated; ulcerative	Well, moderately	Excision	No	Died at age 75	12
12	42	1	Lower extremity		Chronic ulcer	Well	Excision	No	Alive (follow-up 6 years)	12
13	55	2	Upper and lower extremities	Unaffected skin; chronic ulcer	Hyperkeratotic; fungating	Well	Excision, amputation	No	Unknown (lost to follow-up)	12, 13, 15, 16
14	58	1	Lower extremity		Verrucous; exophytic	Well	Excision	No	Alive (follow-up 4 years)	19

SCC squamous cell carcinoma; MMS Mohs micrographic surgery

Discussion

The age of onset, number, location, clinical appearance and course of SCCs in EB patients differs from the general population. EB patients develop their first SCC at a younger age than non-EB patients.[5,6] Patients with severe RDEB are at risk at an even younger age than JEB patients. In the former, the most common 5-year interval during which a first SCC arose was 20-25 years, with the youngest reported case being only six-years old.[5,20] The age at which SCC develops in JEB patients ranged from 28-70 years. Patients with JEB and RDEB are at risk of developing multiple, primary SCCs with a median of 2 and 3-3.5 per patient, respectively.[5] In RDEB the most common site in which SCCs develop is within chronic skin wounds (86.7%-100%) and long-term cutaneous scars (20%-26.7%).[5] They are nearly always located on the extremities, particularly over bony prominences, where the most trauma occurs and consequently the most blisters.[1,5] This study reveals that this also applies to JEB patients, with the most likely site being on the lower extremities. In the general population, most SCCs occur on areas of the body that are frequently exposed to sunlight, such as the head, neck and back of the hands, and they usually present as red scaly nodules with irregular or indistinct borders.[1,6] In EB patients the clinical appearance of SCCs is less obvious and they are not easily distinguished from other wounds on their skin.[6] Another phenomenon seen in JEB as well as in RDEB patients is that SCCs have an aggressive course with a tendency to metastasize irrespective of the histopathological stage of differentiation.[5]

There are many theories concerning the pathogenesis of SCCs in EB patients. One associates scarring and repetitive tissue stress with the development of SCCs. In RDEB patients, there is a correlation between the risk of developing SCC and the severity and extent of ulceration and scarring, and in patients suffering from other cicatrizing dermatoses, e.g. burn scars, a high incidence of similar SCCs is seen at a young age.[1,21] *Goldberg's tissue stress theory* proposes that repetitive tissue ulceration leads to a loss of cellular memory and differentiation, promoting tumor development.[22] Healing with dermal scar formation in RDEB is associated with a persistent growth-activated immunophenotype of epidermal keratinocytes, which could have malignant potential.[23] In burn scar- and EB- related SCCs, mutations are found in the Fas gene and p53 tumor suppressor gene, associated with apoptosis and tumorigenesis, respectively. These are not found, or differ from mutations found in conventional SCCs.[24-26]

Furthermore, obliterated lymphatics by scar tissue leave a depressed immunological state, where tumor cells can grow without normal immunosurveillance mechanisms.[27]

Another theory in the pathogenesis of SCCs in JEB patients is a diminished immunological status, that might be caused by malnourishment and which could allow tumor development and invasion without the normal immunosurveillance mechanisms.[28,29] Various *in vitro* studies have shown that JEB patients have a decreased production of peripheral mononuclear cell cytokines (IL-1, IL-2 and TNF) and a depressed natural killer cell activity, compared to normal control subjects.[28,29]

The pathogenesis of SCCs might also be related to the loss or reduction of LM-332 in JEB patients. LM-332 is not only important in stable adhesion of keratinocytes and hemidesmosome formation, but is also related to migratory situations.[30] Although a downregulation of LM-332 is seen in various tumors,[31-34] it is the general consensus that LM-332 expression is elevated in carcinogenesis and this is correlated with tumor invasiveness and poor patient prognosis.[35-39] Furthermore, several proteolytic fragments of LM-332 stimulate cancer cell migration,[30,39] and enhanced signaling of LM-332 with α3β4 and α6β4 integrins stimulate cell migration, tumor dissemination, tumor invasiveness, cancer cell survival, and tumor angiogenesis.[34,39-41] In RDEB the pathogenesis of SCCs might be linked to the interaction of the non-collagenous (NC1) domain of type VII collagen with LM-332.[35] It is conceivable that the presence of subunits of LM-332 in JEB patients is related to the pathogenesis of SCCs, but further research is needed in this field.

The frequency (25%) of SCC in adult JEB patients seen in our registry is much higher than would be expected from the literature, with only 7 cases reported. From our data, it is likely that JEB patients do have a higher risk of developing SCCs compared to the general population.

Our study results are in contrast to those of Fine *et al.*[5] In their cancer prevalence study, which used the US National EB Registry (US NEBR), only one of the 191 JEB-nH patients developed an SCC, corresponding with a low frequency of 0.5%. However, they also found an increased frequency (4.4%) of SCCs among JEB-H patients, with two out of 45 JEB-H patients developing an SCC.[5] In contrast, the 14 JEB patients reviewed here were all classified as JEB-nH; none were classified as JEB-H. Furthermore, in our EB registry none of the JEB-H patients (n=22) developed an SCC. This may be explained by the short life of these patients, in which they do not have the time to develop an SCC. Indeed, in the study by Fine *et al.*, both JEB-H patients survived to adulthood before developing an SCC.[5] Because the general consensus is that survival to

adulthood of JEB-H patients is a contradiction in terms, it has been proposed that the rare, long-term survival patients in the US NEBR have a limited diagnostic validity.[42] This might mean that the two JEB-H patients who developed an SCC in Fine *et al.*'s study[5] are, in fact, JEB-nH patients. We therefore conclude that adult JEB patients, as in such obligatory classified as JEB-nH, are at increased risk of developing an SCC. On the contrary, as a result of death in infancy in JEB-H patients, they do not have an increased risk of developing an SCC.

The combined increased risk and aggressive course of the SCCs in adult JEB patients has implications. There should be a pro-active attitude towards detecting SCCs in JEB patients, since early detection probably improves the prognosis. Because SCCs can develop in early adulthood, we recommend checking JEB patients from age 25. At our center we check JEB patients every year and inspect their entire skin, paying special attention to their lower extremities. This can be difficult since the SCCs often look like their normal wounds.[43] Suspicious lesions are chronic non-healing ulcers or erosions, hypergranulation, induration and hyperkeratotic, exophytic, verrucous nodules or plaques. Additional clues can be pain, a stinging or burning sensation, and noticeable changes in a lesion.[1,43] Biopsies should be taken of any suspicious lesions and multiple biopsies are necessary to avoid missing malignancies, because tumors often start at the margin of a lesion and only one part of a lesion may undergo malignant transformation.[43] Lymph nodes should be palpated, although in EB patients they are frequently enlarged due to persistent inflammation or chronic infection. The role of sentinel node biopsy in EB patients is still unclear.[44,45] If necessary, further tumor staging should be performed.
The treatment of first choice is excision, although no guidelines for the excision margins are given. The borders of SCCs in EB patients are not clearly defined and special attention should be given to perivascular and perineural growth. In some cases Mohs microscopic surgery is performed to minimize resection margins, although there is no evidence that this technique improves morbidity and mortality over conventional excision.[1,5] The excision site should be observed closely, as there is a high recurrence rate at the same location, despite wide excision with tumor-free margins. If there is an invasive tumor in a limb, amputation is recommended. Although radiotherapy may be beneficial in the palliation of patients with metastatic disease, in dystrophic EB it is considered to be only partially or temporarily beneficial for the treatment of primary SCCs.[1,46]

Acknowledgements

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7

Reply to: “Squamous cell carcinoma and junctional epidermolysis bullosa”

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To the editor

In a commentary to our original research article, "Risk of squamous cell carcinoma (SCC) in junctional epidermolysis bullosa, non-Herlitz type (JEB-nH): report of seven cases and a review of the literature",[1] dr. Fine raises some concerns about possible inaccurate conclusions that we have made in the comparison with his previously published results on the National EB Registry (NEBR) in the US.[2]

In our article we highlight the contrast between the high frequency of developing an SCC seen in our JEB-nH cohort (17.5%; 7 out of 40), and the low frequency seen in the NEBR (0.4%).[2] Although in his response dr. Fine states that he is confident that his data accurately reflects the EB population in the US and other countries, he also admittedly says that with a longer follow-up he is sure that he will see additional SCCs in his JEB-nH cohort, and in such agreeing with us that JEB-nH patients do have an increased risk of developing an SCC.

Dr. Fine's remaining comments revolve around one central question: "can children that suffer from the rare and devastating disease junctional EB, type Herlitz (JEB-H) survive to adulthood?" A 100% childhood mortality rate was seen in the Australasian, Austrian, International, Italian, and Dutch JEB-H cohorts, together comprising a total of 69 JEB-H patients, in which the oldest patient had deceased at the age of 10 years.[3-8] Due to the discrepancy of mortality rates seen in these cohorts, and those seen in the NEBR, Laimer *et al*. suggested that the long-term JEB-H survivors included in the NEBR might reflect a "limited/restricted diagnostic validity/validation."[5] Indeed, the NEBR uses merely non-molecular diagnostic testing in combination with clinical findings to classify patients according to the 2008 classification report.[9] However, in this classification no clear definition of JEB-H is stated. Furthermore, the clinical and non-molecular diagnostic findings reported in this classification, show a distinct overlap between the subtypes JEB-H and JEB-nH, leaving room for different interpretations of the diagnostic findings. As a result, a poor discriminative value, concerning the prognosis between JEB-H and JEB-nH patients, is seen in the NEBR. At the age of 15 years, 66% of the 41 JEB-H patients included in the NEBR had deceased, with an average age of death at three years. In comparison, among the 186 included JEB-nH patients a mortality rate of 51% was seen, with an average age of death around four years. Most deaths occurred in the first two years of life in both the JEB-H as JEB-nH group.[10] In the Netherlands, the definition used to classify JEB-H is the complete absence of functional laminin-332 (LM-332).[3] Recent publications have proposed that this distinction is best made utilizing and

combining immunofluorescence antigen mapping with DNA analysis.[3,5] Clinical findings, especially those seen in newborns, were not helpful in distinguishing JEB-H from other EB subtypes, and also had no prognostic value after the diagnosis was made.[5,11] Using both non-molecular and molecular laboratory tests in all Dutch JEB patients, a nearly perfect distinction was seen in JEB-H and JEB-nH patients, that provided the prognosis (i.e. lethal or non-lethal).[3] In concordance, there have been no case reports of JEB-H survivors with an absent LM-332 immunofluorescence staining with antibody GB3, together with mutations in the genes coding for LM-332 leading to nonsense mediated decay or truncated non-functional LM-332. Although Gache *et al*. did publish a JEB-H patient that showed amelioration with time; according to our criteria this patient is reclassified as JEB-nH, due to illegitimate exon skipping, resulting in truncated functional LM-332.[12] This emphasizes the importance of further RNA analysis in a few extraordinary cases. A correct diagnosis of JEB-H with its associated lethal prognosis is of utmost importance for patients and parents. It facilitates a shift from harmful invasive life-saving treatments, to those that enhance the comfort of JEB-H patients. Also, parents are well informed at the early stage and can be prepared for what is to expected.[11] Although in the 2008 classification report, multiple practical and socioeconomic reasons are given to omit molecular analysis from the classification of EB,[9] we think that no argument can apply to withhold JEB-H patients and their parents from a crucial correct diagnosis and prognosis.

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8

Punch grafting of chronic ulcers in patients with laminin-332 deficient, non-Herlitz junctional epidermolysis bullosa

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Abstract

Background Epidermolysis bullosa (EB) is a genetic, heterogeneous, trauma-induced blistering disease. Patients with laminin-332 (LM-332) deficient non-Herlitz junctional EB (JEB-nH) can have impaired wound healing witnessed by persistent, small, deep ulcers on the hands and feet that adversely affect the quality of life.

Objective To present the results of punch grafting in patients suffering from LM-332 deficient JEB-nH, and to discuss its therapeutic value.

Methods Retrospective analysis of the Dutch EB registry revealed four LM-332 deficient JEB-nH patients that were treated with punch grafting. Punch grafting was performed according to protocol, and the patients were followed-up.

Results In the past 10 years we have treated 23 ulcers in four JEB-nH patients with punch grafting without any complications or adverse effects. The ulcers had on average persisted six years prior to treatment. Healing rate after punch grafting was 70% (n=16), with a mean healing time of 2 months. Thirty percent (n=7) of the treated ulcers did not completely heal, but did show improvement. The recurrence rate after three months was 13% (n=2), and was due to renewed blistering.

Limitations Limitations of the study are the retrospective design, the small amount of patients, the absence of a control group, and a follow-up and ulcer measurement that were not standardized.

Conclusions Punch grafting can be used as a first-line treatment in small persistent ulcers in JEB-nH patients. The method is easy, inexpensive, with little risk of complications, and results in significant healing rates, and improvement in quality of life.

Introduction

Patients with the hereditary disease epidermolysis bullosa (EB) suffer from lifelong trauma-induced blistering of the skin and mucous membranes.[1] Junctional EB, type non-Herlitz (JEB-nH) is an autosomal recessive subtype caused by mutations in the genes encoding the lamina lucida/densa adhesion protein laminin-332 (*LAMA3, LAMB3, LAMC2)*, or the hemidesmosomal proteins type XVII collagen (*COL17A1),* or integrin β4 (*ITGB4*).[2] The laminin-332 (LM-332) deficient patients within this subtype have an impaired wound healing, and develop persistent small ulcers on glabrous skin of palms, soles, and finger tips, and on non-glabrous pretibial skin. At present, there is still no widely available treatment to prevent blistering in EB. The current preferred management strategy for wounds is the application of silicone wound dressings, to provide a moist wound environment and to prevent bacterial contamination.[3] However, this management is rarely associated with accelerated wound healing. In some cases, wounds fail to heal at all and this results in persisting chronic erosions and ulcers.[4] These chronic erosions and ulcers adversely affect the quality of life of EB patients, as they can be painful and result in functional limitations. Skin grafting has been successfully applied in EB patients, ranging from tissue engineered artificial allografts,[5,6] cultured allografts,[7,4] cultured autografts,[8,9] and full-thickness or split-thickness autografts.[10,11] We use the simple and non-expensive punch grafting method in EB patients. A previous publication in a French journal, showed the successful application of this technique in recessive dystrophic EB patients.[12] Punch grafting is a minor surgical procedure in which small full-thickness grafts are harvested with a punch biopsy, whereupon they are placed in the ulcer to promote wound healing.[13-16] The appliance of small punch grafts, aids the healing of chronic ulcers in several ways: it covers the defect with epithelium, it supplies dermis to a deep callus-edged ulcer, it multiplies the circumference of wound edges from which keratinocytes can migrate, and it delivers cytokines and growth factors to enhance wound closure.[16,17] The technique is a derivative of Reverdin's greffe épidermique, in which pinching of the thumb and index finger is used to produce a skin fold, after which small pieces of epidermis are shaved off.[18] A modification of this technique, in which the skinfold is created by lifting the skin with a needle, is called pinch grafting and was first described by Davis.[19] In the past 10 years we have performed punch grafting to treat small, persistent, and quality-of-life-impairing ulcers in patients with LM-332 deficient

JEB-nH. In this article, we present the results and discuss the therapeutic value of this simple grafting technique in this subset of EB patients.

Materials and Methods

Patients

A retrospective study was performed using the Dutch EB Registry comprising a total of 435 patients with 36 JEB-nH patients as of 1 September 2011. Analysis revealed that four patients had undergone punch grafting in the past 10 years, that all suffered from LM-332 deficient JEB-nH. The Dutch EB Registry contains 18 LM-332 deficient JEB-nH patients. The patients were followed up longitudinally. The diagnosis of JEB-nH was established based upon clinical characteristics, immunofluorescence antigen staining, electron microscopy, and mutation analysis (Table 1).

Grafting procedure

As JEB-nH patients have an increased risk of developing squamous cell carcinomas, especially in chronic ulcers, biopsies were taken to exclude malignancy in suspected lesions.[20] Punch grafting was performed according to protocol in the Dutch Center for Blistering Diseases in Groningen, by at least one dermatologist and one registered nurse. Patients were hospitalized for the treatment. The ulcers were pre-treated with individualized wound dressings to obtain granulation tissue. Two hours prior to grafting, the ulcers were dressed with a saline 0.9% compress or wound irrigation solution (Prontosan®, Braun Medical Inc., Bethlehem, Pennsylvania, USA). If insufficient granulation tissue was present, curettage of the wound bed was performed immediately before grafting. Thirty minutes prior to curettage, topical anaesthetic cream (EMLA®, AstraZeneca, London, United Kingdom) was applied to the ulcer. The donor site was localized on the lower abdomen or the thigh, and was disinfected with chlorhexidine 0.5%, after which the donor site was marked and superficial infiltration anaesthetic was applied by injecting xylocaine 2% with epinephrine (1:200.000). In patient 2, grafting was performed under general anaesthesia due to anxiety, and thus local anaesthetic of the donor site was omitted. Depending on the ulcer size, 3 or 4 mm skin was harvested from the donor site with a biopsy punch. The grafts were taken with sterile tweezers and were cut horizontally on a mid-dermal plane with scissors, resulting in a graft consisting of epidermis and a superficial dermal layer. The punch grafts were put on a sterile gauze

drenched in saline 0.9%. The grafts were then placed in the ulcer 2-6 mm apart from each other. After that a silicone foam dressing was put in the ulcer (Mepilex®, Mölnlycke Health Care, Gothenburg, Sweden). In case of a deep ulcer, it was first covered with a silicone exudate transfer dressing (Mepilex transfer®, Mölnlycke Health Care, Gothenburg, Sweden) before the silicone foam dressing (Mepilex®, Mölnlycke Health Care, Gothenburg, Sweden) was applied. The dressings were covered with synthetic cast padding (BSN Delta-rol®, Alimed, inc. Dedham, Massachusetts, USA) and fixated with a cohesive conforming gauze bandage (Peha-haft®, Hartmann international, Heidenheim, Germany). The donor site was either sutured or dressed with a silicone foam dressing (Mepilex® Mölnlycke Health Care, Gothenburg, Sweden) attached by soft silicone tape (Mepitac®, Mölnlycke Health Care, Gothenburg, Sweden). Patients were given strict bed rest for four days, after which they received bathroom privileges until the bed rest was lifted at day six to eight. After 3-4 days the ulcer was first inspected, after which dressing changes were performed every other day. The donor site was inspected when the dressings let loose, after which dressing changes were performed twice a week. The wound dressings that were used are similar as those used directly after transplantation, except in cases of apparent infection, in which the ulcers or donor sites were first covered with an antibacterial contact layer containing silver (Urgotul® Silver, Urgo Medical, Chenôve Cedex, France).

Results

Case reports

Analysis of the Dutch EB Registry revealed four JEB-nH patients who were treated with punch grafting in the past 10 years. The patients are described below, and the details can be found in Table 1.

Patient 1 (EB 132-01)

Patient 1 is a 35-year-old man suffering from localized JEB-nH caused by mutations in *LAMB3* (Table 1). Blistering was present at birth and mainly localized on his hands and feet. At age 31, punch grafting was performed in six ulcers on the plantar side of his right foot and two ulcers on his right shin (Figure 1). The ulcers on his right foot developed four years ago after blistering, and gradually became larger and more painful. Walking was extremely painful, and for the past three years he could only walk for 30 minutes

straight. At home he moved around on his knees. The patient was dependent on pain medication. The ulcers on his right shin had developed recently after blistering. The ulcer sizes varied from approximately 1 to 3 cm in diameter, they all had a red wound bed, and the ulcers on the foot had macerated edges. The patient was given flucloxacillin for two months to prevent wound infection. Forty-eight punch grafts were harvested from the right thigh and transplanted to the ulcers. The donor site was sutured, and it showed a good healing tendency after removal of the sutures at day 10. The dressings of the transplanted ulcers were taken off after three days and transplantation showed a good result with 76% of the grafts revascularized (i.e. changing from a white to a pink/red colour). However, a graft on digit (dig) I of his right foot was lost. Seven days after transplantation another 10 grafts (21%) were lost, but 90% of the remaining grafts showed revascularization. The patient was discharged from the hospital after 12 days and was followed up regularly. The ulcers on his right shin closed after less than one month, however erosions reoccurred after three months due to blistering. The ulcers on his right foot were healed after less than four months, except for the ulcer on dig I, although it did show 80% healing. At this time, the patient was without pain and able to walk long distances. After 25 months the ulcers on his feet were still closed. He experienced no complications or adverse effects of punch grafting.

Patient 2 (EB 048-01)

Patient 2 is a 16-year-old boy suffering from generalized JEB-nH caused by mutations in *LAMA3* (Table 1). In his childhood he developed an aversion against walking, due to blistering of his feet. He has been wheelchair bound for his entire life, only able to walk a few metres. At age 14, three ulcers on his left foot (plantar and dig I) were treated with punch grafts (Figure 2). These ulcers had been present for two to five years, and were very painful, necessitating the use of various pain medications. In the first three days of hospitalization the ulcers were cleaned, producing a red wound base. A swab of the ulcers revealed colonization of *Staphylococcus aureus*, and flucloxacillin was given for one month. Punch grafting was performed under general anaesthesia due to anxiety of the patient. From his right thigh, 44 punch grafts of 3 mm were harvested and transplanted. The donor site was fully healed after 11 days. After four days the ulcers were inspected and all grafts were in place and 80% was revascularized. Six days after transplantation all three ulcers were healed and another five days later physiotherapy was started. During this period, a new blister around the edge of the healed ulcer appeared. After 19 days the patient was discharged from hospital and followed-up

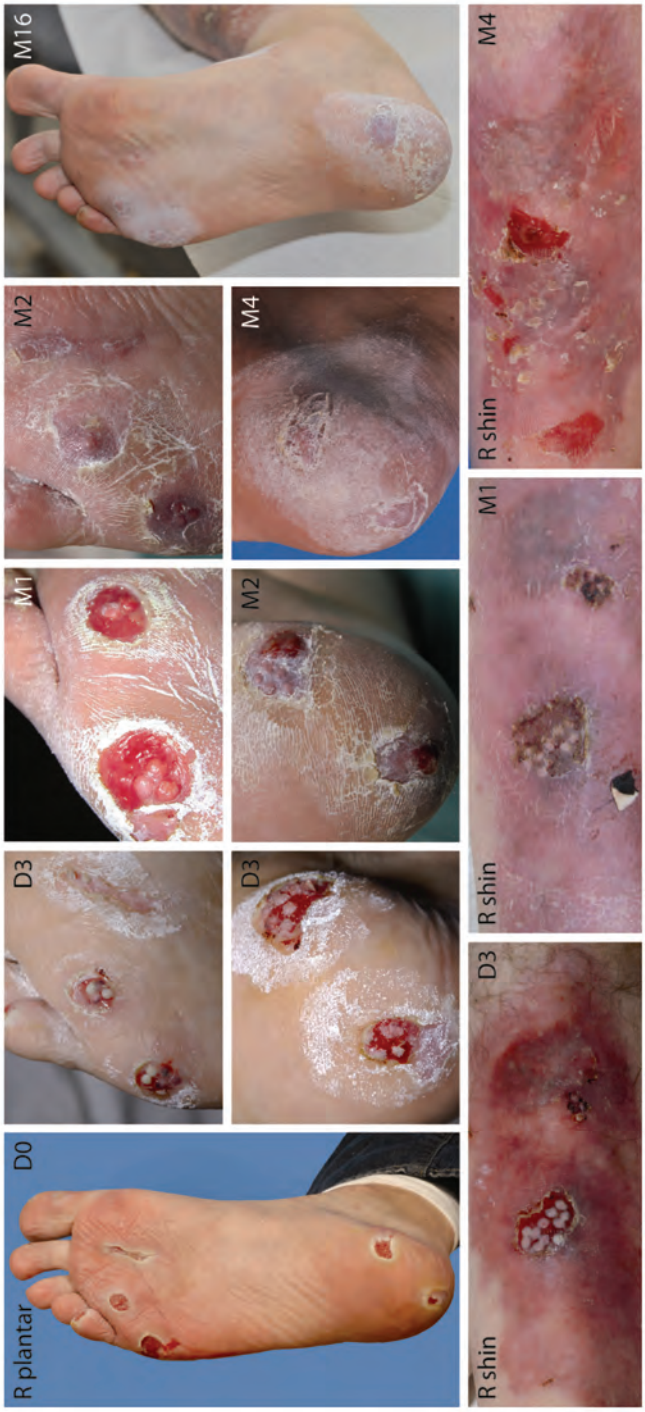

Figure 1. Punch grafting in patient 1. The ulcers on the plantar side of the right foot all healed within four months and remained healed after follow-up. The ulcers on the right shin healed within one month, but recurred after three months due to blistering. Legend: R right; D day; M month

regularly. After one month the ulcers were still closed, although the ulcer on his plantar foot was hyperkeratotic and the newly developed ulcer still persisted. Walking remained too painful for him, and he gradually started to take his pain medication again.

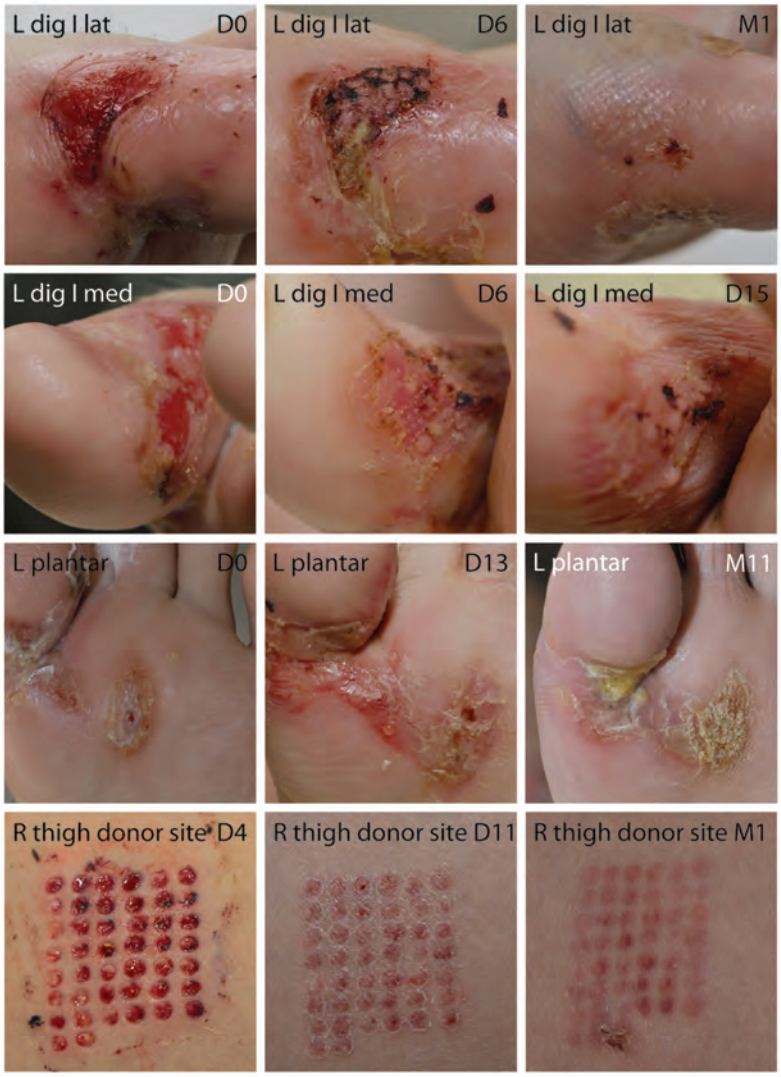

Figure 2. Punch grafting in patient 2. All ulcers on the left foot healed within six days, and remained so after follow-up. On day 13 a new blister appeared on the plantar side of the right foot. The donor site on the right thigh has healed within 11 days. Legend: L left; R right; D day; M month; lat lateral; med medial

Patient 3 (EB 012-01)

Patient 3 is a 63-year-old woman diagnosed with generalized JEB-nH cicatricialis due to *LAMA3* mutations (Table 1).[21] She suffers from generalized blistering that also affects the eyes and mucosa, and that heal with scarring, leading to strictures of the larynx, oesophagus and urethra. At age 27, severe blistering of her hands resulted in flexion contractures of her fingers. To treat this, multiple reconstructive surgeries were performed, in which the defects were covered with numerous split- and full thickness skin grafts. This did not produce the desired result, leaving the patient with six small, persistent ulcers on her right hand, that had been intermittently present for 10 to 17 years at the time of punch grafting at age 61 (Figure 3). These ulcers were painful; especially the ones on her digits limited her in her daily activities, such as applying wound dressings, and buttoning and zipping up her clothes. Twenty-six 3 mm punch grafts were harvested from the right lower abdomen and transplanted. The donor site was sutured and at day 12 the donor site was completely healed. After four days the ulcers were inspected, and all punch grafts were revascularized. The patient was discharged after six days of hospitalization. Follow-up after one month showed that the ulcers on the right palm had completely healed. The ulcers on her digits showed a 25-40% improvement, although in some ulcers crusted grafts were seen.

Patient 4 (EB 043-01)

Patient 4 is a 64-year-old man with localized JEB-nH caused by mutations in *LAMA3* (Table 1).[20] His blistering is mainly restricted to his lower legs, after which he has developed multiple relatively painless non-healing ulcers. His lower legs also show a pasty oedematous circular area with a brown, purple, red and livid discoloration for which he received compression therapy. Most probably this was caused by repeated blistering, but to exclude an effect of a possible venous insufficiency he was regularly examined by duplex sonography. A mild insufficiency of the venous saphena magna of both legs was not seen until the time of his last punch grafting at age 63, and this was treated with echo-foam-sclerotherapy. The patient received his first punch grafting at age 57 for an ulcer on his right medial malleolus, that was present for seven months and that showed no healing tendency. The ulcer was 1 by 3 cm and had a yellow/green dry wound base. A swab of the ulcer showed presence of *Streptococcus group B, Staphylococcus aureus,* and *Pseudomonas aeruginosa*. He was not treated with antibiotics; instead the ulcer was cleaned with wound dressings prior to transplantation for 10 days. Five punch grafts were harvested from the right lower abdomen and transplanted to the ulcer. When the

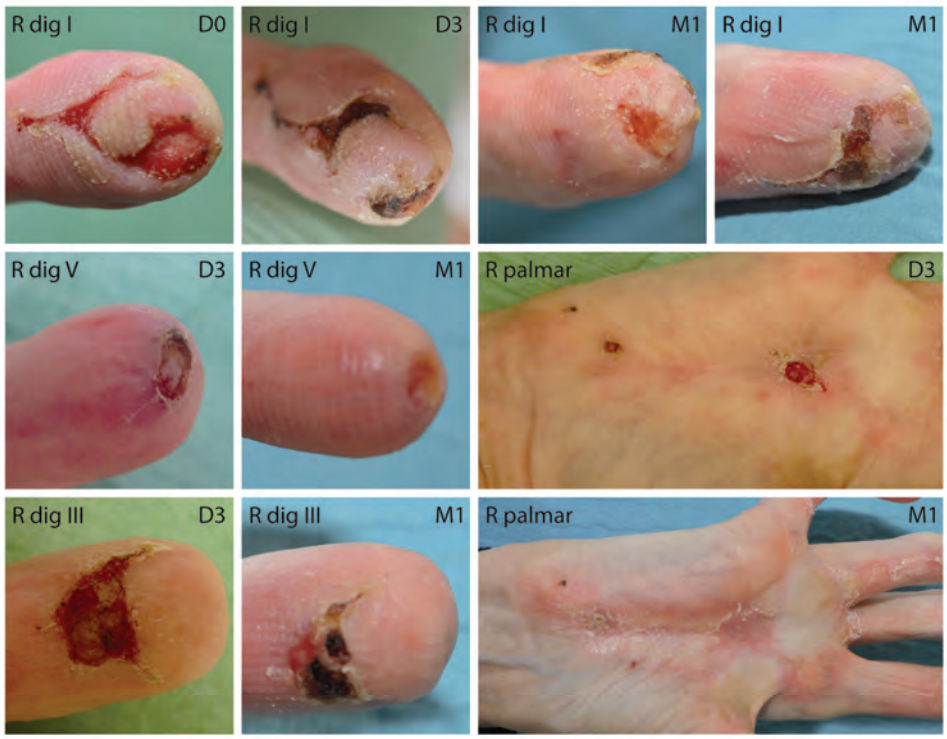

Figure 3. Punch grafting in patient 3. A 25-40% improvement of the ulcers on the digits of the right hand was seen one month after punch grafting. The ulcers on the palm of the right hand healed within one month. Legend: R right; D day; M month; dig digit

ulcer was inspected after four days, all grafts were in place and revascularized. Another three days later the grafts covered around 95% of the ulcer. After 18 days of hospitalization the patient was discharged and follow-up after two and three months showed a 50% healing of the ulcer.

At age 63, punch grafting was performed for two ulcers located on the right and left medial malleolus during a 16-day hospitalization (Figure 4, a). These ulcers had developed two months ago after blistering. The ulcer on the right medial malleolus was a 2.5 by 1.5 deep fibrotic ulcer, and the one on the left medial malleolus was 1 by 1 cm with a red wound base. Twenty 3 mm punch grafts from his lower abdomen were transplanted after curettage of the ulcers. After four days 39% of the grafts on the right medial malleolus was revascularized, and at day eleven 56% was revascularized. The ulcer healed after less then five months and at follow-up after eight months the ulcer remained closed. The grafts on the left medial malleolus showed 100% revascularization

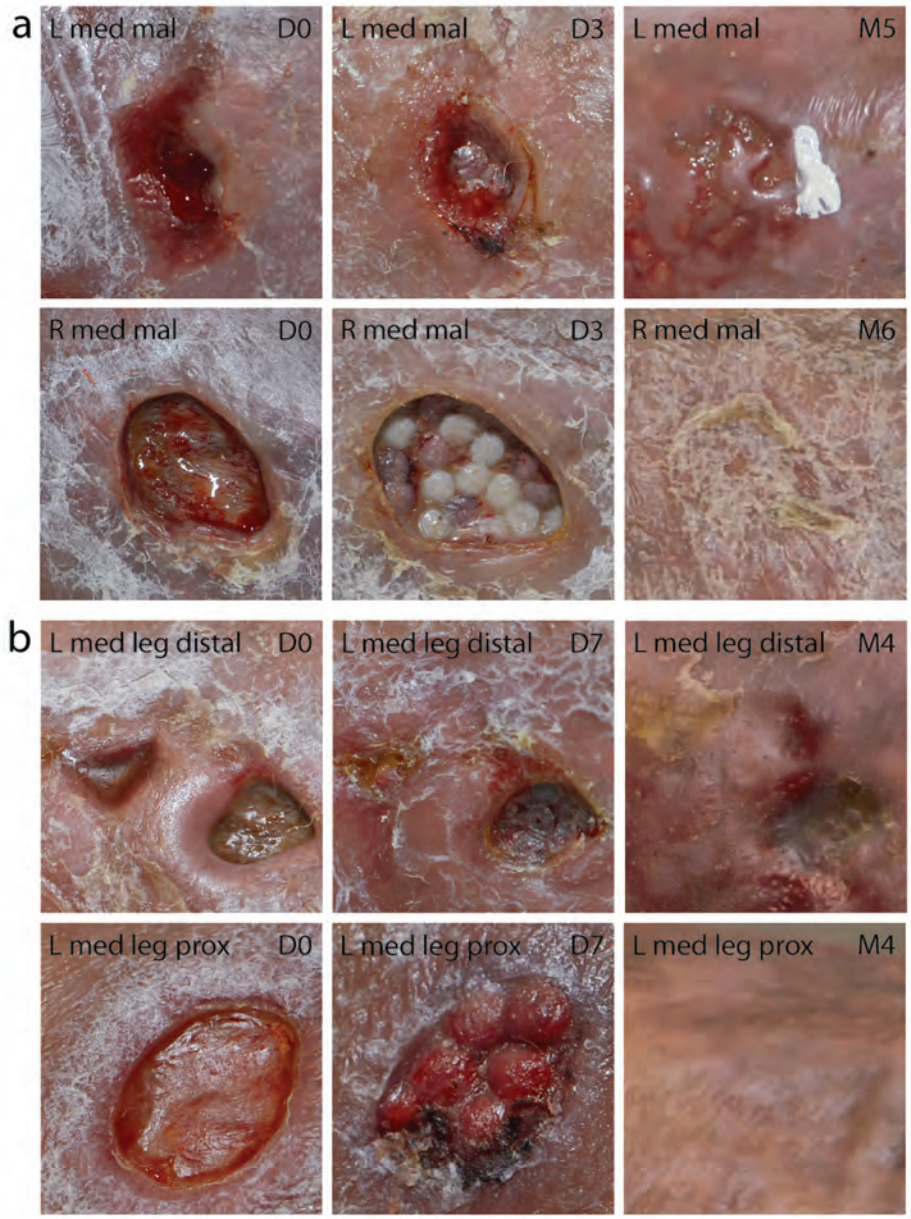

Figure 4. Punch grafting in patient 4. (a) After the second punch grafting, the ulcer on the left medial malleolus remained open due to renewed blistering. The ulcer on the right medial malleolus healed within 5 months. (b) The three ulcers on the left medial lower leg healed within three months after the third punch grafting. Legend: L left; R right; D day; M month; med medial; mal malleolus; prox proximal

after four days, but unfortunately the grafts discoloured again after 11 days. The transplantation did result in a 50% healing of the ulcer, but an exacerbation of blistering on the left medial leg after five months resulted in new ulcers. This has lead to the third punch grafting of three ulcers on his left medial leg (Figure 4, b). The ulcers were approximately 1 cm in diameter and varied from deep to superficial. A swab of the ulcers revealed colonization with *Staphylococcus aureus*, which was treated with ciprofloxacillin for one week. From his left lower abdomen sixteen 3 mm punch grafts were taken and transplanted. Like the first two transplantations, the donor site healed without complications. After five days all grafts were still in place and revascularized. During the 16 days of hospitalization the ulcers showed good healing tendency and at follow-up after four months all ulcers were healed.

Table 1. Characteristics of the patients and ulcers treated with punch grafts

Patient	Diagnosis	Gene	Opera-tion	Age (Y)	Follow-up term	Donor site			Ulcer					
						Location	# Grafts	Healing	No	Location	Size (cm)	Dura-tion	Healing	Recur-rence
1	JEB-nH loc	*LAMB3*	#1	31	25 M	R thigh	48	<2 M	#1	R foot dig V	1x1	4 Y	80%	-
									#2	R plantar radius II	2x0.3	4 Y	7 D	No
									#3	R plantar radius IV	1x1	4 Y	<2 M	No
									#4	R plantar radius V	1x1	4 Y	<2 M	No
									#5	R heel medial	1x1	4 Y	<4 M	No
									#6	R heel lateral	1x1	4 Y	<4 M	No
									#7	Right shin distal	3x2	<3 M	<1 M	3 M
									#8	Right shin proximal	1x1	<3 M	<1 M	3 M
2	JEB-nH gen	*LAMA3*	#1	14	11 M	R thigh	44	11 D	#1	L foot dig I lateral	2x1	5 Y	6 D	No
									#2	L foot dig I medial	2.5x1	5 Y	6 D	No
									#3	L plantar	0.3x0.3	2 Y	6 D	No
3	JEB-nH gen cicatricialis	*LAMA3*	#1	61	1 M	R lower abdomen	26	12 D	#1	R hand dig I distal	1.5x0.5	17 Y	25-40%	-
									#2	R hand dig I proximal	1.3x0.3	17 Y	25-40%	-
									#3	R hand dig III	1.1x0.9	17 Y	25-40%	-
									#4	R hand dig V	0.6x0.3	10 Y	25-40%	-
									#5	R palmar distal	0.9x0.5	17 Y	<1 M	No
									#6	R palmar proximal	0.4x0.3	17 Y	<1 M	No

Table 1 continued

Patient	Diagnosis	Gene	Opera-tion	Age (Y)	Follow-up term	Donor site			Ulcer					
						Location	# Grafts	Healing	No	Location	Size (cm)	Dura-tion	Healing	Recur-rence
4	JEB-nH loc pretibialis	*LAMA3*	#1	57	7 Y	R lower abdomen	5	Yes	#1	R medial malleolus	1x3	7 M	50%	-
			#2	63	10 M	Lower abdomen	20	Yes	#1	R medial malleolus	2.5x1.5	2 M	<5 M	No
									#2	L medial malleolus	1x1	2 M	50%	-
			#3	63	4 M	L lower abdomen	16	<4 M	#1	L medial lower leg distal	0.9x0.8	6M	<4M	No
									#2	L medial lower leg middle	1x0.6	6M	<4M	No
									#3	L medial lower leg proximal	1.1x1.3	6M	<4M	No

JEB-nH junctional epidermolysis bullosa; loc localized; gen generalized; Y years; D days; W weeks; M months; R right; L left; dig digit, - not applicable

Discussion

In this study we present the results of punch grafting in JEB-nH patients. In the past 10 years we have treated 23 ulcers in four patients. All treated patients carried mutations in the genes coding for LM-332, and none of them in the genes coding for the other proteins associated with JEB-nH: type XVII collagen and integrin β4. LM-332 plays an important role in the process of epidermal wound healing. It is secreted by keratinocytes to the underlying basement membrane as a heterotrimer, composed of a laminin α3 (*LAMA3*), laminin β3 (*LAMB3*), and laminin γ2 chain (*LAMC2*).[22] In unharmed epidermis, LM-332 is processed by cleavage of the laminin α3 chain, after which it interacts with hemidesmosomal integrin α6β4 to obtain stable adhesion of basal keratinocytes to the basement membrane.[23,24] If the epidermis is wounded, the hemidesmosomes are disassembled, and unprocessed LM-332 is secreted at the edge of the wound, stimulating migration of keratinocytes.[17,25-27] The role of LM-332 in cell migration, explains why JEB-nH patients with mutations in LM-332 are more likely to develop chronic wounds compared to patients with mutations in *COL17A1*. Especially the laminin α3 chain plays a significant role in cell migration, as cleavage of α3 changes LM-332 from a migratory to a stable state. This might explain why 75% of the JEB-nH patients that we treated carry mutations in *LAMA3,* while *LAMB3* mutations normally cause most JEB-nH cases (~80%).

Complications or adverse effects occurred in none of the treated patients, and in all patients the donor site healed without infection or blistering. The ulcers we treated had a maximum size of 3 by 2 cm. They were all localized on the feet, lower legs, and hands. On average these ulcers existed six years prior to treatment. The healing rate of the treated ulcers was 70% (n=16), with a mean healing time of two months. However, as most patients were discharged from the hospital with not completely healed ulcers, and healing was first recorded when patients came for check-up months later, the true healing time is most probably shorter. Thirty percent (n=7) of the treated ulcers did not completely heal, but did show improvement. This means that all treated ulcers healed or improved. Furthermore, in two patients a (temporary) stop or reduction of pain medication use was achieved, and one patient was able to walk long distances again after three years.

Among the 16 healed ulcers in our patients, the recurrence rate was 13% (n=2). In both ulcers this occurred after three months. The true recurrence rate might be higher,

as in some patients the follow-up time was relatively short. Recurrences, worsening of improved ulcers, or lack of improvement of functional ability after treatment, were most frequently caused by new blistering around or at the location of the ulcer.

All our patients were hospitalized, with a mean hospitalization time of 15 days. Punch grafting can be performed as effectively in an outpatient population as in an inpatient population.[14,28] However, we chose hospitalization as most EB patients live far away from the Dutch EB Center, making it difficult for them to travel for the frequent check-ups in the first weeks.

In the past, tissue engineered artificial allografts,[5,6] cultured allografts,[7,4] cultured autografts,[8,9] and full-thickness or split-thickness autografts[10,11] have already been successfully applied in EB patients. In a Cochrane review concerning venous leg ulcers in a non-EB population, no differences in efficacy were seen in the different types of skin grafting.[29] In the literature, the healing rates of punch- and pinch grafting in non-EB populations with mainly venous, arterial, and mixed leg ulcers ranges from 33% to 61.5%.[14,28,30-34] Several causes could explain the higher healing rate achieved in our population. The ulcers we treated were relatively small; in the non-EB population small ulcers showed a better healing tendency than large ulcers. Furthermore, the patients in the non-EB population were generally older and the aetiology of their ulcer differed. For EB patients the advantage of punch grafting over other autologous grafts, is that the grafts are smaller and thicker; larger grafts are meshed and thinner, and therefore can take longer to heal in deep ulcers or induce prolonged blistering at the donor site.[11,29] For cultured grafts, less or no injuries are made by harvesting donor grafts compared to punch grafting, however producing these cultured grafts is time-consuming, expensive, laborious, and needs special facilities.[29]

With punch grafting the cause of blistering and ulceration is not treated and the risk of recurrences persists after skin grafting. The donor sites of our patients did not contain revertant mosaicism; in patient 1, 2, and 4 this was excluded by immunofluorescence antigen mapping, revealing LM-332 deficiency of sample biopsy.[35] Hopefully in the future punch grafting of revertant skin, or other technologies such as revertant cell therapy or stem cell therapy might help to prevent or treat chronic erosions and ulcers in EB patients.[36-38]

Although the limitations of this study are the retrospective design, the small amount of patients, the absence of a control group, and a follow-up and ulcer measurement that were not standardized, we conclude that punch grafting can be used as a first-line treatment in longstanding ulcers with no healing tendency in JEB-nH

patients. It is an easy, inexpensive method with little risk of complications and results in significant healing rates.

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9

Enamel defects in carriers of a novel *LAMA3* mutation underlying epidermolysis bullosa

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To the editor

Epidermolysis bullosa (EB) is a heterogeneous group of genetic diseases characterized by blistering of the skin and mucous membranes. Junctional epidermolysis bullosa, non-Herlitz (JEB-nH) is an autosomal recessive subtype caused by mutations in the genes *LAMA3*, *LAMB3*, *LAMC2*, or *COL17A1*. The first three genes encode for laminin α3, laminin β3, and laminin γ2, respectively, which together form the heterotrimer laminin-332 (LM-332), whereas *COL17A1* encodes for the homotrimer type XVII collagen (Col17).[1] LM-332 and Col17 are located in the lamina basale, and are important in the epidermal-dermal adhesion. JEB-nH shows blistering after minor trauma, but also extracutaneous symptoms, such as dental abnormalities.[1] Both LM-332 and Col17 are crucial in ameloblast differentiation and enamel formation,[2,3] resulting in enamel defects of the entire dentition in all patients with JEB-nH consisting of hypoplasia, pitting, roughness, thinning or furrowing of enamel.[4] In 1996, McGrath *et al.* reported the first heterozygous carriers of a *COL17A1* mutation who had enamel defects. The carriers of the glycine substitution p.G627V had no signs of skin fragility, but did have extensive enamel hypoplasia and pitting, thought to be caused by the dominant-negative effect of the mutant Col17.[5] In 2007, Murrell *et al*. reported enamel defects in carriers of a *COL17A1* null mutation, which was attributed to haploinsufficiency.[6] Dental abnormalities have not been reported previously in carriers of mutations in the genes encoding LM-332 before. Here we report the first enamel defects in carriers of a *LAMA3* mutation.

Case report

The proband was a 23-year-old woman (EB050) diagnosed with JEB-nH. Starting at the age of two months she showed generalized blistering and erosions that healed with atrophic scarring. She had dystrophic nails, and, occasionally, oral erosions. There was no loss of primary or secondary hair. Her entire primary and secondary dentition was affected with enamel hypoplasia and enamel pits, which were treated with direct composite (Figure 1a-b). Her non-consanguineous parents and her brother had no signs of skin fragility. However, her 53-year-old mother and her 25-year-old brother also had enamel defects in their dentition, consisting of roughness and pits, which had led to a higher susceptibility to caries in both (Figure 1c-d). The mother was treated with

maxillary dentures and numerous restorations (Figure 1c). In contrast, the father's teeth were not affected.

Skin biopsies in the proband and her mother were taken, and immunofluorescence antigen mapping was performed on non-lesional skin. Staining of LM-332 with monoclonal antibody GB3 was slightly reduced in both mother and daughter, while staining for Col17 with monoclonal antibodies NCC-lu-226 and 233 was normal. Genomic DNA of all family members was extracted from peripheral blood. Previous molecular analysis of *COL17A1, LAMB3,* and *LAMC2* yielded no abnormalities in the proband. Screening of *LAMA3* (GenBank accession number NM_000227.3) showed a compound heterozygosity for the novel missense mutation c.4484C>T, and the novel nonsense mutation c.488delG in the proband (methods upon request). The deletion c.488delG in exon 5 creates a frameshift that results in a premature termination codon (p.G163DfsX30), and the mutation c.4484C>T in exon 33 results in a substitution of alanine to valine at position 1495 (p.A1495V). Proving the pathogenicity of the mutation p.A1495V, are two unrelated patients with JEB-nH (EB169-01 and EB173-01) in our EB cohort, who both carry the *LAMA3* mutation p.A1495V. Patient EB169-01 carries it together with the nonsense mutation p.R1126X, and patient 173-01 together with the missense mutation p.R262S (unpublished). Screening of *LAMA3* in the family members of the proband showed that her father, lacking any skin or dental symptoms, was heterozygous for c.4484C>T. Her mother and brother, who lacked skin fragility, but did have enamel defects, showed heterozygosity for c.488delG. To investigate if these enamel defects in the heterozygous carriers of c.488delG could be caused by a dominant-negative effect of an aberrant laminin α3 chain by alternatively spliced cDNA, nested PCRs surrounding both mutations were performed with cDNA isolated from skin biopsies from the proband and her mother (methods upon request). There were no alternatively spliced cDNA products visible on agarose gel (Figure 1e-f). The PCR products were sequenced and cDNA of both alleles of the proband were present (Figure 1g-h). The allele bearing the mutation c.488delG in the mother was reduced compared to the normal wild-type (Figure 1g). Taken this together with the absence of alternative spliced products on agarose gel, we hypothesize that haploinsufficiency of *LAMA3* is the cause of enamel defects in the mother and brother of the proband.

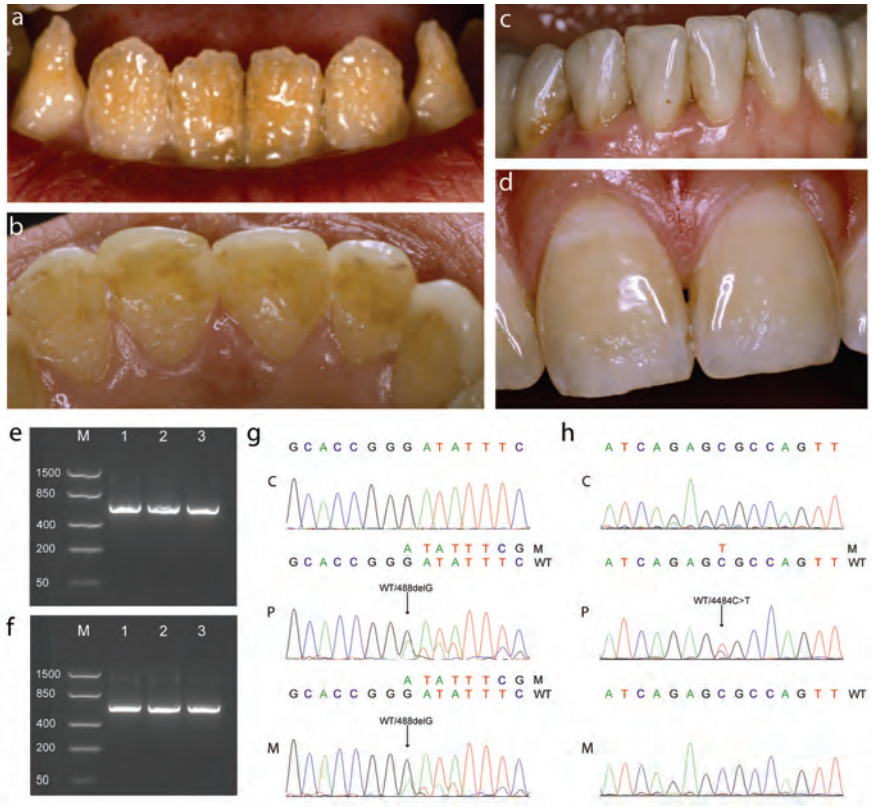

Figure 1. Features of the enamel defects (a-d) and cDNA analysis (e-h). The proband (a,b), her mother (c), and her brother (d) suffered from enamel defects in their dentition. Rough pitted enamel (a) on the buccal side of the mandibular teeth, and (b) on the palatinal side of the maxillar teeth in the proband are seen. The buccal side of the maxillar teeth are treated with direct composite. (c) The enamel defects on the labial side of the mandibular teeth are not visible anymore due to multiple restorations in the mother. (d) In the brother there is typical pitted enamel in the insical area of the central incisors. Agarose gel analysis (e,f) and direct sequencing (g,h) of mRNA amplified by nested PCRs in the proband and her mother. The mutation c.488delG in exon 5 of *LAMA3* has been amplified using a forward primer located in exon 1 (bp 175) and a reverse primer located in exon 8 (bp 815). The mutation c.4484C>T in exon 33 of *LAMA3* has been amplified using a forward primer located in exon 31 (bp 4177) and a reverse primer located in exon 35 (bp 4810). Gel analysis of the exons surrounding (e) mutation c.488delG, and (f) mutation c.4484C>T, showed no alternatively spliced products in the proband (lane 2) and her mother (lane 3), compared to control (lane 1). (g) Direct sequencing of the amplified mRNA showed the presence of mRNA carrying the mutation c.488delG in the proband (P) and her mother (M). (h) In the proband (P) both mRNA with the mutation c.4484C>T as well as mRNA with the mutation c.488delG are produced. The sequence of her mother showed no mutation (M).

Discussion

Enamel formation is a sensitive and complex progress, in which LM-332 is crucial. Enamel is formed in three stages: (1) in the presecretory stage the ameloblasts differentiate in preparation to secrete enamel proteins. In the end of this stage the lamina basale, that supports the ameloblasts, disintegrates; (2) in the secretory stage ameloblasts secrete enamel proteins that mineralize and form enamel crystals; (3) in the maturation stage a lamina basale is deposited, adhering ameloblasts to the underlying enamel surface by hemidesmosomes. To harden the enamel, water and organic material are removed by ameloblasts.[7] LM-332 is expressed by ameloblasts in all stages of enamel formation.[3] In the presecretory and maturation stage LM-332 is a part of the lamina basale. In the secretory stage LM-332 most likely plays a role in the adhesion of ameloblasts to the enamel matrix.[3] In patients with JEB-nH the reduction of LM-332 causes detachment from the underlying matrix and degeneration of ameloblasts. This causes enamel defects, such as pitting.[4,8] The enamel that does form has another chemical composition, as reduction of LM-332 can result in deficiencies in the junctions of ameloblasts that are important in maintaining channels for mineral ions and cell nutrients. This may lead to defective mineral transport or compromised ameloblast metabolism.[8] In teeth of patients with junctional EB it is shown that a significantly reduced mineral per volume content is present compared to control, and that serum albumine is present, which is a known inhibitor of enamel crystal growth.[8] It is possible that in this complex process of enamel formation, in which LM-332 plays such a vital role, there is no coping mechanism that can manage the loss of one *LAMA3* allele, whereas these mechanisms are available in the skin.[6] However, it remains peculiar that enamel defects have never been reported before in carriers of *LAMA3, LAMB3,* or *LAMC2* null mutations. Furthermore, none of the carriers of null mutations in LM-332 in the Dutch EB cohort have noted particular dental problems, however, they have not been systematically examined. It is possible that not all carriers are affected; a phenomenon also seen in carriers of *COL17A1* null mutations.[6,9] Another explanation could be that the enamel defects are so discrete, that they are missed, especially when they are not actively searched for. Furthermore, it is possible that other factors contribute to the enamel defects seen in our carriers, such as the rare inherited enamel defects that occur in the absence of generalized syndromes designated as amelogenesis imperfecta,[10] or

due to environmental factors such as febrile diseases. However, this is less likely in our patients, considering the clinical dental symptoms.[7]

The enamel defects seen in the carriers of *LAMA3* null mutations reported here are comparable to those seen in the carriers of missense *COL17A1* mutations; although the carriers of *COL17A1* null mutations described by Murrell *et al*. also showed horizontal ridging, a feature that was not present in the carriers we described.[5,6]

In conclusion, we have reported: (1) two novel *LAMA3* mutations associated with JEB-nH, and (2) the first carriers of *LAMA3* null mutations to have enamel defects probably caused by haploinsufficiency.

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Junctional epidermolysis bullosa of late onset explained by mutations in *COL17A1*

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Abstract

Background Junctional epidermolysis bullosa of late onset (JEB-lo) is a rare disease characterized by blistering of primarily the hands and feet starting at childhood. The pathogenesis remains unclear.

Objective To clarify the pathogenesis of JEB-lo.

Methods Two JEB-lo patients, a brother and a sister, were examined using electron microscopy (EM), immunofluorescence antigen mapping (IF) and molecular analysis.

Results We found subtle changes in IF and EM. The most remarkable changes were loss of the apical-lateral staining of monoclonal antibodies (mAb) against type XVII collagen (Col17), and a broadened distribution of mAb staining against the ectodomain of Col17, laminin-332, and type VII collagen. Mutation analysis of *COL17A1*, encoding Col17, showed a compound heterozygosity for a novel mutation c.1992_1995delGGGT and the known mutation c.3908G>A in both patients. The deletion c.1992_1995delGGGT results in a premature termination codon and mRNA decay, leaving the patients functionally hemizygous for the missense mutation c.3908G>A (p.R1303Q) in the non-collagenous 4 (NC4) domain of Col17.

Conclusions JEB-lo is an autosomal recessive disorder caused by mutations in *COL17A1*, and subtle aberrations in EM and IF are clues to diagnosis.

Introduction

Junctional epidermolysis bullosa (JEB) is a diverse group of autosomal recessive trauma-induced blistering diseases of the skin and mucous membranes. Junctional epidermolysis bullosa of late-onset (JEB-lo, previously named JEB progressiva) is a rare subtype of JEB.[1] In contrast with other forms of JEB, in which blistering presents at birth, in JEB-lo the symptoms start in childhood or young adulthood.[2] Blistering in JEB-lo is primarily located on the hands and feet, and in a lesser extent on the elbows, knees, and mucosa of the mouth. Other clinical features are dystrophic or absent nails, hyperhidrosis, amelogenesis imperfecta, loss of dermatoglyphic pattern, and slowly progressive skin atrophy.[2,3] Only 22 JEB-lo patients in 12 families have been reported.[3-6] Electron microscopy (EM) and immunofluorescence antigen mapping (IF) have located the blister level in the lamina lucida, but they have not revealed any abnormalities in the epidermal basement membrane zone, although in some patients amorphous electron dense material in the lamina lucida has been observed with EM.[3,7] Since molecular analysis has not been performed in these patients, no gene has yet been linked to this JEB subtype, leaving the pathogenesis unclear.[3-6] Despite the small number of patients known and the unclear pathogenesis, JEB-lo has been recognised as a distinct entity at the International Consensus Meeting of diagnosis and classification of inherited EB in 2008.[1] Here we present two siblings with JEB-lo, in which we found IF abnormalities for type XVII collagen (Col17), laminin-332 (LM-332) and type VII collagen (Col7), EM abnormalities, and mutations in *COL17A1*.

Materials and Methods

Patients

The patients, reported by us previously,[6] are a brother (32 yrs, patient 054-01) and his sister (27 yrs, patient 054-02). JEB was diagnosed on the basis of clinical findings, IF, EM, and molecular analysis. All studies were approved by the UMCG medical ethical committee and performed according to the Declaration of Helsinki principles.

Electron microscopy

For EM 2 mm punch biopsies of perilesional skin and non-lesional skin on the inner upper arm were taken and prepared, as described previously.[8] Ultrathin sections were examined with a Philips CM100 transmission electron microscope.

Immunofluorescence antigen mapping

For IF 4 mm punch biopsies, on the same locations as for EM, were taken and prepared, as previously described.[9] All mAb and their origin have been described previously.[9,10] The skin biopsies were stained for pankeratin (CK1), LM-332 (GB3), laminin α3 chain (BM165), laminin β3 chain (K140), laminin γ2 chain (D4B5), Col17 ectodomain (NCC-Lu-226 (epitope residues 1080-1107), 233 (epitope residues 1118-1143), 1D1 (epitope residues 1357-1387)), Col17 endodomain (1A8c (epitope residues 155-163)), plectin (HD121, 10F6), integrin α6 (GOH3), integrin β4 (58XB4), and Col7 (LH7:2).[10] As the secondary step against these primary mouse monoclonal antibodies, we used Alexa488-conjugated goat anti-mouse IgG. For the rat monoclonal GOH3 we used FITC-conjugated goat anti-rat IgG.[10] The slides were examined with a Leica DMRA fluorescence microscope and images were recorded with a Leica DFC350 FX digital camera. Staining intensity was compared to controls.

Molecular analysis

Genomic DNA was extracted from peripheral blood. The 56 coding exons of *COL17A1* (GenBank NG_007069) were amplified in 48 amplicons with polymerase chain reaction (PCR). The mutation in exon 24 was found with the sense primer 5'-TGAGTGCCTACTAGGTGTC-3' and antisense primer 5'-GCCTCTTCTCTGTGATCCAT-3', and the mutation in exon 52 was found with sense primer 5'- ACCCTGGGAGAGACTCCCTAAC-3' and antisense primer 5'-TGGAGATGCTCCCACGCTCCTT-3'. The amplification was performed with 2 µl sense primer and 2 µl antisense primer in a concentration of 0.5 pmol/µl, 2 µl genomic DNA in a concentration of 40 ng/µl, 10 µl Amplitaq gold® Master Mix (Applied Biosystems, Foster city, CA, USA) and 5 µl distilled water. Amplification conditions were 10 min at 95°C, followed by 5 cycles at 95°C for 5 s, 65°C for 30 s, 72°C for 60 s, followed by 30 cycles at 95°C for 5 s, 60 °C for 30 s, 72°C for 60 s, followed by 15 cycles at 95°C for 5 s, 55 °C for 30 s, 72°C for 60 s, and a final extension at 72°C for 5 min. Afterwards the PCR products were cleaned up with 4 µl Exo-SAP IT by incubating at 37°C for 15 minutes, and inactivated at 80°C for 15 minutes. All amplicons were sequenced directly on an

automated DNA sequencer (ABI 3730 DNA analyzer, Applied Biosystems, Foster city, CA, USA). Verification of mutations was performed by repeating the amplification and sequencing of the relevant exons and its inheritance was verified in both unaffected parents and an unaffected brother.

Results

Clinical findings

Starting at the age of 6 years, blistering occurred on the feet and around the toe- and fingernails, resulting in onychoschisis, longitudinal ridging, distal onycholysis, clubbing, or shedding of the nail plate in a brother (054-01) and a sister (054-02) (Fig. 1d). With age, blistering also occurred on hands, nose and oral mucosa. The pretibial skin was more fragile, with atrophic patches (Fig. 1b). Both patients experienced palmoplantar hyperhidrosis, and loss of dermatoglyphs of the digits (Fig. 1a). Patient 054-01 showed slight hyperkeratosis with a waxy appearance of the hands (Fig. 1a), whereas patient 054-02 had hyperkeratosis limited to the feet. Transverse ridging and enamel pits (amelogenesis imperfecta) could be seen on the surface of the teeth (Fig. 1c). The hair pattern was normal. Nikolsky sign was negative. The non-consanguineous parents and a brother of the patients were unaffected, although the father noticed a composition of the nails in layers.

Electron microscopy

EM of lesional and non-lesional skin of the upper arm in both patients showed interruptions in the lamina densa, but no duplications (Fig. 2c). Focally the lamina densa and sublamina densa zone showed fuzzy electron dense material giving it a cloudy appearance (Fig. 2d). Hemidesmosomes were normal in number and structure. There were no abnormalities of keratin filaments, anchoring filaments, and anchoring fibrils.

Immunofluorescence antigen mapping

IF blister mapping of lesional skin with monoclonal antibodies (mAb) against pankeratin (CK1) and plectin (HD-121) (not shown) stained exclusively the blister roof, whereas mAb against the ectodomain of Col17 (1D1) and LM-332 (GB3) showed “mirror” staining of the roof as well as the floor of the blister in the same intensity (Fig. 2b). IF of non-lesional skin with mAb against pankeratin (CK1), plectin (HD-121; 10F6), integrin α6

(GOH3), and integrin β4 (58XB4) showed no abnormalities in intensity or distribution in both patients (not shown). The mAb against laminin α3 (BM165), laminin β3 (K140), laminin γ2 (D4B5), and Col7 (LH7:2) stained in a normal intensity, but the distribution was changed, with a fuzzy broadened deposition in the sublamina densa zone, that was not seen in the control (Fig. 2a). Staining with mAb 1A8C (Col17 endodomain) showed markedly reduced intensity of the apical-lateral membrane of the basal keratinocytes compared to control, whilst the staining at the dermal-epidermal junction remained bright (Fig. 2a). Staining for the Col17 ectodomain with 1D1, NCC-Lu-226, and 233, did not show any apical-lateral staining of the basal cells (Fig. 2a), while the brightness at the linear dermal-epidermal junction was comparable to the control (Fig. 2a). The mAb to Col17 ectodomain NCC-Lu-226, 233, and to a lesser extent 1D1, also showed a broadened and fuzzy staining of the dermal-epidermal junction, similarly to that as the staining of LM-332 and Col7 (Fig. 2a).

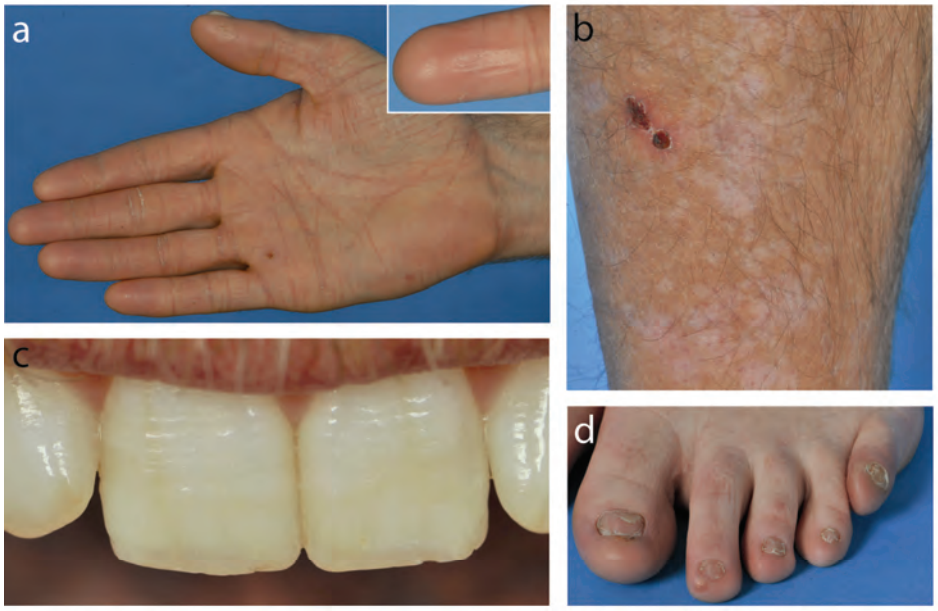

Figure 1. Junctional epidermolysis bullosa of late onset. (a) Loss of dermatoglyphs, waxy hyperkeratosis of the dorsum of the hand in patient 054-01. (b) Atrophic skin on the lower leg of patient 054-01. (c) Transverse ridging and enamel pits of the teeth of patient 054-01. (d) Nail abnormalities of patient 054-02.

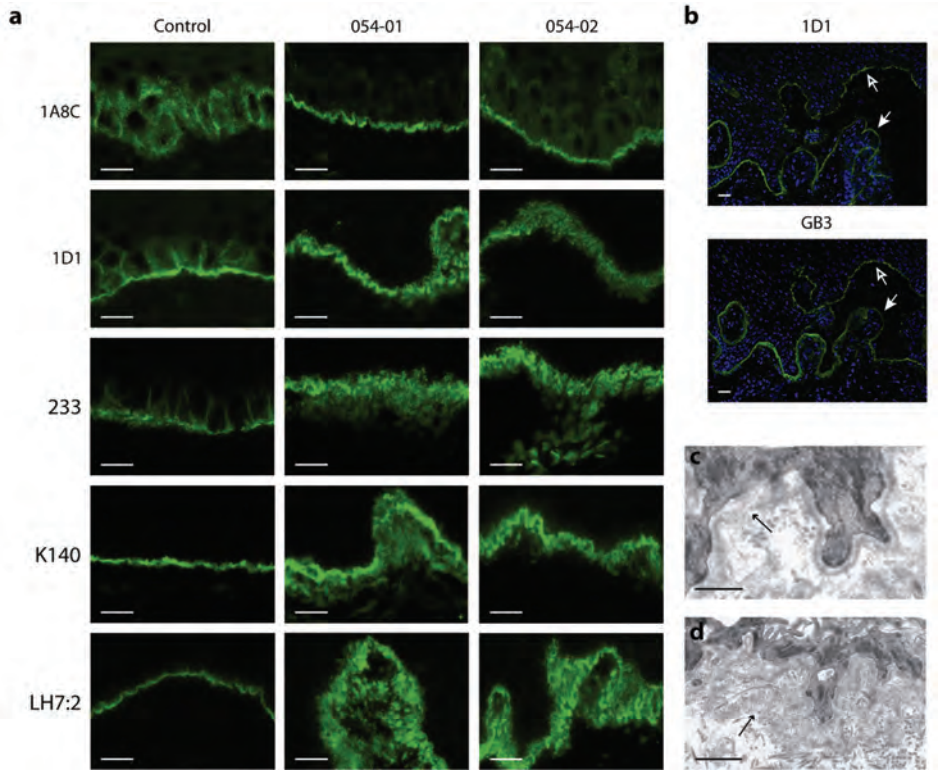

Figure 2. Subtle changes in immunofluorescence antigen mapping and electron microscopy in patients 054-01 and 054-02. (a) Reduced apical-lateral staining with mAb 1A8C for type XVII collagen endodomain of the basal keratinocytes compared to control, especially in patient 054-01, while the linear staining intensity remains along the dermal-epidermal junction. Total loss of apical-lateral staining for type XVII collagen ectodomain with mAb 1D1 and 233 between basal cells in both patients compared to control, while the linear staining shows a fuzzy broadened deposition. Staining for laminin β3 chain (K140), and type VII collagen (LH7:2) also showed a fuzzy broadened deposition in both patients, that was not seen in the control. Scale bar = 10 μm. (b) A similar staining intensity in the roof (open arrow) and floor (solid arrow) of the blister with mAb 1D1 for type XVII collagen, and mAb GB3 for laminin-332, in lesional skin of patient 054-01. Scale bar = 20 μm. (c) An interruption (arrow) in the lamina densa in patient 054-02. Scale bar = 0.5 μm. (d) Electron dense material in the sublamina densa (arrow) in patient 054-02. Scale bar = 1 μm

Molecular analysis

Sequencing analysis of *COL17A1* showed a compound heterozygosity for a novel, maternally derived c.1992_1995delGGGT and a known, paternally derived c.3908G>A mutation (Fig. 3). The unaffected brother showed heterozygosity for the c.3908G>A mutation.

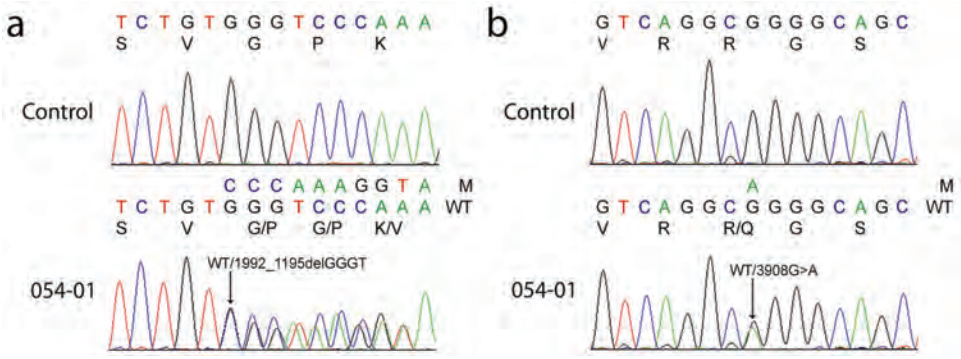

Figure 3. Junctional epidermolysis bullosa caused by compound heterozygous *COL17A1* mutations. (a) DNA analysis revealed a c.1992_1995delGGGT deletion on the maternal allele, and (b) a heterozygous c.3908G>A substitution on the paternal allele in both patients 054-01 and 054-02 in *COL17A1* (GenBank NG_007069).

Discussion

In our two JEB-lo patients we observed subtle changes in IF with respect to the distribution of the mAb staining for Col17. Since loss of apical-lateral staining of 1D1 was described in carriers of *COL17A1* mutations,[11] and autosomal dominant JEB patients,[12] it implies *COL17A1* as a strong candidate for JEB-lo.

The novel, maternally derived *COL17A1* mutation c.1992_1995delGGGT in exon 24 creates a frameshift that results in a preliminary stopcodon (p.G665del_G668VfsX14). The mRNA from this allele will likely be targeted for nonsense mediated mRNA decay, leaving the patient with a functional hemizygosity for the c.3908G>A allele.

The paternal c.3908G>A mutation in exon 52 leads to substitution of an evolutionary highly conserved arginine residue by glutamine (p.R1303Q) in the non-collagenous (NC) 4 domain of Col17. A patient homozygous for this missense mutation has been reported by Schumann *et al*.[13] Their patient, classified with localized JEB, suffered from blistering starting at school age, predominantly at the distal extremities

and, occasionally, of the oral mucosa. During the course of the disease, all nails were lost, and mild skin atrophy developed on the extremities. However, the clinical picture, and especially the childhood onset is much more characteristic and indicative for JEB-lo.[1,11,14-17] Other missense mutations in *COL17A1* have been reported, but not in the NC4 domain of Col17,[18,19] suggesting that p.R1303Q and other missense mutations in the NC4 domain may be specific for JEB-lo.

Col17 is a type II transmembrane protein important in the epidermal-dermal adhesion as a part of hemidesmosomes. Col17 consists of a globular head domain localized in the intracellular region of the hemidesmosome, and an ectodomain containing a linear rod domain penetrating the plasma membrane and crossing the lamina lucida, and a flexible tail domain localized in the lamina densa.[20] The p.R1303Q mutation is predicted to affect protein folding and ligand-binding, and structural analysis has demonstrated increased trypsin-sensitivity probably due to alteration of the NC4 protein structure.[13,21] The mutation is localized in the flexible tail domain, which has a loop that keeps a firm hold on the lamina densa and most likely interacts with LM-332.[13,22] This could well explain the low lamina lucida split in the lesional skin of our patients, witnessed by IF staining of LM-332 in both blister floor and roof. This is unusual for JEB associated with *COL17A1* mutations, and more fitting with JEB associated with mutations in the genes encoding for LM-332.[23] IF mapping has only been performed in three other JEB-lo patients described by Bircher *et al.*,[3] which also showed a low lamina lucida split.

The loss of apical-lateral staining of Col17 in our hemizygous patients is explained by haplo-insufficiency which reduces the production of Col17 to approximately a half, as in carriers of *COL17A1* null mutations.[11] IF demonstrated that the production of Col17 is sufficient for it to be present in hemidesmosomes (BMZ staining), despite the p.R1303Q mutation. However, the mAb staining for Col17 ectodomain showed a fuzzy broadened deposition in the sublamina densa zone. We hypothesize that the mutation p.R1303Q, located in the Col17 ectodomain, results in a disturbed ligand-binding that interferes with a correct build-up of the extracellular matrix. This would also explain the broadened distribution of the mAb staining for LM-332 and Col7; as the Col17 ectodomain has an interaction with LM-332, and thus with Col7.[24] The aberrant structure of the basement membrane could also explain the electron dense fuzzy and cloudy broadening of the lamina densa and its subzone observed in EM.

In conclusion, in this study we have shown that JEB-lo is an autosomal recessive disorder caused by mutations in *COL17A1*, and that subtle changes in EM and IF

mapping for Col17 are a clue to molecular diagnosis. However, alternative pathogeneses are not excluded in JEB-lo, so further research in other JEB-lo patients is required.

Acknowledgements

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11

New versatile monoclonal antibodies against type XVII collagen endodomain that distinguish type XVII collagen-related epidermolysis bullosa subtypes

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Abstract

Background Type XVII collagen (Col17) is a type II transmembrane epidermal-dermal adhesion protein consisting of a 55 kDa endodomain and a 120 kDa ectodomain. Mutations in *COL17A1,* coding for Col17, cause the autosomal recessive blistering disease junctional epidermolysis bullosa, type non-Herlitz (JEB-nH). Immunofluorescence antigen staining (IF) can distinguish between the more severe generalized JEB-nH with (nearly) absent Col17, and the milder localized JEB-nH with a reduced Col17 level. Also, carriers of a *COL17A1* null mutation are detected by a reduced Col17 IF staining. We noticed an imminent extinction of antibodies against the Col17 endodomain, which are the most sensitive antibodies for detecting reduced Col17 expression.

Objective To produce Col17 endodomain antibodies than can diagnose all Col17-related JEB-nH cases and carriers.

Methods Five mouse anti-human monoclonal IgG antibodies, called VK1, VK2, VK3, VK4, and VK5, were produced against a human Col17 endodomain fusion construct. IF staining was performed on respectively generalized JEB-nH, localized JEB-nH, carrier, and healthy control skin. Western blotting and immunohistochemistry (IH) staining were performed on healthy control substrates.

Results All VKs showed absence of Col17 in generalized JEB-nH skin, and reduced Col17 staining in localized JEB-nH and in carriers compared to healthy control skin. All VK monoclonals were effective in western blotting and IH staining.

Conclusion The new VK clones are capable of differentiating all Col17-related JEB-nH cases and carriers from controls, and to distinguish between generalized JEB-nH and localized JEB-nH. Due to its versatility and sensitivity we advise the use of VK for Col17 endodomain staining in the future.

Introduction

Epidermolysis bullosa is a group of heterogeneous blistering diseases of the skin and mucous membranes. To date, mutations in 15 genes causing 29 different subtypes have been identified.[1-3] Mutations in the type II transmembrane protein type XVII collagen (Col17) cause the autosomal recessive subtype junctional epidermolysis bullosa, type non-Herlitz (JEB-nH).[4] JEB-nH is a subtype that is characterized by a junctional split, and is further subdivided in two minor subtypes: 1) generalized JEB-nH (JEB-nH gen) with generalized blistering, atrophic scarring, nail dystrophy, sparse primary hair, absent secondary hair, and enamel defects, and 2) localized JEB-nH (JEB-nH loc) with blisters primarily localized on the hands, lower legs and face, normal primary hair, sparse secondary hair, and enamel defects.[1,5]

Col17 (gene: *COL17A1*) consists of an N-terminal endodomain (466 residues), followed by a transmembrane protein (23 residues), and a C-terminal ectodomain (1008 residues). It is located at both the apical-lateral (AL) and basal surface of basal keratinocytes. AL Col17 is freely soluble in the membrane and can be removed by detergent treatment.[6] It is hypothesized that AL Col17 functions as a reservoir for the formation of new hemidesmosomes.[6-8] At the basal surface Col17 is incorporated into hemidesmosomes, which are multi-protein adhesion complexes important in the adhesion of the epidermis to the underlying dermis.[9-11] The Col17 endodomain is located in the cytoplasmic plaque of the hemidesmosome and binds to the other hemidesmosomal proteins integrin β4, plectin and BP230, while the ectodomain transverses the lamina lucida and interacts with laminin-332.[12-17] The Col17 ectodomain is shed from the cell surface by cleavage at residues 528-547 yielding the soluble 120-kDa linear IgA disease antigen (LAD-1).[18-20] LAD-1 is further cleaved at the C-terminus, resulting in the linear IgA bullous disease antigen of 97 kDa (LABD97) with possible cleavage sites ranging from amino acids 1209-1310.[19,21] The function of ectodomain shedding is not yet fully understood. Hypotheses are that upwards migrating cells cut their anchors, including Col17, or that these are shed into the BMZ to strengthen the epidermal-dermal adhesion.[16,22]

In the diagnostic process of Col17-related JEB-nH cases, immunofluorescence antigen (IF) staining has an important role. It can differentiate between the two JEB-nH subtypes, in which a total absence of Col17 concurs with JEB-nH gen and a reduction of Col17 concurs with JEB-nH loc.[23] Also, heterozygous carriers of a *COL17A1* null

mutation (carriers) can be identified, as they show reduced AL staining of the basal keratinocytes.[5,24] In the past both polyclonal and monoclonal antibodies to the Col17 endodomain and to the ectodomain have been raised (Table 1).[9,21,25-28] However, although the antibodies against the Col17 ectodomain remain available, we noticed an imminent extinction of antibodies against the Col17 endodomain. The antibody 1A8C is not available anymore, and batches of commercially obtained monoclonal antibodies B1052M and V-5-8 appeared nonfunctional in IF staining in our laboratory.[26] To secure the availability of anti-endodomain monoclonal antibodies for diagnostic- and research purposes, we have produced new functional clones that we present here.

Materials and Methods

Monoclonal antibodies

Mouse anti-human monoclonal IgG antibodies were produced for us by ProMab Biotechnologies Inc., Richmond, CA, USA using a human entire endodomain Col17 fusion construct (kindly provided to us by dr. J.C.R. Jones, Chicago, IL, USA).[9] Immunofluorescent analysis showed that five clones stained skin in the typical Col17 pattern, but did not bind to Col17-deficient skin. These clones were named VK1, VK2, VK3, VK4, and VK5. The name VK is derived from Stichting Vlinderkind (Dutch Butterfly Foundation). The antibody staining for the ectodomain of Col17 was 233 (gift from Dr. K. Owaribe, Nagoya, Japan).

Skin substrates

The skin specimens were taken from patients with JEB-nH gen (EB011-01), JEB-nH loc (EB168-01), and a carrier of a *COL17A1* null mutation (father of EB084-01), that were previously published (Table 2).[5,29] The skin specimens were obtained by 4 mm punch biopsies of clinically healthy skin of the upper arm. The control biopsy was taken with a 4 mm punch biopsy from mamma reduction skin from a healthy individual. The skin specimens were snap frozen in liquid nitrogen and stored at -80°C.

Immunofluorescence

Cryosections of 4 µm thickness were mounted on a slide and air dried by a fan for 30 minutes. The sections were incubated with the primary antibodies for 30 minutes at room temperature. VK1 to VK5 were used in a 1:40 dilution with 1% ovalbumin in 150 mM

Table 1. Overview of antibodies against Col17 available and tested in our lab

Name	Specificity (epitope)	Type of antibody	Protein detection	Origin	Effective	Availability	Reference
Jones17	Endodomain	Rabbit polyclonal	Full-length Col17	Dr. J.C.R. Jones, Chicago, IL, USA	+	?	9
1A8C	Endodomain (155-163)	Mouse monoclonal	Full-length Col17	Dr. K. Owaribe, Nagoya, Japan	+	-	26
N18	Endodomain	Goat polyclonal	Full-length Col17	Santa Cruz Biotechnology, Santa Cruz, California, USA	-	+	Commercial
B1052M	Endodomain (1-490)	Mouse monoclonal	Full-length Col17	Santa Cruz Biotechnology, Santa Cruz, California, USA	-	+	Commercial
V-5-8	Endodomain (1-490)	Mouse monoclonal	Full-length Col17	Immundiagnostik AG, Bensheim, Germany	-	+	Commercial
233	Ectodomain (1118-1143)	Mouse monoclonal	Full-length Col17; LAD-1; LABD97	Dr. K. Owaribe, Nagoya, Japan	+	-	26
1D1	Ectodomain (1357-1387)	Mouse monoclonal	Full-length Col17; LAD-1	Dr. K. Owaribe, Nagoya, Japan	+	-	27
Ncc-lu-226	Ectodomain	Mouse monoclonal	Full-length Col17; LAD-1; LABD97	Dr. S. Hirohashi, Tokyo, Japan	+	+	28
E16	Ectodomain	Goat polyclonal	?	Santa Cruz Biotechnology, Santa Cruz, California, USA	-	+	Commercial
97-1	Ectodomain	Mouse monoclonal	LAD-1; LABD97	Dr. J. Zone, Salt Lake City, UT, USA	+	+	21
97-2	Ectodomain	Mouse monoclonal	LAD-1; LABD97	Dr. J. Zone, Salt Lake City, UT, USA	+	+	21
123	Ectodomain	Mouse monoclonal	LAD-1; LABD97	Dr. P. Marinkovich, Stanford, CA, USA	+	+	25

Col17 type XVII collagen; LAD-1 120-kDa linear IgA disease antigen; LABD97 linear IgA bullous disease antigen of 97 kDa; + yes; - no

phosphate buffered saline pH 7.2 (PBS-OVA 1%), and 233 was used in a 1:20 dilution with PBS-OVA 1%. Afterwards the slides were washed for 15 minutes in 150 mM phosphate buffered saline pH 7.2 (PBS). The Alexa488-conjugated goat-anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA) was used as secondary step in a dilution of 1:6000 with PBS-OVA 1%. Afterwards the slides were washed in PBS for 15 minutes. Sections were mounted with Slowfade® Gold antifade reagents (Invitrogen, Carlsbad, CA, USA). The slides were examined with a Leica DMRA fluorescence microscope and photographs were taken with the Leica DFX350 FX digital camera using the Leica Application Suite V3.8. Software (Leica, Wetzlar, Germany). Staining intensity was compared to controls and scored as normal (+++), slightly reduced (++), reduced (+), strongly reduced (±) or absent (-). The pictures were taken with an exposure time mimicking the observations with the naked eye as accurately as possible.

Triton X-100 preparation

A 4 µm cryosection of a control specimen was treated with 0.25% Triton X-100 (Merck KGaA, Darmstadt, Germany) in PBS for 15 minutes. The slide was washed for 15 minutes in PBS and then incubated with VK4 for 45 minutes, omitting fan-drying. Afterwards the slide was washed in PBS for 15 minutes. The secondary step was performed as described above.

Western blotting

SDS-PAGE and western blotting were performed as described before.[20,30] As substrate we used extract from cultured normal keratinocytes. The VK antibodies were used in a 1:300 dilution.

Immunohistochemistry

A 4 mm punch biopsy from mamma reduction skin of a healthy individual was fixed in formaldehyde and embedded in paraffin. Four µm thick sections were cut and stored at room temperature. The slides were deparaffinized and rehydrated in xylene for 3x3 minutes, followed by ethanol 100% for 2x3 minutes, 96% ethanol for 3 minutes, and 70% ethanol for 3 minutes. The slides were then continuously rinsed with demineralised water, until antigen retrieval was performed in 100 mM Tris-HCl buffer, pH 9 by heating in a microwave for 2 times 15 minutes at approximately 98°C. The sections were then incubated for 30 minutes at room temperature with the VK antibodies and 233 in a 1:2 dilution in PBS-OVA 1%. Afterwards the slides were washed for 15 minutes in PBS. Next

they were incubated for 30 minutes with alkaline phosphatase-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) in a dilution of 1:50 in PBS-OVA 1%. Afterwards the slides were washed for 15 minutes in PBS. Color was then developed for 30 minutes by incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl and 5 mM $MgCl_2$. The slides were then mounted with Kaiser's glycerol gelatin (Merck KGaA, Darmstadt, Germany) and examined with a Leica DM2000 microscope. Images were taken with a Leica DFC425C digital camera using the Leica Application Suite V3.7. Software (Leica, Wetzlar, Germany).

Results

Immunofluorescence

All VK antibodies showed a similar positive staining of Col17 at the BMZ and AL area in basal keratinocytes of normal human skin (Figure 1). Staining with VK for 30 minutes in a 1:40 dilution, as described in the protocol, resulted in a similar staining intensity between the BMZ and AL in normal human skin. Reducing the VK dilution or extending the VK incubation time yielded a different staining pattern, with a proportionately larger increase of the BMZ staining intensity compared to the AL staining intensity (Figure 2a-d). Pretreatment with Triton X-100 results in the loss of AL staining, while the BMZ staining was slightly brighter compared to the cryosection with the conventional treatment and fixation (Figure 2e-f).

All the different Col17-related EB cases: JEB-nH gen, JEB-nH loc, and the carrier showed reduced staining at the BMZ and AL compared to control skin (Figure 1). Staining intensity patterns for VK4 are summarized in Table 2. A complete absence of VK staining was seen for JEB-nH gen, and for JEB-nH loc a slightly reduced staining at the BMZ and a reduced AL staining was seen. The VK staining intensity for the carrier was similar to that of JEB-nH loc. Staining with 233 for the Col17 ectodomain in JEB-nH gen showed a reduced BMZ staining and an absent AL staining. For JEB-nH loc and the carrier, 233 staining yielded a (strongly) reduced AL staining, while the BMZ stained as control (Table 2; Figure 1).

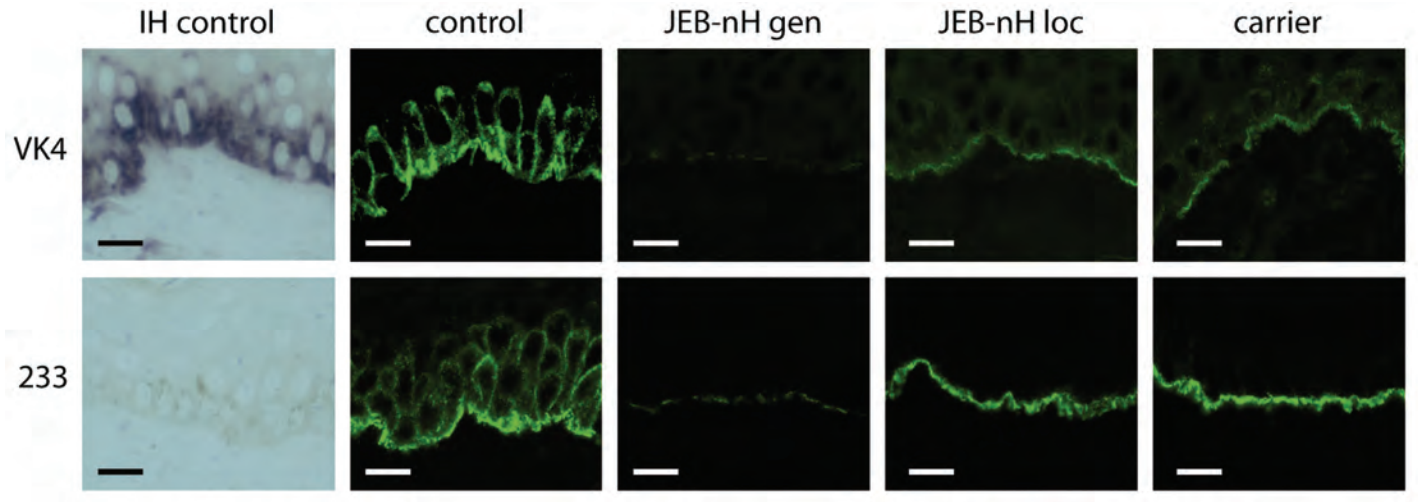

Figure 1. Immunohistochemical (IH) and immunofluorescence (IF) antigen staining with the Col17 endodomain antibody VK4 and the Col17 ectodomain antibody 233. IH staining with VK4 shows positive staining at the basal keratinocytes of a healthy control, whereas 233 is not able to IH stain. For IF staining, a healthy control, and representatives of the Col17-related epidermolysis bullosa subtypes: junctional epidermolysis bullosa, type non-Herlitz generalized (JEB-nH gen), junctional epidermolysis bullosa, type non-Herlitz localized (JEB-nH loc), and a heterozygous carrier of a *COL17A1* null mutation (carrier), are stained with VK4 and 233. With VK4 staining, JEB-nH gen showed absent epidermal basement membrane zone (BMZ) and apical-lateral (AL) staining, and JEB-nH loc and the carrier showed slightly reduced BMZ staining and reduced AL staining compared to control. With 233 staining, JEB-gen showed reduced BMZ and absent AL staining, JEB-nH loc showed normal BMZ and strongly reduced AL staining, and the carrier showed normal BMZ and reduced AL staining compared to control. Scale bar = 10 μm

Table 2. Overview of the representatives of each type XVII collagen-related epidermolysis bullosa subtype and their respective immunofluorescence staining intensities with type XVII collagen endodomain antibody VK4 and type XVII collagen ectodomain antibody 233

EB-nr	Subtype	Molecular analysis of *COL17A1*	VK4 staining		233 staining		Reference
			BMZ	AL	BMZ	AL	
Control			+++	+++	+++	+++	
011-01	Generalized JEB-nH	Homozygous c.2237delG	-	-	+	-	5, 29
168-01	Localized JEB-nH	Homozygous c.4321delT	++	+	+++	-/+	5
084-01	Carrier *COL17A1* null mutation	Heterozygous c.1179delA	++	+	+++	+	5

Staining intensity was compared to controls and scored as normal (+++), slightly reduced (++), reduced (+), strongly reduced (±) or absent (-). JEB-nH junctional epidermolysis bullosa, type non-Herlitz; BMZ basement membrane zone; AL apical-lateral of the basal keratinocytes.

Western blotting

All VK antibodies were effective in western blotting (Figure 3). In particular VK3 and VK5 yielded the best western blotting results.

Immunohistochemistry staining

Immunohistochemistry (IH) staining with all VK antibodies showed positive Col17 staining in basal keratinocytes of normal human skin. IH staining with 233 was negative (Figure 1).

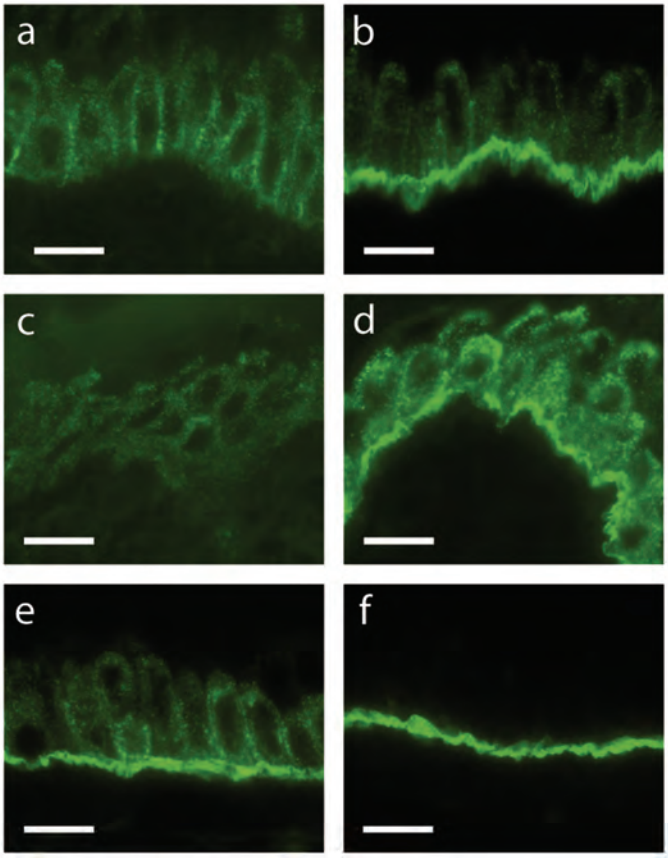

Figure 2. Immunofluorescence antigen staining of healthy control skin, using different incubation times (a, b), different antibody dilution (c, d), and different tissue pretreatments (e, f). Staining of VK2 in a 1:20 dilution for 90 minutes (b) shows an increase of BMZ staining compared to staining for 3 minutes (a). Undiluted staining of VK2 (d) yielded a proportionately larger increase of the BMZ staining intensity than the AL staining intensity, in comparison to VK2 staining in a 1:160 dilution (c). Pretreatment with Triton X-100 results in the loss of AL staining (f), while the BMZ staining was slightly brighter compared to the cryosection that had not been pretreated with Triton X-100(e). Scale bar = 10 µm

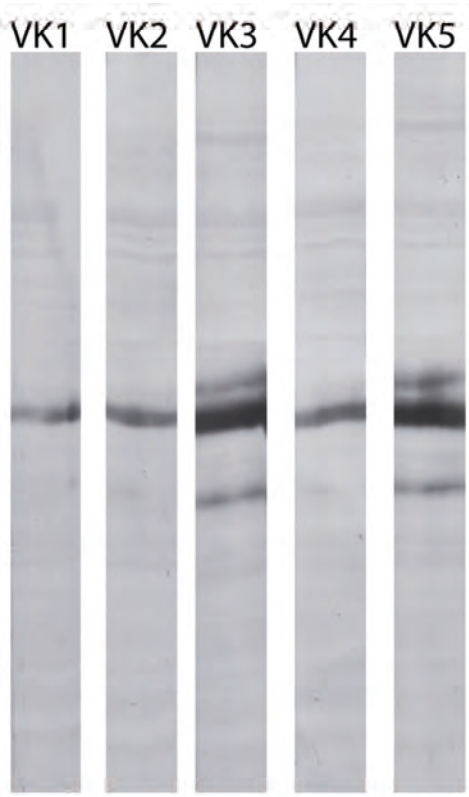

Figure 3. The VK antibodies, especially VK3 and VK5, showed positive western blotting of type XVII collagen.

Discussion

We have developed five new versatile monoclonal antibodies to the endodomain of type XVII collagen that works in IF, IH, and Western blotting. IF staining remains the golden standard for visualizing Col17 in biopsy specimens, however the technique does have some special requirements. A fluorescence microscope is needed, and the obligatory fresh tissue requires special handling, transportation, and treatment. Furthermore, the fluorescence-based signals eventually fade away, excluding permanent archiving. Also, IF staining requires a dark field, so tissue morphology and antigen localization can not be observed together and a level of skill and experience in pattern recognition is required.[31] With VK effective in IH staining many of these drawbacks are surpassed, and a possible application of this technique could be the screening for Col17-related EB cases in laboratories not equipped for IF staining. Western blotting with VK can be valuable in

Col17-related EB research. It enables protein analysis, such as Col17 detection and quantification, and reveals shorter truncated versions.

IF staining with the VK antibodies can distinguish between healthy subjects and all Col17-related EB subtypes and carriers. Furthermore, a good distinction between the (nearly) absent staining in JEB-nH gen and the reduced staining in JEB-nH loc can be made, allowing a first cautious prognosis based on IF. Although the correlation between staining intensity and the severity of clinical symptoms is quite strong,[5,23,32,33] some reserve in interpretation is advisable as also positive or reduced Col17 staining has been reported in JEB-nH gen patients, as has strongly reduced Col17 staining in JEB-nH loc patients.[34] Therefore precaution should always be taken in giving prognosis to parents and patients, and further molecular and protein analysis can help in verification of prognosis.

Staining with VK could not differentiate between the staining pattern seen in JEB-nH loc and carriers of *COL17A1* null mutations. However, these two groups will be best distinguished in their clinical features. Apart from so-called mini-symptoms, such as teeth abnormalities, carriers do not suffer from skin fragility or other clinical symptoms.[32]

The distinction between the control and the different Col17-related EB cases and carriers was more obvious with the endodomain-specific VK antibodies, than with the ectodomain-specific 233 antibody. The AL staining of the basal keratinocytes is more prominent with the VK antibodies and its loss in carriers and JEB-nH loc is easily noticed. At the same time, BMZ expression is decreased. In contrast, the BMZ staining with 233 has slightly to no reduced intensity in carriers/JEB-nH loc compared to controls. This is likely explained by the biological turnover of the different processed forms of the Col17 molecule. After synthesis and assembly of Col17 in the endoplasmatic reticulum, it is transported to the AL cell membrane where it forms a reservoir to be incorporated into newly formed hemidesmosomes at the basal cell membrane.[6-8] In concordance with Hirako *et al*. we showed that the freely soluble AL Col17 can be easily removed with Triton X-100 treatment, leaving only the hemidesmosomal Col17.[6] In the case of reduced Col17 production, such as in JEB-nH loc and in carriers, the AL reservoir will deplete more easily as the available Col17 is needed for incorporation into hemidesmosomes, explaining the loss of AL staining. Depending on the amount of Col17 available, the staining of the hemidesmosomal full-length Col17 might also become reduced, explaining the less intense BMZ VK staining. This stresses the importance of pattern recognition in IF staining for diagnosing Col17-related EB cases and carriers, where the observation of the loss of AL staining might even be more important than the BMZ

staining. After incorporation of Col17 in hemidesmosomes, shedding of the ectodomain yields two more BMZ-located Col17 forms, the 120 kDa LAD-1, and the 97 kDa LABD97.[18-21,35] The latter two proteins will not show up with endodomain specific antibodies, such as VK and the former 1A8C, but ectodomain antibodies as 233 will intensify the BMZ staining as these stain all three forms. Antibodies such as 1D1 that stain the COOH-terminal domain (present in full-length Col17 and LAD-1, but not in LABD97) will stain intermediate.[5] With reduced Col17 production, the BMZ staining with VK against the full-length Col17 is reduced, while the BMZ staining with 233 against full-length Col17, LAD-1, and LABD97, is nearly normal. This discrepancy in loss of staining intensity at the BMZ implicates that full-length Col17 has a different turnover time than LAD-1 and LABD97. We hypothesize that after shedding the Col17 endodomain is resorbed in basal keratinocytes, while the shedded LAD-1 and LABD97 remain settled in the BMZ, where they might exert a still unknown function, and are protected from direct proteolysis.[16,22] Due to a lower turnover rate, LAD-1 and LABD97 are able to accumulate in the BMZ until later degradation, explaining the normal 233 BMZ staining even with reduced Col17. Consequently, antibodies against the Col17 endodomain are the most sensitive in detecting a reduced Col17 expression, as they only detect full-length Col17.

Conclusion

Due to an imminent extinction of antibodies against the Col17 endodomain, we have produced five new monoclonal mouse anti-human IgG antibodies against this domain, called VK1, VK2, VK3, VK4, and VK5. Further testing will provide the exact epitopes of all VK antibodies. We have shown that endomain antibodies are superior to ectodomain antibodies in diagnosing between JEB-nH gen and JEB-nH loc/carriers. We have also shown here that the obtained staining patterns depend on the used staining method, including tissue preparation, incubation time and antibody dilution. Therefore, to obtain optimal interpretable patterns, the IF staining protocol described here should be followed.

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12

A cohort of basal epidermolysis bullosa simplex patients with wild-type *KRT5*, *KRT14*, and *PLEC1* genes: analysis of the remaining EBS candidate genes *COL17A1*, *ITGB4* and *DST*

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To the editor

The trauma-induced genetic blistering disease basal epidermolysis bullosa simplex (EBS) is characterized by a cleavage trough basal keratinocytes, and the majority of cases are caused by mutations in the genes coding for keratin 5 (*KRT5*), keratin 14 (*KRT14*), and plectin (*PLEC1*).[1-4] Some basal EBS cases have been reported carrying mutations in genes that are normally associated with junctional epidermolysis bullosa: *COL17A1* coding for type XVII collagen (Col17) and *ITGB4* coding for integrin β4 (β4).[5-7] Most recently, the dystonin gene *DST* that codes for bullous pemphigoid antigen 1-e (BPAG1-e) was revealed as a new EBS candidate gene.[8,9]

Plectin, BPAG1-e, β4, and Col17 interact with each other in the hemidesmosome.[10,11] Hemidesmosomes are multiprotein complexes that facilitates the stable adhesion between the basal keratinocytes and the underlying basement membrane.[12] Plectin and BPAG1-e are cytoplasmic proteins that belong to the plakin protein family and they provide the link from the hemidesmosomes to the keratin intermediate filaments keratin 5 and 14. Together with integrin α6, β4 forms the non-covalently bound transmembrane heterodimer integrin α6β4. Integrin α6β4 and the glycosylated transmembrane protein Col17, connects the hemidesmosome to the underlying lamina lucida/densa trimeric polypeptide laminin-332 (LM-332).[11]

In our group Bolling *et al*. [2011] have described the molecular analysis of the largest well classified biopsy-confirmed basal EBS cohort (n=64), with 77% of the cases (n=49) caused by mutations in *KRT5* or *KRT14,* and 6% in *PLEC1* (n=4). Further cDNA analysis of *KRT5* and *KRT14* could not detect any large deletions or insertions. This leaves 17% of the cases (n=11) unsolved.[13,14] In this study we investigated the remaining EBS candidate genes (*ITGB4, COL17A1*, and *DST*) in these probands with wild-type *KRT5*, *KRT14*, and *PLEC1* genes.[13,14]

The diagnosis basal EBS was made upon their clinical features and biopsy findings showing a split through the basal keratinocyte. Mutations in *KRT5, KRT14*, and *PLEC1*, were excluded in all probands, as described before.[13,14] Genomic DNA was extracted from peripheral blood. All coding exons of *ITGB4* (GenBank NM_001005731.1), *COL17A1* (GenBank NM_000494.3), and *DST* (GenBank NM_001723.4), including their flanking introns were amplified by polymerase chain reaction and sequenced directly in all 11 probands (methods upon request).

Our cohort consists of a diverse group of patients classified in phenotypical subtypes ranging from EBS-Dowling meara (n=2), EBS-generalized (n=2), and EBS-localized (n=7). Five cases inherit in an autosomal dominant fashion, there are five sporadic cases, and in one case a sibling is also affected. The molecular analysis of *ITGB4*, *COL17A1* and *DST* revealed no pathogenic mutations in our 11 probands.

In this study we show that in the largest biopsy-confirmed EBS cohort, mutations in *ITGB4*, *COL17A1,* and *DST* do not occur. Our results suggest that the occurrence of mutations in these genes is rare in EBS. Consequently, to date only two cases of EBS carrying *COL17A1* mutations, one case carrying *ITGB4* mutations, and two cases carrying *DST* mutations have been reported in the literature.[5-9] However, as cDNA analysis has not been performed, large deletions or insertions are not entirely excluded. This also applies for mutations located in promoter regions. A large *COL17A1* deletion was shown to be pathogenic in the basal EBS patient described by Huber *et al.*,[5] while the remaining four previously described patients carried small deletions or point mutations in *ITGB4*, *COL17A1,* or *DST*.[6-9] The question is whether these genes should be screened in future EBS cases with wild-type *KRT5, KRT14,* and *PLEC1* genes. Two common features in all reported EBS cases caused by *COL17A1, ITGB4,* and *DST* mutations can be distinguished: (1) all are inherited in an autosomal recessive manner, and (2) immunofluorescence antigen staining against Col17, β4, or BPAG1-e was either reduced or absent in the case reports carrying *COL17A1*, *ITGB4*, and *DST* mutations, respectively.[5-9] In contrast, immunofluorescence antigen staining of Col17, β4, and BPAG1-e, that we performed in some of our probands did not show any abnormalities compared to control. To increase the probability of finding a mutation in these genes, molecular analysis could be restricted to some selected cases showing abnormalities in immunofluorescence antigen staining of the respected proteins, and excluding the cases that inherit autosomal dominant.

In our 11 basal EBS families, mutations are excluded by Sanger sequencing in all known genes ever associated with basal EBS. With genetic linkage analysis and next-generation sequencing we hope to further elucidate the molecular pathogenesis and enhance our understanding of basal EBS. Possible candidate genes could be other keratins expressed in basal keratinocytes, such as keratin 15,[15] or other hemidesmosomal proteins, such as integrin α6 or CD151.

In conclusion, we showed that mutations in *ITGB4, COL17A1*, and *DST* did not occur in our basal EBS probands with wild-type *KRT5, KRT14*, and *PLEC1* genes.

Mutations in the *ITGB4, COL17A1*, and *DST* genes are rarely found in basal EBS and should only be screened in some selected cases.

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Health related quality of life in epidermolysis bullosa: assessment in the Dutch population and validation of the Dutch QOLEB questionnaire

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Abstract

Background Defining the health related quality of life (HRQOL) in patients suffering from the heritable heterogeneous trauma-induced blistering disease epidermolysis bullosa (EB) is important for patient care and management, assessing the efficacy of new treatments, and assigning funding to EB. The quality of life in EB questionnaire (QOLEB) is a 17-item EB-specific HRQOL measurement tool.

Objective The aim of this study is to develop a validated and reliable QOLEB in the Dutch language, and to assess the HRQOL in Dutch EB patients.

Methods The QOLEB is translated to the Dutch language according to protocol. Fifty-five adult patients across four EB subtypes completed the QOLEB, Short Form-36 (SF-36), and Skindex-29 twice. Floor and ceiling effects were assessed. The respondent burden was measured with a self-reported completion time. Convergent validity and test-retest reliability was measured with the Spearman's rho correlation coefficient (ρ_s). The discriminative validity was calculated with ANOVA, and the internal consistency with the Cronbach alpha (α).

Results Floor effects were seen in four items; no ceiling effects were present. The respondent burden of time was 8.2 minutes. The QOLEB correlated excellently and well with the Skindex-29 (ρ_s=0.86) and SF-36 physical score (ρ_s=-0.75), respectively, while a moderate correlation with the SF-36 mental score (ρ_s=-0.43) was seen. The discriminative validity between the four different EB subtypes was significant (p=0.002). The internal consistency was excellent (α=0.905), and the test-retest reliability strong (ρ_s=0.88).

Conclusions The Dutch QOLEB is a reliable and valid instrument for the assessment of the HRQOL in adult EB patients.

Introduction

Epidermolysis bullosa (EB) consists of a spectrum of heritable trauma-induced blistering diseases of skin and mucous membranes.[1] Many subtypes exists, and their clinical severity is variable, ranging from an occasional palmoplantar blister to childhood lethality.[1] The health related quality of life (HRQOL) is the impact of a disease on the physical, psychological, and social health of a patient.[2] Defining the HRQOL in EB patients is important in patient care and management.[3] Furthermore, in recent years more focus has been placed on finding specific treatments for EB and measuring the HRQOL can help in assessing the efficacy of these new treatment modalities.[4,5] Also, measuring and comparing the HRQOL to other diseases can help assign funding to the rather unknown and rare disorder, EB.[4,6] Measuring the HRQOL is complicated by the wide range of phenotypes in EB, which all have their own clinical severity.[3] Several studies have focused on qualitatively describing the impact of EB on patients' lives.[7-11] Quantitative measurement of the HRQOL has been performed using both generic- and dermatology-specific instruments in EB patients.[6,12-15] However, due to ceiling effects and content validity issues of these instruments, the accuracy of the measurements are questionable. It has been hypothesized that, especially in the more severe EB subtypes, there has been an overestimation of the HRQOL.[3] To overcome these problems, Frew *et al.* developed and validated an EB-specific HRQOL measurement tool for older children and adults that can be used across all EB subtypes, called the QOLEB (quality of life in EB).[3] Translation of the QOLEB which was created and validated in English in Australia, to other cultures and languages may provide reliable comparisons of the HRQOL in EB and the efficacy of clinical interventions across different countries.[3] The aim of this study was to develop a validated and reliable QOLEB in the Dutch language, and to assess the HRQOL in Dutch EB patients.

Materials and Methods

This study was performed at the Center for Blistering Diseases at the University Medical Center Groningen, which is the single national referral center for EB in the Netherlands. Ethical approval was granted by the Medical Ethical Committee of the University Medical Center Groningen in the Netherlands. A signed consent form was obtained from all participating patients.

Study measurement tools

QOLEB

The English QOLEB is a validated and reliable adult EB-specific HRQOL tool consisting of a 17-item questionnaire, and it was developed by two of the authors (J.W.F and D.F.M).[3] The QOLEB measures two factors: functioning (questions 1-7, 9-10, 12-13, 15) and emotions (questions 8, 11, 14, 16-17). For each question four optional answers exist that are scored from 0 to 3 points, in which a higher score represents a worse HRQOL. The range in the functioning scale is 0-36, in the emotions scale 0-15, and in the overall scale 0-51 points. Recently proposed banding techniques by J.W.F and D.F.M. for the overall severity of the QOLEB score based upon the data reported in the original QOLEB validation are as follows: very mild (0-4 points), mild (5-9 points), moderate (10-19 points), severe (20-34 points), and very severe (35-51 points).[3]

Short Form-36

The Short Form-36 (SF-36) is a 36-item generic HRQOL instrument measuring eight scales: physical functioning (PF, limitations in physical activities because of health problems), role-physical (RP, limitations in usual role activities because of physical health problems), bodily pain (BP, limitations and severity of pain), general health (GH, perceptions of the general health), vitality (VT, energy and fatigue experienced due to health problems), social functioning (SF, limitations in social activities because of physical or emotional problems), role-emotional (RE, limitations in usual role activities because of emotional problems), and mental health (MH, psychological distress and well-being).[16] Each scale is normalized to scores from 0 to 100 points, in which a higher score represents a better HRQOL (contrary to QOLEB). Two summary scales have been developed: the physical component summary (PCS) and the mental component summary (MCS).[17] The SF-36 has been translated and validated to the Dutch language, and age-and gender norm values are available for the Dutch population.[18]

Skindex-29

The Skindex-29 is a 30-item validated and reliable dermatology-specific HRQOL instrument for adults, measuring three factors: emotions (n=10), functioning (n=12) and symptoms (n=7).[19-21] Each item can be answered on a five-point scale ranging from “never” to “all the time”, that are scored from 0 to 100 points. A higher score represents a worse HRQOL (similar to QOLEB). The overall Skindex-29 score indicates that the skin

disease has very little (<5 points), mild negative (6-17 points), moderate (18-36 points), and severe (>37 points) effect on the HRQOL. Different intervals are used for the domains separately but with the same classification: emotions (<5; 6-24; 25-49; >50), functioning (<3; 4-10; 11-32; >33), and symptoms (<3; 4-10; 11-25; 26-49; and >50 for extremely severe).[22] The Skindex-29 has been translated to the Dutch language using a standard protocol.[12]

Translation of the QOLEB to the Dutch language

A forward translation of the QOLEB to the Dutch language was performed by an independent qualified translator. The questionnaire was discussed by experts in EB (W.Y.Y. and M.F.J), who are native speakers in Dutch and fluent in English. Some conceptual changes were made. Content validity was obtained by back-translation to the English language by a different independent qualified translator, to make sure the translated Dutch QOLEB conveys the same meaning as the English QOLEB. The Dutch QOLEB is supplemented in Appendix S1.

Recruitment and design

From the Dutch EB registry, adult patients across a range of subtypes were included to participate in this study. The patients were classified into four main EB subtypes: EB simplex (EBS), junctional EB (JEB), dominant dystrophic EB (DDEB), and recessive dystrophic EB (RDEB). Inclusion criteria were that patients were native speakers of the Dutch language, and that they had an age of ≥ 18 years. The selected patients received an information letter concerning the design, relevance and main goal of the study. A week after receiving this letter, the patients were given further oral information through the telephone and invited to participate in the study. The patients that were willing to participate received the first Dutch QOLEB, Skindex-29, and SF-36. They were requested to complete and return these questionnaires. Four weeks after completing these questionnaires, all patients were asked to complete and return the same questionnaires for a second time. When required and possible, data were clarified through the telephone.

Analysis

Floor and ceiling effects

Floor and ceiling effects for the individual items were considered when 80% or more of the participants scored the highest or lowest possible scores in the first QOLEB.

Respondent burden

The respondent burden was assessed by the self-reported completion time of the first QOLEB, and was considered brief if <15 min.

Validity

The convergent validity was assessed by calculating Spearman's rho correlation coefficient (ρ_s) between the overall score of the first QOLEB and the overall score of the Skindex-29, and the MCS and PCS score of the SF-36.[12] A ρ_s of ≥0.7 was considered as acceptable, and ≥0.8 as excellent. The discriminative validity was calculated between the four main EB subtypes using an analysis of variance (ANOVA). A $p<0.05$ was considered as statistically significant.

Reliability

The internal consistency and construct validity of the Dutch QOLEB was measured with the Cronbach alpha (α) using the data from the first QOLEB. An α of ≥0.7 was considered as acceptable, ≥0.8 as good, and ≥0.9 as excellent. The degree of test-retest reliability was estimated by Spearman's rho correlation coefficient (ρ_s) between the overall scores of the QOLEB questionnaire at the first and second time. A ρ_s of ≥0.7 was considered as acceptable, and ≥0.8 as excellent.

Assessment of QOL

Mean values and standard deviations (SD) were calculated for the scores of all study measurement tools and compared using the t-test and ANOVA with a Bonferroni posthoc test. A $p\leq0.05$ was considered statistically significant.

Results

Study population

As of 1st January 2011, 184 adult EB patients from the Dutch EB Registry met the inclusion criteria and were eligible for participation. We randomly selected 103 patients to be included in the study. Of the 103 patients that were invited by the information letter to participate in the study, 75 patients were willing to co-operate (response rate 73%). The remaining 28 patients did not want to take part in the study or could not be reached by telephone. Of the 75 patients, 55 were included in the study (response rate 73%). The

remaining 20 patients were excluded due to unreturned questionnaires or incomplete questionnaires.

The characteristics of the study population and drop outs, split to EB subtype, are shown in Table 1. Of the 55 included patients, 51% were male and 49% were female. The mean age was 47.6 years (SD±17.1) with a range of 19-85 years. The distribution among EB types was: EBS 29 (53%), JEB 8 (15%), DDEB 13 (24%), and RDEB 5 (9%) patients. All patients were of Dutch ethnicity, except for one Korean, one Surinamese, and one Turkish patient. All patients were native speakers of the Dutch language.

Floor and ceiling effects

Floor effects were seen in four of the 17 items: item 2 (bathing or showering; 82%), item 4 (writing; 84%), item 5 (eating; 80%), and item 12 (modifying house; 82%). No ceiling effects were present in the 17 items (Supplementary Appendix S2).

Respondent burden

The respondent burden of time for the QOLEB was brief, with a mean of 8.2 minutes (SD±5).

Validity

The results of the validity tests are summarized in Table 2. The content validity was addressed through the forward-backward translation of the QOLEB to the Dutch language. The QOLEB correlated well with the SF-36 PCS score (ρ_s=-0.75), and extremely well with the Skindex-29 (ρ_s=0.86). Between the SF-36 MCS score and the QOLEB a moderate correlation was seen (ρ_s=-0.43). A significant discriminative validity between the four different EB subtypes was seen (p=0.002).

Reliability

The results of the reliability tests are summarized in Table 2. The QOLEB showed an excellent internal consistency and construct validity (α=0.905). Furthermore, the internal consistency and construct validity in the separate EB subtypes was good to excellent (range α = 0.85-0.94). The test-retest reliability of the QOLEB was strong (ρ_s=0.88) after an average of 57 (SD±24) days. Also, the test-retest reliability in the separate EB subtypes was good to excellent (range ρ_s=0.82-0.98).

Table 1. Patient characteristics of the study population

	Participants	Drop outs
Patients, *n*	55	48
Age in years, mean ± SD (range)	47.6 ± 17.1 (19-85)	36.9 ± 12.3 (18-67)
Gender, *n* (%)		
Male	28 (51%)	17 (35%)
Female	27 (49%)	31 (65%)
EB subtype, *n* (%)		
EBS	29 (53%)	20 (42%)
EBS-loc	20 (36%)	12 (25%)
EBS-gen-nonDM	2 (4%)	2 (4%)
EBS-DM	4 (7%)	2 (4%)
EBS-AR	3 (5%)	2 (4%)
EBS-MP	-	1 (2%)
EBS-MD	-	1 (2%)
JEB	8 (15%)	8 (17%)
JEB-nH loc	4 (7%)	2 (4%)
JEB-nH gen	4 (7%)	4 (8%)
JEB-lo	-	2 (4%)
DEB	18 (33%)	20 (42%)
DDEB	13 (24%)	14 (29%)
DDEB-pt	3 (5%)	-
DDEB-ac	3 (5%)	7 (15%)
DDEB-gen	7 (13%)	7 (15%)
RDEB	5 (9%)	6 (13%)
RDEB-I	2 (4%)	1 (2%)
RDEB-O	2 (4%)	4 (8%)
RDEB-sev gen	1 (2%)	1 (2%)

EBS, epidermolysis bullosa simplex; EBS-loc, EBS localized; EBS-gen-nonDM, EBS other generalized; EBS-DM, EBS Dowling-Meara; EBS-AR, EBS autosomal recessive; EBS-MP, EBS with mottled pigmentation; EBS-MD, EBS with muscular dystrophy; JEB, junctional epidermolysis bullosa; JEB-nH loc, JEB non-Herlitz localized; JEB-nH gen, JEB non-Herlitz generalized; JEB-lo, JEB of late onset; DEB, dystrophic epidermolysis bullosa; DDEB, dominant DEB; DDEB-pt, DDEB pretibial; DDEB-ac, DDEB acral; DDEB-gen, DDEB generalized; RDEB, recessive DEB; RDEB-O, RDEB generalized other; RDEB-I, RDEB inverse; RDEB-sev gen, RDEB severe generalized.

Table 2.Validity and reliability tests of the Dutch QOLEB

	Dutch QOLEB	Conclusion
Validity tests		
Content validity	n/a	Addressed through forward-backward translation English QOLEB
Convergent validity	Skindex-29: ρ_s =0.86 (p<0.01)	Strong agreement
	SF-36 PCS: ρ_s =-0.75 (p<0.01)	Agreement
	SF-36 MCS: ρ_s =-0.43 (p<0.01)	Moderate agreement
Discriminative validity	p=0.002	Significant
Reliability tests		
Internal consistency and construct validity	All EB subtypes: α =0.905	Excellent
	EBS: α=0.88	Good
	JEB: α=0.85	Good
	DDEB: α=0.87	Good
	RDEB: α=0.94	Excellent
Test-retest reliability	All EB subtypes: ρ_s =0.88 (p<0.01)	Strong agreement
	EBS: ρ_s =0.82 (p<0.01)	Strong agreement
	JEB: ρ_s =0.88 (p<0.01)	Strong agreement
	DDEB: ρ_s =0.82 (p<0.01)	Strong agreement
	RDEB: ρ_s =0.98 (p<0.01)	Very strong agreement

n/a, not available; ρ_s, spearman's rho correlation coefficient, α, Cronbach alpha, EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa; DDEB, dominant dystrophic epidermolysis bullosa; RDEB, recessive dystrophic epidermolysis bullosa; SF-36, Short Form-36; PCS, physical component summary; MCS, mental component summary

Assessment of QOL

Dutch QOLEB

The results of the Dutch QOLEB are listed in Table 3. Overall, the Dutch QOLEB average scores were lower than the ones reported in the original QOLEB study, which included more (n=111) patients and more severe EB patients (8 JEB and 16 RDEB).[3] Females have a lower HRQOL than males, although these differences never reached statistical significance. The milder EB subtypes EBS and DDEB both show a better HRQOL in all scales, compared to the more severe RDEB and JEB. These differences reach significance in the functioning and overall scale, while in the emotions scale significance was not reached (Table 3). According to the categorization of the overall QOLEB score, the Dutch EBS and DDEB cohorts are mildly affected, and the Dutch JEB and RDEB cohorts are moderately affected. None of the patients are very severely

affected, and only 4% were severely affected (Table 4), whereas in the original QOLEB score, several patients with RDEB and JEB were in this range.

The results of the 17 individual QOLEB items are listed in the Supplementary Appendix S2. EB made 71% of the patients feel frustrated, 36% embarrassed, 31% depressed, 27% uncomfortable, and 51% anxious or worried. EB affected patients in their relationship with their friends (31%) and family (25%), although in most patients only a small effect was noticed. Only 16% of all patients were pain free, while 9% experienced constant pain. Twenty percent of the patients were affected in their eating ability, and 19% needed some sort of assistance with bathing or showering. Difficulties in writing were especially prominent in RDEB, with 60% of the patients finding it easier to type than write. Almost all patients were affected in their involvement in sports (95%), with 62% needing to avoid some or all sports. Although 49% of all patients were affected at least a little in their ability to move around at home, patients of the JEB and RDEB subtypes were affected most severely. JEB and RDEB patients also had to make the most modifications in their house. Around 38-40% of the JEB and RDEB patients were affected a lot or severely in their ability to move outside their house, and 40% percent of all EB patients were affected in their ability to go shopping. Of all patients, 33% were financially affected by their EB, and 11% were greatly or severely affected.

Table 3.Mean ± SD QOLEB values by gender and epidermolysis bullosa (EB) subtype

	N	Functioning (range 0-36)	Emotions (range 0-15)	Total (range 0-51)
All	55	7.3 ± 5.4	2.4 ± 2.0	9.6 ± 6.9
Male	28	7.0 ± 5.1	2.0 ± 1.8	9.0 ± 6.3
Female	27	7.5 ± 5.8	2.7 ± 2.2	10.2 ± 7.5
EB subtype				
EBS	29	6.0 ± 4.0	1.9 ± 1.9	7.9 ± 5.3
JEB	8	11.3 ± 5.0	3.9 ± 1.8	15.1 ± 6.4
DDEB	13	5.2 ± 3.3	2.2 ± 2.0	7.3 ± 5.1
RDEB	5	13.4 ± 5.4	3.2 ± 2.4	16.6 ± 11.7

EBS, EB simplex; JEB, junctional EB; DDEB, dominant dystrophic EB; RDEB, recessive dystrophic EB; accolades represent a statistical significant difference of p≤0.05

Table 4. Banding of the QOLEB results by epidermolysis bullosa (EB) subtype

	EBS	JEB	DDEB	RDEB	Total
N	29	8	13	5	55
Very Mild: 0-4 points	8 (28%)	0 (0%)	5 (38%)	1 (20%)	14 (25%)
Mild: 5-9 points	12 (41%)	1 (13%)	2 (15%)	1 (20%)	16 (29%)
Moderate:10-19 points	8 (28%)	6 (75%)	6 (46%)	3 (60%)	23 (42%)
Severe: 20-34 points	1 (3%)	1 (13%)	0 (0%)	0 (0%)	2 (4%)
Very Severe:35-51 points	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

EBS, EB simplex; JEB, junctional EB; DDEB, dominant dystrophic EB; RDEB, recessive dystrophic EB

Short Form-36

The results of the SF-36 are summarized in Table 5. EB has a greater impact on the HRQOL in females, although no significant differences were found. In all SF-36 scales, except for VT, RE, and MCS, JEB and RDEB patients have a lower HRQOL compared to EBS and DDEB patients, reaching statistical significance in the PF scale between EBS and JEB (p=0.025). In comparison to gender- and age matched Dutch normative values, all EB subtypes have a lower HRQOL on the PCS scale, while they are comparable on the MCS scale (Figure 1). Floor effects were seen in item 3j (limitations in bathing or dressing; 84%), and item 5a (cutting down on the amount of time spent on work or other activities due to emotional problems in the last four weeks; 84%). There were no ceiling effects.

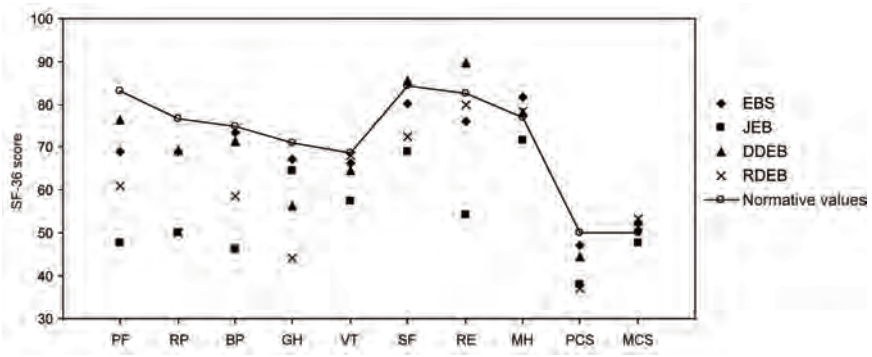

Figure 1. Epidermolysis bullosa (EB) Short Form-36 scores compared to gender- and age matched Dutch normative values. Legend: EBS, EB simplex; JEB, junctional EB; DDEB, dominant dystrophic EB; RDEB, recessive dystrophic EB; PF, physical functioning; RP, role-physical; BP, bodily pain; GH, general health; VT, vitality; SF, social functioning; RE, role-emotional; MH, mental health; PCS, physical component summary; MCS, mental component summary

Table 5. Mean ± SD Short Form-36 values by gender and epidermolysis bullosa (EB) subtype

	N	PF	RP	BP	GH	VT	SF	RE	MH	PCS	MCS
All	55	70.5 ± 25.3	64.5 ± 42.9	67.5 ± 26.2	62.0 ± 22.9	64.8 ± 20.3	79.1 ± 24.4	76.4 ± 39.4	79.1 ± 16.5	44.1 ± 10.8	50.9 ± 9.5
Male	28	74.6 ± 24.7	72.3 ± 43.2	71.0 ± 25.1	66.3 ± 20.5	70.0 ± 19.1	83.9 ± 21.2	79.8 ±37.8	82.4 ±16.9	46.1 ± 10.3	52.5 ± 9.3
Female	27	66.1 ± 25.6	56.5 ± 41.9	64.0 ± 27.3	57.6 ± 24.8	59.4 ± 20.4	74.1 ±26.8	72.8 ± 41.4	75.6 ± 15.6	42.0 ± 11.0	49.2 ± 9.5
EB subtype											
EBS	29	69.0 ± 42.6	69.0 ± 42.6	73.4 ± 24.3	67.1 ± 21.7	66.4 ± 20.7	80.2 ± 23.5	75.9 ± 40.7	81.7 ±18.0	46.9 ± 9.1	50.6 ±10.5
JEB	8	47.5 ± 25.9	50 ± 53.5	46.3 ± 24.8	64.4 ± 22.4	57.5 ± 24.3	68.8 ± 27.5	54.2 ± 50.2	71.5 ±13.4	37.9 ± 11.2	47.6 ± 9.8
DDEB	13	76.2 ±24.4	69.2 ± 37.0	71.2 ± 22.8	56.2 ± 25.9	64.6 ± 18.1	85.6 ± 15.2	89.7 ±28.5	78.2 ±15.0	44.3 ±10.8	52.7 ± 7.6
RDEB	5	61.0 ±36.8	50 ± 46.8	58.5 ± 34.4	44.0 ± 13.4	68.0 ± 20.2	72.5 ± 41.8	80 ± 29.8	78.4 ±15.9	37.1 ±14.9	53.3 ±7.8

EBS, EB simplex; JEB, junctional EB; DDEB, dominant dystrophic EB; RDEB, recessive dystrophic EB; PF, physical functioning; RP, role-physical; BP, bodily pain; GH, general health; VT, vitality; SF, social functioning; RE, role-emotional; MH, mental health; PCS, physical component summary; MCS, mental component summary; accolades represent a statistical significant difference of $p \leq 0.05$

Skindex-29

The results of the Skindex-29 are shown in Table 6. In all Skindex-29 scales, females had a lower HRQOL compared to males (no significance). JEB and RDEB have a lower HRQOL compared to EBS and DDEB in the functioning and symptoms scale, reaching statistical significance in the functioning component between EBS and JEB (p=0.022). RDEB has the best HRQOL on the emotions scale, while JEB has the lowest. There were no floor or ceiling effects seen. According to the Skindex-29 categorization, EBS, DDEB and RDEB have a moderate effect, and JEB has a severe effect on the HRQOL. The greatest effect was seen in the symptom scale, in which EBS has a severe effect, and JEB, DDEB, and RDEB have an extremely severe effect on the HRQOL. Analysis of the individual Skindex-29 items, shows that in the symptoms scale, 31% of the patients answered 'often' or 'all the time' to the item "My skin condition burns or stings", 33% to "My skin itches", 31% to "My skin is irritated", 51% to "My skin is sensitive", and 20% to "My skin condition bleeds". For the functioning scale, 20% of the patients answered 'often' or 'all the time' to the item "My skin condition affects how well I sleep", 22% to "My skin condition interferes with my sex life", and 24% to "My skin condition makes me tired".

Table 6. Mean ± SD Skindex-29 values by gender and epidermolysis bullosa (EB) subtype

	N	Functioning	Emotions	Symptoms	Total
All	55	26.0 ± 22.7	24.9 ± 18.8	45.0 ± 25.4	30.2 ± 20.3
Male	28	25.2 ± 21.7	21.8 ±19.7	43.0 ± 25.9	28.3 ± 20.5
Female	27	26.7 ± 24.1	28.2 ± 17.5	47.1 ± 25.3	32.2 ± 20.4
EB subtype					
EBS	29	19.8 ± 18.1	21.6 ± 17.0	37.4 ± 22.2	24.7 ± 17.6
JEB	8	45.8 ± 16.2	34.4 ± 14.1	55.4 ± 21.3	44.2 ± 13.3
DDEB	13	24.0 ± 25.7	28.5 ± 24.7	50.8 ± 30.6	32.0 ± 25.1
RDEB	5	34.6 ± 22.7	20 ± 14.5	57.1 ± 27.5	35.0 ± 20.3

EBS, EB simplex; JEB, junctional EB; DDEB, dominant dystrophic EB; RDEB, recessive dystrophic EB; accolades represent a statistical significant difference of p≤0.05

Discussion

In this study, we have developed an EB-specific HRQOL measurement tool for Dutch patients by translating and validating the QOLEB into the Dutch language and Dutch EB population, respectively. A total of 55 adult patients across all four subtypes (EBS, JEB, DDEB, and RDEB) have participated to test the reliability and validity of the Dutch QOLEB. Floor effects were present in four items, indicating that only a few patients had limitations in bathing/showering, writing, eating and needing to modify their house, suggesting a poor discriminative value for these items. However, the distribution of our patient population, in which 76% suffer from the milder EB (EBS and DDEB), and only 24% from the more severe EB (JEB and RDEB) subtypes, contributed to these floor effects. This might also explain the lack of ceiling effects seen in the SF-36 and Skindex-29. The Dutch QOLEB is a reliable tool, although the high internal consistency (α=0.905) does suggests some item redundancy. However, this was also seen in the validation of the original QOLEB (α=0.931). The Dutch QOLEB appears to be a valid instrument. The assessment of the convergent validity was performed with the Skindex-29 and SF-36, as they have been proven to be the generic and dermatology-specific HRQOL instruments of choice for dermatologic diseases.[12] A good convergent validity was seen with the Skindex-29 and the physical aspects of the SF-36, but not for the mental aspects of the SF-36. The discriminative validity was significant (p=0.002), and the Dutch QOLEB was also able to discriminate the severe subtypes RDEB and JEB from the milder subtypes EBS and DDEB. Significant differences were seen in the functioning and overall scale, but not in the emotions scale, suggesting one of two explanations: either that the mental burden of EB is similar in milder and more severe subtypes, or that patients with more severe EB subtypes have acquired emotional resilience due to the severe impact of living with EB. This is also seen in the results from a study by Margari *et al*.,[13] who found that 80% of EB patients experienced sub-threshold psychiatric symptoms, in particular depression, anxiety, and behavior disturbances, but that there was no close correlation between these symptoms and the clinical severity of EB.[13] Emotional resilience was not explored by Margari *et al.* but has been proposed by Frew *et al.* to explain the lack of consistent quantitative data regarding emotional burden in EB.[3,13] A complicating factor in evaluating the discriminative factor in EB, is the clinical variety and severity within the four major subtypes.[1] In our study, many patients with a milder “minor” subtype have been included; 69% of the EBS patients suffered from the milder EBS-loc, 50% of the

JEB patients were diagnosed with the less severe JEB-nH loc, 46% of the DDEB patients had the less severe DDEB-pt and DDEB-ac, and only 20% of the RDEB patients suffered from the more severe RDEB-sev gen. This suggests that the HRQOL in our EB cohort might be overestimated.

The results of the HRQOL assessment in our EB population are in concordance with a recent publication of Tabolli *et al,* whom measured the HRQOL of the Italian EB population with different tools, including the Skindex-29 and SF-36.[15] In his study, it was concluded that EB has the greatest impact in patients with a higher perceived disease severity, with a larger skin involvement, with a higher psychological distress measured by the General Health Questionnaire-12, and in females.[15] The latter was also seen in our cohort, in which females have a lower HRQOL in both the functioning/physical as in the emotional/mental domains measured with the QOLEB, Skindex-29, and SF-36. Similar to our Skindex-29 and SF-36 results, Tabolli *et al*. showed that patients suffering from JEB and RDEB-sev gen have the lowest HRQOL, but that no statistically significant differences were seen among the various subtypes. This indicates that the Skindex-29 and SF-36 have a poorer discriminative value compared to the QOLEB. In our study population, a greater variation in the standard deviation was seen in the Skindex-29 and SF-36 compared to the QOLEB, which suggests a higher interpersonal variation per subtype in the first two questionnaires. Our comparison of the SF-36 values to the normal population are also in concordance with Tabolli *et al*., in which the PCS scale shows lower values compared to a normal population, while the values in the MCS scale are quite similar to that of the normal population: EB patients overall therefore seem to be as happy as normal individuals.[15]

Horn and Tidman assessed the HRQOL of EB patients living in Scotland using the dermatology-specific questionnaire Dermatology Life Quality Index (DLQI).[14] They showed that the impairment in HRQOL in patients with RDEB-sev gen exceeded those of any skin disorder previously assessed. The effects of EBS and other subtypes of DEB was similar to that of moderately severe psoriasis and eczema.[14] Comparing our SF-36 results with other dermatological diseases (Supplementary Appendix S3),[23-27] shows that for the PCS scale JEB and RDEB have one of the worst recorded HRQOL, only to be surpassed by psoriatic arthritis.[26] EBS has a HRQOL similar to atopic dermatitis, and DDEB similar to psoriasis.[26] In the MCS scale, DDEB and RDEB have the best recorded HRQOL, while EBS scores similar to occupational contact dermatitis, and JEB to psoriasis and hand eczema.[25-27] In the comparison of the Skindex-29 results with other dermatological diseases (Supplementary Appendix S4),[28-34] the effect of EBS is

comparable to psoriasis on the functioning and symptoms scale.[33] In these scales, RDEB and JEB have the greatest recorded impairments in HRQOL after Hailey-Hailey disease.[31] DDEB is only surpassed by Hailey-Hailey disease and pruritus on the symptoms scale.[30,33] In functioning, the effect of DDEB is comparable to cutaneous T-cell lymphoma.[29] In the emotions scale RDEB and EBS are similarly affected as urticaria, DDEB as connective tissue diseases, and JEB as psoriasis and lichen planus.[33] However, the accuracy of these comparisons might be limited due to the small patient numbers in our JEB and RDEB cohort.

In conclusion, we have shown that the Dutch version of the QOLEB is a reliable, valid, and brief instrument for the assessment of the HRQOL in adult EB patients. The responsiveness of the Dutch QOLEB has not been assessed, and future research should be performed to evaluate this. Also, future development and validation of a QOLEB for children would be of great value.

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Supplementary Appendix

Supplement 1. The quality of life in epidermolyis bullosa (QOLEB) questionnaire in the Dutch language.

Nederlandstalige QOLEB

Beantwoord de volgende vragen over de mate waarin EB van invloed is op uw dagelijks leven. Kies de optie die uw situatie het beste weergeeft. Geef aan het einde aan hoe lang u over het invullen van deze vragenlijst hebt gedaan.

1. Wordt uw bewegingsvrijheid in huis beïnvloed door EB?
   Helemaal niet
   Een beetje
   Heel erg
   Zeer ernstig

2. Hebt u bij het baden of douchen last van EB?
   Nee, geen last
   Ja, ik heb soms hulp nodig
   Ja, ik heb meestal hulp nodig
   Ja, ik heb altijd hulp nodig bij het baden/douchen

3. Hebt u last van lichamelijke pijn als gevolg van EB?
   Geen pijn
   Af en toe pijn
   Regelmatig pijn
   Constante pijn

4. In hoeverre hebt u last van EB als u schrijft?
   Geen last bij schrijven
   Ik vind het moeilijk om de pen vast te houden
   Ik vind typen gemakkelijker dan schrijven
   Ik kan niet schrijven als gevolg van EB

5. In hoeverre hebt u last van EB tijdens het eten?
   Niet, ik eet normaal
   Een beetje
   Heel erg
   Ik ben voor mijn voeding afhankelijk van mijn voedingssonde

6. In hoeverre hebt u last van EB tijdens het boodschappen doen?
   Helemaal niet
   Een beetje
   Heel erg
   Ik heb voortdurend hulp nodig

7. In hoeverre hebt u last van EB tijdens het sporten?
   Geen last
   Ik moet voorzichtig zijn bij het sporten
   Sommige sporten moet ik vermijden
   Ik moet alle sporten vermijden

8. In hoeverre ervaart u frustraties als gevolg van EB?
   Geen frustraties
   Een beetje gefrustreerd
   Heel erg gefrustreerd
   Zo gefrustreerd dat ik eigenlijk altijd boos ben

9. Wordt uw bewegingsvrijheid buitenshuis beïnvloed door EB?
   Helemaal niet
   Een beetje
   Heel erg
   Zeer ernstig

10. In hoeverre is EB van invloed op uw relatie met familieleden?
   Helemaal niet
   Een beetje
   Heel erg
   Zeer ernstig

11. In hoeverre brengen andere mensen u in verlegenheid over EB?
   Helemaal niet
   Een beetje
   Heel erg
   Zeer ernstig

12. Hebt u vanwege EB veel aanpassingen in uw huis moeten aanbrengen of moet u dit nog doen (installeren van hellingbanen, enz.)?
   Helemaal geen aanpassingen
   Een paar aanpassingen
   Veel aanpassingen
   Uitgebreide aanpassingen

13. In hoeverre is EB van invloed op uw relatie met vrienden?
   Helemaal niet
   Een beetje
   Heel erg
   Het vormt een ernstige belemmering in mijn sociale contacten

14. In hoeverre ervaart u bezorgdheid of angst als gevolg van EB?
   Helemaal niet
   Een beetje
   Heel erg
   Extreem veel

15. In hoeverre heeft EB gevolgen voor uw financiële situatie of die van uw familie?
   Geen financiële gevolgen
   Beperkte financiële gevolgen
   Veel financiële gevolgen
   Ernstige financiële gevolgen

16. In hoeverre bent u depressief als gevolg van EB?
   Helemaal niet depressief
   Een beetje depressief
   Heel erg depressief
   Voortdurend zeer depressief

17. In hoeverre zorgen anderen ervoor dat u zich ongemakkelijk voelt (bijvoorbeeld door pesten of staren) als gevolg van EB?
   Helemaal niet
   Een beetje
   Heel erg
   Zo erg dat ik geen sociaal leven heb

Hoe lang hebt u over het invullen van deze vragenlijst gedaan?
…….. minuten
Bedankt voor uw medewerking.

Supplement 2. Results of the 17 individual items of the QOLEB by epidermolysis bullosa (EB) subtype

Q		EBS n (%)	JEB n (%)	DDEB n (%)	RDEB n (%)	Total n (%)
	Total *n*	29	8	13	5	55
1.	Does your EB affect your ability to move around at home?					
	Not at all	16 (55)	3 (38)	7 (54)	2 (40)	28 (51)
	A little	12 (41)	3 (38)	6 (46)	2 (40)	23 (42)
	A lot	1 (3)	2 (25)	0 (0)	1 (20)	4 (7)
	Severely	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2.	Does your EB affect your ability to bath or shower?					
	No, no impact	27 (93)	6 (75)	10 (77)	2 (40)	45 (82)
	Yes, I sometimes need assistance	1 (3)	1 (13)	3 (23)	1 (20)	6 (11)
	Yes, need assistance most of the time	1 (3)	1 (13)	0 (0)	0 (0)	2 (4)
	Yes, I need assistance every time I bath/shower	0 (0)	0 (0)	0 (0)	2 (40)	2 (4)
3.	Does your EB cause you physical pain?					
	No pain	4 (14)	1 (13)	4 (31)	0 (0)	9 (16)
	Occasional pain	19 (66)	2 (25)	2 (15)	3 (60)	26 (47)
	Frequent pain	5 (17)	4 (50)	6 (46)	0 (0)	15 (27)
	Costant pain	1 (3)	1 (13)	1 (7)	2 (40)	5 (9)
4.	How does your EB affect your ability to write?					
	It does not interfere with writing	26 (90)	6 (75)	12 (92)	2 (40)	46 (84)
	I find it difficult to grip the pen	0 (0)	1 (13)	0 (0)	0 (0)	1 (2)
	I find it easier to type than write	3 (10)	1 (13)	1 (7)	3 (60)	8 (15)
	I cannot write due to my EB	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5.	Does your EB affect your ability to eat?					
	No, I eat normally	27 (93)	4 (50)	11 (85)	2 (40)	44 (80)
	A little	2 (7)	4 (50)	2 (15)	2 (40)	10 (18)
	A lot	0 (0)	0 (0)	0 (0)	1 (20)	1 (2)
	I rely on my gastrostomy tube for nutrition	0 (0)	0 (00	0 (0)	0 (0)	0 (0)
6.	Does your EB affect your ability to go shopping?					
	No, not at all	18 (62)	5 (63)	9 (69)	1 (20)	33 (60)
	A little	10 (34)	1 (13)	4 (31)	2 (40)	17 (31)
	A lot	1 (3)	2 (25)	0 (0)	0 (0)	3 (5)
	I need assistance all the time	0 (0)	0 (0)	0 (0)	2 (40)	2 (4)

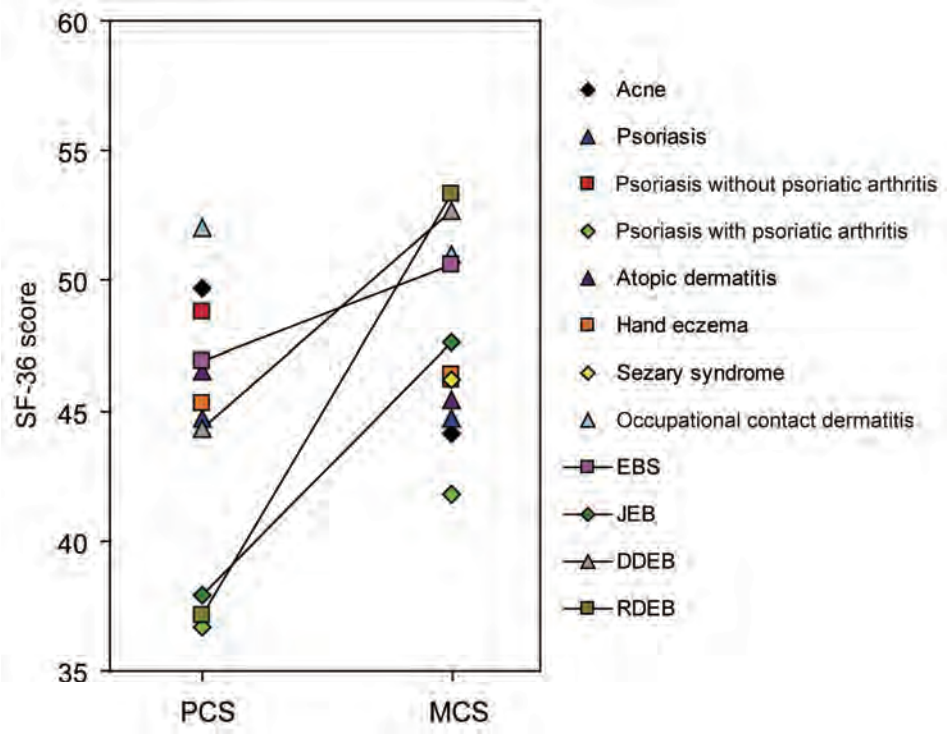
Supplement 2 continued

Q		EBS n (%)	JEB n (%)	DDEB n (%)	RDEB n (%)	Total n (%)
	Total *n*	29	8	13	5	55
7.	How does EB affect your involvement in sport?					
	No impact	2 (7)	0 (0)	1 (7)	0 (0)	3 (5)
	I need to be cautious in sports	8 (28)	0 (0)	8 (62)	2 (40)	18 (33)
	I need to avoid some sports	16 (55)	2 (25)	4 (31)	2 (40)	24 (44)
	I need to avoid all sports	3 (10)	6 (75)	0 (0)	1 (20)	10 (18)
8.	How frustrated do you feel about your EB?					
	No frustration	9 (31)	0 (0)	5 (38)	2 (40)	16 (29)
	A little	18 (62)	8 (100)	8 (62)	2 (40)	36 (65)
	A lot	1 (3)	0 (0)	0 (0)	1 (20)	2 (4)
	So frustrated that I am angry most of the time	1 (3)	0 (0)	0 (0)	0 (0)	1 (2)
9.	Does your EB affect your ability to move around outside of your home?					
	Not at all	4 (14)	0 (0)	6 (46)	1 (20)	11 (20)
	A little	21 (72)	5 (63)	7 (54)	2 (40)	35 (64)
	A lot	4 (14)	2 (25)	0 (0)	2 (40)	8 (15)
	Severely	0 (0)	1 (13)	0 (0)	0 (0)	1 (2)
10.	How does your EB affect your relationships with family members?					
	No impact at all	24 (83)	5 (63)	9 (69)	3 (60)	41 (75)
	A small impact	5 (17)	3 (38)	4 (31)	1 (20)	13 (24)
	A large impact	0 (0)	0 (0)	0 (0)	1 (20)	1 (2)
	A very large impact	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
11.	How embarrassed do people make you feel about your EB?					
	No embarrassment	24 (83)	2 (25)	7 (54)	2 (40)	35 (64)
	A little	4 (14)	6 (75)	5 (38)	3 (60)	18 (33)
	A lot	1 (3)	0 (0)	1 (7)	0 (0)	2 (4)
	Extremely	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
12.	Have you needed to, or do you need to modify your home (installing ramps etc.) due to your EB?					
	No, not at all	25 (86)	4 (50)	13 (100)	3 (60)	45 (82)
	A few	2 (7)	3 (38)	0 (0)	1 (20)	6 (11)
	A lot	2 (7)	1 (13)	0 (0)	1 (20)	4 (7)
	Extensive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

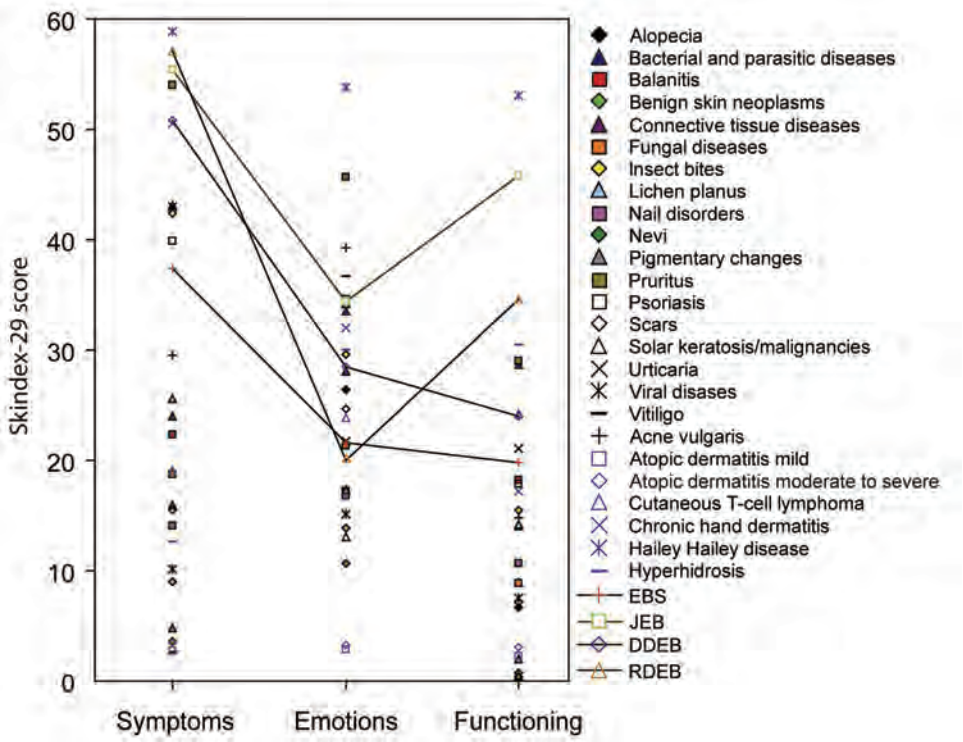
Supplement 2 continued

Q		EBS n (%)	JEB n (%)	DDEB n (%)	RDEB n (%)	Total n (%)
	Total *n*	29	8	13	5	55
13.	Does your EB affect your relationships with friends?					
	No, not at all	21 (72)	4 (50)	10 (77)	3 (60)	38 (69)
	A little	8 (28)	4 (50)	3 (23)	1 (20)	16 (29)
	A lot	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	It severely restricts my social interaction	0 (0)	0 (0)	0 (0)	1 (20)	1 (2)
14.	How worried or anxious do you feel because of your EB?					
	Not anxious at all	15 (52)	3 (38)	7 (54)	2 (40)	27 (49)
	A little	13 (45)	3 (38)	6 (46)	2 (40)	24 (44)
	A lot	1 (3)	2 (25)	0 (0)	1 (20)	4 (7)
	Extremely	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
15.	How are you or your family affected financially by your EB?					
	No financial impact	22 (76)	3 (38)	10 (77)	2 (40)	37 (67)
	Slightly affected	5 (17)	2 (25)	3 (23)	2 (40)	12 (22)
	Greatly affected	2 (7)	2 (25)	0 (0)	1 (20)	5 (9)
	Severely affected	0 (0)	1 (13)	0 (0)	0 (0)	1 (2)
16.	How depressed do you feel because of your EB?					
	Not depressed at all	23 (79)	3 (38)	10 (77)	2 (40)	38 (69)
	A little	6 (21)	5 (63)	3 (23)	3 (60)	17 (31)
	A lot	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Constantly very depressed	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
17.	How uncomfortable are you made to feel by others (e.g. teasing or staring) because of your EB?					
	Not at all	25 (86)	3 (38)	9 (69)	3 (60)	40 (73)
	A little	3 (10)	5 (63)	4 (31)	2 (40)	14 (25)
	A lot	1 (3)	0 (0)	0 (0)	0 (0)	1 (2)
	So much that I don't go out socially	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Q, question; EBS, EB simplex; JEB, junctional EB; DDEB, dominant dystrophic EB; RDEB, recessive dystrophic EB



Supplement 3. Scores of the different Short Form-36 subscales in epidermolysis bullosa (EB) compared to other dermatological diseases. Legend: PCS, physical component summary; MCS, mental component summary; EBS, EB simplex; JEB, junctional EB; DDEB, dominant dystrophic EB; RDEB, recessive dystrophic EB



Supplement 4. Scores of the different Skindex-29 subscales in epidermolysis bullosa (EB) compared to other dermatological diseases. Legend: EBS, EB simplex; JEB, junctional EB; DDEB, dominant dystrophic EB; RDEB, recessive dystrophic EB

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Discussion and future perspectives

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The Dutch junctional epidermolysis bullosa cohort

The Dutch Epidermolysis Bullosa Registry has documented all epidermolysis bullosa (EB) patients that have visited in person the single national EB referral center in Groningen since 1988. When the work on this thesis started, a total of 64 junctional EB (JEB) patients from 57 families were registered and had DNA available for testing (Table 1). Of them, 22 patients from 21 families suffered from JEB, type Herlitz (JEB-H), 27 patients from 22 families suffered from JEB, type non-Herlitz generalized (JEB-nH gen), nine patients from nine families suffered from JEB, type non-Herlitz localized (JEB-nH loc), four patients from four families suffered from JEB with pyloric atresia (JEB-PA), and two patients from one family suffered from JEB of late onset (JEB-lo). With molecular analysis, mutations were found in all JEB patients (Table 1). These mutations were all located in the six known JEB genes: *LAMA3*, *LAMB3*, *LAMC2, COL17A1, ITGB4*, and *ITGA6*.[1-6] Although this excluded the search for novel genes in our JEB cohort, some interesting observations were made regarding clinical and diagnostic features. Here, we present an overview of exceptional cases in our JEB cohort and we suggest some amendments to the International classification of inherited EB that was established by 18 leading authorities in 2007 at the Third international Consensus meeting on diagnosis and classification of EB in Vienna, Austria.[7]

Junctional epidermolysis bullosa, type Herlitz

In **chapter 2** the diagnostic features and mutational profile of all JEB-H patients (n=22) is discussed. As expected, most mutations were located in *LAMB3* (86%), and the rest in *LAMA3* (9%) and *LAMC2* (5%) (Table 1). The nonsense-, frameshift-, and splice site mutations that were found all led to a premature termination codon (PTC), resulting in most likely mRNA decay. In our cohort no apparent genotype-phenotype correlations (i.e. prognosis of lifespan) could be made. However, it is the question whether they are to be expected, as all mutations lead to the loss of LM-332. In the International classification of inherited EB no clear definition of JEB-H is given. In **chapter 2 and 7** we suggest that in the next classification the definition of JEB-H is stated as the complete absence of functional LM-332, and that the diagnosis is established with IF staining combined with molecular analysis.

Table 1. Classification and mutational profile of the Dutch junctional epidermolysis bullosa cohort

Nr	Patient	Diagnosis	Gene	Mutation 1	Consequence 1	Mutation 2	Consequence 2
1	007-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
2	008-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
3	021-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
4	061-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
5	081-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
6	091-01	JEB-H	*LAMB3*	c.786delG	p.K262NfsX134	c.786delG	p.K262NfsX134
7	107-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
8	124-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.3228+1G>T	Splice site
9	126-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.565-2A>G	Splice site
10	142-01	JEB-H	*LAMB3*	c.1978C>T	p.R660X	c.1978C>T	p.R660X
11	176-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
12	187-01	JEB-H	*LAMB3*	c.29-2A>G	Splice site	c.957ins77	p.N345MfsX77
13	188-01	JEB-H	*LAMB3*	c.1289-2_1296del10	Splice site	c.1289-2_1296del10	Splice site
14	200-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
15	206-01	JEB-H	*LAMB3*	c.957ins77	p.N345MfsX77	c.957ins77	p.N345MfsX77
16	212-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
17	238-01	JEB-H	*LAMB3*	c.1045C>T	p.R349X	c.1045C>T	p.R349X
18	263-01	JEB-H	*LAMB3*	c.1903C>T	p.R635X	c.1903C>T	p.R635X
19	269-01	JEB-H	*LAMB3*	c.3928A>T	p.K1310X	c.3928A>T	p.K1310X
20	155-01	JEB-H	*LAMA3*	c.3928A>T	p.K1310X	c.3928A>T	p.K1310X
21	155-02	JEB-H	*LAMA3*	c.1903C>T	p.R635X	c.1978C>T	p.R660X
22	231-01	JEB-H	*LAMC2*	c.31insC	p.L11PfsX43	c.1903C>T	p.R635X
23	011-01	JEB-nH gen	*COL17A1*	c.2237delG	p.G746AfsX53	c.2237delG	p.G746AfsX53
24	025-01	JEB-nH gen	*COL17A1*	c.2237delG	p.G746AfsX53	c.2237delG	p.G746AfsX53

Table 1 continued

Nr	Patient	Diagnosis	Gene	Mutation 1	Consequence 1	Mutation 2	Consequence 2
25	026-01	JEB-nH gen	*COL17A1*	c.1601delA	p.D534AfsX19	c3676C>T	p.R1226X
26	035-01	JEB-nH gen	*COL17A1*	c.2237delG	p.G746AfsX53	c.3676C>T	p.R1226X
27	035-02	JEB-nH gen	*COL17A1*	c.2237delG	p.G746AfsX53	c.3676C>T	p.R1226X
28	084-01	JEB-nH gen	*COL17A1*	c.1179delA	p.A394LfsX9	c.3327delT	p.P1110RfsX21
29	093-01	JEB-nH gen	*COL17A1*	c.3676C>T	p.R1226X	c.4320insC	p.G1441RfsX14
30	117-01	JEB-nH gen	*COL17A1*	c.3131delC	p.P1044QfsX22	c.3131delC	p.P1044QfsX22
31	134-01	JEB-nH gen	*COL17A1*	c.1260delC	p.T421LfsX72	c.3495-3496delCT	p.Ser1166LfsX6
32	208-01	JEB-nH gen	*COL17A1*	c.2237delG	p.G746AfsX53	c.2237delG	p.G746AfsX53
33	248-01	JEB-nH gen	*COL17A1*	c.2576insC	p.G860RfsX105	c.3827insC	p.G1277TrpfsX16
34	266-01	JEB-nH gen	*COL17A1*	c.1259delC	p.T421LfsX72	c.3327delT	p.P1110RfsX21
35	029-01	JEB-nH gen	*LAMB3*	c.628G>A	Splice site	c.628G>A	Splice site
36	029-02	JEB-nH gen	*LAMB3*	c.628G>A	Splice site	c.628G>A	Splice site
37	078-01	JEB-nH gen	*LAMB3*	c.628G>A	Splice site)	c.1903C>T	p.R635X
38	119-01	JEB-nH gen	*LAMB3*	c.31dupC	p.L11PfsX43	c.628G>A	Splice site
39	216-01	JEB-nH gen	*LAMB3*	c.1186_1196del11	p.T396CfsX12	c.1186_1196del11	p.T396CfsX12
40	254-01	JEB-nH gen	*LAMB3*	c.628G>A	Splice site	c.1903C>T	p.R635X
41	299-01	JEB-nH gen	*LAMB3*	c.1106dupG	p.A370SfsX13	c.1106dupG	p.A370SfsX13
42	012-01	JEB-nH gen	*LAMA3*	c.4816-2A>G	skip of exon 36 out of frame	c.4816-2A>G	skip of exon 36 out of frame
43	048-01	JEB-nH gen	*LAMA3*	c.4816-2A>G	skip of exon 36 out of frame	c.4816-2A>G	skip of exon 36 out of frame
44	050-01	JEB-nH gen	*LAMA3*	c.488delG	p.G163DfsX30	c.4484C>T	p.A1495V
45	050-02	JEB-nH gen	*LAMA3*	c.488delG	p.G163DfsX30	c.4484C>T	p.A1495V
46	050-04	JEB-nH gen	*LAMA3*	c.488delG	p.G163DfsX30	c.4484C>T	p.A1495V
47	050-05	JEB-nH gen	*LAMA3*	c.488delG	p.G163DfsX30	c.4484C>T	p.A1495V
48	169-01	JEB-nH gen	*LAMA3*	c.3376C>T	p.R1126X	c.4484C>T	p.A1495V

Table 1 continued

Nr	Patient	Diagnosis	Gene	Mutation 1	Consequence 1	Mutation 2	Consequence 2
49	112-01	JEB-nH gen	*ITGB4*	c.600delC	p.F201SfsX9	c.3040C>T	p.R1014W
50	086-01	JEB-nH loc	*COL17A1*	c.1772-2A>C	skip of exon 22 in frame	c.3327delT	p.P1110RfsX21
51	098-01	JEB-nH loc	*COL17A1*	c.2251C>T	p.Q751X	c.3327delT	p.P1110RfsX21
52	168-01	JEB-nH loc	*COL17A1*	c.4320delT	p.Q1442KfsX70	c.4320delT	p.Q1442KfsX70
53	224-01	JEB-nH loc	*COL17A1*	c.1826G>A	p.G609D	c.2362G>C	p.G788R
54	132-01	JEB-nH loc	*LAMB3*	c.1063T>C	p.C355R	c.1903C>T	p.R635X
55	043-01	JEB-nH loc	*LAMA3*	1076_1077insA	p.E360RfsX4	?	?
56	074-01	JEB-nH loc	*LAMA3*	c.3959T>G	p.L1320X	?	?
57	173-01	JEB-nH loc	*LAMA3*	c.786G>T	p.R262S	c.4484C>T	p.A1495V
58	113-01	JEB-nH loc	*ITGB4*	c.4975G>T	p.E1659X	c.4975G>T	p.E1659X
59	033-01	JEB-PA	*ITGB4*	c.600delC	p.F201SerfsX9	?	?
60	127-01	JEB-PA	*ITGB4*	c.567-2A>G	skip of exon 7 out of frame	?	?
61	129-01	JEB-PA	*ITGB4*	c.113G>T	p.C38F	c.2596C>T	p.Q866X
62	147-01	JEB-PA	*ITGA6*	c.291delC	p.I97MfsX33	c.291delC	p.I97MfsX33
63	059-01	JEB-lo	*COL17A1*	c.1992_1995delGGGT	p.G665fsX16	c.3908G>A	p.R1303Q
64	059-02	JEB-lo	*COL17A1*	c.1992_1995delGGGT	p.G665fsX16	c.3908G>A	p.R1303Q

JEB-H, junctional epidermolysis bullosa, type Herlitz; JEB-nH gen, junctional epidermolysis bullosa, type non-Herlitz generalized; JEB-nH loc, junctional epidermolysis bullosa, type non-Herlitz localized; JEB-PA, junctional epidermolysis bullosa with pyloric atresia; JEB-lo, junctional epidermolysis bullosa of late onset

Junctional epidermolysis bullosa, type non-Herlitz

Junctional epidermolysis bullosa, type non-Herlitz (JEB-nH) is mostly caused by pathogenic *COL17A1*, *LAMB3, LAMC2*, and *LAMA3* mutations.[7] In our JEB-nH cohort not only mutations in these genes were found, but in 6.5% (n=2) of the families a pathogenic mutation in *ITGB4* was shown **(chapter 5)**. An interesting question is the possible phenotype-genotype correlation in *ITGB4* mutations, especially in predicting JEB-PA or JEB-nH. We could not detect any clear-cut correlations; previous efforts were also not conclusive, and it was suggested that the occurrence of pyloric atresia in EB-PA is influenced by environmental factors.[8] We suggest including *ITGB4* as a pathogenic gene for localized and generalized JEB-nH in the next International classification of inherited EB. In cold case JEB-nH patients carrying wild-type *LAMB3, LAMA3, LAMC2*, and *COL17A1* genes, *ITGB4* should be screened for pathogenic mutations. However, for new JEB-nH patients, IF staining for integrin β4 (β4) can designate *ITGB4* as the first candidate gene, as in all cases a reduced β4 staining was seen. The gene profile in our JEB-nH cohort is now completed; however this does not exclude other pathogenic genes and another candidate gene could be *ITGA6*.

According to the classification of inherited EB, JEB-nH loc is only related to mutations in *COL17A1*.[7] However, in our cohort only 44% (4 out of 9) of the JEB-nH loc cases are caused by *COL17A1* mutations (Table 1).[9] The remaining patients carry mutations in *LAMA3* (n=3), *ITGB4* (n=1), and *LAMB3* (n=1). We suggest that the genes coding for LM-332 and for β4 are included as pathogenic genes for JEB-nH loc in the next International classification of inherited EB.

A total absence of Col17 caused by *COL17A1* nonsense mutations, out-of-frame deletions or insertions, and deleterious glycine substitution missense mutations cause generalized JEB-nH.[9-12] In contrast, localized JEB-nH is caused by a reduction of Col17 due to *COL17A1* missense mutations or small in-frame deletions or insertions.[9,10,13,14] For LM-332 deficient JEB-nH, the distinction between JEB-nH gen and JEB-nH loc remains more obscure. The amount of GB3 staining in these patients overlaps each other (Table 2). Of the fourteen LM-332 deficient JEB-nH gen patients, four patients (29%) had strongly reduced, four patients (29%) had reduced, two patients (14%) had slightly reduced, three patients (21%) had normal, and one patient (7%) had an unknown GB3 staining. Compared to the four LM-332 deficient JEB-nH loc patients, three patients (75%) had normal, and one patient (25%) had slightly reduced GB3 staining. Also, there seem to be no clear-cut phenotype-genotype correlations between the LM-332 deficient JEB-nH gen and JEB-nH loc population. Complicating the latter are JEB-nH loc patients

074-01 and 0431-01 who carry a single heterozygous *LAMA3* mutation. In these patients mutations in *LAMB3* and *LAMC2* were excluded. We hypothesize that they carry a large deletion or insertion in *LAMA3* that could not be detected with sequencing analysis.

In **chapter 9** we describe two novel *LAMA3* mutations in a family with JEB-nH gen (family 050). The heterozygous carriers of the mutation c.488delG showed enamel defects without any signs of skin fragility. We conclude that these enamel defects are caused by haploinsufficiency of LM-332; however the exact pathogenesis remains unclear. Further research should be performed to identify why these enamel defects occur, and why they do not occur in all carriers of LM-332 null mutations.

Table 2. Immunofluorescence antigen staining in laminin-332 deficient junctional epidermolysis bullosa, type non-Herlitz patients

Nr	Patient	Diagnosis	Gene	GB3 staining
1	029-01	JEB-nH gen	*LAMB3*	±
2	029-02	JEB-nH gen	*LAMB3*	+
3	078-01	JEB-nH gen	*LAMB3*	±
4	119-01	JEB-nH gen	*LAMB3*	±
5	216-01	JEB-nH gen	*LAMB3*	±
6	254-01	JEB-nH gen	*LAMB3*	+
7	299-01	JEB-nH gen	*LAMB3*	+++
8	012-01	JEB-nH gen	*LAMA3*	+++
9	048-01	JEB-nH gen	*LAMA3*	+
10	050-01	JEB-nH gen	*LAMA3*	+
11	050-02	JEB-nH gen	*LAMA3*	++
12	050-04	JEB-nH gen	*LAMA3*	NA
13	050-05	JEB-nH gen	*LAMA3*	++
14	169-01	JEB-nH gen	*LAMA3*	+++
15	132-01	JEB-nH loc	*LAMB3*	+++
16	043-01	JEB-nH loc	*LAMA3*	+++
17	074-01	JEB-nH loc	*LAMA3*	++
18	173-01	JEB-nH loc	*LAMA3*	+++

JEB-nH gen, junctional epidermolysis bullosa, type non-Herlitz generalized; JEB-nH loc, junctional epidermolysis bullosa, type non-Herlitz localized; NA, not available; Staining intensity was compared to controls and scored as normal (+++), slightly reduced (++), reduced (+), strongly reduced (±) or absent (-); GB3 is the antibody staining against LM-332

JEB with pyloric atresia

Four JEB-PA patients have been included in the Dutch JEB cohort (Table 1). A fifth JEB-PA patient is excluded, as no DNA was available for testing. Pyloric atresia was not surgically corrected, and the patients died an average of five days after birth (range 4-7 days). As expected from the literature, most patients suffered from mutations in *ITGB4* (n=3; 75%), while only one patient had mutations in *ITGA6* (n=1; 25%).[15] With IF staining, we saw a total absence of β4 staining with a reduced α6 staining in two patients carrying *ITGB4* mutations. The other patient with *ITGB4* mutations had a 90% total absence of β4 staining and a strongly reduced α6 staining. The patient affected with *ITGA6* mutations showed a total absence of both the β4 as α6 staining.

In two JEB-PA patients only a single *ITGB4* mutation could be found (Table 1). Most probably they carried a large deletion or insertion in *ITGB4* that could not be detected by our sequencing analysis. Digenec inheritance with *ITGA6* was excluded, as was autosomal dominant inheritance. Two JEB-PA patients carried novel mutations: (1) patient 129-01 carried the novel *ITGB4* missense mutation c.113G>T in exon 3 leading to the substitution C38F, together with the known mutation c.2596C>T in exon 22 that leads to a frameshift and a PTC, and (2) patient 147-01 carried the novel *ITGA6* mutation c.291delC in exon 2 homozygously, leading to a frameshift and a PTC. The genotype-phenotype correlation between lethal and non-lethal JEB-PA is not completely elucidated, and it is hypothesized that the type of mutations (nonsense versus missense) as well as the specific sites in which the mutations reside are associated in this correlation.[8]

JEB of late onset

In **chapter 10** we reveal that JEB-lo is caused by *COL17A1* mutations. We hypothesize that p.R1303Q and other missense mutations in the NC4 domain of Col17 are specific for JEB-lo; in a previous study, a patient carrying the mutation p.R1303Q homozygously showed clinical symptoms matching JEB-lo.[14] Unfortunately, no other missense mutations in the NC4 domain have been described. Molecular analysis of other JEB-lo patients should be performed, to test this hypothesis and to possibly reveal other genes involved in JEB-lo. Some questions regarding the pathogenesis of specific clinical features in JEB-lo still remain, such as why clinical symptoms start at a later age, and why dermatoglyphs are lost.

The role of immunofluorescence antigen mapping in the diagnosis of junctional epidermolysis bullosa

Transmission electron microscopy (TEM) was the first laboratory test available for the diagnosis of EB. Besides distinguishing the cleavage level seen in blisters, it can show ultrastructural changes specific for some EB subtypes.[16] With the advent of IF antigen mapping, a faster and cheaper method became available for the primary diagnosis of EB.[17] Mapping a blister with IF by staining with a series of antibodies provides a major cleavage level, and thus a major EB subtype. Furthermore, a reduced, absent or abnormal staining pattern of non-lesional skin can appoint an affected protein and candidate gene. IF antigen mapping seems to be the superior diagnostic tool over TEM. Yiasemides *et al*. compared TEM with IF antigen mapping in a prospective cohort of 33 suspected EB cases. IF antigen mapping was both more sensitive (97% vs. 71%) as more specific (100% vs. 81%) than TEM in diagnosing EB. Furthermore, appointing a major EB subtype to these cases was more sensitive with IF staining (97%) compared to TEM (80%). More specifically, for appointing JEB as the major EB subtype, IF had a specificity of 100% and a sensitivity of 90%.[18] The question remains whether IF staining can provide a classification to a specific JEB subtype, a prognosis, and a candidate gene.

A total absent staining of laminin-332 with antibody GB3 is concurrent with the diagnosis JEB-H.[17,19] However, in a few JEB-H cases a strongly reduced GB3 straining was seen **(chapter 2)**, a staining pattern also seen in JEB-nH gen cases (Table 2). In these cases, further molecular analysis, multiple biopsies, and cDNA analysis should determine the definite diagnosis. In **chapter 3** we have shown that JEB-H patients with a strongly reduced GB3 staining have a 2-fold higher average life expectancy, compared to patients who have absent GB3 staining (10.6 vs. 5.3 months). This difference was not statistically significant (p=0.08), although this could be explained by the small amount of patients included in the strongly reduced GB3 staining group (n=2). Larger JEB-H cohorts are needed to obtain statistical power for prognostic factors in this group.

For LM-332 deficient JEB-nH patients, there is no distinct correlation seen between the amount of GB3 staining and the occurrence of the milder JEB-nH loc and the more severe JEB-nH gen (Table 2). Although a (strongly) reduced GB3 staining is only seen in JEB-nH gen, a slightly reduced and normal GB3 staining can both be seen in JEB-nH gen as in JEB-nH loc. Thirty-three percent of the JEB-nH cases showed a normal GB3 staining, with the consequence that no candidate gene will be found in these cases.

Staining for Col17 shows an abnormal staining pattern in carriers of a *COL17A1* null mutation, JEB-lo patients, JEB-nH loc patients, and JEB-nH gen patients compared to control.[9] In **chapter 11** we show that Col17 staining can be used to distinguish JEB-nH gen from JEB-nH loc cases. Furthermore, in **chapter 10** we show that subtle changes in Col17 staining, can appoint *COL17A1* as the candidate gene for JEB-lo.

In all our JEB-PA patients an abnormal α6 and β4 staining was seen, indicating the candidate protein integrin α6β4. Also, *ITGB4*-associated JEB-nH cases show an abnormal β4 staining compared to normal, making IF a useful diagnostic tool in appointing *ITGB4* as the candidate gene in JEB-nH patients **(chapter 5)**. As a prognostic factor, it has been postulated that a total absence of either α6 or β4 staining is concurrent with lethal JEB-PA, and a reduced staining with non-lethal JEB-PA. However, no significant differences were seen in the correlation between the amount of α6 and β4 staining and the survival of 26 JEB-PA cases reported in the literature.[20] Most patients with a total absence of either the α6 or β4 staining did not survive, although there was an exception to this rule. A reduced α6 or β4 staining is not conclusive in JEB-PA, as it concurs with both lethal as non-lethal cases.[20]

IF staining was able to appoint candidate genes in all our JEB patients, except in 33% of the LM-332 deficient JEB-nH patients. In IF staining not only abnormalities in the affected protein can be seen; abnormalities can also be present in other interconnecting JEB proteins. In these cases, the most suspected protein with the most evident IF changes should be appointed as the candidate protein. Mapping of the blister level with IF can also provide diagnostic clues; a low junctional split, characterized by LM-332 both in the blister floor and roof, is suspicious for LM-332 involvement, while a split high in the lamina lucida, characterized by LM-332 only present on the blister floor, suspects involvement of hemidesmosomal proteins. If TEM and IF blister mapping shows a low junctional split, and IF staining of non-lesional skin shows no deviations, the genes coding for LM-332 should be the first candidate genes. IF staining is not adequate in distinguishing the mutant polypeptide subunit in multimeric proteins, such as in LM-332 and α6β4. In these cases, multistep testing should be performed in the order of likelihood. For LM-332, *LAMB3* should be first tested, followed by *LAMC2* or *LAMA3*. In the case of α6β4, *ITGB4* should be tested before *ITGA6*.[21] The use of IF antigen staining as a prognostic factor and to classify specific JEB subtypes, is mainly possible for Col17-related EB cases. Apart from some exceptions, such as a total absence of LM-332 in JEB-H, no firm correlation exists between the LM-332 and α6β4 staining with a certain prognosis or specific JEB subtype.

In conclusion, IF staining is an important diagnostic tool for EB. In diagnosing JEB, a broad series of antibodies should be tested and we propose the use of at least the following antibodies: GB3 against LM-332, VK against Col17 endodomain, 58XB4 against β4 ectodomain, 450-11A against β4 endodomain, GOH3 against α6 ectodomain, and 1A10 against α6 endodomain.

Concluding remarks

Studying our JEB cohort has brought us new insights into this disease. It has become clear that the most accurate diagnosis is made in combining IF staining with mutation analysis. Combining our findings in the Dutch JEB cohort, together with the International classification of inherited EB, we propose a JEB classification as summarized in Table 3. However, many questions regarding JEB still remain, especially concerning the genotype-phenotype correlations that are discussed above. Recently, two dynamic online databases were launched comprising the phenotypic information of dystrophic epidermolysis bullosa (DEB) patients linked with their respective *COL7A1* mutations.[22,23] One of the major aims of these databases is to provide help in diagnosing and genetic counseling of future DEB patients.[22] Creating a similar database for all JEB-related genes in the future will help us to better understand the genotype-phenotype correlations.

Table 3. Clinical and diagnostic features of junctional epidermolysis bullosa subtypes

Subtype	Onset	Clinical features	Gene	Inheri-tance	IF	TEM
JEB, Herlitz	Birth	Lethal in childhood; extensive generalized blistering; dyspnea; anemia; failure to thrive; nail abnormalities; enamel hypoplasia	*LAMB3 / LAMC2 / LAMA3*	AR	LM-332 absent or strongly reduced	Lamina lucida split; HD reduced number and hypoplastic; absent subbasal dense plates
JEB, non-Herlitz, generalized	Birth	Generalized blistering; enamel hypoplasia; nail abnormalities; primary hair (incomplete) alopecia; secondary hair absent; atrophic scarring; corneal blistering.	*COL17A1 / LAMB3 / LAMC2 / LAMA3 / ITGB4*	AR	Col17 absent; LM-332 reduced or normal; β4 reduced	Lamina lucida split; HD normal or HD reduced number and hypoplastic
JEB, non-Herlitz, localized	Birth	Blistering hands and feet; enamel hypoplasia; nail abnormalities; normal primary hair; sparse secondary hair	*COL17A1 / LAMB3 / LAMA3 / ITGB4 / (LAMC2?)*	AR	Col17 reduced; LM-332 reduced or normal; β4 reduced	Lamina lucida split; HD normal or HD reduced number and hypoplastic
JEB with pyloric atresia	Birth	Frequently lethal; pyloric atresia; aplasia cutis; enamel hypoplasia; nail abnormalities; urinary tract stenosis	*ITGB4 / ITGA6*	AR	α6β4 absent or reduced	HD reduced number and hypoplastic
JEB, inversa	Birth	Blistering intertriginous areas; atrophic scarring; enamel hypoplasia; dystrophic nails	*LAMB3? / LAMC2? / LAMA3?*	AR	?	Junctional split
JEB, late onset	Childhood – young adulthood	Blistering hands and feet; hyperhidrosis; loss of dermatoglyphs; skin atrophy; enamel hypoplasia; nail abnormalities	*COL17A1*	AR	Col17 staining normal in intensity but altered distribution	Electron dense cloudy broadening of (sub)lamina densa (zone).
LOC syndrome	Birth	Hoarse cry after birth; chronic mucosal, laryngeal and ocular granulation tissue; enamel hypoplasia	*LAMA3*	AR	LM-332 stains in normal intensity and normal distribution	Normal number of HD; Some HD plaques small lacking normal subbasal dense plates; reduced number anchoring filaments

JEB, junctional epidermolysis bullosa; LOC, laryncho-onycho-cutaneous; AR, autosomal recessive; HD, hemidesmosome; IF, immunofluorescence antigen staining; LM-332, laminin-332; Col17, type XVII collagen; β4, integrin β4; α6β4, integrin α6β4; TEM, transmission electron microscopy;

Laminin-332 in wound healing and carcinogenesis

The most characteristic feature of LM-332 deficiency is skin fragility, due to the loss of its adhesive role in hemidesmosome-adhesion complexes. Paradoxically, LM-332 also has an important role in the migration of keratinocytes, making it a significant contributor in wound healing and carcinogenesis. In **chapter 8**, JEB-nH patients with chronic ulcers are treated with punch grafting. The patients suffered from an impaired wound healing due to LM-332 deficiency, mostly caused by mutations in *LAMA3*. In the process of wound healing, cell motility is desirable for wound closure. Basal keratinocytes secrete unprocessed LM-332 around the wound edge. In unprocessed form, the LG3 domain of the laminin α3 chain favors interaction with integrin α3β1 (α3β1). This interaction stimulates the mitogen activated protein (MAP) kinase signaling cascade to promote epithelial cell migration and proliferation. Upon wound closure the LG4-5 domain of the laminin α3 chain is cleaved by plasmin. This changes the favored interaction of LM-332 with α3β1 to an interaction with α6β4, after which hemidesmosomes are formed.[24-32] LM-332-α3β1 interaction enhances plasmin production, making the process self-regulatory with an inhibitory feedback loop.[24,33] Wound healing shares similarities with carcinogenesis, as both require cell proliferation and migration. The role of LM-332 seems to be more complicated in carcinogenesis. Cell migration and invasion are important stages in tumor progression and establishing metastasis. The basement membrane zone (BMZ) has traditionally been viewed as a protective barrier, and its proteolytic degradation by metalloproteinases merely as a way to facilitate tumor spread.[34] This view proves to be wrong, as in a great diversity of cancers expression of LM-332 is elevated and is considered as a diagnostic factor related to tumor invasiveness.[35-38] Especially the γ2 chain, or its proteolytic fragment, are overexpressed in the vicinity of the tumor front in many cancers, and this has been related to tumor invasiveness.[38,39] It is shown that the γ2 chain can be cleaved between domain III and IV by MT1-MMP (matrix metalloproteinase), MMP-2,-3,-8,-13,-14,-20, and BMP (human bone morphogenetic protein)-I, II, and II, inducing epithelial cell migration.[40] Furthermore, domain III of the laminin γ2 chain harbors EGF-domains that can directly interact with epidermal growth factor (EGF) receptors, inducing cell migration.[34,38,40,41] It remains unknown whether LM-332 processing or interaction with other proteins is necessary for the laminin γ2 chain to activate EGF receptors.[34] Another question is whether tumors excrete the laminin γ2 chain in monomeric form.

In prostate carcinoma cells it is shown that the laminin β3 chain is cleaved by MT1-MMP and that this causes cell migration in vitro.[42] Also hepsin protease, which is over expressed in 90% of the prostate tumors, cleaves the laminin β3 chain, leading to increased migration.[40,43] Domain III-V of the laminin β3 chain is essential for the development of squamous cell carcinoma.[34,40] This domain interacts with the NC1 domain of Col7 and promotes carcinogenesis via activation of the phosphatidylinositol-3 kinase (PI3K) pathway, leading to protection from apoptosis and increased tumor invasion.[44]

The cleaved LG4-5 domain of the laminin α3 chain, that is not detectable in mature tissue, is highly expressed in mainly the leading edges of carcinomas, where it stimulates the PI3K pathway, MMP activity, tumor invasion, and tumor growth.[40,45] Also, TGFβ-1 enhances cell migration by promoting interaction of the LG4-5 domain with heparin sulfate proteoglycan.[41] Furthermore, the LG3 domain of the laminin α3 chain interacts with α3β1 and thereby activating several signaling pathways: MAP kinase, PAK1, RAC1, and GTPase RhoA, leading to cancer cell proliferation, motility, and lamellipodia.[34,38] The interaction of the LG1-2 domain of the laminin α3 chain with α6β4 leads to phosphorylisation of receptor tyrosine kinases, such as ErbB2, EGF receptors, and Met, which activates the RAS pathway leading to cell proliferation and migration.[34,38]

Considering the importance of (increased) expression of LM-332 in cell migration and carcinogenesis, it seems paradoxical that LM-332 deficient JEB-nH patients have an increased risk of developing invasive metastatic squamous cell carcinoma **(chapter 6)**, whereas they also suffer from an impaired wound healing **(chapter 8)**. Further research in the tumor micro-environment of LM-332 deficient JEB-nH keratinocytes should be performed to gain more clarity on this subject.

Herlitz junctional epidermolysis bullosa: from care to cure

Chapters 3 and 4 focus on the care for JEB-H patients and their parents. The most important message is that comfort care should be the mainstay of treatment in these patients, as JEB-H is a lethal disease with no cure available at this moment. However, in the last few years much progress has been made towards finding a cure for EB. Although most research in this field focuses on recessive dystrophic EB (RDEB) and many hurdles still have to be taken, our hope is that a cure for JEB-H will become

available in the future. Here we discuss possible treatment options in protein replacement, gene, and cell therapy. [46]

Protein replacement therapy

With protein replacement therapy wild-type protein is administrated to the skin by topical application or local injection.[46] Culturing JEB keratinocytes in a LM-332 enriched extracellular matrix makes adhesion of these keratinocytes possible.[47] Igoucheva *et al*. has ex vivo transfected recombinant β3 polypeptide into the endoplasmic reticulum of β3 null keratinocytes, which led to restoration of LM-332 assembly, secretion and deposition in the BMZ.[48] The proof-of-principle of LM-332 replacement has been established; however some questions arise about its use in JEB-H.

In JEB-H not only the skin and mucous membranes are affected. Extracutaneous symptoms, such as blistering in the gastrointestinal- and respiratory tract are deleterious and contribute to failure to thrive and respiratory failure, which are the most common causes of death in these patients **(chapter 3)**. Local administration of LM-332 will only treat cutaneous symptoms. Future research should focus on the effects and side effects of systemic infusion of LM-332. Research is also needed in assessing the duration of the therapeutic effect, which will depend on the stability and turn-over rate of recombinant LM-332 in the BMZ.[48] For DEB, replacement of Col7 has been performed in various murine models, which resulted in the formation of anchoring fibrils and a conversion of the DEB phenotype. The incorporation of Col7 remained stable for 2-3 months.[49,50] Collagens are known as stable long-lived and persistent proteins that have a slow turn-over time and a long half-life.[50] The half-life for LM-332 has not been identified yet, but it will determine the treatment frequency and thereby the clinical feasibility of the therapy.[46,48]

Gene therapy

In gene therapy a wild-type gene is transferred to cells carrying mutations in that gene. Most research has focused on DEB, characterized by reduction or absence of Col7 (gene: *COL7A1*). DEB keratinocytes or fibroblasts transfected with the *COL7A1* gene showed synthesis and secretion of Col7 in culture, and transplantation of cultured epidermis onto immunodeficient mice showed the formation of anchoring fibrils.[51-54] LM-332 is only produced by keratinocytes and since epidermal cells have a turnover of 3-4 weeks, epidermal stem cells should be treated to obtain a long-term sustainable therapeutic effect.[55] In 1998, Dellambra *et al*. reported the first successful retroviral

transduction of *LAMB3* in human laminin β3-null keratinocytes. Epidermal stem cells were treated and the laminin β3-transduced keratinocytes were able to synthesize, assemble and secrete LM-332, restore keratinocyte adhesion, and assemble hemidesmosomes ex vivo.[56] Robbins *et al*. cultured *LAMB3* transduced human LM-332 null keratinocytes. Epidermis was generated ex vivo and transplanted on immunodeficient mice. This resulted in phenotypic normal skin with sustained laminin β3 expression and the formation of hemidesmosomes.[57] In 2006, the first laminin-β3 deficient JEB-nH patient was treated with ex vivo gene therapy. Epidermal stem cells of the patient were retrovirally transduced with *LAMB3* cDNA, and used to culture epidermal grafts. Nine grafts were transplanted onto surgically prepared non-healing lesions on the patient's legs. For over one year the epidermis remained intact with a normal dermal-epidermal junction, vector-derived laminin β3 transcripts were present in virtually all cells of the regenerated epidermis, and IF antigen mapping showed laminin β3 staining as control.[58]

With in vivo gene therapy, vectors carrying wild-type cDNA are directly applied to the therapeutic site. This method omits the need of culturing epidermis, and is therefore less time-consuming and laborious. The first in vivo gene therapy for EB in mammals was performed by Woodley *et al*., who intradermally injected a self-inactivating lentiviral vector expressing *COL7A1* in human DEB skin that was grafted on immunodeficient mice. Transduction of dermal cells was accomplished and this led to production of Col7 and formation of anchoring fibrils for up to three months.[59] A disadvantage of in vivo gene therapy is its inability to specifically target epidermal stem cells. To overcome this problem, Endo *et al*. has performed intra-amniotic in vivo gene therapy in a murine JEB-H model with a lentiviral vector expressing *LAMB3* under control of a human keratin 5 promoter. To reach epidermal stem cells located in the basal layer, transduction should be performed in a period of the fetal development in which the epidermis consists of one layer. In the murine model this was 8 days after conception. In the human fetal development, this corresponds with the 21-55th day after conception. Unfortunately, at such early stage prenatal diagnosis is not yet available. The treatment did restore LM-332 expression as showed by IF staining, and improved the skin phenotype in 12% of the treated mice. However, the treatment did not affect the survival of the mice.[60]

One of the main problems associated with gene therapy are safety issues concerning the vectors used. Retroviral vectors are inserted uncontrolled into the human genome, with a high chance of deregulating neighboring genes.[61] Moreover, these vectors have a high tendency to target growth-controlling genes and proto-oncogenes.[62]

Occurrence of hematological malignancies has been reported in X-linked severe combined immunodeficiency patients treated with gene therapy.[63] Furthermore, potentially dangerous clonal imbalance was seen in patients treated for chronic granulomatous disease.[64] Another concern associated with vectors is over expression of the transfected gene. In the case of *LAMB3*, not only the basal cell layer, but all epidermal cell layers will produce laminin β3. Although laminin β3 is normally not excreted in monomeric form, an excess of intracellular laminin β3 chains has occasionally been observed in suprabasal cells. To overcome these safety and specificity issues, self-inactivating vectors that are under the control of a promoter for a gene specific for the basal cell layer have been produced. DiNunzio *et al*. developed a self-inactivating lentiviral vector expressing *LAMB3* under the control of the *K14* gene.[65] Also, non-viral gene delivery with ΦC31 bacteriophage integrase, transposons, or zinc-finger nucleases seem to be promising safer techniques.[66,67]

Another strategy in gene therapy is targeting RNA. In 2001, Gache *et al*. reported about the amelioration of disease in a patient with JEB-H. This patient carried heterozygous mutations in *LAMB3* leading to nonsense mediated decay: (1) c.1587delAG in exon 13 leading to a frameshift and PTC in exon 14, and (2) c.1903C>T in exon 14 leading to the nonsense mutation R635X. Over time, mutation c.1587delAG showed illegitimate skipping of exon 14 and thereby restoring the open reading frame.[68] In the patient described by Gache *et al*., it was shown that with this truncated laminin β3 chain, lacking 127 amino acids, the patient was able to synthesize, assemble, and secrete LM-332 that could form well-structured hemidesmosomes. Over time, symptoms reduced and at age 14 this patient suffered from sporadic blistering and was in good general health.[68] With antisense oligonucleotides (AONs), exon skipping can be induced to restore a disrupted reading frame as demonstrated in Duchenne muscular dystrophy.[69,70] As 74% of the JEB-H patients in the Netherlands is affected with a nonsense mutation in exon 14 of *LAMB3* (c.1903C>T; p.R635X) in at least one allele **(chapter 2)**, developing an AON around this exon could prove to be beneficial for many JEB-H patients. Skipping of exon 14 results in an out-of-frame deletion, therefore multi-exon skipping of exon 14 and 15 leading to an in-frame deletion of 180 amino acids could be a possibility.[71] Other AONs could also be developed for other less common JEB-H mutations. Further research should demonstrate the feasibility and the efficacy of these truncated LM-332 chains. Also questions regarding the administration of AONs remain, as AONs require lifelong periodic treatments and distribution to the epidermis is needed.

Cell therapy

Intra-dermal injection of either wild-type or gene-corrected DEB fibroblasts in murine models, results in a stable expression of Col7 at the dermal-epidermal junction and normalizes the DEB phenotype.[72-74] In RDEB patients, a single intradermal injection can increase Col7 for up to three months. Because the injected fibroblasts were not detectable anymore after two weeks, it is hypothesized that fibroblast injection induces subclinical inflammation leading to an upregulation of HB-EGF (heparin-binding epidermal growth factor-like growth factor) resulting in the increase of the recipients own *COL7A1* mRNA levels. This suggests that fibroblast therapy is most useful in RDEB patients with baseline Col7 expression.[75,76] LM-332 is only produced by keratinocytes, and epidermal cell therapy is more difficult. For example, it requires epidermal wounding for successful engraftment. Jiang *et al*. treated chronic wounds of two JEB-H patients with artificial skin bio-equivalents, which resulted in 77% and 11% wound healing after three weeks. In the patient with the best results a normalized weight gain and Hb level were also seen.[77] However, no information on the long-term follow-up and long-term treatment efficacy, especially the survival, were given. Most likely the efficacy of epidermal engraftment will be too localized to cure JEB-H. This problem could be circumvented by working with systemic cells that have long-lasting effects, such as stem cells. Bone marrow derived stem cells (BMSC) have shown to migrate to sites of injury and contribute to tissue repair.[78,79] They are capable of differentiating into epithelial lineage and after bone marrow transplantation (BMT) these cells are able to engraft in the skin of the recipient.[79-82] In *COL17A1* and *COL7A1* knockout mice, allogeneic BMT induced donor-derived keratinocytes, that produced Col17 and Col7, respectively, recovered hemidesmosomal structure and anchoring fibrils, respectively, and improved the skin phenotype and survival.[83,84] In 2005, Kopp *et al*. performed allogeneic hematopoietic stem cell transplantation in a JEB-H patient. Four weeks after transplantation the patient showed a more rapid progression of skin blistering with loss of two thirds of the total epidermis, after which skin from the same donor was transplanted resulting in complete healing with excellent biomechanical results. Unfortunately, the patient succumbed to pulmonary failure caused by uncontrollable infections.[85] Allogeneic BMT has also been performed in RDEB patients. Wagner *et al*. has reported the treatment of seven RDEB children aged 15 months to 14.5 years. One of the patients died before BMT, from complications of the immunomyeloablative chemotherapy. The six treated patients all showed an improved skin phenotype between 30-130 days after transplantation, and in five patients an increase of Col7 was seen. However, in none of

the patients a normalization of anchoring fibrils was detected. One patient died 183 days after BMT due to graft rejection and infection. Some other side effects were mucositis, renal insufficiency, hyperbilirubinemia, and opportunistic infections.[86] Despite the high mortality and morbidity rate, BMT seems to be a promising therapy for JEB-H. The risks associated with BMT might be reduced in the future, by the use of a reduced immunomyeloablative conditioning regimen.[86] It is not yet clear which bone marrow cells are involved in the therapeutic effect, although it seems that both bone marrow-derived hematopoietic stem cells as bone marrow-derived mesenchymal stem cells are able to produce Col17 and Col7.[55,83,87] Furthermore, other stem cells, such as adipose tissue stem cells and umbilical cord stem cells, are also able to differentiate into epithelial lineage, and they might prove to be useful in future stem cell transplantation.[55]

Another promising technique in the treatment of JEB-H is the utilization of the patients own cells to generate induced pluripotent stem cells (iPSCs). After the proof-of-principle was delivered in murine models,[88] it is shown that human wild-type or RDEB fibroblasts and keratinocytes can be de-differentiated to iPSCs and differentiate back into keratinocyte lineage.[79,89] Autologous stem cell therapy could be performed with genetically corrected iPSCs, which eliminates the issue of rejection of grafts.[90] A point of concern in iPSCs are the retroviral vectors that are used to reprogram the differentiated cells and to genetically modify autologous mutated cells, which could induce carcinogenesis.[90] However, a new non-integrating reprogramming protocol based on mRNA has already been shown to be successful in inducing iPSCs.[91] Moreover, revertant mosaic cells could be used to induce iPSCs, and thereby circumventing the necessity of genetically modifying autologous cells.[55] However, although revertant mozaicism has been described in JEB-nH and DEB patients, it has not yet been reported in JEB-H patients.[92,93]

Concluding remarks

Although many hurdles still have to be taken, protein replacement-, gene-, and cell-therapy offer new treatment opportunities for curing JEB-H. Pitfalls in most therapies are safety issues, the ability of systemic application, and the duration of effectiveness. Therefore, stem cell transplantation seems to be the most promising therapy in JEB-H. Another point of concern is a possible autoimmune response against LM-332. As JEB-H patients lack LM-332, auto-antibodies could be formed if immunocompetent cells do not recognize LM-332. This could abolish the effectiveness of the treatment and possibly induce auto-immune diseases associated with auto-antigens against LM-332, such as

anti-laminin-332 mucous membrane pemphigoid and cicatricial pemphigoid.[94,95] This has also been a point of concern in treating RDEB patients with a total absence of Col7. In experiments with immunocompetent *COL7A1* knockout mice, it has been shown that 60-100% of the mice develop antibodies against Col7.[49,50] Although these antibodies did not result in any side-effects and did not prevent the incorporation of Col7 at the BMZ, further research for LM-332 is needed.[49,50]

The Dutch basal epidermolysis bullosa simplex cohort

In **chapter 12** we have tested all known basal EB simplex (EBS) genes (*DST, ITGB4*, and *COL17A1*) in 11 basal EBS patients with wild-type *KRT5, KRT14*, and *PLEC1* genes.[96-102] In none of the patients, pathogenic mutations were revealed in any of these genes, leaving the pathogenesis unclear. However, the sensitivity of gene screening is not 100%. The mutation detection rate in *PLEC1*, *ITGB4* and *COL17A1* ranges from 83-95%.[21] In our patients gDNA was tested with PCR amplification and direct sequencing, however, this method fails to detect large deletions or insertions.[103] For keratin genes the mutation detection rate is even lower and estimated around 67%.[21] In our patients, next to gDNA testing, cDNA analysis of *KRT5* and *KRT14* was performed to detect large deletions or insertions. However, heterozygous in-frame deletions of small exons, and intronic mutations that produce an alternative splice site, would still be missed with this method.[102,103] To fully exclude mutations in all known basal EBS genes in our patients, further cDNA and protein analysis should be performed.[103]

To find novel genes associated with basal EBS in our probands, a screening method combining intra- and interfamilial genetic linkage analysis with whole-genome next-generation sequencing could be applied.[104] Other possible candidate genes have been discussed by Bolling.[103] Mutations might be found in *KRT15* (coding for keratin 15), although *KRT15* analysis in the Scottish *KRT5* and *KRT14* wild-type EBS population did not reveal any pathogenic mutations.[105] Keratin 15 is a type I keratin expressed by basal keratinocytes of stratified epithelia and is absent in differentiating keratinocytes. Although its expression is more restricted than keratin 14, an upregulation of keratin 15 is seen in the absence of keratin 14.[105,106]

Besides the hemidesmosomal proteins Col17, β4, BPAG1-e, and plectin, the remaining hemidesmosomal components α6 and CD151 could also be involved in the pathogenesis of EBS. Mutations in *ITGA6* have been associated with JEB-PA.[5]

However, it is not inconceivable that mutations in *ITGA6*, just as in *ITGB4*, can lead to a mild phenotype without pyloric atresia.[98] Karamatic Crew *et al*. showed that two Israeli siblings, carrying homozygous nonsense mutations in *CD151,* suffered from pretibial epidermolysis bullosa, nail dystrophy, and one sibling had dystrophic teeth.[107] The question is whether *CD151* mutations can cause basal EBS. Electron microscopy in the two siblings showed a junctional split. Furthermore, the *CD151* mutations in these patients resulted in nephrotic syndrome causing end-stage renal failure, neurosensory deafness, and β-thalassemia minor. These symptoms all lack in our wild-type basal EBS patients.[107]

Other possible candidates are genes involved in desmosomes, adherens junctions, tight junctions and gap junctions. Some of these genes are downregulated in *KRT5* and *KRT14* mutational cell lines, showing a relation between them in cell-cell and cell-substrate adhesion interaction. Loss of these proteins could lead to a similar basal EBS phenotype.[108]

Furthermore, post-translational modification of keratin filaments and epigenetics could be involved in the pathogenesis of basal EBS.[103,109,110]

Epidermolysis bullosa: the quality and impact on life

Health related quality of life (QOL) encompasses the physical, psychological and social aspects of an individual's well-being.[111,112] In the past decade, there has been more focus on the impact that EB has on patients, their parents and health care professionals (HCP) (reviewed by Pagliarello *et al.*)[113] The research performed in this field can be classified into qualitative (descriptive) studies and quantitative studies using generic and dermatology-specific tools.

Epidermolysis bullosa and the impact on patients

In 2004, two patients suffering from dominant dystrophic EB (DDEB) and RDEB published their personal experiences with their disease, focusing on the impact on their daily lives and their way to overcome their limitations with a positive attitude and motivation.[114,115] Another qualitative study by Dures *et al*. focuses on the psychosocial impact of EB in adult patients using semi-structured interviews.[116] The perceived impact of EB on patients' lives ranged from those who found that EB intruded into all areas of their lives through those who have learned to live with it. Talking to others about the

challenges of living with EB could be beneficial. Also, patients felt restricted by their symptoms and societies' attitude, resulting in social exclusion in some cases.[116] In a similar fashion van Scheppingen *et al*. identified the main problems experienced by children suffering from EB.[117] Children reported problems with having an itchy skin, being in pain, having difficulties with participation, lack of understanding of others and the feeling of being different.[117] Comparable results were found by Williams *et al*. in a similar study focusing specifically on children suffering from EBS.[118]

Quantitative measurement of the impact of EB using generic and dermatology-specific tools has been performed in both children and adult patients. Margari *et al*. assessed the psychiatric symptoms in both groups using a diversity of psychological and psychiatric generic tools together with semi-structured interviews.[119] EB had a negative impact on the QOL in 82% of the patients. Furthermore, 80% of the patients experienced sub-threshold psychiatric symptoms, in particular depression, anxiety, and behavior disturbances. Interestingly, there was no close correlation observed between these symptoms and the clinical severity of the EB.[119] Fine *et al*. assessed the mobility, activities of daily living and pain in children suffering from EB, using standardized questionnaires.[120] The level of independence for daily living activities was better in the children suffering from the milder EBS and DDEB, compared to those suffering from JEB and RDEB. The vast majority of children suffered from pain, with the RDEB patients experiencing the most pain.[120] Horn and Tidman measured the QOL in EBS and DEB patients using the Dermatology Life Quality Index (DLQI) and the Children's Dermatology Life Quality Index (CDLQI).[121] The impairment of QOL was highest in patients suffering from RDEB severe generalized, and the scores exceeded those of any skin disorder previously assessed. The effects on QOL in EBS, DDEB, and RDEB generalized other were comparable to that of moderately severe psoriasis and eczema.[121] The assessment of QOL with the Short Form-36 (SF-36) and Skindex-29 in EB patients was performed by Tabolli *et al*.[122] EB patients showed statistically lower values in the physical components of the SF-36 compared to a normal population, while the values in the mental components were similar. EB had the greatest impact on QOL in patients with a higher perceived disease severity, with a larger skin involvement, with a higher psychological distress measured by the General Health Questionnaire-12, and in females. Although patients suffering from JEB and RDEB severe generalized showed the lowest QOL, no statistically differences were seen among the various EB types and subtypes.[122]

Although generic and dermatology-specific QOL measurement tools, such as the DLQI, SF-36, and Skindex-29, allows us to compare QOL scores with other diseases,

issues concerning the content validity and ceiling effects have been raised.[112] It has been postulated that using these tools, the QOL has been overestimated, especially in severely affected EB patients. The DLQI for example assesses the impact of the disease over the last week. As EB is a lifelong disease, patients have grown accustomed to the implications of the disease and perceive the impact over the last week marginal.[112] To encounter these problems and to generate an accurate and sensitive tool to monitor QOL over time, Frew *et al*. developed an English EB-specific QOL questionnaire for adults, called the QOLEB.[123] In **chapter 13** we have successfully translated and validated the QOLEB into the Dutch language and assessed the QOL in Dutch EB patients. In the future, applying the QOLEB in clinical trials will provide the dimension of QOL in outcome measurements. Furthermore, the translation of the QOLEB in multiple languages will allow the comparison of QOL in EB throughout the world and also in clinical trials. Also, future development and validation of a QOLEB for children would be of great value.

Epidermolysis bullosa and the impact on parents

The presence of a severely affected child with EB has profound effects on the marriage of the parents, such as lack of interest in participating in activities as couples.[124] This could influence the choice of having more children and even contribute to a divorce.[124] Tabolli *et al*. showed that the family burden of EB is comparable to caregivers of cancer patients and patients with leg ulcers.[122] In children with EB, the family burden increases when parents have an increased perceived disease severity, if patients have an increased body surface involved, and if parents suffer from depression or anxiety. On the contrary, the family burden was independent from EB type or subtype.[125] In regards to what parents of a child with EB perceive as problematic, are the burden of EB on their child's life, and with their child being different and suffering from pain.[126] Furthermore, parents suffered from problems with the burden of care for their child and their family. They faced feelings of uncertainty, restrictions in their employment and social life, difficulties in organizing care, physically and mentally never being off-duty, and feelings of guilt and growing apart of their family.[125,126] Also, parents experienced problems with ignorance and lack of skills of HCP and indicated that they needed to be better informed.[125-127] All these studies have been performed in parents with children suffering from non-lethal EB subtypes. In **chapter 4** we discussed the needs of parents of children suffering from lethal EB and we give some guidelines in caring for these parents. Although some overlap exists with the problems described above, some specific issues

concerning the end-of-life are identified. However, if a cure becomes available for lethal EB, the guidelines should be adjusted to suit this situation.

Epidermolysis bullosa and the impact on health care professionals

Dures *et al*. has shown that supporting patients suffering from EB can deeply affect specialist HCP.[128] The problems identified are feeling sorrow during death and palliative care, frustration and guilt at the limited effectiveness of treatment, witnessing struggles of the patients they have built relationships with, and difficulties with switching off from work. Another problem experienced by HCP is the rarity of EB. Patients depend on the help of their specialist HCP, and HCP can face the unwillingness of other non-specialist HCP to get involved. Nevertheless, the support of team members and meetings with their supervisor, help HCP to cope with these feelings. Furthermore, HCP experience working with EB patients as meaningful and rewarding.[128]

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Summary

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Epidermolysis bullosa (EB) is a heterogenous group of inherited diseases characterized by trauma-induced blistering. The International classification of inherited EB distinguishes four major EB types based upon the ultrastructural level of blistering: EB simplex (EBS) with an intra-epidermal cleavage, junctional EB (JEB) with a junctional cleavage, dystrophic EB (DEB) with a dermal cleavage, and Kindler syndrome with a mixed cleavage.[1] JEB inherits autosomal recessive and is further subdivided into six minor subtypes, all with their own clinical and diagnostic features. Mutations in genes coding for integrin α6β4 (α6β4), type XVII collagen (Col17), and laminin-332 (LM-332) underlie the pathogenesis of JEB. The transmembrane proteins α6β4 and Col17 are part of hemidesmosomes, which are multi-protein complexes that mediate the adhesion of epithelial cells to the underlying basement membrane, by linking keratin intermediate filaments to the lamina lucida/densa protein LM-332.

Since 1988, over 400 EB patients have visited the Center for Blistering Diseases at the University Medical Center Groningen, where they have been carefully examined, diagnosed, treated, and followed-up. This Dutch EB registry (DEBR) provides us with a great insight into the spectrum of EB in the Netherlands, such as the epidemiology, clinical features, diagnostic features, mutational profile, disease progression, and therapeutic options. The aim of this thesis was to distill and summarize this information for JEB, so that this knowledge can function as a guide for health care professionals to base future decisions to enhance the care for patients.

JEB, type Herlitz (JEB-H) is a subtype that leads to childhood lethality and is caused by a total absence of functional LM-332. In **chapter 2 and 3** we described the diagnostic features and long-term follow-up of the 22 JEB-H patients included in the DEBR from 1988 to 2011. Eighteen of these patients were born in the Netherlands, allowing us to calculate the Dutch JEB-H incidence rate of 4.0 per one million live births and the carrier frequency of a JEB-H mutation in the Dutch population of one in 249. Diagnostic testing with transmission electron microscopy (TEM) showed absent or attenuated hemidesmosomes. With immunofluorescence (IF) antigen staining an absent (90.9%) or strongly reduced (9.1%) LM-332 staining with monoclonal antibody GB3 was seen. Molecular analysis revealed that 86.4% of the patients carried mutations in *LAMB3*, encoding for the laminin β3 chain, 9.1% carried mutations in *LAMA3*, encoding for the laminin α3 chain, and 4.5% carried mutations in *LAMC2*, encoding for the laminin γ2 chain. All identified mutations, including several novel nonsense mutations, led to a premature termination codon (PTC), resulting in mRNA decay and loss of functional LM-332. Important in the diagnosis of JEB-H, is the differentiation from the non-lethal

subtype JEB, type non-Herlitz (JEB-nH). This distinction can not be made with TEM analysis or in cases with a strongly reduced GB3 staining. Therefore, all diagnostic tools should be evaluated to clarify the diagnosis JEB-H. Clinical symptoms seen in JEB-H patients were blistering of skin and mucous membranes, hypergranulation, nail deformities, cutis aplasia, enamel hypoplasia and hoarseness. The most common complications were failure to thrive, anemia, and dyspnea/stridor. All patients succumbed to the disease before the age of three years, with an average age of death at 5.8 months (range 0.5-32.6 months). The pattern of weight gain was the only predictor of lifespan that we could identify. The causes of death were failure to thrive (40.9%), respiratory failure (27.3%), pneumonia (9.1%), dehydration (9.1%), anemia (4.5%), and sepsis (4.5%). One patient suffered from uncontrollable discomfort and weakening and newborn euthanasia was performed on the parents' request. Due to the lethality of the disease, we advise that medical procedures intended to extend life, such as blood transfusion, tracheostomy, nasogastric feeding tubes, gastrostomy, parenteral feeding, intubation, mask ventilation, and heart massage, are not performed, as they compromise the comfort of the JEB-H child. The mainstay of treatment should be comfort care, such as pain medication, dressing changes, laxatives and nebulizers. An early diagnosis of JEB-H is important, as it allows the treatment to be switched from aggressive life-extending treatments to comfort care.

The process that parents of a child with lethal EB go through is a painful and difficult path. Our job as health care professionals (HCP) is not only to provide the best care for the child, but also to guide the parents in this difficult time. To improve this, it is important to know what parents want, expect, and appreciate from HCP. In **chapter 4** we have identified the needs of parents with children suffering from lethal EB by performing semi-structured in-depth interviews with these parents. Parents indicated that they have the need (1) for a fast and correct referral to a specialized EB clinic, (2) to be informed as honestly as possible about the diagnosis and lethal prognosis, (3) to have a structured network of care givers in the palliative care, (4) to be involved in the care and the medical decisions involving their child, (5) to be informed about the end-of-life and to discuss euthanasia, (6) for guidance and to have remembrances of their child, and (7) for genetic counseling.

The subtype JEB-nH is usually caused by mutations in the genes coding for LM-332 and Col17. In the literature one JEB-nH case has been reported caused by *ITGB4* mutations,[2] a gene encoding integrin β4 and normally associated with EB with pyloric atresia (EB-PA). In **chapter 5** we present two new JEB-nH cases carrying two novel

ITGB4 mutations. We conclude that *ITGB4*-associated EB may be without pyloric atresia and cause a generalized or a localized pattern of blistering. IF antigen mapping can be helpful in appointing *ITGB4* as the first candidate gene. In our JEB-nH cohort, the incidence of pathogenic *ITGB4* mutations is 6.5%, indicating that if integrin β4 staining is abnormal, or if mutations in *LAMA3*, *LAMB3*, *LAMC2*, or *COL17A1* are excluded, *ITGB4* should be evaluated next.

In **chapter 6** we reviewed all documented JEB patients who developed a squamous cell carcinoma (SCC), both from our EB center as those reported in the literature. In our JEB cohort, seven patients had a history of developing one or more SCCs. The frequency of developing an SCC among adult JEB patients in our center was 25%. In the literature, we found seven case reports of JEB complicated by SCC, bringing the total number of documented cases to 14.[3-10] All patients were adults and classified as JEB-nH. The first SCC developed at an average age of 50 years (median 52 yrs, range 28-70 yrs). In nine patients, multiple primary SCCs occurred, with a total of 45 SCCs. The SCCs are most often located on the lower extremities, in areas of chronic blistering, long-standing erosions or atrophic scarring. The treatment of first choice is surgical excision, although no guidelines for the excision margins are given. Three (21%) patients developed metastases and died on average 8.9 years after diagnosis of the initial SCC. Because SCCs can develop in early adulthood, we recommend checking the entire skin of JEB patients from age 25. Although SCCs can look like normal wounds, suspicious lesions are chronic non-healing ulcers or erosions, hypergranulation, induration, and hyperkeratotic, exophytic, verrucous nodules or plaques. Additional clues can be pain, a stinging or burning sensation, and noticeable changes in a lesion. Multiple biopsies of suspicious lesions should be taken to avoid missing malignancies.

In **chapter 7** we replied to dr. Fine's commentary on our article,[11] "Risk of squamous cell carcinoma in junctional epidermolysis bullosa, non-Herlitz type: report of seven cases and a review of the literature" **(chapter 6)**,[12] in which he raised concerns about possible inaccurate conclusions that we have made in comparison with the National EB Registry (NEBR) in the US. The commentary revolves around one central question: "can children that suffer from JEB-H survive long enough (i.e. adulthood) to develop SCCs?" The NEBR uses merely non-molecular diagnostic testing in combination with clinical findings to classify patients according to the 2008 classification report. However, in this classification no clear definition of JEB-H is stated and the clinical and non-molecular diagnostic findings show a distinct overlap between the subtypes JEB-H and JEB-nH, resulting in a poor discriminative value seen in the NEBR concerning the

prognosis (i.e. lethality) between these two subtypes.[13] The criterion that we use to diagnose JEB-H is the total absence of functional LM-332. It has been shown that this distinction is best made utilizing and combining IF antigen staining with DNA analysis.[14,15] Using this diagnostic criterion and tools in all Dutch JEB patients, provides a nearly perfect distinction in JEB-H and JEB-nH patients, that provided the prognosis (i.e. lethal or non-lethal). We therefore concluded that the long-term JEB-H survivors in the NEBR are misclassified and that JEB-H patients do not survive long enough to develop SCCs.

LM-332 plays an important role in the process of epidermal wound healing. A disruption of wound healing in LM-332 deficient JEB-nH patients can cause persistent, small, but deep ulcers on hands and feet, that adversely affect the quality of life. We have treated these patients with punch grafting, a minor surgical procedure in which small full-thickness skin grafts are harvested with a punch biopsy, whereupon they are placed in the ulcer to promote wound healing. In **chapter 8** we presented the results of punch grafting and discuss its therapeutic value. In the past 10 years we have treated 23 ulcers in four JEB-nH patients with punch grafting without any complications or adverse effects. The ulcers had on average persisted six years prior to treatment and had a maximum size of 3 by 2 cm. Healing rate after punch grafting was 70% (n=16), with a mean healing time of 2 months. Thirty percent (n=7) of the treated ulcers did not completely heal, but did show improvement. Furthermore, in two patients a (temporary) stop or reduction of pain medication use was achieved, and one patient was able to walk long distances again after three years. The recurrence rate after three months was 13% (n=2), and was due to renewed blistering. We concluded that punch grafting is an easy, inexpensive, and effective first-line treatment for small persistent ulcers in JEB-nH patients.

LM-332 and Col17 are crucial in ameloblast differentiation and enamel formation, resulting in enamel defects of the entire dentition, consisting of hypoplasia, pitting, roughness, thinning or furrowing of enamel, in all patients with JEB-nH. In **chapter 9** we reported two related heterozygous carriers of the novel *LAMA3* deletion c.488delG, who did not suffer from skin fragility, but did show enamel defects in their dentition, consisting of roughness and pits, which led to a higher susceptibility of caries in both. As the mutation c.488delG creates a frameshift resulting in a PTC and mRNA decay, and as alternatively spliced cDNA products could not be found in cDNA analysis, we hypothesized that haploinsufficiency of laminin α3 is the cause of the enamel defects in these carriers, and that in the complex process of enamel formation, in which LM-332

plays such a vital role, there is no coping mechanism that can manage the loss of one *LAMA3* allele, whereas these mechanisms are available in the skin. However, it remains peculiar that enamel defects have never been reported before in carriers of *LAMA3, LAMB3,* or *LAMC2* null mutations.

In **chapter 10** we revealed that the rare disease JEB of late onset (JEB-lo) is caused by mutations in *COL17A1*. Two JEB-lo patients, a brother and a sister, suffered from blistering on the feet and around the toe- and fingernails starting at the age of six years. With age, blistering also occurred on hands, nose and oral mucosa. Other clinical features were nail deformities, pretibial atrophic patches, palmoplantar hyperhidrosis, loss of dermatoglyphs, and amelogenesis imperfecta. With IF antigen staining and TEM subtle changes were found. The most remarkable changes were loss of the Col17 apical-lateral staining, and a broadened distribution of staining against the ectodomain of Col17, LM-332, and type VII collagen (Col7). Mutation analysis of *COL17A1* showed a compound heterozygosity for a novel mutation c.1992_1995delGGGT and the known mutation c.3908G>A in both patients. The deletion c.1992_1995delGGGT results in a PTC and mRNA decay, leaving the patients functionally hemizygous for the missense mutation c.3908G>A (p.R1303Q). We hypothesize that the mutation p.R1303Q, located in the Col17 ectodomain, results in a disturbed ligand-binding that interferes with a correct build-up of the extracellular matrix. This would also explain the broadened distribution of the IF staining against LM-332 and Col7; as the Col17 ectodomain has an interaction with LM-332, and thus with Col7. Although we have elucidated the pathogenesis of JEB-lo, alternative pathogeneses are not excluded, so further research in other JEB-lo patients is required.

In **chapter 11** we presented five new monoclonal antibodies (mAb) against the endodomain of Col17, called VK1, VK2, VK3, VK4, and VK5 that are effective in IF staining, immunohistochemistry staining, and western blotting. IF staining with the VK antibodies can distinguish between healthy subjects and all Col17-related EB subtypes and carriers. Furthermore, a good distinction between the (nearly) absent staining in generalized JEB-nH and the reduced staining in localized JEB-nH and carriers can be made. In this chapter we also present a biological turn-over model for Col17. Synthesized Col17 is transported to the apical-lateral cell membrane where it forms a reservoir to be incorporated into newly formed hemidesmosomes at the basal cell membrane. In the case of reduced Col17 production, such as in localized JEB-nH and in carriers, the apical-lateral reservoir will deplete more easily as the available Col17 is needed for incorporation into hemidesmosomes, resulting in a loss of apical-lateral

staining. This stresses the importance of pattern recognition in IF staining, where the observation of the loss of apical lateral staining might even be more important than the basement membrane zone (BMZ) staining. After incorporation of Col17 in hemidesmosomes and migration of the basal keratinocytes, shedding of the ectodomain yields two more BMZ-located Col17 forms: LAD-1 and LABD97. After shedding they accumulate and remain settled in the BMZ until later degradation. This model indicates that mAb against the Col17 endodomain are the most sensitive in detecting a reduced Col17 production, as they only detect full-length Col17, while mAb against the Col17 ectodomain detect both the full-length Col17 as the shedded products.

In **chapter 12** we discussed the Dutch basal EBS cohort comprising of 64 biopsy-confirmed probands. Seventy-seven percent of the cases (n=49) are caused by mutations in the genes coding for the intermediate filaments keratin 5 (*KRT5*) or keratin 14 (*KRT14*), and 6% of the cases (n=4) are caused by mutations in the gene coding for the hemidesmosomal plectin (*PLEC1*).[16,17] This leaves 17% of the cases (n=11) unsolved, consisting of two EBS-Dowling-meara, two EBS-generalized, and seven EBS-localized patients. Molecular analysis of the remaining genes that have ever been associated with basal EBS: *COL17A1*, *ITGB4*, and *DST* (encoding the hemidesmosomal bullous pemphigoid antigen 1-e), did not reveal any pathogenic mutations in our unsolved cases.[18-20] Mutations in these genes are rarely found in basal EBS, and as these reported cases all inherited in an autosomal recessive manner and IF antigen staining of the respected proteins was either reduced or absent, molecular screening could be restricted to some selected cases that show abnormalities in IF antigen staining, and excluding the cases that inherit autosomal dominant. In the future, we hope that genetic linkage analysis and next-generation sequencing will further elucidate the molecular pathogenesis of these unsolved cases and will enhance our understanding of basal EBS.

The health related quality of life (HRQOL) is the impact of a disease on the physical, psychological, and social health of a patient.[21] Defining the HRQOL in EB patients is important for patient care, assessing the efficacy of (new) treatments, and assigning funding to EB. An English EB-specific HRQOL measurement tool called the QOLEB (quality of life in EB) has been developed in Australia.[22] It consists of a 17-item questionnaire and measures two factors: functioning and emotions. In **chapter 13** we have translated the QOLEB into the Dutch language, and we show that the Dutch QOLEB is a reliable and valid instrument for the assessment of the HRQOL in adult EB patients. The 55 participating patients were classified into four main EB subtypes: EBS,

JEB, dominant DEB (DDEB), and recessive DEB (RDEB). The Dutch QOLEB was able to discriminate the severe subtypes RDEB and JEB from the milder subtypes EBS and DDEB. Significant differences were seen in the functioning and overall scale, but not in the emotions scale, suggesting that the mental burden of EB is similar in milder and more severe subtypes. According to the categorization of the overall QOLEB score, the HRQOL of the Dutch EBS and DDEB cohorts is mildly affected, and the HRQOL of the Dutch JEB and RDEB cohorts is moderately affected.

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Nederlandse samenvatting (Dutch Summary)

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Epidermolysis bullosa (EB) bestaat uit een heterogene groep van erfelijke blaarziektes. EB wordt veroorzaakt doordat verschillende “plakeiwitten” in de huid niet of verkeerd aangemaakt worden. Na wrijving of druk ontstaat hierdoor blaarvorming van de huid en slijmvliezen. Welke eiwitten aangedaan zijn en waar deze in de huid liggen, bepalen het niveau van de blaarvorming. De Internationale classificatie van erfelijke EB onderscheidt vier EB groepen op basis van het blaarniveau: EB simplex (EBS) met een intra-epidermale splijting (in de opperhuid), junctionele EB (JEB) met een junctionele splijting (in de overgang tussen de opperhuid en lederhuid), dystrofische EB (DEB) met een dermale splijting (in de lederhuid) en Kindler syndroom met een gemengde splijting.[1] Deze EB hoofdgroepen zijn verder onderverdeeld in vele subtypes, elk met hun eigen klinische en diagnostische kenmerken. Om de diagnose EB te stellen zijn verschillende diagnostische onderzoeken mogelijk. Met transmissie elektronenmicroscopie (TEM) en immunofluorescentie (IF) microscopie kunnen huidbiopten onderzocht worden. Bij TEM wordt er zodanig ingezoomd op de huid, dat afwijkingen in celstructuren gezien kunnen worden. Met IF kleuring worden antilichamen gebruikt die aan een specifiek eiwit blijven plakken. Deze antilichamen worden fluorescerend gemaakt, zodat een afwezigheid of reductie van dat eiwit aangetoond kan worden. Tevens kan met TEM en IF een blaarniveau vastgesteld worden. Met mutatie analyse worden er fouten (mutaties) opgespoord in de genen die coderen voor de specifieke “plakeiwitten”. Junctionele EB (JEB) is een autosomaal recessief overervende aandoening en wordt veroorzaakt door mutaties in de genen die coderen voor de eiwitten integrine α6β4 (α6β4), type XVII collageen (Col17) en laminine-332 (LM-332). De transmembraan eiwitten α6β4 en Col17 maken deel uit van hemidesmosomen. Dit zijn adhesie complexen bestaande uit meerdere eiwitten, die de epitheelcellen aan het onderliggende basaalmembraan hechten. Dit doen ze door keratinefilamenten van de basale epitheelcellen te verbinden met LM-332. LM-332 is een trimeer bestaande uit een laminine α3, laminine β3 en laminine γ2 keten, en is gelokaliseerd in de lamina lucida/densa waar het zorgt voor de verankering met de dermis.

Sinds 1988 hebben meer dan 400 EB patiënten het Centrum voor Blaarziekten in het Universitair Medisch Centrum Groningen bezocht, waar zij zorgvuldig zijn onderzocht, gediagnosticeerd, behandeld en opgevolgd. Het Nederlandse EB Register (DEBR) geeft ons inzicht in het spectrum van EB in Nederland, zoals de epidemiologie, klinische kenmerken, diagnostische kenmerken, mutatie profiel, het ziekteverloop en de therapeutische mogelijkheden. Het doel van dit proefschrift is om deze informatie voor JEB te destilleren en samen te vatten, zodat deze kennis als gids kan functioneren voor

gezondheidsprofessionals om in de toekomst beslissingen op te baseren, zodat de zorg voor patiënten verbeterd kan worden.

JEB, type Herlitz (JEB-H) is een subtype dat leidt tot sterfte op kinderleeftijd en wordt veroorzaakt door een totale afwezigheid van functioneel LM-332. In **hoofdstuk 2 en 3** worden de diagnostische kenmerken en lange termijn follow-up beschreven van de 22 JEB-H patiënten die van 1988 tot 2011 in de DEBR zijn geïncludeerd. Achttien van deze patiënten zijn geboren in Nederland, zodat de Nederlandse JEB-H incidentie van 4,0 per miljoen levendgeborenen en de dragerschapfrequentie van een JEB-H mutatie in de Nederlandse bevolking van één op de 249 berekend kon worden. Diagnostisch onderzoek met TEM toonde afwezige of aangedane hemidesmosomen. Met IF werd een afwezige (90,9%) of sterk gereduceerde (9,1%) LM-332 kleuring met het monoklonaal antilichaam GB3 gezien. Mutatie analyse toonde aan dat 86,4% van de patiënten mutaties had in het gen *LAMB3*, dat codeert voor de laminine β3 keten, 9,1% had mutaties in het gen *LAMA3*, dat codeert voor de laminine α3 keten, en 4,5% had mutaties in het gen *LAMC2*, dat codeert voor de laminine γ2 keten. Alle geïdentificeerde mutaties, waaronder ook enkele nooit eerder beschreven mutaties, leiden tot een vroegtijdige stopcodon, resulterend in voortijdig mRNA verval en het compleet verlies van functioneel LM-332. In de diagnostiek is het belangrijk om JEB-H te onderscheiden van het niet dodelijke subtype JEB, type non-Herlitz (JEB-nH). Dit onderscheid kan niet worden gemaakt met IF analyse in gevallen met een sterk gereduceerde GB3 IF kleuring. Het is daarom van belang dat alle diagnostische instrumenten geëvalueerd worden om de diagnose JEB-H te stellen. De klinische symptomen die in onze JEB-H patiënten aanwezig waren, zijn blaarvorming op de huid en slijmvliezen, hypergranulatie, nagelafwijkingen, aplasia cutis (afwezigheid van de epidermis), glazuurafwijkingen en heesheid. De meest voorkomende complicaties waren ‘failure to thrive’ (het achterblijven van de groei), bloedarmoede en kortademigheid. Alle patiënten overleden aan de ziekte vóór hun derde levensjaar, gemiddeld na 5,8 maanden (range 0,5-32,6 maanden). Het patroon van gewichtstoename was de enige voorspeller van de levensduur die we konden identificeren. De doodsoorzaken waren ‘failure to thrive’ (40,9%), kortademigheid (27,3%), longontsteking (9,1%), uitdroging (9,1%), bloedarmoede (4,5%) en sepsis (4,5%). Eén patiënt leed aan uitzichtloos, ondraaglijk, onbehandelbaar lijden en verzwakking, waarna op verzoek van de ouders euthanasie werd gepleegd. De JEB-H patiënten ondergingen verschillende medische interventies, zoals bloedtransfusies, tracheotomie (kunstmatige beademing door een opening in de luchtpijp), neussonde, gastrostomie (voeding door een opening in de maag), parenterale voeding (voeding via

een infuus), intubatie (kunstmatig beademen door een buis die in de luchtpijp is aangebracht), ballon ventilatie en hart massage. Vanwege de letaliteit van de ziekte, adviseren wij dat deze medische interventies, die bedoeld zijn om het leven te verlengen, niet worden uitgevoerd, omdat deze het comfort van JEB-H patiënten verminderen. De belangrijkste behandeling is comfortzorg, zoals pijnstilling, verbandwisselingen, laxeermiddelen en vernevelaars. Een snelle diagnose van JEB-H is belangrijk, omdat er dan omschakeling van levensverlengende behandelingen naar comfortzorg kan plaatsvinden.

Voor ouders is het verliezen van een kind met letale EB een pijnlijk en moeilijk proces. Onze taak als gezondheidsprofessionals is niet alleen om zo goed mogelijk voor het kind te zorgen, maar ook om de ouders zo goed mogelijk te begeleiden in deze moeilijke tijd. Om dit te verbeteren, is het belangrijk om te weten wat ouders willen, verwachten, en waarderen van gezondheidsprofessionals. In **hoofdstuk 4** hebben we de behoeftes van ouders met kinderen die lijden aan letale EB geïdentificeerd door semigestructureerde diepte-interviews met hen te voeren. Ouders gaven aan dat ze behoefte hebben (1) aan een snelle en correcte doorverwijzing naar een gespecialiseerde EB kliniek, (2) om zo eerlijk mogelijk te worden geïnformeerd over de diagnose en infauste prognose, (3) om in de palliatieve zorg een gestructureerd netwerk van zorgverleners te hebben, (4) om betrokken te zijn bij de zorg en de medische beslissingen betreffende hun kind, (5) om te worden geïnformeerd over het levenseinde en de mogelijkheden van euthanasie, (6) aan begeleiding en om herinneringen te hebben aan hun kind, en (7) aan erfelijkheidsadvies.

Het subtype JEB-nH wordt doorgaans veroorzaakt door mutaties in de genen die coderen voor LM-332 en Col17. In de literatuur is één JEB-nH geval bekend die wordt veroorzaakt door *ITGB4* mutaties.[2] Dit gen codeert voor integrine β4 en is normaal gesproken geassocieerd met EB met pylorus atresie, oftewel een afsluiting van de slokdarm naar de maag (EB-PA). In **hoofdstuk 5** presenteren we twee nieuwe JEB-nH gevallen die veroorzaakt worden door twee nooit eerder beschreven *ITGB4* mutaties. We concluderen dat *ITGB4*-geassocieerde EB kan voorkomen zonder pylorus atresie en een gegeneraliseerde of gelokaliseerde blaarvorming kan veroorzaken. IF kleuring kan behulpzaam zijn in het aanwijzen van *ITGB4* als het eerste kandidaatgen. In onze JEB-nH cohort is de incidentie van pathogene *ITGB4* mutaties 6.5%, hetgeen aangeeft dat, indien de IF kleuring van integrine β4 abnormaal is, of indien mutaties in *LAMA3*, *LAMB3*, *LAMC2*, of *COL17A1* geëxcludeerd zijn, *ITGB4* het volgende kandidaat gen is.

In **hoofdstuk 6** worden alle gedocumenteerde JEB patiënten beschreven die ooit de huidtumor plaveiselcelcarcinoom (PCC) hebben ontwikkeld, zowel van ons EB centrum als degene die in de literatuur beschreven zijn. In ons JEB cohort hebben zeven patiënten een voorgeschiedenis van één of meer PCCs. De frequentie van het ontwikkelen van een PCC bij volwassen JEB patiënten is in ons centrum 25%. In de literatuur zijn er zeven JEB patiënten met een PCC beschreven, waardoor er in totaal 14 gedocumenteerde patiënten zijn.[3-10] Alle patiënten waren volwassen en geclassificeerd als JEB-nH. De eerste PCC ontwikkelde op een gemiddelde leeftijd van 50 jaar (mediaan 52 jaar, range 28-70 jaar). Negen patiënten ontwikkelden meerdere primaire PCCs, met een totaal van 45 PCCs. De PCCs zijn meestal gelokaliseerd op de benen, in gebieden met chronische blaarvorming, of in gebieden met lang bestaande erosies of atrofische verlittekening. De behandeling van eerste keus is chirurgische excisie. Er zijn echter geen richtlijnen voor de excisiemarges. Drie (21%) patiënten ontwikkelden uitzaaiingen en overleden gemiddeld 8,9 jaar na de diagnose van de initiële PCC. Aangezien PCCs zich al vroeg in de volwassenheid ontwikkelen, adviseren wij om de gehele huid van JEB patiënten te controleren vanaf het 25^{e} levensjaar. PCCs kunnen eruit zien als normale wonden, maar verdachte lesies zijn vaak chronische niet-genezende ulcera of erosies, hypergranulatie, induratie, en hyperkeratotische, exophytische, verruceuze nodi of plaques. Extra aanwijzingen zijn pijn, een stekend of branderig gevoel en merkbare veranderingen in een lesie. Om geen maligniteiten te missen moeten het liefst meerdere biopten uit een verdachte lesie genomen worden.

In **hoofdstuk 7** geven we een reactie op het commentaar van dr. Fine op ons artikel,[11] "Risk of squamous cell carcinoma in junctional epidermolysis bullosa, non-Herlitz type: report of seven cases and a review of the literature" **(hoofdstuk 6)**,[12] waarin hij de zorg uit over mogelijk onjuiste conclusies die we hebben getrokken in vergelijking met het Nationale EB Register (NEBR) van de Verenigde Staten. Het commentaar draait rond de centrale vraag: “Kunnen kinderen die lijden aan JEB-H lang genoeg overleven (tot de volwassenheid) om een PCC te ontwikkelen?” In de NEBR worden slechts niet-moleculair diagnostische onderzoeken (dat wil zeggen geen mutatie analyse) in combinatie met klinische bevindingen gebruikt, om patiënten in te delen op basis van het internationale consensus classificatie uit 2008. Er staat in deze classificatie echter geen duidelijke definitie van JEB-H. Tevens staat er in deze classificatie, wat betreft de klinische en niet-moleculaire diagnostische bevindingen, een overlap tussen JEB-H en JEB-nH. Hierdoor berekent de NEBR een zwak discriminerende waarde tussen deze twee subtypes wat betreft de prognose/letaliteit.[13] Het criterium dat wij gebruiken om de

diagnose JEB-H te stellen is de totale afwezigheid van functioneel LM-332. Dit onderscheid wordt het beste gemaakt door de combinatie van IF kleuring en mutatie analyse.[14,15] Met behulp van deze diagnostische criteria en onderzoeken is er wat betreft de prognose (letaal versus niet letaal) bij de Nederlandse JEB patiënten een bijna perfect onderscheid te maken tussen JEB-H en JEB-nH patiënten. Wij concluderen daarom dat de overlevende JEB-H patiënten in de NEBR verkeerd zijn geclassificeerd en dat JEB-H patiënten niet lang genoeg overleven om een PCC te ontwikkelen.

LM-332 speelt een belangrijke rol in het proces van de epidermale wondgenezing. Een verstoring in de wondgenezing kan in LM-332 deficiënte JEB-nH patiënten leiden tot persisterende, kleine en diepe ulcera op handen en voeten, die een negatieve invloed hebben op de kwaliteit van leven. We hebben deze patiënten behandeld met biopttransplantaties. Dit is een chirurgische ingreep waarbij kleine huidtransplantaten van volledige dikte met een ponsbiopt worden geoogst, waarna ze in het ulcus worden geplaatst om de wondgenezing te bevorderen. In **hoofdstuk 8** presenteren we de resultaten en de therapeutische waarde van biopttransplantaties. In de afgelopen 10 jaar hebben we 23 ulcera in vier JEB-nH patiënten met biopttransplantaties behandeld zonder enige complicaties of bijwerkingen. De ulcera bestonden gemiddeld zes jaar voorafgaand aan de behandeling en hadden een maximale grootte van 3 bij 2 cm. Zeventig procent (n=16) van de ulcera genazen gemiddeld 2 maanden na biopttransplantatie. Dertig procent (n=7) van de behandelde ulcera genazen niet volledig, maar vertoonden wel verbetering. Bij twee patiënten was er tevens een (tijdelijke) stop of vermindering van het gebruik van pijnmedicatie en één patiënt was na drie jaar weer in staat om lange afstanden te lopen. Het recidiefpercentage was na 3 maanden 13% (n=2) en dit was te wijten aan hernieuwde blaarvorming. We concluderen dat biopttransplantatie een makkelijke, goedkope en effectieve eerstelijns behandeling is voor kleine persisterende ulcera in JEB-nH patiënten.

LM-332 en Col17 zijn cruciaal in de differentiatie van ameloblasten (cellen die het glazuur van de tand vormen) en de vorming van het glazuur. Een verstoring van dit proces in JEB-nH patiënten resulteert in glazuurdefecten in het hele gebit, bestaande uit hypoplasie, putjes, ruwheid, groeven en het dunner worden van het glazuur. In **hoofdstuk 9** bespreken we twee heterozygote dragers van de nooit eerder beschreven *LAMA3* deletie c.488delG, hetgeen betekent dat bij hen op één allel van het *LAMA3* gen de pathogene mutatie c.488delG aanwezig is, terwijl op het andere allel geen pathogene mutaties aanwezig zijn. Deze dragers hadden geen last huidafwijkingen, maar wel van glazuurdefecten in hun gebit, bestaande uit ruwheid en putjes die hebben geleid tot een

verhoogd risico op cariës in beiden. Wij veronderstellen dat haploïnsufficiëntie (als er maar één functionerende kopie van het gen aanwezig is) van de laminine α3 keten de oorzaak is van deze glazuurafwijkingen, aangezien de mutatie c.488delG een frameshift creëert, die resulteert in een vroegtijdige stop codon en mRNA verval, en omdat er in de cDNA analyse geen alternatief gekliefde producten werden gezien. We hypothetiseren dat er in het complexe proces van glazuurvorming, waarin LM-332 een belangrijke rol speelt, er geen dosis compensatie mechanisme bestaat voor het verlies van één *LAMA3* allel, terwijl deze mechanismen wel beschikbaar zijn in de huid. Het blijft echter merkwaardig dat er nooit eerder glazuurdefecten zijn gemeld bij dragers van mutaties in *LAMA3, LAMB3* of *LAMC2* die leiden tot haploïnsufficiëntie.

In **hoofdstuk 10** laten we zien dat de zeldzame ziekte “JEB of late onset” (JEB-lo) wordt veroorzaakt door mutaties in *COL17A1*. Twee JEB-lo patiënten, een broer en een zus, hebben vanaf hun zesde levensjaar last van blaren op de voeten en rond de nagels. Op latere leeftijd kregen zij ook blaren op de handen, neus en het mondslijmvlies. Andere klinische kenmerken waren nagelafwijkingen, atrofische huid op de scheenbenen, glazuurafwijkingen en excessief zweten op de handpalmen en voetzolen. Met IF kleuring en TEM werden er subtiele veranderingen gevonden. De meest opvallende veranderingen zijn het verlies van de Col17 kleuring in het apicaal-laterale membraan van de basale epitheellaag en de verbrede distributie van de kleuring van het Col17 ectodomein, LM-332 en type VII collagen (Col7). Mutatie analyse van *COL17A1* toonde in beide patiënten de nooit eerder beschreven mutatie c.1992_1995delGGGT op het ene allel en de bekende mutatie c.3908G>A op het andere allel. De mutatie c.1992_1995delGGGT resulteert in een vroegtijdige stopcodon en mRNA verval, waardoor de patiënten functioneel hemizygoot zijn voor de missense mutatie c.3908G>A (p.R1303Q). We hypothetiseren dat de mutatie p.R1303Q, die gelegen is in het Col17 ectodomein, resulteert in een verstoorde interactie met zijn bindingspartners en daardoor interfereert met een correcte opbouw van het extracellulaire matrix. Dit zou ook de verbreding van de IF kleuring van LM-332 en Col7 kunnen verklaren, aangezien het ectodomein van Col17 een interactie heeft met LM-332 en daardoor ook met Col7. Hoewel we de pathogenese van JEB-lo hebben aangetoond, zijn alternatieve pathogeneses niet uitgesloten en is verder onderzoek in andere JEB-lo patiënten noodzakelijk.

In **hoofdstuk 11** presenteren wij vijf nieuwe monoklonale antilichamen tegen het endodomain van Col17, genaamd VK1, VK2, VK3, VK4, en VK5. Deze zijn effectief in IF kleuring, immunohistochemie kleuring, en western blotting. IF kleuring met de VK

antilichamen kan onderscheid maken tussen gezonde proefpersonen en alle Col17-gerelateerde EB subtypes en dragers van *COL17A1* mutaties (dragers). Tevens is het mogelijk om een onderscheid te maken tussen de (bijna) afwezige kleuring in gegeneraliseerde JEB-nH en de verminderde kleuring in gelokaliseerde JEB-nH en dragers. In dit hoofdstuk presenteren we ook een model van de biologische turnover van Col17. Nadat Col17 gemaakt is, wordt het getransporteerd naar het apicaal-laterale celmembraan waar het een reservoir vormt om in nieuw gevormde hemidesmosomen ter plaatse van het basale celmembraan te worden ingebouwd. In het geval van een verminderde Col17 productie, zoals in gelokaliseerde JEB-nH en carriers, zal het apicaal-laterale reservoir sneller slinken, aangezien het aanwezige Col17 nodig is voor incorporatie in hemidesmosomen. Hierdoor is er een verlies van de apicaal-laterale kleuring van Col17 te zien en dit is voor de patroonherkenning in IF onderzoek van groot belang, waarbij de observatie van het verlies van de apicaal-laterale kleuring mogelijk zelfs belangrijker is dan het verlies van de kleuring van de basaalmembraan zone (BMZ). Na de incorporatie van Col17 in hemidesmosomen en migratie van basale keratinocyten, wordt het ectodomein van Col17 gekliefd en levert dit twee extra Col17-vormen in de BMZ op: LAD-1 en LABD97. Nadat ze gekliefd zijn, accumuleren zij in de BMZ tot latere degradatie. Dit model toont aan dat monoklonale antilichamen tegen het Col17 endodomain het meest gevoelig zijn bij het detecteren van een gereduceerde Col17 productie, omdat deze alleen het ongekliefde Col17 herkennen, terwijl de antilichamen tegen het Col17 ectodomain zowel het ongekliefde als de gekliefde producten herkent.

In **hoofdstuk 12** bespreken we de Nederlandse basale EBS cohort die bestaat uit 64 indexpatiënten. De blaren van deze patiënten laten een splijting door de basale epitheelcel zien. Zevenenzeventig procent van de patiënten (n=49) hebben mutaties in de genen die coderen voor de keratinefilamenten keratine 5 (*KRT5*) of keratine 14 (*KRT14*) en 6% van de patiënten (n=4) hebben mutaties in het gen dat codeert voor het hemidesmosomale eiwit plectine (*PLEC1*).[16,17] De pathogenese van 17% van de patiënten gevallen (n=11) is nog onopgelost. Deze groep bestaat uit twee EBS-Dowling-Meara, twee gegeneraliseerde EBS en zeven gelokaliseerde EBS patiënten. Mutatie analyse van de resterende genen die ooit in verband zijn gebracht met basale EBS: *COL17A1, ITGB4,* en *DST* (die codeert voor het hemidesmosomale eiwit BPAG1-e), lieten geen pathogene mutaties in deze patiënten zien.[18-20] Mutaties in deze genen worden zelden aangetroffen in basale EBS, en omdat de bekende gevallen allemaal autosomaal recessief overerven en IF kleuring van de desbetreffende eiwitten bij alle

gevallen abnormaal was, zou de mutatie analyse beperkt kunnen worden tot een geselecteerd aantal gevallen waarbij afwijkingen in IF kleuring worden gezien en alle gevallen die autosomaal dominant overerven te excluderen. In de toekomst hopen we dat linkage analysis en next-generation sequencing de pathogenese van deze onopgeloste gevallen kan ophelderen en daarmee ons inzicht in basale EBS kan verbreden.

De gezondheidsgerelateerde kwaliteit van leven (KvL) is de impact die een ziekte heeft op de fysieke, psychische en sociale gezondheid van een patiënt.[21] Het definiëren van de KvL bij EB patiënten is belangrijk voor de patiëntenzorg, het beoordelen van de effectiviteit van (nieuwe) behandelingen en het toekennen van financiering voor EB. In Australië is specifiek voor EB patiënten een Engelse KvL meetinstrument ontwikkeld, de QOLEB (quality of life in EB) genaamd.[22] Het is een vragenlijst bestaande uit 17 items en deze meet twee factoren: het functioneren en emoties. In **hoofdstuk 13** hebben we de QOLEB in het Nederlands vertaald en laten we zien dat de Nederlandse QOLEB een betrouwbaar en valide instrument is om de KvL van volwassen EB patiënten te beoordelen. De 55 patiënten die hebben deelgenomen aan de studie zijn verdeeld in vier EB subtypen: EBS, JEB, dominant DEB (DDEB), en recessief DEB (RDEB). De Nederlandse QOLEB is in staat om de meer ernstige subtypes RDEB en JEB te onderscheiden van de wat mildere subtypen EBS en DDEB. Significante verschillen werden gezien in de functioneren schaal en de totale schaal, maar niet in de emoties schaal. Dit suggereert dat de mentale belasting van EB gelijk is in de mildere en meer ernstige subtypes. Volgens de Nederlandse QOLEB is de KvL van de Nederlandse EBS en DDEB patiënten gering aangetast en is de KvL van de Nederlandse JEB en RDEB patiënten matig aangetast.

Referenties

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