# Substantiating atypical phenotypes of epidermolysis bullosa

Gostynska, Katarzyna Barbara

Chapter 7

# Discussion and future perspectives

Katarzyna B. Gostyńska

Centre for Blistering Diseases, Department of Dermatology, University of Groningen, University Medical Centre Groningen, Groningen, the Netherlands

## SUBSTANTIATING ATYPICAL PHENOTYPES AND BEYOND

The aim of this thesis was to further our understanding of the divergent phenotypes seen in the inherited blistering disease, epidermolysis bullosa (EB). In this final chapter, I would like to extract the main findings of the studies presented in **Chapters 2-6** and discuss them in a broader perspective with focus on future research of EB.

The *PLEC* gene was a prominent centre of focus for the study of a novel subtype of EBS (**Chapter 2**) and the solved cold case of EBS with muscular dystrophy (EBS-MD) (**Chapter 3**). For this reason, this chapter begins with a collective discussion on how these two studies impact the current understanding of the plectinopathies and genotype-phenotype correlations in EBS-MD. Next, I will discuss the annotation of the EB phenotype and what this could mean for EB diagnostics based on conclusions drawn in **Chapters 5** and **6.** Furthermore, I describe the changes that have taken place in the time that this thesis was finalized with regards to mutation analysis and how the described intronic mutations (**Chapters 3** and **4**) can be sequenced. Finally, I conclude with recommendations for studying unsolved EB cases in the future

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## THE PLECTINOPATHIES AND ALTERNATE SPLICING OF *PLEC*

Plectinopathies observed in humans can be divided into isoform specific and non-isoform specific. The currently known disease phenotypes are presented in **Chapter 2**.

### Unique plectin isoforms 1a and 1c

Plectin is a key cytolinker protein mediating cell-cell adhesion in an array of tissues including, but not limited to skin, muscle, and myocardium. [1,2] Consistent with this, mutations in the plectin gene *PLEC*, can result in multi-organ disease primarily manifesting with skin fragility and myopathy. [2] Multiple gene transcripts are generated from the single *PLEC* gene, which differ in their N-terminal sequences; specifically unique first exons. These transcripts are then translated into eight different tissue specific isoforms in humans. [3,4] The most prominently expressed isoform of plectin in mouse and human keratinocytes *in-vitro* is plectin 1a, followed by plectin 1c, 1, 1f and 1d. [5]

In **Chapter 2**, the objective of our study was to characterize phenotypic and biologic findings of two patients with an isoform specific homozygous nonsense mutation in isoform 1a of *PLEC.* The homozygous mutation caused EB simplex (EBS) without muscular dystrophy, which we classified as a new skin-only plectinopathy, EBS-plectin 1a. These results implied that the plectin 1a isoform is the dominant plectin isoform in the basal epidermis supported by the following findings:

- An identical pattern and intensity of pan plectin staining (targeting all isoforms of plectin) and isoform specific 1a staining at the basement membrane zone (BMZ) of healthy skin. This meant that the protein being expressed at the BMZ is for the greater part composed of the 1a isoform.
- The diminished plectin 1a mRNA (messenger RNA) expression seen in patient keratinocytes and fibroblasts.
- An 84% reduction of total plectin polypeptide expression in keratinocyte extracts observed in the two studied patients.

These data suggest that a homozygous nonsense mutation affecting the specific 1a isoform reduces total plectin in the basal epidermis under the threshold of functional and structural compensation by other isoforms and/or other cytolinker proteins. Previously, the group of Prof. Wiche in Vienna first suggested that the 1a isoform is the crucial plectin isoform expressed by epidermal keratinocytes, which recruits polypeptides for hemidesmosome (HD) formation. [6] They performed extensive phenotypic analysis of the knock-in Ogna mouse that, like our patients, exhibited skin-only EBS without muscular dystrophy. The Ogna mouse carries an autosomal dominant missense mutation in the plectin rod domain (c.5998C>T, p.Arg2000Trp). Although not isoform specific, studies on the Ogna mutation disclosed distinct functions and cellular sub-localization of main plectin skin isoforms, 1a and 1c. [6] The Ogna missense mutation affecting the common plectin rod resulted in defective HD formation, which occurred through a degraded 1a, and not 1c isoform. This resulted in an exclusive skin fragility phenotype. Evidently, when 1a is affected, be it through an isoform specific mutation or through the Ogna mutation, a skin phenotype will occur sparing muscle.
When considering the epidermis as one entity, plectin isoform 1c predominates, accounting for 80% of plectin expression in the epidermis. [6,7] This ratio differs when looking at cultured primary keratinocytes, which offer more than 50% expression of isoform 1a. [8] As primary keratinocytes lack suprabasal layers, they express plectin 1a as their dominant isoform, which is consistent with our findings that 1a is dominantly expressed in the basal epidermis. Evidently, isoform 1c is expressed in the suprabasal layers of the epidermis and in striated and cardiac muscle, and nerves. However, to date, there have been no reported mutations of suprabasal cleavage with muscle dystrophy and neuropathy in patients affected with *PLEC* mutations. The 1c expression in the suprabasal layers may be redundant, and fully compensated for by other cytolinker proteins. [9]
Plectin isoform 1c is also found to be the only major isoform in neurons of the central nervous system. Fuchs et al. investigated plectin 1c deficient mice, and observed decreased motor nerve conduction velocity identified in the sciatic nerve, and no skin fragility. [10] Similar investigations as those done in **Chapter 2** could be performed should a patient present with an isoform specific mutation in isoform 1c.
Currently, there is much on-going research aimed at the individual characterization of the remaining specific plectin isoforms. [9,11,12] Recent work done in Vienna by the group of Prof. Wiche, has shown in murine models that isoform 1 of plectin is involved in myonuclear morphology organization in the desmin network in murine models. Skeletal myocytes deficient in isoform 1, would be expected to display impaired stability. [11] Furthermore, the 1b and 1d isoforms have been linked to mitochondrial dysfunction and Z-disk myopathy respectively. [9,12] Human phenotypes caused by isoform specific mutations in isoforms 1, 1b and 1d remain to be reported. As the expression of these isoforms is concentrated in muscular tissues, a skin phenotype occurring from isoform specific mutations would not be expected owing to an intact isoform 1a.

## ALTERNATE SPLICING IN EBS-MD AND RODLESS PLECTIN

The most well known form of alternate splicing in EBS plectinopathies occurs in EBS with muscular dystrophy (EBS-MD). [3,13] Alternate splicing of exon 31 in *PLEC*, encoding almost the complete rod domain has been observed previously in different tissues in rat and murine

models. [3] The function of the rod in plectin conforms to that of other dimeric structures; the coiled-coil mediates dimerization with other plectin isoform monomers. Dimerization is acknowledged to be essential for the functioning of the plectin protein. [6] The non-coiled plakin repeat domains of plectin are also known to dimerize, while the globular end domains have been shown to participate in protein alignment. [14] Both these domains may therefore contribute to (functional) dimerization in the rodless plectin mice and humans. [15,16]

The fact that EBS-MD patients maintain expression of a rodless isoform of plectin provokes the question if the rodless plectin variant has a specific function and in turn, if this can be exploited for therapy. [3,17] Rodless plectin has been detected in rat tissues, mouse brain, human keratinocytes, and skeletal muscle. [18,19] In human keratinocytes, the ratio of full length to rodless plectin is approximately 21:1, however it differs between tissues and cell types. [19] In EBS-MD, rodless plectin is expressed, whereas in EBS-PA, all plectin expression is lost. [20] The group of Prof. Sonnenberg in Amsterdam recently investigated whether the presence of rodless plectin compensates for full-length plectin in EBS-MD in turn ameliorating the phenotype when compared with EBS with pyloric atresia (EBS-PA). [20] Rodless knock-in mice (rodless plectin mice) did not show skin blistering or myopathy after extensive follow-up. Furthermore, ultrastructurally, hemidesmosomes were normal in number, distribution and morphology. Immunofluorescence staining of muscle showed a normal plectin pattern. A discriminating factor, which should be mentioned, is that the rodless plectin and mRNA expressed in rodless plectin mice was significantly higher than that of full-length plectin in their murine counterparts. This observation could partially contribute to the efficacy through which rodless plectin compensates for loss of full-length plectin in the rodless plectin mice. Accordingly, it is possible to hypothesize that increasing the amount of rodless plectin in patients with EBS-MD might further ameliorate symptoms in these patients. If this is true, future research should be undertaken to investigate whether treatment strategies focussed on promoting exon 31 skipping, where the majority of EBS-MD mutations are located. This in turn would produce more rodless plectin possibly having a therapeutic effect in EBS-MD patients. Currently, antisense oligonucleotide mediated exon skipping is in the preclinical stages of research for dystrophic EB and a similar approach might be used in the case of plectin in EBS-MD. [21]

## RESCUE OF LETHAL EBS BY ALTERNATE SPLICING

Autosomal recessive nonsense or frameshift mutations in common plectin exons preceding and following exon 31 of *PLEC* have been reported to result in lethal EBS-PA. [21] In EBS-PA, plectin expression in skin is lost and death occurs shortly after birth in contrast to EBS-MD, in which patients maintain some plectin skin expression and display moderate skin involvement, surviving to early adulthood. [17,19] This classification was put into question while studying the proband described in **Chapter 3**. Due to a homozygous intronic deletion, the wild-type splice site in exon 8 of *PLEC* was compromised, with as a consequence intronic retention and a premature termination codon (PTC). However, translation still appeared possible using an alternate 5' splice site, resulting in a plectin protein that was 4 amino acids shorter. The patient developed EBS-MD and not, as expected from the localization of the *PLEC* mutation, lethal EBS-PA. The almost full-length protein, produced by virtue of the

alternate splice site, not only saved the patient from developing a lethal variant of EBS, but also resulted in a clinically milder, forme fruste of EBS-MD.

**A novel physiologic plectin isoform**

A serendipitous finding in **Chapter 3** was that the internally truncated protein produced by the patient was also a physiological isoform found in skin, striated muscle and myocardium of healthy individuals. The missing four amino acids (299-302) encode the last portion of the actin-binding domain (ABD) in full-length plectin (see **Chapter 1**, Figure 3). [22] The interaction of plectin with integrin α6β4 secures attachment of the intermediate filaments to the extracellular matrix. Plectin accomplishes this by connecting to the integrin α6β4 unit through the ABD. Earlier work has shown that the key ABD residues, which bind plectin to the β4 subunit are 148-150. [23] Thus, the four that are missed in the shortened transcript would theoretically not abolish this key interaction. Additional transfection studies would reveal whether lack of these four amino acids results in a detrimental effect on the hemidesmosome *in vitro* or if their presence in plectin is functionally redundant.

The functionality of the shortened transcript in target tissues is unknown. Further studies investigating other alternative plectin transcripts and their significance is warranted. Ideally, RT-PCR of mRNA extracted from cultured keratinocytes, myocardium, and muscle samples of a large population of healthy individuals covering the entire cDNA of *PLEC* could be performed to identify other alternate transcripts.

## DEEP PHENOTYPING OF EPIDERMOLYSIS BULLOSA

Historically, annotation of phenotype in genetic disease has varied in literature, often seen as incomplete or imprecise. Mutation reports in EB seldom describe precise clinical and biological characteristics in a structured and consistent manner. As shown in **Chapters 2-6**, subtle differences in both clinical and cellular phenotype of patients affected with one main subtype of EBS, but occurring from mutations in two different genes, can assist the dermatologist with quick diagnosis, can help prognostic advice and can better assist in confirming genetic variants.

The precise analysis of individual elements of a phenotype has been defined as 'deep phenotyping' where all individual elements encompassed within a phenotype are assessed. [23] Affected individuals are grouped together based on their common characteristics. Their genomic information can then be used to look for, among others, common causal variants, response measurement to treatments, and epigenetic data markers. [24]

In the following paragraphs I will briefly discuss genotype-phenotype correlations described in **Chapters 5** and **6**.

**Enamel and laminin-332 mutations**

In **Chapter 5**, we embarked on deep phenotyping of enamel pitting, a specific trait seen in junctional epidermolysis bullosa (JEB). Focal enamel abnormalities, in a subtle, localized form were manifested by parents of JEB patients, who were heterozygous *LAMA3* functional null mutation carriers. This allowed us to consider this feature in a unique, isolated state; in the absence of skin blistering or other JEB traits. The young child affected with JEB in Family 351 described in **Chapter 5**, had inherited a splice-site mutation in *LAMA3*, from her

mother who had enamel abnormalities. The observation of less than 2% α3 chain expression in the child indicated that the splice-site mutation c.4684+1G>A carried by her mother affected with dental abnormalities, behaves like a null mutation. This fact alone was one step forward in suggesting that half a dose of the α3 chain of laminin-332 will compromise the architecture of enamel, however not of skin. Further studies are warranted which would quantify laminin-332 in carriers, beginning with western blotting using keratinocyte extracts. Earlier reports of abnormal dentition in heterozygous carriers of *LAMB3* mutations show much more severe dental abnormalities than our reported *LAMA3* mutation carriers.[25-27] In the *LAMB3* carriers, the extent of dental abnormalities was classified as amelogenesis imperfecta (AI), whereas in *LAMA3* carriers, dental aberrations were localized and asymptomatic.[25-27] The precise reason for the difference in severity between the two genes is unknown. Most striking is that all reported *LAMB3* mutations associated with AI are thought to escape nonsense-mediated mRNA decay (NMD), therefore generating a truncated protein (see Table 1 in **Chapter 5**). All but one, are located in the last exon[25-27] Subsequently, a mutant protein is likely expressed, which exerts a dominant negative effect more detrimental than haploinsufficiency of *LAMA3* on the laminin heterotrimer in enamel. A dominant negative interference on a structural polypeptide interferes with not only its own function but of that of the product of the healthy allele. It is well known that dominant negative effects in structural polypeptides such as collagens are more destructive than one null allele in the same gene.[28] In both situations, skin fragility is absent. The lack of skin phenotype in these carriers might be explained by compensation by other basement membrane proteins, which are not available in enamel. Another explanation might be associated with laminin-332 secretion in target tissues. In keratinocytes, laminin-332 assembly begins with the formation of first a dimer composed of the β3 and γ2 chains, after which the α3 chain becomes incorporated.[29] Considering this, the initial dimerization might be crucial for laminin-332 mediated ameloblast differentiation in enamel tissue, and severely compromised by a primarily defective dimer through the β3 chain. The fatal subtype of JEB-generalized severe is caused by null mutations in *LAMB3*.[30] Both parents of children affected with JEB-generalized severe are obligate heterozygous carriers, however to date, there are no reports of AI or other enamel abnormalities in this population. There are several possibilities for this. Carriers are not screened specifically for enamel abnormalities. Another reason might be that certain mutations in the JEB population affect the amino terminus of the laminin-332 heterotrimer, which is not involved in binding of the three subunit chains.[25] These mutations are less likely to lead to expression of a mutant protein and would rather cause knockout of the one allele. If this should happen, subtle enamel changes such as in the *LAMA3* carriers might be expected and could be missed. It is of course plausible that not all carriers have abnormal dentition, a phenomenon seen in *COL17A1* null mutation carriers.[31]

**Pruritus and dystrophic epidermolysis bullosa**

Itch in dystrophic epidermolysis bullosa (DEB), as described in **Chapter 6**, is a challenging phenotypic trait to measure and quantify. Most EB patients experience itch, as is expected in the situation of chronic wound healing.[32] A recently performed systematic review of genotype-phenotype correlations of published epidermolysis bullosa pruriginosa (EBP) cases done by the group of Prof. Pope confirms that the majority of reported cases are associated

with glycine substitution mutations, followed by in-frame insertions in *COL7A1*.[33] This supports the initial finding of EBP in our two patients affected with the heterozygous mutations: c.6227G>T, p.Gly2076Val (Patient 1) and c.6128G>A, p.Gly2043Glu (Patient 2). The subtle difference from classic EBP seen in two young women with epidermolysis EBP-excoriée was carefully considered, because of the immense psychosocial impact and atypical clinical presentation. What was different here than for classic DEB pruritus was that itch was felt on clinically healthy, non-lesional skin provoking habitual scratching. This excoriée variant goes one step further towards deep phenotyping than the classic description of pruritus. Further analyses of pruritus encompassing precise onset, alleviating and aggravating factors and response to treatment in EB are important, because of the dramatic impact on quality of life. Classification is well underway for chronic pruritus, and the use of grouping pruritic complaints based on skin changes should be regularly employed for better targeted therapy development.[34]

**Deep phenotyping and the cold cases**

When considering cold cases, meticulous analysis of clinical and biological traits, in the form of deep phenotyping has in previously reported cases helped identify candidate genes. Ultimately, better classification of phenotypic traits in each subtype of EB should be the goal of treating centres. This is an immense challenge due to a number of issues. The phenotype can drastically change from that seen in the neonate vs. adult patients. Skin changes can be subtle, go unnoticed by both the clinician and patient themselves, and can vary between family members affected by the same genetic mutation. Therapy of EB will no doubt be aimed at implementing a personalized approach as the extent of affected skin, presence and absence of extracutaneous features, and age of onset all vary between subtypes. Characteristics, such as likelihood of aggressive cancer from chronic cutaneous and extracutaneous wounds in recessive DEB, might intensify screening regimens in these patients. A second factor such as propensity to colonization with certain forms of bacteria might direct antibiotic therapy over the course of the patient's life.

The concept of epigenetics can also be considered as a part of deep phenotyping.[35] A term with many definitions, the 'epi' is the Greek word referring to 'over' or 'outside of'; the inheritable traits, which cannot be explained by changes in a DNA sequence. One of the best examples includes altered DNA methylation affecting gene transcription. Others, such as environmental and developmental factors may influence EB disease phenotypes but would not be revealed by any form of DNA sequencing. To date there have been no reports of epigenetic phenomena in EB.

One way to structurally organize phenotypic findings as a global EB community may be through the Human Phenotype Ontology (HPO). The HPO is a platform, which aims to computationally organize phenotypic data from all forms of human disease.[36,37] HPO includes a standardized vocabulary of more than 11,000 entries describing defects of human disease conditions.[36] The value of deep phenotyping was exemplified in the study performed by Westbury et al. of a patient population affected with bleeding and platelet disorders.[34] By precisely characterizing the patient phenotype they assigned specific HPO terms to affected individuals. They showed that with statistical cluster analyses, causal gene variants were likely to cluster based on their common HPO-coded phenotypes. Although this route

to organizing phenotype within the spectrum of EB is an interesting one, there are several obstacles to overcome. Bleeding and platelet disorders are much more commonly encountered than EB, which may impede large-scale analyses. Sample sizes for such analyses must be large and heterogeneous enough in order to significantly facilitate locus identification and later gene discovery. Collective collaboration of EB centres would also assist in data collection however it would still remain challenging to find multiple affected individuals with same phenotype. Also, as described in this thesis, many pathogenic variants in EB genes are extremely rare **(Chapters 2** and **3)** and using HPO terms for deep phenotyping would not assist in identifying pathogenic variants in these families.

## REVOLUTIONIZING GENOME DIAGNOSTICS IN EB

### Next generation sequencing

In recent years, sequencing of individual candidate genes in inherited diseases caused by multiple genes using Sanger sequencing (SS) has slowly become phased out, and replaced with the introduction of Next Generation DNA sequencing (NGS). [38,39] One of the first successful clinical applications of NGS was in a rare disease called Miller Syndrome. [40]
NGS is a catch-all term referring to modern sequencing technologies, which all have in common parallel gene sequencing of DNA. This can be done using the entire genome termed whole genome sequencing (WGS) or specifically the protein- coding component of the genome, the exons, in whole exome sequencing (WES). WGS screens more than 3 billion nucleotides of human DNA, identifying anywhere between 2-3 million variants in an individual, whereas WES scans the exons of human genes (less than 2% of the genome) and discloses approximately 20,000-30,000 variants.
The concept of NGS entails: reading (sequencing) DNA, identifying variations in DNA, comparison of variations with a reference library and filtering the data set based on different factors. For a detailed review of NGS technologies see Metzker et al., 2010. [41] In the following paragraphs, the advantages of using WES in EB diagnostics and research will be discussed, followed by the limitations together with suggestions for overcoming them.

### Advantages of WES

**Parallel, multiple gene sequencing:** EB is an extremely heterogeneous skin disease owing to the diversity of the many involved genes. WES, in the form of targeted gene panels in EB is defined by parallel analysis of all protein encoding regions (exons) and flanking introns of the 18 EB genes, providing an attractive option for effective molecular diagnosis. With the number of pathogenic genes continuously growing, targeted gene panels offer the most efficient molecular analysis over SS. [42] This has caused a drastic shift when approaching classic EB diagnosis. The advantage over SS is the time saved with parallel scanning of all genes in one test, vs. individual sequencing of genes one by one. The quality of WES is constantly improving and the idea to perform WES standard for all diseases and eliminate targeted disease panels is also becoming an option.
**Comprehensive coverage of exons:** Current genetic knowledge is majorly limited to exons, facilitating data sorting of relevant and irrelevant variants. Variants are disclosed in all known EB genes, which will show not only causative variants, but polymorphisms carried in other EB genes. In addition to identifying pathogenic variants, polymorphism will be

sequenced which might be of interest when studying genetic modifiers.[43] An example of this was shown when Sproule et al. studied a JEB murine model homozygous for a JEB generalized intermediate *LAMC2* mutation.[43] They showed that seemingly innocuous allelic variants of *COL17A1* were a strong genetic modifier in this form of EB, and affected the BMZ adhesion in the presence of the *LAMC2* mutation.[43] Such genetic modifiers involved in EBS might include proteins influencing keratinization, wound healing and hemidesmosomal stability. Aside from known EB genes, collective polymorphisms in genes related to epithelial structural and functional proteins might contribute to the clinical presentation of EB. An example of this possibility was reported recently by Posalvafi et al., while studying the genetic cause of patients with inherited cardiomyopathies for which there are over 70 disease-causing genes. They identified a variant cluster of (mostly) missense mutations encoding the plectin rod domain in the *PLEC* gene in Dutch and UK patient cohorts.[44] Based on their findings, they hypothesized that these specific genetic variations might impact cellular junctions and increase susceptibility for cardiomyopathy.[44]
Genetic modifiers might be more useful when studying phenotypic variability within families, but WES will identify these variants and may even reveal bigenic causes of EB. However, proving causality of a detected variant is enormously difficult and deems convincing functional analysis.

**Limitations of WES**
Although WES has revolutionized genome diagnostics, there are certain drawbacks which should be mentioned.
**Data interpretation:** Although limited to exons, thus to 2% of the human genome, data interpretation in WES is the biggest challenge in both the clinical and research setting. Filtering of variants is possible with different algorithms that can classify variants as benign, likely benign, variants of unknown significance, or pathogenic. To do this, generally speaking the data interpretation consists of assessing pathogenicity by using prediction software, segregation analysis within families, and checking frequency of the suspected variant in population databases. Certain types of mutations remain difficult to capture even with NGS technologies, such as insertion/deletion mutations.

In **Chapter 4**, SS had repeatedly failed to disclose pathogenic mutations in *KRT5*. We performed WES using three family members furthest away from one another in the pedigree in order to minimize the number of shared variants between individuals. Regardless, after employing standard filters, these three individuals still shared hundreds of variants which had to be sorted. It was later determined that the forward read of the primer pair covering the region of the intronic mutation in initial SS provided a double sequence, whereas the reverse had missed the mutation.

When a suspected variant is found with sequencing techniques, the pattern of inheritance may be difficult to determine and even WES does not overcome this hurdle. Identification of a heterozygous variant in an autosomal dominantly inherited disease in a family may not necessarily indicate pathogenicity and this problem is not overcome by WES. An example of this with relation to EB genes occurs in mutations in *KRT14.* Heterozygous nonsense or

frameshift mutations in the first codons of *KRT14* result in Naegeli-Franceschetti-Jadassohn syndrome, which was hypothesized to arise from haploinsufficiency of keratin 14. However, heterozygous carriers of recessive nonsense *KRT14* mutations, which when inherited in a autosomal recessive form cause EBS, are clinically healthy putting this theory into question. [45,46]

**Missed noncoding regions:** With the exception of the splice site regions, variations in non-coding regions will be missed. These include intronic sequences, together with promoter and regulatory untranslated regions of genes. As presented in **Chapters 3** and **4** of this thesis, intronic mutations may, however, harbour pathogenic mutations. DNA sequencing with SS and WES is directed to sequence the protein encoding regions of the genome, the exons and exon/intron flanking sequences spanning 50-100 nucleotides. [47] The region of interest however, which is chosen for interpretation are the flanking sequences, typically 5-10 nucleotides at the intron/exon border. Intronic mutations are pathogenic in different situations. Typically, mutations affecting 5'or 3' conserved di-nucleotides in splice sites can lead to aberrant splicing. [48] Mutations affecting the di-nucleotides GT and AG, which are conserved at the 5'and 3' splice sites respectively can result in intron retention (**Chapter 3**), exon skipping (**Chapter 4**). In both cases of pathogenic intronic mutations described in this thesis, there was overwhelming biological evidence pointing to candidate genes *PLEC* and *KRT5/KRT14*. If intronic mutations are far enough into the intron such as presented in Chmel at al., [49] they will be missed in WES. The described intronic mutation in **Chapter 4** was located at the +2 nucleotide affecting intron 6. The proximity to the exon/intron border allowed it to be detected by WES.

**Incidental findings:** The discovery of pathogenic variants not associated to disease remains an ethical issue in NGS practise, i.e. incidental (secondary) findings of (amoung others) neurodegenerative disease and cancer. There is no international consensus as to what information must be communicated back to the physician (issuing the WES) and patient. [38,41]

**Overcoming the hurdles of WES**

**Directed data interpretation and limiting incidental findings:** As mentioned earlier, the number of genes analysed for each patient is limited by employing targeted capturing such as in the targeted gene panels. Generally speaking, data interpretation begins with routine filters, which allow separation of sequenced variants occurring in healthy populations from those sequenced in patients. A directed choice of standard filters virtually eliminates the chance of incidental findings because of the strict selection of genes. Currently, variants are screened against an in house reference database of sequenced individuals, and other databases in order to eliminate as many benign variants as possible which are present in individuals not affected with the disease. Afterwards, variants in genes screened by targeted panels are considered. Supporting information on phenotype and biopsy findings obtained in the clinic prior to sequencing would indicate a subtype of EB such as EBS, with for example a strong suspicion of a keratin mutation. Variants occurring in these genes (ie. *KRT5* and *KRT14*) would first be investigated and eliminating the chance of incidental findings. Finally, segregation analysis would follow to assess whether all affected individuals within a family carry the suspected variant(s). Causality of the variant is also supported by identical pattern of inheritance within all affected individuals.

**Sequencing noncoding regions:** Should DNA sequencing with WES fail to reveal suspected variants and the clinical findings strongly indicate a certain candidate gene, noncoding regions can be screened in order to exclude mutations lying outside of exons. A possible method is by performing RT-PCR on mRNA isolated from skin and/or blood. An aberrant splicing pattern could point to an intronic mutation. When this method is employed for all mRNA isolated from target tissue, this technique is referred to as RNA sequencing (RNA-seq), which is based on generating copy DNA (cDNA) sequences assembled from RNA isolated from target tissue (skin or epithelia).[50,51] Subsequently, from the mRNA library is produced and compared to a reference sequence. As opposed to traditional hybridization based methods, there is no need for probe design. RNA-seq can reveal transcriptional start sites, alternative promoter sequences and inform on premature transcription termination.[52] Also, information of different mRNA isoforms derived from alternative splicing (occurring for example in *PLEC* and *COL7A1*) are disclosed.[52] Gene expression in organisms varies within cells, changes with different stimuli such as inflammation and can vary over time with development of an organism. RNA-seq can quantify transcript level and monitor changes seen in different developmental stages. RNA-seq might be an interesting platform in particular to study EB phenotypes in which amelioration of disease is traditionally seen over time (EBS, generalized severe **Chapter 4**).

## SKIN BIOPSIES ARE NOT OBSOLETE IN EB

With the emergence of NGS techniques, the primary emphasis is being put on molecular diagnosis instead of on skin biopsies. Since a simple blood test can be used to screen for a genetic mutation and make an EB diagnosis, should the dermatologist still take a skin biopsy? This largely depends on the particular patient, clinical presenting symptoms, disclosed genetic mutation, or lack thereof. As presented in **Chapter 1**, biological clues have lead to the discovery of causative genes in many cases. Other important indications are the following:

**Quick EB diagnosis**: Skin microscopy techniques still offer the quickest diagnosis of EB with immunofluorescence antigen mapping (IF), within 24hrs which can later be confirmed with transmission electron microscopy (TEM) (the Groningen experience). This short period may be a strong argument for taking a skin biopsy when there is clinical suspicion of EB in a neonate. Although currently, therapy is supportive for all newborns presenting with EB (with the exception of JEB-severe generalized for which it is palliative in the Netherlands[30,53]), a diagnosis or exclusion thereof within 24 hrs can avoid the standard current waiting time of up to two months for a mutation result.

**Novel splice-site mutations:** Acquirement of skin biopsies is essential is when mutation analysis reveals splice-site mutations (and example is shown in **Chapter 4**). The consequences of splice-site mutations on RNA must be assessed in order to determine the resulting effect on expressed protein.

**Verification of causal genetic variants**: Conclusions drawn from IF and TEM together with phenotype assessment will first of all point out the initial candidate genes suspected of containing mutations. This way the clinical geneticist can focus on variants in genes associated with certain subtypes. Also, the number of genes in which noncoding regions should be checked (i.e. introns) is then specified. When NGS techniques reveal variants, these variants still need to have proven pathogenicity. Biopsy data aids in determining if the variant found is in fact the cause of the patient's disease.

**Skin protein expression**: The expression level of the affected protein in the epidermis determines classification and prognosis in EB. For instance, in DEB, it has been shown that expression levels of type VII collagen correlate with the extent of the phenotype. [54] By obtaining an approximation of type VII collagen expression by IF, the clinician can approximate onset and extent of extracutaneous lesions. This is an important factor to consider for genetic counselling and prognosis in rare cases of DEB without an identified *COL7A1* mutation.

**Research:** Aside from diagnostics, the value of EB skin biopsies in dermatology research must not be overlooked. This is beautifully exemplified by the discovery of the phenomenon of revertant mosaicism in skin, in which protein expression is restored in healthy looking skin patches. [55] Furthermore, overexpression of epidermal proteins such as keratin 15 in a homozygous *KRT14* null mutation patient, first brought to light the fact that a compensation mechanism occurred in these basal keratins, thus expanding knowledge of keratinocyte biology. [56]

# FINAL CONCLUSIONS ON CHANGING EB DIAGNOSTICS AND THE COLD CASE APPROACH

Over the last couple of years, tremendous progress has been made in the diagnosis of EB. When work began on this thesis, each patient was diagnosed in the same fashion (see **Chapter 1**). With the growing knowledge on the genetic heterogeneity of EB and the introduction of NGS techniques, the classic diagnostic algorithm is changing.

In the future, the ideal diagnostic process of EB would consist of evaluation of phenotype and a diagnosis including subtype based on a genetic mutation with a DNA sample obtained by minimally invasive techniques such as a blood or saliva sample. However, as already mentioned, IF still offers the quickest diagnosis therefore should be done concurrently to obtaining a DNA sample when suspecting EB in a neonate. The proposed model described in Figure 1 is purely for obtaining a genetic diagnosis. In most cases of EB, knowledge of the pathogenic mutation suffices for diagnosis. Skin biopsies may later be taken when prognostic advice deems them necessary, for example in dystrophic EB where type VII collagen expression in skin correlates with severity of phenotype. This information has been collected in the International DEB Patient Registry (www.deb-central.org) to facilitate genetic counselling and prognostic advice for dermatologists and clinical geneticists around the world. [57]

The two most important conclusions learned from studying atypical phenotypes and cold cases described in this thesis, which are relevant for cold case studies in the future, are:

1) Necessity to re-evaluate candidate genes based on biological evidence (skin biopsies and phenotype)

2) Non-coding regions of genes may contain pathogenic variants

Based on these conclusions, the following cold case approach is proposed in Figure 2. Re-evaluation of phenotype is essential and differential diagnoses of EB should be considered and excluded. In some cases, as described in **Chapter 5**, evaluation of family members of affected patients for epithelial abnormalities might aid in identifying candidate genes such as *LAMA3* or *LAMB3* in JEB.

Skin biopsies should be re-evaluated for level of skin cleavage, protein expression, ultrastructural findings in TEM (Figure 2:II). Nanotomy, large-scale Google Earth-like online imaging using TEM, has recently been used to extensively study skin and mucosa of other blistering diseases including pemphigus vulgaris and pemphigus foliaceus. [58] The development of an ultrastructural nanotomy map of EB characteristics might assist in this. Based on these first two steps, candidate genes will most likely be pinpointed (Figure 2:III). By a strong suspicion of candidate genes such as described in **Chapters 3** and **4**, (re)sequencing of genes including exons, introns, and other non-coding regions is warranted (Figure 2:IVa). At this point, should no mutations be found in the most suspected candidate gene, WES combined with segregation analysis to identify the new causative loci would then be the following step to assess genes outside the scope of targeted disease panels. If unsuccessful, WGS can be done (Figure 2:IVb).

Although not described in this thesis, other cold cases of EBS in the Groningen cohort have been solved by simple re-sequencing of candidate EBS genes and by reconsideration of

differential diagnoses. Nonetheless, there are still families affected with EB who remain unsolved genetically. The possibility of a new EB gene still exists. A single report of homozygous nonsense mutations in *CD151* encoding tetraspanin have been previously implicated in pretibial epidermolysis bullosa in two Israeli siblings. Both reported patients had extracutaneous disease such as renal failure and deafness. Although tetraspanin is a component of mature hemidesmosomes in the skin, *CD151* null mice show no signs of skin fragility. [59] In light of the most recently discovered EBS gene, *EXPH5*, [60] the search for new EB genes should focus on proteins indirectly involved in cell-cell adhesion of epithelial tissue, such as Slac2b.

## FINAL NOTE

EB is a disease like no other. All these options present exciting challenges for future research of EB and there is still much work left to be done. By fully characterizing the disease on a biological and molecular level, potential therapies can be better aimed at a personalized approach, which ultimately will benefit the most important members of the research team, our patients.

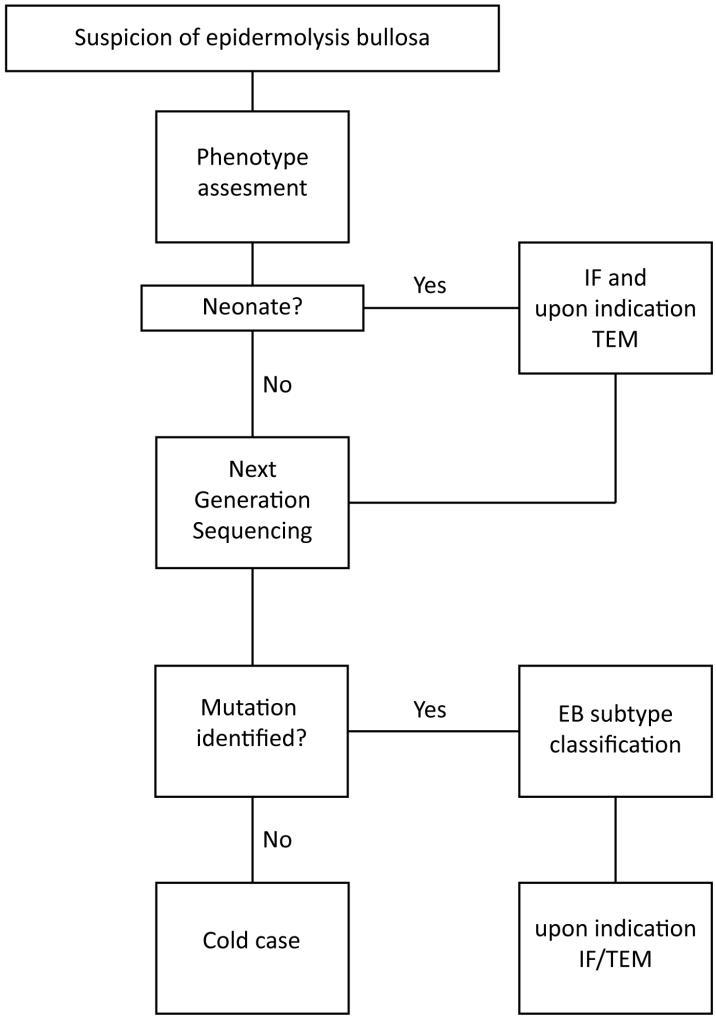


**Figure 1**. Proposed diagnostic algorithm of epidermolysis bullosa (EB). This model shows the proposed diagnostic path when suspecting EB in light of NGS techniques. IF: Immunofluorescence antigen mapping, TEM: Transmission electron microscopy

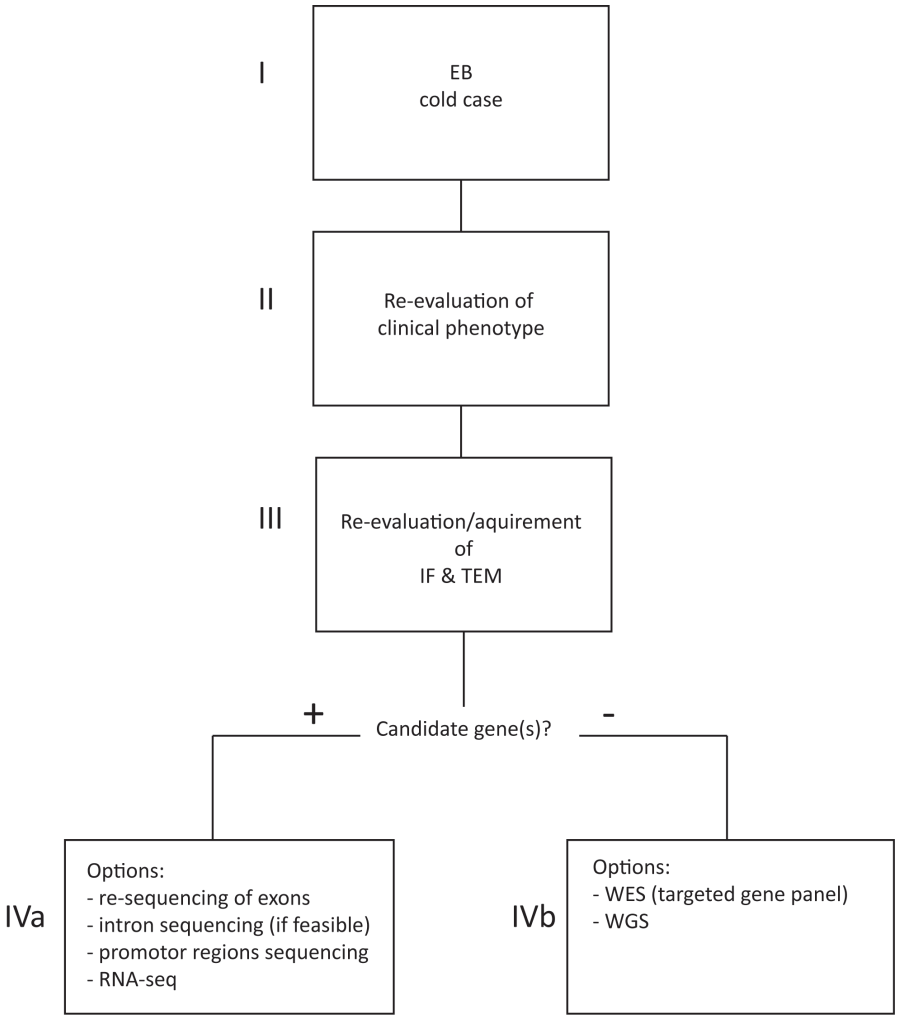


**Figure 2**. The Cold Case approach. Described are the steps in order of relevance when undertaking a cold case of EB. IF: Immunofluorescence antigen mapping, TEM: Transmission electron microscopy, WES: Whole exome sequencing, WGS: Whole genome sequencing

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