ALLOGENEIC CELL-BASED THERAPIES FOR INDIVIDUALS WITH RECESSIVE DYSTROPHIC EPIDERMOLYSIS BULLOSA

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ALLOGENEIC CELL-BASED THERAPIES FOR INDIVIDUALS WITH RECESSIVE DYSTROPHIC EPIDERMOLYSIS BULLOSA

A thesis submitted to the Faculty of Medicine of King's College London for the degree of Doctor of Philosophy

by

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DECLARATION

The work presented here is my own and all experiments, except where acknowledged in the text, were performed by myself.

Gabriela Petrof

February 2017

E-THESIS DECLARATION

Gabriela Petrof

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Relevant publications

1. Bone marrow transplantation in epidermolysis bullosa. Abdul-Wahab A, **Petrof G**, McGrath JA. 2012. *Immunotherapy* 4(12): 1859-67.

2. Serum levels of high mobility group box 1 correlate with disease severity in recessive dystrophic epidermolysis bullosa. **Petrof G**, Abdul-Wahab A, Proudfoot L, Pramanik R, Mellerio JE, McGrath JA. 2013. *Exp Dermatol* 22(6): 433-45.

3. Fibroblast cell therapy enhances initial healing in recessive dystrophic epidermolysis bullosa wounds: results of a randomized, vehicle-controlled trial. **Petrof G**, Martinez-Queipo M, Mellerio JE, Kemp P, McGrath JA. 2013. *Br J Dermatol* 169(5): 1025-33.

4. Cell therapy in dermatology. **Petrof G**, Abdul-Wahab A, McGrath JA. 2014. *Cold Spring Harb Perspect Med* 4(6): pii: a015156.

5. Potential of systemic allogeneic mesenchymal stromal cell therapy for children with recessive dystrophic epidermolysis bullosa. **Petrof G**, Lwin SM, Martinez-Queipo M, Abdul-Wahab A, Tso S, Mellerio JE, Slaper-Cortenbach I, Boelens JJ, Tolar J, Veys P, Ofuya M, Peacock JL, Martinez AE, McGrath JA. 2015. *J Invest Dermatol* 135(9): 2319-21.

Abstract

Epidermolysis bullosa (EB) is a clinically and genetically heterogeneous group of inherited blistering skin diseases that affect 500,000 individuals worldwide. The clinically more severe recessive dystrophic (RDEB) variant affects ~5% of EB individuals with a prevalence of 8 per one million of the population. RDEB is caused by loss-of-function mutations in the type VII collagen gene, *COL7A1*, which leads to reduced or absent type VII collagen (C7) and structurally defective anchoring fibrils at the dermo-epidermal junction (DEJ). From a clinical perspective, individuals with RDEB have fragile skin and are susceptible to blistering following minimal trauma, which leads to poor wound healing, scarring, contractures and oesophageal strictures. The major cause of mortality in RDEB is metastatic cutaneous squamous cell carcinoma (SCC). At present, care is mainly supportive and there are no effective treatments for this debilitating disease.

The main therapeutic challenge, therefore has been to develop gene, protein, cell and drug therapies that are safe, effective and affordable. The basis of this thesis is to evaluate safety and efficacy of allogeneic cell-based therapies and attempt to elucidate their mechanism of action in wound healing in RDEB.

To examine if allogeneic fibroblasts promote healing of chronic wounds in RDEB I intradermally injected allogeneic fibroblasts around the wound margins in an individual with RDEB. I demonstrated that these injections result in Heparin-Binding EGF-like Growth Factor encoding gene (*HBEGF)* and *COL7A1* upregulation followed by C7 production. These led to reduction in wound size by 30% at 8 months post injections. HB-EGF, a member of the EGF family, has been implicated in RDEB-associated SCC. I also assessed whether another growth factor, EGF, which is commercially available for human

use and which is related to HB-EGF, could upregulate *COL7A1* expression in RDEB epidermal cells. I demonstrated *in vitro* that 100ng/ml EGF at 90 minutes and 10ng/ml at 15 minutes led to 3-fold and 5-fold *COL7A1* upregulation in RDEB keratinocytes and fibroblasts respectively. HB-EGF also led to *COL7A1* upregulation in RDB keratinocytes (6-fold) but to a lesser extent in RDEB fibroblasts (2-fold).

To evaluate the effects of intradermally injected allogeneic fibroblasts in a larger number of RDEB individuals, I conducted a prospective, randomised, vehicle-controlled, phase II clinical trial. Twenty-six wounds in 11 adults with RDEB were injected. Fourteen wounds received fibroblasts and 12 were injected with vehicle only. I showed that allogeneic fibroblasts are safe and lead to a greater reduction in erosion area compared to vehicle within the first 28 days following treatment with a single set of injections to the wound margins, but not thereafter.

Finally, I explored the safety and potential of intravenously administered allogeneic bone marrow-derived mesenchymal stromal cells (BM-MSCs) in children with RDEB in an open-label trial. I showed that the infusions were well-tolerated, with no serious adverse events. Although, there was no increase in C7 deposition, children and their parents reported improved wound healing, reduction in blister numbers and pruritus, increased skin resilience to trauma, and reduced pain during dressing changes. In addition, significant reduction in circulating inflammatory cytokines (IL-10, $p<0.001$; IFN-γ, $p=0.04$ and IL-17A, $p=0.03$) was observed.

Taken together, these data reveal new insights into the mechanisms of action of allogeneic cell-based therapies in RDEB and provide evidence for their efficacy in wound healing and reducing morbidity in the context of clinical trials.

Abbreviations

AE	Adverse event
ATMP	Advanced therapy medicinal product
BDN	Bullous dermolysis of the newborn
BEBSS	Birmingham Epidermolysis Bullosa Severity Score
BMT	Bone marrow transplantation
BMZ	Basement membrane zone
BSA	Bovine serum albumin
CB	Cord blood
CCMO	Central Committee on Research Involving Human Subjects
CHMP	Committee for Medicinal Products for Human Use
CI	Confidence interval
CRISPR	Clustered regularly interspaced short palindromic repeats
CRF	Clinical Research Facilities
CRP	C-reactive protein
CXCR-4	Chemokine receptor type 4
C7	Collagen VII
DAMP	Damage-associated molecular pattern
DAPI	4', 6-diamidino-2-phenylindole
DDEB	Dominant dystrophic epidermolysis bullosa
DEB	Dystrophic epidermolysis bullosa
DEBRA	Dystrophic Epidermolysis Bullosa Research Association
DEJ	Dermo-epidermal junction
DEXA	Dual-energy X-ray absorptiometry
DIF	Direct immunofluorescence
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethyl sulphoxide

DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
EB	Epidermolysis bullosa
EBDASI	Epidermolysis Bullosa Disease Activity and Scarring Index
EBOS	EB Oropharyngeal Severity
EBS	Epidermolysis bullosa simplex
EC	Extracellular
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunoabsorbent assay
EMLA	Eutectic Mixture of Local Anaesthetics
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum
FISH	Fluorescence in situ hybridisation
FN	Fibronectin
FSH	Follicle-stimulating hormone
GCSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GLP	Good laboratory practice
GS	Glycine substitution
GSIS	Global severity and improvement score
GSS	Global severity score
HB-EGF	Heparin-Binding EGF-like Growth Factor
HLA	Human leukocyte antigen
HMGB-1	High-mobility group box 1
HS	Hidradenitis suppurativa
IC	Intracellular
IIF	Indirect immunofluorescence

IL	Interleukin
IMP	Investigational Medicinal Product
iPSC	Induced pluripotent stem cells
iscorEB	Instrument for Scoring Clinical Outcome of Research for EB
ITT	Intention to treat
JEB	Junctional epidermolysis bullosa
LD	Lamina densa
LH	Luteinising hormone
LL	Lamina lucida
MHC	Major histocompatibility complex
MHRA	Medicines and Healthcare Products Regulatory Agency
MP	Mottled pigmentation
MSCs	Mesenchymal stromal (stem) cells
MUSE	Multilineage-differentiating stress-enduring
NC	Non-collagenous
NHS	Normal human skin
NS	Not significant
OCT	Optimal Cutting Temperature compound
Paeds QoL	Paediatric quality of life
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR-α	Platelet-derived growth factor receptor alpha
PLA	Proximity-ligation assays
PM	Plasma membrane
PSF	Pain Sleep and Fatigue
PTC	Premature termination codon
PTM	Pre-trans-splicing molecule
PV	Pemphigus vulgaris
qRT-PCR	Quantitative real-time reverse transcription PCR

RAGE	Receptor for advanced glycation end products
RDEB	Recessive dystrophic epidermolysis bullosa
RNA	Ribonucleic acid
RPM	Revolutions per minute
SAE	Serious adverse event
SCC	Squamous cell carcinoma
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDF1-α	Stromal derived factor 1 alpha
SEM	Standard error of the mean
SLAM	Signalling lymphocyte activating molecule
SLE	Systemic lupus erythematosus
TALENs	Transcription activator–like effector nucleases
TBSA	Total body surface area
TEAE	Treatment-emergent adverse event
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TSG-6	Tumour necrosis factor-stimulated gene 6 protein
UMC	University Medical Center
ZFNs	Zinc-finger nucleases

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1.1 The cutaneous dermo-epidermal junction

1.1.1 Structural features of the cutaneous basement membrane zone

The basement membrane zone (BMZ) of the skin consists of an extremely complex network of interconnecting proteins that has a typical morphological appearance at the ultrastructural level (Eady *et al.* 1994; McGrath *et al.* 1994a). It consists of at least 20 distinct structural macromolecules that form a complex attachment zone at the dermal-epidermal interface (Lai-Cheong *et al.* 2011) (**Figure 1.1**).

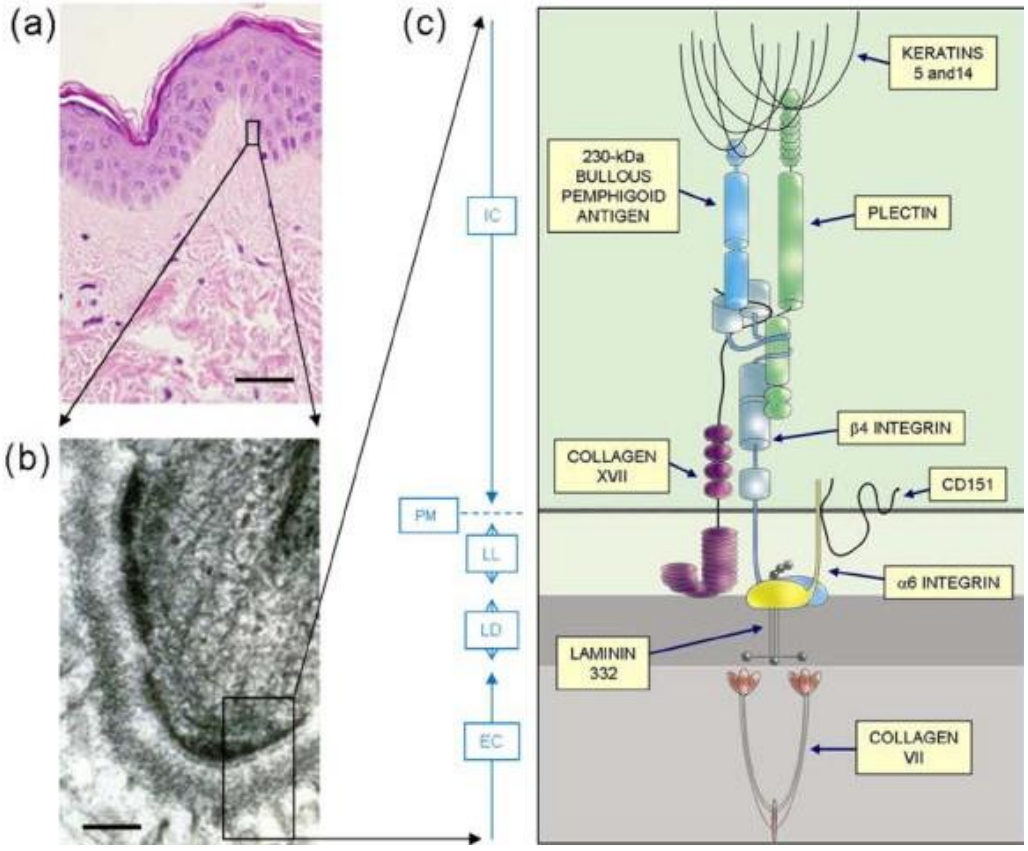


Figure 1.1 The structure of the dermo-epidermal junction and the molecular basis of the major forms of EB.
(a) Light microscopy image of the skin; the boxed area indicates the dermo-epidermal junction. (The section is stained with haematoxylin & eosin; scale bar = 50 μm); (b) Transmission electron microscopy image of the dermo-epidermal junction; hemidesmosome-anchoring fibril attachments are boxed (scale bar = 0.1 μm); (c) schematic representation of the protein organisation of dermo-epidermal attachment complexes; RDEB results from mutations in *COL7A1* encoding C7, the major component of anchoring fibrils. (IC = intracellular; PM = plasma membrane; LL = lamina lucida; LD = lamina densa; EC = extracellular). (Amended from *Trends Mol Med*. 2011 March; 17(3): 140–148).

On the epidermal side of the cutaneous BMZ, one can recognise the underlying BMZ components (Peltonen *et al.* 1989; Kurpakus *et al.* 1991; Sonnenberg *et al.* 1991). The hemidesmosomal proteins form a network that extends from the intercellular milieu of the basal keratinocytes to the extracellular space and physically secures the attachment of the epidermis to the underlying basement membrane. The core of the cutaneous BMZ consists of the basement membrane, which by transmission electron microscopy (TEM) can be divided into two distinct layers. The upper, electron lucent layer, is known as the lamina lucida and the lower portion, which is electron-dense, is known as the lamina densa. The lamina lucida is 20 to 40 nm thick and is traversed by anchoring filaments (5 to 7 nm in diameter), rich in laminin-332 and laminin-311 which interact with the extracellular domain of α6β4 integrin subunits at the cell surface of keratinocytes to form an adhesion structure with the hemidesmosomes. Between the hemidesmosomes, anchoring filaments are less abundant.

The lamina densa has a thickness which varies with age (30 to 60 nm) and is mainly composed of type IV collagen but contains also laminin-511 and laminin-322, nidogen, and heparan sulphate proteoglycans. It constitutes the intermediate anchorage zone for the anchoring filaments originating from the epidermis and the anchoring fibrils originating from the fibrillar zone of the dermis. The lamina densa is thicker in males compared to females, while the lamina lucida has a similar thickness in both sexes.

The fibrillar zone includes the anchoring fibrils which are 20 to 60 nm thick, and which enlarge at their extremities and possess central cross-banding with an irregular periodicity, either dense and thick or clear and thin (Burgeson 1993). The anchoring fibrils are composed of C7. They make the link between the lamina densa and the anchoring plaques of the papillary dermis or, more commonly, form intermingled loops

which join two different areas of the lamina densa and thus provide a bridge that is transversed by dermal interstitial collagens (type I and III).

1.2 Type VII collagen (C7)

C7 is the major, if not exclusive, component of the anchoring fibrils (Sakai *et al.* 1986). C7 molecules consist of a central collagenous, triple-helical segment flanked by the non-collagenous NC-1 and NC-2 domains. (Sakai *et al.* 1986). Cloning of the human C7 collagen gene and the corresponding complementary DNA (cDNA) indicated that the initially synthesised C7 subunit polypeptide, the pro-α1(VII) chain is a complex modular protein consisting of a central 1530-amino acid triple-helical domain (Christiano *et al.* 1994b; Christiano *et al.* 1994c). However, unlike interstitial collagens, the repeating Gly-X-Y sequence of the triple-helix is interrupted by 19 insertions or deletions of amino acids. These interruptions provide flexibility to the rod-like collagen molecules and also provide sites susceptible to non-specific proteolytic cleavage of the primary sequence. Notably, in the middle of the triple-helical domain, there is a 39-amino acid non-collagenous "hinge" region that is susceptible to proteolytic digestion with pepsin. The amino-terminal NC-1 domain of C7, approximately 145 kDa in size, consists of submodules with homology to known adhesive proteins, including segments with homology to cartilage matrix protein, nine consecutive fibronectin type III-like (FN-III) domains, a segment with homology to the A domain of von Willebrand factor, and a short cysteine and proline-rich region (Christiano *et al.* 1992). The carboxy-terminal non-collagenous domain, NC-2 is approximately 30 kDa, and it contains a segment with homology to the Kunitz protease inhibitor molecule (Greenspan 1993; Christiano *et al.* 1994b). In addition to playing a role in dystrophic epidermolysis bullosa (DEB), C7 has

been found to play a role in acquired blistering disorders, such as epidermolysis bullosa acquisita (Woodley *et al.* 2005; Chen *et al.* 2012; Vorobyev *et al.* 2015), bullous systemic lupus erythematosus (SLE) (Chan *et al.* 1999) and, more recently, inflammatory bowel disease (Chen *et al.* 2002b; Hundorfean *et al.* 2010).

1.3 Inherited epidermolysis bullosa

1.3.1 History of EB

The term epidermolysis bullosa (EB) was first introduced in 1886 (Koebner 1886). Since the introduction of the term EB there have been decades of diagnostics and research in EB. The first half of the 20th century was significant for defining phenotypes followed by recognition of the three major subtypes (simplex, junctional and dystrophic) in the 1960s (Pearson 1962). TEM established distinct planes of cleavage for these categories of EB. In the 80s and 90s, the advent of monoclonal and polyclonal antibodies to components of the cutaneous BMZ led to rapid diagnosis using skin immunohistochemistry (Heagerty *et al.* 1986; Leigh *et al.* 1988; Schofield *et al.* 1990), and identification of putative candidate genes. Currently, there are four main categories of EB (those listed above, and Kindler syndrome) with pathogenic mutations reported in 18 different genes (Fine *et al.* 2014) (**Table 1.1**).

Table 1.1 Known genes implicated in EB.
EBS, Epidermolysis bullosa simplex; *JEB*, Junctional epidermolysis bullosa; DEB, Dystrophic epidermolysis bullosa

Level of skin cleavage	Major EB type	Targeted protein	Mutated gene (s)
Intraepidermal	**EBS**	Transglutaminase 5	*TGM5*
		Plakophilin 1	*PKP1*
		Desmoplakin	*DSP*
		Plakoglobin	*JUP*
		Keratin 5	*KRT5*
		Keratin 14	*KRT14*
		Exophilin 5 (Slac2-b)	*EXPH5*
		Plectin	*PLEC*
		Bullous pemphigoid antigen-1 (BPAG1; BP230)	*DST*
Intralamina lucida	**JEB**	Laminin-332	*LAMA3, LAMB3, LAMC2*
		Collagen XVII	*COL17A1*
		Integrin α6β4	*ITGA6, ITGB4*
		Integrin α3 subunit	*ITGA3*
		Laminin-332, isoform α3A chain	*LAMA3A*
Sublamina densa	**DEB**	Collagen VII	*COL7A1*
Mixed	**Kindler**	Fermitin family homologue 1 (kindlin-1)	*FERMT1 (KIND1)*

Early observations on disease pathomechanisms were followed by identification of genetic mutations in candidate genes with diagnostic implications (Christiano *et al.* 1993; Hilal *et al.* 1993; Varki *et al.* 2006; Varki *et al.* 2007). Establishment of DNA-based prenatal testing revolutionised disease diagnostics and led to preimplantation genetic diagnosis as a further choice for couples at risk for recurrence of EB (McGrath *et al.* 1996; Fassihi *et al.* 2006). The early 21st century saw the development of preclinical model systems (Heinonen *et al.* 1999; Palazzi *et al.* 2000; Cao *et al.* 2001; Fritsch *et al.* 2008) and enabled early proof-of-principle clinical trials. Over the past 5 years, phase I/II clinical trials, which are discussed in Section 1.4, have introduced molecular therapies into the clinical setting. EB has served as a prototype of heritable skin diseases in which significant progress has been made over the past few decades from a condition defined purely by clinical description to an entity with profound understanding of the molecular defects at the genomic level.

1.3.2 Epidemiology of inherited epidermolysis bullosa

Inherited EB encompasses genetically transmitted diseases with the unifying characteristic feature being the presence of recurrent blistering and erosions, due to minor mechanical trauma, that affects certain epithelial-lined tissues and the skin.

Calculating the incidence of EB can be difficult and data vary within different populations worldwide. In the United States the incidence of EB is estimated at approximately 20 per one million live births with a prevalence of 8 per 1 million of the population (Fine 2010). EB simplex represents 92% of the total of EB cases, dystrophic EB 5%, junctional EB 1%, whereas the remaining 2% are still unclassified. Prevalence of Kindler syndrome is unknown, although more than 250 cases have been reported to date. These estimates are

very similar to the European and Japanese EB Registries (Inaba *et al.* 1989; Pavicic *et al.* 1990; McKenna *et al.* 1992; Castiglia and Zambruno 2010; Duipmans and Jonkman 2010). Of note, a greater prevalence of EBS has been reported in Scotland of at least one in 30,000 individuals (Horn *et al.* 1997). It is unclear whether this reflects possible greater accessibility of EBS patients for identification and recruitment in the Scottish Registry or the presence of some underlying genetic differences which might distinguish the Scottish EB population from those in other geographic regions. An important unifying finding among all populations is the lack of any EB predilection by gender or ethnicity (Fine and Mellerio 2009a; Fine and Mellerio 2009b).

1.3.3 Classification of EB

There have been several advances in the classification of EB since the early ultrastuctural plane of cleavage subtyping that began in 1962 (Pearson 1962). The first international consensus meeting on diagnosis and classification of EB was held in 1989 (Fine *et al.* 1991). Early classifications were based on ultrastructural determination of the plane of cleavage, as well as immunohistochemical labelling for basement membrane proteins, and were further subclassified on the basis of genetic mode of transmission and clinical phenotype, resulting in 23 EB phenotypes. The second meeting, in 1999, took into account newly described clinical entities and the results of early mutational analyses (Fine *et al.* 2000). During the third meeting, Kindler syndrome was included as a fourth subtype within the EB groups (Fine *et al.* 2008). Of note, the proposed classification separated EB into four major types, depending on the level of blistering: EB simplex (blistering within the epidermis), junctional (cleavage within the lamina lucida), dystrophic (separation below the lamina densa) and Kindler syndrome (multiple and variable levels). Patients

were then further distinguished by minor and major subtypes; Notably, EB simplex was separated in two subgroups (basal and suprabasal). A new classification system ("onion skin approach") was introduced in 2013, during the most recent international consensus meeting (Fine *et al.* 2014). The new system takes into account the investigative resources (or lack thereof) available to the clinician and the different "onion layers" can include the EB type, mode of inheritance, phenotype, immunofluorescence, antigen mapping findings, and mutation(s) present in each patient, and is the one used throughout this thesis. Nevertheless, EB is still separated into four major groups, based on the level within which blisters develop. The next level of subclassification takes into account the clinical phenotypic features present in a given patient, most notably the distribution (localised vs generalised), and severity of cutaneous and extracutaneous disease involvement. Each patient then can be further subclassified on the basis of the mode of transmission and, if identifiable, by the specific gene involved (**Table 1.2**).

Table 1.2 The 2014 proposed "onion skin" terminology–representative examples.
DDEB, dominant dystrophic epidermolysis bullosa; EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa; RDEB, recessive dystrophic epidermolysis bullosa; MP, mottled pigmentation; BDN, bullous dermolysis of the newborn.

2013 "onion skin" nomenclature (examples)
EBS localised, normal keratin 5 and 14 staining, *KRT5* or *KRT14* mutation (specify type)
EBS generalised severe, normal keratin 5 and 14 staining, *KRT5* or *KRT14* mutation (specify type)
EBS generalised intermediate, normal keratin 5 and 14 staining, *KRT5* or *KRT14* mutation (specify type)
EBS-MP, normal keratin 5 staining, *KRT5* mutation (specify type)
JEB generalised severe, laminin-332 absent, *LAMA3*, *LAMB3*, or *LAMC2* mutations (specify type)
JEB generalised intermediate, laminin-332 or collagen XVII reduced staining, *LAMA3*, *LAMB3*, *LAMC2*, or *COL17A1* mutations (specify type)
RDEB generalised severe, C7 absent, *COL7A1* mutations (specify type)
RDEB generalised intermediate, C7 reduced staining, *COL7A1* mutations (specify type)
RDEB-BDN, granular intraepidermal C7 staining, *COL7A1* mutations (specify type)
DDEB generalised, normal C7 staining, *COL7A1* mutation (specify type)

1.3.4 Molecular pathology of DEB

DEB can be inherited in an autosomal dominant (DDEB) or recessive (RDEB) pattern. All forms of DEB are caused by mutations within *COL7A1*, which encodes for the major component of the anchoring fibrils, C7 (Sakai *et al.* 1986; Keene *et al.* 1987). As of 2011, a web-based DEB patient registry (http://www.deb-central.org) contains worldwide information on 1039 DEB patients and their 659 *COL7A1* mutations (last accessed 17th March 2017) (van den Akker *et al.* 2011; Wertheim-Tysarowska *et al.* 2012). These include nonsense, missense, deletion, insertion, insertion-deletion, splice-site and regulatory mutations. Mutations occur throughout all 118 exons and flanking introns of *COL7A1*; most of which are family specific, with few recurrent or hotspot mutations. Recessive forms of DEB are associated with bi-allelic loss-of-function mutations. (**Table 1.1**).

1.3.5 Genotype-phenotype correlation in DEB

Phenotypes may show intra- and interfamilial variability with the same pathogenic variant (Murata *et al.* 2000; Nakamura *et al.* 2004). The dominant forms of DEB typically have a heterozygous glycine substitution (GS) affecting one allele and sited within the collagenous domain of C7, usually leading to a relatively mild phenotype (Varki *et al.* 2007). In contrast, the recessive forms of DEB frequently result from premature termination codons (PTC), frameshift or splice site mutations on both alleles of *COL7A1* (Christiano *et al.* 1993; Hilal *et al.* 1993; Christiano *et al.* 1994a; Hovnanian *et al.* 1994). Patients with RDEB-SG show a clinically severe phenotype because of a complete absence of C7 and total loss of functional anchoring fibrils. Patients with less severe generalised RDEB show a milder clinical picture with various amounts of C7.

Intermediate RDEB is caused by a combination of PTC, missense and splice site mutations on both alleles of *COL7A1*. Some GS mutations can underlie both dominant and recessive forms of DEB (Almaani *et al.* 2009). In part, genotype-phenotype correlation depends on the nature of the substituted amino acid, the position of the GS in C7, as well as the biochemical changes to the collagen helix (Almaani *et al.* 2011).

1.3.6 Clinical manifestations and complications of DEB

The clinical hallmarks of DEB are trauma-induced blisters and healing with scarring. The main clinical subtypes of DEB are dominant DEB and recessive DEB. There can be significant overlap in the clinical features between the subtypes. Patients with dominant DEB generally have milder clinical phenotypes, which range from mainly acral involvement to disseminated blistering and scarring. Severe mucosal involvement, however, is rare, and the teeth are usually normal. Blistering typically starts at birth or soon thereafter, but the disease activity may diminish with advancing age. There is a predilection for the extremities in severe cases, but mechanical stress can induce blisters anywhere on the body. Scars, milia, and dystrophy or loss of nails are common. Dystrophic nails, especially toenails, are often seen and may be the only manifestation of DDEB (Dharma *et al.* 2001; Sato-Matsumura *et al.* 2002; Tosti *et al.* 2003). A rare variant is EB pruriginosa which is characterised by intense pruritus and nodular prurigo-like lesions mainly localised to the extremities (McGrath *et al.* 1994b). Most cases of EB pruriginosa are autosomal dominant although some are autosomal recessive. DDEB has an excellent prognosis with normal life expectancy. Unusually, onset of the clinical manifestations of EB pruriginosa may be delayed with signs only starting in adult life.

Generalised severe RDEB, on the other hand, is characterised by extensive blisters resulting in scarring and contractures. Pseudosyndactyly resulting in mitten deformities of hands and feet start in childhood and is unique to RDEB. There is extensive mucosal involvement, particularly in the oral cavity, the oesophagus and the eyes. Feeding can be problematic due to poor dentition, microstomia, inability to move the tongue due to scarring and oesophageal strictures. Perianal involvement often leads to painful defaecation, which results in severe constipation. Further extracutaneous manifestations are discussed in Section 1.3.7. RDEB individuals have significant lifelong morbidity, with complications such as poor mobility, poor vision and dependence on others for their daily personal and skin care. The leading causes of death are sepsis, metastatic squamous cell carcinoma and renal failure.

Intermediate RDEB, has similar clinical manifestations to RDEB-SG but the blistering is less severe and less extensive. Patients usually have a better prognosis, survive longer, but the risk of SCC remains elevated.

1.3.7 Clinical features and complications of RDEB

RDEB is a multi-system, severely debilitating, disease that can have a variable clinical picture (**Figure 1.2**) that is mainly determined by the causative genetic mutations in *COL7A1* and the amount of functional C7 protein each individual is able to make.

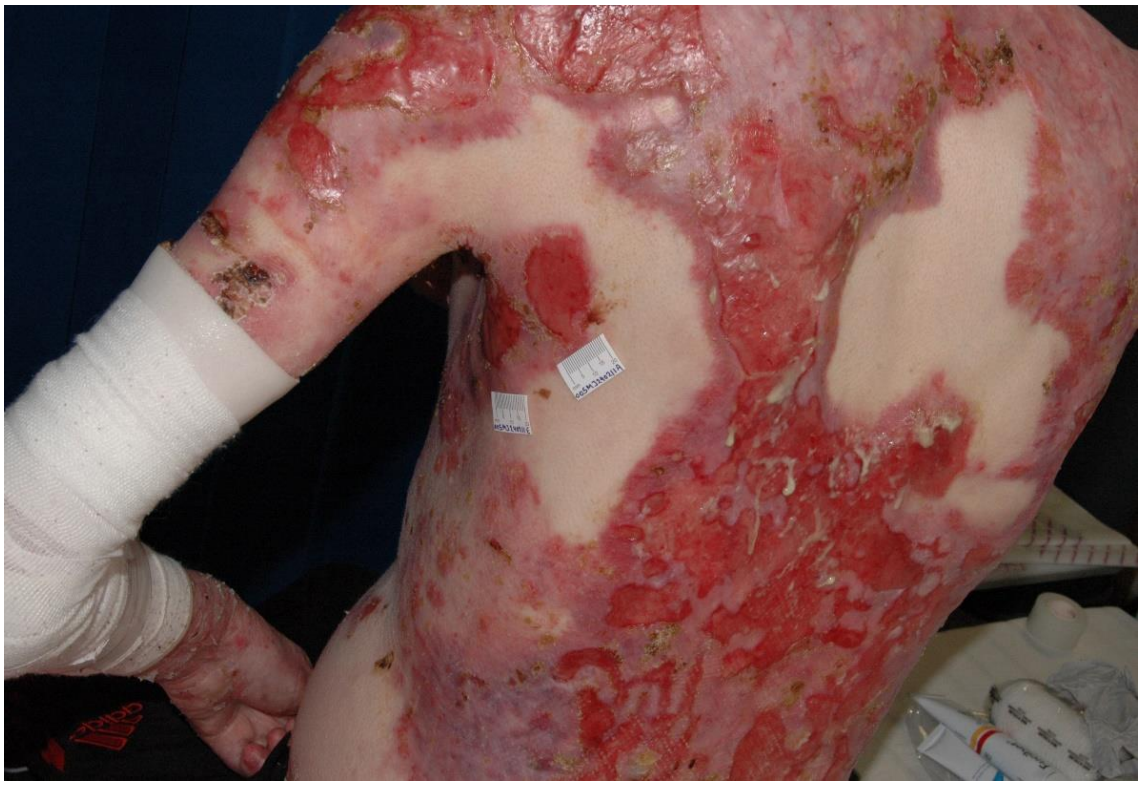


Figure 1.2 Clinical appearance of an individual with severe generalised RDEB.
There is extensive skin involvement with multiple non-healing erosions.

1.3.7.1 Cutaneous involvement

Patients with severe RDEB have extensive skin involvement. They have intact blisters and open wounds at various stages of healing. These wounds occur anywhere on the body but tend to be more pronounced on trauma-prone areas such as knees, feet, hands and elbows, and skin overlying bony prominences. Many wounds heal with scarring leading to contractures, most notably of the hands and feet. Scarring and contractures lead to loss of functional digits as these become encased in scar tissue – this is known as mitten deformity or pseudosyndactyly (McGrath *et al.* 1992a). Recurrent wound infections are also a common problem because of extensive areas of denuded skin and the accumulation of serum and moisture on the surface which enhances the growth of bacteria (Pillay 2008).

1.3.7.2 Gastrointestinal involvement

Gastrointestinal complications frequently affect the mouth, where ankylostomia and microstomia – as a result of scarring and oral mucosa fragility, lead to poor oral hygiene with resultant loss of dentition. Loss of teeth makes mastication difficult and dentures are usually contraindicated because of the mucosal fragility (Serrano-Martinez *et al.* 2003). Malnutrition is therefore common and compounded by increased nutritional requirements due to the burden of multiple chronic wounds. Oesophageal erosions, web formation and strictures cause severe dysphagia which further exacerbates the malnutrition (Azizkhan *et al.* 2006). Gastric reflux is a common problem that also increases oesophageal damage and which requires management with proton-pump inhibitors (Freeman *et al.* 2008). Chronic constipation occurs as result of blistering of the anal margins causing pain on defaecation and recurrent wounds over that area. The gut can also show inflammatory changes leading to diarrhoea (Shah *et al.* 2007).

1.3.7.3 Chronic anaemia

Most individuals with severe generalised forms of RDEB have anaemia, although the precise aetiology of anaemia, which often has an iron-deficiency profile, is not entirely clear. It is postulated that the main cause is continuous blood loss from open wounds, gastrointestinal erosions, poor nutritional intake and anaemia of chronic disease (Mellerio *et al.* 2007). In some cases, the anaemia can be severe, with haemoglobin levels as low as 5-6 gm/dl, and often refractory to oral iron (Fridge and Vichinsky 1998).

1.3.7.4 Dilated cardiomyopathy

Cardiomyopathy, which is frequently asymptomatic, is a not uncommon pathology in RDEB. The aetiology of this complication is not properly understood but possible causes could be deficiencies in carnitine and selenium, drug toxicity caused by amitriptyline, infection, chronic anaemia and iron overload (Lara-Corrales *et al.* 2010; Lara-Corrales and Pope 2010; Taibjee and Moss 2010). Dilated cardiomyopathy can be fatal in some cases and therefore patients require surveillance with echocardiography.

1.3.7.5 Osteoporosis

Almost all patients with severe RDEB suffer from osteoporosis from an early age, often accompanied by vitamin D deficiency. Bone loss occurs as a result of lack of weight-bearing exercise, generalised inflammation, malnutrition and lack of adequate exposure to sunlight (Bruckner *et al.* 2011; Fu *et al.* 2011). Regular DEXA scans can be useful for detecting osteopaenia and osteoporosis. If present, calcium and vitamin D supplementation should be commenced, and the serum bone profile monitored (Martinez and Mellerio 2010). Long bone and vertebral fractures may also occur in patients with the severe forms of EB (Reyes *et al.* 2002; Cheung 2014).

1.3.7.6 External eye disease

The most common ocular finding in RDEB is corneal blister formation, manifesting as painful corneal erosions, followed by corneal scarring. Symblepharon formation has a higher incidence in RDEB compared to other subtypes of EB and approximately half of all people with RDEB have some degree of ectropion, although this tends to be less severe

than the ectropion complicating junctional EB (Fine *et al.* 2004). Sometimes, lacrimal duct obstruction is seen, especially in the inversa type of RDEB. The incidence of impaired vision is also increased in RDEB, attributed in some cases to severe corneal scarring (Smith *et al.* 2009).

1.3.7.7 Genitourinary tract involvement

Genitourinary tract involvement in EB is not uncommon and in some cases, it can result in end stage renal failure. Pathology may be broadly divided into problems resulting in obstruction or disease primarily affecting the renal parenchyma. Most genitourinary complications reported have involved junctional EB patients. Ulceration and scarring of the glans penis and labia may occur in the inverse form of dystrophic EB (Farhi *et al.* 2004; Almaani and Mellerio 2010; Kajbafzadeh *et al.* 2010). Urethral meatus stenosis is the commonest genitourinary complication, occurring in 8% of RDEB patients (Rubin *et al.* 2007). Severe constipation in patients with RDEB may also result in urinary outflow obstruction with hydroureter and hydronephrosis (El Shafie *et al.* 1979; Price *et al.* 2001). The main causes of renal parenchymal disease are: post-infectious glomerulonephritis, mesangial IgA nephropathy and secondary renal amyloidosis (Almaani and Mellerio 2010).

1.3.7.8 Growth and pubertal delay

Profound growth retardation occurs early in the course of severe RDEB and at least half of children show signs of malnutrition (Colomb *et al.* 2012). Two consequences of inadequate nutrition is pubertal delay and short stature. Females with severe forms of EB

and pubertal delay have been found to have hypogonadotrophic hypogonadism due to reduced serum luteinising hormone (LH) and follicle-stimulating hormone (FSH) (Pass *et al.* 2009; Martinez *et al.* 2010), although males with this complication may express low androgen levels.

1.3.7.9 Pain and pruritus

EB is a painful condition. The expanding and often tense bullae resulting in erosions and chronic wounds cause considerable pain and discomfort. Sources of long-term chronic pain include dental and periodontal disease, the formation of scar tissue and contractures causing immobility and pain on extension of the affected joints (Denyer 2012; Goldschneider *et al.* 2014).

Pruritus is a common problem in people with EB (van Scheppingen *et al.* 2008). Pruritus correlates with EB severity and extent of skin damage, with severe RDEB individuals being more severely affected compared to EB simplex (Devries *et al.* 2004). The main reasons for itch in EB are still unclear but it is postulated that dry skin, increased heat and moisture from dressings contribute to the intensity of itch. On the other hand, pruritus can be caused by wound healing and inflammation leading to a vicious itch-scratch-blister cycle of skin damage and skin healing (Danial *et al.* 2015a).

1.3.7.10 Squamous cell carcinoma (SCC)

Malignancy, typically SCC, is the major cause of premature mortality in RDEB. SCC was first documented in association with EB in 1913 (Reed *et al.* 1974). SCCs often arise in areas of non-healing cutaneous wounds. Often, they occur at multiple sites simultaneously and can affect all skin types, in contrast to the non-EB population. The

sites of the SCCs are mainly in areas prone to maximal trauma and scarring. Clinically, early SCCs can be difficult to distinguish from reactive proliferation in the setting of chronic wound healing (**Figure 1.3**).

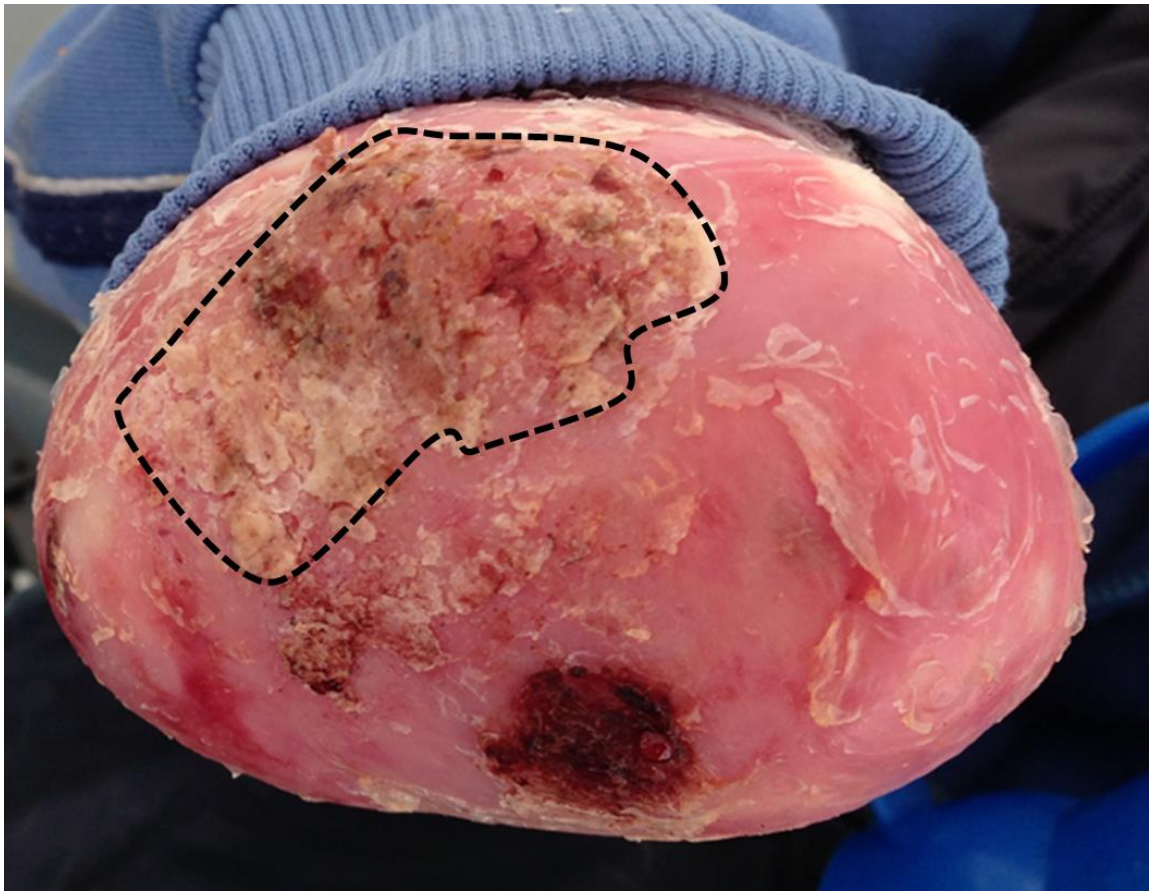


Figure 1.3 Squamous cell carcinoma (SCC) on the scarred, mitten-like hand of a 30-year old individual with RDEB-SG.
The dashed line represents clinically the SCC which was subsequently excised and histologically confirmed as a moderately differentiated SCC.

Multiple biopsies may be needed to establish a diagnosis of SCC (Mellerio *et al.* 2016). Initial SCCs are often well-differentiated but there is clearly a widespread field effect associated with chronic inflammation in RDEB as multiple primary SCCs, separated by shorter time intervals, and with increasing loss of differentiation, occur. SCCs arising in RDEB are biologically aggressive with most people developing metastatic disease within

5 years after diagnosis of the initial SCC. The data from the USA National EB Registry (1986-2006) demonstrate that at least one SCC will arise in at least 80% and 90% of RDEB-SG patients by ages 45 and 55, respectively (Fine *et al.* 2009c). The mortality rate from SCC in this type of EB is 38.7% by age 35 years, 70% by 45 years and 78.7% by 55 years. This is in contrast with an estimated lifetime risk of only 7%-11% within the white non-EB American population (Miller and Weinstock 1994). Furthermore, the overall mortality rate from SCC (0.26 per 100,000 per year) within the non-EB American population is also extremely low (Weinstock *et al.* 1991).

1.3.8 Measuring clinical severity and activity in EB

The development of a severity score for EB is challenging because one needs to take into account the multiple systems involved and the different subtypes. Since the first scoring tools were developed (Tamai *et al.* 2003; Moss *et al.* 2009), there was criticism that it did not discriminate between activity and permanent damage and irreversible scarring. The Birmingham EB severity score (BEBSS) (Moss *et al*. 2009) takes into account eleven items, including area of damaged skin, involvement of nails, mouth, eyes, larynx and oesophagus, scarring of hands, skin cancer, chronic wounds, alopecia and nutritional compromise. The score is simple to define, easy to use and applicable to all types of EB. Its limitations are that only one item takes into account disease activity (c.f. the other components that reflect disease severity) and not all items are applicable to all types of EB; i.e pseudosyndactyly is only applicable in RDEB. Over the past few years with the development of clinical trials there has been intense discussion amongst EB experts about the development of other validated tools as quantitative ways of assessing therapy-

associated changes in clinical status for all types of EB. Recently, two new scores have been introduced. The Epidermolysis Bullosa Disease Activity and Scarring Index (EBDASI) has the capacity to distinguish activity scores that are responsive to therapy separately from damage (Loh *et al.* 2014). However, it has not been tested in severe RDEB and takes longer to complete than the BEBSS. Another group attempted to develop an instrument that would serve as an outcome measure for clinical research studies (Schwieger-Briel *et al.* 2015). The iscorEB (Instrument for Scoring Clinical Outcome of Research for Epidermolysis Bullosa), in contrast to previous scoring models (Tamai *et al.* 2003; Moss *et al.* 2009), includes not only the perceptions of disease severity of EB healthcare professionals, but also patient and parent perception of disease severity.

Almost all EB types show some degree of oropharyngeal involvement and therefore the EB Oropharyngeal Severity (EBOS) score was developed specifically to measure oropharyngeal disease activity and structural damage (Fortuna *et al.* 2013a; Fortuna *et al.* 2015b). In addition, EB-specific quality of life evaluation tools have also been developed (Horn and Tidman 2002; Frew *et al.* 2009), but they are still evolving and have their own limitations.

1.3.9 Management of DEB

1.3.9.1 Principles of wound care

The presence of multiple wounds of varying duration and ability to heal makes management of DEB difficult and complex. The underlying principle of erosion management is to apply an atraumatic dressing to protect the wounds and facilitate healing as well as prevent pain and bleeding on removal (Caldwell-Brown 1992). Blisters occur following trauma and minimal friction. Blisters are not self-limiting and will extend

rapidly if left intact. Intact blisters should be lanced at their lowest point to facilitate drainage and limit tissue damage (Denyer 2010).

Management of DEB must address skin colonisation and infection, offer protection from trauma, avoid contractures and reduce pruritus. Dressings are often extensive and large sizes must be sought in order to avoid blistering where two smaller dressings join. Exudate can be copious and needs careful containment to avoid maceration and leakage. Odour can be a feature and must be addressed to avoid embarrassment and social compromise although eradication of smell can be impossible (http://www.debra-international.org/med-professionals/clinical-practice-guidelines-cpgs-for-eb/wound-care.html).

1.3.9.2 Pain management

Pain is a feature of all types of EB. Pain management should be individual and person-focused. The use of recognised age-appropriate pain score tools, if available, is recommended (Melzack 1987; McCormack *et al.* 1988). Best practice guidelines have been developed and published by the patient advocacy group, Dystrophic epidermolysis bullosa Research Association (DebRA) International, as part of a project to improve care of EB individuals (Goldschneider *et al.* 2014). Pain in DEB has several aspects and all of them should be addressed. It is divided into acute pain related to wounds, procedures (dressing changes, blister lancing and bathing) and chronic pain (wounds, abdominal pain, dental and constipation).

Recommendations for wound pain include: treating infections, topical therapies for pain and systemic pharmacologic therapy for both acute and chronic forms of skin pain. For

localised wound pain, topical lidocaine jelly (2% concentration) has been used in some cases. In adults, topical opioids (morphine sulphate in hydrogel) with dressing changes can be used on individual wounds (Twillman *et al*. 1999; LeBon *et al*. 2009). Topical anaesthetics are routinely used in children prior intravenous access and skin biopsies.

Systemic analgesics include use of paracetamol, non-steroidal anti-inflammatory drugs and opioids. Transmucosal analgesics (including intranasal fentanyl and transbuccal opioids) should be considered for short procedures such as dressing changes while midazolam should be considered for anxiety prior procedures. The IV form of ketamine given orally has been used with variable success for chronic pain (Blonk *et al.* 2010).

Psychological therapies for pain management have been shown to modify pain intensity, reduce related distress, decrease pain-related functional disability, and improve pain coping (Goldschneider *et al.* 2014). Dressing changes and blister lancing can cause anxiety and pain. Analgesia should be given beforehand and tense blisters should be lanced. Leaving the wounds uncovered causes pain and therefore, new dressings should be ready. Distraction is helpful especially in children. Rectal analgesics are not recommended due to the fragility of the perianal skin. **Table 1.3** summarises the pain management options for different modalities of pain.

Table 1.3 Summary of pain management treatments.

Topical treatments	• Occlusive and non-adherent dressings • Adhesive removers • Topical lidocaine • Topical morphine • Lubrication and bath additives
Systemic treatments	• Paracetamol • Non-steroidal anti-inflammatory drugs • Opioids • Ketamine
Non-traditional systemic treatments	• Tricyclic antidepressants • Anticonvulsants
Psychological techniques	• Relaxation • Self hypnosis • Cognitive behavioural therapy

1.3.9.3 Dental care

The approach to dental care has dramatically changed over the last 30 years. It was initially considered extraction of all teeth to be the treatment of choice for patient with RDEB (Crawford *et al.* 1976). More recently, it was illustrated that it is possible to manage dental abnormalities more conservatively (Wright *et al.* 1993). Recently it has been demonstrated that caries can be prevented by regular dental follow-up, oral hygiene, professional cleaning and fluoride therapy (Fortuna *et al.* 2013b; Fortuna *et al.* 2015a). As part of a multidisciplinary approach, patients should be reviewed by a dentist regularly. Prevention of dental disease is the main aim. Tooth brushing, even for severe RDEB is possible and recommended. Toothbrushes with small heads, soft and short bristles are available. Daily exercises can also help to improve/maintain the mouth opening (Kramer *et al.* 2012).

1.3.9.4 Management of pseudosyndactyly

Neonates with RDEB-SG are frequently born with wounds extending over their limbs, hands and feet, caused by intrauterine movement and delivery trauma. In many cases careful dressing of these wounds, with attention paid to separating the digits, can prevent early fusion.

Degloving injuries following trauma are not uncommon and these also require immediate action to separate the digits to prevent digital fusion (**Figure 1.4**).

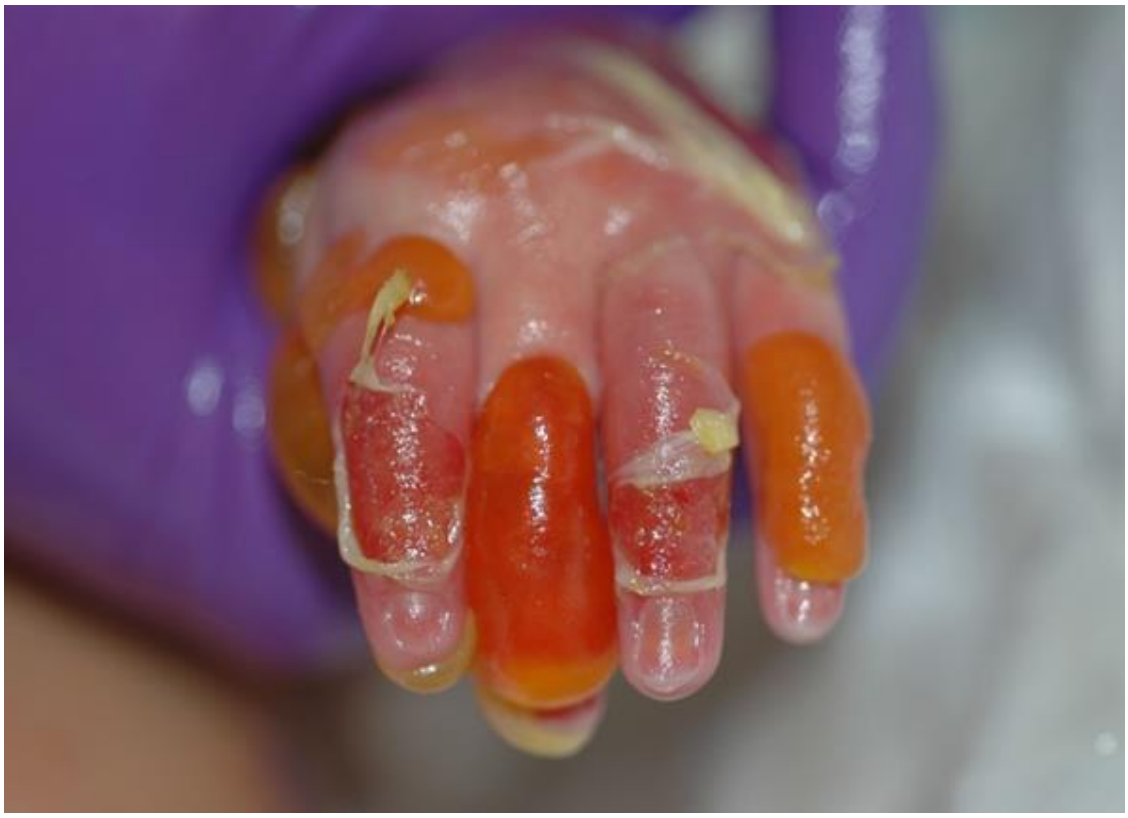


Figure 1.4 Degloving injury.
Severe ulceration of the hand with degloved fingers (courtesy of Dr Jemima Mellerio).

Despite these measures, over time and following repeated trauma the web spaces are gradually lost and digital fusion and contractures will develop. Surgery is usually successful in releasing the contractures and separating the fingers, but this is complex and requires skin grafting, repeated general anaesthesia and compliance with postsurgical splinting (Terrill *et al.* 1992; Bernardis and Box 2010; Luria *et al.* 2014). In many patients, the process of fusion and contractures begins again within a short time. Some adult patients may decline hand surgery because of the need for repeat procedures, preferring instead to manage with hands that may in some cases exhibit complete 'mitten deformity' (**Figure 1.5).**

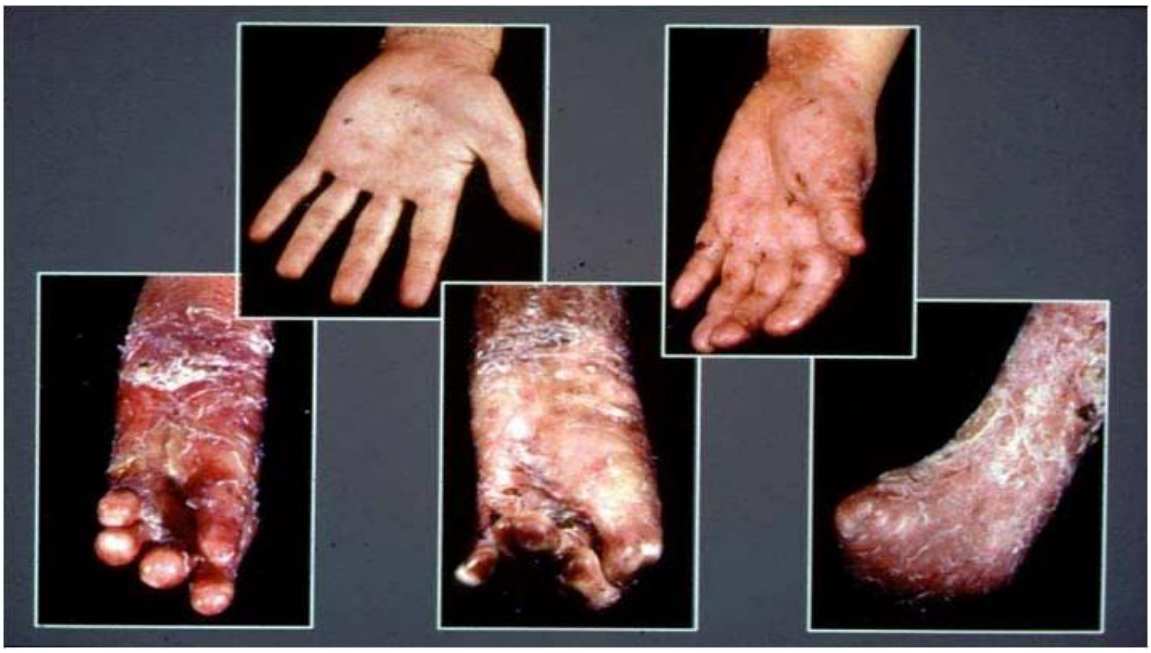


Figure 1.5 Different levels of pseudosyndactyly in DEB individuals.
These photos show very mild level of pseudosyndactyly (top left), to mitten deformity of the hand in the bottom right corner in RDEB-SG.

1.3.9.5 Management of pruritus

Pruritus is one of the most challenging aspects of the management of DEB. Intense itch provokes scratching leading to further cutaneous damage.

Almost healed wounds are particularly pruritic and scratching can lead to wound breakdown. Apart from skin breakdown, intense pruritus can be part of the pain spectrum and can lead to insomnia and depression (Danial *et al.* 2015a; Danial *et al.* 2015b). This is particularly marked in EB pruriginosa. If the skin is dry it tends to be itchier too. In DEB, one should aim for a balance between moisturising without making it prone to blistering. Menthol containing, oil-based products may be useful to relieve itch (www.debra-international.org for best practice guidelines).

Non-pharmacological treatments include cognitive behavioural therapy, hypnosis and meditation (Chida *et al*. 2007). Habit reversal is a specific behavioural intervention where

specific behavioural techniques are developed as a competing response to the urge to itch and is frequently combined with cognitive behavioural therapy and relaxation (Azrin and Nunn 1973).

1.4 Therapies for RDEB

1.4.1 Gene therapy

Given that keratinocytes and fibroblasts are responsible for the synthesis of all the key adhesive proteins within the BMZ, attempts at gene therapy for EB are likely to focus on genetic modification of these cells. There are two main strategies that are used for cutaneous gene delivery. In the first, *in vivo* strategy, the corrected gene is directly delivered into the skin by topical application or by intradermal injections, such as by using biolistic particle bombardment ("gene gun") (Furth 1997; Woodley *et al.* 2004b). In the second, *ex vivo* strategy, genetically corrected autologous or allogeneic keratinocytes are cultured *in vitro* and grafted onto the patient (Featherstone and Uitto 2007). Most gene therapy efforts initially were being focused on *ex vivo* transfer of the wild-type *COL7A1* cDNA into affected keratinocytes or fibroblasts to correct the RDEB phenotype (Murauer *et al.* 2011).

Integrating *COL7A1* cDNA into human C7-null RDEB keratinocytes has been achieved with a variety of vectors (Chen *et al.* 2002a; Ortiz-Urda *et al.* 2002; Gache *et al.* 2004). Retroviral vectors are the most widely used vehicles in human gene trials (Khavari *et al.* 2002; Gache *et al.* 2004). The use of these vectors, however, has been limited by the large size (9.2 kb) of the *COL7A1* cDNA (Goto *et al.* 2006b). The application of large viral vectors to gene therapy also raises some safety concerns and plasmid-based approaches

offer poor efficiency for stable gene transfer. The development of lentiviral vectors holds promise for gene transfer in skin diseases as shown by the successful *in vivo* transduction of a C7 lentiviral vector containing full-length *COL7A1* transgene into RDEB keratinocytes and fibroblasts in animal studies (Chen *et al.* 2002a; Ortiz-Urda *et al.* 2002; Baldeschi *et al.* 2003). Limitations of these therapies are a) the short-term correction of phenotype (Goto *et al.* 2006a), b) production of truncated C7 (if a modified mini-gene is used to reduce cDNA size) that may induce dominant-negative interference in DDEB (Chen *et al.* 2000), c) unstable accommodation of the large *COL7A1* transgene in viral vectors, and d) ectopic expression of the recombinant C7 in all layers of the transgenic epidermis (Baldeschi *et al.* 2003; Gache *et al.* 2004).

After transcription, most pre-RNAs are processed through splicing to become a mature RNA. There are two types of splicing: cis- and trans-splicing. Trans-splicing involves two pre-RNA molecules, whereas cis-splicing occurs within a single pre-mRNA. Trans-splicing aims to reduce the size of the cDNA transcript by recombining an endogenous target pre-mRNA and an exogenously delivered RNA molecule called the pre-trans-splicing molecule (PTM). A partial, wild-type coding sequence from the PTM is then inserted to replace disease-causing sequences in order to generate a new, reprogrammed wild-type mRNA to correct the disease phenotype (Wally *et al.* 2008). Although keratinocytes produce more C7 *in vivo*, gene-corrected RDEB fibroblasts have been shown to produce greater c7 at the mouse DEJ compared to gene-corrected RDEB keratinocytes and might have greater therapeutic potential (Ryynanen *et al.* 1992; Ortiz-Urda *et al.* 2003; Goto *et al.* 2006a).

One further innovation is gene editing, a new means of silencing or repairing genes that has relevance to both RDEB and DDEB. This technology and its potential applications to *COL7A1* and DEB are discussed in Section 6.4.3.1.

1.4.1.1 Induced pluripotent stem cells (iPSCs)

Induced pluripotent stem cells are reprogrammed from somatic cells and have the potential to differentiate into any cell type. The reprogramming process is initiated by the introduction of certain embryonic transcription factors (*c-MYC*, *SOX2*, *OCT4* and *KLF4*). The development and use of iPSCs first emerged in 2006 with the pioneering and remarkable demonstration that somatic cells, such as skin fibroblasts (Yamanaka and Takahashi 2006), epidermal keratinocytes (Aasen *et al.* 2008), and hair follicle outer root sheath (Aasen and Izpisua Belmonte 2010) could be reprogrammed into pluripotent cells with many of the properties and characteristics of embryonic stem cells derived from the inner cell mass of an embryo. So far, murine studies have successfully generated iPSCs in culture from multipotent keratinocyte lineages capable of forming a fully developed epidermis (Bilousova *et al.* 2011). Subsequently, Itoh *et al*. reported successful generation of iPSCs from healthy human skin fibroblasts and individuals with RDEB (Itoh *et al.* 2011). These two studies used application of specific proteins to allow differentiation of iPSCs into keratinocytes. A third study (Tolar *et al.* 2011c) took a different approach using direct injections and teratoma formation which allows spontaneous differentiation of iPSCs into an epidermis. Another group generated patient-derived *COL7A1*-corrected epithelial keratinocyte sheets for autologous grafting (Sebastiano *et al.* 2014). Revertant mosaicism (see Section 1.4.1.2) offers a unique opportunity for generation of iPSCs for patient-specific stem cell therapy, perhaps paving the way for personalised therapy in EB (Tolar *et al.* 2014; Umegaki-Arao *et al.* 2014).

1.4.1.2 Revertant mosaicism and natural gene therapy phenomenon

One striking observation in recent years has been that some patches of skin in patients with various inherited skin diseases can undergo spontaneous correction of the genetic defect underlying the skin pathology, a concept known as revertant mosaicism or "natural gene therapy". This was first noticed in the late 1990s in a patient with non-Herlitz junctional EB (now classified as generalised intermediate junctional EB) due to autosomal recessive mutations in *COL17A1*, encoding type XVII collagen (Jonkman *et al.* 1997). Revertant mosaicism is a naturally occurring process characterised by spontaneous genetic repair that can lead to a partial or complete reversal of an affected to a wild-type phenotype. In addition to generalised intermediate junctional EB, other variants of EB, including EB simplex, RDEB and Kindler syndrome have all shown evidence of revertant mosaicism (Jonkman *et al.* 1997; Darling *et al.* 1999; Hirschhorn 2003; Pasmooij *et al.* 2005; Almaani *et al.* 2010; Kiritsi *et al.* 2012; Lai-Cheong *et al.* 2012). Likewise, skin diseases such as ichthyosis with confetti and dyskeratosis congenita (Choate *et al.* 2010; Jongmans *et al.* 2012; Choate *et al.* 2015), have also been shown to display this phenomenon. There is evidence that natural gene therapy may occur in all patients with generalised intermediate junctional EB (Pasmooij *et al.* 2012) and in an increasing number of individuals with dystrophic EB (Pasmooij *et al.* 2010; van den Akker *et al.* 2012; Kiritsi *et al.* 2014). The predominant mechanisms of gene correction include back mutation, gene conversion, intragenic recombination and second-site mutation (Jonkman *et al.* 1997; Jonkman and Pasmooij 2009). The genetic correction appears to be limited to keratinocytes rather than fibroblasts or other skin cell populations, perhaps reflecting higher proliferation rates in keratinocytes (Jonkman *et al.* 1997). The main clinical consequence of revertant mosaicism is the phenotypic improvement of disease, although the timing of the reversion may influence the extent of revert mosaicism

and the severity of the condition, i.e. early reversion during embryonic development is more likely to result in larger revertant patches.

1.4.1.3 Revertant cell therapy for epidermolysis bullosa

The opportunity to expand keratinocytes derived from a patch of revertant mosaicism creates a translational opportunity for revertant cell therapy that is personalised and patient-specific. The key advantage of this approach is that the cells are naturally corrected and therefore additional genetic manipulation should not be necessary. The first attempt at revertant cell therapy was reported in an individual with revertant mosaicism in junctional EB (Gostynski *et al.* 2009). Revertant keratinocytes from this patient were isolated, expanded into epidermal sheets and grafted onto a de-epidermised area of mutant skin. In culture, ~30% of keratinocytes displayed reversion but, for reasons unknown, fewer than 3% of the corrected cells persisted in the graft and thus clinically, the graft remained fragile and skin integrity did not improve. Nevertheless, punch grafting techniques have been used to successfully implant reverted skin into unreverted sites (Gostynski *et al.* 2014).

1.4.2 Protein therapy

The main determinant of the blistering phenotype in DEB is the absence or reduced expression of C7. Therefore, therapeutic approaches for direct administration of recombinant human C7 at the DEJ are also of interest. Studies of *Col7a1* null mice that were treated with intradermal injections of recombinant human C7 successfully incorporated into the DEJ and improved the phenotype for up to 2 months (Woodley *et*

al. 2004a; Remington *et al.* 2009). This also translated to increased survival rate for DEB mice. Topical application of human recombinant C7 (rC7) accelerated wound healing (Wang *et al.* 2013) in mice and intravenously administered rC7 homed to engrafted RDEB mouse skin and restored C7, anchoring fibrils, and epidermal–dermal adherence (Hovnanian 2013; Woodley *et al.* 2013). Intravenous administration of rC7 in a spontaneous animal model of inbred Golden Retriever dogs with mild RDEB revealed no side effects and led to reduced wound erythema and blistering (Palazzi *et al.* 2000). However, the mechanisms of this process remain to be elucidated as well as the necessary amount of rC7 that is needed for the best and safest result, taking into account the potential for development of antibodies to C7 (Woodley *et al.* 2014).

1.4.2.1 HMGB-1

The high mobility group (HMG) proteins were discovered and isolated 40 years ago from calf thymus (Goodwin et al. 1973). They comprise three families: HMG-A, HMG-N and HMG box (HMGB) proteins. The HMGB family consists of the HMGB-1, HMGB-2, HMGB-3 and HMGB-4 members. HMGB-1-3 contain two DNA-binding domains (HMG boxes A and B). HMGB-1, also known as amphoterin, is most abundantly expressed and plays a key role in nuclear homeostasis, inflammatory response, tissue repair (Wang et al. 1999) and cancer (Sims et al. 2010; Tang et al. 2010; Andersson and Tracey 2011). It is primarily a DNA-binding nuclear protein, which is released actively during inflammatory responses and tissue repair as well as passively during cell death (Scaffidi et al. 2002; Bianchi 2004). Although predominantly located in the nucleus, HMGB-1 can also translocate to the cytoplasm, as well as the extracellular space via multiple pathways. There, HMGB-1 serves as a damage-associated molecular pattern

molecule (DAMP) or alarmin to trigger an immunological response to injury or inflammation (Oppenheim and Yang 2005; Andersson and Tracey 2011). This process occurs primarily, but not exclusively, by binding to the receptor for advanced glycation end product (RAGE) (Hori et al. 1995) and toll-like receptors (TLR) 2 and 4 (Park et al. 2006; Riuzzi et al. 2006; van Beijnum et al. 2008). Quantitating HMGB-1 levels in biological fluids is key to exploring its role in physiological and pathological immune responses. As an alarmin, HMGB-1 has the capacity to drive pathogenesis in multiple inflammatory and immune-mediated systemic skin conditions where it can correlate with disease severity such as systemic lupus erythematosus, lupus nephritis (Popovic et al. 2005; Jiang and Pisetsky 2008; Abdulahad et al. 2011; Ma et al. 2012; Zickert et al. 2012), primary Sjogren's (Dupire et al. 2012) and toxic epidermal necrolysis (Nakajima et al. 2011). In vasculitic diseases, levels of HMGB-1 might also serve as a marker of disease activity (Wibisono et al. 2010; Bruchfeld et al. 2011; Henes et al. 2011; de Souza et al. 2013).

Murine studies have shown that HMGB-1 is rapidly released from hypoxic keratinocytes (e.g. from free skin grafts or from within blister roofs) and that, when released into the circulation, HMGB-1 can mobilise reparative epithelial progenitor cells (Lin−/PDGFRα+) from within the MSC-BM population (Tamai et al. 2011). The in vivo mobilisation of these cells and the pathways implicated in that process are yet to be fully determined although there is preliminary evidence for this BM-skin repair axis (Iinuma et al. 2015). The same study identified blister fluid and serum as the main pools of HMGB-1 in three RDEB subjects (Tamai et al. 2011).

1.4.3 Drug therapy

Several studies have suggested that anti-inflammatory antimicrobials may promote wound healing and decrease blister formation in patients with EBS (Fine and Eady 1999; Retief *et al.* 1999; Veien and Buus 2000; Weiner *et al.* 2004). A proof-of-concept study in RDEB showed improved wound healing with oral trimethoprim compared to placebo (Lara-Corrales *et al.* 2012) although further studies are required to prove potential benefit. Antisense-mediated modulation of splicing where an exon is skipped, in order to restore a disrupted reading frame, appears to have an application in selected DEB patients (Goto *et al.* 2006b). More recently, a case study of an individual with a severe form of JEB, with compound heterozygous nonsense mutations in *LAMA3* (R943X/R1159X), was rescued by spontaneous read-through of the R943X allele, providing evidence that this phenomenon may lead to milder phenotypes than are usually associated with nonsense mutations (Pacho *et al.* 2011). The authors also investigated the effects of gentamycin on read-through of the termination codon (TAG) in that patient and found that read-through can be increased up to six-fold. An attempt to explore the potential of thymosin β4, to promote wound re-epithelialisation in EB was initiated in 2005, but unfortunately the study was terminated due to lack of subject recruitment (Fine 2007). Sulphoraphane, a compound found in broccoli, was found to reduce blistering in an EBS mouse model (Kerns *et al.* 2007). Reports of systemic granulocyte colony-stimulating factor (GCSF) (Fine *et al.* 2015), mycophenolate mofetil (El-Darouti *et al.* 2013), the tumour necrosis factor- α (TNF-α) inhibitor, Etanercept (Gubinelli *et al.* 2010), vitamin E (Ayres 1986) and chloroquine (Baer 1961) have shown variable therapeutic impact in DEB.

1.4.4 Fibroblasts and the skin

1.4.4.1 Fibroblasts and their role in wound healing

After skin injury, several events are initiated including inflammation, granulation tissue formation, angiogenesis, tissue contraction and tissue remodelling (Clark 1989). Crucial to all of these events is the interaction of cells with the extracellular matrix (ECM). After a blood clot has formed during the inflammatory response, white blood cells invade the wound region by migrating through the ECM. Fibroblasts subsequently migrate into the region and begin to replace the blood clot with collagen. Fibroblasts biochemically alter the ECM by degrading the fibrin and producing collagen (Lorenz *et al.* 1992). While new tissue is being generated, endothelial cells migrate into the region forming new vasculature, angiogenesis. In the final phase of wound healing, the composition of the ECM is modified over a period of months, again with key roles by dermal fibroblasts.

Five (not exclusive) mechanisms exist about ECM-fibroblast interactions: First, ECM structure influences fibroblast direction, a phenomenon known as ‘contact guidance’ (Clark *et al.* 1990); secondly, the ECM affects the speed of the fibroblast migration (Wojciak-Stothard *et al.* 1997); thirdly, the ECM alters the production of different proteins by the fibroblasts (Clark *et al.* 1995); fourthly, the ECM alters fibroblast behaviour, and finally enabling fibroblasts to organise the thin collagen fibrils into the fibrous structures more typical of unwounded dermis (Ehrlich and Krummel 1996).

1.4.4.2 Topography and fibroblasts

The skin is a prime example of an organ with clear spatial patterns of morphological and functional specialisation. For instance, it is remarkable that terminal hairs grow on top of

the head but not on the palm (Osterfield *et al.* 2003). Moreover, a large number of skin diseases show striking specificity for particular anatomical sites. Although fibroblasts from different anatomical sites are morphologically similar, it has been previously shown that fibroblasts from varying sites exhibit differences in their gene expression depending on the anatomic site of origin (Chang *et al.* 2002). For example, fibroblasts derived from palms and soles are different from those from other body sites in terms of *KRT9* induction in non-palmoplantar keratinocytes (Yamaguchi *et al.* 1999). This observation was confirmed by others (Rinn *et al.* 2006), as well as in observations that adult fibroblasts retained features of the embryonic HOX code, the spatial pattern of expression of a family of transcription factors that delineate positional identity (Rinn *et al.* 2008). The expression pattern of *HOX* genes in skin fibroblasts is systematically related to their anatomical site of origin. Fibroblasts from different anatomical sites may even be considered distinct cell types because their gene expression programmes are as diverse as cells from different haematopoietic lineages (Rinn *et al.* 2006). Cells more closely located to each other are more likely to have shared local interactions, and therefore may have greater similarity to each other in gene expression. For instance, cells from the hand and feet may share a distinct gene expression signature that reflects their spatial position on distal limbs even though these two body parts are spatially far apart. Positional identity is also essential during wound healing and regeneration. In regenerative therapies based on stem cells, fibroblasts may play similar instructional roles by providing the appropriate stem cell niche for stem cell differentiation (Watt and Hogan 2000). As well as regional differences in fibroblasts, different populations of fibroblasts have been detected in the papillary and reticular dermis (Driskell *et al* 2013). Papillary dermal fibroblasts have been implicated in hair regeneration while reticular dermal fibroblasts have a more prominent role in wound healing and scarring.

1.4.4.3 Autologous vs allogeneic fibroblasts

Fibroblasts used in tissue engineering may be allogeneic or autologous. In contrast to allogeneic cells, autologous fibroblasts carry no risk of rejection or risk of cross-infection. However, there is often a delay in culturing autologous cells in order to obtain sufficient cell numbers, whereas allogeneic cells can be cryopreserved and therefore are more readily available. There have also been several studies investigating the immunological impact of allogeneic cells on the recipient. One study looked at the persistence of allogeneic fibroblasts in an acute wound (porcine model) and found that after 1 week, allogeneic fibroblasts were not detectable by polymerase chain reaction (Price *et al.* 2004). Apligraf® (Novartis, Basel, Switzerland) is a living skin substitute composed of allogeneic keratinocytes and allogeneic fibroblasts and its application in acute human wounds showed that neither foreign cell population was detectable beyond 6 weeks (Griffiths *et al.* 2004). It has therefore been suggested that allogeneic cells are eventually silently replaced by host cells. In addition, large trials involving grafting of allogeneic skin equivalents onto venous ulcers did not reveal evidence of rejection clinically or immunologically in the patients (Falanga *et al.* 1998). The ideal commercial cellular therapy is pre-prepared, allogeneic, off-the-shelf, easy to store and deliver.

1.4.4.4 Murine studies of fibroblast cell therapy for EB

Several murine studies have focused on developing cell therapies for RDEB. C7 is synthesised and secreted by basal keratinocytes and by dermal fibroblasts (Stanley *et al.* 1985; Regauer *et al.* 1990; Woodley *et al.* 2003; Goto *et al.* 2006a; Ito *et al.* 2009), and given that fibroblasts are much easier to isolate and maintain in culture than keratinocytes, fibroblasts present an attractive target for cell-based therapies in RDEB mouse models.

Wild type and *COL7A1* gene-corrected human RDEB fibroblasts overexpressing C7 have been injected intradermally into immunodeficient mouse skin or into transplanted human RDEB skin equivalents (Ortiz-Urda *et al.* 2003; Woodley *et al.* 2003). Both these interventions led to sustained human C7 deposition and new anchoring fibril formation at the DEJ. It was also shown that intradermal injections of wild-type fibroblasts, i.e. without *COL7A1* correction, could correct the RDEB skin pathology, but only when injected at sufficient cell density, i.e., 5×10^6 cells versus 1×10^6 cells (Ortiz-Urda *et al.* 2003; Woodley *et al.* 2003). Increase in C7 at the DEJ after intradermal injections of wild-type human fibroblasts was also confirmed in studies using a hypomorphic c7 mouse model of RDEB that expresses c7 at ~10% of normal levels (Fritsch *et al.* 2008; Kern *et al.* 2009). Moreover, intravenously injected wild-type or *COL7A1* gene-corrected human RDEB fibroblasts could home to wounded mouse skin and improve wound healing (Woodley *et al.* 2007). Collectively, these murine studies provide a rationale for the use of allogeneic wild-type or *COL7A1* gene-corrected fibroblasts in humans with RDEB.

1.4.4.5 Mode of action of allogeneic fibroblast cell therapy in DEB

Transcriptomic analysis of serial skin biopsies following injection of allogeneic fibroblasts in one subject with RDEB revealed that expression of *HBEGF* was upregulated and that expression levels mirrored those seen for *COL7A1* (Nagy *et al.* 2011). *In vitro* studies showed that HB-EGF could upregulate *COL7A1* expression in both normal control and RDEB keratinocytes and fibroblasts and that this was preceded by increased expression of *JUN* and *FOS*, components of the AP1 transcription factor that is known to bind to and upregulate *COL7A1*. The interpretation of the *in vivo* situation was that allogeneic fibroblasts were able to induce upregulation of *HBEGF*, predominantly in

neighbouring keratinocytes. The HB-EGF could then lead to a sustained increased in C7 by acting in an autocrine, paracrine and juxtacrine manner to upregulate *COL7A1*. The new C7 was thought to be predominantly recipient-derived, i.e. mutant but partially functional. A slight increase in *HBEGF* and *COL7A1* expression was also noted after injection of saline into skin, although upregulation was less marked than following fibroblast injection and was for a shorter duration. It is possible that saline induces an irritant reaction associated with a relatively milder upregulation of similar growth factors and cellular responses to fibroblasts. It is also plausible that the donor fibroblasts can release wild-type full length C7 for incorporation into the DEJ during the time (few days) the cells are present in the RDEB skin. Evidence for this "direct release" theory is supported by studies in mice (Kern *et al.* 2009), although both mechanisms are not mutually exclusive. Although often thought as differentiated somatic cells, cultured skin fibroblasts may also contain a sub-population of cells with stem cell properties, capable of differentiation into mesoderm, endoderm and ectoderm. Termed MUSE (multilineage-differentiating stress-enduring) cells, these cells are also found in the skin, bone marrow and umbilical cord (Wakao *et al.* 2011; Liao *et al.* 2014).

1.4.4.6 Fibroblast cell therapy in DEB

Initial studies of allogeneic fibroblast injections in RDEB focused on responses at the DEJ in intact skin. However, the major clinical burden in affected individuals is poor wound healing. The putative upregulation of *HBEGF* (Iwamoto and Mekada 2000) following allogeneic fibroblast injection (Nagy *et al.* 2011) may have benefits for wound healing beyond upregulation of C7, given that HB-EGF has been shown to promote keratinocyte migration (Shirakata *et al.* 2005). However, one caveat may be the effects

of HB-EGF on epidermal growth factor receptor (EGFR) signalling and the risk of malignancy (Zenz *et al.* 2003; Kivisaari *et al.* 2010). Given that individuals with RDEB have an increased incidence of squamous cell carcinoma, especially in chronic wounds (McGrath *et al.* 1992b; Pourreyron *et al.* 2007; Tsukada *et al.* 2012), further safety monitoring of allogeneic fibroblasts injected into RDEB wounds was required. At the bedside, allogeneic fibroblasts have been used off-licence to treat a number of patients with RDEB (McGrath and Almaani unpublished data). The clinical impression and experience indicates that for a subgroup of individuals with RDEB, notably those with mild to moderate disease severity and some baseline expression of C7 at the DEJ, allogeneic fibroblast therapy may be useful clinically, in contrast to those individuals with more severe disease that lack C7. For the latter individuals, alternative cell therapy or other strategic approaches that target stem cell correction may be more appropriate.

1.4.4.7 First-in-human study of allogeneic fibroblasts in RDEB

Based on the mouse data, a proof-of-concept study in 5 RDEB individuals demonstrated that a single intradermal injection of allogeneic fibroblasts (5×10^6 cells injected into the superficial dermis over a ~$1cm^2$ area) increased *COL7A1* expression for at least 3 months (the end point of that study) in most individuals (Wong *et al.* 2008). The study also demonstrated the low immunogenicity of allogeneic fibroblasts and lack of host response at an immunological and histological level. The injected cells were not detectable at 2 weeks post-injection, the time-point at which an increase in C7 protein at the DEJ was seen. Of note, the increase in C7 was most apparent in RDEB individuals who had some baseline expression of C7 compared to those who had a complete absence of the protein. However, the increase in C7 was not accompanied by the formation of new, normal-

appearing anchoring fibrils: there was an increase in the number of fibrillar structures below the lamina densa of the basement membrane but these were somewhat wispy and lacked characteristic ultrastructural features of mature anchoring fibrils. Nevertheless, there was some subjective evidence for increased epidermal-dermal adhesion following the allogeneic fibroblast injections (Wong *et al.* 2008). The increase in *COL7A1* and C7 protein expression beyond the physical presence of the injected allogeneic cells, however, were thought to be due to a paracrine effect induced by the fibroblasts. A subsequent study was able to show that a single injection of allogeneic fibroblasts could increase *COL7A1* expression for 3-6 months and C7 protein for 9-12 months (Nagy *et al.* 2011). That study also identified that the paracrine effect was probably mediated by HB-EGF (Nagy *et al* 2011).

1.4.5 BMT and the skin

BM stem cells have a fundamental role in generating erythrocytes, leukocytes and platelets, but also demonstrate plasticity in being able to show lineage differentiation into tissues of mesodermal, endodermal, and ectodermal origin, including skin (Grove *et al.* 2004). Some sub-populations of BM cells can differentiate into keratinocytes (Badiavas *et al.* 2003; Badiavas and Falanga 2003; Borue *et al.* 2004; Fathke *et al.* 2004), and in humans who have undergone BMT, donor cells that have differentiated into keratinocytes can be detected in the epidermis for at least 3 years (Korbling *et al.* 2002). Further studies have shown that the BM is also a source of fibroblast-like cells in the dermis (of haematopoietic and mesenchymal lineages) and that the number of these cells increases after skin wounding (Fathke *et al.* 2004; Ishii *et al.* 2005; Sasaki *et al.* 2008; Yew *et al.* 2011; Tamama and Kerpedjieva 2012). BM cells also contribute to skin development:

infusion of BM cells *in utero* in mice leads to accumulation of a sub-population of these cells in the dermis, particularly around developing hair follicles (Chino *et al.* 2008). Overall, however, BM-derived keratinocytes appear to be an extremely rare finding, perhaps contributing only ~0.0001–0.0003% of all cells in the new epidermis (Tamai *et al.* 2011; Iinuma *et al.* 2015). The physiological role of BM cells in epithelial regeneration therefore has been questioned, although recent studies focusing on RDEB have started to provide new insight into key BM cells and mechanisms germane to skin repair and regeneration.

1.4.5.1 Murine studies of bone marrow cells and skin repair

Initial attempts to use BM cells to correct the inherent skin fragility of RDEB were made in the hypomorphic *col7a1* knockout mouse that recapitulates some of the features of the human disease (Heinonen *et al.* 1999; Tolar *et al.* 2009). Murine studies were undertaken to explore the skin-bone marrow repair mechanisms, relevant to correcting the C7 deficiency in RDEB (Tolar *et al.* 2009). First, congenic (inbred mouse strains) wild-type bone marrow cells were given to *col7a1* deficient mice and it was shown that a bone marrow subpopulation (CD150+ CD48–) migrated to injured skin and secreted C7 with partial anchoring fibril restoration (Tolar *et al*. 2009). Non-manipulated BM cells failed to rescue the RDEB mice but alternative use of the signalling lymphocyte activating molecule (SLAM)-positive subpopulation of BM cells resulted in new C7 and presence of donor cells at the DEJ. Following infusion of SLAM-positive BM cells, there was improved healing of blisters on the mouth, paws and histological evidence of rudimentary anchoring fibril formation (Tolar *et al.* 2009). This functional correction of C7 in a mouse model of RDEB, alongside other supportive data that identified potential keratinocyte

progenitors within the mesenchymal stem cell (MSC) population (Chino *et al.* 2008), led to the first human BMT trial in children with severe RDEB, discussed in Section 1.4.5.3.

Other studies have also shown correction of RDEB skin defects with BM cells, that can occur after *in utero* infusion of BM cells (Chino *et al.* 2008), and that BM-derived MSCs can stimulate secretion of C7 with partial restoration of the damaged BMZ and less blister formation (Alexeev *et al.* 2011). In a different mouse that lacks type XVII collagen, a model for junctional EB-GI, both haematopoietic and non-haematopoietic BM stem cells have been shown to correct basement membrane protein expression and lead to phenotypic rescue (Fujita *et al.* 2010). Collectively, these murine studies provide a scientific rationale for translation of BM cell therapies into human clinical trials. Moreover, several additional mouse models for different forms of EB have been developed that may also be useful for assessing BM cell therapy, including the hypomorphic c7 mouse (Fritsch *et al.* 2008) and a hypomorphic model for junctional EB-GI with spontaneous mutations in the *Lamc2* gene (Bubier *et al.* 2010).

1.4.5.2 Epithelial progenitors within sub-populations of bone marrow stem cells

A recent study has confirmed that BM contains a specific population of epithelial progenitors and that these are mobilised and recruited by specific key factors (Tamai *et al.* 2011). Tamai *et al*. (2011) used an irradiated mouse model in which green fluorescent protein-labelled BMT was performed. Skin grafting in this mouse showed that BM-derived keratinocytes are not of haematopoietic origin but instead are derived from a specific subpopulation of Lineage negative, platelet-derived growth factor receptor alpha-positive (Lin-/PDGFRα+) BM cells. This is still a somewhat heterogeneous cell population that represents ~1 in 450 BM cells. Indeed, these cells may show lineage

overlap with other defined subpopulations of mesenchymal stromal cells (MSCs), including multi-potent adult progenitor cells, Lin-/SCAR1+/CD45- very small embryonic-like stem cells in BM, CD45- unrestricted somatic cell stems in cord blood, and nestin-positive cells that maintain the stem cell niche with haematopoietic stem cells in BM (Kucia *et al.* 2006; Morikawa *et al.* 2009).

The study by Tamai *et al*. (2011) proposed that the skin graft acts as a hypoxic bioreactor, rapidly releasing the non-histone nuclear protein, high mobility group box-1 (HMGB-1). Following skin grafting, HMGB-1 levels in serum increased and HMGB-1 was shown to mobilise the Lin-/PDGFRα+ cells from the BM and recruit these cells along concentration gradient to the area of hypoxic keratinocytes. Differentiation of these cells into keratinocytes was clearly demonstrated. These cells were also shown to persist in the skin after several renewals of the murine epidermis, data which support engraftment of a murine BM population that has generated keratinocyte stem cells. The study also showed that in RDEB the main source of HMGB-1 was hypoxic keratinocytes in blister roofs. Moreover, when *col7a1* -/- murine skin was grafted, new c7 protein was expressed in the grafted skin, co-localising with the GFP +ve Lin-/PDGFRα+ cells.

1.4.5.3 First BMT in RDEB

Following the effectiveness of BM stem cells in murine RDEB, a clinical trial of whole BMT was performed in children with RDEB (Wagner *et al*. 2010). The approach used high dose chemotherapy to immunoablate individuals with RDEB to permit more reliable lymphohaematopoietic engraftment. The BM was not filtered so that all the haematogenous populations could be preserved, allowing for the possibility that non-haematopoietic MSC populations might also be needed for clinical benefits. Seven

patients entered the trial and 6 underwent BMT. All individuals had some clinical improvement and 5 of the 6 showed increased C7 at the DEJ. So far, only the results for the first seven patients have been reported in the scientific literature (Wagner *et al.* 2010). This first clinical trial of BMT for RDEB showed that donor cells homed to injured skin, C7 expression increased, anchoring fibrils gradually appeared and the response was sustained for many years after (current longest follow up is for 8 years, Jakub Tolar, personal communication).

Before the BMT, each individual had more than 50% of their skin covered with blisters and erosions. Three of the 6 individuals showed dramatic clinical improvement, such that less than 10% of the body surface area remained affected. In the other 3 subjects, there was a moderate improvement with less than 25% of the body surface area affected. Despite the clinical improvement some toxicity was noted. One patient died before the BMT because of heart failure, possibly related to cyclophosphamide toxicity and pre-existing renal failure. Another individual died 6 months after transplantation due to infection secondary to graft failure. To try to reduce morbidity and mortality, additional protocols have been developed to use reduced intensity conditioning rather than full myeloablation. Modifications to the original protocol, such as the additional subsequent infusion of MSCs from the original donor, have also been adopted.

1.4.5.4 Mode of action of BMT in skin repair

The precise mode of action of BMT leading to clinicopathological improvement in RDEB is not known. High levels of chimerism (donor and recipient cells) were noted in the skin after BMT, with donor non-haematopoietic cells being the probable source of new C7 at the DEJ (Tolar and Wagner 2013a). Notably, although there were some haematopoietic

cells in the skin (CD45+), the majority were non-haematopoietic, non-endothelial cells (CD45-, CD31-) (Tolar and Wagner 2013a). In spite of the increased C7, however, there was a lack of normal anchoring fibrils on transmission electron microscopy. Although the lack of these adhesion structures may relate to gradual maturation of new anchoring fibrils (as has been observed for several months following burns) (Compton et al. 1989), it is plausible that some of the increased C7 may reflect an upregulation of mutant but partially functional endogenous C7, similar to the proposed mode of action for allogeneic fibroblast therapy (Nagy et al. 2011). Clearly, however, there is more to the reparative story in that the transplanted RDEB individual who did not show any increase in C7, also showed a sustained clinical response in skin healing and integrity at the DEJ. This may indicate the incorporation or availability of additional extracellular matrix proteins that can contribute to better skin repair and adhesion, although such a possibility remains speculative. Functional improvement in skin adhesion in patients following BMT was shown by progressive increase in suction blister times (the time it takes to form a blister following placing a negative pressure suction cup on the skin surface) (Wagner et al. 2010; Tolar and Wagner 2013a). Although the mode of action of BMT in skin repair in EB continues to be investigated, current clinical data provide evidence for a sustained functional improvement in the majority of patients.

1.4.5.5 Mesenchymal stromal cells

MSCs are multipotent stem cells that can be found in the bone marrow, skeletal muscle, adipose tissue, synovial membranes, dental pulp, periodontal ligaments, cervical tissue, menstrual blood, Wharton's jelly, umbilical cord, umbilical cord blood, amniotic fluid, placenta, and fetal tissues (Montesinos *et al.* 2009; Najar *et al.* 2010). MSCs are of non-

haematopoietic lineage that are present in tiny quantities in the circulation (1 in every 10^4 nucleated cells). MSCs have been shown to differentiate into a number of different cell types of stromal lineage including skin cells, osteoblasts, adipocytes and chondrocytes. Despite functional heterogeneity, MSCs express similar panels of markers. The Mesenchymal and Tissue Stem Cell Committee of the International Society for cellular Therapy has proposed four criteria for the determination of MSCs. These include: 1) the plastic adherence of isolated cells in culture, 2) the expression of CD105, CD73 and CD90 in more than 95% of cultured cells, 3) lack of expression of haematopoietic cell markers including CD34, CD45, CD14 or CD11b, CD79a or CD19 and HLA-DR in more than 95% of the cultured cells and 4) the capacity to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici *et al.* 2006).

One of the reasons favouring clinical application of MSCs is their low immunogenicity and lack of requirement for pre-conditioning (Le Blanc *et al.* 2003a; Le Blanc *et al.* 2003b). Adult human MSCs are reported to express intermediate levels of major histocompatibility complex (MHC) class I antigens but do not express human leukocyte antigen (HLA) class II antigens on the cell surface (Le Blanc *et al.* 2003a). Therefore, they are unlikely to be rejected or cause graft versus host disease. Cultured MSCs are known to exert beneficial effects on wound healing via a number of mechanisms *in vitro* (Kim *et al.* 2009; Walter *et al.* 2010; Yoon *et al.* 2010). The effects go beyond differentiation into the cells of the damaged tissue (i.e. direct cellular tissue repair), as they have shown not only to have an immunomodulatory effect both *in vitro* and *in vivo*, but also to have trophic effects through the release of various growth factors and cytokines with both paracrine and autocrine functions (Prockop 2009). These factors can inhibit fibrosis and apoptosis, enhance angiogenesis and stimulate mitosis. Therefore, permanent

engraftment of MSCs is not a prerequisite for therapeutic benefit. In fact, it has become increasingly clear that transient engraftment or delivery of MSCs can exert a favourable effect through secretion of cytokines and paracrine factors which engage and recruit some of the recipient's own cells towards tissue repair.

Allogeneic MSCs, have also anti-inflammatory properties. MSCs can home to damaged tissue and release various immunomodulatory factors that influence the behaviour of dendritic cells, T-cells and natural killer cells. MSCs can reduce B-cell proliferation, monocyte maturation, secretion of IFN-γ and TNF-α while promoting induction of T-regulatory cells and secretion of anti-inflammatory IL-10 from macrophages in direct contact with MSCs (Wang *et al.* 2008). Although the precise micro-environmental contributions to tissue repair are not fully known, a more detailed understanding of the trophic mechanisms associated with MSCs in tissue regeneration is likely to lead to further exploration of their clinical utility. In general, whatever immune conditioning or cell population is being planned for use as cell therapy, for diseases such as EB, it is evident that treatment that is both systemic and given at an early age is likely to have most clinical benefit, certainly for patients with widespread disease.

1.4.5.6 First-in-man studies of mesenchymal stromal cell therapy in DEB

In RDEB animal models it had been shown that MSCs contribute to skin regeneration and accelerate wound healing. Non-tissue matched intradermal human 0.5 x 10^6 allogeneic MSCs or vehicle were injected intradermally in two individuals with RDEB (Conget *et al.* 2010). C7 replenishment was confirmed at 12 weeks as well as improved wound healing at the MSCs injected wound; with the clinical effects lasting for up to 4 months. No adverse effects were noted in the injected skin or systemically. Successful

topical use of BM cells to heal chronic wounds in other diseases has been previously reported (Badiavas and Falanga 2003; Humpert *et al.* 2005; Falanga *et al.* 2007), but Conget *et al* (2010) pioneered the use of intradermal MSC injections in humans with RDEB.

1.4.6 Umbilical cord blood transplantation and the skin

Cord blood (CB) and other parts of the umbilical cord, such as the Wharton's jelly or tissues associated with the placenta, are also rich sources of stem cells. As well as haematopoietic stem cells, CB is an important source of other progenitor cells, as well as MSCs, of very small embryonic/epiblast -like stem cells and unrestricted somatic stem cells, which may have individual or collective value in regenerative medicine. Using some stem cell populations, notably the MSCs, a number of clinical studies have been initiated, mostly to repair tissue, including some studies on skin. The first transplant in humans using CB was reported in 1989 with the use of HLA-matched sibling to treat Fanconi anaemia (Gluckman *et al.* 1989). Subsequently, CB banks were established for the collection and cryopreservation of cells and more than 20,000 CB transplants have been carried out worldwide. MSCs from CB are similar to BM-derived MSCs in having low immunogenicity and are already being used in several regenerative medicine studies. That said, comparison of UC cells versus BM stem cells in individuals with RDEB has shown better skin engraftment with a BM-derived population (Tolar *et al.* 2012) and therefore the clinical utility of cord cells in EB remains to be determined in future clinical trials. Preclinical data, however, have shown that unrestricted somatic stem cells can express c7 and accelerate wound healing, improve wound healing and form new skin appendages (Liao *et al.* 2014).

1.5 Research aims and objectives

Allogeneic fibroblasts have been injected previously into the skin of individuals with RDEB and have shown potential in *COL7A1* upregulation and C7 increase at the DEJ in intact skin. At the start of this thesis work in 2010, there had been no published reports on the effects of allogeneic fibroblasts on wound healing in RDEB. The precise mechanism through which allogeneic fibroblasts may have a beneficial impact on RDEB had not been fully characterised. Moreover, therapeutic use of BM-MSCs in RDEB had not been tested beyond the initial studies in two patients (Conget *et al.*, 2010).

The key therapeutic objectives of cell therapy for RDEB must be to improve wound healing as well as to improve overall health, given that RDEB as a systemic disease that might benefit from early use of systemically delivered therapies.

The primary aim of this work described in this thesis was to assess the safety and efficacy of allogeneic fibroblasts and BM-MSCs in wound healing in adults and children with RDEB. It also aimed to assess the feasibility of such treatments in the context of well-designed clinical trials and translation of the results in the routine clinical setting. The work comprises functional studies on the potential mechanism of allogeneic fibroblasts in RDEB, as well as two completed early phase clinical trials, one of intradermal allogeneic fibroblasts in RDEB wounds in adults, and the other of intravenous allogeneic MSCs in children with RDEB.

1.5.1 Hypotheses

The first hypothesis is that intradermal allogeneic fibroblasts can enhance wound healing in RDEB.

The second hypothesis is that intradermally injected allogeneic fibroblasts enhance wound healing by increasing the expression of *HBEGF* and *COL7A1* expression and in individuals with RDEB. HB-EGF and EGF cytokines can increase C7 and

The third hypothesis is that, BM-MSCs, when delivered systemically, have a potential therapeutic and anti-inflammatory effect that can improve wound healing and quality of life in children with RDEB.

1.5.2 Objectives

- To ascertain whether intradermal injections of allogeneic fibroblasts around wound margins *in vivo* result in *HBEGF*, *COL7A1* upregulation and C7 production. To investigate the optimal dose and time-points at which recombinant growth factors HB-EGF and EGF have the largest effect on *COL7A1* expression *in vitro*.

- To study the wound healing rate of chronic wounds after injections of allogeneic fibroblasts or vehicle in the context of a prospective, phase II, randomised and blinded clinical trial.

- To assess the safety and efficacy of systemic allogeneic BM-MSCs in children with RDEB in the context of an open label clinical trial.

2.1 Research Ethics

Clinical trial specific regulatory approvals are mentioned in the relevant chapters. The mechanistic studies were performed using research ethics approval by Guy's Research Ethics Committee, Guy's and St Thomas' NHS Trust (Characterisation of molecular and structural skin abnormalities in inherited skin disorders, Ref: 07/H0802/104). For experiments with skin samples from healthy volunteers an informed consent form for 'discarded skin' was used (Immunopathogenesis of Psoriasis/Inflammatory Skin Disease, Ref: 06/Q0704/18). Written informed consent was obtained from all patients and healthy volunteers.

2.2 Laboratory techniques

2.2.1 Cell culture

2.2.1.1 Isolation of primary keratinocytes from skin biopsies

Skin punch biopsy specimens were transported in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, UK) and 10% fetal bovine serum (FBS) (Invitrogen) and stored at 4^{o}C for up to 24 hours. Before starting the isolation, a 60 mm Culture dish was coated with 1.7 mls of coating matrix and 17 µl of dilution medium (Invitrogen) and incubated for 30 minutes at room temperature (RT). The residual coating matrix was removed and the coated dishes could be stored at 4^{o}C for several days. Each biopsy specimen was placed in an uncoated 100 mm bacterial petri dish and kept moist with some medium. The subcutaneous fat and any loose connective tissue were removed using

tweezers and a scalpel until only the thin epidermis and the dermis remained. Depending on the biopsy size, samples were cut in smaller pieces. The pieces were placed in a 15 ml falcon tube with 10 mls of dispase 2 (Stemcell Technologies, Grenoble, France) and stored at 4^{o}C overnight. The overnight digested tissue pieces were processed further by holding the edge of the dermal part of the tissue with one set of tweezers and the thin epidermal part with another set of tweezers and the epidermis was slowly peeled off. The epidermis was transferred into another dish with Epilife medium (Invitrogen) with S7 supplement (Invitrogen). The dermis was preserved for fibroblast isolation, as described in Section 2.2.1.2. The epidermis was cut using a sterile scalpel into small pieces. The pieces were placed in a falcon tube containing 15-20 mls TrypLE express (Invitrogen). The samples were incubated at 37^{o}C for 20 minutes in a shaker or in a tube with gentle manual mixing every 5 minutes. An equivalent amount (15-20 mls) of DMEM with 10% FBS was then added to the above mix and pipetted vigorously up and down to inactivate the trypsin. The solution was passed through a 100 μm mesh filter into a new 50 mls falcon tube to remove undigested pieces of tissue. The mixture was centrifuged at 1500 RPM for 5 minutes. The supernatant was removed and the pellet was resuspended in 5 mls Epilife medium. The keratinocytes were subsequently seeded out in 5 mls Epilife for each 60 mm bacterial culture dish and 50 μl (1/100 of the medium volume) of 10,000 units penicillin, 10,000 g streptomycin and 25 g amphotericin B/ml (Invitrogen) was added. The culture dishes were stored at 37^{o}C and 5% CO_2. The medium was changed the next day and subsequently every 3 days paying close attention to cell density. Adherent cells could be seen in 2-3 days.

2.2.1.2 Isolation of primary fibroblasts from skin biopsies

The above steps were followed until the separation of the dermis and epidermis. After the separation, the dermis was placed in DMEM culture medium with 10% FBS. Using sterile forceps and a scalpel, the dermis was cut into 1.0 mm^3 pieces. Each piece was dried out slightly with circular movements on a dry culture dish and placed directly on a 100 mm culture dish, whose base was previously scraped with a scalpel to assist cell adherence. Up to 10 pieces were placed in each 100 mm dish. The pieces were left to dry, but not completely, for a few minutes and then gently, 12-15 mls of DMEM with 10% FBS was added, as well as 1/100 of this volume of 10,000 units penicillin, 10,000 g streptomycin and 25 g amphotericin B/ml and stored in a 37^{o}C with 5% CO_2 incubator. The cell medium was replaced with fresh medium every 5 days and growth was seen within 10-14 days.

2.2.1.3 Maintenance and passage of cells

Primary cell cultures were maintained by changing growth media three times per week for fibroblasts and every two days for keratinocytes on average. When culture dishes reached 80-90% confluence, the cells were passaged. A cell suspension was obtained following incubation for 10 minutes in 5 ml of trypsin. An equivalent volume of culture medium containing 10% FBS was added to neutralise trypsin activity and the mixture was centrifuged at 1000 RPM for 5 minutes. The filtrate was discarded by inversion and the cell pellet was re-suspended in fresh culture media. Cells were re-plated onto new bacterial culture dishes using a split ratio of 1:4 as the seeding density.

2.2.1.4 Cryopreservation and recovery of cells

A cell suspension was obtained and subsequently re-suspended in 10% FBS/Dimethyl sulphoxide (DMSO) (Fisher Scientific, Leicestershire, UK) in 1.2 ml cryovials. The cells were frozen down initially at -80ºC overnight in a freezing container 'Mr Frosty' (Fisher Scientific) and then transferred to liquid nitrogen for long term storage. *Cell* s were recovered by thawing in a 37ºC water bath. The mixture was centrifuged at 1000 RPM for 5 minutes, re-suspended in fresh culture medium and re-plated in a bacterial culture dish.

2.2.1.5 Prevention and testing for infection

To prevent contamination of cultured epidermal cells by infection, the cells were maintained in a good laboratory practice (GLP) facility and cultures in dedicated class II hoods. All surfaces were carefully disinfected with Virkon (Antec International, Suffolk, UK); disposable sterile equipment was used and solutions containing antibiotics prior to use. Mycoplasma testing of cell cultures was performed periodically (**Figure 2.1**). To do this, cells were centrifuged at 1500 RPM and 1ml of supernatant was removed for testing. The supernatant was centrifuged at maximum speed for 30 minutes and the majority of the supernatant was removed leaving the pellet ~ 20 µl. The pellet was re-suspended in 50 µl of TE buffer (10Mm Tris-HCL with 1mM EDTA) with 20 µl proteinase K. The samples were incubated for 1 hour at 55ºC and the enzyme deactivated at 98-100ºC for 10 minutes using dry heating blocks. The PCR mix was prepared using 2.5 µl 10x buffer, 1 µl 2mM dNTPs, 0.125 µl Taq polymerase, 13.875 µl nuclease-free H_2O and 1.25 µl

forward and reverse primers per reaction. The following primer pair was used (van Kuppeveld *et al.* 1992).

Forward (GPO-3): 5'GGGAGCAAACAGGATTAGATACCCT-3'

Reverse (MGSO): 5'-TGCACCATCTGTCACTCTGTTAACCTC-3'

A GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Warrington, Cheshire, UK) was used. The amplification conditions were 94ºC for 1 minute, 55 ºC for 1 minute and 72ºC for 1 minute for a total of 35 cycles.

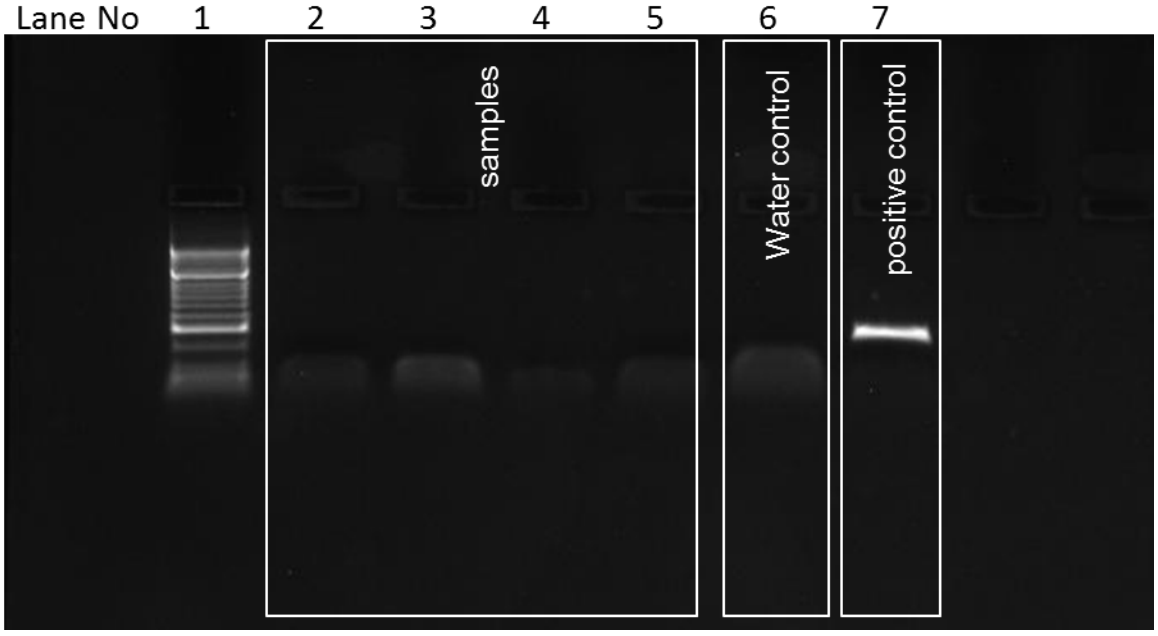


Figure 2.1 PCR testing for mycoplasma contamination.
Supernatants from 4 different primary cell cultures were subjected to the same sample preparation and PCR amplification of each sample were run with the primers mentioned in the text (Lanes 2-5 inclusive). The size of the *Mycoplasma hominis* target amplicon (Lane 7) corresponds to the 270bp band represented by the provided hyperladder II (Lane 1).

2.2.2 Stimulation of fibroblasts and keratinocytes with growth factors

Normal human dermal fibroblasts and epidermal keratinocytes were isolated from 6mm punch skin biopsies, as described previously in Sections 2.2.1.1 and 2.2.1.2. The skin originated from discarded skin of healthy individuals undergoing plastic or reconstructive surgery. For the experiments, first and second-passage fibroblasts and keratinocytes were plated in 100 mm bacterial culture dishes. Subsequently cells were plated at 30,000 cells/well into 12-well plates. After being plated, fibroblasts were maintained in DMEM containing 10% FBS for 24 hours, then media was changed to DMEM with 1% FBS to slow growth on day two and media was changed to DMEM without FBS for two hours prior each growth factor stimulation. Keratinocytes were maintained in Epilife with S7 supplement for two days prior the experiment and media was changed to Epilife without supplement S7 two hours prior growth factor stimulation. One, 10 and 100 ng/ml of rhHB-EGF and EGF were added to the cells (Recombinant growth factors listed in **Table 2.1**). Well duplicates were used for each growth factor dose and time-point, including untreated cells. Samples were collected 15, 90 and 180 minutes after the application of HB-EGF and EGF. Quantitative real-time PCR was performed with RNA isolated at the indicated times after recombinant growth factor stimulation.

Table 2.1 Human recombinant growth factors.

Human recombinant growth factors	Supplier	Catalogue number
HB-EGF	R&D systems	259-HE
EGF	Sigma-Aldrich	E9644

2.2.3 RNA isolation from skin biopsy specimens and cell monolayer cultures

Skin biopsy specimens were placed in RNAlater (Ambion, Austin, TX). The samples were stored overnight at 4^{o}C and then stored at -20^{o}C until processing.

2.2.3.1 Tissue disruption

All samples were processed using a polytron homogeniser (Fisher Scientific). A maximum of four samples were processed at one time to minimise RNA degradation. Initially, three round bottom 14 mls tubes were prepared, one with 1 ml 1% SDS, one with 1 ml 100% ethanol, and one with 4 mls dH_2O. The polytron was washed in each tube in that sequence and the last wash was always dH_2O. One ml lysis buffer was added in an extra round bottom 14mls tube. The RNAlater with the skin biopsy was emptied into a sterile culture dish and the skin biopsy gently wiped dry with paper. Potential technical problems arose when the small biopsy specimens became lodged within the homogeniser

probe. This problem was minimised by cutting the 4mm diameter biopsy in 4-5 pieces prior to the addition of lysis buffer. The tube containing the skin sample and lysis buffer were kept on ice at all times. The polytron was used to homogenise the samples and overheating was avoided by turning it off every 10-15 seconds. Between samples the probe was rinsed three times as described above (in 1% SDS, 100% ethanol and dH_2O) to avoid cross contamination.

2.2.3.2 Cell membrane disruption

The culture media was removed from the culture dishes and the cells were washed with PBS. For a 12-well plate 300 µl of lysis buffer were used per well. After 2-3 minutes, the mixture was pipetted up and down a couple of times and then collected in an eppendorf and kept on ice.

2.2.3.3 RNA extraction

RNA extraction was performed using the Ambion® mirVana™ miRNA isolation kit (Life Technologies, Paisley, UK). The bench was cleaned with RNase zap (Invitrogen), 70% ethanol and dH_2O, in that order. The samples were kept on ice. MiRNA homogenate additive was added at $1/10^{th}$ of the lysate volume (around 400 µl) and mixed by vortexing the tube for 15 seconds. The mixture was left on ice for 10 minutes. An equal volume of acid-phenol:chloroform from the bottom phase of the bottle was added to the initial lysate volume (400 µl) and vortexed for 15 seconds to mix. The samples were centrifuged at 10,000 RPM for 5 minutes at room temperature to separate the aqueous and organic phases. Then the aqueous (upper) phase was removed carefully without disrupting the

lower phase, and transferred to a new eppendorf. At that stage the elution solution (30 µl per tube) was preheated to 95^{o}C. Next, 1.25 volumes of 100% ethanol were added to the aqueous phase recovered previously (e.g for 300 µl, add 375 µl ethanol). For each sample, a filter cartridge was placed into each of the collection tubes supplied. Up to 700 µl of the lysate/ethanol mix was pipetted onto the filter cartridge. If I had volumes larger than 700 µl, I applied the mixture in successive applications to the same filter. The tube was centrifuged at 10,000 RMP for 1 minute to pass the mix through the tube. The flow through was discarded and the above repeated if more than 700 µl were available. Next, 700 µl miRNA wash solution 1 (supplied by the manufacturer) was applied to the filter cartridge and the tube centrifuged for 30 seconds. The flow-through from the collection tube was discarded and the filter cartridge replaced into the same collection tube. Then, 500 µl of wash solution 2/3 (supplied by the manufacturer) was applied and the tube centrifuged for another 30 seconds. A centrifuge with a second 500 µl wash solution 2/3 was repeated. The flow-through was discarded and an extra spin was performed to clear the washing buffer. After discarding the flow-through from the last wash, the filter cartridge was replaced in a new collection tube. At that stage, 30 µl of the pre-heated elution solution was applied, the cap was closed and the tube was left at room temperature for 1 minute. The tube was centrifuged for a further 1 minute at 10,000 RPM to recover the RNA. The elute was collected and stored at -80^{o}C.

2.2.3.4 RNA quantification and quality control

RNA concentration was determined using a Nanodrop ND1000 UV-Vis spectrophotometer (Labtech International Ltd). The absorbance of each sample at 260 and 280 nm was also assessed using the same instrument.

2.2.4 Reverse transcription

cDNA was synthesised from RNA extracted from cell monolayer cultures using the high capacity reverse transcription kit (Applied Biosystems). RNA samples were taken out of -80ºC and allowed to thaw on ice for 10 minutes. For a 10 µl master mix reaction per well, the components used included 2 µl of 10x RT buffer, 0.8 µl of 25xdNTP Mix, 2 µl of 10x RT Random Primers, 1 µl multiscribe RT and 4.2 µl nuclease-free H_2O. The amount was added to each well of a 96-well plate and an equal amount of RNA (10 µl) was added to the master mix. The plate was centrifuged at 10,000 PRM for 15 seconds. The reverse transcription was performed in a thermal cycler at 25ºC for 10 minutes, 37ºC for 120 minutes, 4º C for 4 seconds, then held at 4ºC and transferred to -20ºC for storage.

2.2.5 Quantitative-PCR (q-PCR) using Taqman

Total mRNA levels were assessed by multiplex real-time PCR using Taqman Gene Expression Assays (Life Technologies). The PCR reaction comprised the components: 1 µl of 20x Taqman Gene Expression Assay (*COL7A1* Hs01574745_g1, *HB-EGF* Hs00181813_m1 as FAM target probes), 1 µl of endogenous control VIC probes (*18sRNA* and *GAPDH* for Chapter 3), 10 µl 2 x Taqman Gene Expression Master Mix, 7 µl RNAase free H_2O (Qiagen, Crawley, UK) and 2 µl cDNA. All Taqman probes were purchased from Applied Biosystems. All PCR reactions were carried out in triplicate, in 96-well plates (Thermo Scientific ABgene, Ashford, UK) covered by optical adhesive lids and samples were amplified in an Applied Biosystems 7900HT Sequence Detection System. Target gene amplification was performed under the following cycling conditions: 95ºC for 10 minutes and then 95ºC for 15 seconds, 60ºC for 1 minute for a total of 40 cycles. Target gene expression was normalised to that of endogenous genes (*18S*).

2.2.5.1 Interpretation of q-PCR data

qPCR results were analysed using SDS 2.4 software (Life Technologies). Real-time data were expressed as mRNA expression fold changes using the comparative C_T ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak 2008). The ΔΔCt value was calculated using the formula: ΔΔCt= (*C*T, Target - *CT*, Endogenous) Treated - (*C*T, Target - *CT*, Endogenous) Untreated. Bar graphs, error calculations and t-tests were performed using Microsoft Excel 2010 and GraphPad Prism software (version 5.03, GraphPad, San Diego, CA).

2.2.6 Immunohistochemistry

2.2.6.1 Immunofluorescent staining of skin specimens

Normal human skin was used as positive controls and for negative controls the same sections without primary antibody incubation were used. OCT-embedded skin biopsies were cut using a cryostat at 5 μm thickness and stored at -80^{o}C. Sections were air dried for 30 minutes, if freshly cut. If taken out of the freezer they were left for 10 minutes at room temperature. Hydrophobic circles were marked around the tissue sections using a pen (Abcam, Cambridge, MA, USA) and slides were dried for 5 minutes. The sections were then fixed with paraformaldehyde for 5 minutes. All sections were washed in PBS for 5 minutes and then blocked for 20 minutes with blocking buffer containing: 10% goat serum with 0.1% bovine serum albumin diluted in PBS (BSA/PBS) and 0.1% Tween20. Primary and secondary antibodies (**Table 2.2**) were diluted in blocking buffer and incubated for at least 20 minutes on ice. The secondary antibody was stored in the dark. The sections were washed once in PBS for 5 minutes. PBS was gently removed with paper. Following primary antibody incubation for 30 minutes at RT, sections were washed thoroughly with three changes of five minute PBS washes and the appropriate

goat secondary antibody conjugated with Alexa Fluor 488 was applied for 30 minutes at room temperature in the dark. Sections were washed in two changes of PBS for 5 minutes covered from light. Then sections were lastly rinsed in distilled H_2O for 5 minutes also protected from the light. Sections were allowed to air dry, were then mounted with DAPI and a cover glass was gently applied. The sections were left overnight at room temperature in the dark. The sections were kept at 4^{o}C for up to 7 days and at -80^{o}C for longer. IgG1 (Sigma-Aldrich, Dorset, UK) was used as isotype control and for negative control normal human skin sections without primary antibody incubation.

Table 2.2 Table with primary and secondary antibodies.

Antibody	**Species**	**Conjugate**	**Supplier**	**Catalogue number**
Collagen VII clone LH 7.2	Mouse monoclonal	-	Sigma-Aldrich	C805
IgG	Goat anti-mouse	Alexa Fluor 488	Thermo-Scientific	A-11001

2.2.7 Preparation of skin biopsies for fluorescence *in situ* hybridisation (FISH)

The skin samples were transported in 10% neutral buffered formalin. Within 48 hours tissue was immersed in sequential 70%, 100% ethanol, xylene and paraffin wax using an automated tissue processor. Subsequently, it was embedded in paraffin wax, allowed to cool at room temperature and was stored at room temperature. Paraffin-embedded

samples were cut at 5 μm using a Leica Microtome RM 2125 (Leica Microsystems, Milton Keynes, UK). Sections were picked up on silane coated slides (Sigma-Aldrich).

The paraffin-embedded sections were de-waxed by incubation in xylene for 5 minutes at room temperature and then through graded ethanol immersions (of 2 minutes each), beginning with 100%, followed by 90%, 70% and finally 50% ethanol. Slides were washed in water and then immersed in Mayer's haematoxylin (Sigma-Aldrich) for 3 minutes, and then washed and immersed in 0·5% eosin Y (VWR International Ltd, Lutterworth, UK) staining solution for 3 minutes. Sections were mounted by covering with 22 x 50 mm coverslips (VWR) using DPX in xylene mountant (Sigma-Aldrich) and then visualised.

2.2.8 Serum isolation from whole blood

Blood was collected in a gold top BD Vacutainer® tube with Hemogard™ Closure (BD Diagnostic Systems, Oxford, UK). The tube was left at room temperature for 20 minutes prior to centrifuging. Subsequently, the tube was centrifuged for 15 minutes at 3000 RPM at 4°C. Once the serum was isolated, it was aliquoted and stored at -80°C.

2.2.9 Serum cytokine measurement

Serum cytokine levels were measured by using a Milliplex kit for interferon-γ (IFN-γ), interleukin-17A (IL-17A), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), and tumour necrosis factor-α (TNF-α) (Millipore Ltd, Watford, UK). Serum samples were prepared as detailed in Section 2.2.8. Samples were thawed completely and mixed well by vortexing and centrifuging prior use. Sixty μl from each vial of antibody-

immobilised beads was added to the Mixing bottle and brought to a final volume of 3 mls with Bead diluent. The mixed beads were vortex well. The quality controls, the wash buffer, the Serum Matrix and the human cytokine standard were prepared as per the manufacturer's instructions. After preparation of all reagents, the assay was conducted. Two hundred µl of Wash Buffer were added per well and the plate was shaken for 10 minutes at room temperature. Subsequently, the Wash buffer was decanted and 25 µl of Standard or Control was added to the appropriate wells. The same amount of Assay Buffer was added to the background and sample wells. Twenty-five µl of the Serum Matrix, was added to the background, standards and control wells. The same amount of undiluted sample was added to the sample wells and finally, 25 µl of the Mixed beads to each well, while shaking the plate intermittently. The plate was sealed and wrapped with foil and incubated overnight at 4^{o}C or for 2 hours at room temperature. Afterwards, the well contents were removed and the late washed using a hand-held magnetic plate washer (Millipore Ltd). Twenty-five µl detection antibodies were added per well and incubated at room temperature for 1 hour. Without aspirating, 25 µl of streptavidin-Phytocoerythrin was added per well, incubated for 30 minutes at room temperature and washed twice with 200 µl Wash Buffer. Hundred and fifty µl of Sheath fluid was added to all wells and the plate was run on Luminex® FLEXMAP 3D® System (Millipore Ltd). Each multiplex assay was performed in duplicate on two different occasions according to the manufacturers' specifications. Standard curves for each cytokine, using each kit, were generated by using the reference cytokine concentrations supplied by the manufacturer. Raw data (mean fluorescent intensity) from all kits were analysed by MasterPlex Quantitation Software (MiraiBio, Inc., Alameda, CA, USA) to obtain concentration values.

2.3 Wound measurement methodology

A number of wound measurement devices and software products were reviewed to find the most suitable wound measurement product. ElixrTM (previously known as WITA) was selected because it had the advantage of a no touch technique reducing patient discomfort. It combines a pattern recognition software algorithm with artificial intelligence to analyse digital erosion images (iCLRTM technology, Imago Care Ltd). A digital photograph was taken of each erosion included in the study, and uploaded onto a secure hospital personal computer. The image was subsequently calibrated and the erosion edge was traced with a computer mouse. Erosion surface area was then calculated by the software (**Figure 2.2**). The software has good intra-user reproducibility and I performed all the tracing and measurements myself. Approval was sought and gained by the GSTT Business Management team for the software to be installed onto specific NHS computers.

Figure 2.2 Demonstration of the wound measurement software.
(A) Once the digital photo is uploaded on the digital planimetry software, calibration with the ruler in the picture and mapping of the wound edge follows. (B) Analysis of the wound surface area and (C) tissue analysis.

2.4 Suction blisters

Suction blister time was measured using a two-chamber negative pressure device with three 3mm orifices (NP2 model, Electronic Diversities, Finksburg, USA). The Negative Pressure Cutaneous Suction System is a self-contained instrument package. The blisters are created through the use of suction chambers that are attached to the patient's skin. Briefly, the numbered chambers are connected to the appropriate chamber control channel. Once the chamber is secured to the patient's skin, the device is turned on at a pressure of 12–15 mm Hg. This pressure creates a suction blister in a healthy person in 60 minutes. The application of negative pressure from the instrument console, to the chamber interior, causes the patient's skin to be gently drawn through the openings in the orifice plate approximately the size of the opening(s) in the orifice plate. The procedure caused no discomfort to the children and the discomfort was minimal to the parents. A video of the procedure can be found in Tolar *et al* (2013a).

2.5 Assessment tools

2.5.1 Paediatric Quality of Life questionnaire (PaedsQL)

The PaedsQL™ Measurement Model is a modular approach to measuring health-related quality of life in healthy children and adolescents and those with acute and chronic health conditions. It integrates both generic core scales and disease-specific modules into one measurement system. The parent and child completed the questionnaires independently of one another. If the child or parent had a question about what an item means or how they should answer it, I repeated the item to them verbatim. I asked them to answer the item according to what they think the question means. If they had trouble deciding on an

answer, I asked them to choose the response that comes closest to how they feel. The child and/or the parent had the option of not answering a question if they truly did not understand the question.

For the child, the PaedsQL asks questions about how he/she feels and what they think about their health. For the parent, it assesses health-related quality of life in their children. It contained questions about their child's physical, emotional, social, and school functioning in the past one month. According to the dedicated website (http://www.pedsql.org/score.html) the PaedsQL™ Generic Core Scales are:

- **Brief** (23 items)
- **Practical** (Less than 4 minutes to complete)
- **Flexible** (Designed for use with community, school, and clinical pediatric populations).
- **Developmentally Appropriate** (Ages 2-18; Child Self-Report Ages 5-7, 8-12, 13-18; Parent Proxy-Report Ages 2-4, 5-7, 8-12, 13-18).
- **Multidimensional** (Physical, Emotional, Social, School Functioning).
- **Reliable** (Total Scale Score: 0.88 Child Self-Report; 0.90 Parent Proxy-Report).
- **Valid** (Distinguishes between healthy children and children with acute and chronic health conditions; distinguishes disease severity within a chronic health condition).
- **Responsive** to clinical change over time.

2.5.2 Pain sleep and fatigue questionnaire

This is a non-validated, EB-specific questionnaire developed by the Paediatric Psychologist Consultant, Dr Christina Liossi, at Great Ormond Street Hospital. The questionnaire was developed because there was no existing, validated questionnaire to use. The questionnaire scores the pain intensity for different pain sources such as the skin, muscle and bones, mouth, teeth, eyes, bowel opening, urination and dressing changes.

Sleep and fatigue are also assessed with several questions. Full questionnaire is included in the CD-ROM enclosed.

For both the children's and parents' questionnaire the score is calculated by computing the total score for all pain intensity related items and all other items are reported separately (if at all).

2.5.3 Global severity score

The GSS or global impression scale, is a well-established rating tool applicable to several skin diseases and it is easy to use (. It is a 5-point scale which provides a snapshot of disease severity. The purpose is to make a clinical global judgement about the disease severity across various time points. There is no validated GSS specific to EB. A generic GSS was adjusted for the purpose of the EBSTEM clinical trial. It contains a global severity section which takes into account the area of skin affected, how inflamed and how painful the skin is. It also contains a second part about global improvement since the treatment given in the EBSTEM trial. The same elements as above are assessed.

2.5.4 Birmingham Epidermolysis Bullosa Severity Score (BEBSS)

The BEBSS is a validated EB severity scoring system and was considered the best available for the purpose of the clinical trials included in this thesis. Eleven items were scored: area of damaged skin, involvement of nails, mouth, eyes, larynx and oesophagus, scarring of hands, skin cancer, chronic wounds, alopecia and nutritional compromise. Area is allocated 50 points, and the 10 other items 5 points each, giving a maximum score of 100.

2.5.5 Blister manual

The blister manual used in the EBSTEM trial was developed by Dr Jemima Mellerio for the study. People with EB pierce their blisters and do their dressings for different parts of the body on different days, e.g arms and legs on one day, trunk the next day. Blisters present on each day were recorded and then totaled once all body areas are included. A body site was only counted once. Images of different size blisters were included to demonstrate tense and flat blisters. If blisters were in clusters, if the skin between them was not red or crusty, each blister was included in the count. Individuals were advised to photograph any blisters they were not sure if they needed inclusion or not.

3 MODE-OF-ACTION OF ALLOGENEIC CELL THERAPY IN RDEB

3.1 Intradermal injections of allogeneic fibroblasts in a person with RDEB

3.1.1 Introduction

It has been previously demonstrated that intradermal injections of allogeneic fibroblasts in RDEB intact skin can increase C7 expression at the DEJ, although that study did not disclose how long the benefits were sustained for or indicate a therapeutic mode of action (Wong *et al.* 2008). A further study attempted to elucidate the mechanism of action of allogeneic fibroblast injections in an individual with RDEB who was compound heterozygous for a nonsense mutation and a splice-site mutation (p.Arg682*/IVS87+4A →G) in *COL7A1* (Nagy *et al.* 2011). That study showed that a single fibroblast injection in an individual with RDEB could lead to increased C7 at the DEJ for ~9 months and increased *COL7A1* expression for ~3 months. Transcriptomic data demonstrated that the elevated *COL7A1* expression is associated with increased expression of *HBEGF* and, *in vitro*, incubation of keratinocytes and fibroblasts with HB-EGF leads to increased *COL7A1* expression. It was then hypothesised that injection of allogeneic fibroblasts led to upregulation of *HBEGF in vivo* in an autocrine manner (Hashimoto *et al.* 1994; Goishi *et al.* 1995) (**Figure 3.1**).

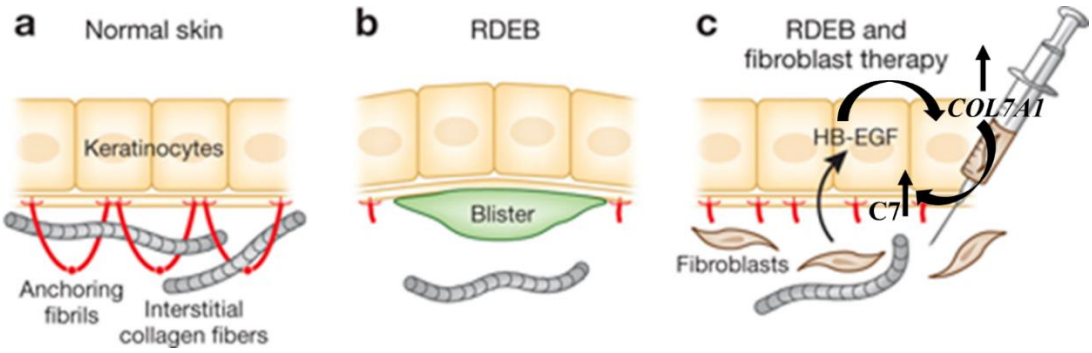


Figure 3.1 Schematic representation of the possible mode-of-action of fibroblast injections at the DEJ. (a) Normal skin; (b) blister formation in a patient with RDEB; and (c) postulated mechanism by which fibroblast therapy may ameliorate the blistering tendency. (a) In normal skin, keratinocytes synthesise C7 molecules (red), which assemble into anchoring fibrils. These fibrils entrap the interstitial collagen fibres in the dermis, securing the stable association at the dermal–epidermal junction. (b) In most patients with RDEB, there are only a few rudimentary anchoring fibrils, allowing formation of blisters below the lamina densa as a result of minor trauma. (c) Allogeneic fibroblasts injected directly into the dermis elicit an undetermined reaction that leads to synthesis of HB-EGF, which upregulates the synthesis and assembly of the patient's own mutated C7. The increase in the rudimentary anchoring fibrils, which are partially functional, partially stabilises the association of epidermis to the underlying dermis and ameliorates the blistering tendency.

These data on mode-of-action of allogeneic fibroblasts however, are limited to observations in one patient. Although that study (Nagy *et al.* 2011) provided novel and significant insight into the mechanism of allogeneic fibroblasts, questions about mechanism remained unanswered, such as what was the optimal dose of HB-EGF underlying C7 responses, or, whether other growth factors might play a role in upregulating *COL7A1* expression.

After the Wong *et al* study (2008) showed potential for wound healing, allogeneic fibroblasts were used as an off-label indication in 12 individuals with RDEB (McGrath and Almaani, unpublished data). Specifically, similar numbers of cells (5×10^6 per centimetre of wound margin) were injected intradermally into the edge of chronic wounds. Serial photographs showed that RDEB wounds can heal rapidly following this intervention (**Figure 3.2**).

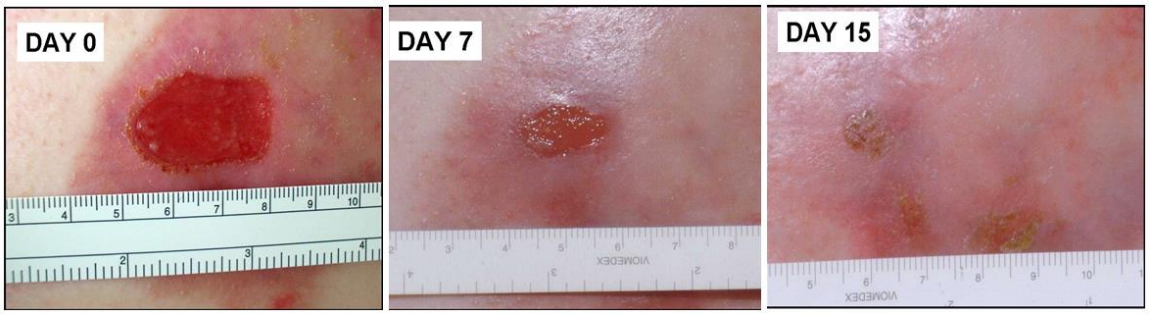


Figure 3.2 Photos showing off-licence use of fibroblasts in a RDEB wound.
Day 0 represents a wound on the day of injections. Injections were given to the margins of this wound (but not into the eroded area). Reduction in size can be seen on Day 7 with complete closure at Day 15 (Photographs kindly provided by Dr Almaani, and Professor McGrath).

Based on these observations and the transcriptomic data from Nagy *et al*. (2011), I aimed to: a) ascertain whether intradermal injections of allogeneic fibroblasts around wound margins *in vivo* result in *HBEGF*, *COL7A1* upregulation and C7 production, b) the optimal dose and time-points of HB-EGF stimulation of normal and RDEB epidermal cells to induce *COL7A1* expression *in vitro* and c) whether EGF stimulation of epidermal cells *in vitro* has the same effect on *COL7A1* expression as HB-EGF.

To demonstrate the first objective, and following informed consent, I selected a female individual with RDEB-SG with an open wound on her mid/lower back which was present and non-healing for approximately 15 years. The wound edges appeared clean with minimal exudate and were covered with an absorbent soft silicone foam dressing (Mepilex®, Mölnycke Health Care, Dunstable, UK) which was changed daily. The rest of her skin was mildly affected with another large wound on the back of her neck and smaller wounds on her knees. She was not on antibiotics and had no background of malignancy. She was of Pakistani origin with a homozygous acceptor splice site mutation in *COL7A1* (IVS13-1G>C). She had negative indirect immunofluorescence for both IgG and IgA antibodies (screened on normal human skin, split-skin and monkey oesophagus

substrates) at baseline and 3 months post fibroblast injections. Approximately, 80 cm of wound margin was injected. All injections were given at the same single occasion and no further fibroblast injections were administered. She was injected with a total of 20 vials of 20 x 10^6 cells/ml (Vavelta, Intercytex Ltd, UK) receiving 5 x 10^6 cells per linear centimetre of wound margin. EMLA was used before and Entonox during the injections. The procedure caused significant localised pain to the patient which subsided rapidly after the completion of injections. Initially, weekly telephone calls confirmed that she was recovering well and had no significant adverse reactions. During the course of the upcoming weeks she reported tightness at the wound margins and increased exudate from the wound surface, which needed change of dressing twice daily. The patient remained well and also managed to complete her college exams and driving lessons.

On Day 60 post fibroblast injections, 4mm diameter skin biopsies for direct immunofluorescence were taken from 3 different sites as indicated in **Figure 3.3**. The time-point was selected based on the finding from the Nagy *et al* study (2011) that *COL7A1* mRNA is increased between Day 15 and Day 90. Initially I examined the effects at a protein level with direct immunofluorescence, which was performed for samples from all three sites indicated in **Figure 3.3**.

3.1.2 Statistical analysis

Statistical analyses were performed using Microsoft Excel and GraphPad Prism software (version 5.03, San Diego, CA). The error bars represent standard error of the mean. The graphs represent results from three technical replicates.

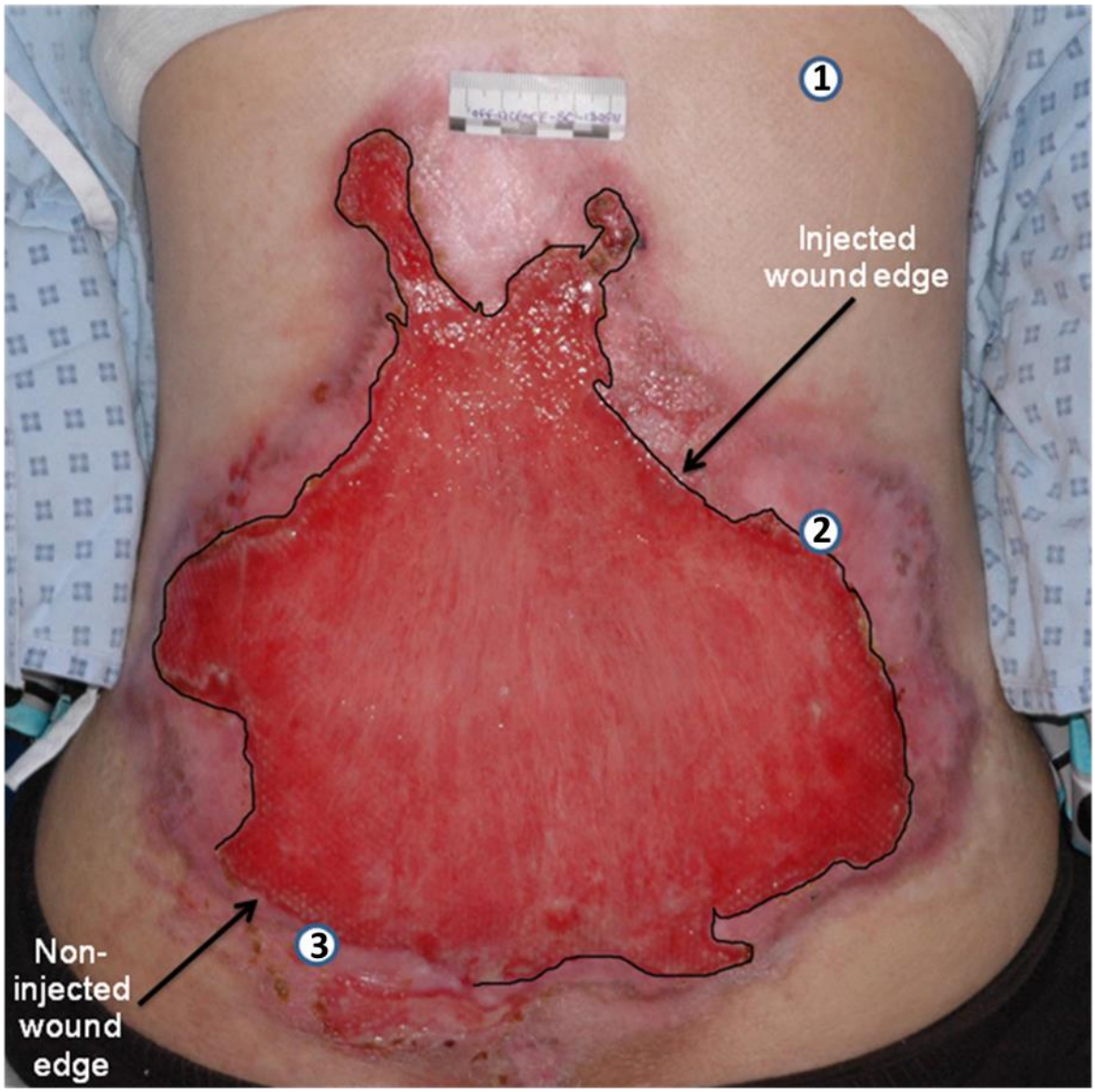


Figure 3.3 Photograph showing the biopsy sites on the back of an individual with RDEB.
The injected and non-injected wound margins are drawn and indicated with arrows. The black line indicates where the cells were injected. The white circles represent the three areas where the skin biopsies were taken from. Site 1 is non-lesional skin away from the wound margin. Site 2 is adjacent to the injected wound margin. Site 3 is adjacent to the non-injected wound margin.

3.1.3 Results

Immunolabelling for C7 revealed linear C7 labelling at the DEJ in normal human skin (NHS). There was some baseline C7 staining in the unblistered, non-lesional skin (Site 1) at Day 60 which was reduced compared to NHS. C7 staining at the injected wound edge (Site 2) was present at Day 60 which was more intense than the patient's normal looking skin but, as expected, reduced compared to normal skin. Site 3 at Day 60 had similar C7 intensity staining to the patient's non-lesional skin (**Figure 3.4**).

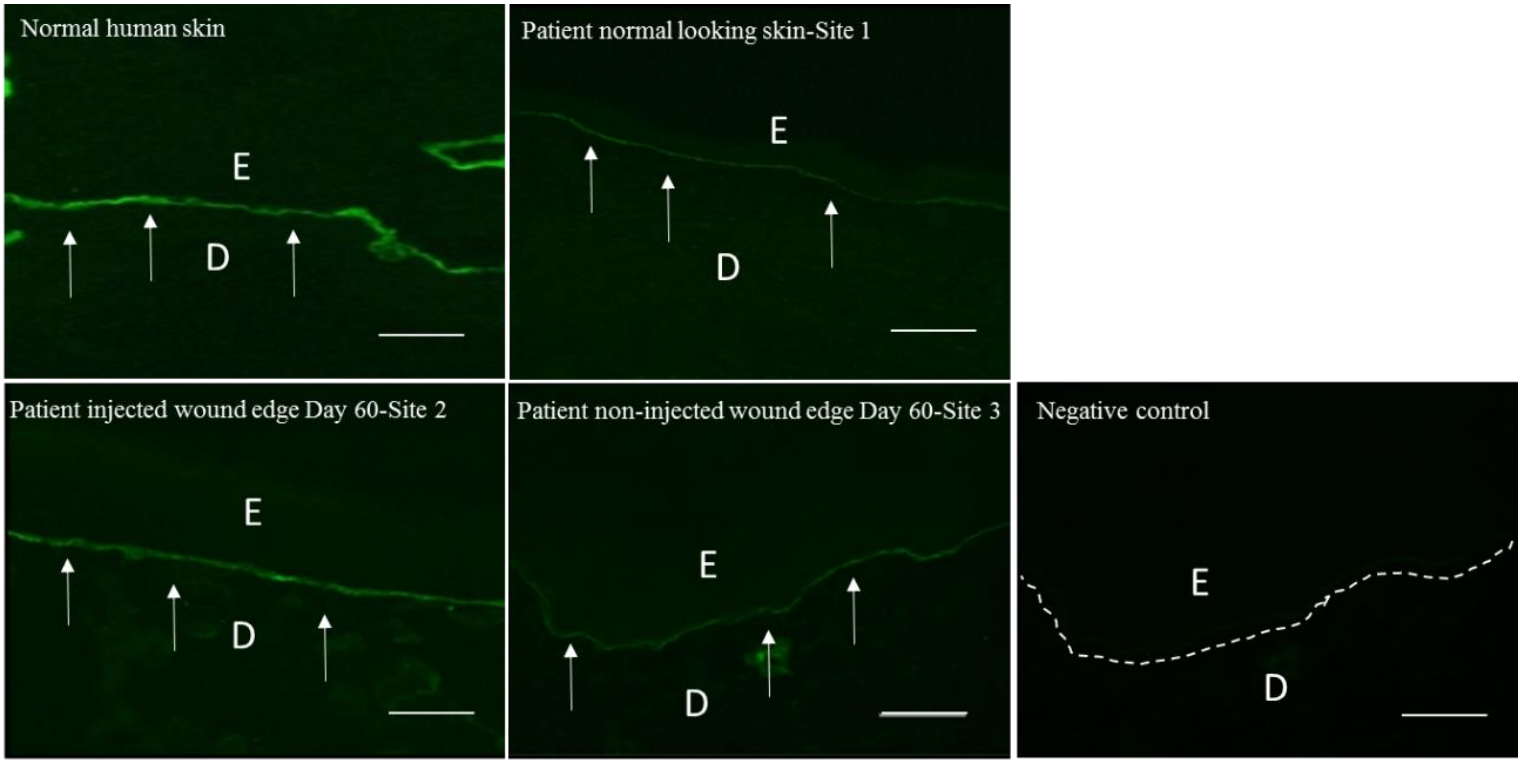


Figure 3.4 C7 immunofluorescent staining of the biopsy sites.
Immunolabelling for C7 using the monoclonal LH7.2 antibody shows some baseline C7 linear staining for the normal looking skin (Site 1) which is reduced compared to normal human skin. At Day 60 the injected wound edge (Site 2) demonstrates increased staining intensity as compared to the patient's normal looking skin. Some C7 deposition is also evident at Day 60 at the non-injected wound edge. (Bar represents 100μm). E=epidermis; D=dermis; dashed lines represent DEJ.

The immunofluorescence findings were supported by the relative mean immunofluorescence intensity of C7 at the DEJ (**Figure 3.5**). The non-injected wound edge (Site 3) at Day 60 had similar immunofluorescence intensity to the normal looking skin (Site 1).

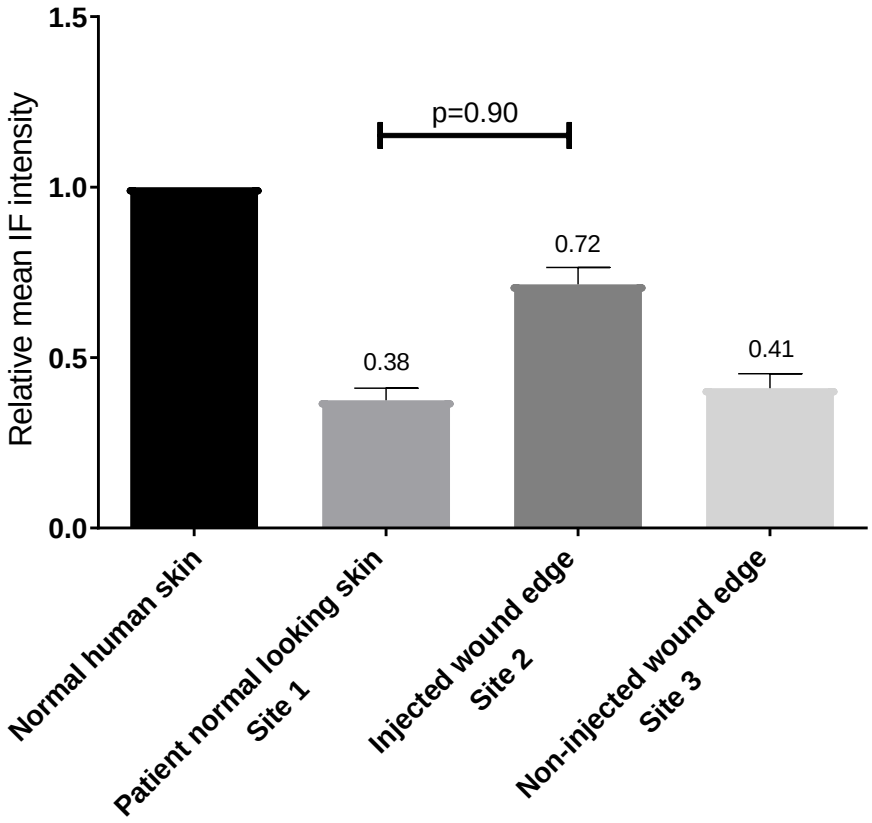


Figure 3.5 C7 immunofluorescence intensity of the biopsy sites.
Using identical image settings demonstrated that the relative mean fluorescence intensity of C7 fluorescence staining at the DEJ increased on Day 60 after treatment with fibroblasts (Site 2) compared with untreated, non-blistered skin (Site 1). Error bars represent standard deviation. Numbers show the relative immunofluorescence inactivity compared to normal human skin.

RNA was extracted and levels of *COL7A1* and *HBEGF* were measured with real-time PCR. **Figure 3.6** shows 4-fold increased, but not significant, expression of *COL7A1* at Day 60 at Site 2 compared to the patient's unblistered skin (Site 1) following a single series of injections of allogeneic fibroblasts. *COL7A1* mRNA levels also increased, at a lesser level, 2-fold at the non-injected wound edge (Site 3) as compared to the patient's unblistered skin (Site 1). Site 2 (injected wound edge) showed 1.8-fold change, which was not significant, in *COL7A1* expression as compared to the non-injected (Site 3) wound edge. The *COL7A1* expression at the injected wound edge (Site 2) was approximately half that of normal human skin. In all samples the *COL7A1* expression levels were less these of normal human skin, which is consistent with the immunolabelling and clinical findings.

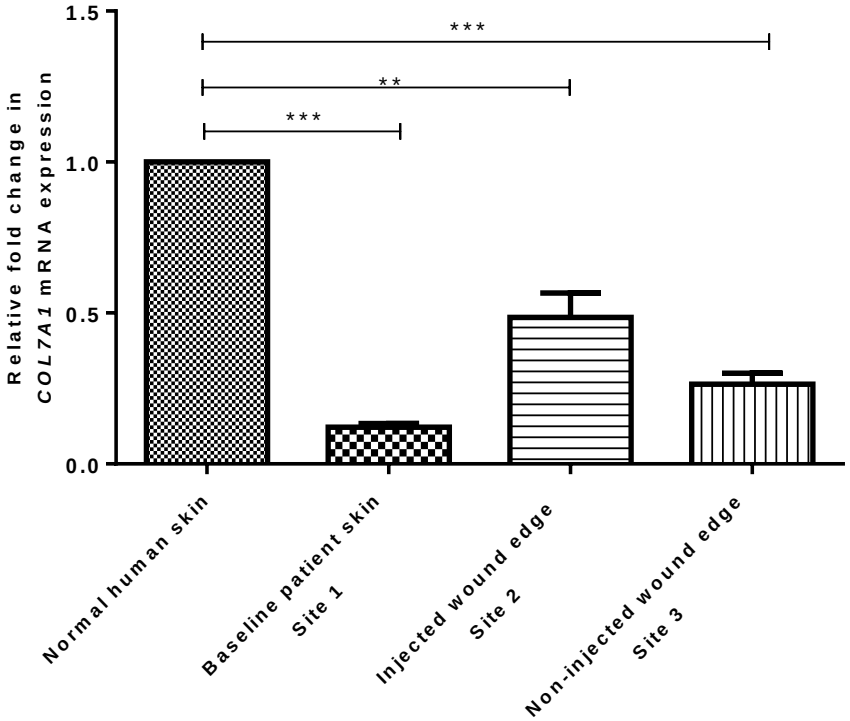


Figure 3.6 qPCR for *COL7A1* expression at Day 60 following a single series of injections of allogeneic fibroblasts.
*** $p<0.001$, ** $p<0.05$.

Compared to the patient normal skin (Site 1), increase ($p<0.05$) in *HBEGF* expression was seen in the injected wound edge (Site 2) (**Figure 3.7**).

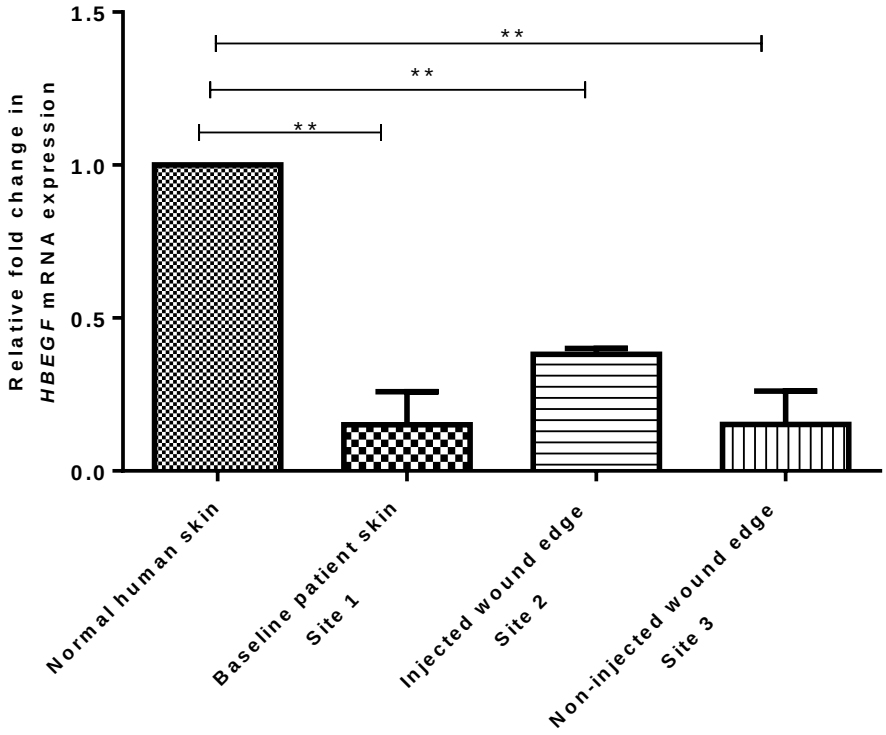


Figure 3.7 q-PCR for *HBEGF* expression at Day 60 following a single series of injections of allogeneic fibroblasts.
**p <0.05

Figure 3.8 shows a series of wound photographs, which demonstrate the reduction in wound size over the course of 8 months. During this period the patient required two courses of oral antibiotics for wound infections and had one iron infusion. She also reported that the healed wound edges broke down secondary to minimal trauma, i.e from scratching during her sleep. Despite the above, there was a 30% reduction in the wound surface area at Day 233 after the single series of fibroblast injections. This time-point at 8 months was selected based on the finding of the Nagy *et al* study (2011) that C7 labelling persisted for at least 270 days.

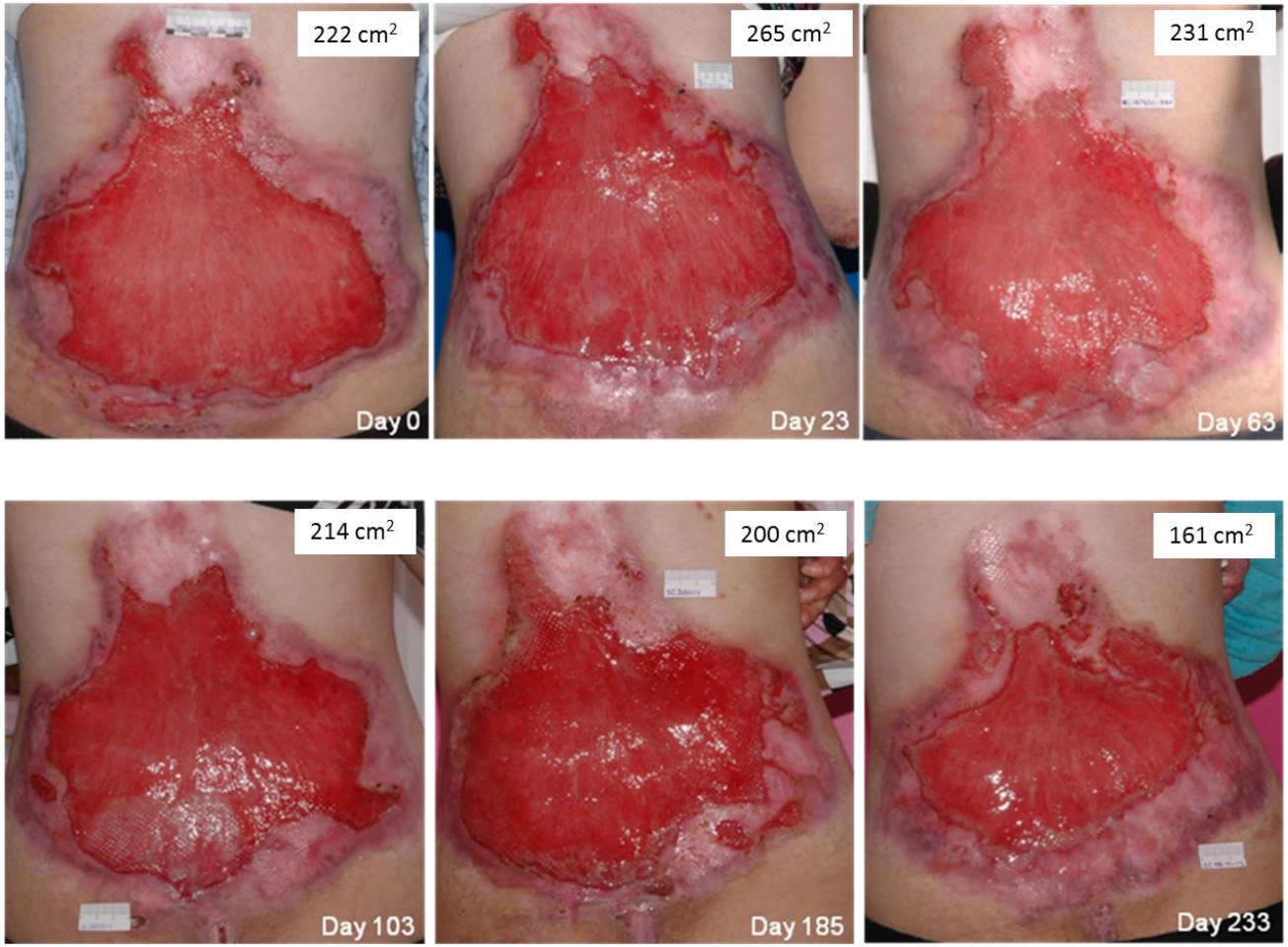


Figure 3.8 Photographs showing the progress of the RDEB wound over the course of 8 months**.** The patient had not seen any clinical improvement for the past 15 years.

3.2 Growth factor experiments

3.2.1 Introduction

The mechanism leading to formation of new C7 remains largely unknown. The same group that tested the effect of fibroblasts into intact (non-blistered) RDEB skin performed gene expression profiling to show that, among other factors, the expression of heparin binding–EGF-like growth factor (HB-EGF) was increased after intradermal injections of fibroblasts (Nagy *et al.* 2011). Therefore, they hypothesised that this molecule may be a key factor stimulating the patient's own keratinocytes to increase synthesis of mutant, but partially functional, C7. The study also showed that recombinant HB-EGF protein can increase *COL7A1* gene expression in normal or RDEB keratinocytes and fibroblasts and that this may provide an explanation for how allogeneic fibroblasts improve skin function in RDEB. In recognising the potential of HB-EGF to upregulate C7, it also has to be borne in mind that, given the increased risk of SCC in RDEB, that HB-EGF has been implicated in RDEB-associated SCCs by stimulating cell proliferation and survival (Kivisaari *et al.* 2010). HB-EGF is a member of the EGF family, which includes: EGF, TGFα, amphiregulin, epiregulin and betacellulin (Higashiyama *et al.* 1991). Apart from HB-EGF, none of the other EGF family members was upregulated in the transcriptomic data in the Nagy *et al* (2011) study. EGF is commercially available in spray form (1 mg/20 ml) (Easyef, Daewoong Pharmaceutical Ltd, South Korea). This product has been used in phase III clinical trials and preliminary results have shown positive effects in treatment of diabetic foot ulcers (Tuyet *et al.* 2009).

Based on the RDEB case study results presented in Section 3.1 and the Nagy *et al* paper (2011), I designed a number of *in vitro* experiments to assess the effect of HB-EGF and EGF on normal and RDEB keratinocytes and fibroblasts. The 1 and 10 ng/ml doses were tested in addition to the dose of 100 ng/ml for both growth factors. The 1 and 10 ng/ml

doses were selected based on the Nagy *et al* paper (2011) The dose of 100 ng/ml was added, as it was more biologically relevant based on mouse and rat data. EGF doses of 10ng/ml have been used in mouse colon cell lines (Frey *et al*. 2004). In a radiotherapy-induced oral mucositis rat model, survival rate improved when rats were treated with 50 μg/ml or 100 μg/ml rhEGF (Lee et al. 2007). In a mouse model of small intestinal irradiated mucosa regeneration of villi was noticeable in mice treated with more than 0.2 mg/kg rhEGF, and the villi recovered fully in mice given more than 1 mg/kg rhEGF (Lee *et al*. 2008).

The normal human cells were derived from discarded human skin after plastic surgery. The RDEB keratinocytes were derived from an individual with compound heterozygous *COL7A1* mutations: c.3632insC (p.Gln1211fs*8) and c.520G>A (p.Gly174Arg) The RDEB fibroblasts were derived from an individual with the homozygous *COL7A1* mutation c.8506insC (p.Val2836fs*12). Cells were isolated as described in **Section 2.2.1**. Data represent the average of three technical replicates of a representative experiment.

3.2.2 Statistical analysis

Statistical analyses were performed using Microsoft Excel and GraphPad Prism software (version 5.03, San Diego, CA). The error bars represent the standard error of the mean. The graphs represent results from three technical replicates.

3.2.3 Results

Following addition of HB-EGF, *COL7A1* upregulation was observed at 15 minutes and persisted for at least 180 minutes in both normal cells types (**Figure 3.9** and **Figure 3.10**).

Maximum effect was seen at 90 minutes for both cell types at the dose of 10 ng/ml for the fibroblasts and at the dose of 1ng/ml for the keratinocytes.

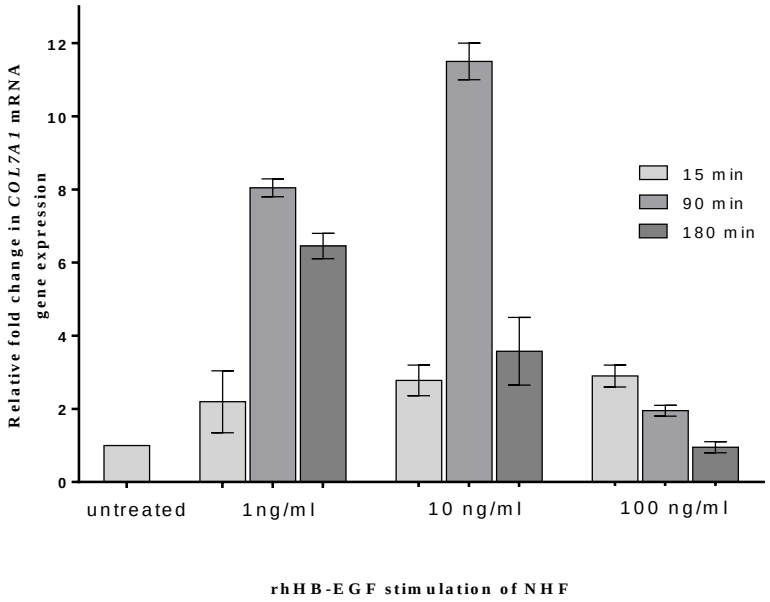


Figure 3.9 Normal human fibroblasts (NHF) treated with recombinant human HB-EGF (rhHB-EGF). Graph shows the relative fold change in *COL7A1* expression after stimulation of NHF with rhHB-EGF. Error bars represent standard error of the mean (SEM).

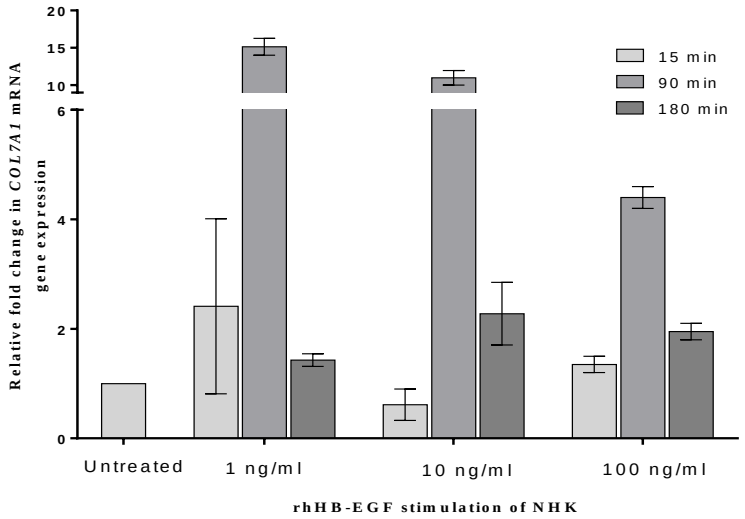


Figure 3.10 Normal human keratinocytes (NHK) treated with recombinant human HB-EGF (rhHB-EGF). Graph shows the relative fold change in *COL7A1* expression after stimulation of NHK with rhHB-EGF. Error bars represent standard error of the mean (SEM).

COL7A1 was upregulated at 90 minutes for all EGF doses for both normal cells but to a lesser degree compared to HB-EGF stimulation at 1 and 10ng/ml. (**Figure 3.11** and **Figure 3.12**). The effect was greater at the dose of 10 ng/ml for both normal keratinocytes and fibroblasts.

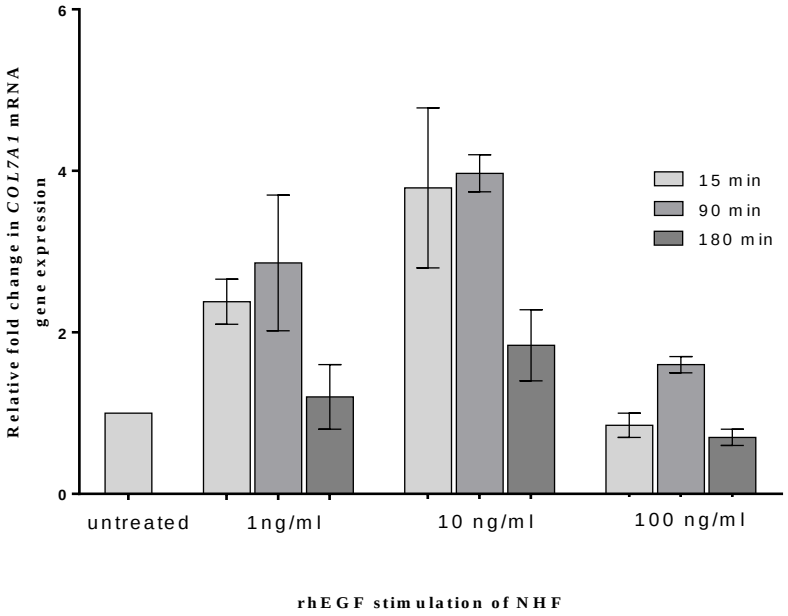


Figure 3.11 Normal human fibroblasts (NHF) treated with recombinant human EGF (rhEGF). Graph shows the relative fold change in *COL7A1* expression after stimulation of NHF with rhEGF. Error bars represent standard error of the mean (SEM).

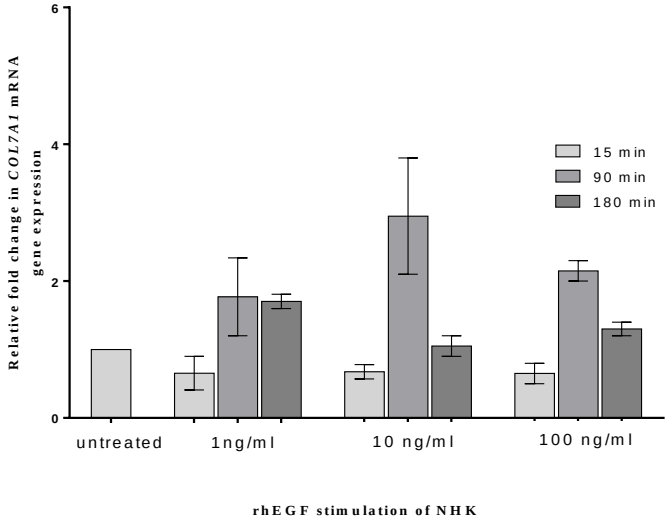


Figure 3.12 Normal human keratinocytes (NHK) treated with recombinant human EGF (rhEGF). Graph shows the relative fold change in *COL7A1* expression after stimulation of NHK with rhEGF. Error bars represent standard error of the mean (SEM).

Following addition of HB-EGF, *COL7A1* upregulation (up to 2-fold) was observed at 15 minutes for all doses in RDEB fibroblasts (**Figure 3.13**) and at 15 minutes for the dose of 1ng/ml, at 90 and 180 minutes for the doses of 1 and 10ng/ml for RDEB keratinocytes (**Figure 3.14**). The effect was greater in the RDEB keratinocytes (up to 6-fold) at 15 minutes. The higher dose of 100 ng/ml HB-EGF had an inhibitory effect on *COL7A1* expression in the RDEB keratinocytes at 15 and 90 minutes.

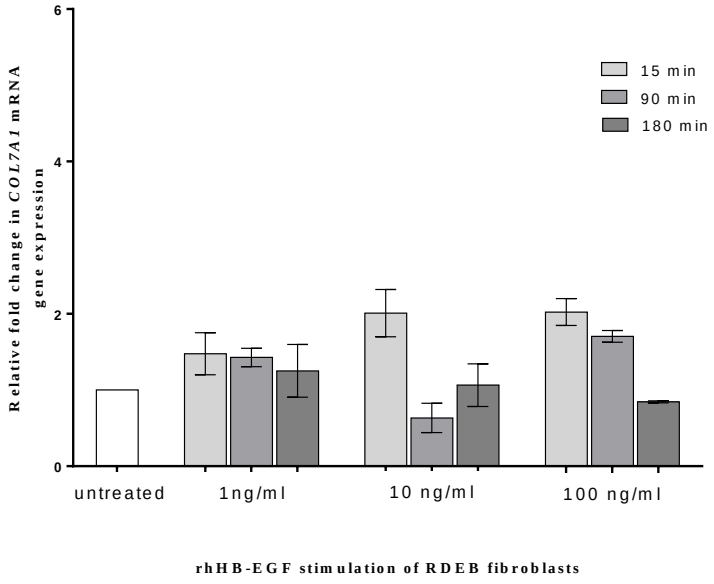


Figure 3.13 RDEB fibroblasts treated with recombinant human HB-EGF (rhHB-EGF).
Graph shows the relative fold change in *COL7A1* expression after stimulation of RDEB fibroblasts with rhHB-EGF. Error bars represent standard error of the mean (SEM).

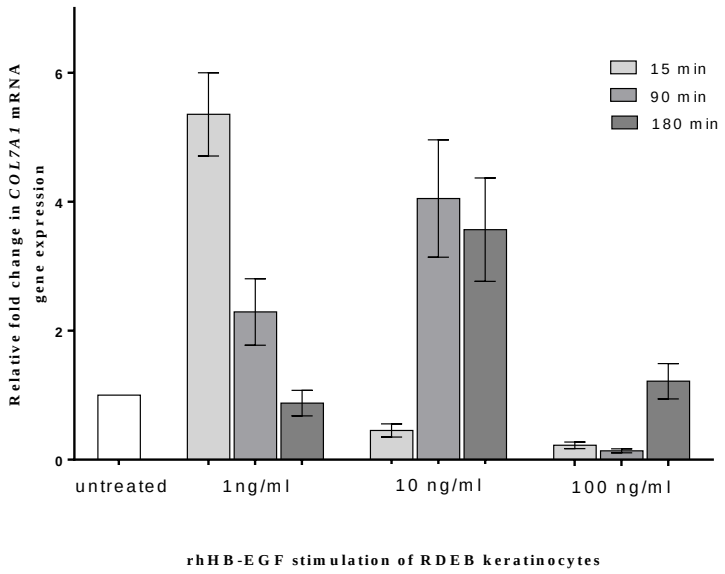


Figure 3.14 RDEB keratinocytes treated with recombinant human HB-EGF (rhHB-EGF).
Graph shows the relative fold change in *COL7A1* expression after stimulation of RDEB keratinocytes with rhHB-EGF. Error bars represent standard error of the mean (SEM).

EGF addition to RDEB fibroblasts led to *COL7A1* upregulation at 15 minutes at the dose of 1 and 10 ng/ml and at 90 minutes at all doses. EGF addition to RDEB keratinocytes led to *COL7A1* upregulation at 90 minutes for all doses but to a lesser degree than for the RDEB fibroblasts. For the RDEB fibroblasts, EGF led to greater *COL7A1* upregulation as compared to the HB-EGF stimulation (**Figure 3.15** and **Figure 3.16**). For RDEB keratinocytes, HB-EGF had a greater effect than EGF at 15 minutes at the dose of 1ng/ml and at 90 and 180 minutes for the dose of 10ng/ml.

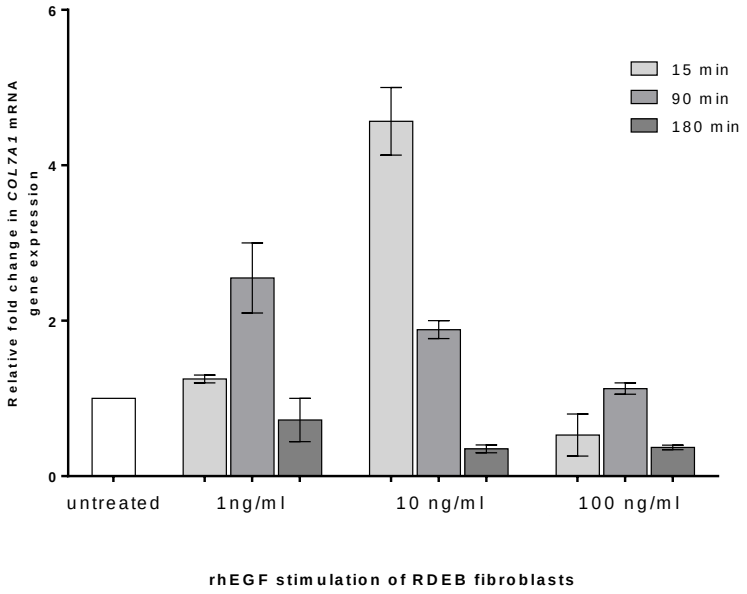


Figure 3.15 RDEB fibroblasts treated with recombinant human EGF (rhEGF).
Graph shows the relative fold change in *COL7A1* expression after stimulation of RDEB fibroblasts with rhEGF. Error bars represent standard error of the mean (SEM).

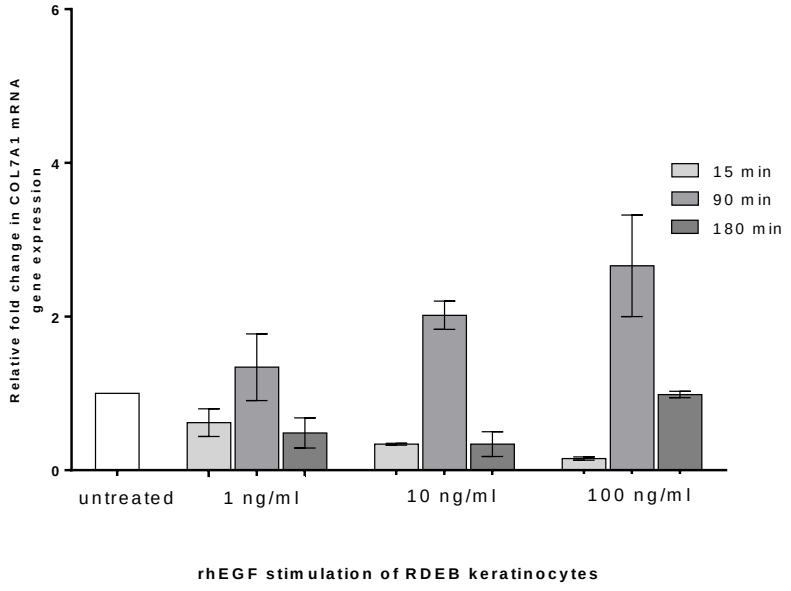


Figure 3.16 RDEB keratinocytes treated with recombinant human EGF (rhEGF).
Graph shows the relative fold change in *COL7A1* expression after stimulation of NHK with rhEGF. Error bars represent standard error of the mean (SEM).

3.3 Discussion

Chronic, non-healing wounds are hallmark features of RDEB. I demonstrated that intradermal injections of allogeneic fibroblasts around wound margins in an individual with RDEB result in improved wound healing at 8 months. Multiple separate injections given at one time point at a density of 5 million cells per linear cm resulted in 30% reduction of the wound size on this individual's back. The patient had clearly described that the wound has never healed or reduced in size for more than a few days over the past 15 years. The clinical appearance of the wound changed after the injections. There was initial deterioration at Day 23 and the wound size reduced slowly after that time-point. The deterioration could have been due to mechanical trauma and complicated by a wound infection. There was a 2-fold upregulation of *HBEGF* by Day 60 compared to the patient's baseline skin and non-injected wound edge. There was a 4-fold upregulation of *COL7A1* at the injected wound edge. The *COL7A1* upregulation was not parallel to the wound size reduction and lagged. This could be because protein expression follows gene upregulation. This would be consistent with findings in the Nagy *et al* study (2011), where C7 labelling was increased and maintained for at least 270 days, while *COL7A1* gene expression after initial increase returned to baseline at Day 180.

There was also a 2-fold *COL7A1* upregulation at the non-injected wound edge. One can speculate that this was a result of cell spillage from the adjacent injected edge because the biopsies were taken post injections from the injected and non-injected wound edges. The *COL7A1* upregulation was closely matched by C7 production at the dermo-epidermal junction. The injected wound edge had increased C7 IF intensity which however, was around 75% of that of normal human skin. This would explain the fact that the healed wound edges were covered with easily damaged skin as described by the patient herself. The fact that there was no statistically significant difference in the IF intensity between

the patient's normal looking skin and the injected wound edge could be because the patient had some baseline C7 expression as seen in **Figure 3.4**. The fact that there was some C7 staining at the non-injected wound edge could be from an indirect effect on the wound that extends from the adjacent injected areas or from local spillage of the injected fibroblasts to the adjacent skin area.

The clinical improvement of 30% reduction in wound size at Day 233 had a significant impact on the patient's daily routine by making it more manageable because of requiring smaller dressing sizes. The patient was dependent on her mother for the dressing change on her back and after it has reduced in size she was able to change the dressing herself on occasion. Although the patient reported the healed edges were friable and would break down easily if injured, i.e. by scratching, they would heal faster than what she anticipated compared to other wounds on her body.

The role of HB-EGF and EGF in skin wound healing has been well established (Brown *et al*. 1989; Shirakata *et al*. 2005), but there is evidence that HB-EGF is implicated in the pathogenesis of SCC in RDEB and the over-expression correlates with poor prognosis (Kivisaari *et al.* 2010). EGF, on the other hand, enhances acute wound healing by stimulating epidermal and dermal regeneration and not contributing to malignant transformation (Berlanga-Acosta *et al*. 2009). The microarray data from the Nagy *et al* study (2011) did not identify EGF as significantly upregulated post allogeneic fibroblast injections. The growth factor experiments described in this thesis, demonstrated that treatment with HB-EGF and EGF lead to *COL7A1* mRNA upregulation in normal and RDEB keratinocytes and fibroblasts. The optimal dose of HB-EGF with the highest *COL7A1* gene upregulation in normal keratinocytes was 1ng/ml at 90 minutes (15-fold upregulation) closely followed by 10ng/ml at 90 minutes (12-fold upregulation). For normal fibroblasts, the optimal dose was 10mg/ml at 90 minutes (12-fold). The effect was

smaller in RDEB cells. For RDEB fibroblasts, the dose of HB-EGF with the highest COL7A1 upregulation was 10 and 100ng/ml at 90 minutes (2-fold). For RDEB keratinocytes, the optimal dose was 1ng/ml at 15 minutes (6-fold). The optimal dose of EGF for highest *COL7A1* upregulation was 10ng/ml at 90 minutes (4-fold) for normal keratinocytes and 10 ng/ml at 15 minutes (5-fold) for normal fibroblasts. For RDEB keratinocytes the optimal EGF dose was 100ng/ml at 90 minutes (3-fold) and 10ng/ml at 15 minutes (5-fold) for RDEB fibroblasts. The higher dose of EGF required for the RDEB keratinocytes could be because they are less sensitive compared to fibroblasts to the effects of EGF.

HB-EGF is known to be an autocrine growth factor for human keratinocytes (Hashimoto *et al.* 1994) which would explain the more potent effect on *COL7A1* upregulation in the control and RDEB keratinocytes compared to EGF. The inhibitory effect of 100 ng/ml HB-EGF on *COL7A1* expression in the RDEB keratinocytes at 15 and 90 minutes could be due to an autocrine/paracrine inhibitory signalling of HB-EGF at higher doses. This was not observed with the addition of EGF and one can speculate that this is either due to higher affinity for the binding receptor or upregulation via a different pathway. EGF is known to activate predominantly EGF-R receptor complexes (Holbro *et al*. 2003); whereas HB-EGF binds to tyrosine kinase receptors (Junttila *et al*. 2000). Collectively, the current experiments show differential induction of *COL7A1* by low and high doses of EGF concentrations, but the underlying mechanisms remain to be elucidated.

4.1 Introduction

A therapeutic challenge has been to develop new gene, protein, cell and drug therapies for RDEB. Since the late 1980s, cell therapy clinical trials to improve wound healing in RDEB have involved several modalities, including autologous and allogeneic keratinocyte grafts and non-adherent dressings (Carter *et al.* 1987; McGrath *et al.* 1993; Falabella *et al.* 2000; Blanchet-Bardon and Bohbot 2005), as well as cultured dermal substitutes (Hasegawa *et al.* 2004). Given the orphan disease status of EB, most clinical trials have involved only a few affected individuals and the majority has failed to demonstrate clear beneficial clinical outcomes.

One recent innovation has been the use of fibroblast cell therapy. Fibroblasts have the capacity to synthesise adhesive components of the skin basement membrane zone including C7, as well as potentially augmenting wound healing (El Ghalbzouri and Ponec 2004). In 2003, two research groups showed that human RDEB fibroblasts, first corrected for the *COL7A1*, and then injected into the dermis of immune-deficient murine models of RDEB, could increase C7 expression at the DEJ and thereby restore the inherent basement membrane defect in RDEB (Ortiz-Urda *et al.* 2003; Woodley *et al.* 2003). Woodley *et al.* (2003) also showed that similar changes in C7 expression were possible using wild type control human fibroblasts (i.e. without *COL7A1* correction), if the fibroblasts were used at sufficient cell density (5 x 10^6 cells worked, but 1 x 10^6 did not). Based on these findings, in 2008, a proof-of-concept study in five subjects with RDEB demonstrated that a single intradermal injection of 5 x 10^6 allogeneic fibroblasts increased *COL7A1* and C7 expression in the skin of some individuals for at least 3 months, the endpoint of that study (Wong *et al.* 2008). An extended study in one of these individuals demonstrated that a

single injection of allogeneic fibroblasts could increase *COL7A1* gene expression for 3–6 months and C7 protein for 9–12 months (Nagy *et al.* 2011). Most of the initial human studies were performed in intact, unwounded skin, although some of the mouse studies showed that intravenously injected *COL7A1* gene-corrected fibroblasts could home to RDEB wounds and promote wound healing (Woodley *et al.* 2003). Based on these studies and Dr Almaani's unpublished data about wound healing after intradermal injections of allogeneic fibroblasts, I used the same cells in an individual with RDEB (described in **Chapter 3**) and demonstrated that they can promote wound healing *in vivo* and *in vitro*. The *COL7A1* upregulation, followed by C7 increased labelling led to reduction in wound size reduction in this one patient. These results were encouraging but preliminary. The need to assess the effect of intradermal injections of allogeneic fibroblasts on erosion healing in RDEB individuals led to the design and conduct for a prospective, randomised, vehicle-controlled trial.

4.2 Study ethics

The protocol was approved by the U.K. National Research Ethics Committee London-Dulwich (ref: 10/H0808/146) and the U.K. Medicines and Healthcare Products Regulatory Agency (EudraCT number: 2010-023121-38). The trial was registered with www.controlled-trials.com (ISRCTN67757229). The trial was conducted in accordance with the Declaration of Helsinki principles and the principles of the International Committee on Harmonisation Tripartite Guideline on Good Clinical Practice. All participants provided written informed consent.

4.3 Participants and interventions

Participants of either sex (aged 16–70 years) were invited to participate if they had a diagnosis of RDEB, based on clinical and molecular findings and had at least five open skin erosions on the limbs or the trunk, each with a surface area between 5 and 50 cm^2. Full inclusion and exclusion criteria are listed in **Table 4.1**. One minor and two substantial protocol amendments were submitted after the trial started. These changes included adding the option of home follow-up visits, expanding the age range, and exclusion of subjects with positive indirect IF for C7 antibodies.

Table 4.1 Inclusion and exclusion criteria.

Inclusion criteria
• Subjects with a clinical diagnosis of RDEB
• Subjects who are aged ≥ 16 and ≤ 70 years
• Subjects with at least five open skin erosions, which are located on the limbs or the trunk, each with a surface area between 5 and 50 cm^2. The size of these erosions must not exceed 50 cm2 at baseline.
• Subjects who have voluntarily signed and dated an informed consent form prior to the first study intervention
• Subjects, who are, in the opinion of the investigator, able to understand the study, cooperate with the study procedures, and are willing to be available for all the required follow-up visits
Exclusion criteria
• Subjects who have received immunotherapy/chemotherapy within 60 days of enrolment into this study
• Subjects with a known allergy to any of the constituents of the product
• Subjects with known or suspected malignancy

- Subjects with intolerance or allergy to additional study associated drugs/therapies (e.g. local anaesthetics, etc.)
- Subjects who have taken systemic antibiotics within 7 days
- Subjects taking immunosuppressive therapy including systemic steroids (e.g. oral prednisolone > 40 mg for more than 1 week) within the 30 days of the first treatment or planning immunosuppressive therapy at any time during the study. Intranasal/inhaled steroids are acceptable
- Subjects who have taken any other investigational product within 90 days prior to screening or planned use of any other investigational product during the study period
- Subjects who are pregnant, planning pregnancy and women of childbearing potential who are not abstinent or practicing an acceptable means of contraception, as determined by the investigator, for the duration of the treatment phase
- Subjects with abnormal laboratory findings considered clinically significant
- Subjects with a known history of poor adherence/compliance with medical treatment or follow-up
- Subjects who are unable to understand the aims, objectives and follow-up treatment
- Subjects with known alcohol or narcotic drug dependence.
- Subjects who have previously been screened on more than two occasions, or who have previously been treated under this protocol.
- Subjects who have previously been treated with allogeneic fibroblasts and who have an immune response to C7, with autoantibodies detected by immunofluorescence microscopy.

4.3.1 Randomisation and masking

Eligible erosions were randomised according to a computer generated 1:1 block randomisation schedule, which was provided in a sealed envelope by the trial statistician, assigning fibroblasts or vehicle for each erosion. The fibroblast suspension had a cloudy appearance, while the vehicle was clear. The trial sponsor, the statistician, myself, conducting the follow-up visits and erosion measurements, and all participants with

RDEB, were masked to treatment allocation until data verification was completed and the database locked.

4.3.2 Investigational medicinal product

The cell product (ICX-RHY-013) consisted of a suspension of allogeneic human dermal fibroblasts in HypoThermosol-FRS (BioLife Solutions Inc., Bothell, WA, U.S.A.) presented in a sterile solution. ICX-RHY-013 is designated as an orphan medicinal product by the European Medicines Agency for the treatment of EB. The contents of HypoThermosol-FRS, the vehicle, are listed in **Table 4.2**. The human dermal fibroblasts were isolated from neonatal foreskin from donors whose mothers had been screened for a range of diseases (syphilis, HIV 1/2, hepatitis B and C, HTLV-1/2 and CMV). The fibroblasts were cryopreserved, thawed and expanded in culture under good manufacturing practice principles at Intercytex Ltd, Manchester, U.K. The product was quality control tested, qualified person released, and shipped in prepacked vials to the Guy's Hospital pharmacy at 2–8°C. Cell viability was checked before dispatch, including following passage through a 21-G needle, and following one episode of warming to room temperature. Each vial contained 20 x 10^6 cells/mL and the dose used was 0.25 mL of 20 x 10^6 cells/mL (ICX-RHY-013) per linear cm of erosion margin, or 0.25 mL vehicle per linear cm of erosion margin. The cells had a shelf-life of 11 days, were stored at 2–8°C, and allowed to warm to room temperature 20-30 minutes before administration.

Table 4.2 Vehicle - HypoThermosol®-FRS constituents.

Dextran-40
Potassium chloride
Potassium hydrogen carbonate
Glucose (dextrose)
Mannitol
Calcium chloride
Magnesium chloride
Potassium hydroxide
Sucrose
KH_2PO_4
Sodium hydroxide
Lactobionic acid
Adenosine
Glutathione
HEPES
Trolox

A 10-point scale (verbal numeric rating scale) was used to record the pain associated with each study erosion. Trial subjects were asked to complete a questionnaire and score each erosion by completing a 5-point scale for each of the following categories: care, daily function, appearance and concerns (**Table 4.3**).

Table 4.3 Quality of life measurements.
A sample table from the case report file representing the questionnaire filled by a patient with three erosions at baseline and week 12. For each erosion the subject was asked to complete a 5-point scale for each of the listed categories.

Treated Erosions	CARE	DAILY FUNCTION	APPEARANCE	CONCERNS
1	3	2	1	1
2	3	3	2	2
3	3	2	2	2
4				
5				
6				
7				
8				
9				
10				

INSTRUCTIONS:

- Please think about each of your treated erosions, and then score each of the categories from 1-5, where 1 indicates that the erosion has minimal impact and 5 indicates maximum impact. If the erosion does not affect you at all in this category, please enter 0.

- Care – please think about how easy or difficult it is to dress the erosion, and how time consuming it is.

- Daily Function – please think about the impact this erosion has on your movement, sleeping, grooming, getting dressed and working.

- Appearance – please think about your feelings about the appearance of this erosion, the quality of the skin and how fragile it is.

- Concerns – please consider how anxious you feel about the erosion, and how you feel about other people's reaction to it.

4.4 Study design

The participants attended for a screening visit 4 weeks (±2 weeks) prior to the injections at the Clinical Research Facilities (CRF) unit at Guy's Hospital, London, UK. They were seen by me for initial assessment and confirmation of eligibility for the study. At the screening visit, inclusion and exclusion criteria were verified, informed consent was obtained, physical examination was performed, vital signs noted and use of any concomitant medications was recorded. Documentation, photography and measurement of erosions were performed, and baseline serum biochemistry and haematological laboratory parameters were tested, including serum testing for anti-C7 antibodies. At visit 2 (the day of injections, designated day 0), the screened erosions were photographed and measured by me to ensure they still met the inclusion criteria. The fibroblasts or vehicle were then injected intradermally into the mid-to-superficial dermis at the erosion margins under prior local anaesthesia with lidocaine/prilocaine cream (EMLA^{TM}, AstraZeneca, Wilmington, NC, U.S.A.) and simultaneous nitrous oxide and oxygen gas (Entonox^{TM}, BOC Healthcare, Worsley, U.K.) inhalation, as required. Injections were administered by the principal investigator (Professor McGrath) at the CRF unit at Guy's Hospital, London, in the presence of two CRF nurses. The injections were administered by the principal investigator so I, who was performing the subsequent wound measurements, was blinded to treatment. All injections of fibroblasts and vehicle were given using the same approach for each injection: drawing up the vial contents into a 2-mL syringe using a 19-G sterile needle and then injecting into the skin using a 21-G needle.

All participants continued standard skin care and regular dressing changes. Subjects were subsequently assessed at Days 7, 14, 28, 56, and at 3 and 6 Months after the injections on Day 0 (**Figure 4.1**). All assessments were done by me, who was blinded to treatment assignment, and were undertaken at the CRF or at the participant's home. Assessment

parameters included erosion photography and measurements of erosion size at all visits, safety monitoring blood tests (including IIF for serum C7 antibodies) at Day 28 and 3 Months and quality of life questions at Day 0 and 3 Months. Adverse events, erosion pain and concomitant medications were recorded at each visit. Each subject was loaned a digital camera to take weekly photos of the treated erosions for up to 3 months and diary cards to record the date any erosions healed as well as adverse events.

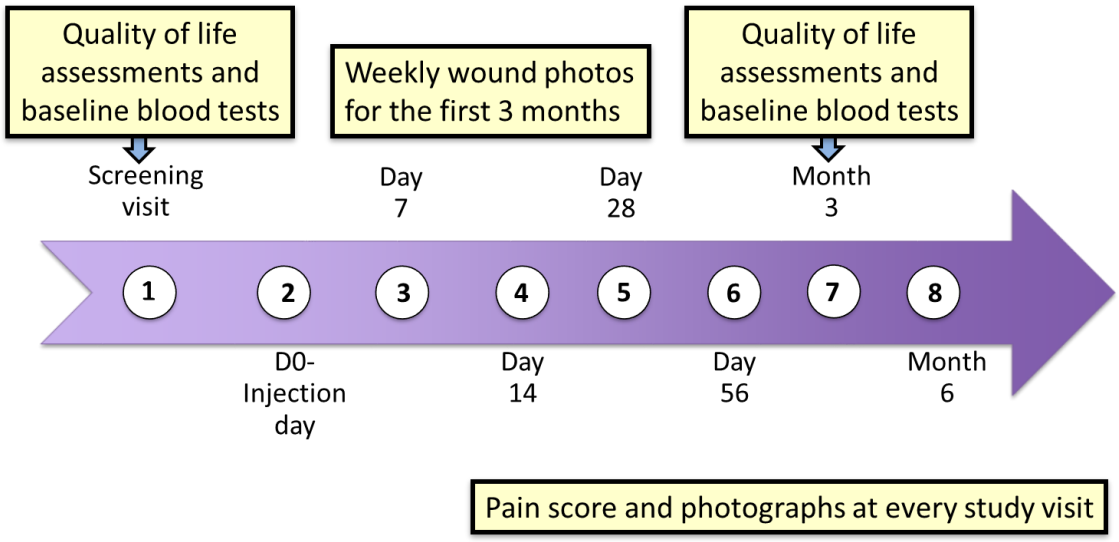


Figure 4.1 Trial design schematic**.**

4.5 Endpoints

The initial primary outcome measure was the time to complete closure of erosions treated with fibroblasts or vehicle. After consultation with the U.K. Medicines and Healthcare products Regulatory Agency (MHRA), however, and while the study was still blinded, the primary/secondary endpoint designation was changed to a hierarchy of outcomes as per the Committee for Medicinal Products for Human Use (CHMP) guideline on clinical trials in small populations (CHMP/EWP/83561/ 2005) (source www.ema.europa.eu). The first endpoint was the mean change in treated erosion area (cm^2) from baseline in erosions treated with fibroblasts compared with those treated with vehicle for up to 6 months. Other endpoints, in order, were percentage change in erosion area over treated areas, time to erosion closure, erosion recurrence (time to recurrence, if healed), and clinical assessment by an independent assessor of the treated area compared with baseline (using a 5-point scale: 1, a lot worse; 2, a bit worse; 3, much the same; 4, a bit better; 5, much better), erosion pain, and healed/not healed for each erosion.

4.6 Statistical analysis

Final statistical analysis was based on the CHMP guideline on clinical trials in small populations. The initial statistical analysis plan was based on the assumption that for 80% power, 20 subjects with an average of six erosions per person would have to be randomised and treated. The power calculation was based on the fact that 80% power is considered the ideal power for a study to detect an effect and 20% chance of error. Owing to the rarity of RDEB, however, as well as the inevitable small recruitment numbers for a rare disease, and the number of screened erosions that were excluded, a consultation with the MHRA led to a protocol amendment, with the aim of making the most efficient use of the dataset. There were no known prognostic variables that might be predictors of

outcome. The analysis of the study was on a comparative basis, with two-sided statistical significance tests performed at the 5% level. A two-way ANCOVA model was used to analyse the main efficacy endpoint of average percentage erosion area reduction from Day 0 to the 6-month assessment period. The ANCOVA model included treatment and patient as factors in the model and baseline area as the covariate. Erosion area and % change in erosion area were summarised at each study visit and analysed using analysis of covariance, with baseline area as a covariate. The study analysis was carried out when all patients completed the study and the database was locked. The study was only unblinded once the database was locked. The full analysis set comprised all erosions that received treatment. All statistical analyses were performed and all data appendices were created using the SAS (version 9.1.3; SAS Institute, Cary, NC, U.S.A.) statistical software.

4.7 Trial recruitment and logistics

Following regulatory and ethics approvals, databases of individuals with EB were reviewed in the three U.K. adult specialist centres (London, Solihull and Edinburgh) and 104 subjects with RDEB were invited to participate between February and December 2011. **Figure 4.2** shows the flow of patients through the study.

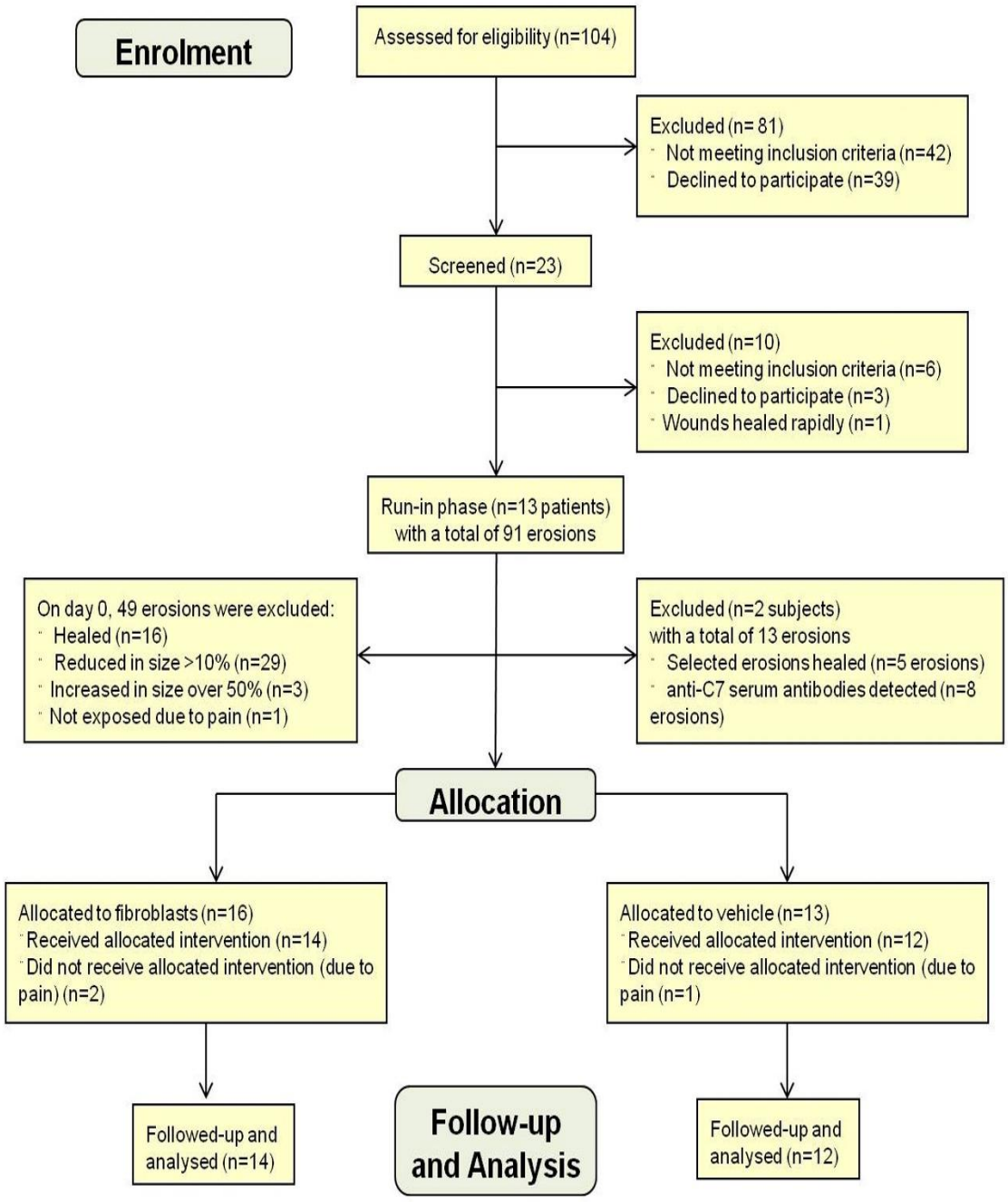


Figure 4.2 Study flow and patient disposition.
The majority of the subjects initially invited to participate were not screened because they either did not meet the entry criteria or declined to participate. Of those screened, additional subjects were excluded because their wounds were not chronic (present for at least 4 weeks), or wounds healed spontaneously, or wounds were too small (< 5 cm^2) or too large (> 50 cm^2), or they had fewer than five wounds (based on initial power calculation).

4.8 Results

Of the 104 individuals assessed for eligibility, 23 potentially met the entry criteria and were invited for screening. After screening, 13 subjects with a total of 91 erosions entered the run-in phase and 11 subjects were treated with a total of 26 erosions. Baseline characteristics of the study population are listed in **Table 4.4**.

Table 4.4 Baseline characteristics of the study participants and their wound measurements.
(#) Also affects intron 3 donor splice site. (1) Based on indirect immunofluorescence staining. The skin C7 expression has been estimated as +, 5–25% of wild type; ++, >50% of wild type; and +++, >75% of wild type.

Study ID	***COL7A1* DNA mutation**	**Age (years) /Sex**	**C7 skin expression**	**Erosion surface area measurements (in cm^2)**								
				Treatment	*Screening*	*Day 0*	*Day 7*	*Day 14*	*Day 28*	*Day 56*	*Month 3*	*Month 6*
P1	1) c.3054delA; p.Leu1018Leufs*8 2) c.3054delA; p.Leu1018Leufs*8	25/F	Absent	ICX-RHY-013	9.2	8.5	1.9	2.5	0	5.1	1.2	3.0
				Vehicle	14.2	26.7	17·6	8·4	0	1.5	3.8	0.9
P2	1) c.3632insC; p.Gln1211fs*8 2) c.520G>A; p.Gly174Arg	27/M	Absent	ICX-RHY-013	23.7	25.3	15.5	22.6	25.5	19.0	21.6	7.8
				Vehicle	21.9	25.8	21.6	15.2	6.5	3.5	0	0
P3	1) c.4172insC; p.Pro1391Glnfs*10 2) c.4172insC; p.Pro1391Glnfs*10	24/M	Absent	ICX-RHY-013	30.3	29.7	6.1	3.3	0	0	0	0
				ICX-RHY-013	10.9	42.0	27.9	29.8	20.5	78.6	24.1	8.5
				Vehicle	22.1	36.7	35.4	39.0	45.4	39.3	24.2	23.3
P4	1) c.7786delG; p.Gly2596Valfs*35 2) IVS53+1G>T	40/M	No IF (clinically severe)	ICX-RHY-013	6.3	18.0	13.0	17.2	7.7	7.3	4.4	15.1
				Vehicle	27.4	33.4	30.7	35.6	36.5	34.7	25.6	9.4
P5	1) c.1732C>T; p.Arg578X 2) IVS20+2T>C	41/M	++	ICX-RHY-013	26.7	26.0	15.7	5.3	16.4	41.7	4.0	13.0
				Vehicle	13.6	17.4	4.6	3.4	3.8	33.8	13.0	0
P6	1) c.186delG; p.Gly62Alafs*39 2) IVS79+1G>C	23/M	Absent	ICX-RHY-013	10.1	8.2	6.3	6.0	5.2	11.0	5.3	6.7
				ICX-RHY-013	7.4	20.2	17.5	19.7	18·4	30·3	28·5	16·7
				ICX-RHY-013	7.1	18.7	5·8	6.0	5.1	24.3	13.0	8.4
				Vehicle	14.8	13.7	9.8	10.3	9.9	15.0	11.2	12.0

Study ID	*COL7A1* DNA mutation	Age (years) /Sex	C7 skin expression	Erosion surface area measurements (in cm^2)								
				Treatment	*Screening*	*Day 0*	*Day 7*	*Day 14*	*Day 28*	*Day 56*	*Month 3*	*Month 6*
				Vehicle	15.1	14.2	10.8	11.0	10.7	13.1	10.0	1.5
P7	1) c.425A>G; p.Lys142Arg* 2) c.425A>G; p.Lys142Arg*	31/M	Absent	ICX-RHY-013	18.4	17.4	4.7	6.9	4.8	4.0	1.8	0
				Vehicle	14.2	14.3	7.5	9.6	6.5	5.1	1.4	0
P9	1) c.6022C>G; p.Arg2008Gly 2) c.7249C>T; p.Gln2417*	43/F	No IF (clinically severe)	ICX-RHY-013	5.1	6.6	0	3.8	0	2·5	4·8	0·9
				Vehicle	9.1	10·4	6·5	11·1	6·1	5·3	2·1	6·7
P10	#1) c.425A>G; p.Lys142Arg 2) c.565C>T; p.Gln189*	19/M	No IF (clinically severe)	Vehicle	6.4	5·9	2·6	0	0	0	0	0
P11	1) c.5047C>T; p.Arg1683* 2)c.5720_21GA>AT; p.Gly1907Asp	52/M	+++	ICX-RHY-013	17.3	30.1	20.8	22.3	17.1	3.6	3.2	2.4
				ICX-RHY-013	15.4	14.7	13.4	14.0	14.9	0.3	3.8	7.4
				Vehicle	7.8	15.3	18.6	19.7	29.4	12.8	3.7	2.6
P13	1) c.1732C>T; p.Arg578* 2) c.2965T>G; p.Trp989Gly	32/M	+++	ICX-RHY-013	8.0	8.1	1.4	1.1	1.2	10.4	2.0	3.4
				Vehicle	9.0	10.5	2.6	1.1	2.3	0.7	0.9	1.7

Between screening and day 0, I excluded two of these recruits because in one case the selected erosions had healed spontaneously and in the other case basement membrane binding serum anti-C7 antibodies were detected. Eleven subjects and a total of 29 erosions were randomised to receive either allogeneic fibroblasts or vehicle. Three erosions were not treated because subjects found injection of other erosions too painful. The remaining 26 erosions were injected and comprised the full analysis set; 14 erosions received active treatment and 12 erosions vehicle. There were no subject dropouts and data for all endpoints were available for all 26 erosions at 6 months (**Figure 4.2**). The final trial subjects attended the 6-month follow-up visit in May 2012. No protocol deviations were reported.

4.8.1 Safety analysis

The safety analysis included a total of 15 treated erosions in the fibroblast group and 12 treated erosions in the vehicle group. For the purpose of the adverse event reporting, we included a single erosion that was only partially (20%) treated (incomplete injections due to pain). This action explains the discrepancy in the numbers above (14 vs 15). Of the 15 erosions in the active group and the 12 erosions in the control group, nine (60%) and five (42%) erosions, respectively, had at least one local treatment-emergent adverse event (TEAE). The majority of the local TEAEs involved procedural complications (such as pain, erythema, and pruritus). When subject follow-up was completed, seven of the nine (78%) in the active group and five of six (83%) in the vehicle group local TEAEs had resolved. Two of the nine erosions (22%) in the fibroblast group and one of the five erosions (20%) in the vehicle group had a local continuing TEAE that required no follow-up.

4.8.2 Wound healing in RDEB erosions after fibroblast/vehicle injections

Serial measurements of erosion surface areas following a single injection of fibroblasts/vehicle are recorded in **Table 5.4**. For the primary analysis, fibroblast injections produced a greater, but not statistically significant, reduction in erosion area than did vehicle alone during the first 28 days. The mean erosion area (in cm^2) for the active group at day 0 was 19.5 (SD 10.3) vs. 18.7 (SD 9.7) for the vehicle group. At day 7 it was 10.7 (8.2) vs. 14.0 (10.9); at day 14 it was 11.5 (9.3) vs. 13.8 (12.3); and at day 28 it was 9.8 (8.7) vs. 13.1 (15.2). These changes over the first 28 days are illustrated in **Figure 4.3**.

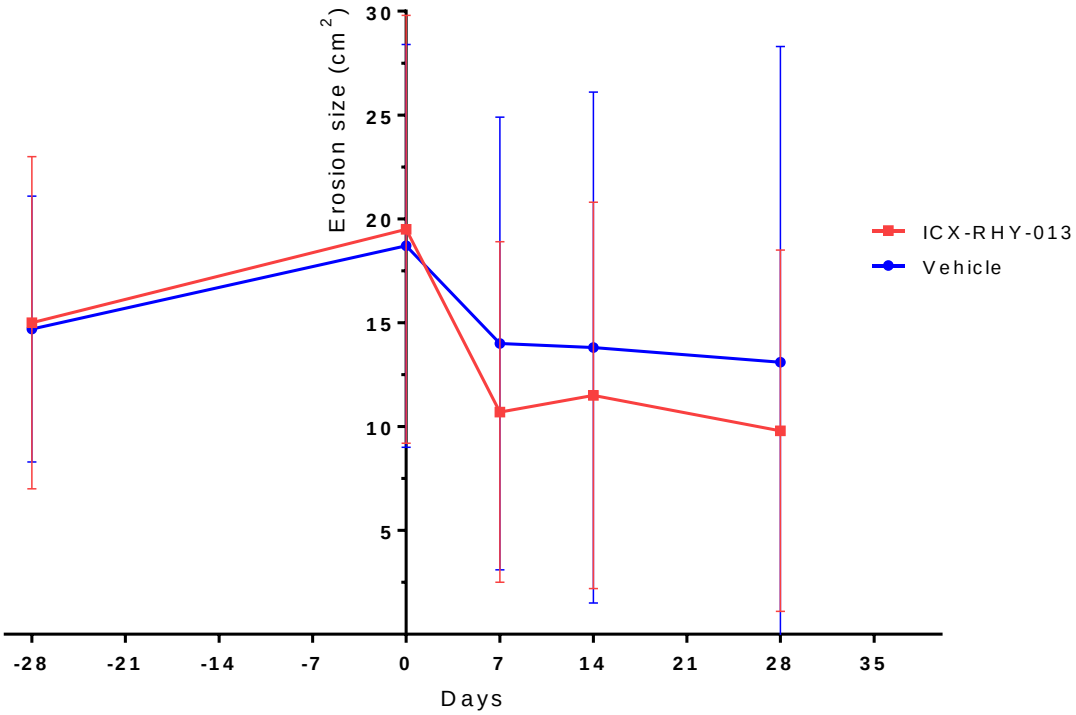


Figure 4.3 Reduction in erosion size during the first 28 days.
ICX-RHY-013: allogeneic fibroblasts.
Error bars represent the standard deviation (SD).

Figure 4.4 and **Figure 4.5** demonstrate the clinical response to fibroblast or vehicle injections in two trial participants.

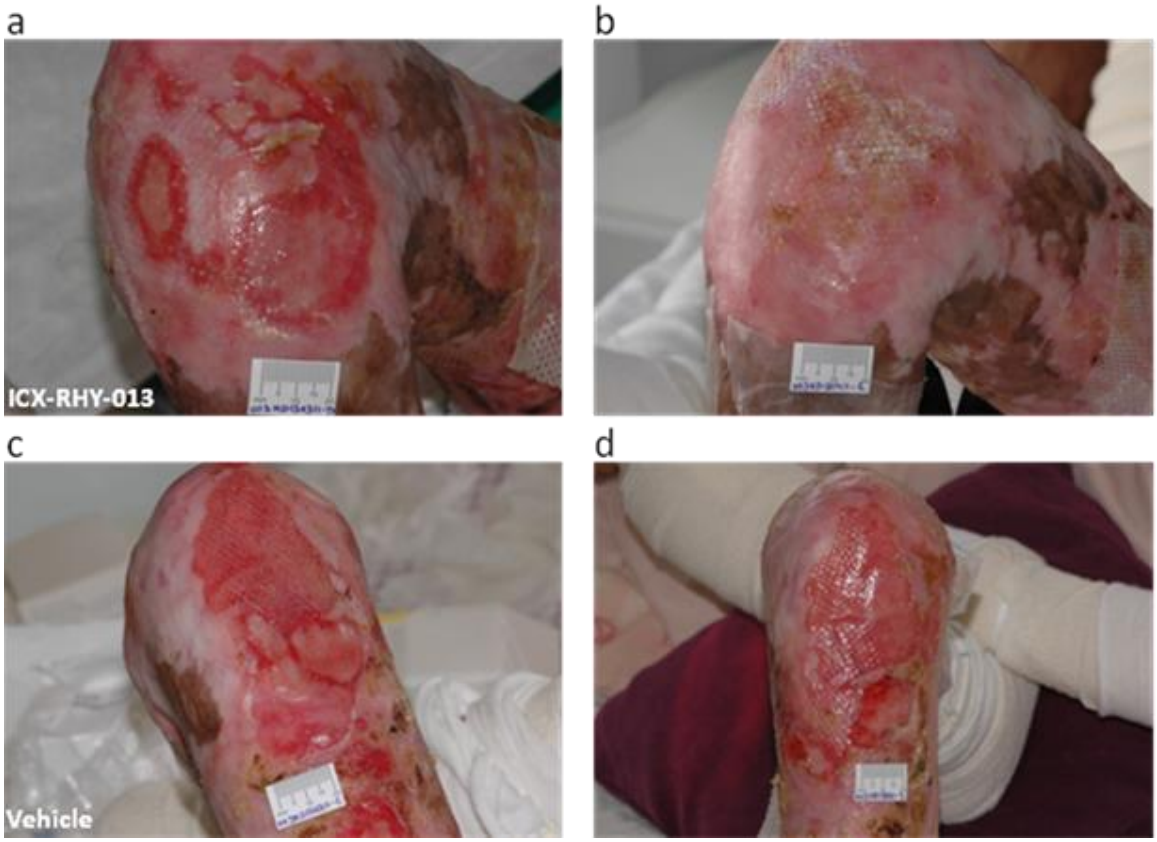


Figure 4.4 Clinical response to fibroblast/vehicle injections in trial subject P3.
After the data was locked and the trial was unblinded, subject P3 was shown to have had two erosions treated with fibroblasts and one with vehicle. Picture (a) represents a randomised erosion treated with fibroblasts on day 0 (recorded as 29.7 cm^2 on that day in Table 5). Picture (b) shows the same erosion at Day 28 (0 cm^2). Picture (c) represents the randomised erosion, of the same trial subject, treated with vehicle alone on day 0 (recorded as 36.7 cm^2 on that day in Table 5). Picture (d) shows the same erosion at Day 28 (45.4 cm^2).

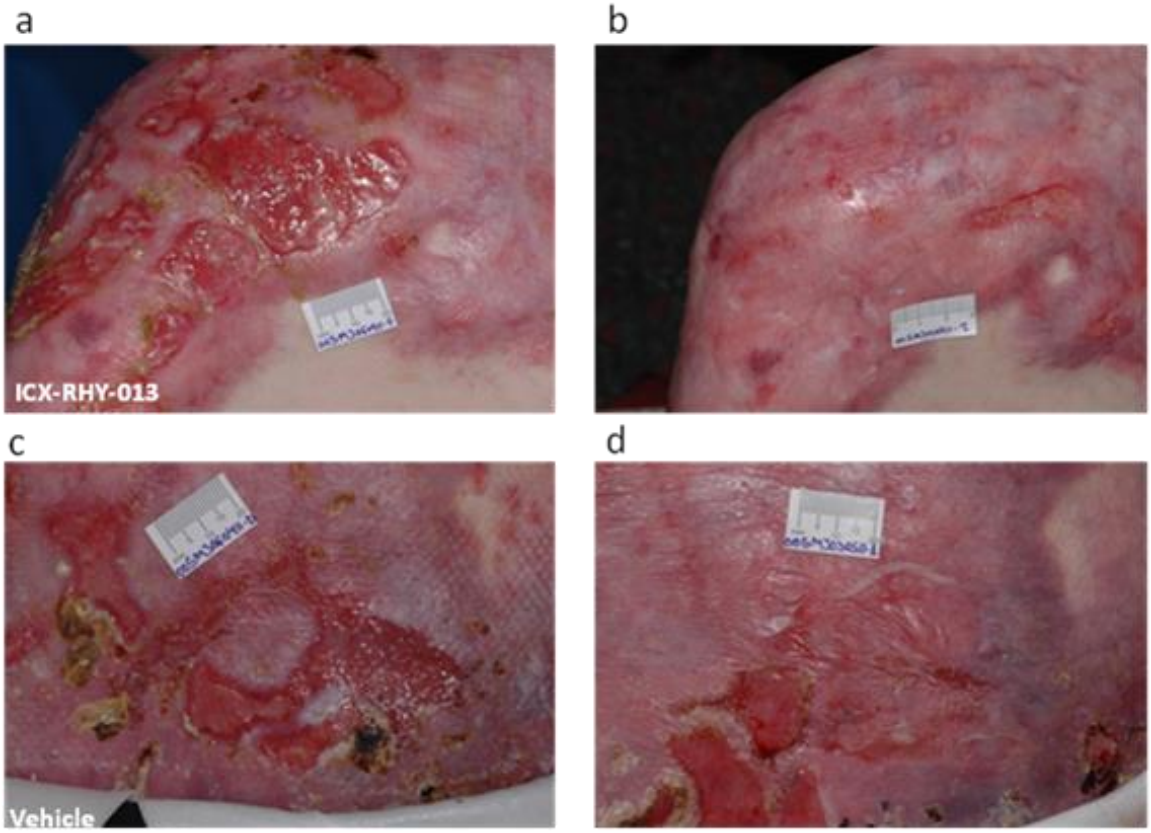


Figure 4.5 Clinical response to fibroblast/vehicle injections in trial subject P5.
After the data was locked and the trial was unblinded, subject P5 was shown to have had one erosion treated with fibroblasts and one with vehicle. Picture (a) represents the randomised erosion treated with fibroblasts on day 0 (recorded as 26.0 cm^2 on that day in Table 5). Picture (b) shows the same erosion at Day 28 (16.4 cm^2). Picture (c) represents the randomised erosion, of the same trial subject, treated with vehicle alone on day 0 (recorded as 17.4 cm^2 on that day in Table 5). Picture (d) shows the same erosion at day 28 (3.8 cm^2).

Initially, percentage erosion area decreased rapidly after active treatment. For the active group vs. vehicle, mean (SD) reduction in erosion area at day 7 was 50% (29%) vs. 32% (29%); at day 14 it was 43% (32%) vs. 34% (42%); and at day 28 it was 54% (36%) vs. 38% (57%). This statistically not significant difference between the groups, however, was lost after 28 days. At 6 months, the mean erosion area size was 6.7 (5.4) cm^2 in the active treatment group and 4.8 (7.1) cm in the vehicle group. The percentage change in erosion area for the fibroblast and vehicle groups did not differ significantly at 6 months ($p = 0.42$). Mean treatment difference between fibroblasts and vehicle was -23.5% [95% confidence interval (CI) -43.5 to -3.5, $p = 0.025$] at day 7; -19.2% [95% CI -41.7 to 3.4, $p = 0.089$] at day 14; -28.8% (95% CI -65.6 to 8.0, $p = 0.11$) at day 28. The estimated difference between the groups was 1.15 (95% CI -4.7 to 7.0, $p = 0.68$) at 6 months, thus showing no significant difference at this later time point (**Table 4.5**). On the basis of Kaplan–Meier estimates, the 4-week healing rate was 21.4% for the active group vs. 16.7% for the vehicle group.

Table 4.5 Effect of ICX-RHY-013 versus vehicle on absolute and percentage change in erosion area.

	Day 0	Day 7	Day 14	Day 28	Day 56	Week 12	Week 26
Erosion area (in cm^2) over 26 weeks							
Mean (SD) (ICX-RHY-013)	19.5 (10.3)	10.7 (8.2)	11.5 (9.3)	9.8 (8.7)	17.0 (21.6)	8.4 (9.4)	6.7 (5.4)
Mean (SD) (vehicle)	18.7 (9.7)	14.0 (10.9)	13.8 (12.3)	13.1 (15.2)	13.7 (14.3)	8.0 (9.1)	4.8 (7.1)
Treatment difference (ICX-RHY-013 – Vehicle)							
Difference (95%CI)	-	-4.39 (-8.91 to 0.12)	-3.85 (-9.95 to 2.25)	-5.20 (-14.78 to 4.37)	2.49 (-11.01 to 15.99)	-0.09 (-7.59 to 7.42)	1.15 (-4.65 to 6.95)
p value	-	0.056	0.20	0.26	0.70	0.98	0.68
	Day 0	**Day 7**	**Day 14**	**Day 28**	**Day 56**	**Week 12**	**Week 26**
Percentage change in erosion area (in cm^2) over 26 weeks							
Mean (SD) (ICX-RHY-013)	-	-49.8 (29.4)	-42.8 (32·2)	-54.4 (36.0)	-18.4 (65.0)	-55.9 (39.6)	-61.2 (29.3)
Mean (SD) (vehicle)	-	-31.9 (28.6)	-33.5 (41.5)	-37.8 (57.1)	-33.1 (58.7)	-62.8 (33.3)	-75.8 (30.6)
Treatment difference (ICX-RHY-013 – Vehicle)							
Difference (95%CI)	-	-23.50 (-43.48 to -3.51)	-19.15 (-41.66 to 3.36)	-28.83 (-65.63 to 7.97)	8.61 (-38.09 to 55.32)	3.28 (-26.27 to 32.83)	10.33 (-16.24 to 36.91)
p value	-	0.025	0.089	0.11	0.70	0.81	0.42

4.8.3 Clinical appearance of erosions after fibroblast/vehicle injections

Clinical assessment of erosion photographs by a blinded independent dermatologist (Dr Anna Martinez, Great Ormond Street Hospital, London, U.K.) showed that 78.6% and 92.8% of the erosions treated with fibroblasts were deemed to show clinical improvement from baseline at day 28 and 6 months, respectively (**Table 4.6**). The comparative figures for the vehicle group were 66.7% and 83.3%. Statistically, none of these differences was significant at either time point, despite the higher percentages in the active cell-treated erosions.

Table 4.6 Independent, blinded to study treatment assessment of clinical photographs of study erosions.

	Day 7	Day 14	Day 28	Day 56	Week 12	Week 26
Total erosions treated with ICX-RHY-013 (n=14)						
Much better	5 (35.7%)	7 (50.0%)	7 (50.0%)	6 (42.9%)	8 (57.1%)	8 (57.1%)
A bit better	5 (35.7%)	3 (21.4%)	4 (28.6%)	1 (7.1%)	2 (14.3%)	5 (35.7%)
Much the same	4 (28.6%)	4 (28.6%)	3 (21.4%)	4 (28.6%)	3 (21.4)	1 (7.1%)
A bit worse	0	0	0	2 (14.3%)	1 (7.1%)	0
A lot worse	0	0	0	1 (7.1%)	0	0
Total erosions treated with vehicle (n=12)						
Much better	3 (25.0%)	5 (41.7%)	5 (41.7%)	5 (41.7%)	7 (58.3%)	9 (75.0%)
A bit better	3 (25.0%)	1 (8.3%)	3 (25.0%)	1 (8.3%)	3 (25.0%)	1 (8.3%)
Much the same	5 (41.7%)	6 (50.0%)	3 (25.0%)	5 (41.7%)	2 (16.7%)	2 (16.7%)
A bit worse	1 (8.3%)	0	1 (8.3%)	1 (8.3%)	0	0
A lot worse	0	0	0	0	0	0
Estimated odds ratio (ICX-RHY-013 – Vehicle)	2·26	1·83	1.65	0.80	0.76	0.57
(95%CI)	(0.54 to 9.55)	(0.42 to 7.96)	(0.39 to 6.94)	(0.19 to 3.33)	(0.17 to 3.44)	(0.11 to 2.93)
p value	0.27	0.42	0.50	0.76	0.73	0.50

4.8.4 Pain scores and quality-of-life measurements

Pain associated with the fibroblast-injected erosions decreased compared with the vehicle group within the first 28 days although this was not significant ($p = 0.17$ at day 28), and with no differences between the two groups for the rest of the study visits (**Table 4.7** and **Table 4.8**). Quality-of-life scores in all categories decreased in both the cell and vehicle groups during the course of the study (**Table 4.9**, **Table 4.10**, **Table 4.11** and **Table 4.12**); there was no meaningful difference in the quality-of-life measures between groups.

Table 4.7 Erosion pain scores in the intention-to-treat (ITT) population.

	ICX-RHY-013								Vehicle							
	Day 0 Pre-treatment	Day 0 Post-treatment	Day 7	Day 14	Day 28	Day 56	Week 12	Week 26	Day 0 Pre-treatment	Day 0 Post-treatment	Day 7	Day 14	Day 28	Day 56	Week 12	Week 26
Total erosions	14	14	14	14	14	14	14	14	12	12	12	12	12	12	12	12
Mean	2.3	3.5	1.0	1.0	1.4	1.8	1.4	1.4	3.3	3.1	1.0	1.8	2.2	1.4	2.0	1.2
Median	1.5	2.5	1.0	0.0	1.0	1.0	0.5	1.0	3.0	2.0	0.5	0.0	0.5	0.0	0.0	0.5
SD	2.5	3.1	1.1	2.0	1.9	2.4	2.0	1.7	2.8	3.0	1.1	3.0	2.8	2.7	3.2	1.6
25% quartile	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0
75% quartile	5.0	6.0	2.0	1.0	2.0	3.0	2.0	2.0	6.0	6.5	2.0	3.0	4.5	1.5	3.5	2.0
Minimum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Maximum	7.0	9.0	3.0	7.0	7.0	7.0	6.0	6.0	7.0	7.0	3.0	8.0	7.0	9.0	10.0	4.0

Table 4.8 Erosion pain statistical analysis.

* By Analysis of Covariance with baseline pain and patient in model. # By Wilcoxon Rank Sum test

	Day 0 Post-treatment	Day 7	Day 14	Day 28	Day 56	Week 12	Week 26
Estimated treatment difference (ICX-RHY-013 - Vehicle)	-0.48	0.16	-0.64	-1.12	0.51	-0.73	-0.24
SEM	1.00	0.39	0.87	0.77	1.18	1.05	0.58
Lower 95% CI	-2.65	-0.69	-2.51	-2.78	-2.04	-3.01	-1.50
Lower 95% CI	1.68	1.00	1.24	0.55	3.05	1.54	1.01
Statistical significance of treatment difference *	p=0.64	p=0.70	p=0.48	p=0.17	p=0.67	p=0.50	p=0.68
Statistical significance of treatment difference #	p=0.40	p=0.36	p=0.48	p=0.42	p=0.19	p=0.47	p=0.20

Table 4.9 Quality of Life Assessment of Care: Intention-to-treat (ITT) Population.

	ICX-RHY-013				Vehicle			
	Day 0		Week 12		Day 0		Week 12	
Total erosions treated	14	(100.0%)	14	(100.0%)	12	(100.0%)	12	(100.0%)
Score 0	2	(14.3%)	2	(14.3%)	3	(25.0%)	3	(25.0%)
Score 1	2	(14.3%)	7	(50.0%)	2	(16.7%)	6	(50.0%)
Score 2	2	(14.3%)	3	(21.4%)	1	(8.3%)	2	(16.7%)
Score 3	3	(21.4%)	0	(0.0%)	3	(25.0%)	1	(8.3%)
Score 4	4	(28.6%)	2	(14.3%)	2	(16.7%)	0	(0.0%)
Score 5	1	(7.1%)	0	(0.0%)	1	(8.3%)	0	(0.0%)

Table 4.10 Quality of Life Assessment of Daily Function: Intention-to-treat (ITT) Population.

	ICX-RHY-013				Vehicle			
	Day 0		Week 12		Day 0		Week 12	
Total erosions treated	14	(100.0%)	14	(100.0%)	12	(100.0%)	12	(100.0%)
Score 0	3	(21.4%)	4	(28.6%)	2	(16.7%)	4	(33.3%)
Score 1	2	(14.3%)	3	(21.4%)	1	(8.3%)	3	(25.0%)
Score 2	2	(14.3%)	3	(21.4%)	2	(16.7%)	3	(25.0%)
Score 3	1	(7.1%)	3	(21.4%)	2	(16.7%)	2	(16.7%)
Score 4	0	(0.0%)	1	(7.1%)	0	(0.0%)	0	(0.0%)
Score 5	6	(42.9%)	0	(0.0%)	5	(41.7%)	0	(0.0%)

Table 4.11 Quality of Life Assessment of Appearance: Intention-to-treat (ITT) Population.

	ICX-RHY-013				Vehicle			
	Day 0		Week 12		Day 0		Week 12	
Total erosions treated	14	(100.0%)	14	(100.0%)	12	(100.0%)	12	(100.0%)
Score 0	1	(7.1%)	5	(35.7%)	1	(8.3%)	5	(41.7%)
Score 1	3	(21.4%)	2	(14.3%)	2	(16.7%)	3	(25.0%)
Score 2	3	(21.4%)	1	(7.1%)	1	(8.3%)	1	(8.3%)
Score 3	0	(0.0%)	2	(14.3%)	2	(16.7%)	1	(8.3%)
Score 4	1	(7.1%)	2	(14.3%)	0	(0.0%)	1	(8.3%)
Score 5	6	(42.9%)	2	(14.3%)	6	(50.0%)	1	(8.3%)

Table 4.12 Quality of Life Assessment of Concerns: Intention-to-treat (ITT) Population.

	ICX-RHY-013				Vehicle			
	Day 0		Week 12		Day 0		Week 12	
Total erosions treated	14	(100.0%)	14	(100.0%)	12	(100.0%)	12	(100.0%)
Score 0	3	(21.4%)	8	(57.1%)	4	(33.3%)	6	(50.0%)
Score 1	4	(28.6%)	2	(14.3%)	3	(25.0%)	3	(25.0%)
Score 2	1	(7.1%)	1	(7.1%)	1	(8.3%)	1	(8.3%)
Score 3	0	(0.0%)	0	(0.0%)	0	(0.0%)	1	(8.3%)
Score 4	3	(21.4%)	2	(14.3%)	1	(8.3%)	0	(0.0%)
Score 5	3	(21.4%)	1	(7.1%)	3	(25.0%)	1	(8.3%)

4.9 Discussion

A single intradermal injection of human allogeneic dermal fibroblasts into the margins of chronic erosions in subjects with RDEB produced a greater reduction in erosion area, time to healing, and in proportion of wounds healed, compared with vehicle within the first 28 days following treatment. Although the study failed to reach statistical significance because of the small number of treated erosions, the results suggest that a greater number of chronic RDEB erosions can be expected to heal within 4 weeks following fibroblast injection (21.4% vs. 16.7%). Thereafter, the study showed no significant differences between fibroblasts and vehicle, although both led to clinical improvement over 6 months compared with baseline. Subjective pain scores were reduced in the fibroblast group during the first 28 days, and independent assessment of wound healing appearance scored better in the fibroblast group at day 28 and 6 months although these differences did not reach statistical significance. No significant differences were seen between the two groups for other measures.

One clear limitation of the study is that the natural history of wound healing in RDEB is unknown. It has generally been assumed that the extensive skin and mucous membrane fragility inherent to RDEB leads to non-healing and persistent erosions. However, the pre-screening data showed that is not the case. More than 58% of the chronic erosions screened in this study were found to have healed or reduced in size by > 10% between screening and day 0, and therefore did not meet the set inclusion criteria for the trial. In future, interventional wound healing studies in RDEB should have a third arm that includes erosions without any intervention to document spontaneous wound healing.

Finding that erosion size reduced in both the fibroblast and vehicle groups raises the question of whether some of the clinical response may be due to the injection itself and/or the vehicle alone. Indeed, one other study in chronic wounds in RDEB has shown similar

changes for both allogeneic fibroblasts and vehicle, although that trial used a different source of cells, vehicle and injection protocol (Venugopal *et al.* 2013). Nevertheless, previous studies have shown that injection of saline into RDEB skin can lead to upregulation of *COL7A1* expression although this was less in both amount and duration compared with fibroblast injection (Nagy *et al.* 2011).

One further limitation of this trial was the selected erosion range of 5–50 cm^2 and the intention to treat these with a single injection. This study indicates that the fibroblasts appeared to accelerate wound healing in the first 28 days but not thereafter and that a single-injection approach, therefore, may be suitable for smaller wounds but not for larger erosions. These observations suggest a probable need for re-treatment, perhaps every 4 weeks, to try to sustain the rate of re-epithelialisation. On a practical level, however, injection pain was a major issue in the study. Delivering the cells and vehicle using a syringe/ hypodermic needle was often very painful especially in areas of scarring that had developed as a consequence of the disease. Future clinical applications of intradermal cell therapy delivery in RDEB, therefore, should address this and are likely to need an alternative delivery system, possibly using microneedles or other methods.

The pain associated with intradermal injection questions whether an alternative approach such as topical use of cells onto the erosion surface might suffice? Topical use of cell therapy has been used with some success in chronic venous ulcers (Kirsner *et al.* 2012), but in RDEB the results have mostly been disappointing. Wounds in RDEB typically show colonisation or infection with a range of Gram positive or negative bacteria, as well as abnormal granulation tissue, and therefore cells or recombinant growth factors applied topically have often proved a sub-optimal therapeutic approach. Nevertheless, use of cultured dermal substitutes has been reported to improved wound healing in some cases of RDEB (Falabella *et al.* 2000; Fivenson *et al.* 2003; Natsuga *et al.* 2010), although loss

of the grafted material and failure to heal wounds is common in clinical practice. In addition, the considerable cost of these dermal substitutes has limited their clinical uptake.

The main finding of the trial is that intradermally injected allogeneic fibroblasts can accelerate the rate of wound healing in RDEB wounds. The clinical utility might be extended further provided a less painful means of intradermal delivery could be achieved. For individuals with more severe forms of RDEB, however, the considerable extent of the erosions means that local injections of fibroblasts might only offer limited clinical benefits and an alternative systemic treatment would be preferable.

5 ALLOGENEIC BM-MSCS FOR CHILDREN WITH RDEB

5.1 Introduction

Clinical experience of RDEB suggests that a systemic therapy, delivered early in life, might offer the best disease-modifying potential. The first systemic therapeutic trial for children with RDEB was reported in 2010 (Wagner *et al.* 2010). Six children received BMT following myeloablative chemotherapy. Five out of six children showed significant clinical improvement, with reduction of blisters and improved wound healing with sustained benefits beyond 12 months in some cases (longest follow up is now 8 years and improvement has been maintained). This form of therapy, however, was considered high risk due to the conditioning chemotherapy required. One patient died due to cardiomyopathy before transplantation and one developed severe chemotherapy-related sepsis. Since then, over 20 patients with RDEB had received BMT; although the clinical data have yet to be published. Data presented in abstract form suggest that changing the conditioning to a reduced intensity conditioning regime might be associated with lower morbidity and mortality but conclusive data are awaited.

With regard to alternative systemic cell therapy the only reported clinical experience is with allogeneic MSCs given intravenously in adults with RDEB. Again, the main data have been reported only in abstract form, although a more detailed clinical synopsis has been published very recently (El-Darouti *et al.* 2015) In this study, 14 RDEB individuals received intravenously $2x10^6$ cells/kg of BM-MSCs, with or without cyclosporin, some of whom showed clinical improvement and no SAEs. The relative contributions of the BM-MSCs or the cyclosporine in some cases, however, were not clear. MSCs represent a heterogeneous collection of mostly non-progenitor connective tissue cells that are

structurally and functionally different from self-renewing stem cells and progenitors. Initially considered to be a population of stromal cells supporting and organising parenchymal frameworks, several studies have identified important roles for MSCs in modulating tissue inflammation and promoting tissue repair, including skin wounds (Chen *et al.* 2008; Prockop 2009; Tolar *et al.* 2010; Tolar *et al.* 2011a). Indeed, there are around 200 open clinical trials using BM-MSCs for specific disease indications on www.clinicaltrials.gov. Precisely how MSCs impact on the process of tissue repair is not fully known, although immunomodulatory changes (T-cells, dendritic cells), a stimulatory paracrine function, and local immunosuppressive changes, have been observed (Nauta and Fibbe 2007; Walter *et al.* 2010; Bianco *et al.* 2013; Fibbe *et al.* 2013). Moreover, within murine bone marrow, a sub-population of MSCs (still heterogeneous but positive for PDGFRα), has been shown to contribute directly to epithelial repair in skin (Tamai *et al.* 2011).

Emerging data from the cardiovascular field demonstrated that culture medium conditioned by human MSCs significantly reduced infarct size by approximately 50% in animal models of myocardial ischaemia injury when administered intravenously in a single bolus (Lai *et al.* 2011). The conditioned medium consisted 10% of an endosomal origin protein, known as exosome. The therapeutic activity of the human MSCs was attributed directly to the exosomes (Lai *et al.* 2010). Exosomes are secreted vesicles that contain proteins and RNA and serve as mediators of paracrine effect and promote communication between cells. The implications of this hypothesis is that if we are able to identify these specific molecules, we could replace transplantation of MSCs with administration of their secreted exosomes, overcoming the limitations associated with the transplantation of viable replicating cells.

Although the skin blistering in RDEB is primarily induced by trauma, the failure of wounds to heal quickly and the tendency for the repair process to break down due to further mechanical injury and secondary bacterial skin infections, typically leads to acute and chronic inflammation in the skin. Transcriptomic studies in RDEB wounds have identified elevated levels of pro-inflammatory cytokines and matrix metalloproteinases, enzymes that breakdown collagen and elastic tissue in skin (Nagy *et al.* 2011). Clinically, prolonged skin inflammation leads to scarring, contractures and an increased risk of developing squamous cell carcinomas, particularly in areas of chronic inflammation. Thus, innovative therapies that reduce skin inflammation in RDEB potentially may have positive clinical benefits in reducing disease burden. Assessing the safety, feasibility and potential benefit of intravenous infusions of allogeneic BM-MSCs to children with RDEB is the subject of this clinical trial.

5.2 Study design and participants

This open-label phase I/II trial was approved by the UK Medicines and Healthcare Products Regulatory Agency (MHRA), with EudraCT number: 2012001394-87. The UK National Research Ethics Committee London Bloomsbury provided ethics and site-specific approvals (Ref: 12/LO/1258) for Great Ormond Street Hospital. The trial was registered prospectively with www.controlled-trials.com (ISRCTN46615946). Participants were invited to participate if they were 1–17 years old and had a genetically confirmed diagnosis of RDEB with partial or complete deficiency of C7 in their skin. Participants were enrolled after written informed consent of the parents and written informed assent from the child (if over five years old) was also obtained. Skin biopsies obtained for previous diagnostic testing (as part of routine clinical care) were used as

baseline samples for DIF for C7 and TEM for anchoring fibrils. Children were recruited between July and October 2013 and all BM-MSC infusions per individual were completed by December 2013. The study was initially designed for the children to be followed up clinically for 24 months after their last infusion of BM-MSCs. Due to lack of serious adverse events (SAEs) observed, however, and positive outcomes noted by the children and their parents, a substantial protocol amendment approved shortening study completion and clinical observation to 12 months after each child's last infusion.

5.3 Production of MSCs

Bone marrow-derived MSCs are classified as an advanced therapy medicinal product (ATMP) and the cells were manufactured according to Good Manufacturing Practice (GMP) guidelines. BM-MSCs from the bone marrow of two healthy unrelated donors (male donor aged 2 years and female donor aged 10 years) were isolated, cultured and packaged at the GMP-licensed Cell Therapy Facility at University Medical Center Utrecht (UMC), The Netherlands. Use of these donor cells was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO, Biobanking bone marrow for MSC expansion, NL41015.041.12). Cells were cryopreserved with 10% DMSO. HLA typing was performed on donor cells but not the recipients of the MSCs. The cells were screened against an infectious disease panel in accordance with the EU directive 2006/17 (EUD 2006/17/EC). DNA from both donors was screened for *COL7A1* mutations and none were found. The pre-packed, cryopreserved cells were stored at the Camelia Botnar Cell Laboratories at Great Ormond Street Hospital in liquid nitrogen.

5.3.1 Dose of MSCs and infusion schedule

Each child in the trial received three separate intravenous infusions of same donor BM-MSCs on Day 0, 7, and 28, at a dose of 1-3x10^6 cells/kg. Each child received either 20x10^6 (body weight 5-20 kg) or 40x10^6 cells (body weight 20.1-40kg) depending on their weight. The infusions were administered as day-case procedures at the Somers CRF at Great Ormond Street Hospital. Cryopreserved cells were transferred from the Camelia Botnar Cell Laboratories in a canister filled with liquid nitrogen. The bags containing BM-MSCs were thawed in a 37^{o}C water bath and immediately infused over 10 minutes via a peripheral cannula. Vital signs (blood pressure, respiratory rate, heart rate, pulse oximetry and temperature) were checked before administration of the cells and thereafter every 15 minutes for one hour after the infusion and on discharge. Premedication with chlorphenamine was given intravenously 30 min before the cell infusions.

5.4 Objectives of trial

The primary objective was to assess safety and feasibility. Secondary objectives were to assess efficacy on clinical and functional outcomes, as well as skin pathology. I assessed participants by conducting six follow up visits over six months and then two further safety visits up to 12 months after the last infusion. Structured phone interviews to obtain qualitative data were held at nine months. Skin samples were analysed by DIF and TEM (at screening and at Day 60) at the National Diagnostic Epidermolysis Bullosa Laboratory at St Thomas' Hospital (Viapath, London, UK). Clinical assessment and photographs were undertaken for all participants at each visit. The BEBSS, a Global Severity and Improvement Score (GSIS) questionnaire, a Pain Sleep and Fatigue (PSF) assessment, and a Paediatric Quality of Life (Paeds QoL) assessment, were completed as per protocol

(**Figure 5.1**). Blister counts and clinical photographs were done by the parents during dressing changes and the data and images were reviewed during each visit.

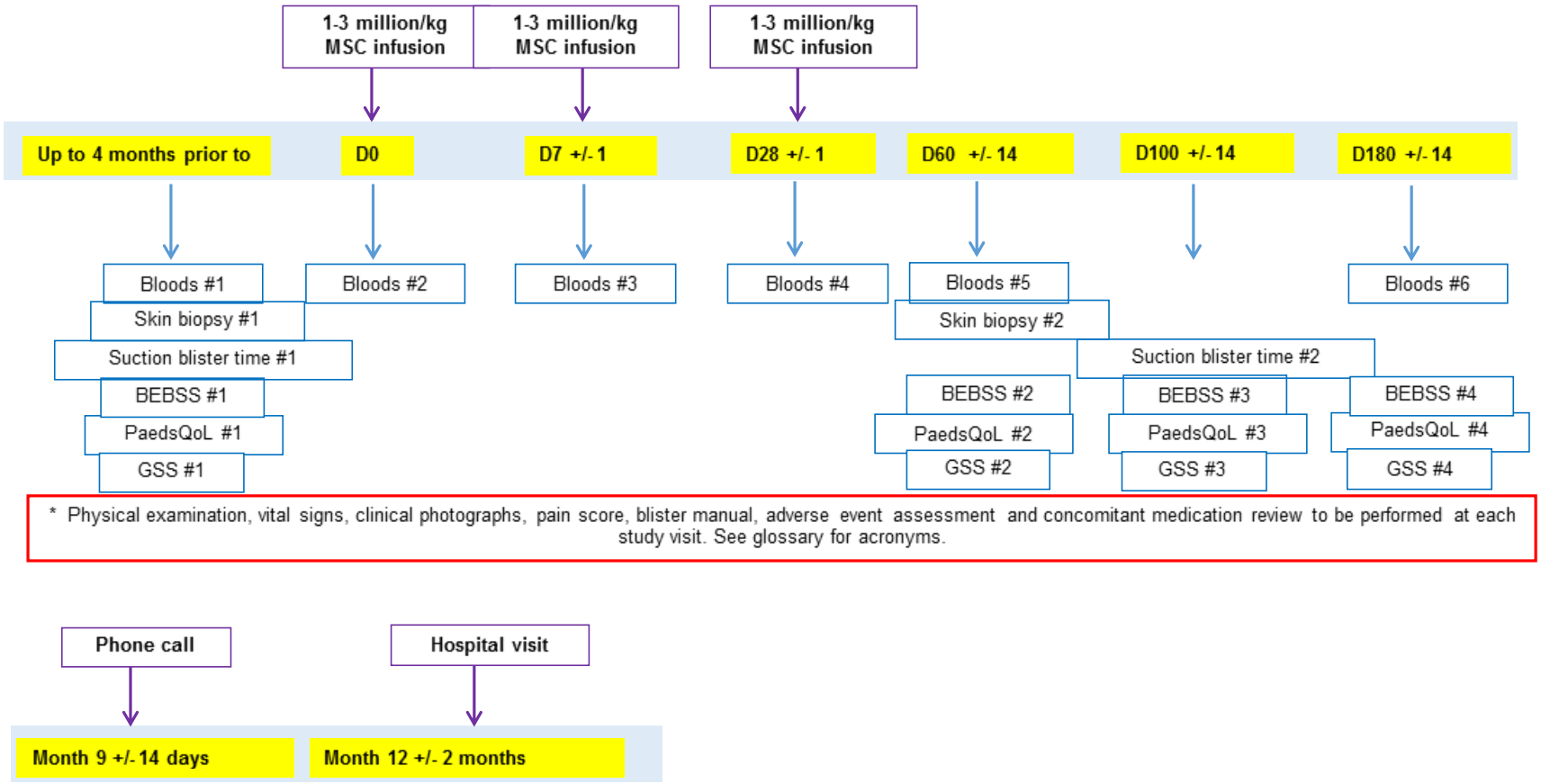


Figure 5.1 Trial flowchart summarising the trial interventions and assessments per visit.

5.5 Blood and skin profiling

Blood samples for haematology and biochemistry were taken and analysed at screening, Day 0, 7, 28, 60 and 180 at the Great Ormond Street Hospital pathology laboratories. Sera were analysed for C7 antibodies by IIF and ELISA at screening and Day 60 (Immunobiology Laboratory at St Thomas' Hospital). Inflammatory serum cytokine levels for interleukin IL-4, IL-6, IL-10, IL-17A, interferon-gamma (IFNγ) and tumour necrosis factor alpha (TNFα) were also analysed at screening, Day 0, 7, 28, 60 and 180 (Flow Cytometry facility, Guy's Hospital). No donor MSC-specific antibodies were tested for in any of the recipients' sera. For cases in which the BM-MSC donor cells were sex-mismatched, quantitative donor analysis using FISH was performed on tissue sections (Department of Cytogenetics, Guy's Hospital). Suction blister times were performed at screening and Day 100.

5.6 Statistical analysis

No formal sample size calculations were done because this was primarily a safety clinical trial. The paired t-test was used to compare mean differences in continuous outcomes between two chosen time points for normally distributed differences. The Wilcoxon signed rank test was used for data that were not normally distributed or which could not be transformed. The 2-sample test was used to compare differences in change of percentage BEBSS total body surface area (TBSA) between children aged ≤ two years and aged three years or more. Results are reported as estimates with 95% confidence intervals. P values are given but should be regarded as indicative as the study was not powered for efficacy. The scales of the paediatric quality of life questionnaire (PaedsQL) differed depending on the age of the child, and ranged from either 0–84 (aged 2–4 years)

or from 0–92 (aged 5–13 years). In order to make the scales comparable across all children, the scores for the younger children (ranged 0–84) were rescaled to 0–92 by multiplying by 92/84 (Varni *et al.* 1999; Varni *et al.* 2002; Varni *et al.* 2003). For the child version of the Pain Sleep and Fatigue Questionnaire, only patients aged >6 years were eligible to complete these. Children who had completed the questionnaire for all the seven visits were included in the analysis (n=3/10). One patient did not complete the questionnaire at visit 1 (baseline) but completed it at subsequent visits. Analyses were performed using the Stata statistical software (StataCorp. 2013, version 13.0).

5.7 Results

5.7.1 Clinical safety

Eleven children with RDEB were screened for inclusion into the trial. One child was excluded because of both positive ELISA for C7 antibodies and positive IIF with binding to skin DEJ (base of salt split skin). Ten children were enrolled at Great Ormond Street Hospital (London, UK). Participants fulfilled the eligibility criteria, had a mean age of 4.6 years (range 1-11), and had a clinical and genetic diagnosis of RDEB. There were a total of 163 adverse events (AEs) as summarised in **Table 5.1**, **Table 5.2** and **Table 5.3**. Initially two SAEs were reported but were subsequently downgraded in line with an updated protocol (version 4.0, 1st August 2014). Twenty-percent (32/163) of the AEs were definitely related to the infusion of BM-MSCs and comprised: DMSO odour, nausea, abdominal pain and bradycardia. DMSO odour was noted following 28 of the 30 infusions and lasted for up to 48 hours. Mild nausea occurred during two infusions; abdominal pain and bradycardia were observed during two other infusions. All AEs resolved within 15 minutes without treatment. For the remaining AEs, 84 were mild

(51%), 46 were moderate (28%) and 1 (0.6%) was severe, consistent with complications related to RDEB and the children's age-related events, rather than the BM-MSCs. The mild/moderate AEs included vomiting and pain on swallowing due to oesophageal strictures, corneal abrasions, recurrent spontaneous and trauma-induced blistering, wound infections and age-related accidental injuries. No adverse events resulted in either discontinuation or reduction in the dose of the study drug. Thirty infusions were administered as planned and all follow up visits were completed. The intravenous administrations of BM-MSCs, including cannulation, were well tolerated.

Table 5.1 Summary of adverse events.

	N	%
Total number of patients in study	10	100
Number of patients who experienced adverse events	10	100
Total number of adverse events reported	163	100
	Number of events	**%**
Intensity		
Mild	101	62.0
Moderate	59	36.0
Severe	3	2.0
Serious		
Yes	0	0.0
Relationship to study drug		
Definitely	32	20.0
Possibly	3	2.5
Likely	1	0.6
Unlikely	4	1.8
Not related	123	75.0
Outcome		
Resolved	153	94.0
Continuing, but no further follow up required	10	6.0
Frequency		
Single occurrence	144	88.0
Intermittent	14	9.0
Continuous	5	3.0
Action taken		
None	107	65.0
Required concomitant medication	56	35.0

Table 5.2 Intensity of adverse events by relationship to MSC infusion.
MSC: Mesenchymal stromal cells; *The 2 adverse events with severe intensity were Dimethyl Sulphoxide (DMSO) odour.

Intensity	Relationship to MSC infusion (n (%))					
	Definitely	**Possibly**	**Likely**	**Unlikely**	**Not related**	**Total**
Mild	18 (18.0)	3 (3.0)	0 (0.0)	3 (3.0)	77 (76.0)	101 (62.0)
Moderate	12 (20.0)	0 (0.0)	1 (1.7)	1 (1.7)	45 (76.0)	59 (36.0)
Severe	2 (67.0)*	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.0)	3 (2.0)
Total	32 (20.0)	3 (1.8)	1 (0.6)	4 (2.5)	123 (75.0)	163 (100)

Table 5.3 Adverse event by system organ class and relationship to MSC infusion.

MSC: Mesenchymal stromal cells; EB: Epidermolysis Bullosa; DMSO: Dimethyl sulphoxide.

			Relationship to MSC infusion					
System organ class	**Adverse event**	**Number of patients**	**Definitely**	**Possibly**	**Likely**	**Unlikely**	**Not related**	**Total number of events (N)**
Total number of patients in study		**10**						**163**
Patients who experienced adverse events		**10**						
Ear, Nose and Throat	Total in class	4	0	0	0	0	4	4
	Epistaxis	1	0	0	0	0	1	1
	Sore throat	3	0	0	0	0	3	3
Eyes	Total in class	5	0	0	0	0	24	24
	Conjunctivitis	1	0	0	0	0	1	1

			Relationship to MSC infusion					
System organ class	**Adverse event**	**Number of patients**	**Definitely**	**Possibly**	**Likely**	**Unlikely**	**Not related**	**Total number of events (N)**
	Corneal abrasion	4	0	0	0	0	20	20
	Sore eyes	1	0	0	0	0	3	3
Dermatological	Total in class	8	0	3	1	3	23	30
	Spontaneous skin/mucosal blisters and wounds	7	0	2	0	0	12	14
	Trauma induced skin/mucosal blisters and wounds	2	0	0	0	0	4	4
	Dry skin	2	0	0	0	0	2	2
	Fine hair growth	1	0	1	0	0	0	1
	Milia	1	0	0	0	1	0	1
	Pruritus	4	0	0	1	1	2	4
	Rash	2	0	0	0	1	3	4

			Relationship to MSC infusion					
System organ class	**Adverse event**	**Number of patients**	**Definitely**	**Possibly**	**Likely**	**Unlikely**	**Not related**	**Total number of events (N)**
Lymph nodes	Total in class	1	0	0	0	0	1	1
	Lymphadenopathy	1	0	0	0	0	1	1
Gastrointestinal	Total in class	9	3	0	0	1	20	24
	Abdominal pain	1	1	0	0	0	0	1
	Gastro-oesophageal reflux	1	0	0	0	0	1	1
	Constipation	2	0	0	0	0	2	2
	Diarrhoea	5	0	0	0	0	9	9
	Increased appetite	2	0	0	0	1	1	2
	Nausea	2	2	0	0	0	1	3
	Vomiting	5	0	0	0	0	6	6

			Relationship to MSC infusion					
System organ class	**Adverse event**	**Number of patients**	**Definitely**	**Possibly**	**Likely**	**Unlikely**	**Not related**	**Total number of events (N)**
Respiratory	Total in class	3	0	0	0	0	4	4
	Cough	3	0	0	0	0	4	4
Cardiovascular	Total in class	1	1	0	0	0	0	1
	Bradycardia	1	1	0	0	0	0	1
Genitourinary system	Total in class	1	0	0	0	0	1	1
	Reduced urine output	1	0	0	0	0	1	1
Musculoskeletal	Total in class	1	0	0	0	0	1	1
	Joint pain	1	0	0	0	0	1	1
Infections	Total in class	8	0	0	0	0	20	20
	Fever	2	0	0	0	0	2	2
	Respiratory tract infections	5	0	0	0	0	10	10

			Relationship to MSC infusion					
System organ class	**Adverse event**	**Number of patients**	**Definitely**	**Possibly**	**Likely**	**Unlikely**	**Not related**	**Total number of events (N)**
	Skin infection	5	0	0	0	0	7	7
	Urinary tract infection	1	0	0	0	0	1	1
Medical and surgical procedures	Total in class	5	0	0	0	0	6	6
	Oesophageal dilatation	4	0	0	0	0	4	4
	Routine surgical procedure Related to complications of EB	1	0	0	0	0	1	1
	Dental procedure	1	0	0	0	0	1	1
Accidental injuries	Total in class	5	0	0	0	0	18	18
	Accidental injuries	5	0	0	0	0	18	18
DMSO odour	Total in class	10	28	0	0	0	0	28

			Relationship to MSC infusion					
System organ class	**Adverse event**	**Number of patients**	**Definitely**	**Possibly**	**Likely**	**Unlikely**	**Not related**	**Total number of events (N)**
	DMSO odour	10	28	0	0	0	0	28
Mood	Total in class	1	0	0	0	0	1	1
	Irritability	1	0	0	0	0	1	1

5.7.2 Blood monitoring

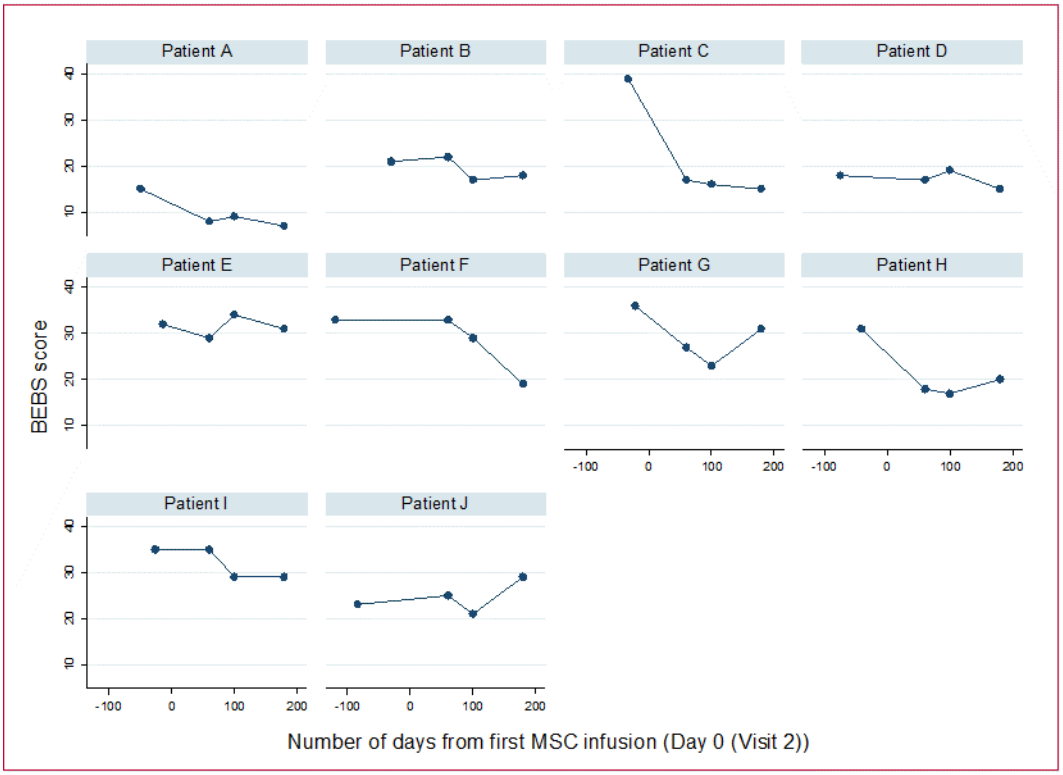
Blood monitoring assessments did not reveal any adverse impact of the BM-MSCs on renal, liver or bone marrow function. Due to skin fragility and scarring, venous access was difficult, thus some bloods tests were unobtainable. I did not identify any rash or signs of allergic reaction during the infusions. Anti-C7 antibodies were detected by serum ELISA at baseline in 9/10 participants and levels remained similar after treatment. None of these positive sera showed binding to the skin basement membrane by IIF. Following MSCs, there were no major changes in the ELISA or IIF data (**Table 5.4**). Skin biopsies revealed no change in C7 deposition and no new formation of anchoring fibrils at Day 60 when compared to baseline. FISH analysis of skin specimens from four children who received sex-mismatched BM-MSCs taken on Day 60 did not show evidence of donor cell chimerism for sex-mismatched donor cells.

Table 5.4 Summary of anti-BP180, anti-BP-230 and anti-C7 antibody levels (in units) by ELISA in the sera of the children.
BP180 = bullous pemphigoid antigen-180; BP230= bullous pemphigoid antigen-230 C7=collagen VII

Patient ID	**Pre-treatment (screening)**			**Post-treatment (Day 60)**		
	BP180	**BP230**	**C7**	**B180**	**BP230**	**C7**
A	42	29	13	27	34	13
B	68	66	35	58	50	23
C	32	32	15	54	31	11
D	97	68	24	97	97	28
E	2	2	1	2	3	1
F	45	48	10	42	40	13
G	60	41	29	52	50	17
H	42	28	16	51	48	19
I	28	28	4	32	29	4
J	70	47	20	48	46	18
005–excluded	132	94	52	–	–	–

5.7.3 Clinical response

The BEBSS and Global Severity Scores (GSS) were completed for all 10 participants. Mean disease severity, as measured by total BEBSS, improved by 5.2 points (95% CI -10.7, 0.3; p=0.06) and also mean BEBSS total body surface area (TBSA%) showed improvement by 5.9 points at Day 60 (-15.3, 3.5; p=0.19). The improvement was sustained, and at Day 180 had improved by 6.9 points for the BEBSS (-12.7-1.1; p=0.03) and 8.9 points for the TBSA (-18.9, 1.1; p=0.07). Mean reduction in TBSA was greater in children under the age of two years (n=4) as compared to baseline by 4.4 (-31.7, 23) at both Day 60 and by -9.9 (-33.9, 14.0) on Day 180 (**Figure 5.2** and **Figure 5.3**).



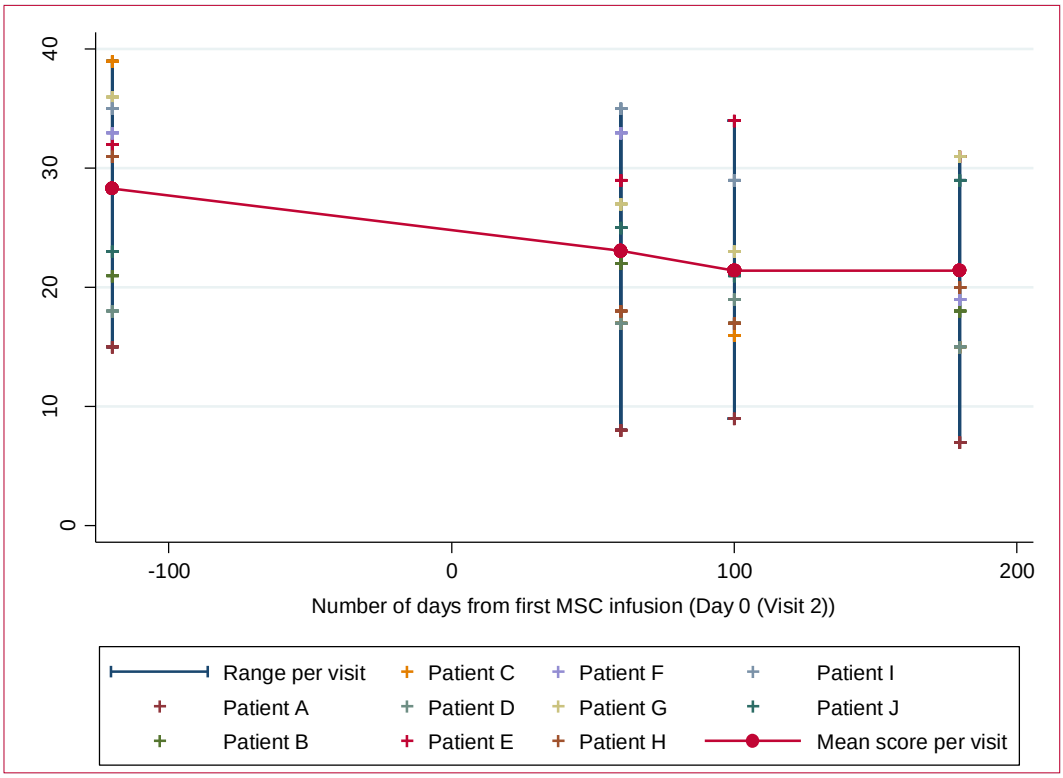


Figure 5.2 Disease severity score.
Birmingham Epidermolysis Bullosa Severity Scores (BEBSS) for each patient (N=10) by number of days from first MSC infusion (top); Distribution of BEBSS, with means and range per visit by number of days from first MSC infusion (N=10) (bottom).

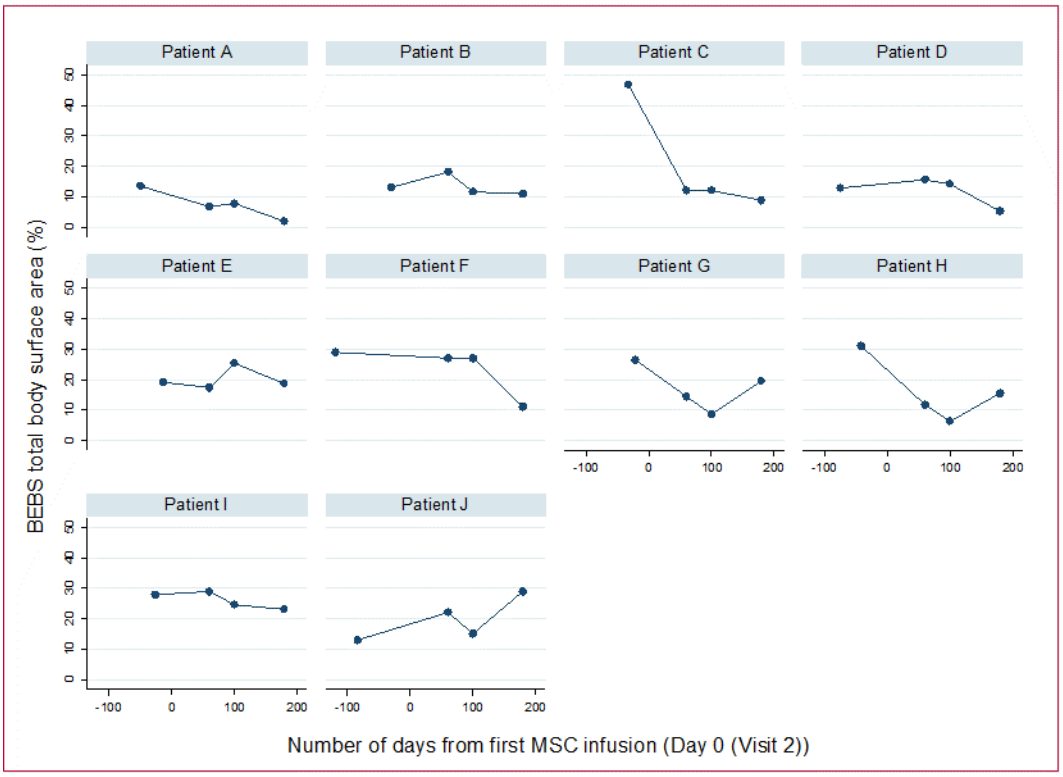


Figure 5.3 Percentage total body surface area (TBSA%) affected by EB.
This is calculated from BEBSS for each patient (N=10) by number of days from first MSC infusion.

Mean global severity score reduced from 7.0 to 4.6 at Day 60 with mean difference from baseline -2.4 (-3.4, -1.4; p<0.001) and to 5.4 at Day 180 with mean difference from baseline 1.6 (-3.0, -0.24; p=0.03) (**Figure 5.4**).

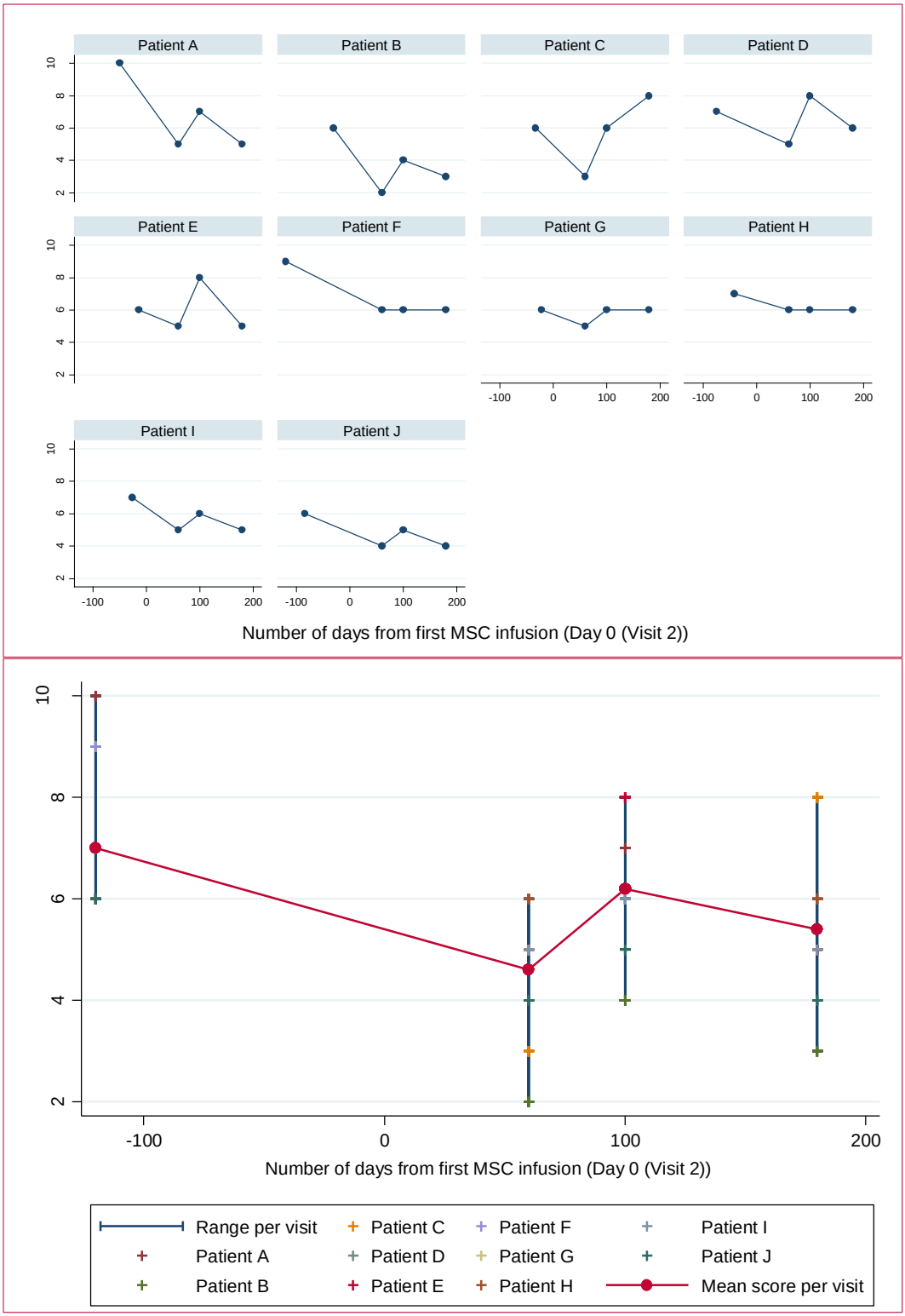


Figure 5.4 Global severity score.
Global Severity Scores for each patient (N=10) by number of days from first MSC infusion (top); Distribution of global severity scores, with means and range per visit by number of days from first MSC infusion (N=10) (bottom).

Pain, sleep and fatigue questionnaires were completed independently for children over 6 years old (n=4) as well as by the parents. Mean pain score reported by parents showed reduction from 26.1 at baseline to 20.6 at Day 60 (-16.3, 5.3, p=0.28) and to a lesser degree 23.1 at Day 180 (-14.7, 8.7, p=0.58). Mean pain score reported by children aged 6 or over (n=3) showed no change from baseline 20 to 20 at Day 60 but reduced to 11.3 by Day 180 (-33.2, 15.8, p=0.27) (**Figure 5.5**).

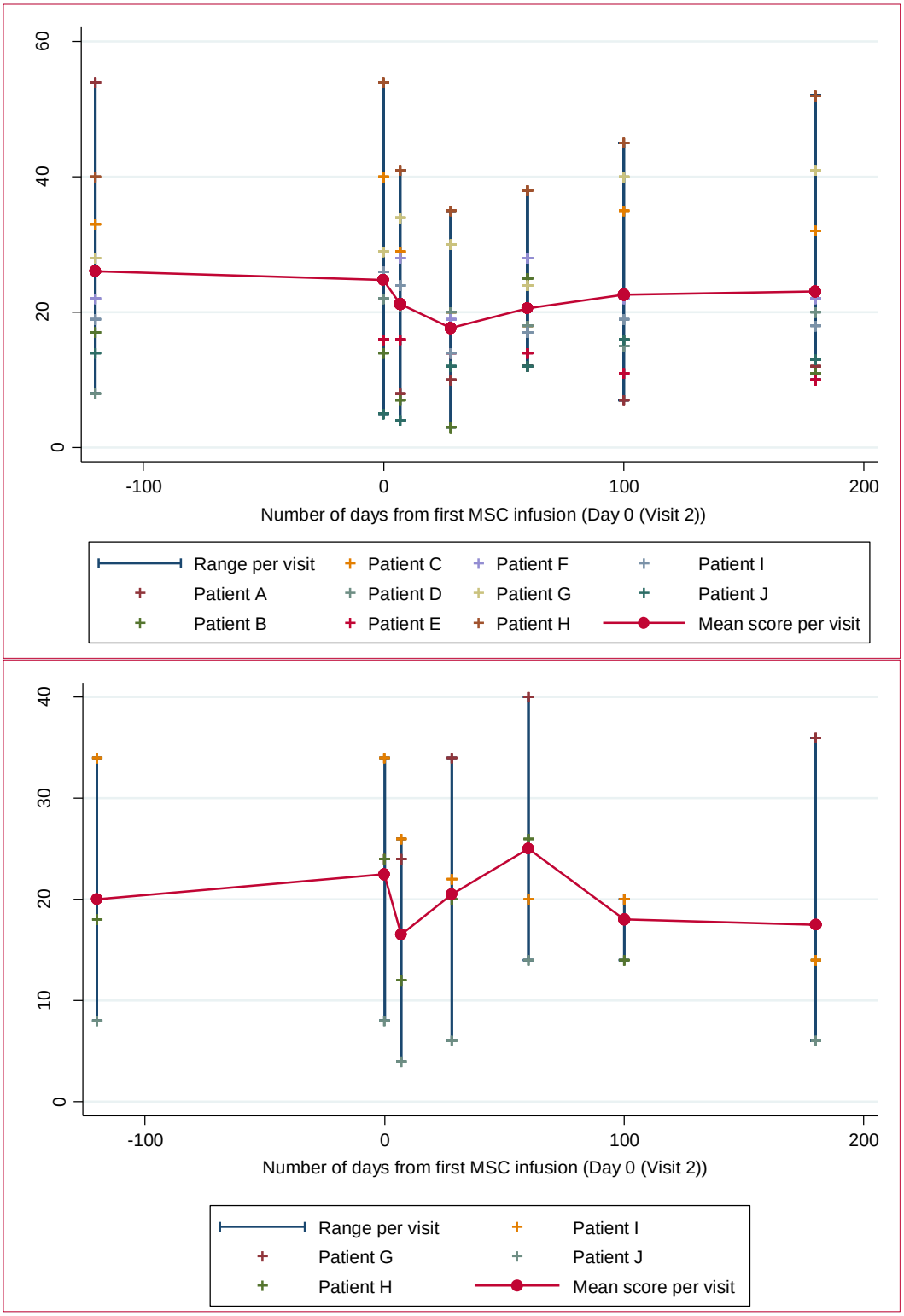


Figure 5.5 Parent and child versions of pain scores from Pain, Sleep and Fatigue Questionnaire.
Top = parent: Graph showing distribution of scores with means and range by number of days from first MSC infusion (N=10). Bottom = child: Graph showing distribution of scores with means and range by number of days from first MSC infusion (N=4). *Patient G was < 6 years at baseline and so was not eligible to complete the questionnaire at visit 1 but completed it at subsequent visits.

Paeds QoL (Parent version) was completed for children over two years (n=8); and Paeds QoL (Child version) was completed by children over five years (n=5) as per questionnaire guidance. Similarly, mean quality of life score (higher is worse) reported by parents showed improvement from 41.9 at baseline to 37.5 at Day 60 (-8.1, -0.7, p=0.03) and to a lesser degree 39.0 at Day 180 (-7.5, 1.8, p=0.19) (). Mean age-adjusted quality of life score reported by children aged 5 or above (n=5) showed improvement from 32.4 at baseline to 27.2 at Day 60 (-25.6, 15.2, p=0.52) and to a lesser degree 29.6 by Day 180 (-18.6, 13.0, p=0.65) (**Figure 5.6**).

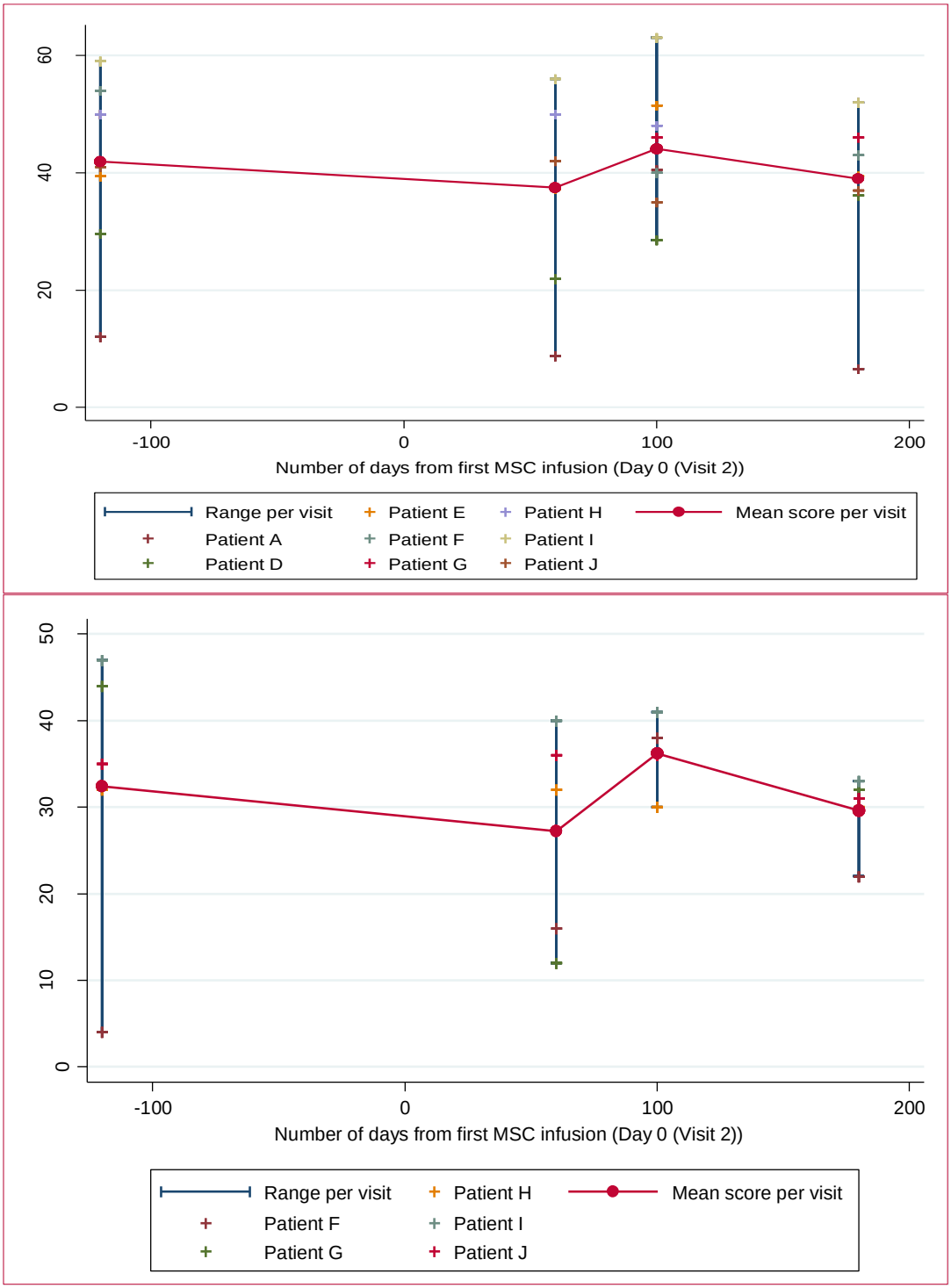


Figure 5.6 Parent and child versions of paediatric quality of life scores (PaedsQL).
Top = parent. Graph shows distribution of scores with means and range by number of days from first MSC infusion (N=8). *PaedsQL parent version can only be completed for children over 2 years.
Bottom = child. Graph showing distribution of scores with means and range by number of days from first MSC infusion (N=5).

Mean pruritus score (children's report) showed improvement by 1.3 (-4.2, 1.5; p=0.18) by Day 60 and this was sustained at Day 180 (-4.2, 1.5, p=0.18) (**Figure 5.7**).

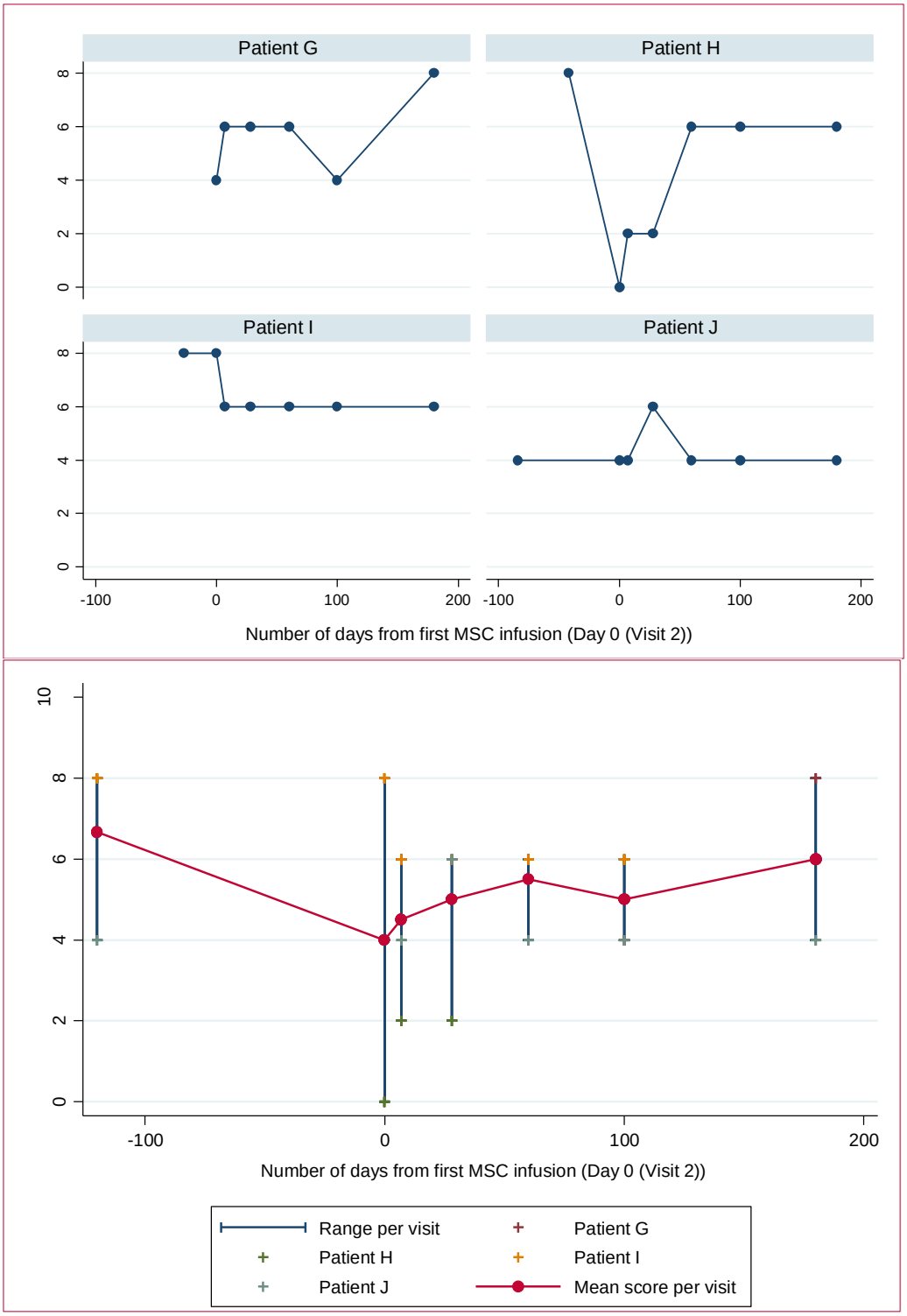


Figure 5.7 Pruritus scores by number of days from the first MSC infusion.
Top graph shows pruritus scores (pruritus scale of the pain, sleep and fatigue questionnaire); for each patient (N=4). Bottom graph shows distribution of scores with means and range by number of days from first MSC infusion (N=4).
*Patient G was < 6 years at baseline and so was not eligible to complete the questionnaire at visit 1 but completed it at subsequent visits.

Median blister count at baseline was 5.5 and reduced to 3.5 by Day 60 and remained thus at Day 180 (**Figure 5.8**).

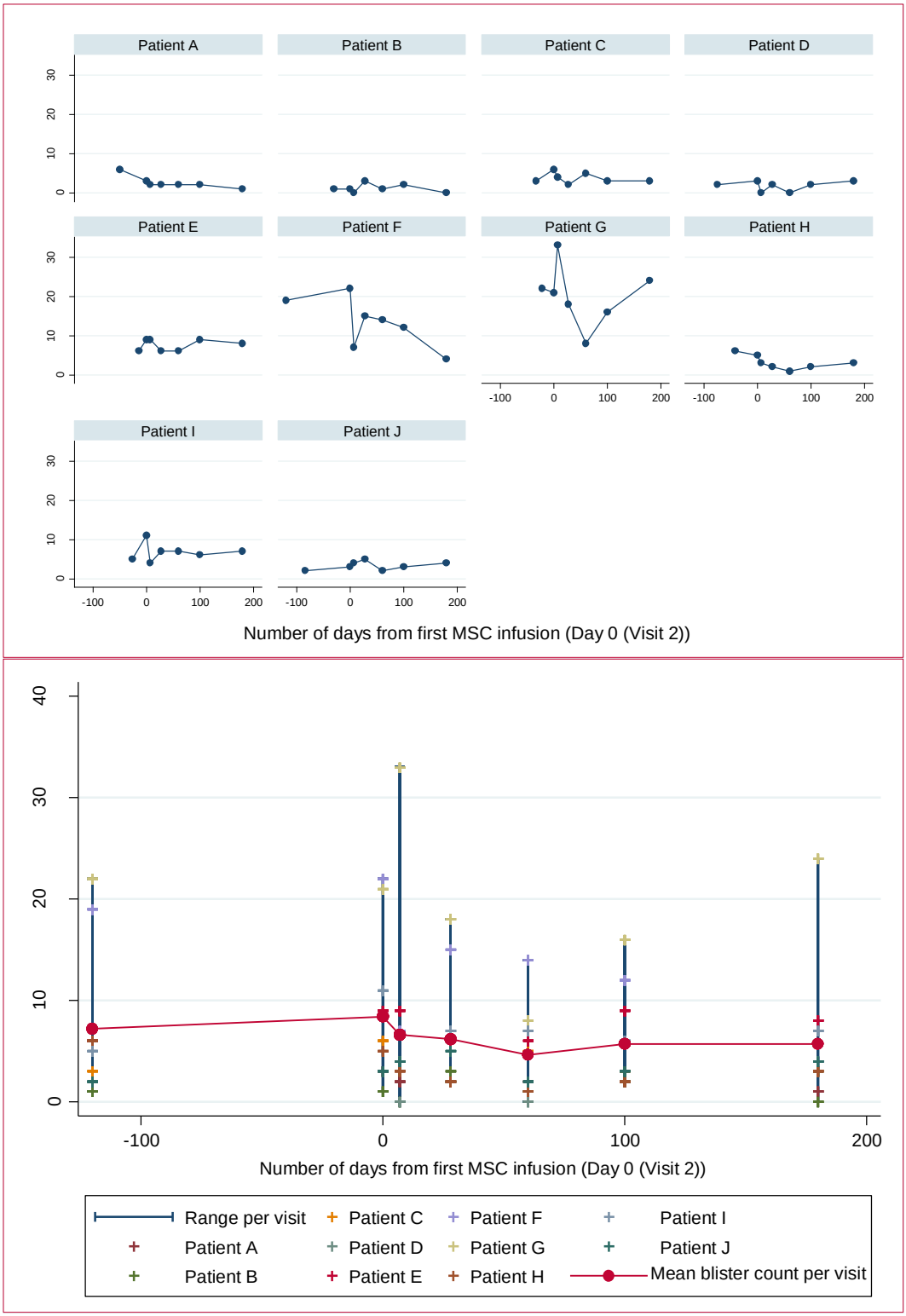


Figure 5.8 Blister count.
Distribution of blister count for each patient (N=10) by number of days from first MSC infusion (top); distribution of blister count with means and range per visit by number of days from first MSC infusion (N=10) (bottom).

Serial clinical photographs of skin erosions revealed improved wound healing and lessening in skin redness **Figure 5.9**, **Figure 5.10** and **Figure 5.11**. A summary of the clinical secondary outcome measures is shown in **Table 5.5**.

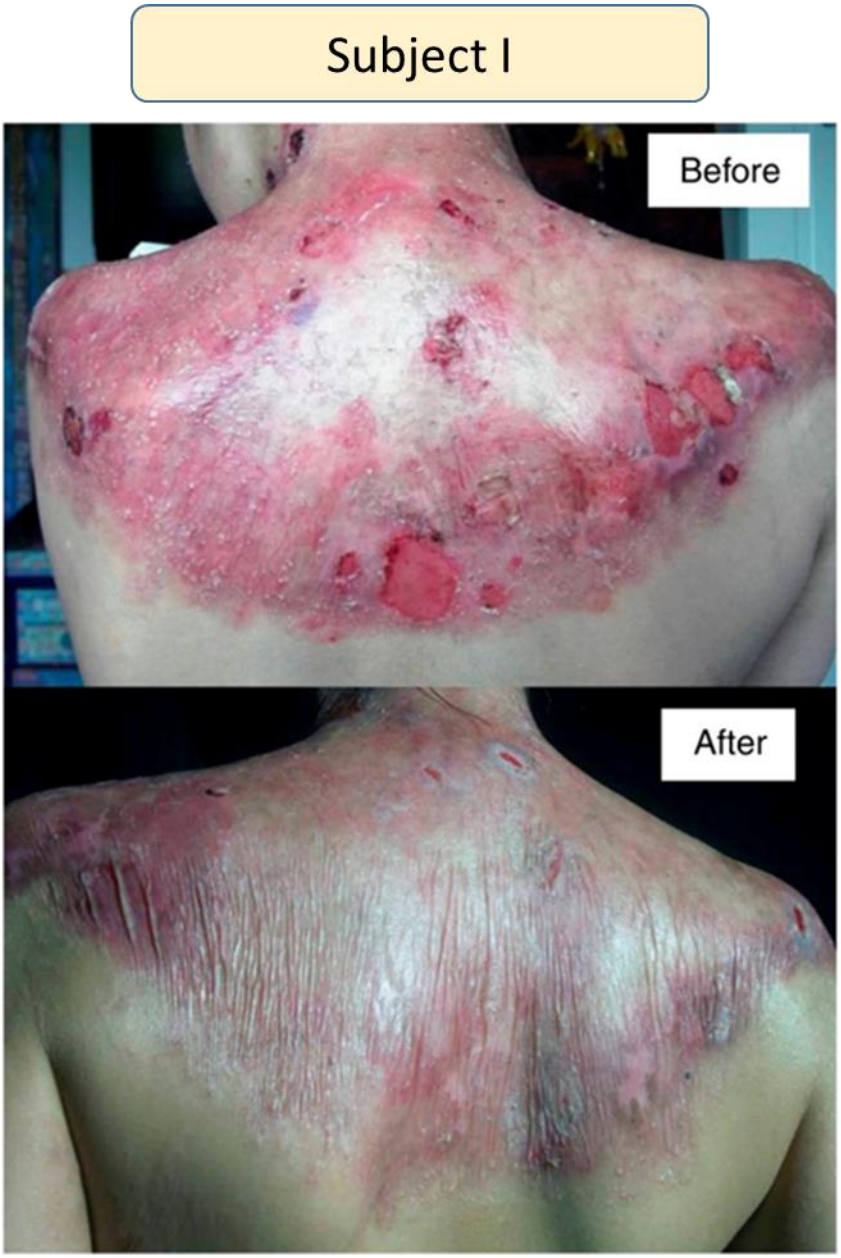


Figure 5.9 Improved wound healing and reduced skin erythema 8 weeks after the third infusion of BM-MSCs in subject I.

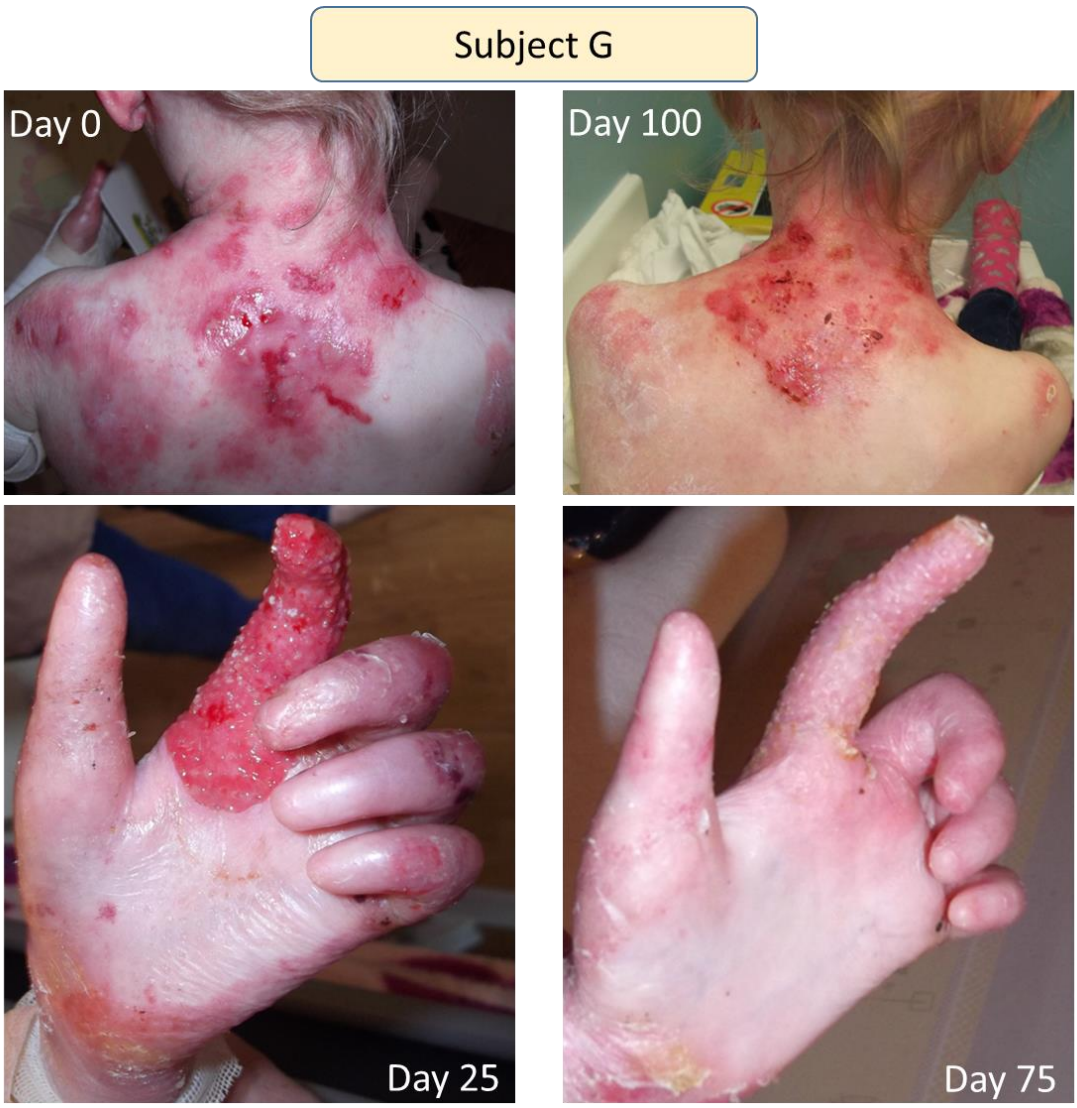


Figure 5.10 Clinical appearances in Subject G following BM-MSCs.

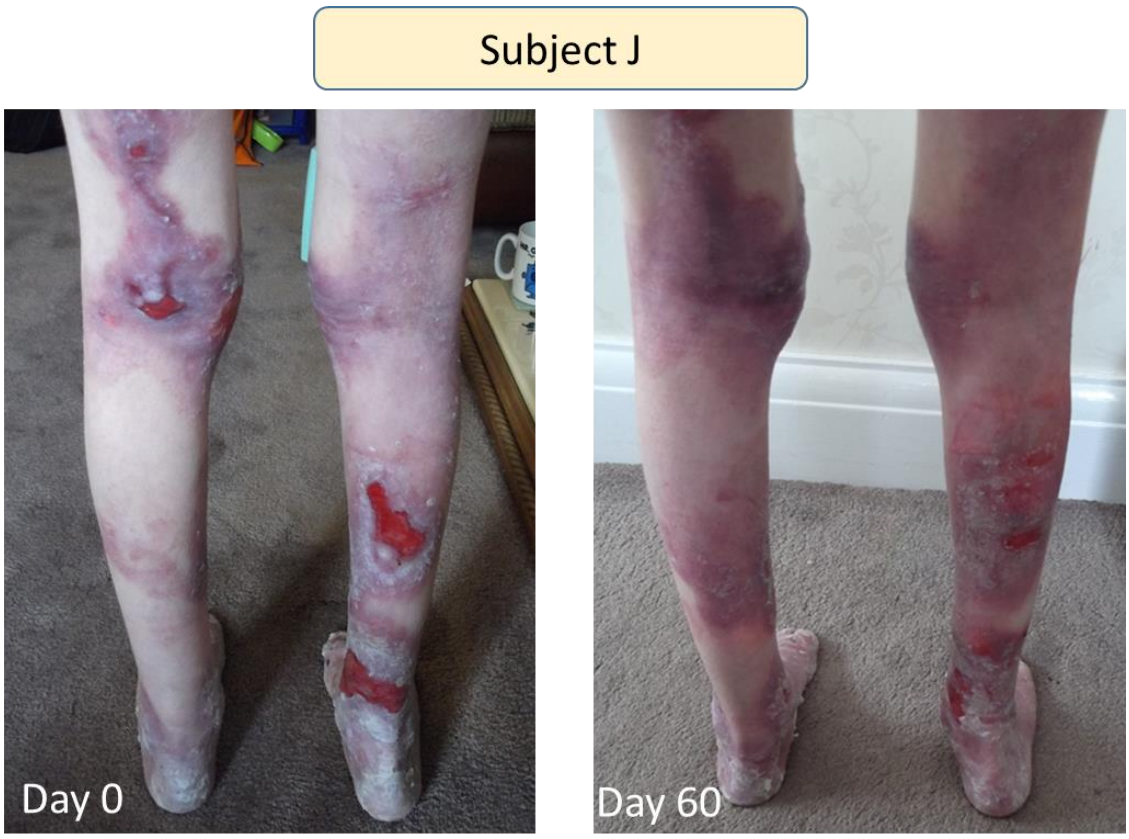


Figure 5.11 Clinical appearances in Subject J following BM-MSCs.

Table 5.5 Summary of baseline characteristics and clinical secondary outcome measures.
BEBSS: Birmingham Epidermolysis Bullosa Severity Score, scale range: 0-100: TBSA: Total Body Surface Area; Global Severity Score Scale range: 0 – 12; PaedsQL™: Paediatric quality of life questionnaire - parent version: child aged 2-4 years (range:0-84), 5-7 years (range:0-92), and 8-12 years (range:0-92) and child version: child aged 5-7 years (range:0-92) and 8-12 years (range:0-92); Pain scale range: 0-80; Fatigue score scale range: 0-10; Pruritus score scale range: 0- 10. **Child was aged < 6 years at baseline. Abbreviations: M= male; F=female; CA=Complete absence; PA=Partial absence, IF=immunofluorescence; NA= not applicable.

	Subject A	**Subject B**	**Subject C**	**Subject D**	**Subject E**	**Subject F**	**Subject G**	**Subject H**	**Subject I**	**Subject J**
Clinical characteristics										
Age (years)	1	1	1	1	4	7	5	7	10	11
Sex	M	M	M	F	M	F	F	F	F	M
Body mass index (kg/m^2)	17	15	15	17	15	13	14	12	15	14
Molecular characteristics										
***COL7A1* mutations**	(+/-) c.425A>G; p.Lys142Arg, (+/-) c.1939C>G; p.Ser609*	(+/-) c.425A>G; p.Lys142Arg, (+/-) IVS5+1G>A	(+/-) c.3840delC; p.Thr1280Thrfs*33 (+/-) c.4037delA, p.Lys1346Argfs*51	(+/-) c.1573C>T; p.Arg525* (+/-) IVS79+1G>C	(+/-) c.3293delAC; p.Tyr1098*1, (+/-) c.4894C>T; p.Arg1632*	(+/-) c.4621delG, p.Gly1541Thrfs*67 Other mutation not identified	(+/-) c.1732C>T, p.Arg578*; (+/-) c.5047C>T, p.Arg1683*	(+/-) c.409C>T, p.Arg137* (+/-) c.6269delC	IVS23-2A>G; c.4317delC; p.Pro1441Leufs*271	(+/+) c.7787delG, p.Gly2596Valfs*34
Skin C7 protein expression by IF	CA	PA	PA	CA	CA	CA	CA	CA	CA	CA
Disease Severity										
BEBSS	15	21	39	18	32	33	36	31	35	23

	Subject A	Subject B	Subject C	Subject D	Subject E	Subject F	Subject G	Subject H	Subject I	Subject J
BEBSS TBSA (%)	13·5	13	47	12·8	19	29	26·5	31	28	13
Global severity score	10	6	6	7	6	9	6	7	7	6
Blister count	6	1	3	2	6	19	22	6	5	2
Pain sleep and fatigue questionnaire										
Pain score Child version (≥6 years)	NA	NA	NA	NA	NA	NA	NA**	18	34	8
Pain score Parent version	17	17	33	8	26	22	28	40	19	14
Fatigue score Child version (≥6 years)	NA	NA	NA	NA	NA	NA	NA	2	6	2
Fatigue score Parent version	3	2	0	1	6	4	5	5	3	1
Pruritus score Child version (≥6 years)	NA	NA	NA	NA	NA	NA	NA	8	8	4
Quality of life questionnaire										
PaedsQL score (Child version)	NA	NA	NA	NA	NA	4	44	32	47	35
PaedsQL score (Parent version)	12	NA	NA	30	39	54	50	50	59	41

Mean suction blister time increased from 613.2 seconds at baseline to 716.4 by Day 100 (p=0.12). Eight out of 10 children showed increased times following BM-MSCs (**Figure 5.12**).

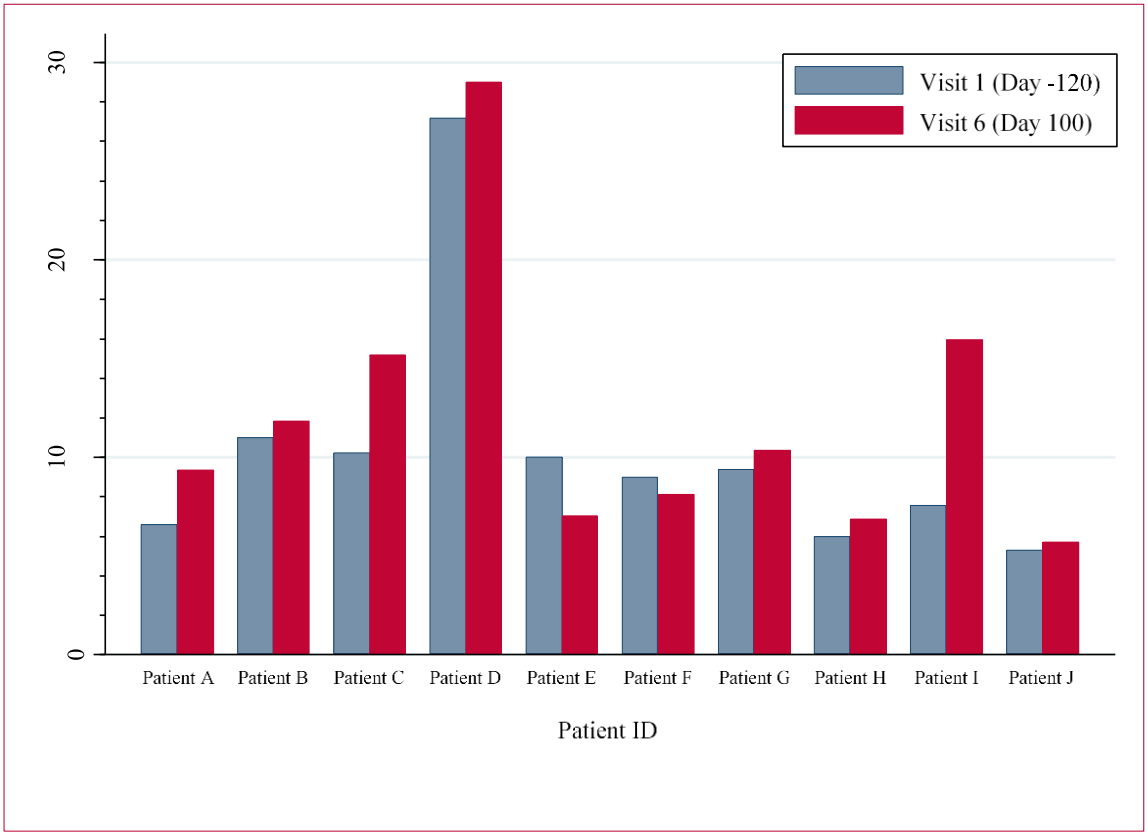


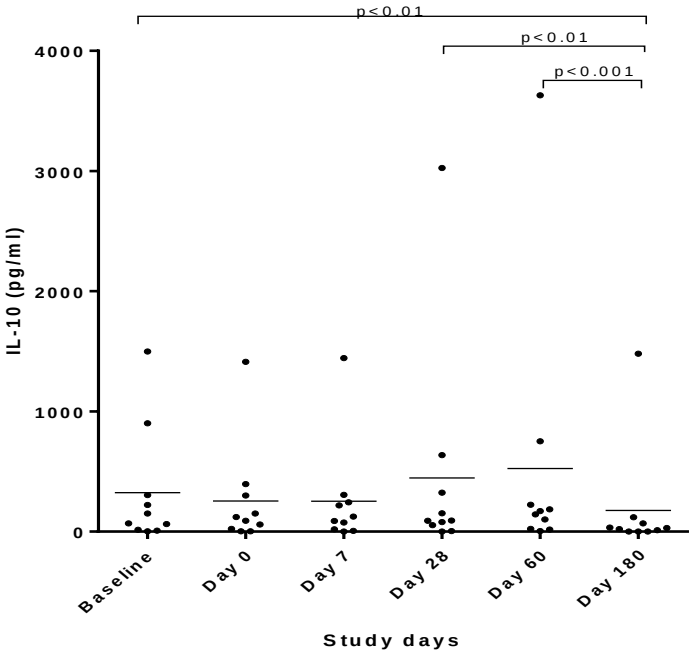
Figure 5.12 Suction blister times.
Three areas of skin, each 3-millimeter diameter, on the thigh were exposed to continuous negative pressure (12 inches of mercury). Time to blister formation was determined with a stop watch. Two measurements, one pre-infusion (Visit 1) and one 100 days post-infusion (Visit 6) are shown all trial children. Results indicate increased skin resistance after infusion for all patients apart from patient E and F.

5.7.4 Cytokines / inflammatory profiles

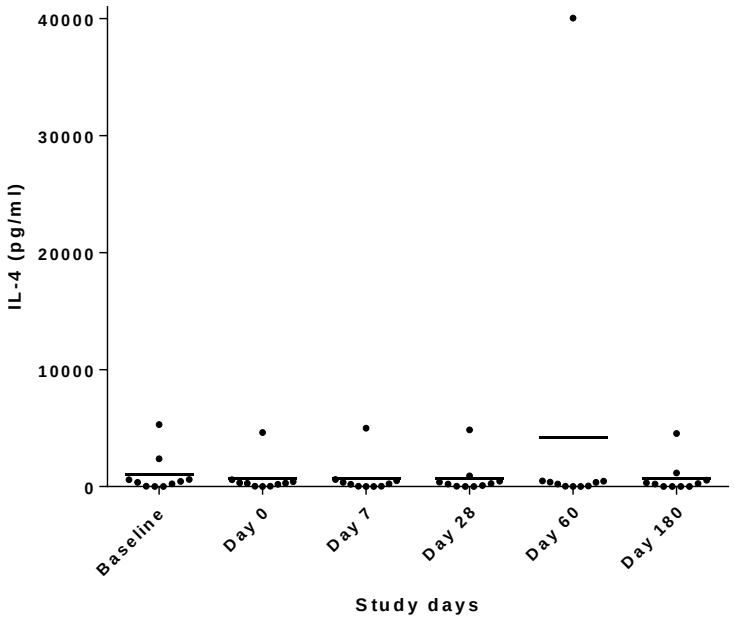
Mean serum IFN-γ levels reduced from 1,871 (95% CI 11, 3,730) pg/ml at baseline to 337 (-26, 700) pg/ml at Day 180 (p=0.09); IL-17A levels reduced from 668 (-149, 1,485) pg/ml at baseline to 49 (-0.65, 100) pg/ml at Day 180 (p=0.21); IL4 levels remained essentially unchanged from 993 (-196, 2,182) pg/ml at baseline to 700 (-296, 1,697) pg/ml at Day 180 (p=0.83); IL-6 levels decreased from 312 (8, 616) pg/ml at baseline to 106 (-17, 229) pg/ml at Day 180 (p=0.47); IL-10 levels decreased from 323 (-29, 676) pg/ml at baseline to 176 (-152, 505) pg/ml at Day 180 (p<0.01) and TNF-alpha levels decreased from 123 (16, 230) pg/ml at baseline to 22 (9-36) pg/ml at Day 180 (p=0.63). The reduction in cytokine levels between Day 60 and Day 180 was significant for IL-10 (p<0.001), IFN- γ (p=0.04) and IL-17A (p=0.03) (**Figure 5.13**).

Figure 5.13 Inflammatory serum cytokines.
Levels of interleukin (IL)-10 (A), IL-4 (B), IL-10 (C), IL-17A (D), Tumour necrosis factor (TNF)-alpha (E) and Interferon (IFN)-gamma (F) (human T-cell and B-cell cytokines) in sera of the trial patients (•). Mean value indicated by line (–).

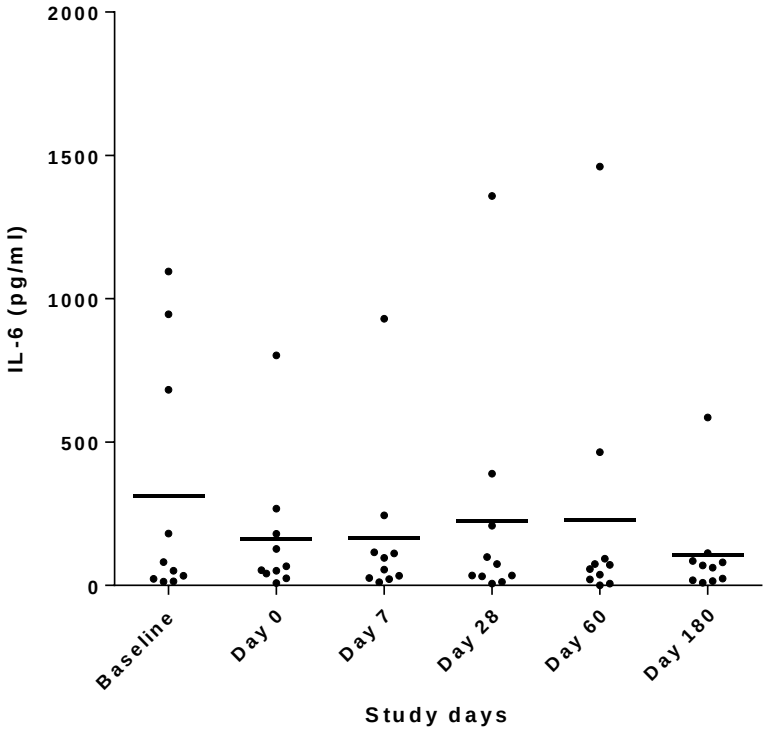
A.



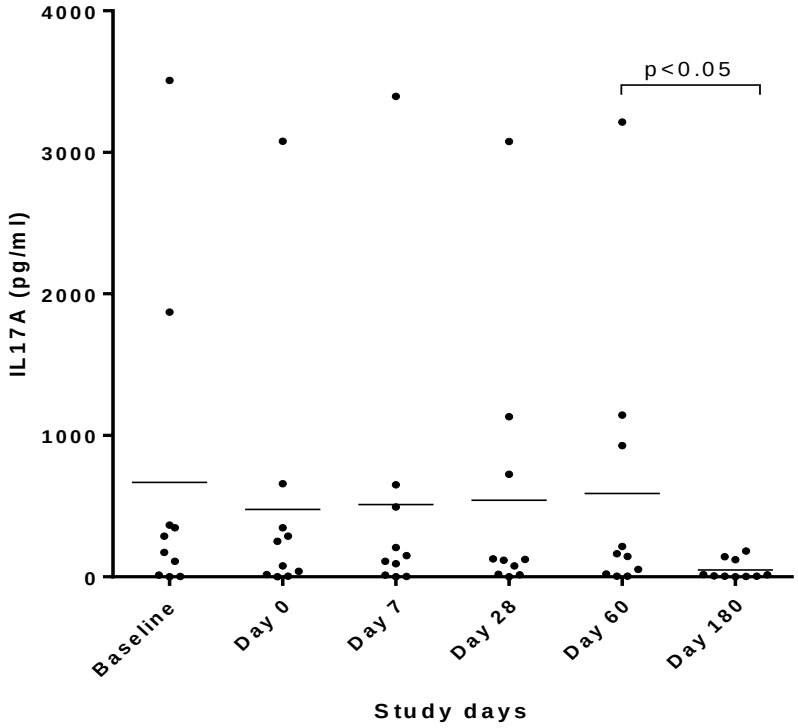
B.



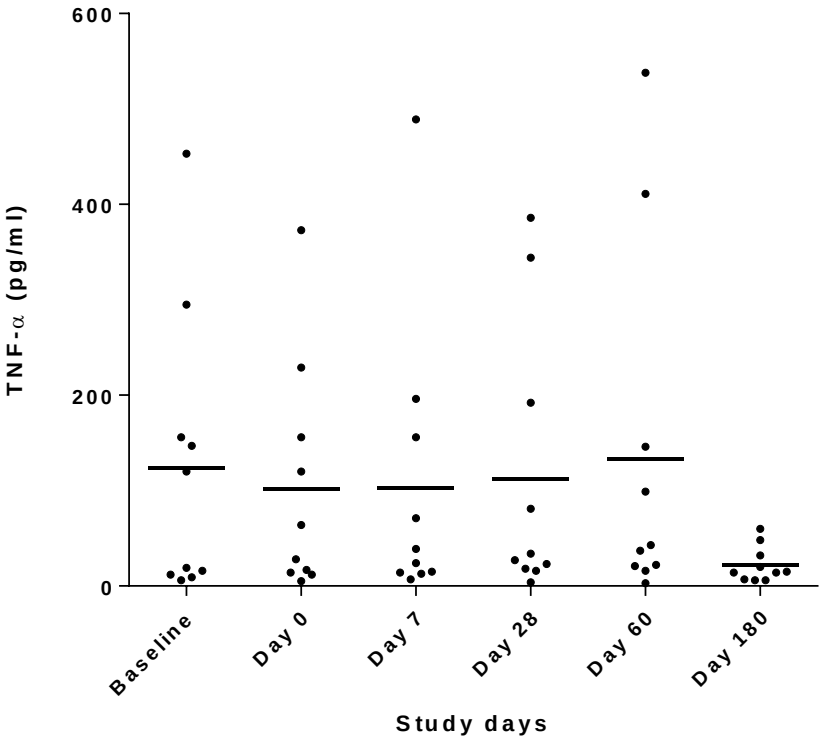
C.


2000
1500
1000
500
0
IL-6 (pg/ml)
Baseline
Day 0
Day 7
Day 28
Day 60
Day 180
Study days

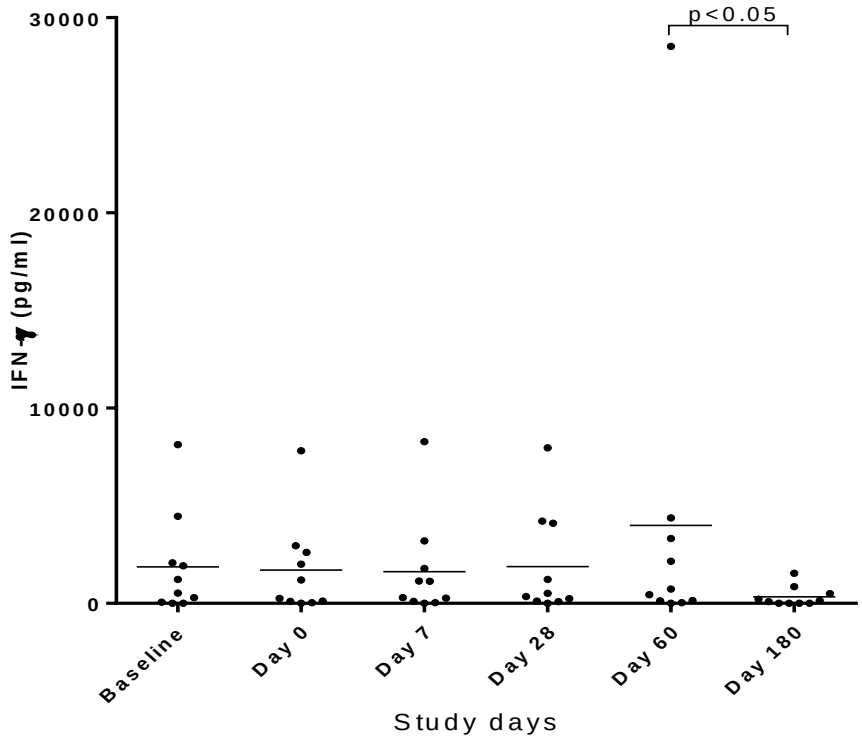
D.


4000
3000
2000
1000
0
IL17A (pg/ml)
p<0.05
Baseline
Day 0
Day 7
Day 28
Day 60
Day 180
Study days

E.


600
400
200
0
TNF-α (pg/ml)
Baseline
Day 0
Day 7
Day 28
Day 60
Day 180
Study days

F.


p<0.05
30000
20000
10000
0
IFN-γ (pg/ml)
Baseline
Day 0
Day 7
Day 28
Day 60
Day 180
Study days

5.7.5 Descriptive data

Semi-structured telephone interviews were conducted with the parents of all trial participants at 9 months after the last MSC infusion. The parents recalled their experience of caring for their children with RDEB prior to and during the clinical trial. The rate of wound healing improved with chronically ulcerated areas of skin beginning to heal. The general improvement to skin condition, together with increase in skin resilience in trauma, enabled the children to participate more fully in play and family life.

"There was an improvement in the colour of her skin and we noticed how quickly everything healed. I am sure [name of patient] was in less pain. [name of patient] was more able to cope with her [sibling] being rougher with [name of patient]. We could reduce the oramorph by a fifth before the bandage changes. I am sure she was experiencing less pain. [name of patient]'s skin was more resistant so she was more prepared to let her sister fling her about the room, you know, like big sisters do. Or maybe it was because she was in less pain. [the skin] could bump but not blister. Or if her sister was doing 'row row row', it would leave finger marks on her [previously before the clinical trial], but not [now, during the clinical trial]. [name of patient's sibling] was just braver, more able to exist as a functional sister. It was very important for us that [name of sibling] was able to interact with her more like normal siblings. It makes you realise how many times you say stop, don't do that, how you are always on edge"

Some parents reported a reduction in the amount of the time required to provide skin care for their children. The amount of dressings required has also reduced. A parent reported about 50% reduction in dressings.

One parent described he often need to return home to assist with his child's skin care prior to the clinical trial. During the clinical trial, he saw a reduction in unscheduled absence from work as his child's skin condition improved. One parent reported that the

improvement to her child's skin condition was one of the key factors that enabled her to take up part-time employment after the clinical trial commenced.

"[I took time off work] 4 or 5 times a month. I have to change a shift, ring a colleague and disrupt a shift. I haven't taken any days off [since the clinical trial started]. You can see the difference."

The improvement to the children's RDEB has led to improved quality of family life with two families reporting they went abroad for holidays and one family reporting regular visits to the zoo since the clinical trial began, which they would not have otherwise done if their children's skin condition did not improve.

"As you can imagine, his skin was all healed up. We were able to put him in the water. Every single day, he was in the ocean. We had to do the dressings everything but the difference was that he can do that and he didn't feel pain. [He had] some areas with little blisters. He was very happy to be in the water. That's why we'd try what we can to go on holiday again. [the clinical trial made a] big difference for him."

The parents of all the children had a more positive outlook for the future of their child with the parents of one child stated that the improvement to their child's RDEB condition was a contributing factor to their decision to have another child.

"Before we even had [name of child] we wanted 3 or 4 children–it was never an option to have just 1 child. If things had been really bad with [name of child], like she wasn't going to walk, I don't think we would have had another child. It's very difficult to know. The fact that we made the decision to have the second one [child] was because of the hope we had from the trial and it certainly has contributed to our decision."

5.8 Discussion

This was an open, uncontrolled clinical trial of intravenous infusions of BM-MSCs in children with RDEB. Availability of BM-MSCs as a pre-manufactured, quality controlled product without HLA matching makes it a safe therapeutic option for children with this severe genetic skin condition. The administration of 1–3 million cells/kg in 3 infusions over 30 days was well tolerated and without significant AEs. Children (>6 years of age) and their parents directly reported increased speed of wound healing, reduction in blister numbers, reduction in pruritus, increased skin resistance to trauma and reduced pain during dressing changes. All parents reported improvement of their children's skin disease, more evident after the second or third infusions. The degree and duration of clinical improvement was variable, ranging from 3–6 months after the first infusion.

No increase in C7 deposition or the formation of new anchoring fibrils was seen at Day 60 after the first infusion. Thus, there is no evidence to indicate that allogeneic MSCs directly recover the inherent skin pathology in RDEB. The mechanism of action through which the MSCs improve wound healing in RDEB is not known but the benefits appear to be indirect and trophic in nature.

The cytokine data showed reduction in immune and pro-inflammatory serum levels of IFN-γ, IL-6, IL-17A, IL-10 and TNF-α. IL-4 levels remained essentially unchanged. More specifically, higher levels of IFN-γ levels are found in DEB subjects, whereas IFN-γ blockers have been successfully proposed as possible pharmacological approach for DEB patients (Skurkovich and Skurkovich 2007). IL-6 is predominantly a pro-inflammatory cytokine, whose levels are higher in DEB patients with a more severe phenotype (Odorisio *et al*. 2014). IL-6 levels also positively correlate with disease severity (Kawakami *et al*. 2005; Annicchiarico *et al.* 2015). Therefore, one can postulate

that the reduction in its levels, although non-significant as in this study, could suppress the inflammatory cascade seen in RDEB. Interestingly, IL-17, a major inflammatory cytokine, was found to be elevated in the blister of patients with bullous pemphigoid and led the IL-17-induced MMP-9 associated inflammatory response (Le Jan *et al*. 2014). IL-17 and MMP-9 also positively related to treatment response (Le Jan *et al*. 2014). MMP-9 has been identified as potential therapeutic targets in DEB patients (Lettner *et al*. 2015) and therefore, the reduction in IL-17A levels, detected in this study, could be relevant. Levels of IL-10, an anti-inflammatory cytokine, initially increased by Day 60 and dropped at Day 180. Finally, TNF- α levels decreased in the study patients, which would be expected, if we assume that the BM-MSCs infusions triggered a systemic anti-inflammatory response. The finding that the majority of the cytokine levels reduction occurred between 2 and 6 months is perhaps unexpected. I can speculate that the MSCs alter the innate immunity system balance and lead to ongoing activation of an anti-inflammatory cascade which lasts beyond the presence of the cells in the circulation. There are still very limited data about the levels and role of these cytokines in EB and the majority of data are extrapolated from other inflammatory skin diseases.

Collectively, the tolerance data appear encouraging, although it should be noted that a zero event rate for a serious AE in just 10 patients is compatible with an upper 95% confidence interval of over 30%.

The natural history of generalised RDEB is one of progressively worsening blistering, scarring and contractures; spontaneous improvement is very rare and limited to cases of bullous disease of the newborn, or subjects with atypical *COL7A1* mutations that lead to leaky splice sites or in-frame exon skipping, or individuals who develop skin patches of revertant mosaicism, none of which were present in the trial participants. In this early phase trial, safety was the primary outcome, therefore, it was not powered to determine

efficacy and to demonstrate benefit. The changes observed in pain scores, BEBSS and BEBSS TBSA, while not conclusively indicating benefit, are promising and the results will inform the design of a more definitive future trial. With regard to descriptive data and potential clinical impact, parents noted significant reduction in pruritus, and pain reductions that allowed children to bathe and perform other activities previously unthinkable due to painful wounds. Increased energy levels and improved appetites were also evident. The parents perceived skin redness, itching, skin resilience, wound healing and pain control were the key areas of noticeable change to their children's disease. Although healing of individual wounds can occur spontaneously in RDEB, in this study there was clinical improvement of the total body surface area as well as objective increased suction blister times signifying increased skin resilience in 8/10 children. The rate of wound healing improved with chronically ulcerated areas of skin beginning to show signs of healing, often for the first time in months or years. The general improvement in skin condition, together with increase in skin resilience to trauma, enabled the children to participate more fully in play and family life. Overall, the changes in efficacy outcomes were promising, although it should be remembered that this is an unblinded study of participants who are keen to help, thus giving a potential for positive information bias.

The small sample size and the lack of a control group are limitations to this study. RDEB is a rare genetic skin disease with an incidence of 1 in 17,000 live births and therefore an underpowered study was justified with the trend of the results presented being more helpful in data interpretation of secondary outcome measures compared to absolute p-values. Inclusion of a control group raised both ethical and practical concerns: it was considered unethical for children to participate in a study in which they would receive a non-active substance and be subjected to skin biopsies and multiple blood tests.

Moreover, the preservative in the BM-MSCs is DMSO which produces an odour shortly after infusion and therefore providing an adequate control was deemed impractical.

Aside from this trial, the only other study reporting both cutaneous and systemic positive outcomes for RDEB has been the report of whole BMT following myeloablation (Wagner *et al.* 2010). However, there was a high mortality rate of >20% in that cohort. Reduced intensity conditioning regimens for BMT are being studied in other clinical trials although detailed safety and efficacy data for those treatments have not yet been published. There were no safety concerns in the use of allogeneic BM-MSCs in children with RDEB in this trial and there were suggestions of clinical benefit. Intravenous infusions of allogeneic un-matched BM-MSCs, without any pre-conditioning, are safe and appear to improve some of the clinical manifestations of RDEB until such a time that more curative therapies are developed.

6 CONCLUSIONS AND FUTURE DIRECTIONS

6.1 General discussion

RDEB is an inherited trauma-induced blistering skin disease caused by mutations in *COL7A1*. The need for a safe, effective and ideally systemic therapy for this debilitating disease has always been great.

The focus of this thesis has been to assess the effect of allogeneic cell-based therapies in RDEB, both at preclinical and clinical levels. The aim was to improve the understanding of the mechanism of action of intradermal injections of skin-derived fibroblasts and their effect on wound healing in individuals with RDEB. I also attempted to investigate the role of EGF in *COL7A1* expression in normal and RDEB skin cells using *in vitro* studies. The effect of allogeneic fibroblast injections on intact skin had only been assessed in a previous study of five adult individuals with RDEB (Wong *et al.* 2008). Based on this single, proof-of-concept study and the findings of mine and other mechanistic studies (Nagy *et al.* 2011) I coordinated and conducted a prospective, blinded, vehicle-controlled clinical trial to assess the effect on wound healing and safety of intradermal injections of allogeneic fibroblasts. I managed to demonstrate that a single series of fibroblasts injections enhances wound healing compared to vehicle in the first 28 days. However, RDEB is a genetic, systemic disease so early intervention and a systemic approach to treatment is the key. Thereafter, I assessed the safety and efficacy of intravenous infusions of BM-MSCs in children with RDEB. Although MSCs have been used in many medical conditions, they had only been administered intradermally in two individuals with RDEB (Conget *et al.* 2010). The study, described in this thesis, was the first to assess the effect of these cells in the context of a prospective, open label study in children with RDEB.

6.2 Summary of key findings

A single series of intradermal injections of allogeneic fibroblasts around the margins of a chronic RDEB wound led to *HBEGF* and *COL7A1* increase, albeit not statistically significant, in mRNA expression compared with normal looking RDEB skin at the injected edge. This finding added further support to earlier studies in another individual with RDEB showing increased *HBEGF* expression after injections in intact skin (Nagy *et al*. 2011). These molecular changes were supported by IF and clinical findings. C7 staining at the injected site was increased at approximately 75% of normal human skin. This led to clinical improvement of the wound size and reduction in size over 8 months.

The *in vivo* experiments were followed by *in vitro* studies, where I was able to show that HB-EGF could upregulate *COL7A1* expression in both normal control and RDEB keratinocytes and fibroblasts, notwithstanding its association with malignant processes (Kivisaari *et al*., 2010). It is also of interest that RDEB cells, especially fibroblasts, lack responsiveness to HB-EGF, when compared with normal human epidermal cells. A closely related growth factor, EGF was also found to upregulate *COL7A1* in both normal control and RDEB fibroblasts. EGF was more potent than HB-EGF in upregulating *COL7A1* in RDEB fibroblasts *in vitro*. The novel finding that EGF plays a role in *COL7A1* upregulation in RDEB epidermal cells could have implications about its potential use alone or in combination as a therapeutic tool in non-healing RDEB wounds. With regard to individuals living with RDEB, the question of whether intradermal injections of allogeneic fibroblasts can accelerate erosion closure had not been answered at the start of this thesis. Most of the initial human studies were performed on intact, unwounded skin, although some of the mouse studies showed that intravenously injected

COL7A1 gene-corrected fibroblasts could home to RDEB wounds and promote wound healing (Woodley *et al.* 2003). I therefore conducted a prospective, randomised, double-blind, vehicle-controlled trial of individuals with RDEB to assess the effect of intradermal injections of allogeneic fibroblasts on erosion healing. A single series of intradermal injections of human allogeneic dermal fibroblasts into the margins of chronic erosions in subjects with RDEB produced a greater, but not statistically significant, reduction in erosion area, time to healing, and in proportion of wounds healed, compared with vehicle within the first 28 days following treatment. Although the study failed to reach statistical significance because of the small number of treated erosions, the results suggest that a greater number of chronic RDEB erosions can be expected to heal within 4 weeks following fibroblast injection (21·4% vs. 16·7%). Thereafter, the study showed no significant differences between fibroblasts and vehicle, although both were associated with clinical improvement over 6 months compared with baseline.

RDEB is a life-long condition that affects people from birth. Therefore, studies in children had long been anticipated in an attempt to assess treatment safety in this population and to ameliorate the condition early in life. The phase I, proof-of-principle study was designed and conducted by me to assess the safety and efficacy of intravenous MSCs in 10 RDEB children. The infusions were tolerated well with no safety concerns. No increase in C7 or formation of new anchoring fibrils was seen. Parents reported reduced skin redness and inflammation, improved wound healing, reduction in pruritus, increased skin resistance to trauma and reduced pain during dressing changes. But it should be remembered that this is an unblinded study of participants who are keen to help, thus giving a potential for positive information bias.

Collectively, these findings provide some insight into the mode-of-action of intradermally administered allogeneic fibroblast. Furthermore, allogeneic cell therapies

were tested in adults and children with RDEB proving that they are safe to use and have a positive, but non-significant, clinical impact on the disease severity. Further studies are required to elucidate the underlying mechanisms and test new treatments in individuals with RDEB.

6.3 Limitations and challenges

Conducting clinical trials in the NHS setting is challenging and the process can be laborious. In particular, recruiting trial participants in a rare disease, such as RDEB, poses three main challenges, subject availability, access and knowledge.

The first challenge is study participant availability. There are three independent adult and paediatric EB centres in the UK, in London (the largest adult and paediatric centre), Solihull (adult centre), Birmingham (paediatric centre) and Edinburgh (both adult and paediatric centre). All of them have their own databases and access to them is restricted for clinical purposes, unless the Investigator holds an honorary contract with the relevant NHS Trust. There is a national database for all UK individuals diagnosed with EB but once again access is password restricted and it can only be used for clinical reasons and not to identify potential research subjects for clinical trials – all factors that generate logistical and practical difficulties. The RDEB individuals are located across the UK; they can be difficult to reach and to attend scheduled study-related hospital visits. In many cases, during the study, the study visits had to be coordinated with regular hospital appointments to ensure compliance and availability. **Figure 6.1** shows the number of places I had to travel to, for the purpose of the fibroblast trial, in order to ensure all trial data is collected when patients could not travel to London.

Figure 6.1 Map showing the areas where I had to travel to conduct the home visits during the fibroblast trial.

Lack of availability for study participation and scheduled trial reviews can also be due to illness, since RDEB individuals are chronically 'ill'. Many of them develop recurrent wound infections, undergo complex eye or hand surgeries with long recovery periods and essentially any illness has a much greater impact on their well-being with a prolonged

recovery period, compared to individuals with other illnesses. In the fibroblast study, these concurrent illnesses played a large role in subject selection because, according to the study entry criteria, study subjects should not have taken antibiotics within 1 week of screening and injections. Even when geographical location and illness do not pose a problem, the participation of an RDEB individual depends greatly on the availability and well-being of their carer. In the severe cases of RDEB, parents are usually full-time carers, who have to care for other household members and may themselves become unwell. This was also taken into account in my study. Moreover, a number of people with RDEB (usually with the generalised intermediate form rather than the generalised severe) declined to participate in the study because they were in full-time occupation.

A third challenge to patient recruitment was the limited knowledge about the IMP. Some RDEB individuals were familiar with the fibroblast cell therapy option, but a large number, especially female subjects, were concerned about local skin pain as a result of the injections. Also the fact that the fibroblasts remain an investigational drug for treatment of chronic wounds without the assurance of clinical effectiveness was a negative factor for several, mostly young, potential study participants.

In the paediatric trial, the parents were highly motivated and fewer challenges in recruitment were encountered. Early phase interventional trials in paediatric population are fewer compared to adult populations due to safety considerations. Mine was the first European medicinal clinical trial in the paediatric RDEB population; therefore, anticipation and eagerness for participation promoted recruitment. Parents were understandably anxious about the phase 1/2 trial, since the IMP was a new, experimental form of systemic treatment in this disease. The consent process took much longer and in cases of children over the age of five, assent was also required.

From the beginning of the fibroblast trial, a somewhat unexpected phenomenon became apparent, namely the natural history of some seemingly chronic wounds to actually undergo dynamic changes in wound size and healing, showing in some cases complete healing within a few weeks. This phenomenon of natural healing in RDEB has not been documented previously according to my knowledge. The patients though, were aware of it, and reported that most of their skin wounds would heal spontaneously and subsequently break down again. Notably, within a particular area, the healing of older wounds was often compounded by the appearance of adjacent and sometimes overlapping newer wounds (**Figure 6.2** and **Figure 6.3**). Perhaps it would be more appropriate rather than describing individual chronic wounds, to refer to areas of chronic trauma that break down and heal continuously. Further discussions with the patients and their carers revealed that the majority of wounds follow a cyclical pattern of wound formation and healing. Initially, a blister develops after minimal trauma or spontaneously, and then after a few days the roof of the blisters becomes detached and a new wound forms. The wound remains open for days to weeks (depending on disease severity) and subsequently shows signs of gradual healing with reduction in size and usually crusting. The same cycle is repeated endless times during the life of an RDEB patient. Most of wounds screened for this trial were well-defined but others had irregular edges and it was difficult to distinguish precisely where one wound ended and another one started.

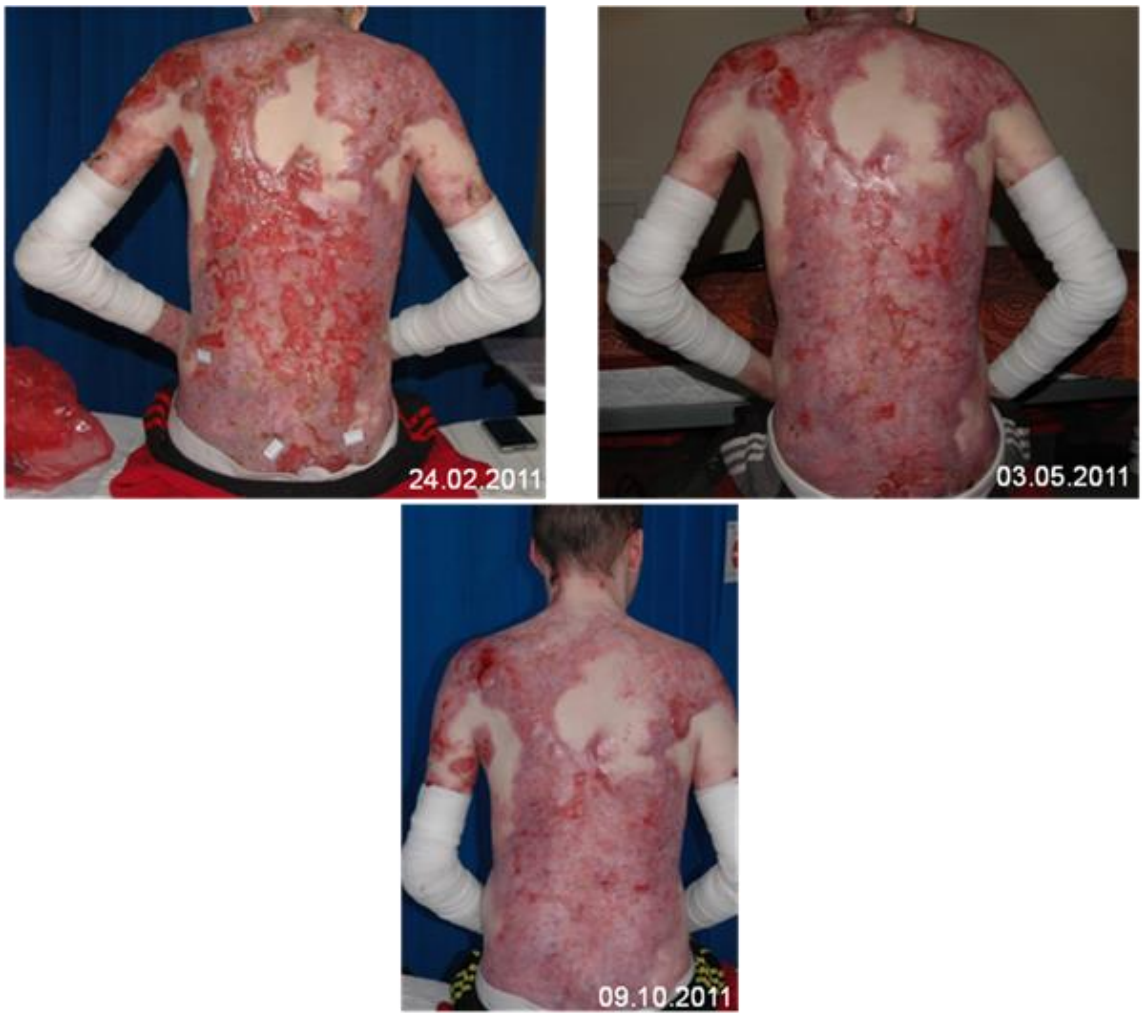


Figure 6.2 An individual with severe generalised RDEB with evidence of spontaneous wound healing on his back.

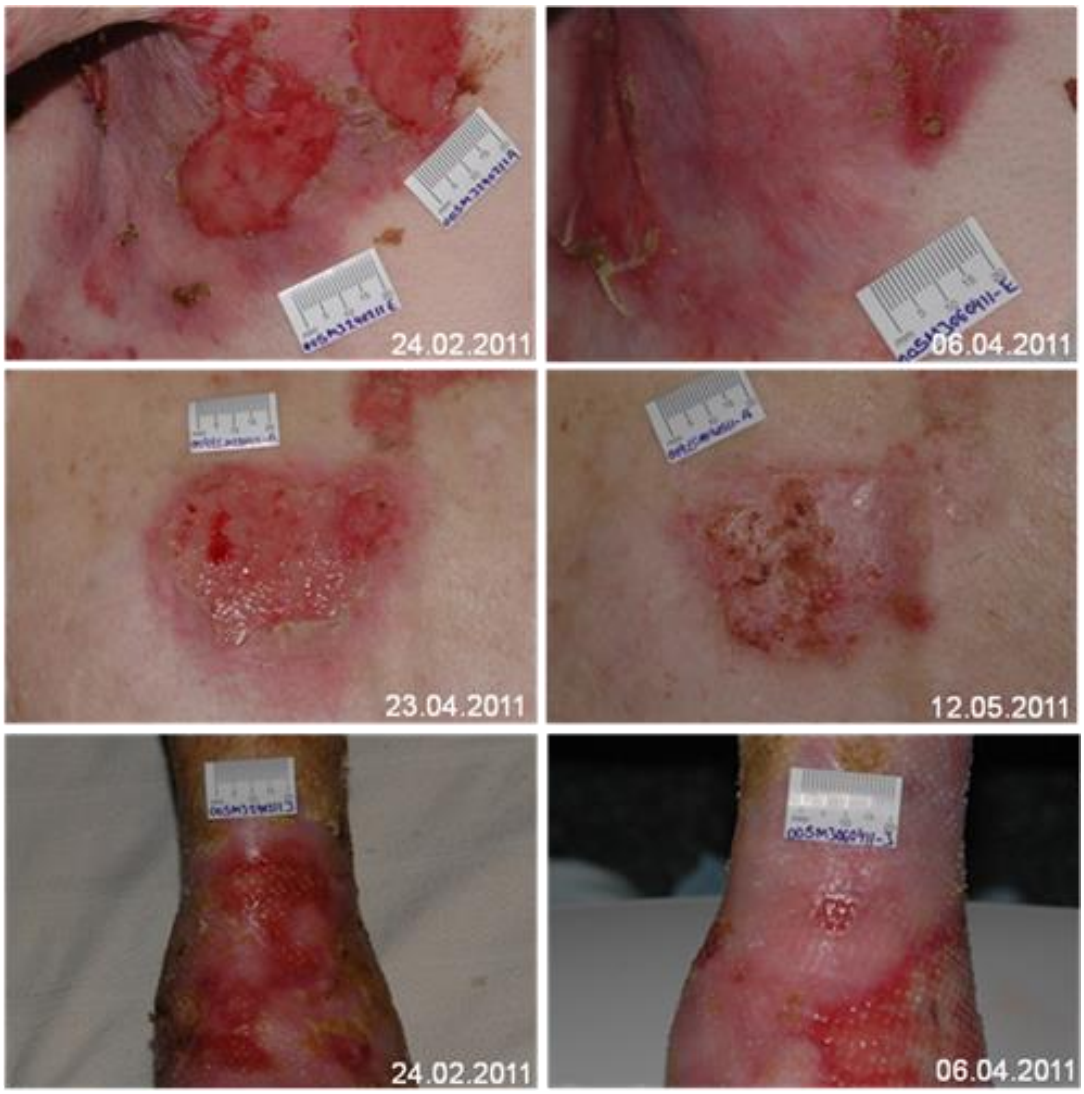


Figure 6.3 Further examples of spontaneous wound healing of wounds screened for the fibroblast trial.

In the fibroblast trial, despite use of topical anaesthesia, in individuals with more severe disease, associated with scarring and contractures, pain was a major issue. One of the reasons for severe pain during the intradermal injections was the temperature of the cells. The fibroblasts/vehicle are kept a 4^{o}C and could only remain out of the fridge for a limited time (< 1 hour) in order to be viable, so they were injected cold into the dermis, which caused significant pain. An additional reason for the pain is the fact that the fibroblast preparation is very concentrated and generates a 'pressure' feeling when injected in the

dermis, even more so when the area has chronic scarring. Lastly, the delivery method via a hypodermic needle is another cause for pain, especially when multiple injections are needed in order to cover the, quite often, irregular wound margins. On the other hand, the pain usually subsides within a few minutes after the end of the injections and no additional analgesia is needed.

Although skin biopsies were not obtained during the fibroblast trial, the phenotype and genotype correlation allowed me to postulate about baseline C7 levels in some RDEB individuals. Also some patients had direct immunofluorescence performed in the past for previous diagnostic studies and I was aware of their baseline C7 levels. In Chapter 3, I demonstrated increased C7 labelling at Day 60 (close to 75% of normal human skin) compared to the patient's un-blistered skin following a single series of injections of allogeneic fibroblasts. In some cases, prolonged wound healing was also seen in patients with no detectable baseline C7, which was partially unexpected. Previous murine studies using fibroblast injections showed that new C7 protein can be generated in the absence of baseline C7 (Ortiz-Urda *et al.* 2003). Also, a pilot study in two patients with severe RDEB suggested that intradermal injection of allogeneic mesenchymal stem cells into chronic ulcerated sites can accelerate re-epithelialisation of these wounds even in individuals with no detectable C7 at baseline (Conget, *et al.* 2010).

6.4 Future directions

6.4.1 Systemic HMGB-1 for milder variants of RDEB

Local administration of HMGB-1 was shown to promote tissue regeneration in myocardial infarction or diabetic ulcer by attenuating the inflammation or promotion of angiogenesis (Straino *et al.* 2008; Takahashi *et al.* 2008). BM-MSCs secrete the TNF-α-

TSG-6 protein (Choi *et al.* 2011) and promote IL-10 production, both of which possess anti-inflammatory properties (Nemeth *et al.* 2009). It has also been previously shown that skin grafts, including RDEB skin, release HMGB-1, which mobilises BM-derived PDGFRα^{+} MSCs into the circulation, which in turn play a major role in skin regeneration (Tamai *et al.* 2011). In the context of RDEB, BM-derived PDGFR-α+ MSCs have been shown to migrate to RDEB mouse skin and restore c7 at the BM (Iinuma *et al.* 2015). These cells are recruited to damaged skin by release of HMGB-1 from hypoxic keratinocytes in RDEB blister roofs, with involvement of a stromal derived factor 1 alpha (SDF1-α) / C-X-C chemokine receptor type 4 (CXCR-4) signalling axis (Iinuma *et al.* 2015). Nevertheless, the precise nature and mechanism(s) of the anti-inflammatory responses induced by the MSCs in RDEB skin are not known. Recently, it was demonstrated, in a skin injury mouse model, that systemic administration of HMGB-1 mobilised endogenous PDGFRα^{+} BM-MSCs into the circulation, in skin grafts and suppressed inflammation (Aikawa *et al.* 2015). Both of these studies provide encouraging data about the systemic use of HMGB-1 for some forms of RDEB and also several other non-EB wound healing or skin tissue injury pathologies, including common conditions such as chronic venous leg ulcers.

6.4.2 BMT revisions

6.4.2.1 BMT protocol refinement

Strategies to improve BMT outcomes include reduced intensity conditioning, and selection of more appropriate BM cell subpopulations, such as MSCs or PDGFRα+ cells. Reducing the degree of immunomyeloablative conditioning used for transplantation must be weighed against the patient's likelihood of developing graft-versus-host disease, a

major immune complication associated with whole BMT. BMT protocol changes have been made to reduce side-effects and improve the speed of recovery. For example, the intensity of the conditioning regimen has been reduced from a combination of busulfan, fludarabine, and cyclophosphamide to combination therapy with fludarabine and low doses of cyclophosphamide and radiation (Tolar and Wagner 2013a). Of the 20 patients with RDEB who received blood and marrow transplantation, five died from disease progression or complications of transplantation, and all those treated with a non-myeloablative regimen are alive (Tolar and Wagner 2013a). The new conditioning regimen is associated with substantially less toxicity, but although disease amelioration is still substantial, it may be less impressive than the original myeloablative protocols used, although full outcomes have yet to be published. Clinical application of BMT is also being expanded to generalised severe and generalised intermediate junctional EB, although RDEB remains the predominant clinical indication for now.

6.4.2.2 Co-infusion of MSCs

In view of the high mortality of the initial BMT protocols, MSCs were added as part of the more recently revised treatment regime. This was based on the theory that co-infusion of allogeneic MSCs post BMT can shift from a pro-inflammatory to an anti-inflammatory environment. This could be achieved by reducing early toxicity due to GvHD. (Tolar *et al.* 2011b). This was likely to make the BMT safer, especially in the setting of RDEB, where large areas of skin consist of open wounds colonised with bacteria. Co-infusion of MSCs early after BMT was also thought to promote engraftment. Therefore, infusion of MSCs was added to subsequent protocols of BMT and cord blood trials (Professor M. Jonkman, personal communication) that used reduced intensity conditioning. The data available so far in abstract form show that the addition of MSCs does not appear to have

added clinical benefits, although the reduced intensity conditioning has reduced patient mortality (Tolar *et al*. 2015).

A potential solution sought is the co-administration of MSCs to promote engraftment, exhibit local immunosuppressive effects and support local progenitor cells (Perdoni *et al.* 2014). Expression of TNFα-TSG-6 by MSCs has been associated with both improved wound healing and anti-inflammatory effects at wound sites (Qi *et al.* 2014). Notably, exposure of MSCs to TGF-β, TNF-α or SDF1-α has been shown to upregulate *col7a1* expression and c7 protein secretion in a time and concentration-dependent manner (Perdoni *et al.* 2014). In theory, this should bring C7 levels toward the 30% of the amount of wild-type C7 that is believed to be adequate for preventing blistering in RDEB (Fritsch *et al.* 2008). Other studies have investigated pre-conditioning of MSCs for potential clinical benefit in RDEB. Moreover, these cytokines also lead to increased MSC production of the anti-inflammatory protein tumour necrosis factor-stimulated gene 6 protein (TSG-6) that has already been implicated in the indirect trophic benefits of allogeneic MSCs (Pittenger *et al.* 1999; Lee *et al.* 2009). Thus, future clinical trials are likely to assess systemic delivery of *COL7A1*-supplemented autologous RDEB MSCs, with possible pre-conditioning.

6.4.3 Gene replacement trials

6.4.3.1 Gene editing

The limitations of localised cell and protein therapies, haematopoietic cell transplantation, and gene therapy vectors have made gene-editing nucleases an attractive option for RDEB therapy. These reagents can be rationally designed and engineered to mediate a break in the DNA strand at a user-defined locus resulting in a 100- to 1000-

fold rate increase in homologous recombination (Taghian and Nickoloff 1997; Donoho *et al.* 1998). Candidate platforms for gene editing include zinc-finger nucleases (ZFNs), transcription activator–like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease system. ZFNs, TALENs, and CRISPR-Cas9 function similarly, in that they all bind a user-defined DNA sequence and mediate a break in one or both of the DNA strands. However, their architecture and engineering processes differ greatly.

ZFNs are heterodimeric DNA-binding proteins fused to a *fok*I nuclease domain and co-localise to a target site to mediate a DNA break. The relatively small size of ZFN arrays makes them highly attractive for use for many gene delivery platforms; however, the challenges of requisite purchase or the acquisition of specialised starting materials and generation methodologies could restrict their widespread use. Similar to ZFNs, TALENs function as dimeric proteins and are tethered to the *Fok*I nuclease; however, the region in which DNA cleavage occurs is larger (Christian *et al.* 2010; Cermak *et al.* 2011). TALENs comprise repeating elements that each contact a single base of DNA. The most recently described gene-editing platform is the highly user-friendly CRISPR-Cas9 system (Jinek *et al.* 2012). Nuclease-free targeting of the *COL7A1* locus has also been demonstrated by Sebastiano *et al* (2014), the corrected cell types in these experiments were iPSCs derived from RDEB keratinocytes and fibroblasts. The corrected iPSCs were then transformed into corrected keratinocytes, which were used to create autologous epithelial grafts with normal C7 composition.

6.4.4 iPSCs

iPSCs represent, in principle, a nearly inexhaustible source of somatic cell types for tissue regeneration and *ex vivo* modelling of genetic disease (Hanna *et al.* 2007; Thatava *et al.* 2011; Yusa *et al.* 2011). Skin cells from RDEB and JEB patients can be reprogrammed to pluripotency, thereby providing new tools with which to investigate the mechanisms underlying EB pathology *in vitro* (Tolar *et al.* 2011c; Tolar *et al.* 2013b). Furthermore, keratinocytes isolated from an unblistered patch of skin from a patient with RDEB, were reprogrammed into iPSCs (Tolar *et al.* 2014). The healthy patch of skin was determined to be a result of somatic mosaicism, and iPSCs cells derived from this healthy patch produced functional, biologically relevant levels of C7. RDEB iPSCs cells can also be differentiated into keratinocytes and fibroblasts, and can be used to construct full-thickness three-dimensional skin equivalents (Itoh *et al.* 2011; Uitto *et al.* 2012; Itoh *et al.* 2013). Finally, differentiation of iPSCs to non-haematopoietic MSCs has been reported and represents another option to address the systemic manifestations of RDEB, although further studies are required to ascertain whether the iPSC–MSC described in these studies are similar to the mouse Lin-PDGFRα+ mesenchymal cells in their capacity to migrate to wounds and mediate C7 deposition (Zhao *et al.* 2015).

6.4.5 Revertant mosaicism

In EB, revertant mosaicism, the coexistence of mutant cells carrying germline mutations and revertant cells that have spontaneously corrected the germline mutation by a somatic reverse mutation, has been described in patients with mutations in the *COL17A1*, *COL7A1*, *FERMT1*, *KRT14*, and *LAMB3* genes (Kiritsi *et al.* 2014). Autologous punch grafting of healthy looking but genetically affected skin to treat chronic wounds in patients with the junctional form of EB showed initially a high healing rate of 70% but

recurrence of blisters was seen in all grafted areas (Yuen *et al.* 2013). Transplantation of revertant skin in an individual with junctional EB, resulted in revertant epidermis which expressed amounts of laminin-332 comparable with normal-appearing human skin (Gostynski *et al.* 2014). DNA analysis of the revertant skin cells confirmed that the acceptor sites were populated by the transplanted revertant keratinocytes. The grafted site remained blister-free for at least 18 months. This study raised hopes for using revertant cells to treat larger areas of mutant skin. The major challenges would be to find new methods for higher *in vitro* expansion of revertant keratinocytes and identifying the revertant skin patches.

6.4.6 Protein therapies for RDEB

The use of human recombinant C7 protein as a potential treatment for patients with RDEB was first described in 2004 with the demonstration that intradermal injections of the protein could localize to the BMZ and produce functional anchoring fibrils in murine and human RDEB skin models (Woodley *et al*. 2004a). Recent studies aiming to address the stability of c7 *in vivo* have demonstrated that both endogenous and exogenous C7, administered via fibroblasts or MSCs, have a half-life of about one month (Kühl *et al*. 2016). Subsequent studies extended these findings into a col7a1−/− murine model, showing similar benefit (Remington *et al*. 2009). Although these mice developed antibodies against the C7 protein, this did not preclude formation of anchoring fibrils and improvement of the disease phenotype. Given the presumed advantage of a systemic intravascular delivery method—which could theoretically provide recombinant C7 protein to all mucocutaneous lesions rather than being restricted to injection sites—this same group provided evidence in 2013 that intravenous infusions of recombinant C7 could home to the DEJ and form anchoring fibrils in an RDEB skin mouse model

(Woodley *et al*. 2013). Although these preclinical studies hold translational potential and provide proof-of-concept for recombinant protein-based therapies, the likely need for repeated injections, with the associated costs and the possibility of anti-C7 antibody formation, may limit the efficacy of this approach (Bruckner-Tuderman 2009).

In the case of RDEB, protein replacement consists in delivering recombinant human C7 protein locally or systemically. Injection of C7 corrected the subepidermal blistering and restored C7 expression in the BMZ for at least 2 months after a single injection. These results were supported further by intradermal injection of human C7 in a murine model of RDEB (Remington *et al.* 2009). The injected human C7 incorporated stably into the BMZ of RDEB mice, formed anchoring fibrils, and corrected the disease phenotype, leading to markedly prolonged survival. Remarkably, the treated RDEB mice developed circulating anti-human C7 antibodies that did not bind to the mouse's BMZ, nor did it prevent anchoring fibrils formation. Finally, treatment of the mice with an anti-CD40L mAb prevented the production of anti-human C7 antibodies.

The question whether a structural protein of the skin such as C7 would be able to home to skin, incorporate into the BMZ, and form anchoring fibrils after systemic injection had never been addressed previously. Indeed, protein replacement therapy is usually used in systemic diseases such as haemophilia or in lysosomal storage disorders, in which systemic delivery of a normal recombinant protein into the circulation allows it to reach therapeutic levels in the relevant target organs (Desnick and Schuchman 2012).

To answer this question, Woodley *et al.* (2013) used venous tail injection of human recombinant C7 in two immunodeficient murine models. In the first model, human C7 was injected intravenously into wild-type mice with full-thickness skin wounds. C7 homed to the wounded skin only, where it formed anchoring fibrils as shown by electron

microscopy, and it improved wound healing. In the second model, C7 was injected intravenously in a murine model with grafted murine RDEB skin. C7 homed only to the RDEB skin graft, and it restored DE adhesion, C7 expression, and anchoring fibrils formation. In both of these models, the contribution of human C7 to anchoring fibrils formation was confirmed by labelling using gold particles attached to an antibody specific for human C7. Remarkably, the injected human C7 remained stably incorporated in the BMZ for at least 3 months after one injection. Translation of these results from a mouse model weighing 35 grams injected with 60 μg of C7 protein to children and adults weighing 35–70 kg would need a dose of 60–120 mg of C7 per infusion, respectively, which does seem theoretically achievable.

Despite the initial preclinical positive studies, human trials of C7 protein therapy were put on hold in March 2016, because of unexpected toxicity in later animal studies (http://debraofamerica.tumblr.com/post/125355034512/shire-research-update).

6.4.7 Drug and small molecules

It is also likely that some individuals with EB will benefit from a new group of compounds that have the capability of inducing read-through of nonsense mutations (Arakawa *et al.* 2003; Cardno *et al.* 2009). Several recessive forms of EB involve loss-of-function mutations. Notably, in RDEB, ~15% of all pathogenic mutations involve nonsense mutations (Dang and Murrell 2008) and these are likely to be a focus for read-through therapeutics. Such drugs can increase mRNA expression by ~5-10%, leading to increased protein synthesis (Rowe and Clancy 2009). Given that mouse models of RDEB have indicated that it is necessary to have 30-40% of the normal levels of c7 at the DEJ to prevent blistering (Fritsch *et al.* 2009; Kern *et al.* 2009), the use of a systemic read-

through drug in appropriate patients may help contribute towards this target and complement other therapies.

Gentamicin, an aminoglycoside antibiotic, and its derivatives are old drugs traditionally used to resolve serious Gram-negative bacterial infections and are known to be ototoxic and nephrotoxic in some patients. Interestingly, this class of antibiotics has been shown in some cases to "read-through" premature stop codons generated by nonsense mutations in certain types of gene defects. Approximately 10–25% of RDEB patients have nonsense mutations resulting in a truncated C7 or no C7 at all. Woodley *et al* (2013) identified, and characterised 22 RDEB patients, of whom two had nonsense *COL7A1* mutations. They cultured their keratinocytes and fibroblasts with varying doses of aminoglycoside antibiotics. Without aminoglycosides, these cells synthesised no C7. By contrast, in the presence of noncytotoxic doses of aminoglycosides, both the RDEB cultured keratinocytes and the fibroblasts synthesised and secreted full-length C7 at a level between 10 and 35% that of normal cells (Cogan *et al.*, 2014). The C7 generated was structurally identical to normal C7 and incorporated correctly into the DEJ of a human skin equivalent *in vitro*. RDEB cells expressing C7 also exhibited a reversal of the abnormal cellular motility that is characteristic of RDEB cells.

A more recent study looked at the association of scarring and fibrosis in RDEB and how this is regulated by TGF-β expression and its role as phenotype modulator (Odorisio *et al.* 2014). Based on evidence that losartan has an anti-fibrotic effect in diseases such as Marfan syndrome (Lacro *et al.* 2014), Nystrom *et al* investigated the effect of losartan on TGF-β in a mouse model of RDEB (Nystrom *et al.* 2015). The group managed to show that losartan reduced tissue inflammation by reducing TGF-β levels; reducing the expression of TNF-α in RDEB paws and as a result reducing fibrosis progression i*n vivo*. The delay of mitten deformity formation by a 7-week treatment in the c7 hypomorphic

mice corresponds roughly to a delay of 2 years in patients with severe RDEB (Fritsch *et al.* 2008) A major limitation is, however, that it does not reduce skin blistering, the primary manifestation of RDEB.

Furthermore, a phase 3 comparative study of the healing of chronic wounds of RDEB individuals using decellularised human amniotic membrane and standard of care wound dressing Mepitel® (www.molnlycke.com) is undergoing in Paris, France (NCT02286427; https://clinicaltrials.gov).

Progress has also been made in EBS treatment. *In vitro* studies on EBS keratinocytes showed a significant upregulation of IL-1ß, while treatment with anti-IL-1ß antibody successfully resulted in reduced IL-1ß and stabilisation of the intermediate filament network (Wally *et al.* 2013b). Based on these preclinical findings, a double-blinded, randomised, placebo-controlled pilot study investigated the use of a topical 1% diacerein, a prodrug of the IL-1 converting enzyme inhibitor rhein (Moldovan *et al.* 2000). The investigators managed to show a 66-78% reduction in blister numbers in 2 out of 4 patients (Wally *et al.* 2013a).

7 APPENDIX A

7.1 Contents of enclosed CD-ROM

The CD-ROM enclosed with this thesis contains documents used in the clinical trials described in Chapter 5 and Chapter 6. The CD-ROM contents are in Adobe Acrobat format, i.e. PDF. To view this material, the Acrobat Reader browser plug-in is required. The plug-in can be downloaded from Adobe's website: http://www.adobe.com/.

The main menu is divided into two sections, which include documents related to the Fibroblast trial (Chapter 5) and the EBSTEM trial (Chapter 6). Specifically, for Chapter 5 these include the trial protocol, participant information sheets, letters of invitation and a trial advert to improve recruitment. There are also samples of the case report form which was used to record the trial visit activities for each participant and the patient diary which was given to each trial participant on Day 0.

The second section includes documents related to the EBSTEM trial (Chapter 6). These comprise of the trial protocol, age-appropriate participant information sheets, the case report form, participant diary and additional trial-specific documents. These include quality of life, pain sleep, fatigue and severity questionnaires and an EB-specific blister count manual. The section also contains documents relating to the BM-MSCs storage, management and release as well as management of the study biological samples.

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