Translational trials in wound healing

by

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Declaration

I, Muholan Kanapathy, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Date Signature

Mr Muholan Kanapathy *MD MRCS MSc*

Thesis Abstract

This work explores the role of gap junctional proteins (GJP) in wound healing in two clinical settings: venous disease and epidermal grafting.

Chronic wounds and ulcers are common and a feared problem particularly in the elderly, causing pain and disability. Treatment costs are estimated at £2-3 billion to the NHS with a further loss of 2 million workdays per year. Varicose veins are the major contributor to the prevalence of ulcers affecting about 0.3-0.5% of the population at any point of time.

The expression of GPJ; connexins 43, 30 and 26 were explored in a cross-sectional study of patients with varicose veins at different stages of venous disease (CEAP stage). A stepwise increase in GJPs overexpression was seen corresponding to the clinical CEAP stage of the disease, supporting their role in the disease mechanism and as a biomarker of wound healing. This is also the first-time varicose veins were shown to be associated with poor wound healing.

Concurrently, with the introduction of a new wound healing system for epidermal grafting, a sequential program of research was developed. Initially, a systematic review using Cochrane methodology on epidermal grafting for wound healing, and a pilot case series to evaluate the novel surgical technology. Following positive outcomes; a patient reported outcome measure and cost evaluation study was performed.

Combining these data, a pilot randomised controlled trial was performed to compare efficacy of epidermal grafting to standard of care. Alongside, translational studies on GJP were undertaken to outline the cellular mechanism of action of epidermal grafts.

These data led to the development of a wound healing group at UCL and subsequent engagement with the MRC UCL clinical trials team to design a novel platform trial to further assess epidermal grafting. This platform will investigate the molecular mechanism of action and explore the most appropriate use for this technology. A NIHR EME and a collaborative industry grant is in progress.

Acknowledgements

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Thesis Statement

This thesis is divided into three parts. The first part explored the expression of gap junctional proteins in venous disease. The work done in this part formed the groundwork aimed at learning steps involved to perform a robust translational trial which requires thorough clinical and laboratory methodology. Having learned from this, I then designed a series of studies to evaluate a novel surgical technology for wound healing which formed the second part of this thesis. The third part of this thesis contains the overall conclusion and elaborates the future direction for both part one and two.

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Abbreviations

CEAP	Clinical severity, Etiology or cause, Anatomy, Pathophysiology
CENTRAL	Central Register of Controlled Trials
CONSORT	Consolidated Standards of Reporting Trials
CRF	Clinical research folder
Cx	Connexin
ECM	Extracellular matrix
EME	Efficacy and Mechanism Evaluation
EG	Epidermal grafting
GJP	Gap junctional protein
H	Hour
H&E	Haematoxylin and eosin
KD	Kilo dalton
MeSH	Medical Subject Headings
Min	Minute
MMP	Matrix metalloproteases
MOOSE	Meta-analysis Of. Observational Studies in Epidemiology
MRC	Medical Research Council
NIHR	National Institute of Health Research
NHS	National Health Service
NHS R&D	National Health Service Research and Development

NPWT	Negative pressure wound therapy
RFH	Royal Free Hospital
RT	Room temperature
Sec	Seconds
SPIRIT	Standard Protocol Items: Recommendations for Interventional Trials
SSG	Split thickness skin grafting
UCL	University College London
UCLH	University College London Hospital
VV	Varicose vein
VLU	Venous leg ulcer
3D	Three-dimensional

Part 1

Chapter 1

1.0 Literature review on skin changes in chronic venous disease and the role of Connexin in venous ulcer

1.1 Chapter summary

Varicose veins are common, affects 1 in 3 adults, and a feared complication is venous leg ulcer (VLU), which affects about 0.3-0.5% of the population at any point of time. However, the precise mechanism leading to skin changes in venous disease and the subsequent ulceration remains unclear. This chapter explores the mechanism of skin changes and its link with a potential biomarker, the Connexin (Cx) family of gap junctional proteins (GJPs), a known contributor to poor healing of VLUs. The skin changes seen in venous disease progression is attributed to venous reflux and venous hypertension that leads to chronic inflammation and ischaemia-reperfusion cycles, the likely trigger to Cx upregulation in VLUs. We evaluate and explain how these factors lead to skin changes in venous disease and cause upregulation of Cx and its consequences, based on clinical and in vitro studies. There is substantial associative and mechanistic evidence to support the role of Cx in poor healing of VLU and its likely overexpression in the early stages of venous disease. Elucidating the pathogenesis of venous disease with the use of a biomarker can inform clinical practise for a more targeted therapeutic intervention as well as for disease prevention.

1.2 Introduction

About 1 in 3 adults have varicose vein (VV) and over 35,000 VV operations are carried out in the NHS per year[1, 2]. Within patients with VV, 10% may go on to develop skin changes, such as pigmentation and eczema, while 2-6% have a lifetime risk to develop a venous leg ulcer (VLU) [1, 3, 4]. This is likely to increase, in part at least, because of the increasing ageing population and prevalence of obesity[3-5]. VVs are a major contributor to the prevalence of VLU which affects about 0.3-0.5% of the population at any point of time[5, 6]. VLU are the most common type of lower limb ulcer, comprising about 70-80% of the ulcers in the community, with treatment costs of about £2-3 billion and loss of 2 million workdays per year [7-9].

Venous disease derives most commonly from valve incompetence, but can also occur due to an obstruction of outflow; or due to immobility or obesity causing impedance of the mechanical pump generated by the muscles of the lower limb; or the combination of these [4]. In the Caucasian population the pattern of venous disease most commonly occur in superficial veins (45%), solely in the deep veins of the leg (12%) or a combination thereof (43%) [10]. The venous hypertension and the pressure gradient between the deep and superficial venous system leads to the plethora of complications and sequelae observed.

Stage of venous disease can be classified according to the clinical, etiologic, anatomic, and pathophysiologic (CEAP) classification according to the disease severity[11]: C0 = no visible venous disease, C1 = spider veins, C2 = VV, C3 = oedema, C4 = lipodermatosclerosis, C5 = healed VLU, and C6 = active VLU (Figure 1.1). Despite good understanding of the clinical signs and symptoms of each class, the mechanism that influences the progression of skin changes from CEAP class C2 to C6 remains unclear [1, 12, 13] and reliance on the known risk factors will not predict those patients at risk of developing VLUs. Hence, a measurable biological element, a biomarker, that reflects the pathogenesis of the disease and which

correlate to the disease severity and effectiveness of a treatment is needed to guide early intervention of venous disease.

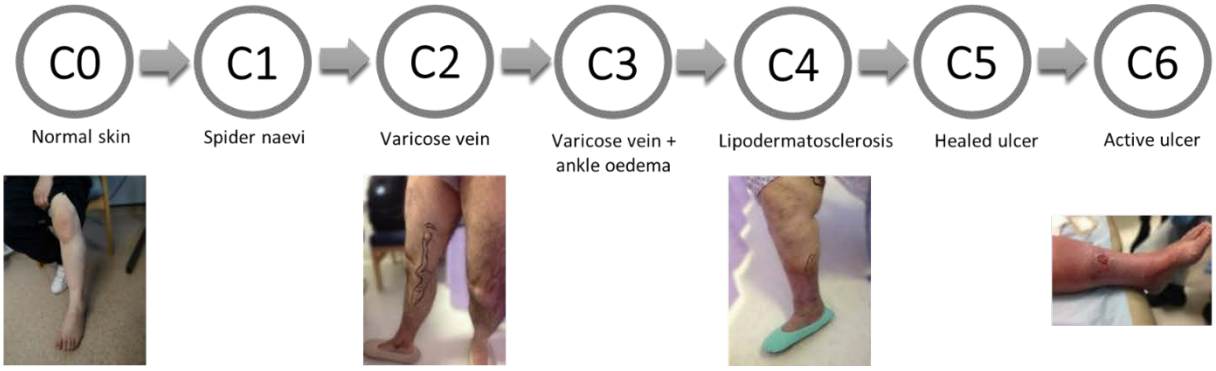


Figure 1.1 Clinical spectrum of venous disease

Venous disease can be classified into seven classes per CEAP Classification, from C0 to C6, according to the disease severity. The CEAP classification along with corresponding clinical photographs are shown here.

This review explores the mechanism underlying skin changes and its link with a potential biomarker for venous disease, the Connexin (Cx) family of gap junctional proteins (GJPs). We first highlight the current understanding on the pathophysiology of the skin changes with venous disease progression. We then explore the role of Cx in VLUs, linking their expression with the pathogenesis of venous disease. We end by proposing models to evaluate the role of GJPs as a biomarker of venous disease progression.

1.3 Skin changes in chronic venous disease

The mechanism leading to changes in skin quality such as reduced elasticity and hyperpigmentation as seen in lipodermatosclerosis remain debatable. Histologically, lipodermatosclerosis is characterised by fibrous scar tissue of the reticular dermis built up of

collagen bundles replacing cellular components, whereas venous ulceration is characterised by the total loss of epidermis and partial loss of the matrix structures in the upper dermis[14]. The chronic venous insufficiency (CVI) related hyperpigmentation, as seen in lipodermatosclerosis, was initially assumed to be due to haemosiderin and/or melanin deposition[15]. The abnormal presence of these pigments in the dermis was explained by extravasation and lysis of red blood cells, followed by decomposition of haemoglobin into haemosiderin, which stimulates melanogenesis[16]. However, in an observational study involving skin biopsies from patients with lipodermatosclerosis, haemosiderin deposition and melanin aggregates were seen in the dermis even despite the lack of evidence of erythrocyte extravasation in 50% of the patients [17]. In a follow-up study performed by the same group involving patients from CEAP class C2-C6, the authors reported that the haemosiderin deposition was obligatory for severe venous disease (C4 onwards) and was not present in the earlier stages [18]. Based on these observations, they hypothesised that the dysregulation of melanin metabolism in advanced venous disease was not a result of erythrocyte extravasation, and instead is likely to be due to chronic inflammation. Inflammatory cytokines and growth factors such as alpha-melanocyte stimulating hormone, endothelin-1, or stem cell factors have been considered as possible mediators[19-21]. This finding was echoed in a larger study that similarly observed extravasation of erythrocytes taking place only in concomitance of severe dermal inflammation [22].

Chronic inflammation, venous dilatation and extravasation have been attributed to venous reflux and venous hypertension, which are the basis of several hypotheses on the pathogenesis of skin changes seen in advanced venous disease (Figure 1.2) [23, 24]. Three main hypotheses were proposed: fibrin cuff deposition, microvascular leukocyte-trapping and leukocyte activation [4]. The initial proposition of fibrin cuff deposition around capillaries secondary to filtration of fibrinogen has been superseded by the theory of chronic inflammation related leukocyte-trapping and neutrophil activation [4, 12]. The presence of chronic inflammation in the walls of VV and the skin of C4-C6 have been well established [12, 25-27].

While none of the histological studies observed the presence of inflammatory cells in the skin of C2, a recent study investigating skin changes in patients with CVI using ultrasound, however, pointed out that inflammation and dermal oedema were present in apparently normal skin of C2 legs[28]. Despite demonstrating the ability to use a non-invasive technique to identify early skin changes in C2, the ultrasonographic findings in this study were not correlated with histological evaluation.

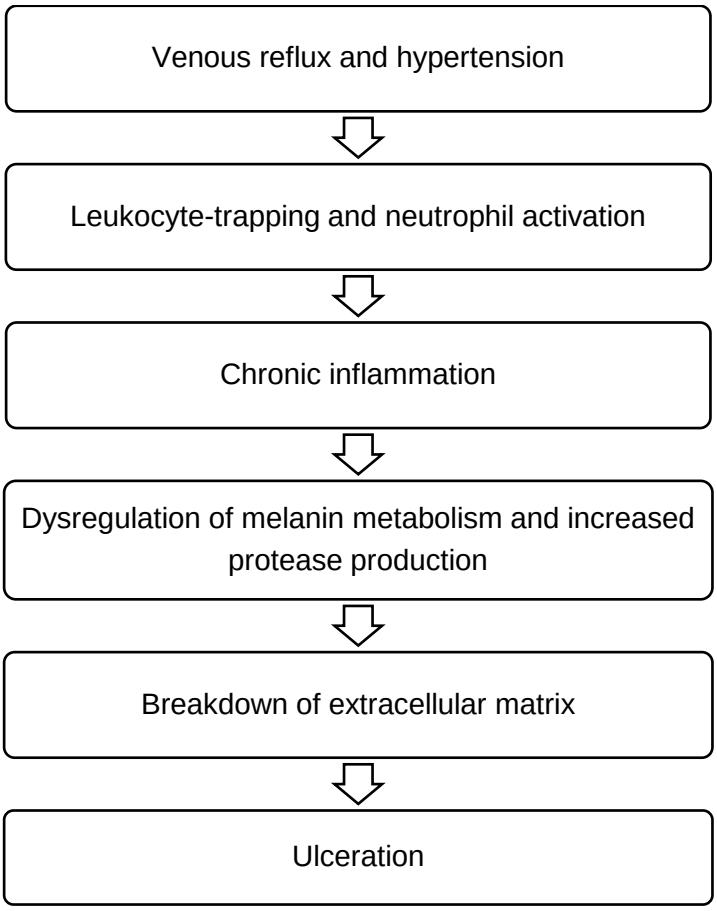


Figure 1.2 Summary of the pathogenesis underlying skin changes and ulcer formation in venous disease.

Flowchart summarised the pathogenesis underlying venous disease and the progression leading to skin changes and ulcer formation.

Chronic inflammation remains the only consistent mechanistic feature between C4 and C6 [29, 30]. The high number of activated neutrophils that exist in the microenvironment of the chronically inflamed ulcers secrete excessive amount of proteases that can cause tissue destruction and persistent inflammation that perturbs healing[12, 31]. The prolonged activity of activated neutrophil proteases, such as the Human Neutrophil Elastase (HNE) and matrix metalloproteinase (MMP), causes degradation of ECM, receptors and growth factors, resulting in ulceration and impedes healing by thwarting cellular migration and attachment [32]. A recent multicenter study comprising 541 patients with CVI of CEAP class C1-C6, have for the first time, showed that each class may be described by expression of specific MMPs[33]. They found that the serum elevation of MMP-2, ADAMTS-1 and ADAMTS-7 correlates with the initial stages of CVI (C1-C2), while the serum elevation of MMP-1, MMP-8, MMP-9, NGAL, ADAM-10, ADAM-17 and ADAMTS-4 were particularly involved in the more advanced stages (C4-C6) [34]. Although the trigger for inflammation and the link between venous hypertension and chronic inflammation remains unclear, this interesting finding suggests that there are indeed early changes despite noticeable histological alteration to the skin, indicating the need to investigate at the cellular and molecular level, hence the need for a biomarker.

1.4 Phases of wound healing

Normal wound healing can be divided into 3 phases: inflammatory, proliferative, and remodelling [35]. Each of these phases will be explored in this section.

1.4.1 Haemostasis and inflammatory phase

The haemostasis and inflammatory phase of wound healing begins immediately following tissue injury. Upon injury, components of the injured tissue, including fibrillar collagen and tissue factor, act to activate the clotting cascade and prevent ongoing haemorrhage. During this process, platelets degranulate to release growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor β (TGF- β)[35]. The result of this process is the

conversion of fibrinogen to fibrin and subsequent polymerization into a provisional matrix which provides scaffolding for cell recruitment and attachment. At the same time, the activation of the clotting cascade also attracts and activates inflammatory cells to the site of injury. Within the first 48 hours, neutrophils infiltrates into the fibrin matrix within the wound cavity to remove dead tissue by phagocytosis and prevent infection besides releasing a variety of proteases to degrade ECM to prepare the wound for healing. However, the prolonged presence of neutrophils in the wound has been proposed to be a primary factor in the conversion of acute wounds to non-healing chronic wounds.

Neutrophils are then replaced by macrophages, which appears 48 to 72 hours post-injury. They are recruited primarily by expression of monocyte chemoattractant protein 1. Macrophages phagocytose debris and bacteria, but are especially critical for the orchestrated production of growth factors necessary to produce ECM by fibroblasts and new blood vessels in the healing wound. Among the chemokines, cytokines, and growth factors present in the healing wound are as follows: vascular endothelial growth factor, fibroblast growth factor 2, platelet-derived growth factor, keratinocyte growth factor, epidermal growth factor, transformation growth factor beta, tumour necrosis factor alpha, granulocyte macrophage colony-stimulating factor, interferon alpha, interleukin 1, 4 and 8, and endothelial nitric oxide synthase [35].

1.4.2 Proliferative phase

Although the phases of wound healing are not exclusive and have features that overlap, it is generally accepted that the proliferative phase occurs from day 4 to 21 following injury. Certain facets of the proliferative phase such as re-epithelialisation could begin almost immediately following injury. Keratinocytes at the wound edge alter their phenotype in the hours following injury. Regression of the desmosomal connections between keratinocytes and the underlying basement membrane frees cells and allow them to migrate laterally. Keratinocytes then move via interactions with ECM proteins (such as fibronectin, vitronectin, and type 1 collagen) via specific integrin mediators as they proceed between the desiccated eschar and the provisional

fibrin matrix beneath [35]. The provisional fibrin matrix is then gradually replaced by a new platform for migration, the granulation tissue, which is composed of three cell types: fibroblasts, macrophages, and endothelial cells. Fibroblasts are the workhorses during the first few days of injury to produce the ECM that fills the healing scar and provides a scaffold for keratinocyte migration. Macrophages continue to produce growth factors such as PDGF and TFG-β1 that induce fibroblast to proliferate, migrate and deposit ECM, as well as produces proangiogenic factors which stimulates endothelial cells to form new vessels.

1.4.3 Remodelling phase

The remodelling phase is the longest component of wound healing which lasts from 21 days up to 1 year and is characterised by the processes of wound contraction and collagen remodelling. Contraction is produced by wound myofibroblasts, which are fibroblasts with intracellular actin microfilaments capable of force generation and matrix contraction. Collagen remodelling involves replacing type III collagen laid down by fibroblasts during proliferative phase to type I collagen [35]. This slow remodelling phase is largely mediated by matrix metalloproteinases (MMPs) that are secreted in large part by macrophages, fibroblasts and endothelial cells. The strength of the wound improves slowly, reflecting the turnover in collagen subtype and increased collagen cross-linking, gradually improving to about 80% of the breaking strength of unwounded skin at 1 year.

1.5 Connexin in venous disease

The regulation of inflammation and tissue repair requires precise local intercellular communication via cell adhesion molecules and cell-cell junctions. The Cx family of GJPs are highly specialised transmembrane channels, which play a pivotal role in the healing of VLUs [36-38]. Cxs are clusters of plasma membrane protein (Figure 1.3), spanning adjacent cell membranes, leaving a 2-4nm extracellular “gap”, hence their name, and are described in terms of molecular mass (Cx43 represents the Cx protein of 43 kDa) [39]. This section will explore

the physiological roles of Cx, their expression and implication in VLU and the association between Cx and pathogenesis of venous disease.

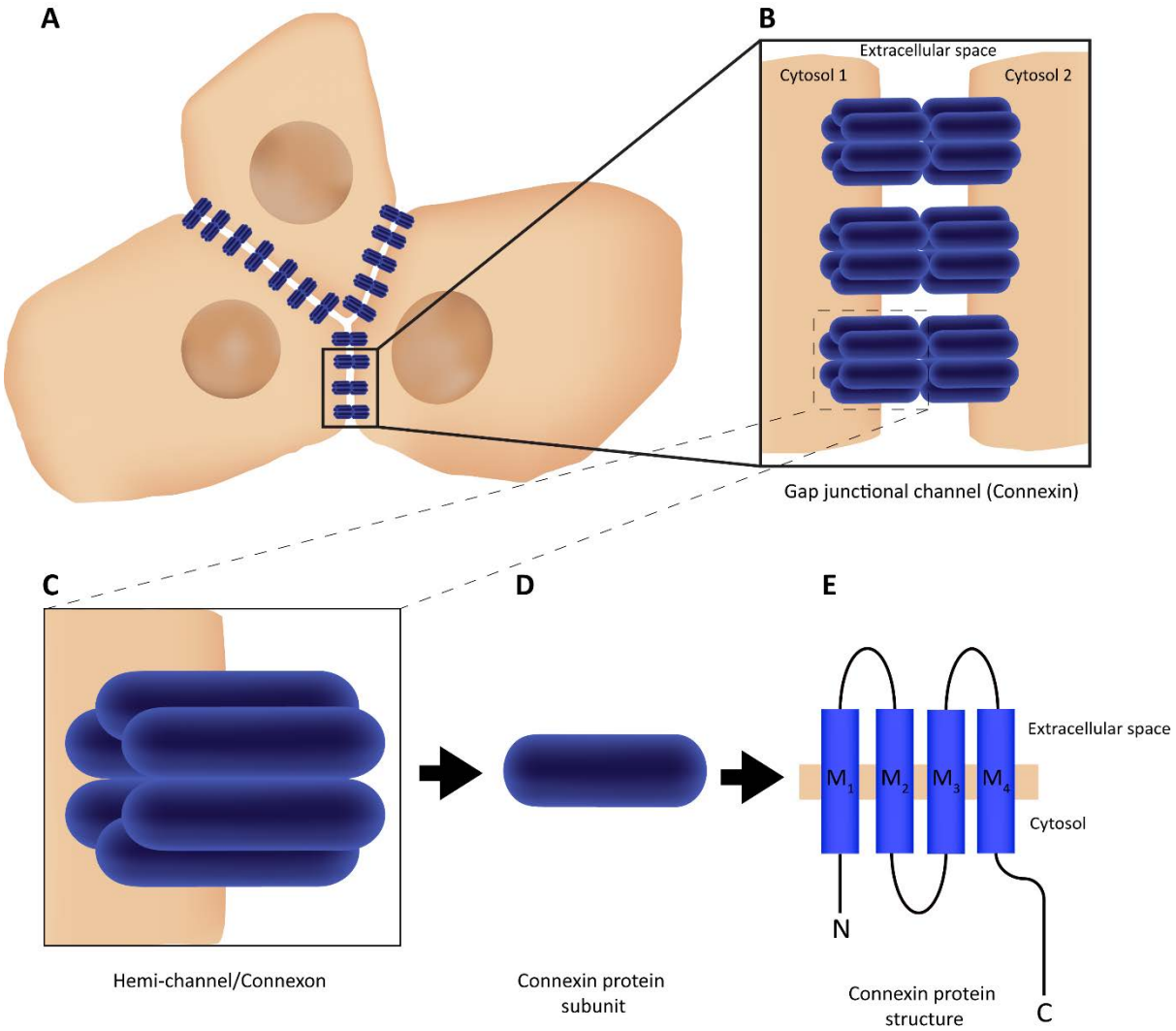


Figure 1.3 The structural organisation of gap junctional protein (Connexin)

(A, B) Cxs are specialised clusters of plasma membrane channels which assist cell-cell communication and exchange of metabolites. (C, D) Each Cx is made of a paired hemi-channel known as a Connexon, which consists of six Connexin protein subunits. (E) Each Connexin protein subunit has four alpha-helical transmembrane proteins, two extracellular loops, a cytoplasmic loop, and a N- and C-terminus located within the cytoplasm. The C-terminus binds to cytoskeletal elements within the cells to regulate cellular migratory properties.

1.5.1 Physiologic roles of Connexin

Cxs facilitate cell-to-cell communication and exchange of ions and metabolites of less than 1kDa in size between adjacent cells [40]. Nine different Cxs are expressed at different levels in human skin (including Cx26, 30, 30.3, 31.1, 32, 37, 40, 43, and 45), with Cx43 being the most ubiquitous, and found in epidermal keratinocytes, dermal fibroblasts, blood vessels, and appendages such as sweat glands, sebaceous glands, hair follicles, mast cells, and activated leukocytes [39-42]. They are present in precise temporal and spatial context within the epidermis and dermis. Intercellular communication mediated by the GJP is important during cellular growth and development as well as in the maintenance of normal metabolism and tissue homeostasis [37, 40, 43]. Cxs also act as a nexus interacting with adhesion molecules (Cadherin, α- and β-catenin), tight junctions (Zonular Occludin-1 (ZO-1) and ZO-2) and cytoskeletal components via the long cytoplasmic C-terminal tail, either directly or via adaptors in a cycle phase-specific manner [34, 44-46]. These multiprotein interaction influences both cell adhesion and cytoskeletal dynamics, which may require to be broken down to facilitate efficient cellular movement in wound healing [34]. In addition to its role in the migratory property of keratinocytes and fibroblasts, Cx is also required in other physiological processes, which include cell differentiation, proliferation, electrical transmission and inflammation[45]. Cxs have a short half-life, and therefore gap junction remodelling constantly occurs with a high turnover rate. For example, the half-life of Cx43 is only 1–3 h, much shorter than the average turnover time for other integral membrane proteins [47]. The dynamic nature of this protein and the regulation of their assembly and turnover therefore are important in the healing of VLUs.

1.5.2 Connexin expression in VLU

In acute wounding, Cx43 starts to downregulate about 6 h after injury, which correlates with the keratinocyte adopting a migratory phenotype as they start to crawl across the wound bed to re-epithelialise the wound[40]. In chronic VLUs, multiple Cxs are abnormally upregulated at

the wound margins and have been implicated in impaired keratinocyte and fibroblast migration, hence poor wound healing [34, 36, 48]. Figure 1.4 (below) summarises the effect of Cx downregulation and upregulation in skin wound healing.

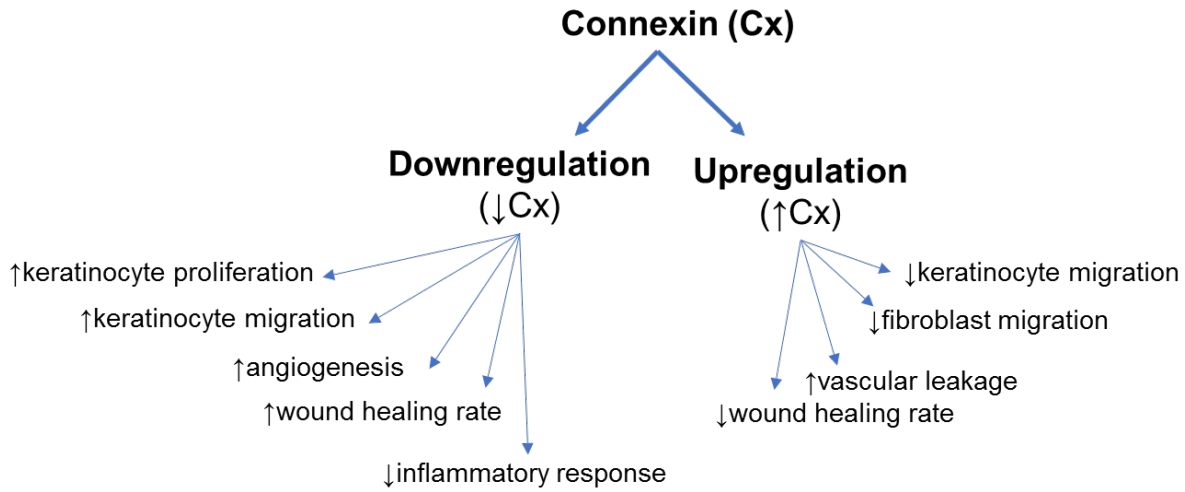


Figure 1.4 The effect of Connexin downregulation and upregulation in skin wound healing

A recent observational study involving wound edge biopsies from patients with VLUs revealed that the principal epidermal and dermal Cxs (Cx43, Cx30 and Cx26) were consistently overexpressed in each patient (Figure 1.5) [36]. The levels of Cx43 at the wound edge epidermis was reported to be on average 14-fold higher than the normal skin from the arm of the same patient ($p<0.001$). Cx30 and Cx26, conversely, had a striking 226-fold and 123-fold increase at the wound edge compared to the matched intact, control skin ($p<0.001$).

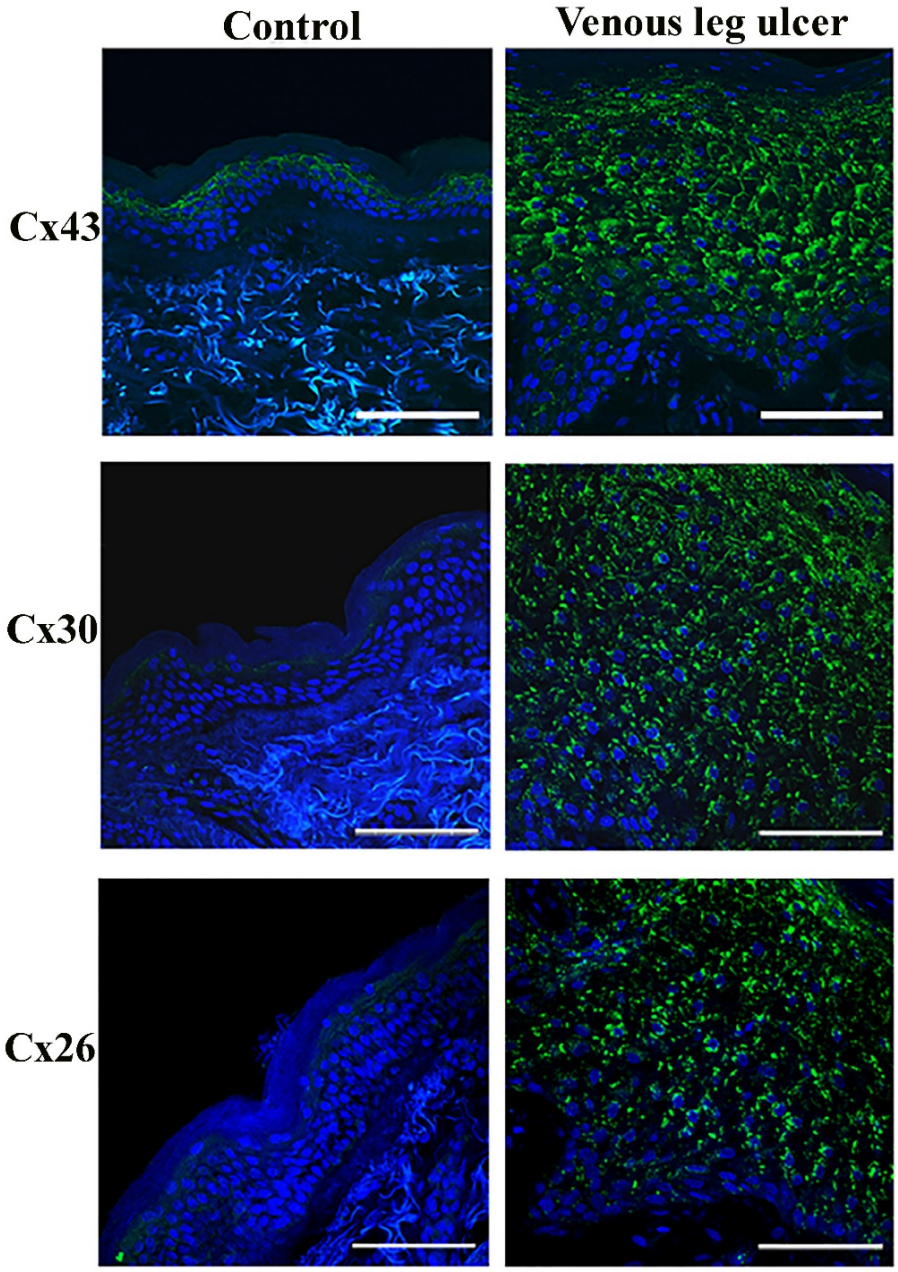


Figure 1.5 Connexin expression in venous leg ulcer

Expression of Cx43, Cx30 and Cx26 in control (unwounded) skin and wound edge of venous leg ulcer. Increased expression of the Cx proteins (green pixels) were seen in the epidermis of venous leg ulcer. Green: Cx43, Cx26 and Cx30; Blue: nuclei. Scale bar = 100µm. Magnification 40x. Figure reproduced with permission from Sutcliffe et al., 2015. Copyright © 2015 John Wiley & Sons, Inc.

The negative effect of Cx overexpression on cellular migration is mediated by both gap-junctional intercellular communication and non-junctional mediated effects. An increase of Cx43 by one-fold reduces cellular migration rate by half [34]. On the other hand, downregulation of Cx by Cx43 antisense in murine and humans have been shown to significantly accelerate wound healing [34]. The attenuation of Cx43 expression leads to reduced cell adhesion via a reduction in adhesion molecule (N-cadherin) expression and activation of regulators of cytoskeletal dynamics (GTPases Rac1 and RhoA), resulting in significantly longer migratory lamellipodial extension, enabling increased cell motility of fibroblasts cultured in-vitro[34]. Besides increasing the migratory activity of fibroblast, promoting Cx43 downregulation at a wound site also promotes angiogenesis, keratinocyte proliferation and migration, and decreases the number of infiltrating neutrophils and macrophages [49].

The precise mechanism which triggers Cx upregulation in VLUs remain to be elucidated, however this is likely to be related to venous hypertension induced ischaemia-reperfusion cycle and chronic inflammation[50].

Under hypoxic conditions, Cx hemichannel opens to allow the release of signalling molecules into extracellular space [51]. The oxidative stress induces opening of the hemichannels, a result of a large drop in extracellular Calcium (Ca^{2+}) concentration and membrane depolarisation, allowing movement of molecules down their respective concentration gradient to establish electrochemical equilibrium [52-54]. Such molecular transport leads to a loss of ionic homeostasis and destabilization of the membrane potential, further influencing and increasing the hemichannel activity, likely resulting in the upregulation of Cx expression. The excessive hemichannel opening is not favourable for cell survival as it contributes to lesion spread, inflammation and direct loss of cells' ability to osmoregulation, leading to cell death [40, 55-57]. This has been reaffirmed through a reduction in cell death, both in vitro and in vivo, with inhibition of hemichannel opening by Cx mimetic peptides [40, 50, 58, 59].

In inflammatory skin disease, dysregulation of Cx hemichannels was observed as evidenced by extracellular ATP leakage and excessive cytosolic influx of calcium[60]. The extravasation of inflammatory cells increases expression of Cxs, especially Cx43, as the channels are involved in the release of cytokines and immunoglobulins[61, 62]. In wounds, treated with Cx43 antisense or mimetic peptide, significant reduction of neutrophils, macrophages, chemokine Ccl2 and cytokine TNF-α were seen, confirming the role of Cx43 expression in neutrophil extravasation and release of inflammatory cytokines[49, 63, 64]. Venous dilatation secondary to venous hypertension could also directly result in the disruption of tight junctions in between endothelial cells along the vessel wall, interfering cellular adhesion and communication [58]. This endothelial disruption results in pathological hemichannel opening, further worsening vascular leakage and inflammatory response[65]. The role of Cxs in vascular leakage have been previously reviewed [66]. A recent study highlighted that Cx37, Cx43, and Cx47 were also involved in the development of vein valves and lymphatic function, emphasising the important role these GJPs play in the formation of VV[67]. While the expression pattern and the effect of Cx gene knockout on embryonic valve development is shown in mouse models, it remains unclear if patients with VV are subjected to congenital mutation of these proteins.

1.5.3 Therapies targeting Connexin in VLU

The ability of GJPs to regulate immune responses, cellular proliferation, migration and apoptosis make them an attractive therapeutic target to improve wound healing. Cx expression can be targeted and manipulated for therapeutic benefit in wound healing via three approaches[65, 68]: 1)targeting transcription/translation modulation (antisense approaches); 2)peptidomimetic approaches (such as Gap26, Gap 27, Peptide5, Gap19 and ACT-1); and 3)non-specific compounds or small molecule approaches (such as fatty acids, volatile anaesthetics, alcohols, steroids, or quinine and derivatives) (Figure 1.6).

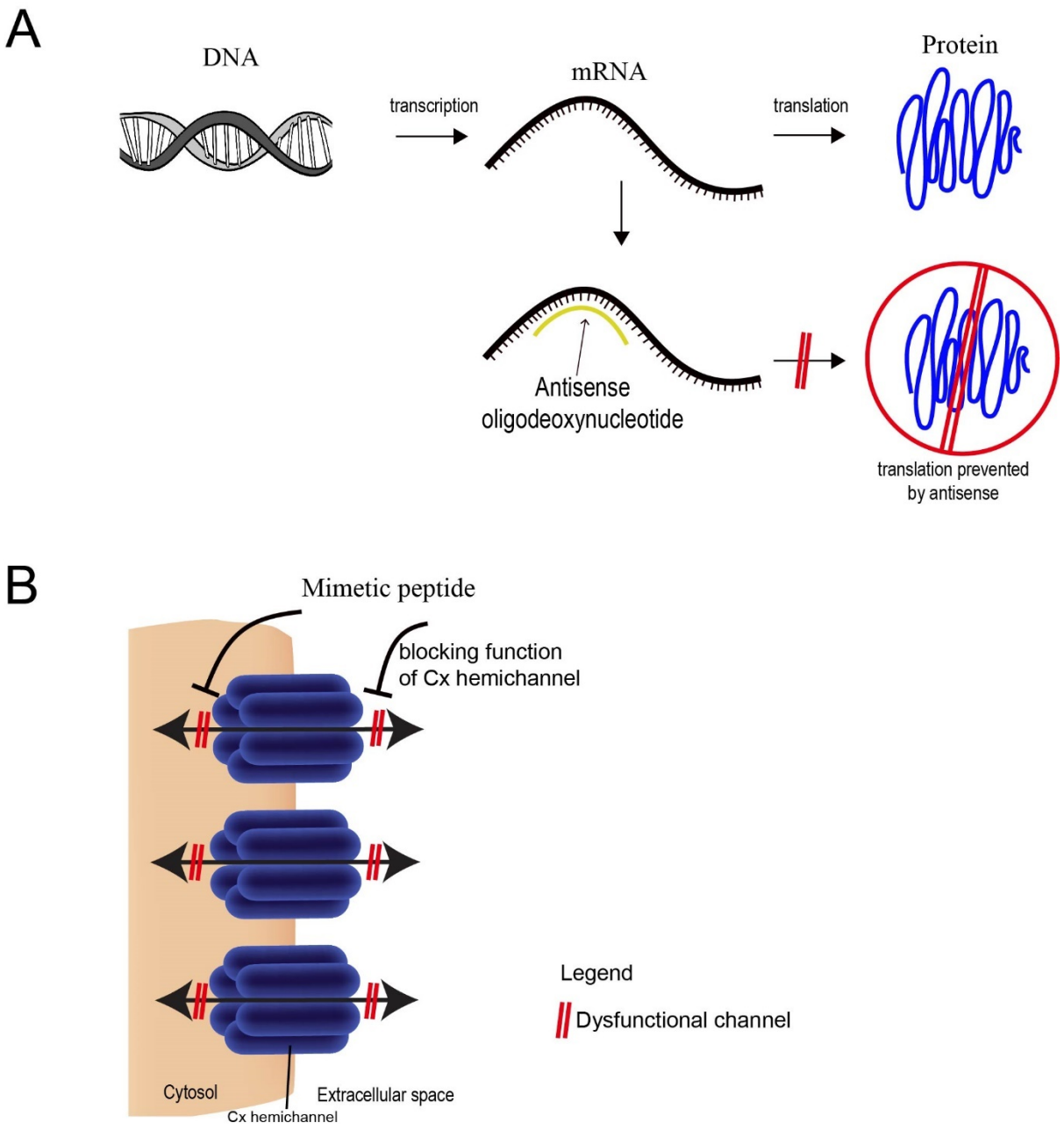


Figure 1.6 Mechanism of action of therapies targeting Cx expression.

(A) Antisense oligodeoxynucleotide binds to mRNA to block Cx protein translation, thus downregulating the protein expression. (B) Mimetic peptides can act either cytoplasmically or extracellularly, blocking the function of Cx hemichannel, hence preventing cell-to-cell communication and exchange of ions and metabolites.

Of these, the antisense is very specific to Cx targets, while peptidomimetics are Cx-specific though not always isoform-specific, and the non-specific compounds often affect multiple channel types[65]. The antisense oligodeoxynucleotide works by binding to the messenger RNA (mRNA) to block Cx protein translation, reducing turnover, thus downregulating the gap junction expression [69]. The peptidomimetics, on the other hand, can either act cytoplasmically (targeting the amino or carboxyl–terminal tails, or the intracellular loop such as Gap19 and ACT-1) or externally (targeting extracellular loops such as Gap26, Gap27 and Peptide5) [70, 71]. These peptides inhibit Cx hemichannel function and are independent of Cx expression, however they can cause a downregulation of Cx proteins in long term and some peptides have been shown to be not Cx specific as is often claimed[50, 70, 72]. Efforts to improve the ability to target the Cx proteins are currently being intensified, with biotech companies developing peptides with several libraries of gap junction modulation compounds. This includes a library of 200 Cx43 interacting compounds, a library related to danegaptide /rotigaptide that contains 500 compounds, including hexapeptides, cyclic peptides, modified dipeptides and small molecules, a library containing of 150 modified Gap-peptides, and many more[72].

Both the peptides and antisense approaches have been investigated in several multi-centre randomised clinical trials for treating VLUs, by using topical application and gel delivery systems, with positive outcomes in accelerating wound healing [59, 65]. These treatments exert effect via four key aspect: 1)enhancing cell migration (epithelial proliferation and granulation tissue formation); 2)reducing oedema; 3)reducing inflammation via targeting the inflammasome pathway; and 4)reducing vascular haemorrhage[34, 49, 59, 65, 70]. Newer tissue engineering approaches, such as sustained release of Cx43 antisense from coated collagen scaffolds, are currently underway [73]. Of course, the downside of all these drugs is that they might not go through the skin barrier and only work on open wounds, unless the molecules are under 500KD that will penetrate the skin and knock down the Cx proteins.

1.6 Models to evaluate Connexin as a biomarker of venous disease

Cx upregulation is likely to take place in the early stages of venous disease as both inflammation and hypoxia underlies the pathogenesis of venous disease. While no notable histological changes are seen prior to C4, the upregulation of the plasma membrane channels can only be confirmed using immunohistochemical staining or gene array study. This can be established by taking skin biopsies from patients across the CEAP class, from C0 to C6, ideally from the distal part of the lower limb and a control biopsy either from the proximal part of the lower limb or from the upper limb. Analysis should include the principal epidermal and dermal Cx, which are Cx43, Cx30 and Cx26. The increased expression of these Cxs in C2 and C4 compared to C0 and control skin can confirm that the upregulation of Cxs take place prior to ulceration as Cxs are currently often only thought to be a feature of chronic wounds. Effort should be taken to standardise the duration of VV among patients within the same class especially in the asymptomatic group. A large population based cohort study should be considered, with age and gender matching to limit heterogeneity within group. Thereafter, if Cxs are overexpressed as early as C2, a longitudinal study should then be undertaken to evaluate if early intervention of VV could downregulate Cx expression, given the short half-life of Cxs. As well as providing insight into the pathogenesis of venous disease, this could also indicate that VV predisposes skin to future ulceration, hence a biomarker for early surgical intervention.

Sequencing technology should also be harnessed to identify alteration at the mRNA level, which may translate into new therapeutic targets to regulate Cx protein expression, for disease prevention or treatment. This can identify at risk patients who would benefit from early treatment with molecular pharmaceutical agents to prevent disease progression, an aspiration towards personalised medical care. Gene analysis could also be performed using blood serum or punch biopsy of the skin and vein wall. This should be correlated with the genetic profile of patients with CVI to look for any association, which could be used to predict patients who are more likely to form future VLUs. Efforts are currently being undertaken to identify genes

involved in CVI, which includes analysis of heredity, differential gene expression and novel metabolic pathways involved in primary VV[74]. Current work on the differential gene expression in patients with VV identified that the up-regulation of extracellular matrix components and cytoskeletal proteins involved in CVI is a pathological response to injury and subsequent repair[74, 75]. However, further in-depth mechanistic study is necessary to further validate this in larger patient cohorts with high-throughput genotyping platforms[74].

1.7 Conclusion

In this chapter, I have highlighted the mechanism underlying skin changes in venous disease and the role of Cxs in VLU and its potential as a biomarker of venous disease. There is substantial evidence to support the role of Cx in poor wound healing of VLU as well as its likely overexpression in the early stages of venous disease. However, more work needs to be done to confirm the expression pattern of the principal Cxs in the pre-wounded skin. Cxs have a potential to provide deeper insight on the mechanism of skin changes in venous disease and assist in early identification of patients at risk of future ulceration. Elucidating the pathogenesis with the use of a biomarker can inform clinical practise for a more targeted therapeutic intervention as well as for disease prevention.

Chapter 2

2.0 Methods and Materials

2.1 Study aim

The aim of this study is to determine and compare the principal epidermal GJP levels in venous disease: Connexin 43, Connexin 30 and Connexin 26. We hypothesise that venous disease affects skin and induces an up regulation of the GJPs from the early stages of the disease. This in turn predisposes skin to poor wound healing.

2.2 Objectives of the study

The primary objective is to determine and compare Connexin levels in human epidermis at different stages of venous disease.

The secondary objectives are to determine if Connexin can be used as a biomarker to identify whether patients with venous disease are at risk of poor wound healing. If we could identify Cx as a biomarker for poor wound healing, this may help identifying patients at risk of venous ulceration. Thereby appropriate treatment could be initiated to treat the patients early and prevent venous ulceration.

2.3 Clinical study protocol

2.3.1 Study Design

We designed an observational study that involves taking skin biopsies from patients undergoing routine elective care. The biopsies were taken at the normal surgical incision site. There were no therapeutic interventions, changes to patient care, or patient treatment options.

Brief description of the study design and research participant's journey throughout the study is illustrated in Figure 2.1 (below).

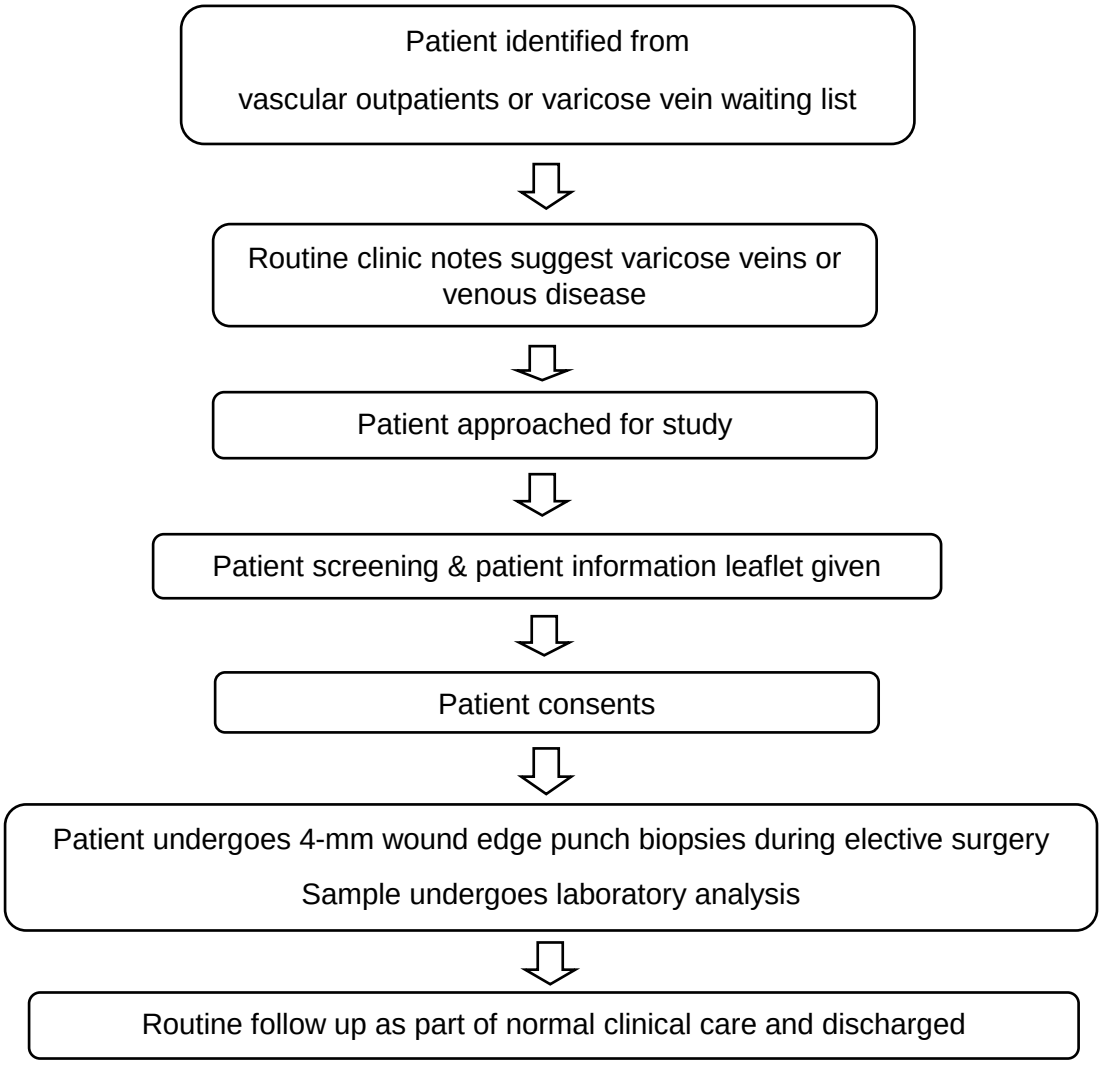


Figure 2.1 Flow chart illustrating patient journey through study

2.3.2 Patient selection

Patients from four main stages of the CEAP classification, namely C0, C2, C4, and C6, were enrolled, with a total of 12 patients in each CEAP class. The total sample size was 48 patients. Patients were eligible for study inclusion if they were aged >18 years and fulfilled the CEAP classification criteria. The inclusion and exclusion criteria are listed in Table 2.1 below.

Inclusion Criteria	Exclusion Criteria
1. Male or female 2. Age 18-90 3. Patients with a venous disease 4. Patients undergoing planned surgery or leg ulcer care	1. Ulcers not due to conditions specified in the inclusion criteria (such as neoplastic or rheumatoid) 2. Presence of arterial disease, connective tissue disorders, systemic inflammatory disorder, diabetic mellitus, cancer and concurrent skin disease 3. Patients known to have a bleeding disorder 4. Previous history of excessive bleeding associated with surgical biopsies or trauma 5. Allergies to local anaesthesia 6. Any other relevant medical concerns causing concern

Table 2.1 Inclusion and Exclusion Criteria for Study Participants

2.3.3 Biopsy acquisition

Paired 4mm punch biopsies of the skin were taken from each patient: one below the knee (15-20cm above the ankle) as the pathological skin and one above the knee (5-10cm above the

knee) as the control skin. Biopsies from the C0 group were taken from patients undergoing total knee replacement surgery while biopsies for the C2 and C4 groups were taken at the endovenous catheter insertion sites during surgery for the treatment of VV. Punch biopsies for the C6 group were performed at the wound edge and 5-10 cm above the knee. The wound edge biopsy was taken at 1mm away from the wound margin to obtain the highest Cx expression, as per our previous protocol[36]. All patients in the C2, C4 and C6 group were scanned with duplex ultrasonography to confirm the presence of venous reflux and screened to exclude mixed arteriovenous disease. Patients from the C0 group were screened to ensure the absence of clinical signs of venous disease.

2.3.4 Ethics

All biopsies were taken after written informed consent was obtained from the patients. This study was executed in accordance with the principles of the Declaration of Helsinki and the recommendations of Good Clinical Practice. Ethical approval was obtained from the National Research Ethics Service Committee London - South East (project ID: 11/LO/1483) and Nanyang Technological University Institutional Review Board (project ID: IRB-2015-05-003) (See Appendix A1). All biopsies were obtained at the University College London Hospital, UK and the Royal Free Hospital London, UK. Preliminary laboratory analysis was performed at the Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, and the final analysis was performed at the University College London, UK under similar laboratory conditions.

2.3.5 Participants Recruitment & Consent

Participants were identified from the vascular outpatient clinic, waiting lists and medical records. Patients were offered participation in the research and given a written patient information sheet (see Appendix A2). This was followed by explanation on the aims, methods,

anticipated benefits and potential hazards of the study. Patients are given sufficient time (offered a minimum of 24 h) to consider whether they wish to participate before written informed consent was obtained (see Appendix A3). All patients were consented and enrolled by myself. A total of 18 months was taken to complete enrolment.

2.3.6 Data collected

Details on patient's demographics, co-morbidities, CEAP classification, wound location, wound size and wound duration were recorded in the wound assessment form (see Appendix A4) prior to biopsy. This data was collected to correlate the clinical information with the histological analysis of the biopsy samples. Clinical photographs were often taken as part of routine care.

2.3.7 Sample size calculation

The sample size was determined through discussion with academic clinical (Professor Toby Richards, Professor Ash Mosahebi) and laboratory supervisors (Professor David Becker). Twelve patients per CEAP class for C0, C2, C4 and C6 were enrolled. The principle factors were pragmatic based on the number of patients that could be screened for inclusion. We considered technical difficulties of obtaining and using the biopsies (such as insufficient biopsies identified at time of microscopy).

2.4 Laboratory study protocol

2.4.1 Biopsy preservation and cryosectioning

All biopsies were fixed overnight in 4% paraformaldehyde, then transferred to 20% sucrose in phosphate buffered saline (PBS), and stored at 4^{o}C until processing. Prior to cryosectioning, tissues were embedded in optimal cutting temperature (OCT) medium (BDH-Poole, UK) and stored at -20^{o}C for 24 h. Frozen sections, 10 µm thick, were obtained using a Leica CM1900 UV cryostat (Leica, Wetzlar, Germany).

A pair of samples from C4, and another from C6, were damaged during the collection process and were not included in the final analysis. Samples included in the final analysis were as follows: C0 (n=12), C2 (n=12), C4 (n=11) and C6 (n=11).

2.4.2 Haematoxylin and eosin (H&E) staining

Cryosectioned slides were removed from -20^{o}C freezer and placed in slide rack and left at room temperature (RT) for 1 min to allow the tissue to thaw and immediately immersed in Acetone for 10 min at -20 ^{o}C to prevent drying. The slides were the placed in xylene for 1-2 min and rehydrated by placing in 95% ethanol followed by 70% ethanol for 1 min each at RT and washed with purified water. Slides were then stained with Hematoxylin solution for 2 min and washed in purified water for 1 min. Slides were then washed with Scotts Tap Water for 1 min and then washed with purified water for another minute. Slides were then dehydrated by placing in 70% and 90% ethanol for 1 min each before counter-staining with Eosin for 30 sec. Slides were then placed in 95% ethanol and 100% alcohol for 1 min and placed in xylene for 5 min before left to air dry for 5 min in a hood. Mounting was then performed using DPX mounting medium and allowed to dry overnight in the hood before imaging.

2.4.3 Slide scanner

Imaging for H&E was performed using a Zeiss AxioScan Z1 slide scanner at 20x magnification at the Division of Biosciences with the assistance of Mr Christopher Thrasivoulou.

2.4.4 Histological analysis

The average epidermal thickness was calculated by dividing the epidermal cross-sectional area by the average epidermal length. Measurements were performed using ImageJ (http://imagej.nih.gov/ij/).

The number of epidermal rete ridges per millimetre were calculated using a selected section (1mm) of the epidermis that best represented the skin section. The average depth of the rete ridge was calculated by dividing the depth of each rete ridge along the selected area by the total number of rete ridges. The epidermal rete ridge depth was defined as the distance between the upper pole of stratum corneum and the rete ridge trough (Figure 2.2).

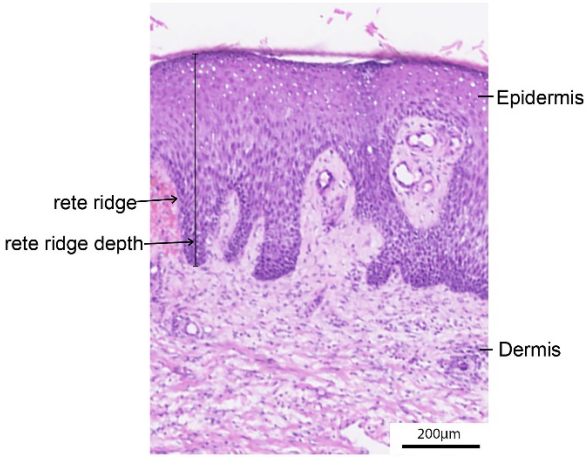


Figure 2.2 H&E section of a skin illustrating rete ridge depth measurement

2.4.5 Immunohistochemistry

Tissue sections were thawed, immersed in PBS to dissolve excess OCT, permeabilized for 15 min in 0.2% Triton X-100 and blocked using 0.1M lysine-PBS for 30 min to block non-

specific binding. Primary antibodies were prepared in PBS: anti-Cx43 (1:4000; C6219, Sigma - Poole, UK), anti-Cx26 (1:200; 10202093, Fisher Scientific, UK), and anti-Cx30 (1:200; 10795723, Fisher Scientific, UK). The tissues stained for Cx43 were incubated with the primary antibody for 1h at RT, while tissues stained for Cx30 and Cx26 were incubated with the primary antibody overnight at 4^{o}C. For negative controls, the primary antibody was omitted from the preparation. The tissues were washed with PBS for 3 x 5 min and stained with secondary antibody (Alexa Fluor 488 goat anti-rabbit, 10729174, 1:400; Fisher Scientific, UK) at RT for 1h. Nuclei were stained using Hoechst (1:10000; 10150888, Fisher Scientific, UK) for 5 min followed by 3 x 5 min PBS washes. Coverslips were mounted using Citifluor (Glycerol/PBS solution, Citifluor Ltd, London, UK) and sealed with nail varnish.

2.4.6 Confocal microscopy

A Leica TCS SP8 confocal microscope (Leica, Mannheim, Germany) (Figure 2.3) was used to obtain 40x images of the epidermis. The 4mm biopsies were examined across their diameter at six locations: Hoescht was excited by a 405nm laser and Alexa Fluor 488 by a 488nm laser. Six images per biopsy were taken to ensure that the staining pattern observed truly represented the distribution of the protein of interest (Figure 2.4). All parameters were kept constant between the patient's control and pathological skin sections to allow direct comparison.

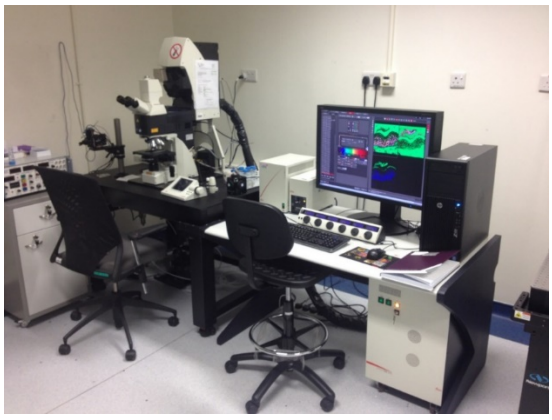
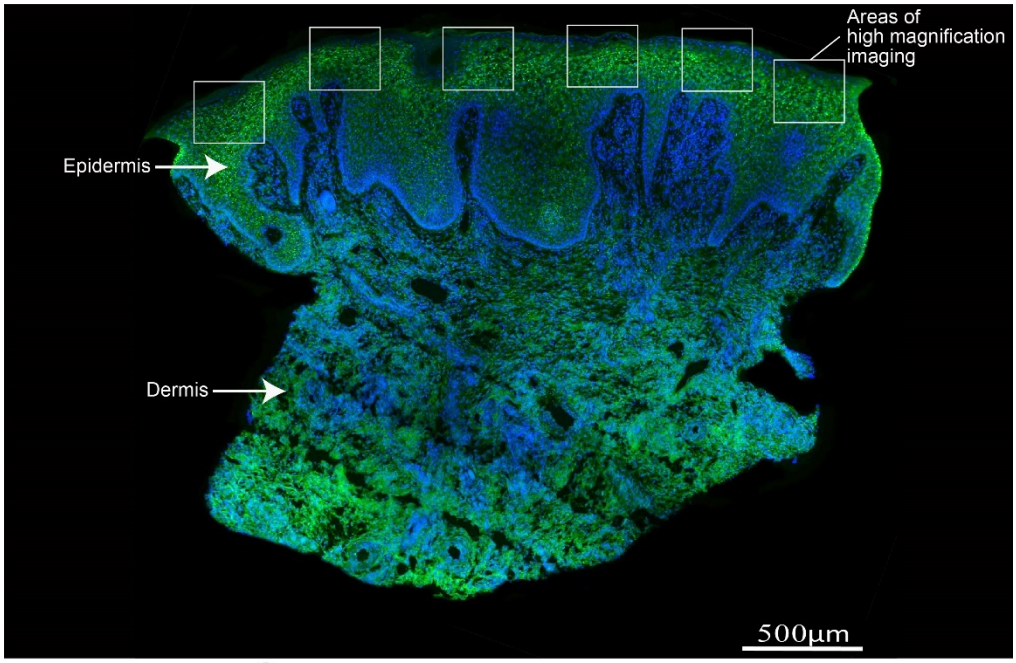
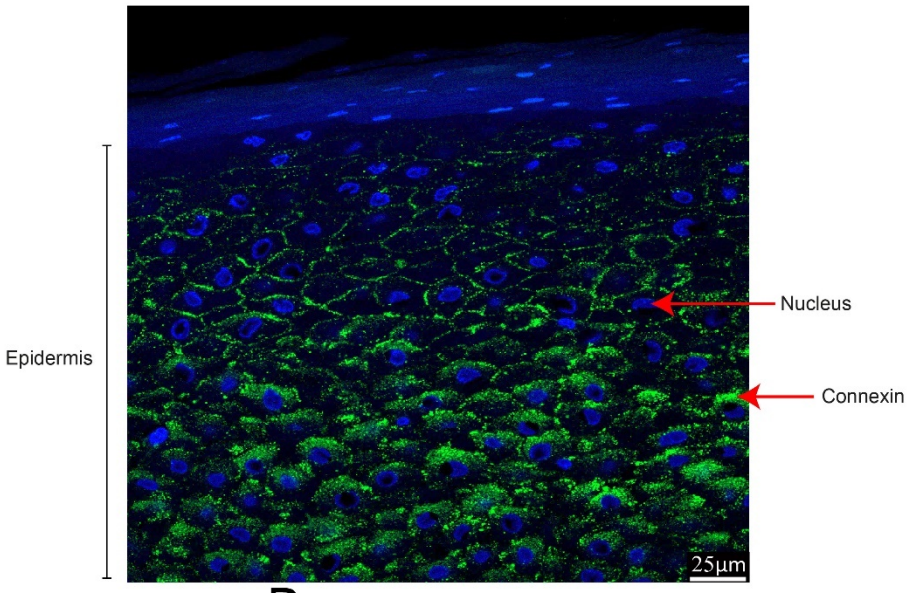


Figure 2.3 Leica TCS SP8 Confocal Microscope used for imaging



A Confocal image of an entire skin section



B High magnification image

Figure 2.4 Confocal image of the epidermis

(A) Confocal image of an entire skin section. High magnification images were taken at six random locations (as represented by the white boxes) along the epidermis to quantify the absolute Cx expression. Scale bar = 500µm. Magnification 10x. (B) High magnification images were used to quantify the Cx levels. Connexins were stained green (excited with 488nm laser) while the nuclei were stained blue (excited with 405nm laser). Scale = 25µm. Magnification 40x.

2.4.7 Connexin quantification

ImageJ was used for Cx quantification. Each image was converted to binary images (black and white) using an identical threshold. Epidermal threshold was kept constant between all images, being set at 80, with a recognised pixel threshold size of 2-infinity used for all images[36]. Regions of interest were manually marked to selectively include the epidermis only, excluding any areas of auto-fluorescence in the stratum corneum.

The Cx levels from the six confocal images from each tissue section were used to quantify the mean Cx expression. This data was presented as 'absolute connexin expression', which was used for statistical analysis and presented in graphs. The corresponding fold-increase data, comparing the Cx expression in the pathological and control skin, was presented in a table as 'mean fold increase'. This was based on each individual's fold difference between the pathological skin section to their matched control, following which the mean fold difference for each group was calculated.

Mean Cx expression per cell was calculated by the ratio of the overall Cx expression to the corresponding number of nuclei present in each tissue section. The average Cx expression per cell was compared between groups.

2.4.8 Statistical analysis

All data were presented as mean ± standard deviation. Statistical differences were determined using paired t-test for paired group and independent t-test for two unpaired groups. For more than two groups, one-way analysis of variance (ANOVA) test, followed by post-hoc Bonferroni test for multiple comparisons, was applied. The relationship between the Cx protein expression in the pathological and control skin was tested by Pearson correlation. Significance was taken at values $p<0.05$. Normality testing was performed using Shapiro-Wilk test; the Cx expression was normally distributed in each class. All statistical analyses were performed using IBM SPSS Statistics 22 software.

Chapter 3

3.0 An observational study of Connexin protein expression in patients with venous disease

3.1 Chapter summary

We investigated the expression pattern of GJP, Connexin (Cx), a known biomarker of poor wound healing, across stages of venous disease. Patients undergoing intervention for VV were assessed according to CEAP classification: C0(n=12), C2(n=12), C4(n=12), and C6(n=12). Paired 4mm punch biopsies were taken from above the ankle (pathological) and above the knee (control). Tissues were stained for H&E, Cx43, Cx30, and Cx26. The pathological skin revealed progressive epithelial hyper-thickening, increase in the number and depth of rete ridges, increased inflammation and loss of dermal architecture with disease progression from C4 onwards. The overall absolute Cx expression and mean Cx expression per cell in the pathological skin similarly increased across the CEAP from as early as C2. Increasing levels of Cx in the control skin was also seen, indicating the progression of the disease proximally. Elevated Cx43 expression had the strongest positive correlation between the pathological and control skin. Cxs were overexpressed as early as C2, suggesting that skin is preconditioned by VV for poor wound healing. The stepwise sequential increase in Cx43, prior to histological changes, suggest that it is a sensitive biomarker for poor wound healing and ulceration. Our finding suggests that VV predisposes skin to poor wound healing, indicating a need for early surgical intervention to prevent ulceration.

3.2 Aim

This chapter aims to observe the expression pattern of the principal epidermal Cx proteins across the CEAP classification in patients with venous disease to better understand the early skin changes and the expression of these proteins in the pre-wounded skin.

As described in chapter two, patients undergoing intervention for VV were assessed according to CEAP classification: C0(n=12), C2(n=12), C4(n=12), and C6(n=12). Paired 4mm punch biopsies were taken from above the ankle (pathological) and above the knee (control). Tissues were stained for H&E, Cx43, Cx30, and Cx26.

3.3 Results

3.3.1 Patient demography

A total of forty-eight patients were enrolled into this study. The average age was 59.2 ± 17.5 years (range: 32-89 years). The demographic data is summarised in Table 3.1 below.

Overall mean age (years)	**66.1 ± 21.1 (range: 32-89 years)**
Mean age per class (years)	
CO	63.6 ± 11.5
C2	44.4 ± 9.0
C4	57.1 ± 13.1
C6	77.3 ± 10.1
Overall Gender	
Male	22 (45.8%)
Female	26 (54.1%)

Table 3.1 Patient demography

Table shows the overall mean age, the mean age for patients in each CEAP class, and the overall gender breakdown. Values represent mean ± standard deviation.

3.3.2 Histological features of skin with disease progression

The histology of the pathological skin revealed distinct and consistent features within each CEAP class. A progressive change in structure is seen with disease severity: progressive epithelial hyper thickening, increase in the depth and number of epidermal rete ridges, increase in inflammatory cells, and loss of dermal architecture in the upper dermis (Figure 3.1, next page). The most prominent change observed was the increase of the epithelial thickness at C6. The number of rete ridges per millimetre of the epidermis was, however, significantly increased in the pathological skin as early as C2 and the depth was significantly increased from C4 onwards. This was accompanied by the loss of dermal architecture.

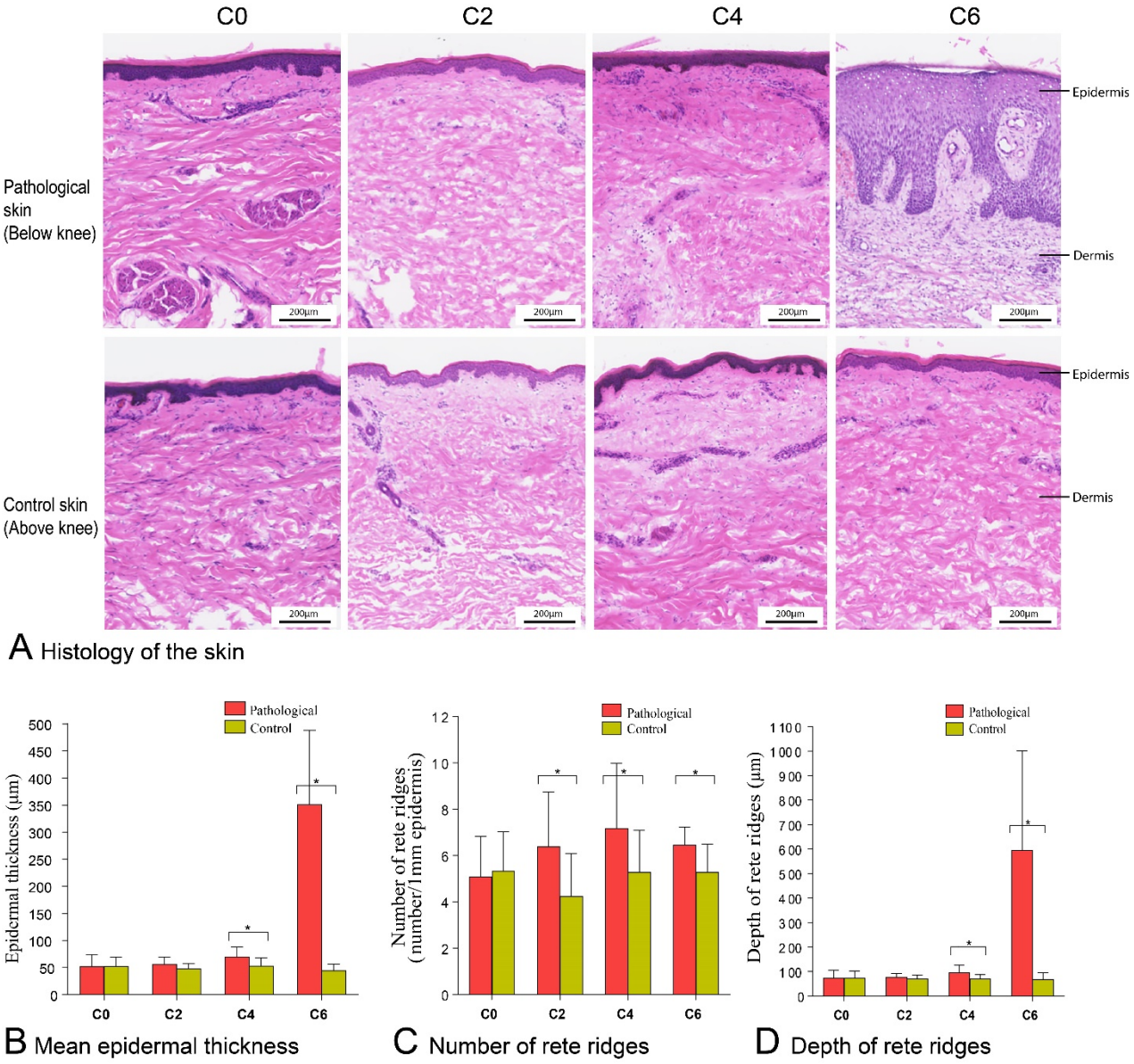


Figure 3.1 Epithelial thickness

(A) Haematoxylin and eosin-stained section of the skin section for each CEAP class. The mean epithelial thickness of each CEAP class is indicated at the bottom of the image. Scale bar = 200μm. Magnification 20x. Bar charts show the (B) mean epithelial thickness, (C) number of epidermal rete ridges, and (D) depth of epidermal rete ridges in each CEAP class. Values represent mean ± standard deviation. *P<0.05 (paired t-test)

3.3.3 Epidermal Cx proteins overexpression

The overall absolute Cx expression for Cx43, Cx30 and Cx26 in the pathological skin were similarly increased across the CEAP class (Figure 3.2, Figure 3.3, Figure 3.4). The overexpression of the Cxs in C6 has been previously described [36]. Interestingly, here we note that the principal epidermal Cxs were significantly overexpressed as early as C2 and C4. No significant overexpression was noted at C0. Cx43 had the highest expression in each class. Cx30 had lesser expression in C0, C2 and C4 but increased significantly in C6, as did Cx26.

The mean Cx expression per cell in the epidermis corresponds to the trend of the absolute Cx expression across the CEAP class. A significant overexpression of the mean Cx per cell was observed as early as C2 for all three Cxs. No significant difference was noted in the Cx expression per cell between the control and pathological skin in C0.

An increasing trend of Cx expression was also observed in the control skin across the CEAP class, suggesting the progression of the disease proximally. A significant increase of Cx43 expression in the control skin was seen from C4 onwards: C4 vs C0 ($p<0.001$) and C6 vs C0 ($p<0.001$). No significant difference was noted between C4 vs C6. For Cx30, significant difference was only observed between C4 vs C0 ($p=0.003$); while for Cx26, significant difference was only observed between C6 vs C0 ($p<0.001$).

Compared to the control skin, Cx proteins were overexpressed multiple fold higher in the pathological skin (Table 3.2). Cx 26 and Cx30 had a greater mean fold increase compared to Cx43 as they were expressed at relatively lower levels in the control skin at each CEAP class. There was a striking and significant 431-fold and 38-fold increase in Cx30 and Cx26 at C6. In contrast, Cx43 was elevated by an average of 6-fold at C6.

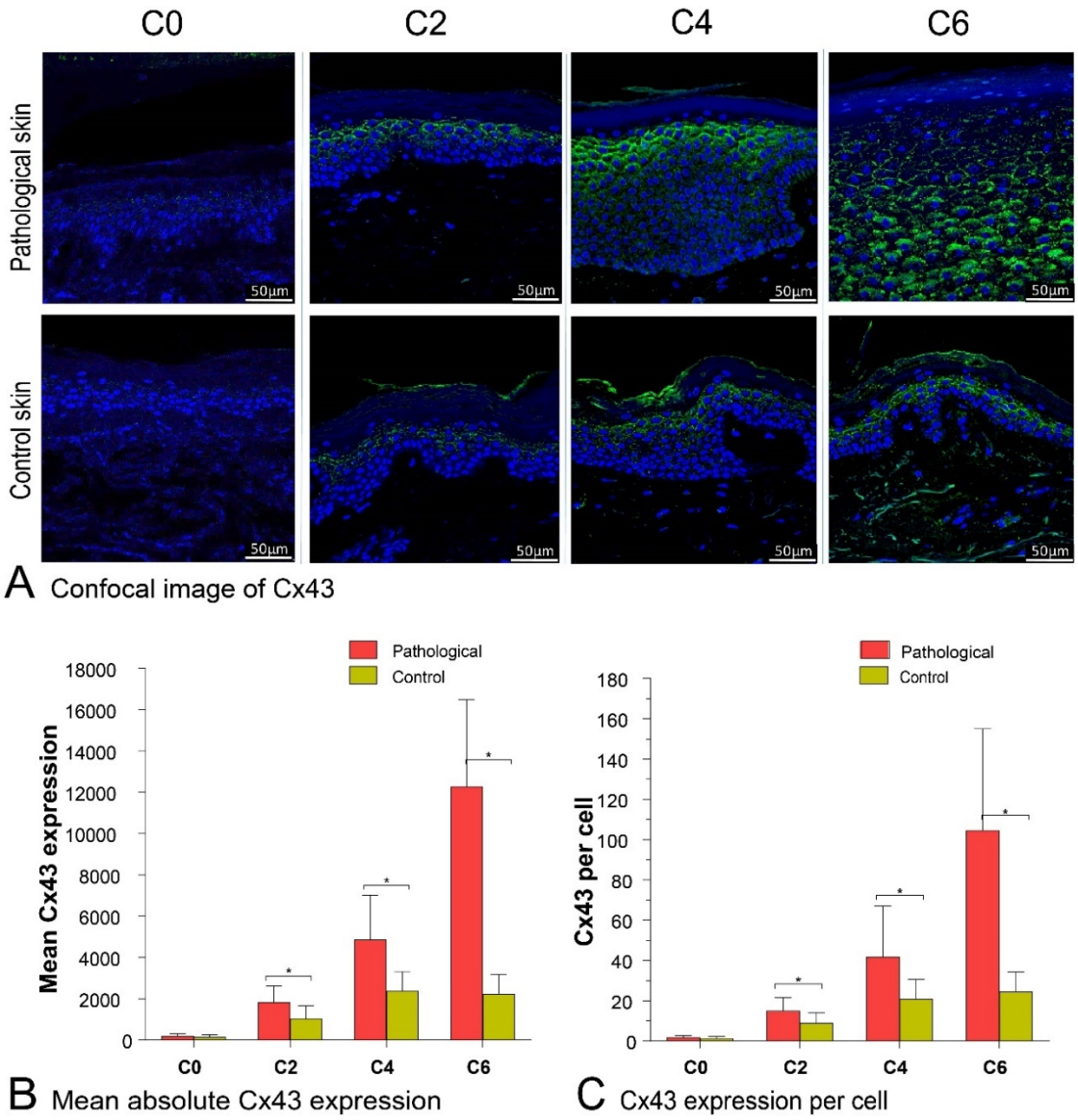


Figure 3.2 Connexin 43 expression across the CEAP classification

(A) Confocal images of Cx43 expression in each group. Scale bar = 50µm. Magnification 40x. (B) Mean absolute Cx expression. (C) Mean Cx expression per cell for each CEAP class. Values expressed as mean ± standard deviation. *P<0.05 (paired t-test)

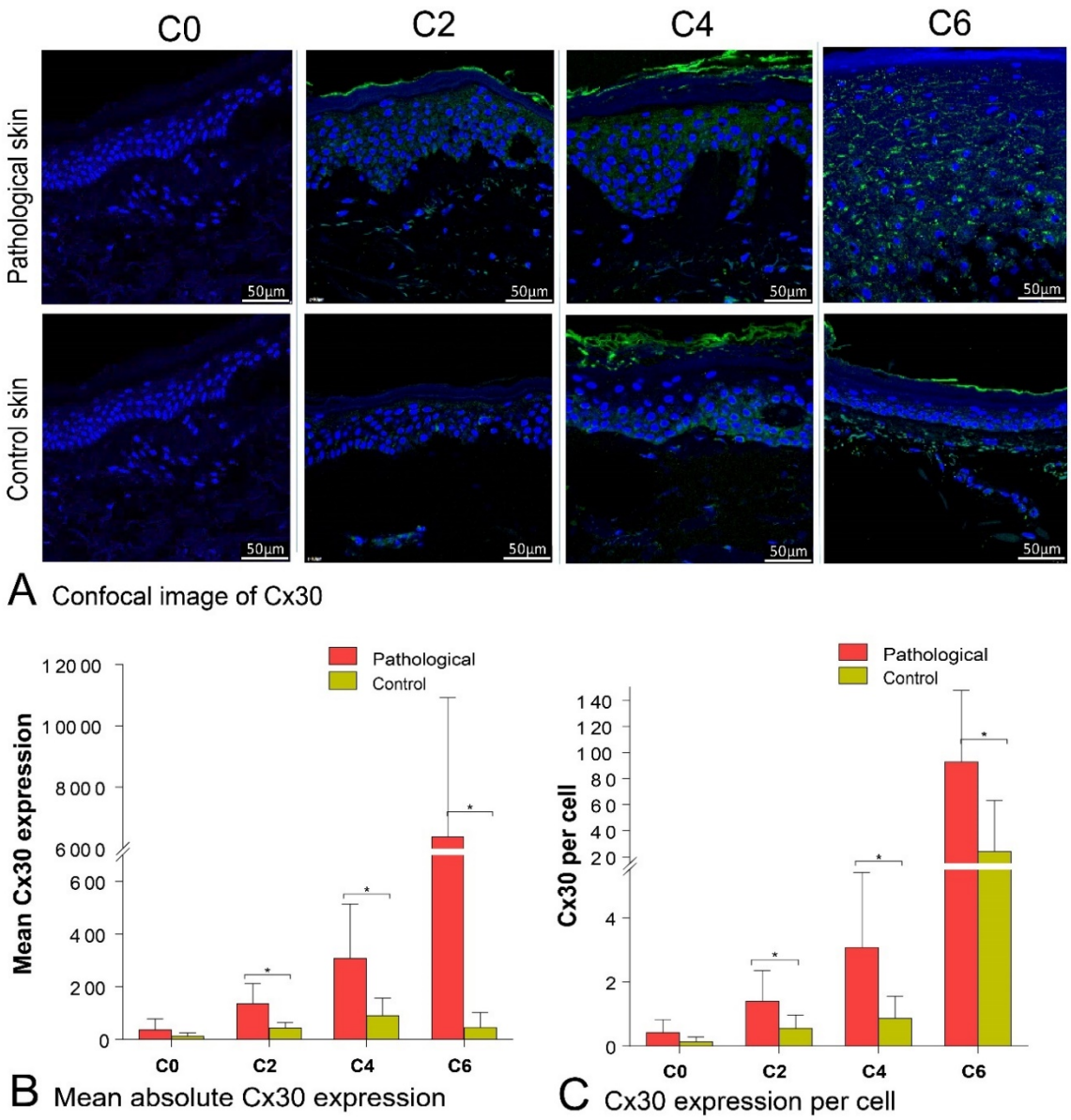


Figure 3.3 Connexin 30 expression across the CEAP classification

(A) Confocal images of Cx30 expression in each group. Scale bar = 50µm. Magnification 40x. (B) Mean absolute Cx expression. (C) Mean Cx expression per cell for each CEAP class. Values expressed as mean ± standard deviation. *P<0.05 (paired t-test)

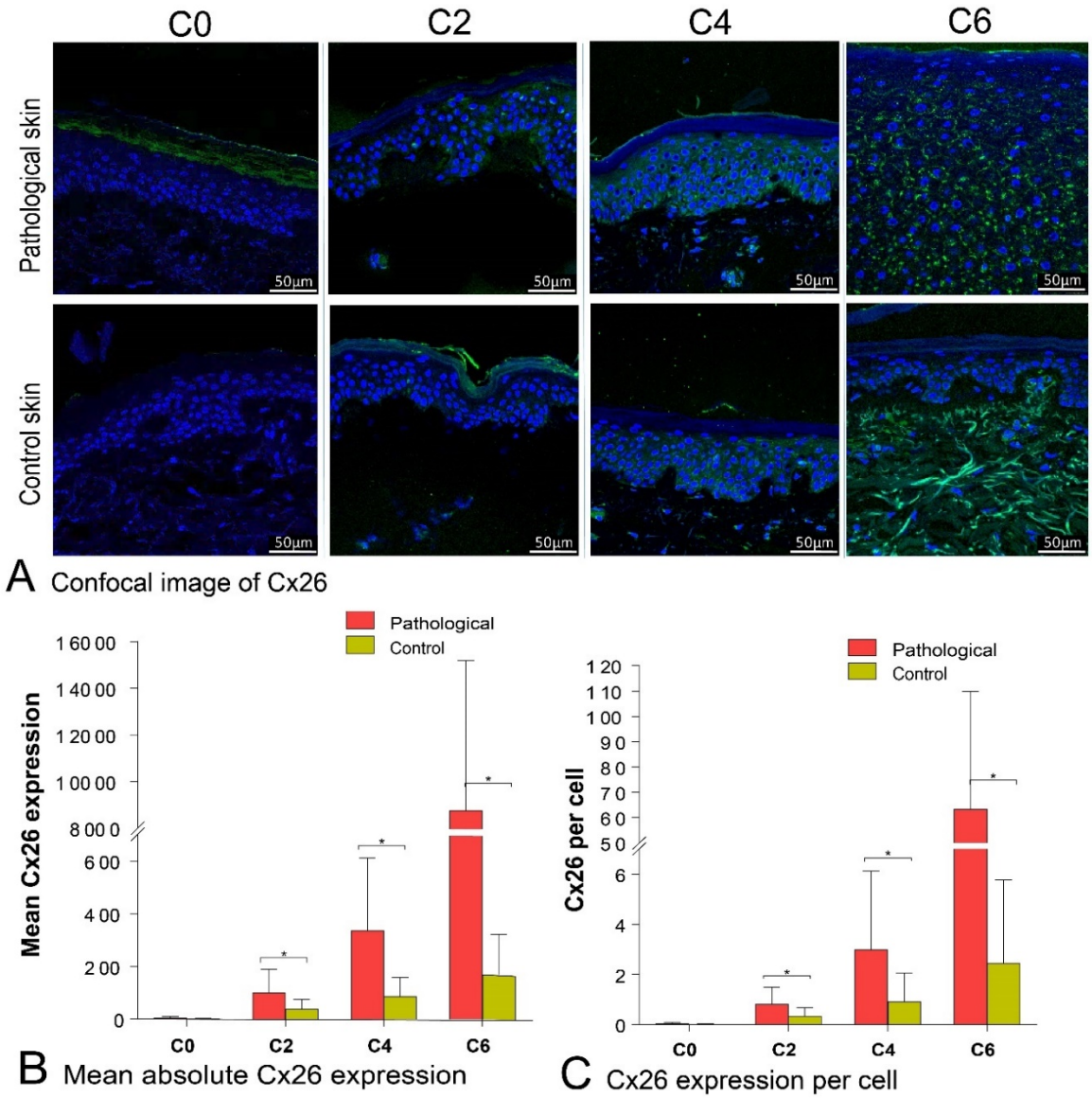


Figure 3.4 Connexin 26 expression across the CEAP classification

(A) Confocal images of Cx26 expression in each group. Scale bar = 50µm. Magnification 40x. (B) Mean absolute Cx expression. (C) Mean Cx expression per cell for each CEAP class. Values expressed as mean ± standard deviation. *P<0.05 (paired t-test)

	C0	C2	C4	C6
Connexin 43	2.03 ± 2.04	2.06 ± 0.76	2.12 ± 0.72	6.52 ± 3.66
Connexin 30	3.03 ± 1.50	3.50 ± 2.24	4.92 ± 4.72	431.80 ± 614.74
Connexin 26	2.27 ± 1.94	2.04 ± 2.85	0.80 ± 3.39	38.14 ± 55.48

Table 3.2 Mean fold increase of the Cx proteins in the pathological skin compared to control skin

Table shows the mean fold increase of Cx43, Cx30 and Cx26 expression in the pathological skin compared to control skin in each CEAP class. Compared to the control skin, the Cx proteins were overexpressed multiple fold higher in the pathological skin. Cx 26 and Cx30 had a greater mean fold increase compared to Cx43 as they were expressed at relatively lower levels in the control skin at each CEAP class. Values represent mean ± standard deviation, corrected to second decimal place.

3.3.4 Distribution pattern of Cx proteins with disease progression

Cx43 was generally expressed in all layers of the epidermis with the highest intensity in the stratum spinosum and lowest intensity in the stratum basale (Figure 3.2, above). The expression pattern changed with disease progression; in C2 and control, the highest expression was seen along the upper portion of the stratum spinosum, in C4, Cx43 was expressed further down the stratum spinosum, approaching the stratum basale, and in C6, Cx43 was expressed throughout the epidermis, producing a “fish scale” pattern.

Similar to Cx43, Cx30 was expressed throughout the epidermis in C6 (Figure 3.3, above). The expression of Cx30 in C0, C2, C4, and control skin was, however, very weak and sporadic. Although expressed with low intensity, it was visible along stratum spinosum and granulosum. Despite no noticeable difference in the distribution pattern in the pre-wounded skin, the intensity was higher in C4. The temporal and spatial expression pattern of Cx26 was similar to Cx30 throughout the four classes (Figure 3.4, above).

3.3.5 Correlation of the Cx protein expression between the pathological and control skin

Compared to the expression pattern of all the Cx proteins, Cx43 had the strongest positive correlation between the expression in the pathological skin and control skin ($r=0.63$, $p=0.001$) (Figure 3.5). This suggests that Cx43 expression increases steadily with the disease progression.

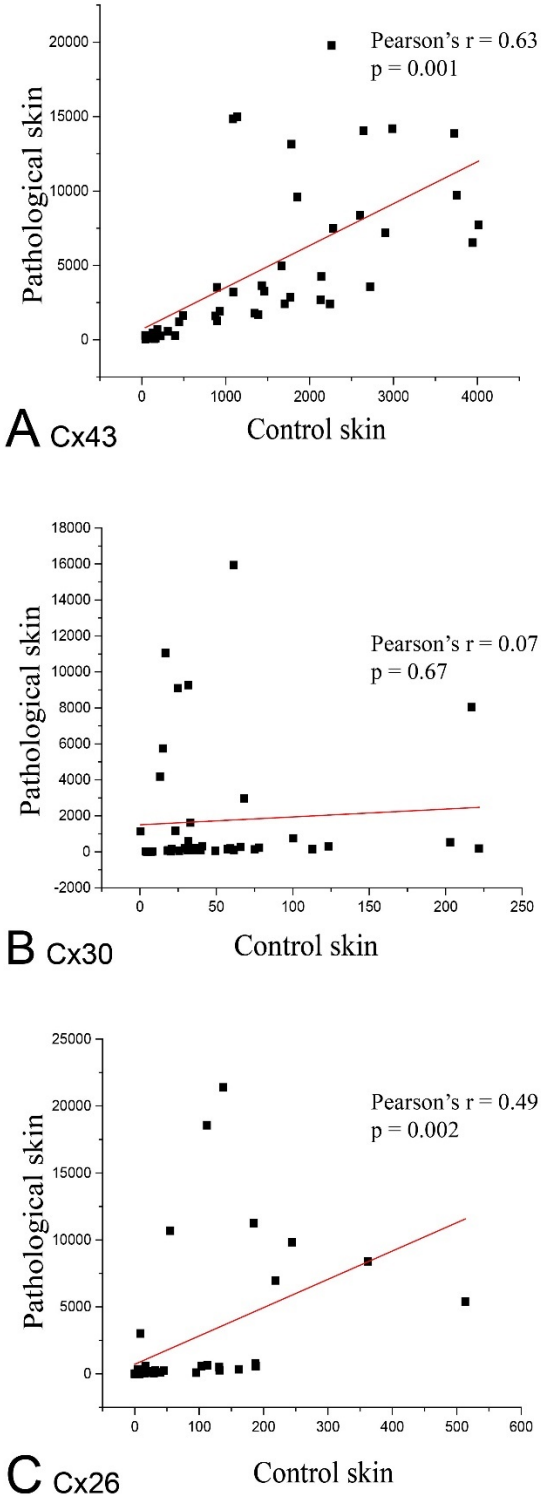


Figure 3.5 Correlation of absolute Cx proteins expression

Pearson's correlation of the absolute Cx proteins expression in the pathological skin versus control skin.

3.4 Discussion

Cx proteins were previously shown to be upregulated in diabetic ulcers, pressure ulcers and VLUs[36]. Here, we sought to understand the expression pattern of the principal epidermal Cx proteins across the stages of venous disease, especially in pre-wounded skin. We demonstrated that there is a stepwise sequential increased expression in the principal epidermal Cx proteins as early as C2. This finding likely suggests that VVs predispose skin to poor wound healing and increase the risk of future ulceration. This is the first time it has been shown that VVs, even as early as C2, are associated with poor wound healing. Additionally, our finding suggests that Cx43 is a sensitive biomarker of venous disease progression. These findings support a conclusion that treating VV early, to improve circulation, could help prevent future ulceration.

Cx43 upregulation in VLUs has been implicated in impaired keratinocyte migration and poor wound healing[34]. The negative effect of the Cx protein overexpression on cellular migration is mediated by both gap-junctional intercellular communication and non-junctional mediated effects. Cx proteins act as nexus interacting with adhesion molecules, tight junctions and cytoskeletal components via the long cytoplasmic C-terminal tail, either directly or via adaptors[34, 44, 45]. An increase of Cx43 by one-fold was shown to halve cellular migration[34]. The striking multiple-fold increase that we observed in C6 could have a profound negative effect on healing. Despite increased absolute Cx levels at C2 and C4, the fold-increases were comparable to that of C0. This is due to the increased Cx levels in the control skin at C2 and C4, signifying the clinical progression of the disease from the distal to proximal part of the lower limb. These skin changes, secondary to venous hypertension, were previously not known to extend proximally as the clinical signs are confined to the medial-distal aspect of the lower limb. The Cx upregulation identified here suggests that skin is preconditioned to poor wound healing and this extend proximally with disease progression. This finding advances our understanding on the pattern of Cx overexpression, which, in the context of VLUs, was previously thought only to be a feature of wound chronicity.

Cx30 and Cx26 were previously only known to be overexpressed at the wound edge and hyperproliferative skin disease. The persistent Cx26 overexpression maintains a hyper-proliferative state, slowing down healing, stalling the transition to the remodelling stage, and leads to immune cell infiltration[76]. We found that Cx30 and Cx26 in the pre-wounded skin were expressed in low levels, but were significantly overexpressed after wounding. The observed upregulation at C2 and C4, which were also related to epidermal hyper-thickening, suggests that the overexpression takes place prior to wounding, contributing to the chronicity of non-healing VLUs.

We observed early histological changes at C2 and C4. The increase in the number and depth of the rete ridges indicates that perfusion of the epidermis is compromised secondary to the recurrent ischaemia-reperfusion cycle; a consequence of venous hypertension. The avascular epidermis is entirely dependent on the highly-vascularised dermis for perfusion. The hypoperfusion in the superficial vessels (nutritive vessels) which happens concurrently with hyperperfusion in the deeper vessels (shunt vessels) stimulates the epidermis to project further into the dermis for perfusion[77]. The increase in epidermal thickness and worsening hypoperfusion could ultimately result in skin breakdown at C6; a consequence of imbalance between supply and demand.

The chronic inflammation seen in C4 has been previously reported by several studies, which documented the presence of inflammatory cells in the skin of patients with lipodermatosclerosis and venous ulcer [12]. The exact mechanism that triggers this chronic inflammation remains unclear, however, it had been hypothesised to occur due to leukocyte-trapping and neutrophil activation secondary to ischaemia-reperfusion cycles, a consequence of venous hypertension [4, 12]. This also leads to leukocyte sequestration, and upon reperfusion as seen with leg elevation, the leukocytes are activated and release reactive species causing further oxidative damage to the ischemic tissue[78, 79]. This cycles could lead to hypoxia though it is not known if prolonged hypoxia is the trigger of this sterile inflammation.

This study has several limitations. Ultrasound duplex assessment was not performed for the patients in the C0 group although the prevalence of venous reflux in the general population is estimated to be about 20 percent[80]. Patients were, however, clinically assessed to ensure absence of signs of venous disease. In patients with leg ulceration (C6), some (segmental) deep venous reflux was seen in 4 of 12 patients. However, there was no difference in the distribution pattern or expression intensity of Cx observed within these patients. Additionally, a formal sample calculation was not performed as the difference in Cx expression between the CEAP classes was previously not known, and this is the first time that it has been established that Cxs were overexpressed prior to wounding.

3.5 Conclusion

This is the first study to show that the presence of VV is associated with elevated Cx levels, likely suggesting that skin is preconditioned for poor wound healing prior to ulceration. The cellular and structural changes correlate with the clinical stage of the disease. Our data suggests that treating VVs early could prevent future ulceration.

Part 2

Summary of Part 2

In this part, I have used GJP to explore the mechanism of healing of a novel technology in wound healing involving epidermal grafting. In order to do this, I have first conducted a systematic review using Cochrane methodology and a descriptive review on the mechanism of wound healing by epidermal graft. I then performed a pilot case series to evaluate the feasibility of using this technology in the outpatient setting, which was followed by a cost analysis study and patient reported outcome measure. Combining all these data, I then designed a randomised controlled trial (RCT) with a translational component to evaluate the efficacy and mechanism of healing of this technology against the current standard of care. Within this RCT, I have employed GJP to explore the mechanism of action of epidermal grafts.

Chapter 4

4.0 Systematic review and mechanistic review of epidermal grafting for wound healing

4.1 Systematic review of epidermal grafting for wound healing

4.1.1 Chapter summary

Epidermal grafting (EG) enables epidermal transfer to the wound with minimal donor site morbidity. However, data to date has been heterogeneous. This study aims to synthesise the current evidence on EG for wound healing to establish the efficacy of this surgical technique. A comprehensive search in the MEDLINE, EMBASE and CENTRAL database was conducted from 1946 until November 2015. The endpoints assessed were; proportion of wounds healed and mean wound healing time. This systematic review was conducted and reported according to the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) guidelines. We identified 1088 articles, of which 6 articles were included in this review; a total of 126 wounds in 107 patients. The mean wound duration was 48.39 weeks (95 percent c.i. 3.35 to 69.88). Of these, 57.0 percent (95 percent c.i. 36.0 to 76.7) of the wounds achieved complete healing. Mean time for complete wound healing was 4.87 weeks (95 percent c.i. 2.57 to 9.27). The mean donor site healing time was 7.25 days (95 percent c.i. 4.9 to 12.54), with no reported donor site morbidity. The current data are small and lacks level 1 evidence.

4.1.2 Introduction

Wound care presents a significant financial and resource burden to the healthcare system, reported to account for over 5 percent of National Health Service (NHS) expenditure. Between £2·3–£3·1 billion is spent in caring for patients with chronic wounds in the United Kingdom alone [9]. Chronic wounds account for a burdening problem with over 100,000 new ulcers anticipated every year, with an ageing population and rising prevalence of obesity and diabetes.

In most cases management is conservative, by wound care and dressings. Intervention by autologous skin grafting is an important modality for wound coverage [35]. Skin grafting can be classified based on the thickness of the harvested skin (Figure 4.1), namely, full thickness skin graft (FTSG), split thickness skin graft (SSG) and epidermal graft (EG) [81, 82].

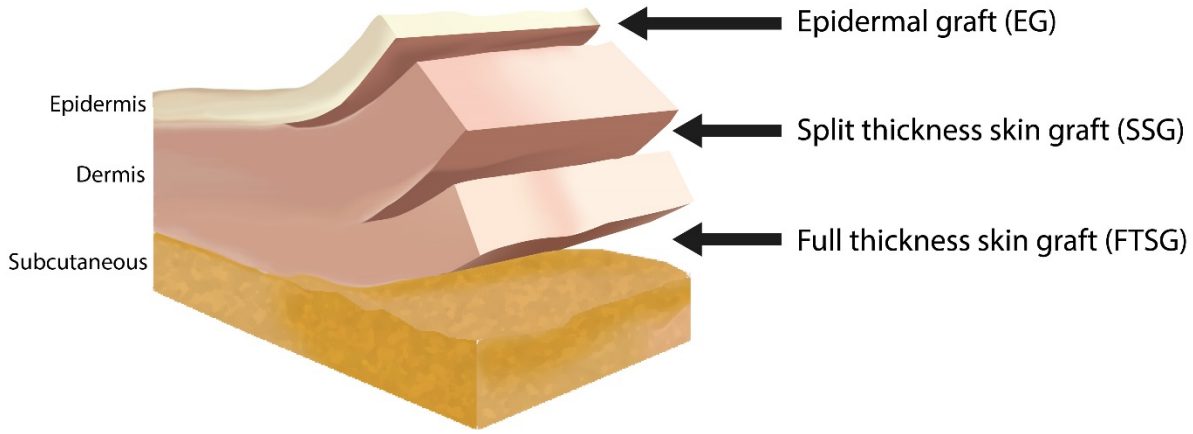


Figure 4.1 Illustration of the skin layers involved in autologous skin graft harvest.

FTSG consists of the epidermis and the entire dermis of the skin. FTSG is normally reserved for smaller wounds as the donor site must be closed primarily. Thus, only selected areas with sufficient skin laxity is suitable for skin harvest, commonly the retroauricular area, cervicopectoral area, and groin [35]. Larger areas can be managed by SSG which involves shaving the epidermis and part of the dermis of the skin. This is best performed by an electric

air dermatome and the donor site regenerates by secondary intention from the residual reticular dermis [35]. SSG is the commonest form of autologous skin grafting performed and can be meshed to cover a wide surface area. A major consideration for SSG is that the donor site may itself develop as a second, often painful wound, which may take more time to heal than the graft site itself and holds the risk of infection and scarring [83]. Both the FTSG and SSG often require hospital admission, even as a day case, anaesthesia, and a period of immobility for some patients.

EG, on the other hand, is an emerging and promising option. EG involves harvesting only the epidermal layer of the skin from the donor site by applying continuous negative pressure on the normal skin to raise blisters. The roof of the blister, which is the epidermis, is then excised and transferred onto the wound. As the dermis in the donor site remains untouched, the skin regenerates itself without scar. This procedure is also often painless as the pain fibres in the dermis are unstimulated, allowing autologous skin grafting in the outpatient setting without administration of anaesthesia and with minimal donor site morbidity [84].

The use of EG for wound healing has been on the rise of late, with several recent publications in the last couple of years. However, data to date has been heterogeneous on the outcome and on when and in which patient group this surgical technique should be employed. This systematic review synthesises the current evidence on EG for wound healing to establish the efficacy of this technique in the clinical setting, by measuring the proportion of wounds healed and the mean wound healing time. It is timely that the evidence is assessed to guide clinical decision making and to further facilitate future research.

4.1.3 Materials and methods

The protocol for this systematic review was registered with the PROSPERO international prospective registration of systematic reviews (registration number: CRD42016033051) (see Appendix B1), and a detailed protocol was peer reviewed and published [85]. There was no deviation from the published protocol. This systematic review was conducted and reported according to the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) guidelines [86].

4.1.3.1 Search strategies

We searched the MEDLINE (OvidSP), EMBASE (OvidSP), and Cochrane Central Register of Controlled Trials (CENTRAL) database from 1946 until November 2015 to identify studies of relevance to this review. The search strategy included a combination of text words and Medical Subject Headings (MeSH) terms relating to the use of EG for treating wounds. No language or publication restrictions were applied. The reference list of all articles included were cross-checked for further articles of relevance. A sample search strategy for MEDLINE (OvidSP) is shown and similar strategy was adapted for other databases.

1. [epidermal graft*] OR [blister graft*] OR [suction blister*] OR [suction graft*]
2. Epidermis/su, tr [Surgery, Transplantation]
3. [1] or [2]

4.1.3.2 Inclusion criteria

The inclusion criteria used were: (i) studies involving adult patients above 18 years old; (ii) EG for wound healing; (iii) English language; (iv) available information containing at least the following: number of subjects, method of EG harvest, and healing time.

4.1.3.3 Exclusion criteria

The exclusion criteria were: case reports or case series of lesser than five patients; studies describing the use of EG in skin pigmentation disorder such as vitiligo; studies combining EG with other treatments such as stem cells or dermal substitutes; and studies describing only the harvest technique without treatment outcome.

4.1.3.4 Outcome measures

The primary efficacy outcome measures were the proportion of wounds healed and the mean wound healing time (time for complete healing). Secondary outcome measures were the mean donor site healing time, need for anaesthesia, economic evaluation based on the cost associated with resources used, health-related quality of life, and proportion of patients with adverse event. Subgroup analysis was performed for the proportion of wounds with complete healing based on the wound aetiology.

4.1.3.5 Study selection

The retrieved articles' titles and abstracts were scanned for potential eligibility, using the predetermined selection criteria, after excluding duplicate records. Full-text review was undertaken for studies that met the inclusion criteria. Abstracts and conference proceeding without full text were not included because of the difficulty in evaluating incomplete information. Ongoing trials without complete data were not included.

4.1.3.6 Data extraction

Data from all full-text articles accepted for final analysis were independently retrieved by two authors (Mr Oliver Smith and myself) using a standardised data extraction form. Discrepancies were resolved by discussion. The following data were extracted: study characteristics (first author, year of publication, country, study design), patient demography (number of studied subjects, sex, mean age, comorbidity, number of wounds treated), wound characteristics (wound aetiology, mean wound duration, mean wound size, pre-grafting wound quality), characteristics of EG harvest technique, use of anaesthesia, outcomes (wound healing time, number and type of wounds with 100 percent healing, number of wounds with 50-99 percent healing, number and type of wounds failed to heal, donor site healing time), and complications or adverse events. Data were extracted from the studies as presented or were calculated (e.g.: mean age and mean wound size).

4.1.3.7 Assessment of risk of bias of included studies

A formal risk of bias assessment was not performed as the included studies were mostly small case series.

4.1.3.8 Data analysis and synthesis

The main outcome measures of the included studies were the pooled estimate of the proportions of wounds healed, the mean wound healing time, and the mean donor site healing time with the corresponding 95 percent confidence intervals (c.i.). Meta-analysis of proportion was performed for the proportion of wounds healed. Meta-analysis of summary was performed for the mean wound and donor site healing time, mean wound size, mean wound duration and mean age of the patients. The clinical and methodological heterogeneity were assessed. Random effects model was used for the pooled estimates as the included studies demonstrated high clinical and statistical heterogeneity[87]. The outcomes were analysed

using StatsDirect Statistical software (StatsDirect statistical software, version 2.8.0; StatsDirect, Altrincham, UK).

4.1.4 Results

4.1.4.1 Literature search results

We found 1088 articles in the MEDLINE database search, 946 articles in the EMBASE database search, and 271 articles in the CENTRAL database search. References from these three searches were combined and after removing the duplicates, 1541 articles were available for title and abstract reviewing. Of these, 1373 articles did not meet the inclusion criteria and were excluded. Following full text review of the remaining 168 articles, 162 articles were excluded as the inclusion criteria was not met. A total of 6 articles were included and formed the basis of this systematic review [88-93] (Figure 4.2). Cross-checking of the reference list revealed that no article was missed by the initial search. Details of the included studies are summarised in Table 4.1.

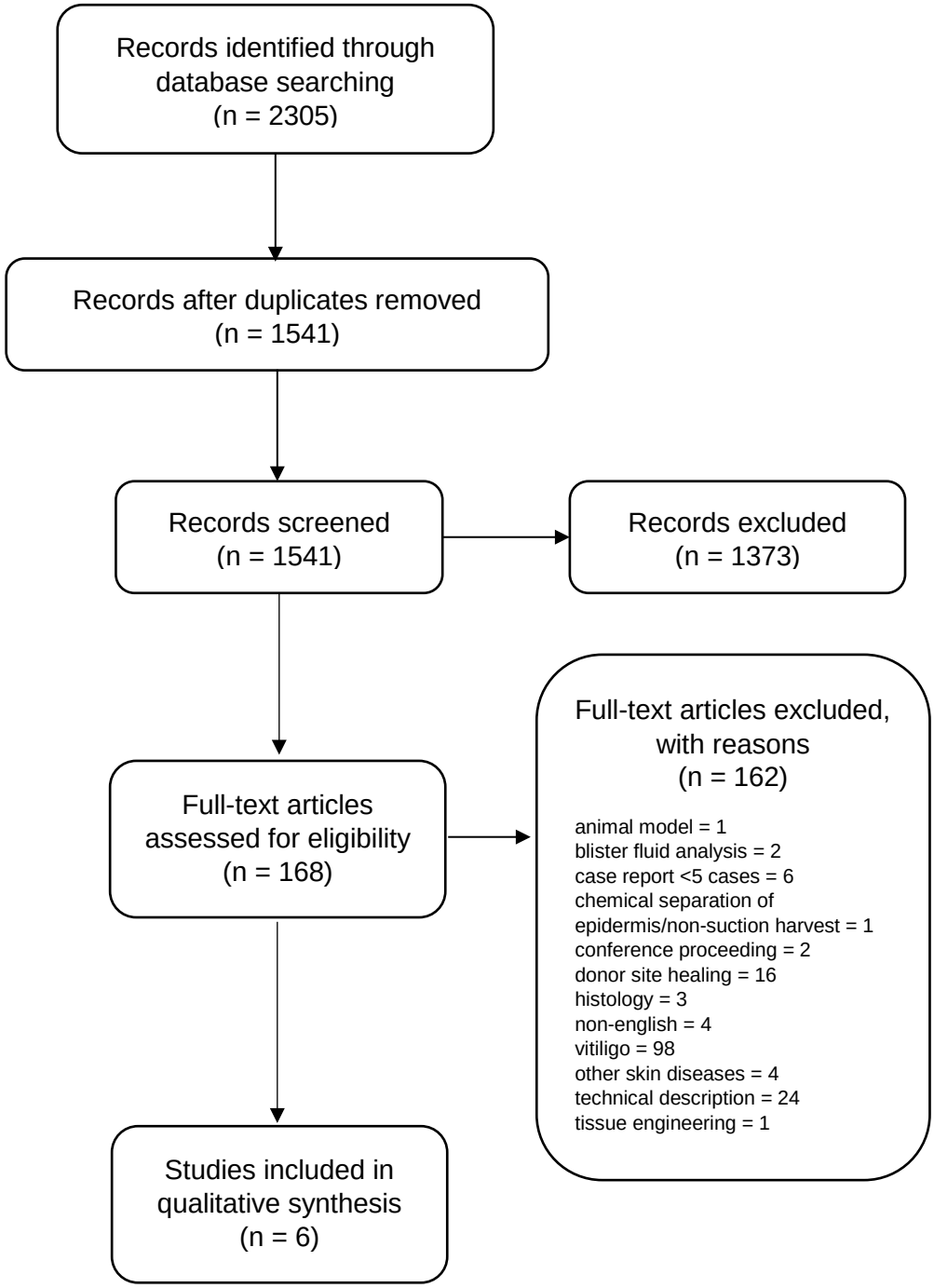


Figure 4.2 Flow diagram of literature search

Citation	Hentzer B et al, 1975[90]	Costanzo U et al, 2006[88]	Hanafusa T et al, 2008[89]	Gabriel A et al, 2014[91]	Richmond NA et al, 2014[92]	Serena T et al, 2015[93]
Year	1975	2006	2008	2014	2014	2015
Country	Denmark	Switzerland	Japan	USA	USA	USA
Study type	Case series	Case series	Case series	case series	Case series	Case series
Number of patients	12	18	61	4	5	7
Male	N/R	5	N/R			
Mean age (year)	N/R	76.5	I/R	50.25	50	45.6
Number of wounds	12	29	69	4	5	7
Mean wound duration (week)	N/R	21±178.7	I/R	106.5±178.7	65.6 ± 72.38	105.6 ± 121.67
Average wound size (cm^2)	N/R	6.70 ± 5	27.80 ± 7.10	N/R	9.56 ± 10.03	30.29 ± 16.07
Epidermal graft harvesting technique						
Device	Suction device Dermovac®	Suction device Dermovac®	Syringe	CelluTome	CelluTome	CelluTome
Negative pressure (mmHg)	250-300 mmHg	200-300	N/R	400-500	400-500	400-500
Duration	1-2 h	2 to 3 h	16-128 min	35-45 min	N/R	25-35 min
Use of anaesthesia	No	No	LA in 27 patients	No	No	No
Donor site dressing	N/R	Antiseptic cream and gauze	N/R	N/R	N/R	N/R

Wound site dressing	Gauze + wet dressing with 2% boric acid.	Non-adherent dressing (Sofra-Tulle), gauze, compression bandage	N/R	Gauze + occlusive dressing (n=3), foam + occlusive dressing (n=1)	Absorbent foam dressing and 4-layer compression bandages	Adhesive dressing + 2 layered compression bandage
Wounds with complete healing	10	16	18	3	3	1
Duration for complete healing (week)	2 ± 1.98	3.6 ± 1.98	8.3 ± 0.9	N/R	8 ± 2.94	4
Wounds with 50-99% healing	1	10	N/R	1	2	5
Number of failure	1	3	N/R	0	0	1
Donor site healing	10 days	N/R	N/R	1-2weeks	1 week	3-4days

Legend: N/R=Not reported I/R=Incomplete reporting LA=local anaesthesia

Table 4.1 Overview of the included studies

4.1.4.2 Wound healing outcome

A total of 126 wounds in 107 patients were treated with EG, with the average wound size of 10.98 cm^2 (95 percent c.i. 2.58 to 46.73). The mean age of the patients was 67.53 years (95 percent c.i. 29.75 to 153.31). The mean wound duration of the 45 reported wounds was 48.39 weeks (95 percent c.i. 3.35 to 69.88).

The number of wounds that achieved complete wound healing was reported in five studies involving 57 wounds. The proportion of wounds that achieved complete healing was 57.0 percent (95 percent c.i. 36.0 to 76.7) (Figure 4.3), with the mean time for complete wound healing of 4.87 weeks (95 percent c.i. 2.57 to 9.27) (Figure 4.4).

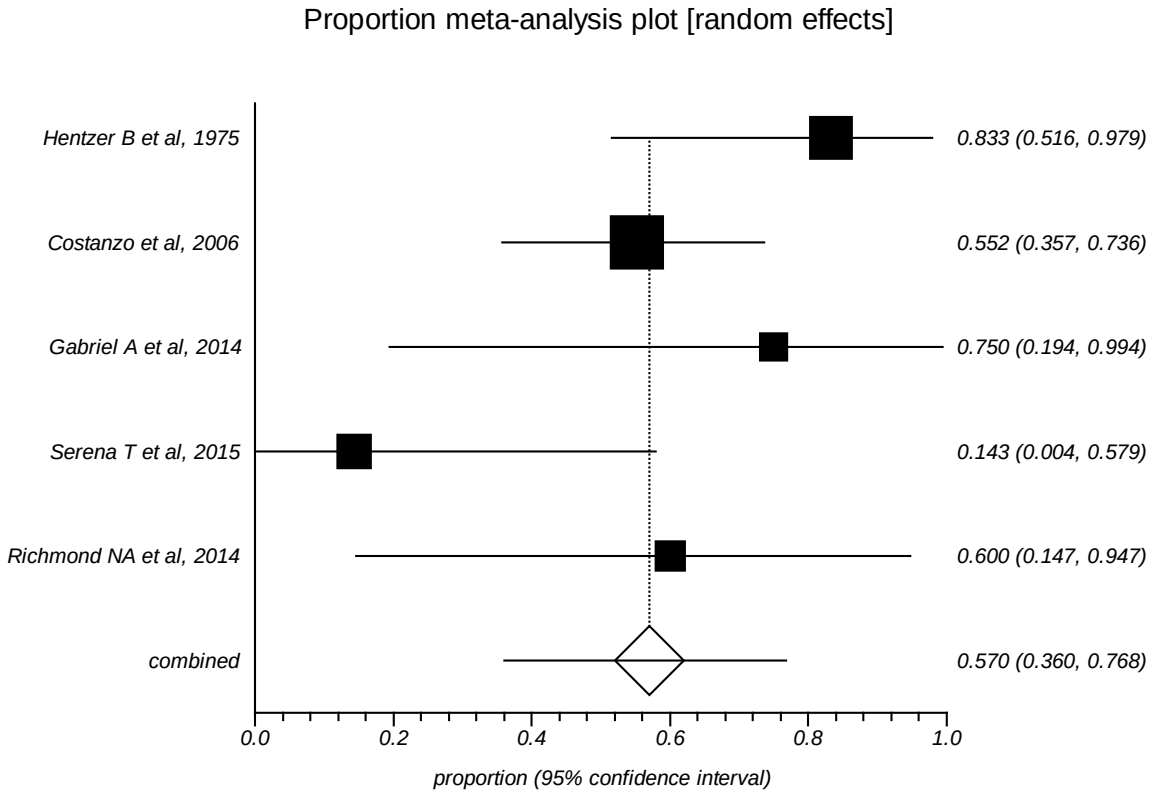


Figure 4.3 Proportion of wounds with complete healing

Meta-analysis of proportion of wounds with complete healing (random-effects plot). Proportions are shown with 95 per cent confidence intervals.

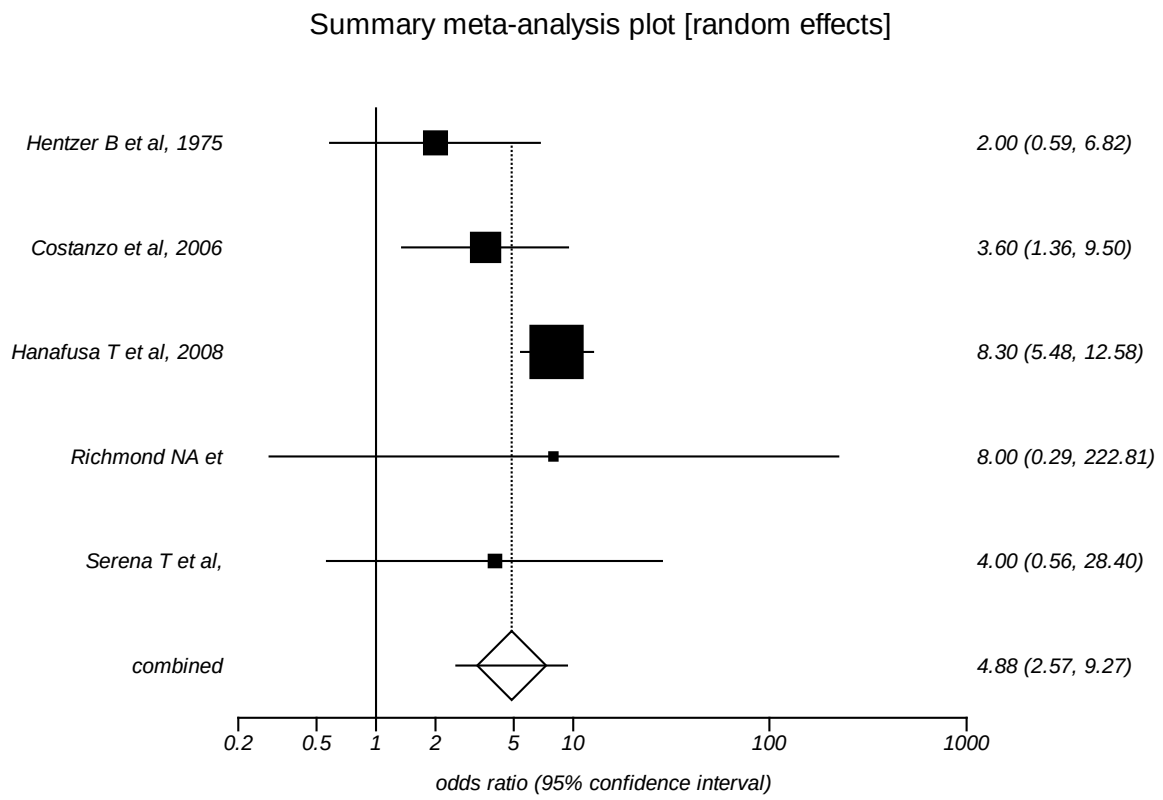


Figure 4.4 Time for complete healing

Meta-analysis of summary of time for complete wound healing (random effect plot). Sumaries are shown with 95 per cent confidence intervals.

EG was used to treat wounds of various aetiologies, duration and size. The following wide range of wounds were treated: venous ulcer (n=34), arterial ulcer (n=2), mixed arteriovenous ulcer (n=3), diabetic ulcer (n=7), vasculitis (n=9), trauma (n=2), burns (n=1), pyoderma granulosum (n=5), and lymphoedema (n=1) (Table 4.2). Most of the treated wounds were chronic wounds (more than or equal to three months in duration), except for 13 wounds that were acute wounds (less than three months in duration). All treated diabetic foot ulcer, arterial ulcer, trauma and burns wounds achieved complete healing. Wounds of other aetiologies demonstrated a lower success rate.

Wound aetiology	Number treated	Completely healed wounds	Percentage healed (%)
Diabetic foot ulcer	7	7	100.00%
Burn	1	1	100.00%
Arterial	2	2	100.00%
Trauma	2	2	100.00%
Venous	34	33	97.06%
Mixed	3	2	66.67%
Pyoderma granulosum	5	3	60.00%
Vasculitis	9	5	55.56%
Lymphatic	1	0	0.00%

Table 4.2 Healing by wound aetiology

Partial healing of between 50-99.9 percent healing was achieved by 34.8 percent (95 percent c.i. 17.6 to 54.4) of the wounds within the follow up duration while failure or no healing (0-49.9 percent healing) was reported in 10.9 percent (95 percent c.i. 4.4 to 19.8) of the wounds. The failures were mainly attributed to wound infection. There was lack of reporting on the time for partial healing and there was no consistency in the follow up duration in the included studies.

4.1.4.3 Donor site healing

Three different EG harvesting systems were used: Dermovac (Oy Instrumentarium, Helsinki, Finland), syringe system, and CelluTome Epidermal Harvesting System (Acelity, San Antonio, Texas) (Figure 4.5).

Figure 4.5 EG harvesting systems used.

(A) Dermovac harvesting system. Figure reproduced from Kiistala U et al., 1967. (B) Syringe system. Figure reproduced from Yamaguchi Y et al., 2004 (C) CelluTome Epidermal Harvesting System. Figure reproduced from Hachach Haram N et al., 2016.

Although these systems harvest different sizes of grafts, they share the similar harvest principle that applies continuous negative pressure on normal skin to raise blisters. The donor site healing time was reported in five studies involving 28 patients, whereby one study used the Dermovac system while two studies used the CelluTome Epidermal Harvesting System.

The pooled mean donor site healing time was 7.25 days (95 percent c.i. 4.9 to 12.54). The donor site healing time of the CelluTome Epidermal Harvesting System alone, which is the latest EG harvesting system, is 5.71 days (95 percent c.i. 2.76 to 11.77). The Dermovac system, which raises blisters measuring 5-15 mm, has a donor site healing time of 10 days [90].

Hentzer et al. reported slight diffuse pigmentation at the donor site while Costanzo et al. reported occasional hypopigmentation by the Dermovac system, but all donor sites healed without scar [88, 90]. The donor site healing time and quality of the donor site healing by the syringe system was not reported by any of the included studies.

4.1.4.4 Use of anaesthesia

Only one study reported on the use of local anaesthesia during graft harvest which used the syringe system. In this retrospective study, Hanafusa et al. compared pain during harvest using syringes of different sizes (5ml, 10ml and 20ml) with and without the use of anaesthesia [89]. Pain at graft harvest was eliminated among patients that received local anaesthesia (n=27), while 50 percent of patients (n=34) without local anaesthesia felt pain. However, the pain severity at harvest was not reported using a validated pain measurement scale. By contrast, the CelluTome Epidermal Harvesting System and the Dermovac system, which harvests multiple small blisters, were reported to be painless even without the administration of local anaesthesia although this was also not reported using a pain measurement scale.

4.1.4.5 Cost, quality of life and adverse events

None of the included articles measured the health-related quality of life or patient satisfaction. The economic evaluation of the various harvesting systems was also not reported. No adverse events were reported in any study.

4.1.5 Discussion

The aim of this systematic review was to evaluate the efficacy of epidermal grafting for wound healing. We found 6 articles, no RCTs exist now. The current evidence on the efficacy of epidermal grafting involves small case-series with huge heterogeneity in the study population.

We found that complete healing was achieved by 57.0 percent (95 percent c.i. 36.0 to 76.7) of the wounds with the mean time for complete healing of 4.87 weeks (95 percent c.i. 2.57 to 9.27). None of the studies compared the healing outcome with conservative management or SSG, which are the current standard of care. The average time for complete donor site healing was 7.25 days (95 percent c.i. 4.9 to 12.54), with no reported donor site morbidity. EG was performed on wounds of various aetiologies, duration and size. The diabetic foot ulcer, arterial ulcer, trauma and burn wounds achieved complete healing while the other wounds had low success rate. Despite demonstrating the wide applicability of this technique, there was lack of consistency in between studies to make a strong recommendation on the type of wound that would best benefit from this treatment. The broad heterogeneity in between studies with large variation in the wound aetiology, patient demographic and harvest technique resulted in a huge difference in the wound healing outcome in between studies, leading to an overall success rate of just over 50 percent.

Although the success rate of EG in direct comparison to SSG is yet to be known, the lack of donor site morbidity and the ability to perform this procedure in the outpatient setting without the use of local anaesthesia are major advantages this technique offers over conventional techniques. The mechanism of healing between EG and SSG is expected to be different, whereby EG is expected to behave more like a bioactive dressing which stimulates the wound bed to regenerate (reviewed in the next section of this chapter). The difference in the success rate between the various wound aetiologies suggest that the EG is sensitive to the

microenvironment of the wound. This also suggests that post grafting wound care should be specific to the wound aetiology.

The various EG harvesting systems used in the included studies varied in the amount of negative pressure generated and the size of graft harvested despite relying on the similar principle. The EG harvesting system that was most widely used in the included studies was the CelluTome Epidermal Harvesting System, which was used in three studies, and is the latest technology for EG. This system has the shortest graft harvest time, fastest donor site healing, no reported donor site morbidity, and can be performed in outpatient setting without anaesthesia. Being an automated system, the procedure is easily reproducible with consistent graft quality. The short harvest time is contributed by the high negative pressure which is applied concurrently with mild thermal energy of 40^{o}C and its design which harvests an array of 128 micro-blisters, each measuring 2mm in diameter and spaced 2mm apart, within an area measuring 5cm x 5cm [91]. The earlier systems used to harvest EG, Dermovac and syringe system, faced several challenges which limits its clinical applicability. The Dermovac, which has an adapter plate that allows user to determine the number and size of blisters to be harvested, has a long harvest time and requires a large equipment [94]. The reliability of the syringe system, on the other hand, had been described to depend on numerous patient and environmental factors, requires skill, time consuming, causes pain and tedious to use with often inconsistent blister shape and size formation [91]. The evolution of the harvesting systems will be thoroughly reviewed in the next section of this chapter.

The evidence in this study is limited by the lack of high-quality, level-1 evidence. The existing studies were mostly small, retrospective case-series that are often at risk of bias. There were no comparative data to evaluate that healing outcome of EG against the current standard of care such as advanced dressings or SSG. Formal bias assessment was not performed due to the study design of the included studies. In terms of the search strategy, as there is no Medical Subject Heading (MeSH) term available for EG, potentially valuable and informative studies published with other keywords may therefore been missed. The definition of wound healing

was also not clearly defined in the included papers although time for 100% wound closure was accepted to fit the definition for the purpose of this review. Further, several different harvesting systems were used in the included studies. Proportion of healing based on the size and depth of wound as well as device utilized were not performed due to incomplete reporting in most of the studies. Similarly, subgroup analysis for healing based on the wound aetiology was not performed due to the broad heterogeneity in the study population. The EG harvested by the different harvest systems were assumed to produce similar graft quality for the purpose of this review as the grafts were all harvested by blister formation.

4.1.6 Conclusion

In conclusion, EG offers a healing rate of over 50 percent and allows painless autologous skin grafting to be performed without donor site morbidity. The rapid donor site healing could have a major impact in the patient's quality of life. Our conclusions are limited by the small size and heterogeneity of the studies and the different techniques of EG. Methodologically sound RCTs to compare EG against SSG or conservative treatment are therefore necessary.

4.2 Mechanistic review on the harvesting systems, the ultrastructure of the graft and mechanism of wound healing by epidermal graft

4.2.1 Chapter summary

The structural difference of epidermal graft in comparison to split thickness skin graft and full thickness skin graft contributes to the mechanism of effect. Whereas skin grafting is an epidermal transfer, little is known about the precise mechanism of wound healing by epidermal graft. This study explored the evolution of the epidermal graft harvesting system over the last five decades, the structural advantages of epidermal graft for wound healing and the current hypotheses on the mechanism of wound healing by epidermal graft. Three mechanisms were proposed: keratinocyte activation, growth factor secretion and re-epithelialisation from the wound edge. We evaluated and explained how these processes work and integrate to promote wound healing based on the current in vivo and in vitro evidence. We also review the ongoing clinical trials evaluating the efficacy of epidermal graft for wound healing. Epidermal graft is a promising alternative to the more invasive conventional surgical techniques as it is simple, less expensive, and reduces the surgical burden for patients in need of wound coverage.

4.2.2 Introduction

The mechanism of wound healing by SSG is well understood, however very little is currently known on the precise mechanism of healing by EG. The EG has been reported to behave more like a tissue engineered skin graft or a cultured keratinocyte sheet [92, 93], which stimulates the wound to regenerate by itself rather than to provide instant wound coverage as seen with FTSG and SSG (see Figure 4.6 for images comparing wound healing by EG and SSG). Cultured keratinocytes have been used for resurfacing burn wounds and in the treatment of skin ulcers since the 1970s [95]. However, the clinical application of the cultured keratinocytes has been limited by the short-term and long-term results: variable graft take rate, limited mechanical resistance, hyperkeratosis, scar contracture, ulceration and blister formation due to reaction towards foreign fibroblasts in feeder media [96-98]. These results, accompanied by the long culture time (typically requiring three to four weeks), the fragility of the sheets, and the high cost, has limited the use of this technique to only specialised facilities [99].

Newer methods developed to overcome these drawbacks, include pre-confluent keratinocytes combined with various delivery systems such as dermal substitute [99], polymer matrix [100, 101], fibrin glue suspension [102], and aerosol spray [103] as well as co-culture with melanocytes [99] require advanced logistics and handling capacity which involves clean room facilities and the use of clinical-grade reagents that are compliant with the Advanced Therapy Medicinal Products (ATMPs) guideline [99]. Similar challenges are faced by tissue engineered skin grafts, which are often not easy to handle, lack durability, are expensive and not available off-the-shelf [104].

EGs are advantageous as they do not require a carrier system, additional culture time, or a specialised facility. Several groups have previously reported good clinical results with the use of EGs for wound healing [91-93]. However, little is known about its mechanism of healing.

The goal of this review is to explore the mechanism of healing by EG. First, the evolution of the harvesting system over the last five decades and the structural advantages of EG will be highlighted before exploring the current hypothesis on its healing mechanism. This review ends with proposing the possible models to study the mechanism of healing by EG along with an overview on the ongoing clinical trials aimed at evaluating the efficacy of EG for wound healing.

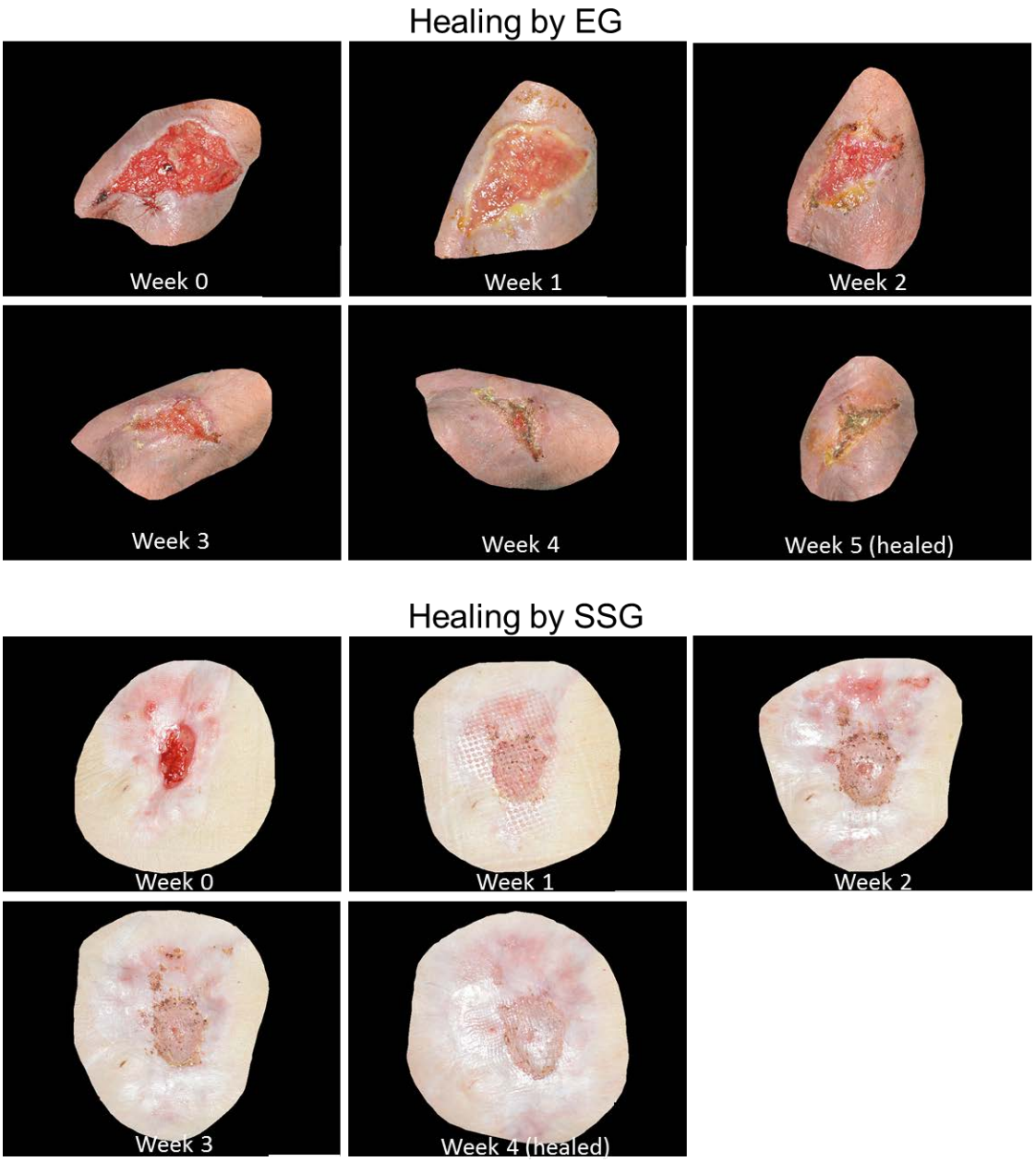


Figure 4.6 3D images of weekly wound healing by EG and SSG.

Wounds treated with EG forms a clear film over the wound a week after grafting. Subsequently, healthy granulation tissue are seen in the wound bed and the wound rapidly re-epithelialises from the edges. On the other hand, SSG integrates with the wound bed a week after grafting and the wound re-epithelialises from the integrated skin graft.

4.2.3 EG harvesting systems

Various EG harvesting devices were used over the last fifty years with clear refinement in technology over the years [84, 105-108]. The three harvesting systems which were most commonly used to harvest EG were the Dermovac system (Oy Instrumentarium, Helsinki, Findland), the syringe system, and the CelluTome Epidermal Harvesting System (Acelity, San Antonio, Texas) (Figure 4.7). These devices rely on the same principle of applying continuous negative pressure onto healthy skin to promote blister formation, although they vary in the amount of the negative pressure generated and the size of graft harvested.

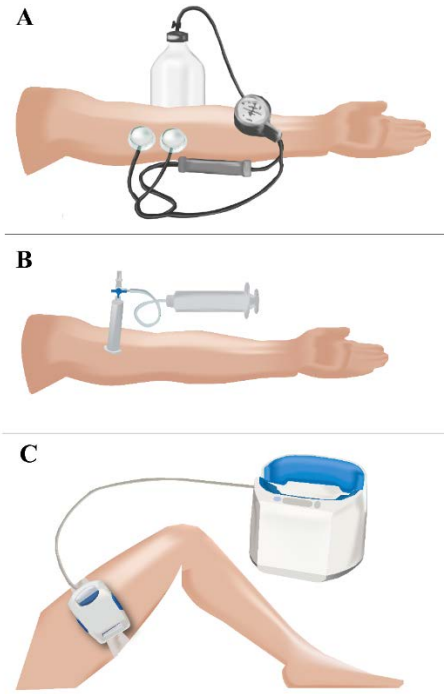


Figure 4.7 Epidermal graft harvesting systems

(A) The Dermovac system consists of a pair of transparent plexiglass suction cups and a handheld pump. (B) The syringe system consists of a small syringe with the piston removed and connected to a larger syringe via a three-way connector. The three-way connector is locked to maintain the negative pressure throughout the procedure. (C) The CelluTome Epidermal Harvesting System consists of a control unit connected to a vacuum head.

The earliest device used was Dermovac, first developed by Kiistala in 1968, which enabled separation of the epidermis from the dermis using purely mechanical forces without causing any chemical or thermal damage [108]. This device consisted of a transparent plexiglass suction cup and a hand pump that generated negative pressure of 250-300 mmHg with a blistering time of about 1 - 2 h (Figure 4.7a, above). The suction cup was equipped with an adapter plate, which allowed the user to determine the number and size of blisters to be harvested. The suction blisters were then excised separately by the surgeon and transferred to the site of interest. Smaller grafts were more convenient as larger grafts tended to curl at the edges, making the transfer challenging [90]. The long harvest time and the size of the equipment meant the techniques did not gain popularity [94].

The EG harvesting system more commonly associated with EG employs syringes [89, 94, 109]. The syringe system was simple, comprising a syringe with the piston removed, placed onto the skin then suction applied through the nozzle. This could be simply achieved by a three-way connector linked to a larger syringe, which had two to three times the suction capacity of the smaller one (Figure 4.7b, above). The syringe system had a blistering time of one hour and raised a blister measuring 1.5 cm in diameter which required surgical excision for transfer [89, 94, 109]. Variations on the system include use of a smaller syringe or subepidermal local anaesthesia infiltration [89]. However, the reliability of the syringe system is dependent on numerous patient and environmental factors[91]. Furthermore, its clinical applicability has been limited by the long harvest time, the requirement for repeated grafting due to the small graft size as well as being tedious with inconsistent blister formation [91].

The most recent harvesting system, which has been commercially developed, is the CelluTome Epidermal Harvesting System [91-93]. This system consists of an automated harvester, a vacuum head and a control unit (Figure 4.7c, above). It combines negative pressure of 400-500 mmHg and temperature of 40^{o}C, allowing 128 micro-blisters (each of 2mm diameter, 2mm apart) to be raised within 30 min [84]. The harvester is equipped with an

in-built blade to excise the roof of the blister and the EG is then transferred by use of an non-adherent dressing to the designated wound site. Being an automated device, it ensures consistency in the graft size and number. In contrast to the previous devices, the shorter harvest time of the CelluTome Epidermal Harvesting System comes from the high negative pressure, which is applied concurrently with mild thermal energy and its design which harvests an array of micro-blisters [91]. It also offers painless graft harvest without anaesthesia, which is easily performed in the outpatient and community setting due to the straightforward nature of the procedure [92, 93]. Serena et al highlighted several advantages of this technique in resource-poor setting, including simplicity, affordability, reproducibility, efficiency and the capacity of non-surgically trained clinician to perform the procedure [93].

4.2.4 Histology of EG

The epidermis is the upper most layer of the skin. The EG harvesting systems separate the epidermis from the dermis at the dermal-epidermal junction (DEJ) while preserving the histological architecture of the epidermis[108]. Ultrastructurally, the DEJ consists of four zones (Figure 4.8): first, the membrane of the basal keratinocytes which contains hemidesmosomes; second, the lamina lucida, an electron-lucent region as seen by electron microscopy, which anchoring filaments traverse; third, the lamina densa, an electron-dense area as seen by electron microscopy; and fourth, the sub-basal lamina which contains anchoring fibrils[110].

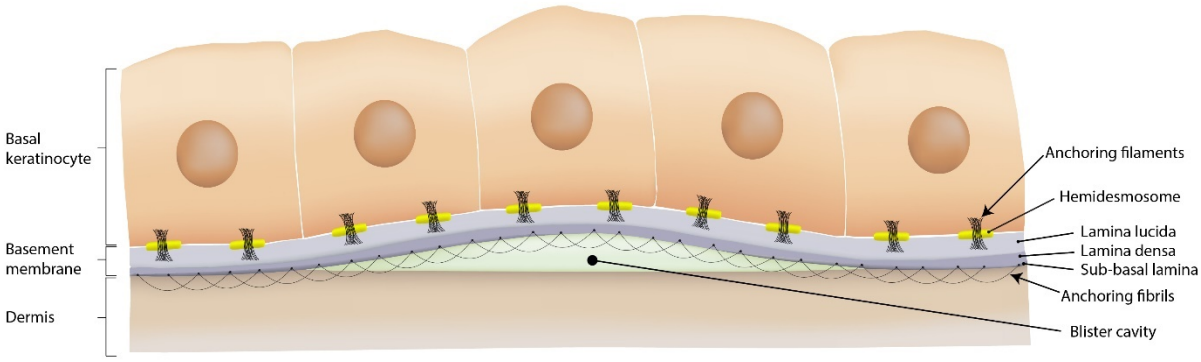


Figure 4.8 Ultrastructure of DEJ and blister cavity

The DEJ consists of four zones: membrane of the basal keratinocytes, lamina lucida, lamina densa, and sub-basal lamina. Hemidesmosomes, present at the dermal pole of the basal keratinocytes, link to anchoring filaments that connect the basal keratinocytes to the lamina lucida. Anchoring fibrils link the lamina densa and the dermal matrix. Continuous negative pressure forms a blister at the level of sub-basal lamina.

The anchoring filament links the basal keratinocytes to the lamina lucida while the anchoring fibrils link the lamina densa to the underlying dermal matrix [110, 111]. Histological study of the EG harvested from seven healthy volunteers showed that the separation is sub-epidermal, at the level of the sub-basal lamina, with a well-defined basement membrane lining the blister [112]. Immunohistochemical staining for collagen type IV, the primary component of lamina densa, further confirmed that the basement membrane components were contained within the micrografts [113, 114].

Electron microscopic analysis of the EG harvested from healthy volunteers using Dermovac at -200 mmHg within 90 to 120 min revealed that the ultrastructure of the epidermis is preserved, although vacuoles were seen within the keratinocyte cytoplasm [115]. Similar finding of vacuoles within the cytoplasm was observed in another study analysing EGs harvested using the syringe system [116]. Despite the presence of vacuoles, the nuclear membrane remained intact [116]. Furthermore, the epidermal cells were found to be viable in a study that analysed EG harvested from healthy volunteers using the CelluTome Epidermal

Harvesting System, which demonstrated the presence of Ki67 stained proliferative cells at the basal layer of the grafts [113]. The presence of the nuclear protein Ki67, which is expressed in cycling cells (G1, S, G2, and M phases) and absent in resting G0 cells, indicates that the proliferative potential of the EG is preserved upon separation [117].

The separation at the DEJ can be accelerated by heat, with the temperature ranging between 40^{o}C to 45^{o}C being reported as the optimal temperature for rapid suction blister formation [118]. In a systematic review on the suction blistering time, skin temperature was identified as the strongest predictor for the blistering time, indicating that the DEJ loses its strength with the increasing temperature due to temperature related detachment of the hemi-desmosomes and/or the inflow of blister fluids [119]. The ability of the CelluTome Epidermal Harvesting System to raise blisters in a short period of time is due to negative pressure coupled with a temperature of about 40^{o}C.

4.2.5 Mechanism of wound healing by EG

The separation at the DEJ maintains the entire ultrastructure of the epidermis, constituents of which contributes to its unique wound healing mechanism. The healing by EG is influenced by the interplay of three main mechanisms: keratinocyte activation, growth factor secretion and re-epithelialisation from the wound edge (Figure 4.9). Each of these mechanisms will be explored in detail in this section.

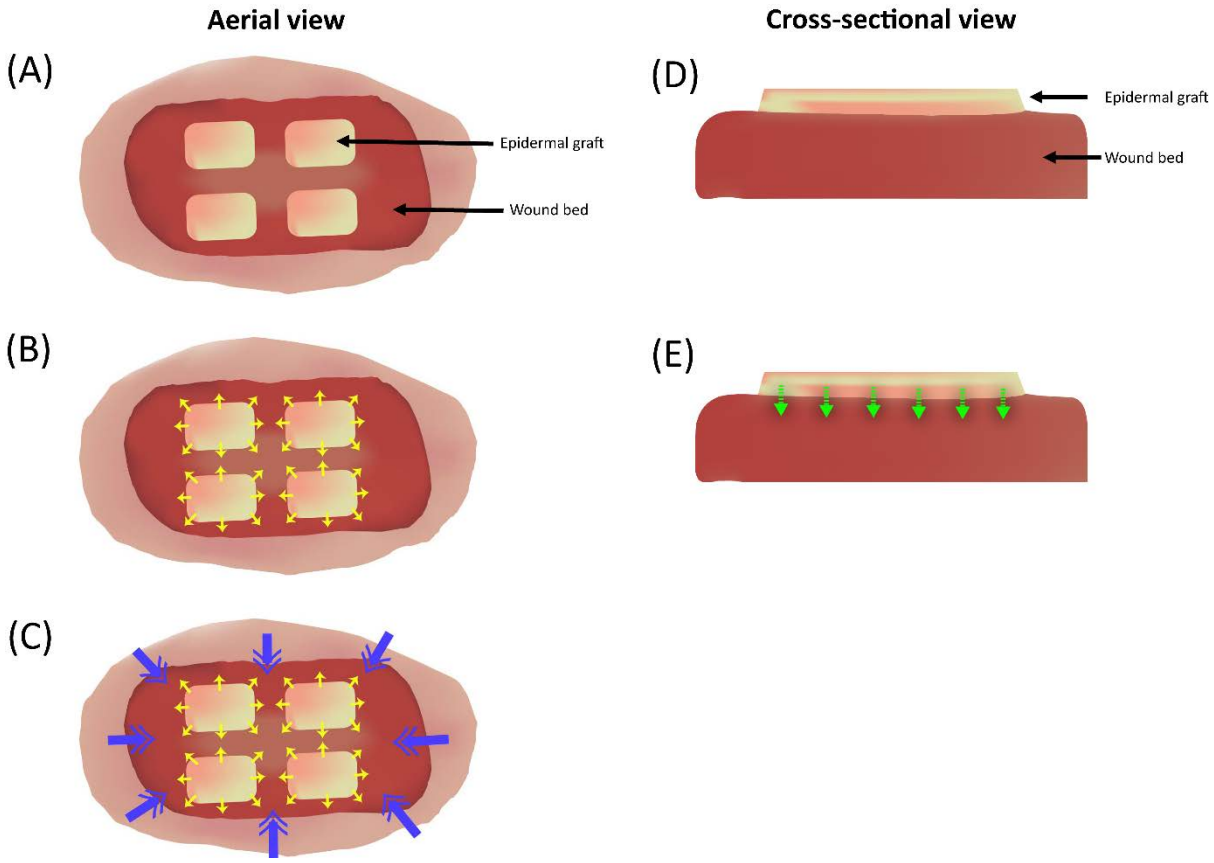


Figure 4.9 Mechanism of healing by EG

(A, B, C) The aerial view of four EGs on a healthy wound bed. (D, E) The cross-sectional view of an EG on a wound bed. Upon grafting (B), the keratinocytes within the EGs are activated and migrate onto the wound bed (yellow arrows resemble keratinocyte migration). The activated keratinocytes concurrently secrete growth factors to the wound bed to stimulate endogenous process of wound healing (E) (green arrows resemble growth factor expression). The activated keratinocytes and the growth factors stimulate the wound edge keratinocytes to migrate into the wound, accelerating re-epithelialisation from the wound edge (C) (blue arrows resemble the migration of the wound edge keratinocyte into the wound).

4.2.5.1 Keratinocyte activation and migration onto the wound bed

The first of these mechanisms is the activation of the basal keratinocytes within the EG. Whilst, keratinocyte activation in response to epidermal injury has been well reviewed (please see references [120, 121]) keratinocyte activation within EG, has not. Keratinocyte activation within EG was proposed to occur in addition to the well understood phases of skin graft healing: plasmatic imbibition, inosculation, and revascularization [122]. The direct interaction between the basal keratinocytes within the EG and the wound bed contributes to this additional phase that is not seen in FTSG and SSG, which instead have a layer of dermis that interacts with the wound bed [122]. This phase was proposed based on the pronounced expression of Ki67 (marker of cell proliferation) and β_1 integrin subunit (a putative keratinocyte stem cell marker) in the basal keratinocyte layer and on the wound bed after epidermal grafting [122]. Both the Ki67 and β_1 integrin were seen in the first week post grafting and disappeared at the fourth week, suggesting that the keratinocyte activation phase begins as part of the inosculatory phase and persists into the early stages of the revascularisation phase. The activated phenotype is also marked by changes in the cytoskeleton and increased expression of the cytoskeletal keratins involved in re-epithelialisation, namely KRT6, KRT16 and KRT17 [120, 121].

Arguably, keratinocyte activation could potentially be initiated upon separation of the EG from the DEJ during the graft harvest. As seen in epidermal injury, the exposure of the keratinocytes to their surrounding initiate the keratinocytes activation cycle[120]. This activation process is achieved by the expression of several cytokines, with interleukin-1 (IL-1) being the most common initiator [120, 121]. This cytokine, which is present in the cytoplasm of the keratinocytes in an unprocessed form, is converted by cellular injury to a processed form and released extracellularly, enabling the surrounding cells to perceive the injury [123]. The IL-1 serves as an autocrine signal to activate the surrounding keratinocytes and as a paracrine signal to the dermal fibroblasts, enhancing their migration, proliferation, and production of dermal extracellular components [120, 124, 125]. The other common initiator of keratinocyte

activation is the pro-inflammatory cytokine, tumor necrosis factor-α (TNFα) [126]. Similar to IL-1, TNFα acts in an autocrine fashion to stimulate keratinocyte migration, and in a paracrine fashion activating fibroblast[126].

The activated keratinocytes are mitotically active and are capable of outgrowth from the multiple small epidermal islands onto the wound bed [113]. The proliferative capacity of these small islands is immense, as exemplified by the ability of Cultured Epidermal Autografts (CEA), harvested from a small area, to rescue patients with burn wounds over 30% of their total body surface area [127]. As the keratinocytes migrate away from the EG, these hyper-proliferative, migratory keratinocytes secrete components of basement membrane into the microenvironment of the wound bed [120]. EG from healthy donors cultured in vitro synthesized and secreted components of basement membrane, whereby fibronectin, laminin 332 and type IV collagen were prominently stained at the expanding peripheries of the epidermal islands compared to the terminally differentiated upper layers of the epidermis [128, 129]. This suggests that keratinocytes deposit basement membrane components on the wound which assist in the anchorage and migration of the keratinocytes [130]. This ability of keratinocytes to secrete products of basement membrane and extracellular matrix is being exploited in efforts at producing tissue engineered skin grafts[131]. These cell-derived matrices are advantageous for bioengineering as they are entirely cell-type specific and are processed and deposited onto the surface containing a full portfolio of ligands such as growth factors and proteoglycans[131]. The synergy between the extra-cellular matrix and cytokines plays a pivotal role in the regulation of keratinocyte proliferation during re-epithelialisation.

4.2.5.2 Expression of cytokines to activate wound bed

Activated keratinocytes are the principal source of cytokines in the epidermis [132]. The cytokines secreted can be broadly divided by their biological activities into three categories: growth factors, interleukins, and colony stimulating factors [132-134]. The production of these

cytokines are mediated by the change in cell cycle, cell-differentiation state, a wide range of biological and physiological agents, and even the cytokines themselves [132].

A number of growth factors, including epidermal growth factor (EGF), transforming growth factor alpha (TGFα), heparin-binding EGF, and keratinocyte growth factor, are known to stimulate keratinocyte motility and proliferation in a wounded epidermis [120, 134]. EG harvested from three healthy donors and cultured in vitro for seven days have been shown to secrete vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), platelet-derived growth factors AA (PDGF AA), platelet-derived growth factors AB/BB (PDGF AB/BB), hepatocyte growth factor (HGF), and granulocyte colony-stimulating factor (G-CSF)[113]. These growth factors are known to modulate wound healing response and are able to stimulate endogenous process of wound healing [92]. Such benefit is seen even with allogenic cell therapy that has shown impressive therapeutic value in wound healing [135]. The allogenic cells, despite not attaching and covering the wound permanently, release growth factors, dermal extracellular matrix and basement membrane components to accelerate epithelialisation from the wound edge and promote granulation formation from the wound bed [135].

It is known that growth factors in combination are more stimulatory for wound healing in vivo than topical application of isolated growth factor therapy [133]. However, the combination of growth factors has to be tailored to the needs of the wound at any given time. This points to the benefit of EGs, which have the potential to deliver a cocktail of growth factors continuously in keeping with the stage of healing.

4.2.5.3 Stimulation of wound edge keratinocytes

Given the potent mitogenic and motogenic effects of the many growth factors, it is likely that the EG enhances wound edge keratinocytes to proliferate and migrate into the wound, stimulating re-epithelialisation from the wound edge [134, 136]. Several authors have reported

that the EG do not exhibit graft take on the underlying wound bed, however observed re-epithelialisation occurs from the wound edge, dubbed the ‘edge effect’ [88, 92]. Gabriel et al. and Serena et al., on the other hand, reported visible graft take and subsequent re-epithelialisation from the wound edge as well as from within the wound bed when the EG exhibited graft take [91, 93]. Costanzo et al similarly reported graft take in 8 out of 29 cases but highlighted that the major effect appears to be the stimulation of re-epithelialisation from the wound edge [88].

For re-epithelialisation to occur from the wound edge, keratinocytes must first disassemble their cell-cell and the cell-substratum adhesion. Numerous regulators modulate the proliferation and migration of keratinocytes during epithelialisation [121]. A key event in breaking the polarity between the tightly organised epithelial cells is the loss of epithelial junctions, mediated by the downregulation of the tight and adherens junction proteins, zonula occludens 1 (ZO-1) and E-cadherin, respectively. These molecules are the transmembrane proteins, which mediate cell-cell interaction and communication [44]. These transmembrane proteins are known to be co-localized and co-assembled in a multiprotein complex with the GJP, Connexins, especially Connexin 43, the most ubiquitous Connexin in the epidermis [44].

Connexins play a vital role in the migratory property of keratinocytes in addition to other physiological processes, which includes cell differentiation, proliferation, electrical transmission and inflammation [40, 45]. Furthermore, Connexins form the centre of a protein complex or “nexus” acting as a master gene that can influence the expression of over 300 other genes at the transcriptional level [137]. The cytoplasmic tail of Connexin 43 is associated with actin cytoskeletal proteins via E-cadherins, ZO-1, α- and β-catenin, either directly or via adaptors [44, 45]. These interactions affect both the cell adhesion and cytoskeletal dynamics and therefore the cell migration and wound healing. In acute wounding, Connexins are downregulated about 6 h after injury which correlates with the keratinocyte adopting a migratory phenotype as they start to crawl across the wound bed to re-epithelialise the wound [40]. The upregulation of Connexin 43, Connexin 30 and Connexin 26 at the wound edge, as

seen in chronic wounds, is known to reduce the migratory activity of keratinocytes and fibroblasts due to the substantially increased adhesion between cells [138, 139].

The modulation of the GJPs by growth factors and cytokines has been reviewed extensively by Schalper et al [140]. The growth factors expressed by the EG are likely to downregulate Connexins at the wound edge, initiating keratinocyte migration. Although the exact type and concentration of growth factors expressed by the EG in vivo is yet to be outlined, the concentration of growth factors expressed by the grafts in vitro suggests that it is likely sufficient to modulate the GJPs at the wound edge [113, 140].

4.2.6 Models to study wound healing mechanism of EG

There is currently a paucity of data on the precise in vivo wound healing mechanism by the EGs. As EG stimulates both the wound edge and wound bed to regenerate, analysis should involve tissues taken from these two locations. This could be performed by taking tissue biopsies prior to treatment and repeated again at week 1 post treatment or done repeatedly at several fixed intervals throughout the treatment. The skin biopsies taken at the wound edge can confirm the activation and proliferation of the keratinocytes upon grafting. This can be done by observing the morphologic changes of the keratinocytes by a simple haemotoxylin and eosin (H&E) staining as well as by immunostaining for proliferative markers and GJPs. The morphological changes and the downregulation of the GJPs can confirm the change of the keratinocytes into a migratory state [48, 138]. Tissue biopsy from the wound bed, on the other hand, will be able to confirm the activation of the wound bed and the presence of components of the basement membrane. Furthermore, staining for keratinocyte markers, such as KRT5, KRT6 and KRT14 can confirm the presence of the graft on the wound bed [141], as several studies have reported that graft take was not clinically visible in most cases [88, 91]. Besides tissue biopsy, non-invasive investigation such as the analysis of wound fluid collected

throughout the treatment will be able to provide invaluable information on the expression of cytokines and growth factors [142]. As well as confirming the type and concentration of growth factors expressed, this will provide insight into the changes in expression with treatment.

Several clinical trials are currently underway to investigate the efficacy of EG in the clinical setting using the Cellutome Epidermal Harvesting System. There is currently a large randomised multicentre controlled trial comparing the safety and effectiveness of EG combined with multi-layered compression therapy for the healing of venous leg ulcers [143]. Similarly, the effectiveness of EG for chronic wounds in the outpatient setting is being investigated by a non-randomised study which compares EG against SSG from historical controls [144]. Besides chronic wounds, the efficacy of EG for wounds secondary to inherited connective tissue disease, epidermolysis bullosa, is also being evaluated [145]. The findings from these high-quality trials will define the efficacy of this technique and further improve our understanding of the mechanism of healing by EG.

4.2.7 Conclusion

The increased number of publications in the last couple of years testifies the growing clinical popularity of this technique as a form of autologous skin grafting in the outpatient setting. In this review, I have highlighted the possible mechanisms of wound healing by EG based on the current in vitro and in vivo evidence. However, more work needs to be done to better understand the mechanism of healing at the cellular level in order to propose an evidence based clinical pathway. The limitations identified here will be addressed in the subsequent chapters.

Chapter 5

5.0 Pilot case series to evaluate epidermal grafting for wound healing in the outpatient setting

5.1 Chapter summary

Current wound management with the use of SSG often requires hospital admission, a period of immobility, attentive donor site wound care and pain management. This study evaluates the feasibility of using a novel epidermal graft harvesting device (CelluTome) which allows pain-free EG in the outpatient clinic setting. A prospective series of 23 patients was performed, involving 10 acute and 13 chronic wounds. All patients were subjected to EG in the outpatient specialist clinic, without the use of anaesthesia, and allowed to return home after the procedure. Completely healed wounds were noted in 20 patients (86.7%). The overall mean time for 50% and 100% reduction in wound size was 2.55 ± 0.97 weeks and 5.10 ± 1.58 weeks respectively. There was no significant difference in healing times between the acute and chronic wounds (50% reduction in wound size; acute 2.22 ± 0.91 weeks versus chronic 2.9 ± 0.94 weeks, p=0.152. Hundred percent reduction in wound size; acute 4.80 ± 1.53 weeks versus chronic 5.40 ± 1.56 weeks, p=0.422). The mean time for donor site healing was 5.57 ± 0.97 days. The mean pain score during graft harvest was 1.78 ± 0.79 and the donor site Vancouver Scar Scale was 0 for all cases at 6 weeks. This automated device offers autologous skin harvesting in the outpatient setting with minimal or no pain and a scar free donor site, equally benefiting both the acute and chronic wounds. It has the potential to save NHS resources by eliminating the need for theatre space and a hospital bed, while at the same time benefiting patient care.

5.2 Introduction

This study evaluates the feasibility of using a novel epidermal graft harvesting device, the CelluTome Epidermal Harvesting System[146], which allows EG to be performed in the outpatient clinic setting, with minimal or no pain, as an alternative to the current wound management methodology.

5.3 Methods

A prospective case series was conducted, from July 2014 to March 2015, at the Royal Free Hospital, London. EG was an established technique in the Trust so a Divisional protocol was developed with trust approval for a new device and all patients registered for a prospective audit.

5.3.1 Patient Selection

Adult patients who had been referred to the department of plastic surgery for consideration for SSG due to difficult or non-healing wounds were considered. The wounds were between 2cm x 2cm and 12cm x 12cm, and had clean and granulating wound beds. Prior to grafting, the wound bed was prepared as per standard clinical practice, either with negative pressure wound therapy or appropriate dressings, until healthy granulation tissue was present. Wound swabs were performed to exclude infection. Details on patient's demographics, co-morbidities, wound aetiology, wound type, wound location, wound size, healing time, pain scores during graft harvest, wound measurements at each visit, and donor site scar quality were recorded. The wound type was classified into acute (<3 months in duration) and chronic (≥3 months in

duration). This study was conducted in the outpatient setting and patients were allowed to return home on the same day after the intervention.

5.3.2 Epidermal graft harvest and post grafting wound care

Epidermal grafts were harvested using an automated harvesting system, the CelluTome Epidermal Harvesting System (Acelity, San Antonio, TX, USA) (Figure 5.1). This device harvests epidermal micrografts, without the use of anaesthesia, via the formation of suction blisters, carried out in the outpatient setting. By combining negative pressure (400 – 500 mmHg) and heat (40^{o}C), this device produces an array of epidermal blisters within 30 to 50 min, providing autologous keratinocytes for grafting. The microdomes were formed at the layer of the lamina lucida of the dermo-epidermal junction[146]. Following the harvest, the grafts were transferred onto a non-adherent silicone dressing (Adaptic Touch, Systagenix) and applied onto the wound. The graft was then secured with a secondary dressing, while the donor site was dressed with an occlusive dressing (Tegaderm Film, 3M). Patients were allowed to return home on the same day after the procedure and were reviewed on day 7±3 post-grafting. The patients were reviewed weekly for a minimum of 6 weeks or until the wound had healed.

During the post-operative review the donor site dressing was removed at week 1 and no further dressing was required. The recipient wound was reviewed by the same clinician for every case to ensure continuity of care and practice as well as reliability in outcome measure assessment. Once the dressing was removed the bed was not tampered with, to allow for the fragile keratinocyte layers to set, and a new dressing was applied usually in the form of a non-adherent silicone dressing, Adaptic Touch (Systagenix), followed by a secondary dressing which usually included iNadine (Systagenix) or Aquacel (Figure 5.2) to deal with the exudate levels. In cases where the exudate level was moderate or high the secondary dressing was changed twice weekly.

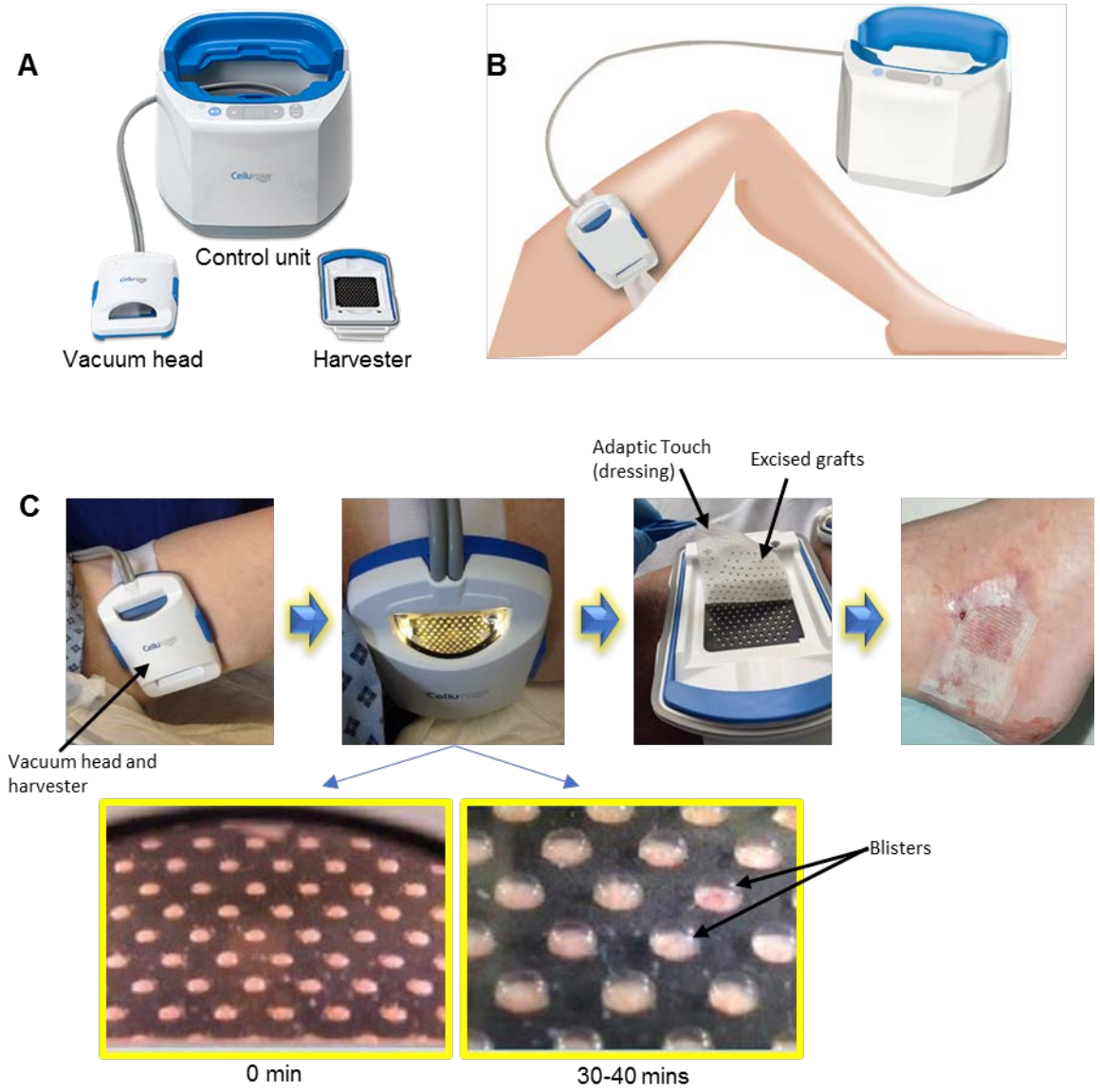


Figure 5.1 Illustration of the epidermal graft harvesting device

(A) The CelluTome epidermal graft harvesting system. (B) Illustration on the application of the harvester and vacuum head onto the patient's thigh. (C) The system was turned on for about 30-40 min to raise an array of blisters (shown in inset). The roof of the blisters was then excised by an in-built blade and the microdomes were transferred on to the wound using a non-adhesive silicone dressing.

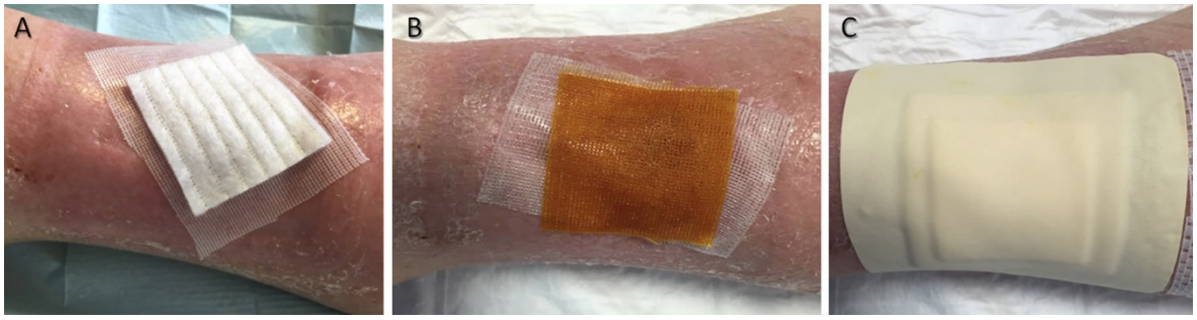


Figure 5.2 Secondary dressings

The secondary dressing used were either (A) Aquacel, or (B) iNadine. (C) Further occlusive dressing was applied on top of either (A) or (B).

5.3.3 Outcome measures

The wounds were measured (length (cm) x width (cm)) and photographed before and after grafting, and at each wound review. The primary outcomes measured were the time taken for 50% and 100% reduction in wound size as well as the time taken for the donor site to heal. A completely healed wound (100% healed) is defined as a wound which does not require further dressing and can be left exposed. The secondary outcomes measured were pain score during graft harvest and donor site scar quality. The pain score was measured using a Numerical Rating Scale, with 0 being no pain and 10 being worst pain. The donor site scar quality was evaluated using the Vancouver Scar Scale (VSS) at 6 weeks post grafting[147]. The VSS assesses 4 variables: vascularity, height/thickness, pliability, and pigmentation. Each variable includes ranked subscales that were summed to obtain a total score ranging from 0 to 13, with 0 representing normal skin and 13 representing maximum alterations of the skin.

5.3.4 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 21 software. The p values of <0.05 were considered statistically significant. Data was presented as the mean ± standard

deviation. The time for reduction in wound size between the acute and chronic wounds, and the size and type of wound were compared using the independent t-test. The Pearson correlation co-efficient was used to determine the association between age and the time for the donor site to heal.

5.4 Results

A total of 23 patients were treated with the EG with an average age of 61.1 years (range: 18-93 years). Of these patients, 10 were male (43.5%) and 13 were female (56.5%) (Table 5.1).

The most common aetiology in this patient cohort were wound dehiscence and trauma (n=8). There were 10 acute wounds (mean duration: 1.45 ± 0.76 months) and 13 chronic wounds (mean duration: 12.8 ± 16.6 months) with the average wound duration of 7.87 months (range: 0.5-60 months) (Table 5.2). The majority of the wounds treated were on the leg (39.1%), followed by foot (13.0%), ankle (13.0%) and thigh (13.0%). The average wound size was 17.1 ± 10.0 cm^2. There was no difference between wound size and type of wound (acute: 19.6 ± 9.8 cm^2 versus chronic: 15.3 ± 9.8 cm^2, p=0.337, t-test).

Number	Patient	Sex	Age	Comorbidities	Wound aetiology	Location of wound	Duration of wound (month)	Wound size (cm^2)	Time for 50% reduction of wound size (weeks)	Time for 100% reduction of wound size (weeks)	Time for donor site healing (days)	Pain score during graft harvest	VSS of donor site scar
1	SB	F	24	Nil	Pyogenic granuloma	Foot	4	6	Failed	failed	5	1	0
2	HB	M	18	Nil	Trauma	Knee	1	13.5	2	5	5	3	0
3	RK	F	85	IHD, CABG, HTN, asthma,	Venous ulcer	Leg	4	6	2	5	7	2	0
4	BL	F	93	Dementia, COPD, HTN, CCF	Trauma	Leg	1	14	1	3	5	1	0
5	JC	M	54	Nil	Amputation stump wound dehiscence	Foot	2	28	2	4	5	2	0
6	LH	F	50	SLE (oral steroids)	Venous ulcer	Ankle	5	8	2	5	7	2	0
7	JZ	M	84	Right hemicolectomy, postop fistula and hernia	Abdominal wound dehiscence	Abdomen	60	15.8	4	8	5	1	0
8	GM	F	62	Bowen's disease, myasthenia gravis (on oral steroids), osteoporosis	Trauma	Leg	1.5	12	2	6	5	2	0
9	RF	M	78	Prostate cancer, myelodysplasia, IHD, PVD, multiple BCC/SCC	Wound dehiscence	Leg	3	27	Failed	failed	5	1	0
10	NS	F	91	Breast cancer, hypertension, smoker, CKD3,	Trauma	Leg	3.5	3	2	3	5	3	0
11	LR	F	64	RA, COPD	Trauma	Ankle	9	40	failed	failed	5	0	0
12	AL	F	26	Anaemia smoker	Burn	Leg	0.5	19	1	3	7	1	0

13	DM	F	50	Breast ca and chemo, previous DVT on warfarin	Dehiscence of LD donor site	Back	4	21	4	8	5	3	0
14	JB	F	52	Hypertension, gastric banding, abdominoplasty, thigh lift	Wound dehiscence	Thigh	1	28	4	8	7	2	0
15	JM	F	76	Cerebral palsy, hypothyroid, osteoporosis	SSG donor site	Thigh	24	18	2	4	5	2	0
16	OM	M	32	Deaf	Trauma	Foot	2	26	3	6	5	2	0
17	KI	M	82	IHD, CABG, HTN, T2DM, RA (on pred + methotrexate), Hypercholesterolaemia, CVA, AAA (4.5cm)	Wound dehiscence	Leg	2	16	3	6	5	1	0
18	BR	M	78	Prev sigmoid ca, colostomy, nec fas abdomen, CVA	SSG donor site	Thigh	36	39	2	5	7	2	0
19	JS	M	88	IHD, CABG, HTN	Trauma	Forearm	0.5	7	3	5	5	2	0
20	SM	M	93	IHD, CABG, HTN, AF	SSG donor site	Leg	3	9	2	3	5	2	0
21	PS	M	28	Nil	Wound dehiscence	Ankle	5	6	2	4	5	1	0
22	LM	F	39	GORD	Wound dehiscence	Arm	4	18	4	6	7	3	0
23	JK	F	58	PE/DVT, PCOS, asthma	Trauma	Pretibial	5	14	4	5	7	2	0

Keys: M=male, F=female, IHD=Ischaemic heart disease, CABG=Coronary artery bypass graft, HTN=hypertension, COPD=Chronic obstructive pulmonary disease, CCF=congestive cardiac failure, SLE=systemic lupus erythematosus, PVD=peripheral vascular disease, BCC=basal cell carcinoma, SCC=squamous cell carcinoma, CKD=chronic kidney disease, RA=rheumatoid arteritis, DVT=deep vein thrombosis, T2DM=type 2 diabetes mellitus, AAA=abdominal artery aneurysm, CVA=cerebrovascular accident, AF=arterial fibrillation, GORD=gastro-oesophageal reflux disease, PE=pulmonary embolism, PCOS=polycystic ovary syndrome, LD=latissimus dorsi, SSG=split skin graft

Table 5.1 Clinical data of patients treated with epidermal graft.

Detailed breakdown of individual patient demographics along with the wound healing characteristics are presented.

Characteristics	Number of patients (%)
Mean age (years)	61.1 ± 24.0
Gender	
Male	10 (43.5%)
Female	13 (56.5%)
Wound aetiology	
Venous ulcer	5 (14.3%)
Arterial ulcer	2 (5.7%)
Burns	1 (2.9%)
SSG donor site	3 (8.6%)
Wound dehiscence	12 (34.3%)
Trauma	10 (28.6%)
Pyogenic Granuloma	1 (2.9%)
Diabetic foot ulcer	1 (2.9%)
Type of wound	
Acute	10 (43.48%)
Chronic	13 (56.52%)
Mean wound duration (months)	7.87 ± 13.7
Anatomical location	
Foot	3 (13.0%)
Ankle	3 (13.0%)
Leg	9 (39.1%)
Knee	1 (4.4%)
Thigh	3 (13.0%)
Abdomen	1 (4.4%)
Back	1 (4.4%)
Arm	1 (4.4%)
Forearm	1 (4.4%)

Table 5.2 Summary of patient demography and wound characteristics.

Summary of patient's demography, overall wound aetiology, type of wound, mean wound duration and anatomical locations of the wounds.

Complete wound healing (100% reduction in wound size) was achieved in 20 patients (86.7%). Of these, 17 patients (85.0%) healed within 6 weeks and 3 patients (15.0%) within 8 weeks.

The mean time for 50% and 100% reduction in wound size were 2.55 ± 0.97 weeks and 5.1 ± 1.58 weeks, respectively. There was no significant difference in healing times between the acute wounds and the chronic wounds (50% reduction in wound size; acute 2.22 ± 0.91 weeks versus chronic 2.9 ± 0.94 weeks, p=0.152. Hundred percent reduction in wound size; acute 4.80 ± 1.53 weeks versus chronic 5.40 ± 1.56 weeks, p=0.422).

The mean time for the donor site to heal was 5.57 ± 0.97 days. There was no correlation between patient's age and donor site healing time (Pearson correlation, p=0.915). The mean pain score during graft harvest was 1.78 ± 0.79 and the donor site Vancouver Scar Scale was 0 for all cases at 6 weeks, whereby all donor sites looked and felt similar to the surrounding skin.

There were three graft failures due to infection. No other complications were experienced by the patients.

5.4.1 Case examples

Case 1: Patient 2/HB.

A healthy young male sustained a traumatic wound over the left patellar region from a motorbike injury. The wound measured 4.5cm x 3.0cm with exposed infra-patellar tendon, requiring surgical debridement followed by four weeks of negative pressure wound therapy (NPWT). The wound granulated well with the NPWT and subsequently underwent EG (Figure 5.3). Complete re-epithelialisation of the wound was noted at 5 weeks post-grafting while the donor site healed within the first week without any noticeable scar at week 6.

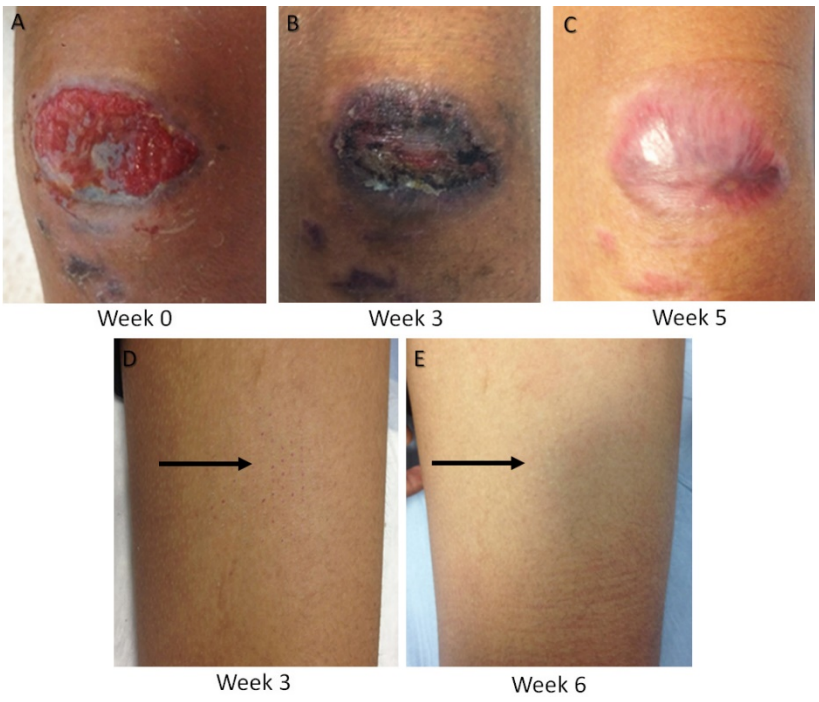


Figure 5.3 The wound and donor site of Patient 2/HB

(A) Healthy granulation tissue was seen on the wound bed after 4 weeks of NPWT. The wound measures 4.5cm x 3.0cm over the left patella region. (B) More than 50% of the wound was re-epithelialised at week 3 post grafting. (C) Complete wound healing was seen at week 5. (D) Minimal scabs were seen at the donor site (black arrow) at week 3. (E) No visible scar was seen at the donor site (black arrow) at week 6. The donor site looks aesthetically similar to the surrounding skin.

Case 2: Patient 7/JZ.

An 83-year-old fit and independent gentleman with history of appendicectomy and right hemicolectomy complicated by an incisional hernia in 2011, referred with chronic non-healing wound over the central abdomen. The wound measured 4.5cm x 3.5cm and was dressed with honey dressings, Inadine (Systagenix) and Silflex (Advancis Medical) prior to EG (Figure 5.4). 50% reduction in wound size was achieved at week 4, and complete wound healing was achieved at week 8.

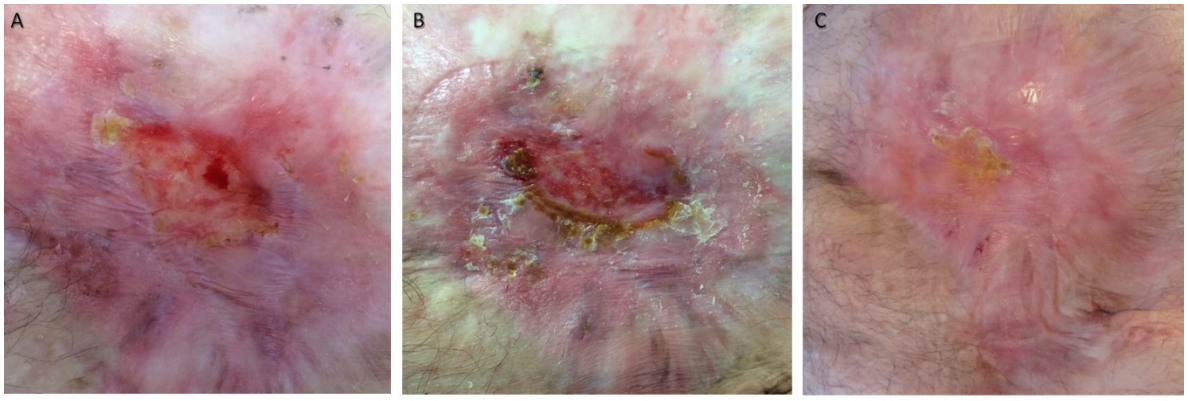


Figure 5.4 The wound of Patient 7/JZ

(A) 4.5cm x 3.5cm superficial, granulating wound over the central abdomen. (B) At week 4, 50% of the wound was re-epithelialised. (C) The wound was completely healed at week 6.

Case 4: Patient 19/JS

An 88-year-old male presented with a two-week history of a right forearm laceration following trauma. The wound measured 2 x 3.5cm, was granulating and had no growth on microbiology swabs. The patient had a past medical history of ischaemic heart disease only and was a non-smoker. Epidermal grafts were taken from the right thigh and applied to the wound. Adaptic touch (Systagenix) dressings were applied. Within three weeks the wound had reduced in size by 50% and within five weeks the wound was 100% healed (Figure 5.5). The donor site healed within six days after harvest.

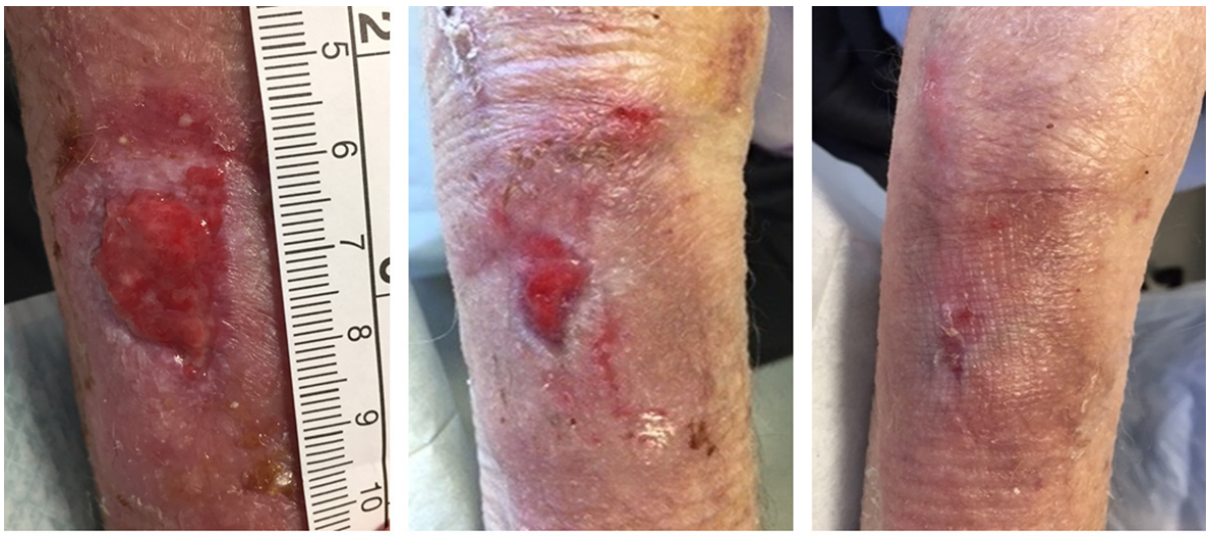


Figure 5.5 The wound of Patient 19/JS

(A) Right forearm 2x3.5cm laceration wound. (B) At 3 weeks post EG the wound was 50% healed; (C) at 5 weeks the wound was 100% healed.

5.5 Discussion

The use of epidermal grafts or blister grafts for the treatment of vitiligo and chronic wounds has already been widely reported but it's use has been limited due to the lack of reproducible and efficient harvesting techniques for the outpatient setting [148, 149]. This study demonstrates the feasibility of using a novel epidermal harvesting device to achieve definitive wound coverage in the outpatient setting.

The CelluTome device produces an array of epidermal microdomes, comprising of epidermis down to the basal layer, immediately available for transfer to the recipient site. Epidermal grafts are made of multi-layered keratinocytes, in which a variety of other cell types with specialised functions are embedded, such as the melanin pigment-producing melanocytes, the immune-competent Langerhans cells, and the neuroendocrine Merkel cell; while its basal layer contains epidermal stem cells [150]. During the early stages of wound healing, keratinocytes begin to migrate from wound edges within 24 h to the wound bed where they proliferate and form new epithelium [151]. Migrating keratinocytes synthesise and deposit a variety of extracellular matrix components, such as laminin, fibronectin, and type IV collagen [152]. In addition, numerous growth factors are also produced, namely, epidermal growth factor (EGF), transforming growth factor alpha (TGF-a), and heparin-binding growth factor (HB-EGF), which acts on the epidermis to drive wound closure [153]. The epidermal grafts, hence act more like a bioengineered skin, stimulating the endogenous process of wound healing.

Another key factor to the success of EG is the ability for basal cell outgrowth from the graft edge and this occurs for up to a 2mm distance [113]. This is intrinsic to the design of the harvester, which consists of 128 micrograft pores set at a 2mm distance apart to allow for the grafts to be raised in this manner (Figure 5.1, above).

Wound healing was a key outcome measured and the results demonstrated that 86.7% of the wounds fully healed with the use of Cellutome. 50% wound healing was achieved within 2.55

± 0.97 weeks and complete wound closure was achieved within 5.1 ± 1.58 weeks. Of the wounds that healed, 56.52% were chronic wounds that were not responding to dressings and conservative management, which potentially implies that the EGs stimulate the healing process in quiescent wounds. The donor site wound healed within 5.57 ± 0.97 days with excellent aesthetic outcome, requiring neither frequent nursing care nor scar management. This result is encouraging as a donor site from a SSG can take up to 21 days to re-epithelialise with current donor site dressing methods[154]. Furthermore, donor site complications such as infection, pain, and hypertrophic scarring can be avoided. Interestingly there was no significant difference in wound healing time between the acute and chronic wounds making the CelluTome equally useful in both types of wounds. The aetiology of the wound and anatomical site also had no significant impact on the wound healing times. This is likely due to the wound bed preparation as all wounds were prepared to have healthy granulating wound bed prior to grafting using standard wound bed preparative methods. Donor site healing was excellent in all patients with all cases scoring 0 on the Vancouver Scar Scale at 6 weeks, implying that the skin looked and felt similar to the surrounding normal skin. As for the pain scores, these were reported to be very low for all patients with a mean pain score during graft harvest was 1.78 ± 0.79 making this a very tolerable technique.

As with all new technologies the costs of intervention need to be assessed. We did not formally undertake a cost analysis. However, the series included 10 acute wounds (mean duration: 1.45 ± 0.76 months) and 13 chronic wounds (mean duration: 12.8 ± 16.6 months) a total of 181 months of dressing care was performed before the intervention of CelluTome. If these wounds had been dressed 2-3 time per week, a total of 1448-5792 dressing changes would have been performed. In total 23 interventions were performed with 200-300 dressing changes in the 8 weeks of care with over two thirds of patients achieving a dressing free (healed) outcome. The intervention and subsequent treatment therefore costing less than 10% of the previous management costs.

Our experience shows that this harvesting device can be introduced routinely in the outpatient setting for both acute and chronic wounds. Once a patient has been assessed they can be invited back to a routine 'cellutome' clinic and undergo the epidermal harvest followed by routine dressing changes by the delivering clinical team. Chronic wounds pose a significant burden on the NHS, representing at least 5.5% of NHS budget expenditure, therefore such technologies that can introduce lasting improvements to wound management should be welcomed[155].

5.6 Study limitation

This is an observational study and therefore prone to selection bias. The data reported includes all cases performed in a sequential manner and patients were identified from routine referrals. Learning curve was experienced in the first few cases and this was attributed to three main points; 1) the quality of the wound bed preparation, 2) ensuring absence of wound bed infection (responsible for three graft failures), and 3) the harvest and post-operative wound care. Following some graft failures due to infection, assessing wounds with a pre-operative swab has become the standard approach. The fragility of the epidermal grafts and keratinocyte sheets that develop in the weeks post-operatively was acknowledged and as such our practice has changed whereby the wound bed is not touched during the first 3-4 weeks of dressing changes. Furthermore, better management of exudate levels with various secondary dressings was achieved which also improved results.

The feasibility of treating acute wounds in the outpatient setting reduces the need for patients to be admitted for autologous skin grafting. In addition, the prospect of using this device in the emergency department for the management of acute wounds could potentially reduce the number of hospital visits. The CelluTome is easy to use and well tolerated by patients. Elderly patients with multiple co-morbidities would benefit from this technique as it does not require

anaesthesia and avoids the complications of bed rest, maintaining patient's independence and quality of life.

5.7 Conclusion

This automated device offers a novel method in autologous skin harvesting resulting in minimal or no pain and a scar free donor site in the outpatient setting. Complete wound coverage is achieved, while maintaining patient independence. It has the potential to save healthcare resources by eliminating the need for theatre space and a hospital bed, while at the same time benefiting patient care.

Chapter 6

6.0 Patient reported outcome measure (PROM) and cost evaluation study

6.1 Chapter summary

This chapter is aimed to compare EG with SSG by evaluating patient reported outcome measures (PROMs) and cost implications of both. Twenty patients answered a graft satisfaction questionnaire which evaluated: donor and graft site noticeability, aesthetic concerns, adverse problems and patient satisfaction. Cost per patient was calculated based on total operative expenses and five clinic follow-ups. In 100% of EG cases there were no donor site noticeability or adverse problems compared to 25% in the SSG group. Complete satisfaction with donor site appearance was observed in 100% of EG cases (50% in the SSG group). Noticeability, adverse problems and overall satisfaction were significantly better in the EG group ($p<0.05$). Graft site parameters were comparable with similar healing outcomes. The estimated cost per patient for EG was £776 and £1487 for SSG with an annual profit to the trust of £41400 based on ten grafts per month. For the right patient, EG with CelluTome provides comparable wound healing with reduced donor site morbidity and higher patient satisfaction.

6.2 Introduction

This chapter aims to evaluate the patient reported outcome and cost efficiency of using CelluTome to provide EG in an outpatient setting against SSG. The systematic use of information from PROMs is known to lead to overall better decision making between doctors and patients and results in patients being more satisfied with their treatment [156]. By comparing PROMs and the cost of EG versus SSG in an analogous cohort of patients, we aim to determine if EG with CelluTome is a viable alternative to current wound management technique besides being cost effective for the NHS and patients.

6.3 Methods

6.3.1 Patient Reported Outcome Measures (PROMs)

Twenty demographically matched patients (ten who had undergone SSG and ten who had undergone EG with CelluTome) were retrospectively selected for inclusion in the study. All patients received grafting once wounds showed healthy granulation tissue with good vascularity. PROMs were assessed using a validated patient skin graft satisfaction questionnaire (Appendix B2). All patients received the questionnaire at least six weeks after their procedure. The questionnaire assessed patient views on donor and graft site, noticeability; problems; concerns about cosmetic appearance; and, overall outcome. The results were found to be non-normally distributed using Shapiro-Wilk normality test and were analysed using the Mann-Whitney U test.

Patients also underwent weekly wound assessment in the dressings clinic to monitor progress of healing.

6.3.2 Cost Analysis

A calculation was made for cost and income for inpatient SSG, an EG with CelluTome and conservative dressing management. The cost of each patient event was calculated using the corresponding Office of Population Censuses and Surveys Classification of Surgical Operations and Procedures (OPCS) codes and overall cost of each treatment event was calculated as an average of the patients included in this study taking into account co-morbidities. The cost and income per patient based on the OPSC code is shown in Table 6.1.

For both the SSG and CelluTome procedures, costing included one initial and four follow-up dressing clinic appointments, which is the average standard practice in our department. For conservative dressing management, costing included one initial and fourteen follow-up dressing clinic appointments, based on the average number of dressing changes for a patient with chronic wound.

An annual cost was also calculated based on an estimate of ten patients per month, which is the expected case volume once the EG service is fully operational.

Patient Event	OPSC Code	Average cost per event (£)	Average income per event (£)	Loss/profit per event
Inpatient SSG	S35.2	1060	1032	-28
EG with Cellutome	S36.8	349	668	319
Initial dressing clinic appointment	S57.4	91	137	46
Follow-up dressing clinic appointment	S57.5	84	79	-5

Table 6.1 Summary of average cost and income per patient event

Average cost per event takes into account the cost of manpower and materials involved per hospital or clinic visit. Average income per event represents the payment received by the trust per hospital or clinic visit. Loss or profit per event represents the difference in the average cost and income per event.

6.4 Results

6.4.1 Patient demography

The demography of patients included in this study is presented in Table 6.2.

	EG patients (n=10)	**SSG patients (n=10)**
Age: Average (range)	74 (50-93)	54.6 (19-94)
Male: Female	3:7	4:6
Aetiology of wound		
Chronic traumatic wound	5	7
Acute wound	3	3
Venous ulcer	2	0
Location of wound		
Leg	9	10
Abdomen	1	0
Average wound size (mean cm^2)	16.5	21

Table 6.2 Demographics of patients included in the study

6.4.2 Patient Recorded Outcome Measures

6.4.2.1 Donor site:

There was no donor site noticeability, adverse problems or concerns in 100% of patients undergoing EG. All patients were either very (80%) or somewhat (20%) satisfied with their donor site outcome. All donor sites healed fully with an average healing time of 5 days.

In comparison, only two patients in the SSG group stated that they did not find their donor site noticeable, with six patients finding it either somewhat or very noticeable. Six of the SSG patients found their donor site to be problematic to some degree. Four patients were unsatisfied to some degree with the overall outcome. However, only two patients were concerned about their donor site appearance. All donor sites healed fully.

Donor site noticeability, adverse problems and overall satisfaction were statistically significantly better in EG ($p<0.05$). Figure 6.1 shows the results of the PROMs for donor sites.

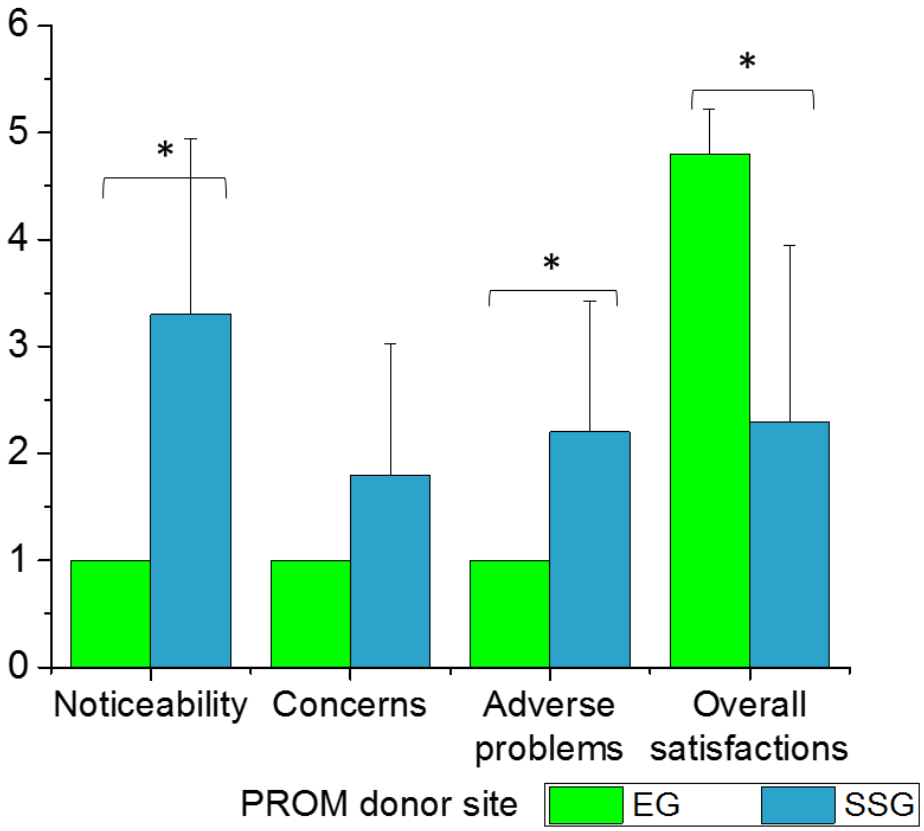


Figure 6.1 PROM for Donor Sites

Data were compared using Mann-Whitney U test. Statistically significant results ($p<0.05$) were highlighted by *.

6.4.2.2 Graft recipient site:

Overall, 80% of the EG patients were completely satisfied with the appearance of their graft site. Only 30% of patients found their graft site very noticeable, whilst 10% found it not noticeable at all. 40% of patients had no concerns or adverse problems with their graft site. Those that complained of it being problematic were referring to the length of time it took for the graft to heal, but none complained about pain or infection.

Eight EG patients had evidence of healing at the graft site, with two having a 50% reduction in wound size and six having a 100% reduction. Average healing time for 100% reduction was six weeks. Two patients had failed grafts, one who had a chronic venous ulcer and one who had a chronic surgical wound following skin lesion excision; both patients were immunocompromised.

The graft site in SSG was noticeable to some degree in all patients, with 70% of patients finding it very or somewhat noticeable. Despite this, the majority of patients (70%) were not concerned at all about their graft site appearance, with only two patients suffering adverse problems. Overall satisfaction rates showed 100% of people were satisfied to some degree. Figure 6.2 shows the results on the PROMs regarding the graft site in the EG and SSG groups. There was no statistically significant difference in the outcomes of the two groups.

Four SSG patients had 100% reduction in wound size, with the other six having at least 50% reduction. Average healing time for 100% reduction was 7 weeks. There were no graft failures. One patient suffered a graft site infection requiring a course of antibiotics. Two patients expressed dissatisfaction about long healing times. A comparison of EG and SSG graft site healing is shown in Table 6.3.

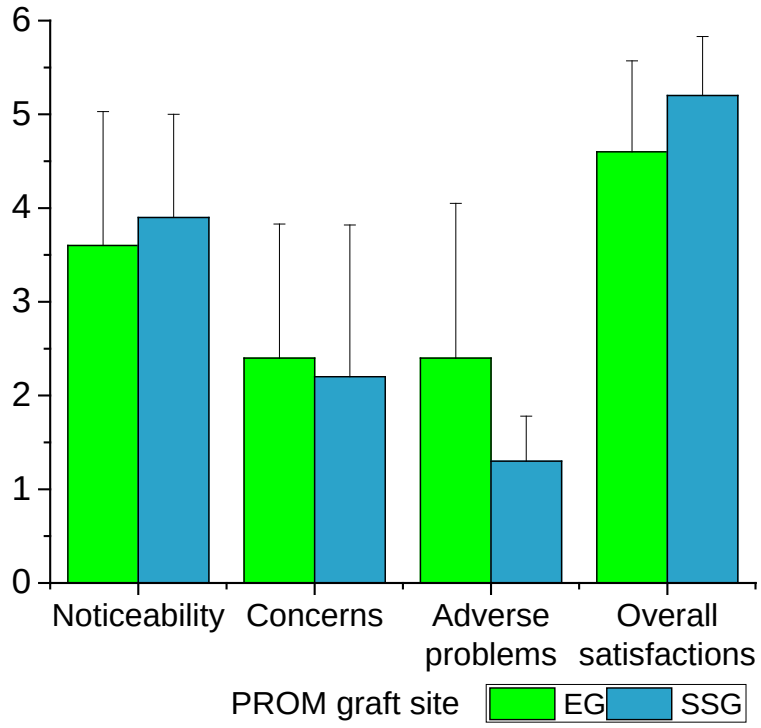


Figure 6.2 PROM for Graft Sites

Data were compared using Mann-Whitney U test. No statistically significant difference was seen between groups.

	100% reduction	50% reduction	Failed grafts	Average time to 100% reduction (weeks)
EG	6	2	2	6
SSG	4	6	0	7

Table 6.3 Comparison of EG and SSG graft site healing outcomes

6.4.3 Cost Analysis

Figure 6.3 shows the overall treatment cost (including dressing clinic follow-up) for an individual patient.

The overall loss or profit per treatment option (including dressing clinic follow-up) for 120 patients annually is summarized in Table 6.4.

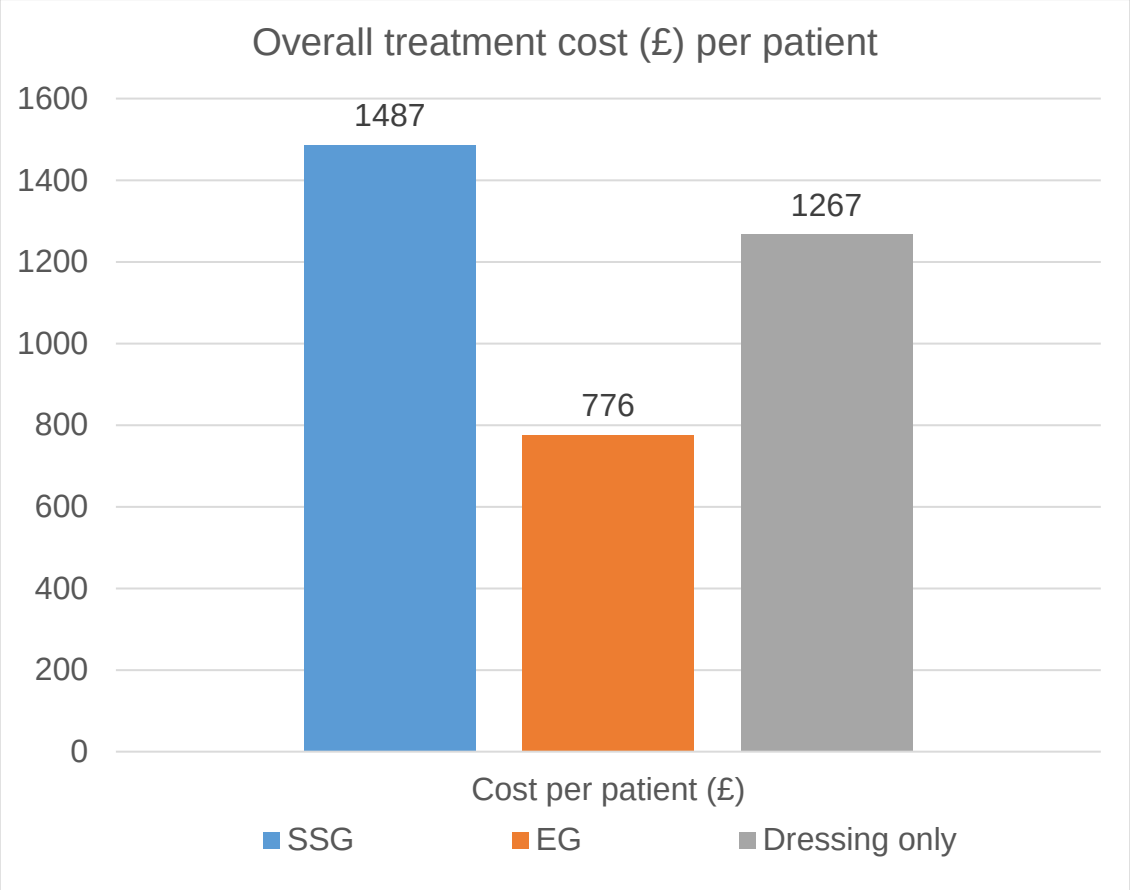


Figure 6.3 Overall treatment cost per individual patient.

The costing for SSG and EG included one initial and four follow-up dressing clinic appointments. For conservative dressing management, costing included one initial and fourteen follow-up dressing clinic appointments.

Treatment Option	Cost per patient (£)	Income per patient (£)	Annual cost for 120 patients (£)	Annual income for 120 patients (£)	Annual loss or profit for 120 patients (£)
SSG + dressing clinic follow-up	1487	1489	178440	178680	-240
EG + dressing clinic follow-up	776	1121	93120	134520	41400
Dressing management only	1267	1243	152040	149160	-2880

Table 6.4 Summary of annual loss/profit per treatment option

Cost per patient takes into account the treatment itself, dressing cost for the treatment and subsequent clinic follow-ups. Income per patient represents the payment received by the trust for the treatment and subsequent clinic follow-ups. Annual cost and income represent the cost and income for a total of 120 patients (10 patients per month). Annual loss or profit is the difference in the annual cost and income for a total of 120 patients.

6.5 Discussion

Management of acute and chronic wounds cost the NHS billions of pounds per annum, and had been identified by the WHO as a significant health problem. Current management options include SSG or conservative management. However, SSG often requires anaesthesia in a setting with access to the correct equipment, and can cause significant donor site morbidity and discomfort for the patient. Conservative dressing management is often an extremely long process requiring many attendances by nurses (either in the community or in a hospital dressing clinic) with varying outcomes and occasional progression to theatre for a SSG or commencement of negative pressure wound therapy.

Our pilot case series (Chapter 5) shown that the CelluTome epidermal graft harvesting device is effective in managing a variety of acute and chronic wounds in a wide range of patients, offering a viable alternative to SSG.

This study is the first to evaluate PROM in patients treated with the CelluTome epidermal harvesting device. Currently there is a growing need for transparency in results and procedural outcomes from both operator and patient's perspective. Chronic non-healing wounds often cause stress and morbidity to patients. Treatment options that do not involve anaesthesia, donor site morbidity and an admission to hospital (whether as a day case or longer stay) should be widely available. PROMs have been used to monitor outcomes for certain procedures in the surgical community for several years now. They have been widely shown to improve public transparency, aid surgeons to improve their practice, to offer patients informed choices about their care, whilst aiding health service commissioners to make sensible funding decisions[157]. Subjective rating scale questionnaires such as the one used in this study are validated tools for assessment of psychological impact of skin grafting and provide a review of a patient's opinion on a specific treatment[158].

We found that 100% of patients who had EG had no concerns, adverse outcomes or issues with noticeability of their donor site, with all patients being either somewhat or very satisfied

with the final outcome and all donor sites showing complete healing. This is in comparison with SSG patients, of which three-quarters found their donor site to be significantly more noticeable, half of whom experienced problems such as on-going pain, requiring analgesia and the donor taking longer than expected to heal, and only half being satisfied with the final outcome. EG patients have reduced discomfort, with previous studies also illustrating rapid healing of the donor site within a few days[92, 93].

There was no statistically significant difference between the EG and SSG group when patients were asked about their opinion of their graft site. 80% of EG patients were completely satisfied with the graft site outcome and the majority of patients showed good evidence of healing at the graft site. These findings illustrate that patient perception of wound site healing with EG is satisfactory and similar to current standard therapy. However, larger studies are required to evaluate the effectiveness of CelluTome derived EG in wound healing compared to SSG and a RCT comparing the two options is essential.

Our study also found that the average cost of using the CelluTome device was considerably less than both SSG and conservative dressing management per individual patient. An annual estimate based on twenty EG patients per month shows a significant saving compared to traditional treatment options. The calculations were estimates and do not represent actual spending; however, they provide an excellent overview of the potential financial benefits of this procedure. A particular benefit of EG is its ease of use in an outpatient setting which is cost effective and more importantly convenient for patients. Serena et al (2015) have also highlighted that the cost and simplicity of the device make it an excellent option for resource-poor nations, as well as developed regions under contemporary austerity pressures[93].

A limitation of the cost analysis is that the ultimate cost of each treatment event was based on an average of the patients in this study according to the OPSC code. Therefore, ultimate revenue of each procedure will be variable as a Health Resource Group code (HRG code) will be calculated for each patient based on the treatments given and co-morbidities. It must also

be highlighted that the costing in this study is based on UK coding procedures and therefore costing will vary in other healthcare systems based on different coding practices per geographical variation.

The small sample size and heterogeneity in patient population are the main limitations of this study, limiting the evidence. Furthermore, as this is a retrospective study with convenient sampling, it raises the question of selection bias. However, given this is a new procedure, there is not yet a large patient population from which to draw data and further studies are required to provide more evidence.

6.6 Conclusion

EG using a CelluTome epidermal graft-harvesting device is associated with a significant improved patient perceived donor site outcome when compared to SSG. This combined with the possible financial benefits and comparable graft site outcomes means EG could be considered as a first line treatment option for both small chronic and acute wounds requiring skin coverage in the right patient.

Chapter 7

7.0 EPIGRAAFT: Epidermal grafting versus split-thickness skin grafting for wound healing: a randomised controlled trial

7.1 Clinical Protocol

7.1.1 Chapter summary

EPIGRAAFT is a randomised controlled trial that compares the clinical efficacy and wound healing mechanism of EG against SSG for wound healing. The co-primary outcome measures are the proportion of wounds healed in 6 weeks and the donor site healing time. The secondary outcome measures include the cellular mechanism of healing, mean time for complete wound healing, patient satisfaction, health care utilisation, cost analysis, and incidence of adverse events. A total of 44 patients were included, 22 in each arm. The healing outcome (proportion of wound healing and healing time) of EG and SSG were similar at week 6. EG had faster donor site healing ($p<0.001$) and lesser donor site morbidity ($p<0.001$). Greater downregulation of GJPs was seen in the EG group, especially in the chronic wounds, suggesting different healing mechanism between these two treatment groups. The massive downregulation in the EG group suggests that it initiates keratinocyte migration from wound edge and activates wound edge keratinocytes into a remodelling state of wound healing. The wound bed biopsies revealed increased inflammatory cells after EG in both acute and chronic wounds, with increased expression of proliferative markers, suggesting the activation of the wound bed. Keratinocyte markers revealed that the EG does not integrate to the wound bed, suggesting that it behaves more like a bioactive dressing instead of a skin substitute as previously believed. The EPIGRAAFT trial outlined that both techniques have comparable clinical efficacy, however EG has superior donor site outcomes and further highlighted that the two autologous skin grafts have unique healing mechanism.

7.1.2 Introduction

SSG is the current standard of care for wound closure for non-healing wounds. Despite SSG being an important modality for wound closure, the donor site becomes a second, often painful wound, which may take more time to heal than the graft site itself and holds the risk of infection and scarring.

This study evaluates the efficacy of EG, using the CelluTome Epidermal Harvesting System, as an alternative to the more invasive SSG (Figure 7.1). In our pilot study carried out using this system (reported in Chapter 5), EG was noted to be an effective method of autologous skin grafting with complete wound healing achieved in two thirds of selected patients with minimal or no pain and a scar free donor site. The ability to perform EG in outpatient setting eliminates the need for a theatre space and a hospital bed, contributing to better patient satisfaction and lower cost (reported in Chapter 6). However, it is not known if EG is an effective clinical alternative to SSG.

The mechanism of wound healing by EG may be different compared to SSG. EG is postulated to promote wound healing by expressing growth factors that accelerate wound healing and encourage keratinocytes to migrate from the wound edge (reviewed in Chapter 4B). We hypothesise that EG has similar wound healing rates to SSG at 6 weeks but with less donor site morbidity. We wish to evaluate the efficacy of EG as an alternative to SSG and to further investigate the mechanism by which each technique achieves wound healing.

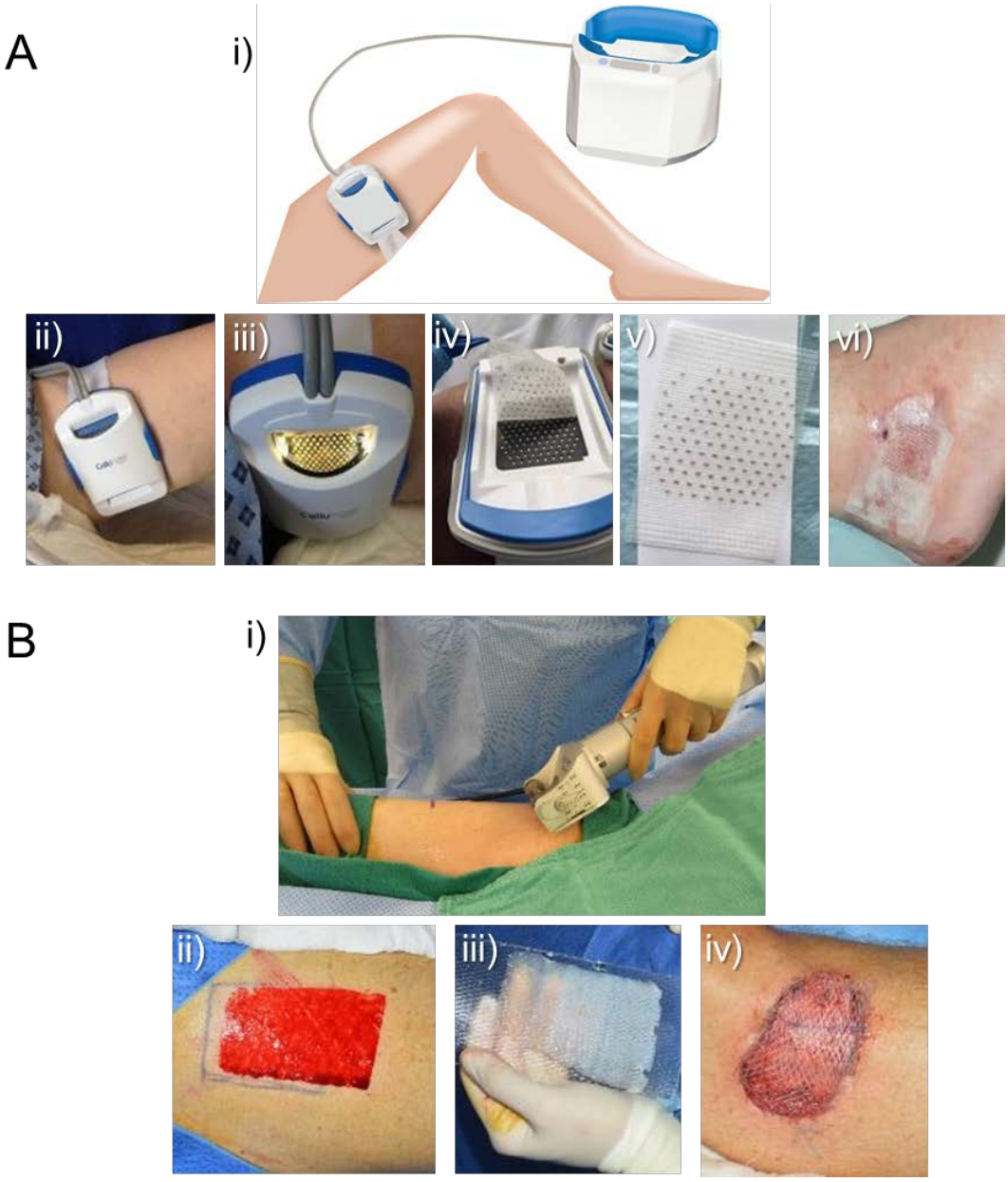


Figure 7.1 Comparison between the two surgical procedures (EG and SSG)

(A) Epidermal grafting. (i, ii, iii) The harvester and vacuum head are strapped to patient's thigh and the device is turned on for 30min, which raises an array of blisters. (iv) The roof of the blister is then excised by an in-built blade and transferred to the wound using a non-adhesive dressing (v, vi). **(B) Split-thickness skin grafting**. (i) Skin is often harvested from patient's thigh using an air dermatome which excises the epidermis and dermis of the skin, resulting in a donor site wound (ii). (iii) The excised skin is then transferred to the wound and secured with sutures (iv).

7.1.3 Trial design

This study was single-centre, prospective, randomised, open-label, non-inferiority, controlled trial with 2 parallel groups. Eligible patients were randomised to EG or SSG using a computer randomisation method. This protocol is reported in accordance to the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) 2013 guideline [159]. Clinicaltrials.gov identifier: NCT02535481 (Date of registration: 11 August 2015)

7.1.4 Research ethics approval

This trial was approved by the National Research Ethics Service Committee London-Fulham (project ID: 15/LO/0556) and from the National Health Service Research & Development Department, Royal Free Hospital (project ID:9417) (see Appendix B3). This trial was conducted in accordance to the Declaration of Helsinki and the recommendations of Good Clinical Practice.

7.1.5 Study setting

Participants were recruited at the Royal Free Hospital (RFH), London which is an academic teaching hospital and is associated with UCL.

7.1.6 Eligibility criteria

Patients referred by consultant plastic surgeons for skin grafting were eligible for this study. Before enrolment, patients were screened for inclusion into the trial and a patient information sheet was given (see Appendix B4). This process involved an explanation of the aims, methods of skin grafting and subsequent wound management, anticipated benefits, and potential hazards of the study. Patients were given sufficient time (offered a period of 24 h or

more if needed) to consider whether they wish to participate (see Appendix B5). Patients were then offered participation in the study and informed consent was obtained. Treatment was given within seven days of patient enrolment.

Inclusion criteria are as follow:

1. Age ≥18 years
2. Wound measuring more than 1cm x 1cm and less than 6cm x 6cm (1 percent total body surface area)
3. Clean, healthy granulating bed
4. Patient understand and willing to participate in the trial and can comply with the weekly visits and follow-up regime

Exclusion criteria are as follow:

1. Infected wound
2. Wound at the plantar of the foot
3. Unsuitable for SSG
4. Previous history of excessive bleeding associated with surgical biopsies or trauma
5. Uncontrolled diabetes mellitus, as measured by HbA1c ≥10 percent
6. Presence of one or more medical conditions including renal, hepatic, hematologic, active auto-immune or immune diseases
7. Use of systemic steroid or immunosuppressant
8. Not fit for surgery (ASA classification ≥4)

7.1.7 Interventions

Wound bed preparation

All wounds were prepared as per normal clinical practise which was either using the negative pressure wound therapy (NPWT) or appropriate wound dressings to achieve a healthy granulating bed. Wound swabs were performed to ensure no bacterial growth. During the time of wound bed preparation, the patients were referred to the research team. When the wound bed was deemed ready for grafting, as agreed between two treating clinicians, patients were screened and offered a patient information sheet for inclusion into the trial. Once the patients were ready for intervention, following review by the study team, patients underwent informed consent and randomisation.

Epidermal grafting

Prior to grafting, the wounds were cleaned using wound irrigation solution by the surgeon and debrided if necessary. The suction head of the CelluTome Epidermal Harvesting System was applied to the donor site (thigh) for 30-40 min to harvest epidermal graft as per protocol [84, 160]. The harvested epidermal grafts were then transferred onto the wound using a non-adhesive silicone dressing (Adaptic Touch, Systagenix). The wound was then dressed with a secondary dressing based on the wound type and exudate amount as deemed appropriate by the treating clinician. The dressings were secured with a crepe bandage or a Mefix dressing (Mölnlycke Health). The donor site was dressed with Tegaderm (3M). The wound and donor site were reviewed on day 7 ± 2 post-grafting, and then on a weekly interval.

Split thickness skin grafting

Patients underwent this procedure in the operating theatre under general or local anaesthesia. The wound was initially debrided by the treating surgeon in a similar manner to the EG group. Skin was harvested from the thigh using an electric air dermatome, set to cut at the thickness of 8-10/1000 inch, which was then meshed by 1:1.5. The wound was grafted and dressed with Adaptic Touch (Systagenix), gauze and a Mefix or wool and crepe bandage depending on the site of the graft. The donor site was dressed with Kaltostat (Alginate dressing) with a 2.5cm overlay beyond the wound margins and secured with Mefix. As per standard clinical practise, the graft was checked at day 7 ± 2, and then on a weekly interval.

Punch biopsy

Skin punch biopsies (4mm) were taken from two locations, at the centre of the wound and at the wound edge, after administering adequate local anaesthesia (1% lidocaine). This procedure was done prior to grafting and repeated at day seven post-grafting. The specimens were placed in a sterile vial containing four percent paraformaldehyde and transferred to the laboratory.

7.1.8 Study Outcome

The co-primary endpoints were the proportion of wounds with complete healing at six weeks post grafting and the time for donor site healing. Complete wound healing was defined as 100 percent re-epithelialisation and not requiring dressings. The assessment of wound healing was done via wound measurement at each review using a three-dimensional (3D) camera (LifeViz 3D camera (from Quantificare)) to obtain high quality, accurate and standardised images for digital measurement of the wound surface area [161]. The 3D photographs of the wounds and the donor sites were taken at each weekly visit and stored in the patients' digital

photo diary. An independent blinded analysis of the photo diary was carried out by 2 plastic surgeons.

The secondary endpoints include the mean time for complete wound healing, donor site healing quality (measured using Vancouver Scar Scale), PROM (assessed using the validated patient skin graft satisfaction questionnaire as used in Chapter 6) [158], healthcare utilisation and cost analysis (measured by the consumables used and the frequency of visits), and the incidence of adverse events. The incidence of serious adverse events (SAEs) include mortality of any cause within the three-months duration from the time of initial therapy, the incidence of device-related adverse events (DAEs) and the incidence of wound-related adverse events (WAEs) occurring within the study duration. The patient skin graft satisfaction questionnaire was completed by the participants at the sixth-week and third-month visit.

Further, we determined the wound healing mechanism of EG compared to SSG by analysing the expression of Connexin proteins (GJP), proliferative marker and keratinocyte markers at the wound edge and the centre of the wound before and after grafting (elaborated in Chapter 7.2).

7.1.9 Participant timeline

The study was opened to recruitment in October 2015 and closed in February 2017. Each patient was followed up weekly for six weeks or until the wound heals. The final review was at the third month from the initiation of treatment. Figure 7.2 summarises the patient's journey throughout the trial.

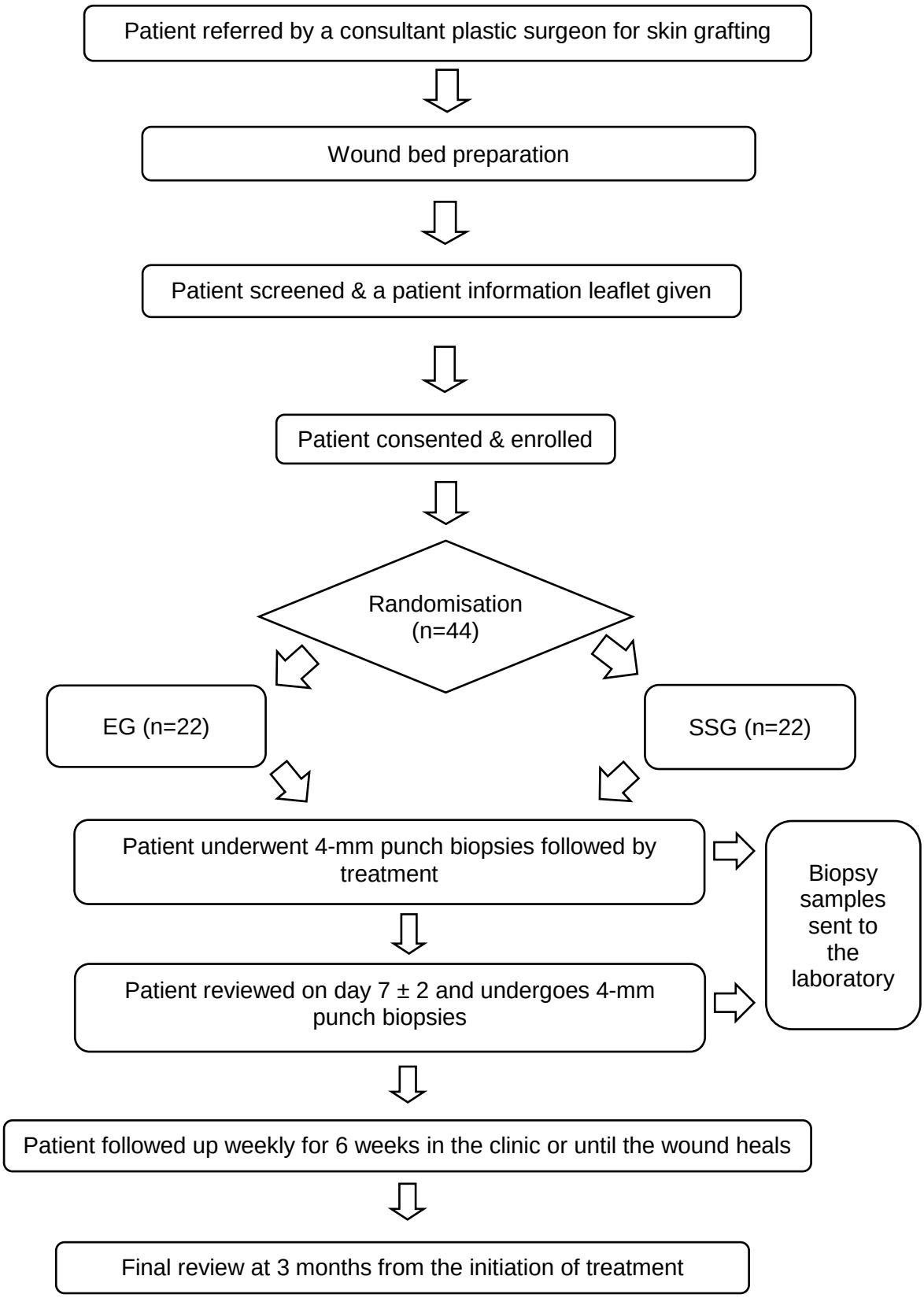


Figure 7.2 Flow chart illustrating patient's journey throughout the study

7.1.10 Sample size

Our pilot study and historical control revealed that both techniques offer the same healing rate at 6 weeks' post grafting, however the donor site morbidity was present in 40% of patients with SSG while only 5% was seen in patients with EG. Donor site morbidity includes discolouration, scarring, pain, and risk of infection.

Given a significance level of 0.05 for 80 percent power, this yields a sample size of 19 patients per group. Taking into consideration the potential dropout rate of 15 percent, the sample size has been adjusted to 22 patients per treatment arm. A total of 44 patients were recruited into the study. The timeline for recruitment was 24 months, although enrolment was completed ahead of schedule.

7.1.11 Randomisation, allocation concealment and blinding

Once consented, patients were randomly assigned to one of the treatment groups. A random allocation sequence was computer generated using SPSS version 24 (IBM, Armonk, NY, USA). The allocation sequence was sealed in opaque, identical envelopes and given to the enrolling researcher upon consenting patients. The surgical team, clinical staffs, and patients were not blinded to the intervention status.

7.1.12 Data collection and management

All data collected was recorded on paper forms and digital clinical research folder (CRF). Any adverse events were recorded and reported to the primary investigators and the institutional ethics committee.

Wounds were assessed and recorded in a wound assessment form at each visit (see Appendix B6). Details on patient's co-morbidities, wound duration and type, and previous wound bed preparation methods were recorded. 3D photographs were used to digitally

measure the wound surface area. The number and cost of outpatient visits were recorded and the type and cost of the dressings used were documented.

7.1.13 Statistical analysis

All analysis was conducted according to the intention-to-treat principle with the use of SPSS version 24 (IBM, Armonk, NY, USA). Patients are evaluated for analysis if they received a study treatment. If the clinical course cannot be fully evaluated, the last point of visit was considered as the last data analysed. Baseline demographics of the two groups were recorded. The continuous variables were compared using independent t-test. The categorical variables were compared using Pearson's chi-square or Fisher's exact test depending on the number of events.

The proportion of wounds healed with each treatment was compared using Fisher's exact tests (expected count was less than 5 in 2 cells). Mean time to wound and donor site healing were determined on the basis of the number of days until complete re-epithelialisation, using Kaplan-Meier analysis of cumulative wound healing, followed by a log rank test.

Donor site healing quality as assessed using Vancouver Scar Scale (VSS) was compared using independent t-test. PROM for wounds and donor site was found to be not normally distributed using Shapiro-Wilk test and the continuous variables were compared using Mann-Whitney U test.

A p value of less than 0.05 was considered significant and all tests were two-sided.

7.2 Laboratory Protocol

7.2.1 Introduction

The laboratory protocol used for tissue biopsy analysis was similar to the protocol used in Chapter 2. Additional protocol specific for this study will be elaborated here.

The punch biopsies were taken at the wound edge and wound bed, prior to treatment and at week 1 post treatment (Figure 7.3). Patients were then followed up for 3 months as per trial protocol.

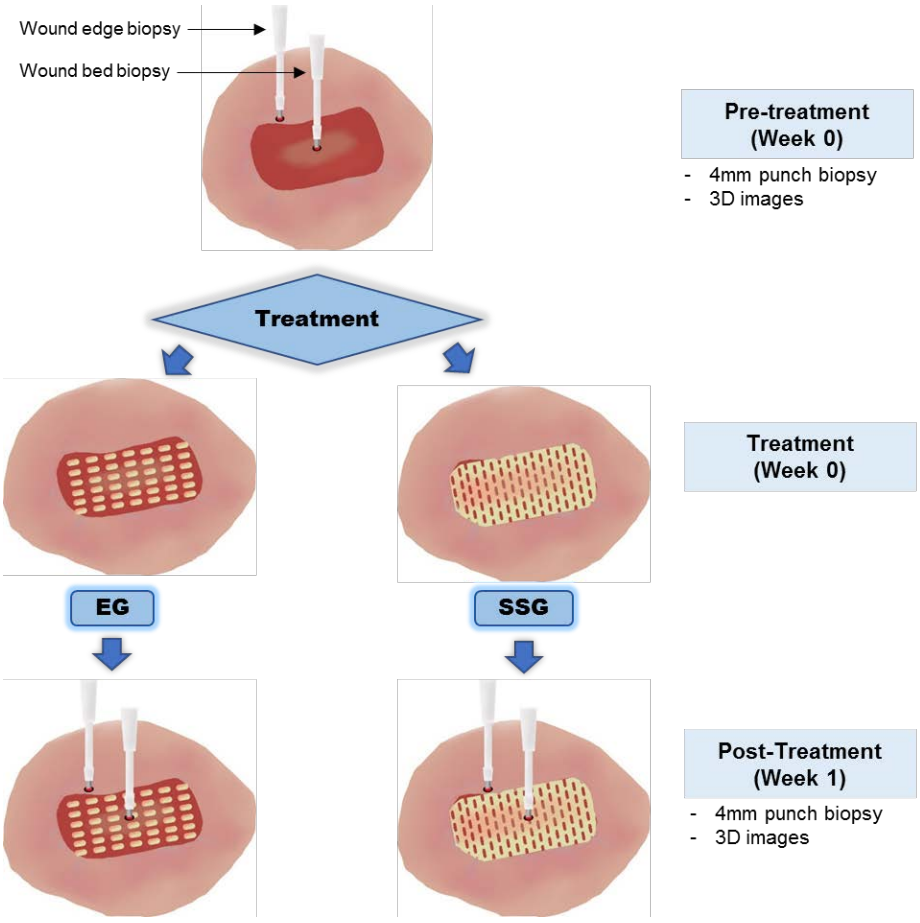


Figure 7.3 Brief illustrative outline of the study protocol

The punch biopsies were taken at the wound edge and wound bed. Following that, patients were randomised to either of the treatment arms. Biopsies were repeated at week 1 post treatment at the two similar locations.

7.2.2 Biopsy preservation and cryosectioning

Samples from a total of 24 patients were analysed. These included 12 patients from EG group and 12 patients from SSG group.

Sample preservation and cryosectioning were similar as performed in Chapter 2.4.1.

7.2.3 H&E staining

H&E staining was performed using the similar protocol as in Chapter 2.4.2 and imaged using Zeiss AxioScan Z1 slide scanner at 40x magnification at the Division of Biosciences, UCL with the assistance of Mr Christopher Thrasivoulou.

7.2.4 Immunohistochemical staining for wound edge biopsies

Tissue sections were thawed, immersed in PBS to dissolve excess OCT, permeabilized for 15 min in 0.2% Triton X-100 and blocked using 0.1M lysine-PBS for 30 min to block non-specific binding. Primary antibodies were prepared in PBS: anti-Cx43 (1:4000; C6219, Sigma - Poole, UK), anti-Cx26 (1:200; 10202093, Fisher Scientific, UK), and anti-Cx30 (1:200; 10795723, Fisher Scientific, UK). The tissues stained for Cx43 were incubated with the primary antibody for 1 at RT, while tissues stained for Cx30 and Cx26 were incubated with the primary antibody overnight at 4^{o}C. For negative controls, the primary antibody was omitted from the preparation. The tissue was washed with PBS for 3 x 5 min and stained with secondary antibody (Alexa Fluor 488 goat anti-rabbit, 10729174, 1:400; Fisher Scientific, UK) at RT for 1h. Nuclei were stained using Hoechst (1:10000; 10150888, Fisher Scientific, UK) for 5 min followed by 3 x 5 min PBS washes. Coverslips were mounted using Citifluor (Glycerol/PBS solution, Citifluor Ltd, London, UK) and sealed with nail varnish.

7.2.5 Immunohistochemical staining for wound bed biopsies

Staining was performed using protocol obtained from our collaborator Dr John Connelly from Blizard Institute, Barts and The London, Queen Mary University of London. Tissue sections were thawed and placed in a staining dish containing Citrate Buffer solution (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) and placed in a water bath at 90^{o}C for 15 min to break protein cross-linking for enhanced staining intensity of antibiotics. Slides were then allowed to cool down at RT for 20 min. Tissues were then rinsed twice with 0.1% PBS Tween 20 and permeabilized for 5 min in 0.2% Triton X-100. Sections were then blocked with 0.25% Gelatin from cold water fish and 10% fetal bovine serum for 60 min. Primary antibodies were prepared in PBS: anti-Ki67 (1:400; rabbit polyclonal, ab15580, abcam, UK), anti-cytokeratin 6 (1:100; mouse monoclonal, ab18586, abcam, UK), and anti-cytokeratin 14 (1:100; mouse monoclonal, ab7800, abcam, UK). Tissue sections were incubated with the primary antibody overnight at 4^{o}C. For negative controls, the primary antibody was omitted from the preparation. The tissue sections were washed with PBS for 2 x 5 min and stained with secondary antibody and incubated at RT for 1h. Alexa Fluor 488 goat anti-rabbit (1:400; 10729174, Fisher Scientific, UK) was used for anti-Ki67, while DyLight 488 goat anti-mouse IgG (1:400; ab96879, abcam, UK) was used for anti-cytokeratin 6, and anti-cytokeratin 14. Nuclei were stained using Hoechst (1:10000; 10150888, Fisher Scientific, UK) for 5 min followed by 2 x 5 min PBS washes. Coverslips were mounted using Citifluor (Glycerol/PBS solution, Citifluor Ltd, London, UK) and sealed with nail varnish.

7.2.6 Confocal microscopy

Leica TCS SP8 confocal microscope (Leica, Mannheim, Germany) was used to obtain 40x images: Hoescht was excited by a 405nm laser and Alexa Fluor 488 by a 488nm laser.

Images for Connexin proteins at the wound edge biopsies were taken at six locations per biopsy (as per chapter 2.4.5) to ensure that the staining pattern observed truly represented the distribution of the protein of interest.

Images for collagen and Ki67 at the wound bed were taken at 3 locations: 500µm from each edge and one at the center of the biopsy, taken with a zoom factor of 2.0 to magnify the nuclei (Figure 7.4). Cytokeratin 6 and cytokeratin 14 were imaged at 3 random locations at the top part of the sections taken from the wound bed (Figure 7.4).

All parameters were kept constant between the week 0 and week 1 skin sections to allow direct comparison.

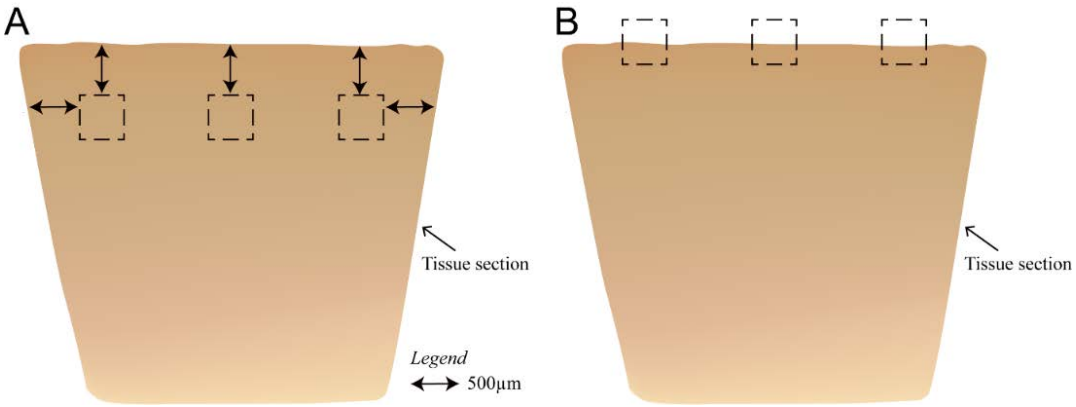


Figure 7.4 Areas images within the skin section

Rectangular areas within the skin section represents the areas imaged for (a) Ki67, and (b) for cytokeratin 6 and cytokeratin 14.

7.2.7 Connexin quantification

ImageJ was used for Cx quantification using similar protocol as described in chapter 2.4.6. The Cx levels of the six confocal images from each tissue section were used to quantify the mean Cx expression. This data was presented as the 'absolute connexin expression'. Subgroup analysis was performed based on wound type; acute (<3 months in duration) and chronic (≥3 month in duration). Statistical analysis was performed for both the absolute connexin expression and subgroup analysis and results presented in graphs.

7.2.8 Assessment for Ki67

The number of nucleus expression Ki67 was manually calculated for each image and presented as ‘absolute Ki67 expression’. Subgroup analysis was performed based on wound type: acute and chronic wounds. Statistical analysis was performed for both absolute Ki67 count and subgroup analysis and results presented in graphs.

Due to damaged sample during processing, only total of 5 patients from the EG group and 4 in the SSG group were included in the final analysis of Ki67.

7.2.9 Assessment for cytokeratin 6 and cytokeratin 14

Qualitative assessment was performed. The captured confocal images were used to evaluate for the presence or absence of the protein of interest.

7.2.10 Statistical analysis

All data were presented as the mean ± standard deviation. Statistical differences were determined using paired t-test for paired group (week 0 Vs week 1). Normality testing was performed using Shapiro-Wilk test; the Cx and Ki67 expressions were normally distributed in each group. All statistical analyses were performed using IBM SPSS Statistics 24 software.

7.3 Clinical results

7.3.1 Patient demography

Forty-four patients were enrolled between October 2015 and February 2017 and randomised, with twenty-two patients in each group. The monthly enrolment progress is summarised in Figure 7.5. The clinical results were reported here as per Consolidated Standards of Reporting Trials (CONSORT) guideline. All randomised patients received the intended treatment (Figure 7.6: CONSORT diagram for the trial). There was no protocol deviation and analysis was performed as per intention-to-treat.

The two groups did not differ in age, sex, or wound size (Table 7.1). The duration of wound in the EG group was significantly longer, with more chronic wounds compared to the SSG group.

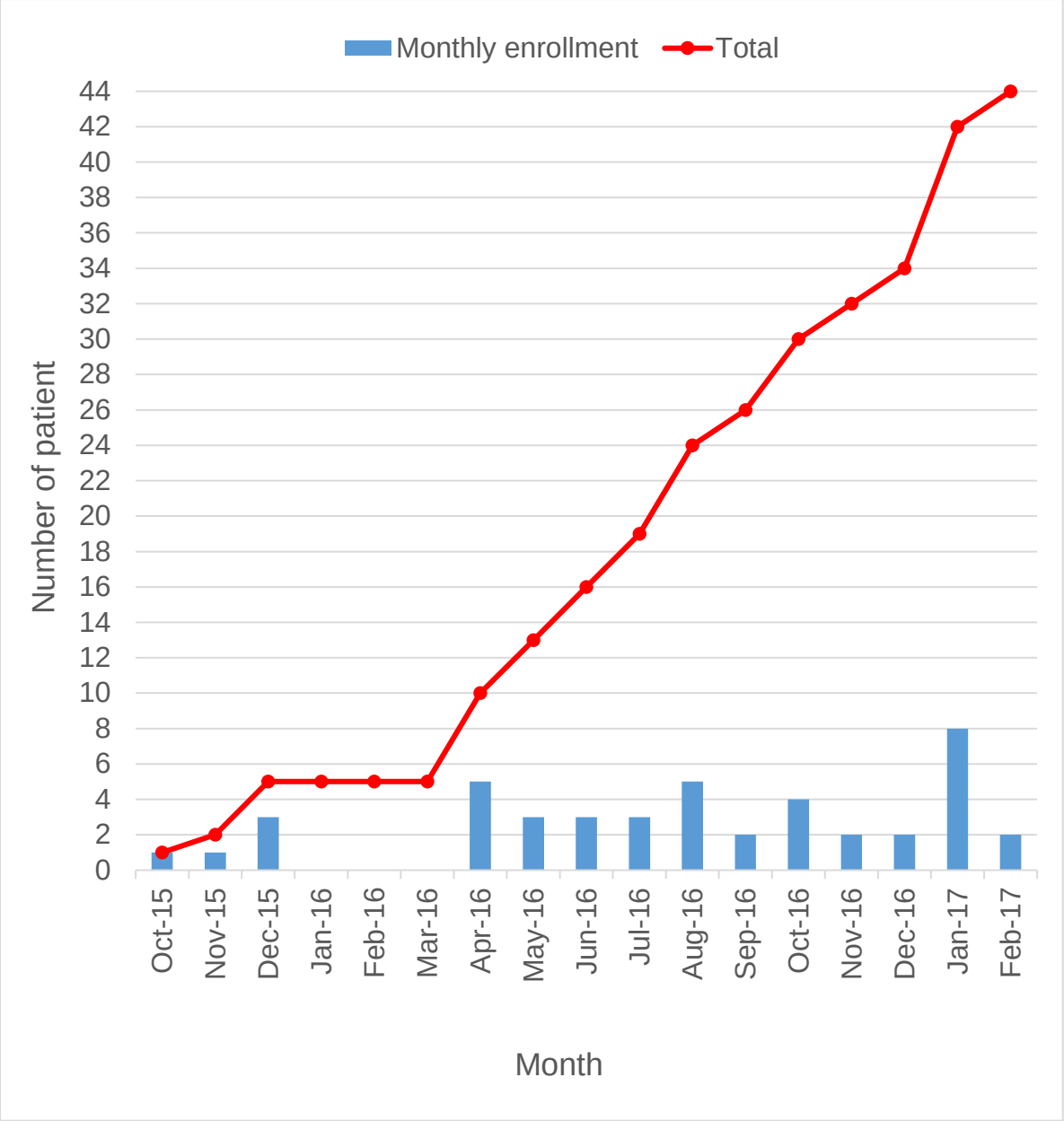


Figure 7.5 Monthly enrolment progress

Summary of the monthly enrolment progress for the 44 patients.

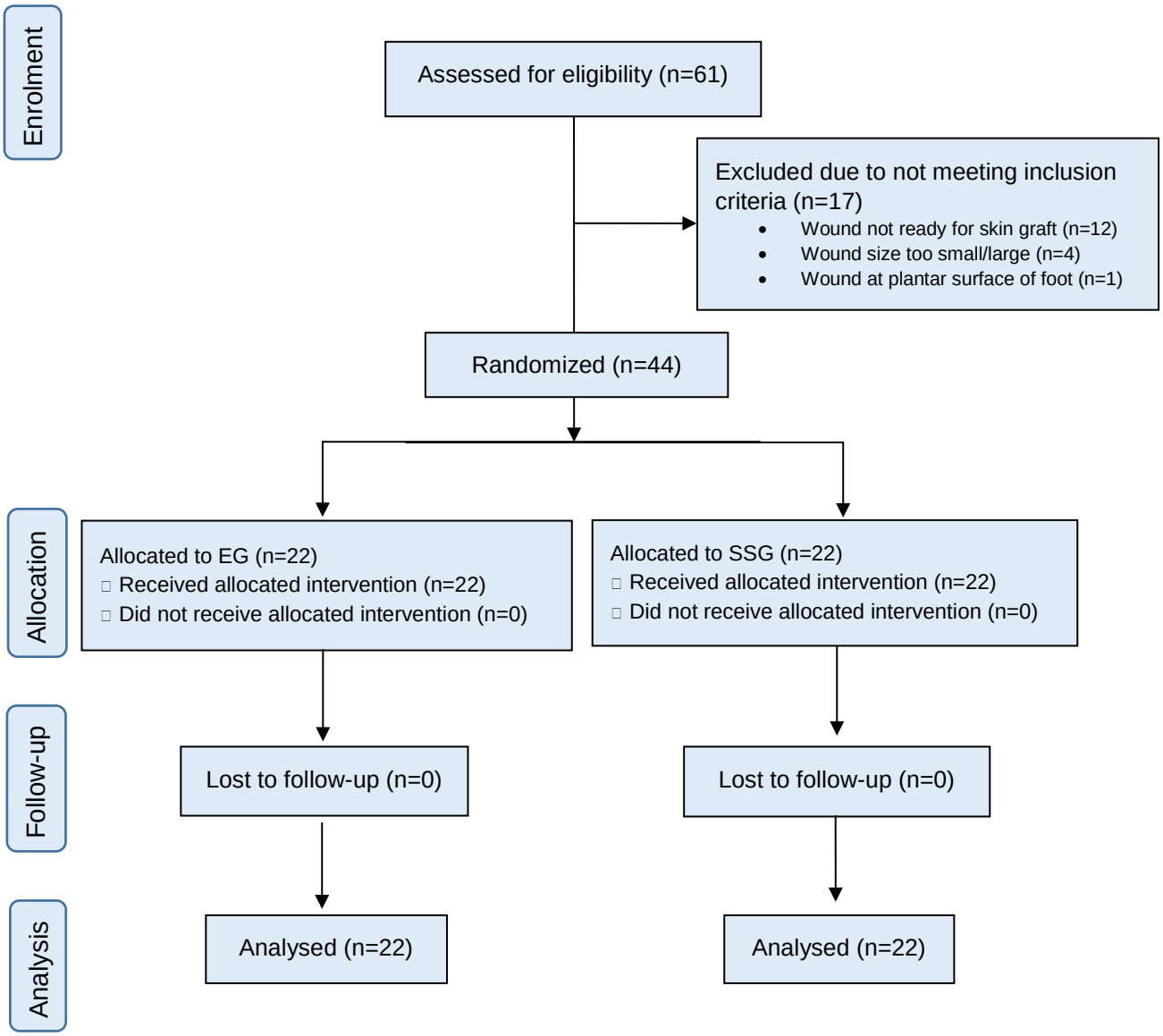


Figure 7.6 CONSORT flow diagram for the trial

	EG	SSG	p (t-test)
Mean age	58 ± 20	59 ± 18	0.86
Sex ratio (Male: Female)	13:9	17:5	0.20
Wound size (cm^2)	12.7 ± 9.9	16.5 ± 11.0	0.24
Duration of wound (week)	48.9 ± 75.4	7.86 ± 16.1	0.02
Type of wound			
Acute	11	18	
Chronic	11	4	
Cause of wound			
Dehiscence	7	2	
Amputation	0	1	
Trauma	11	8	
Debridement	1	3	
Excision	1	7	
DFU	0	1	
Arterial ulcer	1	0	
Radiotherapy	1	0	
Location of wound			
Hand	1	1	
Forearm	0	1	
Foot	3	2	
Ankle	2	0	
Leg	7	9	
Knee	3	3	
Thigh	0	2	
Groin	0	1	
Abdomen	2	1	
Chest	1	0	
Back	1	1	
Breast	1	0	
Scalp	1	1	

Values are mean ± s.d

Table 7.1 Demographics

7.3.2 Wound healing

No statistically significant difference was noted in the proportion of complete wound healing between both groups at 6 weeks (EG: 40.9% vs SSG:59.1%; p=0.366, Fisher's exact test) and 3 months' time point (EG:72.7% versus SSG:90.9%; p=0.24, Fisher's exact test). The Kaplan-Meier mean estimate for complete wound closure was 7.81(95 percent CI 6.44-9.19) weeks for EG versus 6.59 (95% CI 5.29-7.89) weeks in patients who had SSG, with no statistical significance between groups (p=0.12, log rank test) (Figure 7.7).

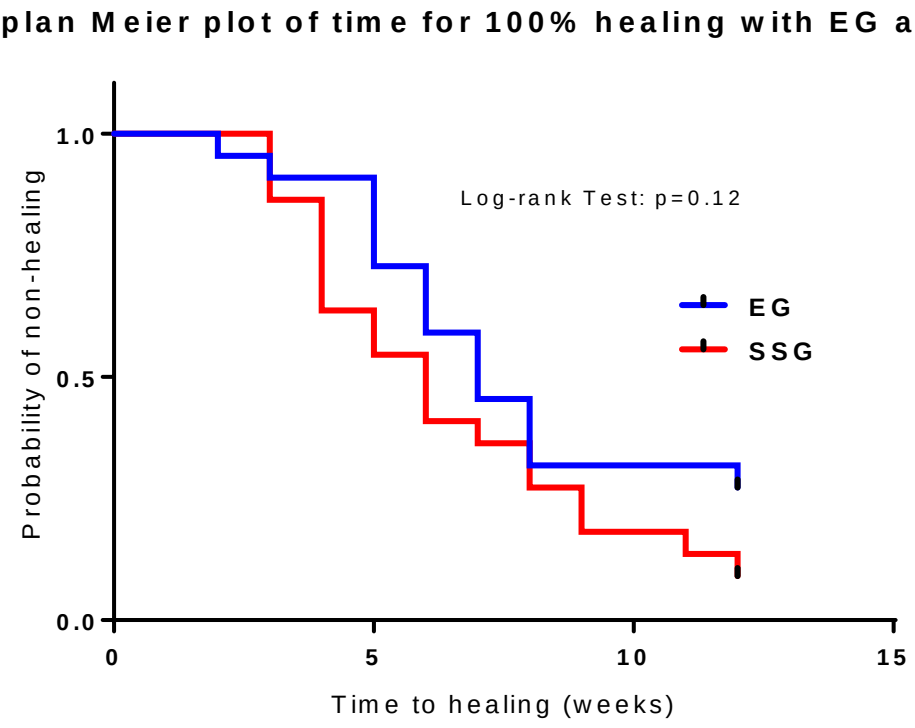


Figure 7.7 Kaplan Meier plot of time for complete wound healing with EG and SSG

7.3.3 Donor site healing

Time for donor site healing was significantly shorter in patients with EG: the Kaplan–Meier mean estimate of time for complete donor site healing was 4.86 (95 percent c.i. 4.41 to 5.32) days versus 21.32 (95 percent c.i. 15.65 to 26.99) days for SSG ($p<0.0001$) (Figure 7.8). All donor sites achieved complete healing for both treatment groups.

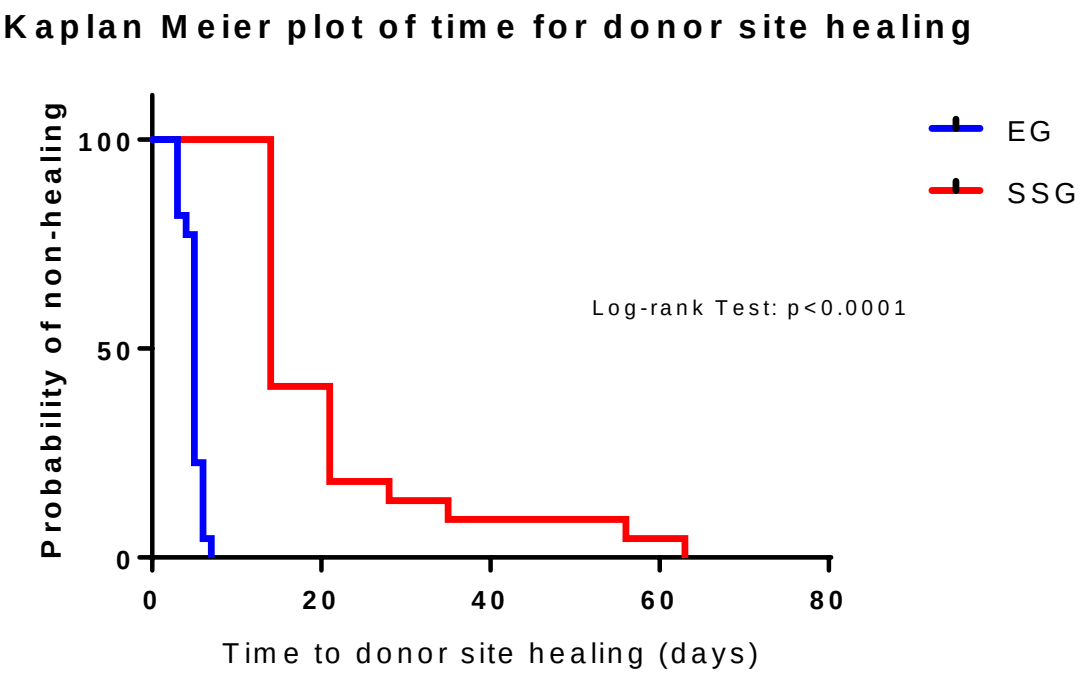


Figure 7.8 Kaplan Meier plot of time for complete donor site healing

7.3.4 Donor site morbidity

Vancouver Scar Scale scores were lower in the EG group than in the SSG group both at 6 weeks (0.14±0.45 vs 3.73±0.69; p=0.001, independent t-test) and at 12 weeks (0.09±0.29 vs 2.91±0.67; p=0.001, independent t-test) (Figure 7.9). Figure 7.10 illustrates the clinical outcome of the donor sites for both treatment groups.

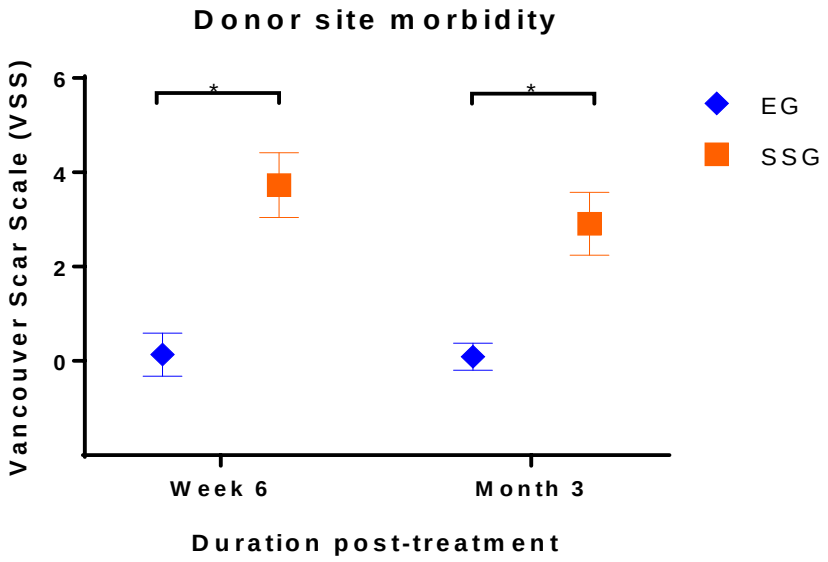


Figure 7.9 Vancouver Scar Scale for donor site morbidity

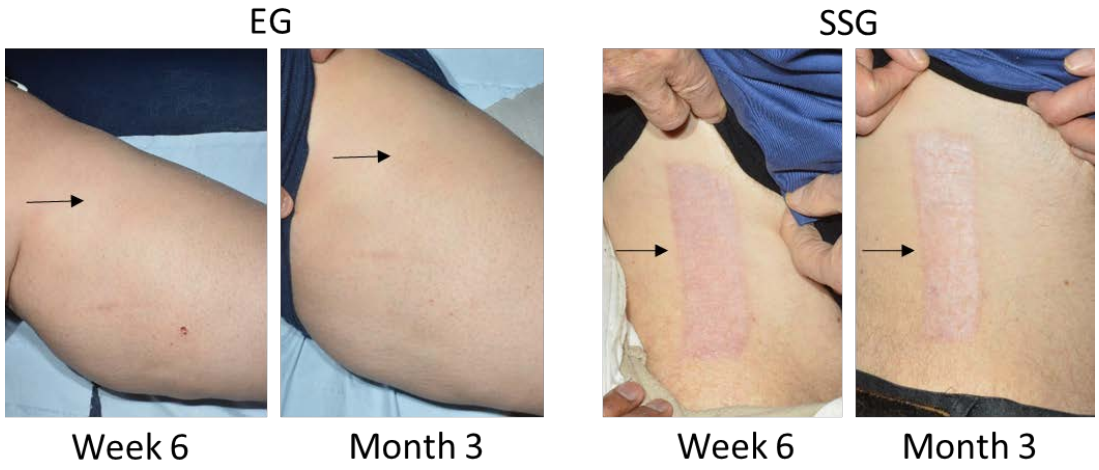


Figure 7.10 Clinical outcome of donor sites for EG and SSG

7.3.5 Patient reported outcome measure (PROM)

PROM of the donor site at week 6 and month 3 reveals that patients in the EG group reported significantly lower noticeability (week 6: p=0.001; month 3: p=0.001, Mann-Whitney U test), concerns (week 6: p=0.001; month 3: p=0.001, Mann-Whitney U test) and adverse problems (week 6: p=0.001; month 3: p=0.001, Mann-Whitney U test), and higher overall satisfaction (week 6: p=0.001; month 3: p=0.001, Mann-Whitney U test) (Figure 7.11).

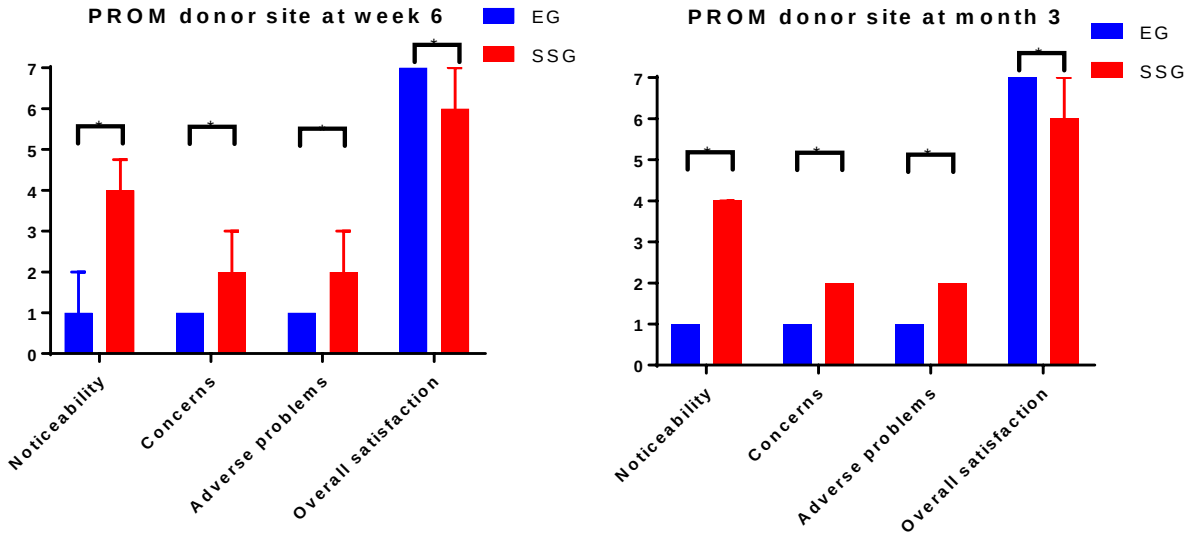


Figure 7.11 PROM of the donor site at week 6 and month 3

PROM of the wound site at week 6 and month 3 reported no significant difference in the wound noticeability(week 6: p=0.266; month 3: p=0.072, Mann-Whitney U test), concerns(week 6: p=0.462; month 3: p=0.056, Mann-Whitney U test), adverse problems(week 6: p=0.470; month 3: p=0.272, Mann-Whitney U test) and overall satisfaction(week 6: p=0.252; month 3: p=0.106, Mann-Whitney U test) (Figure 7.12).

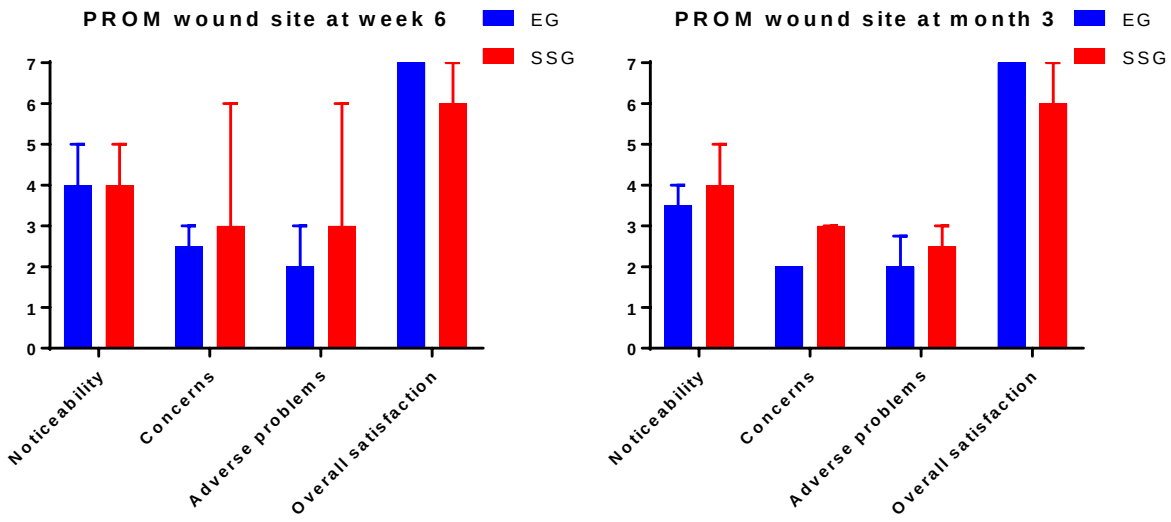


Figure 7.12 PROM of the wound site at week 6 and month 3

7.3.6 Incidence of adverse event

No incidence of adverse events experienced by any patients.

There was no post-operative complication in both groups. All patients were followed up for 12 weeks. During follow-up visits, both groups demonstrated good wound strength, as measured by the assessing clinicians. No recurrent wound was recorded in the EG group, whereas one patient with arterial ulcer in the SSG group developed wound breakdown at the graft site. This patient underwent wound dressings until complete healing.

7.3.7 Health economics

Health economics were not included in this thesis although data was collected. The data is still being analysed and will be included into future publication.

7.4 Laboratory results

7.4.1 Analysis of wound edge biopsy

7.4.1.1 Histological feature of wound edge

The histological observation of the wound edge biopsies did not reveal any obvious change in the wound edge epidermis and dermis after treatment in both groups (Figure 7.13). There was no obvious difference in the thickness of the epidermis or the distribution and depth of rete ridges. No prominent change in the distribution of inflammatory cells or density of collagen fibres in the dermis was observed.

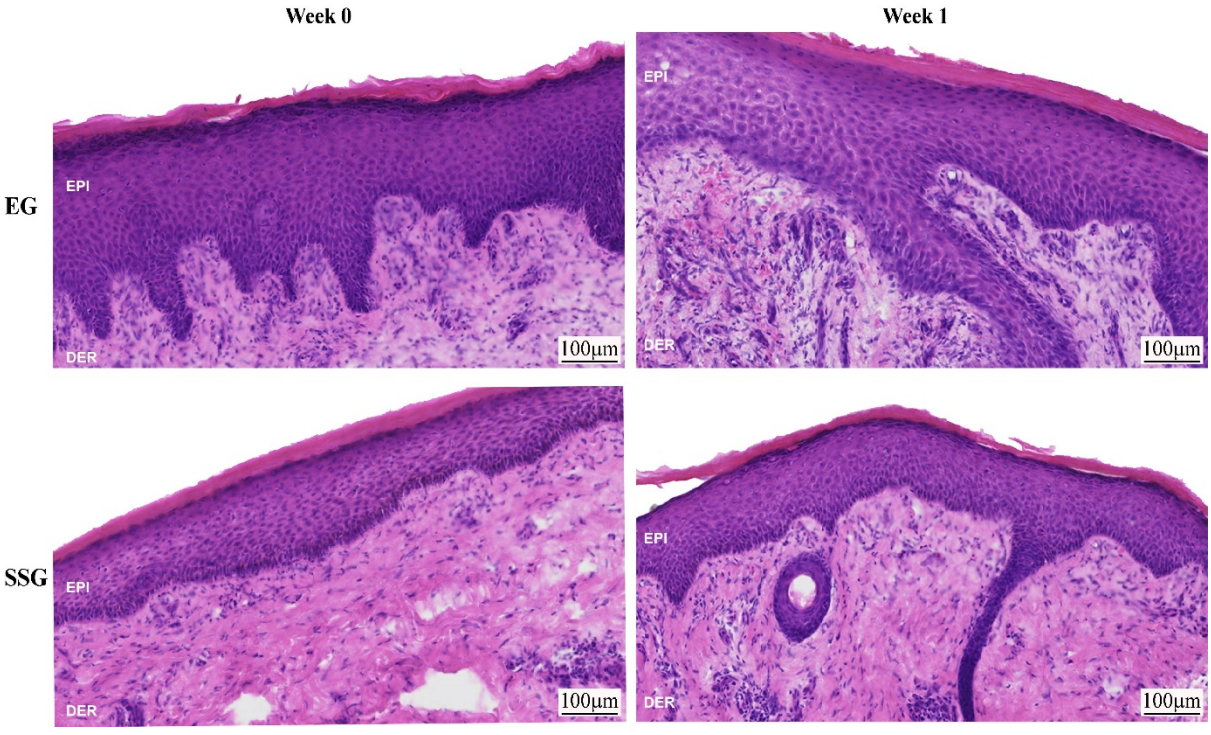


Figure 7.13 Histology of the epidermis and dermis at the wound edge

H&E stained section of the skin biopsies of both treatment groups. No obvious difference was observed in the epidermis and dermis between both treatment groups with treatment. EPI=epidermis, DER=dermis. Scale bar = 100µm. Magnification 20x.

7.4.1.2 Connexin expression at the wound edge

A general downregulation of the Cx proteins were seen at the wound edge after treatment, which was more prominent in the EG group. The pattern of downregulation differed between acute and chronic wounds for all three Cx proteins.

A significant downregulation of Cx43 was seen after EG (p=0.021, paired t-test) (Figure 7.14). Subgroup analysis based on wound type revealed significant downregulation of Cx43 in the chronic wounds after EG (p=0.023, paired t-test). Downwards trend of Cx43 expression was observed in the acute wounds after EG, however this was not statistically significant (p=0.246, paired t-test).

The absolute Cx30 expression demonstrated similar pattern of downregulation as Cx43, with significant overall downregulation in the EG group (p=0.046, paired t-test) (Figure 7.15). Similarly, subgroup analysis revealed significant downregulation in chronic wounds after EG (p=0.027, paired t-test).

Despite Cx26 demonstrated reduction in the mean Cx expression after treatment in both treatment arms, the downregulation was not statistically significant in either groups (EG: p=0.162; SSG: p=0.299, paired t-test), and in either wound types (Figure 7.16).

A change in the distribution pattern of the three Cx proteins were observed after treatment in both groups. Reduced expression of the Cx proteins in the basal layer of the epidermis was observed, which was most prominently observed in Cx43.

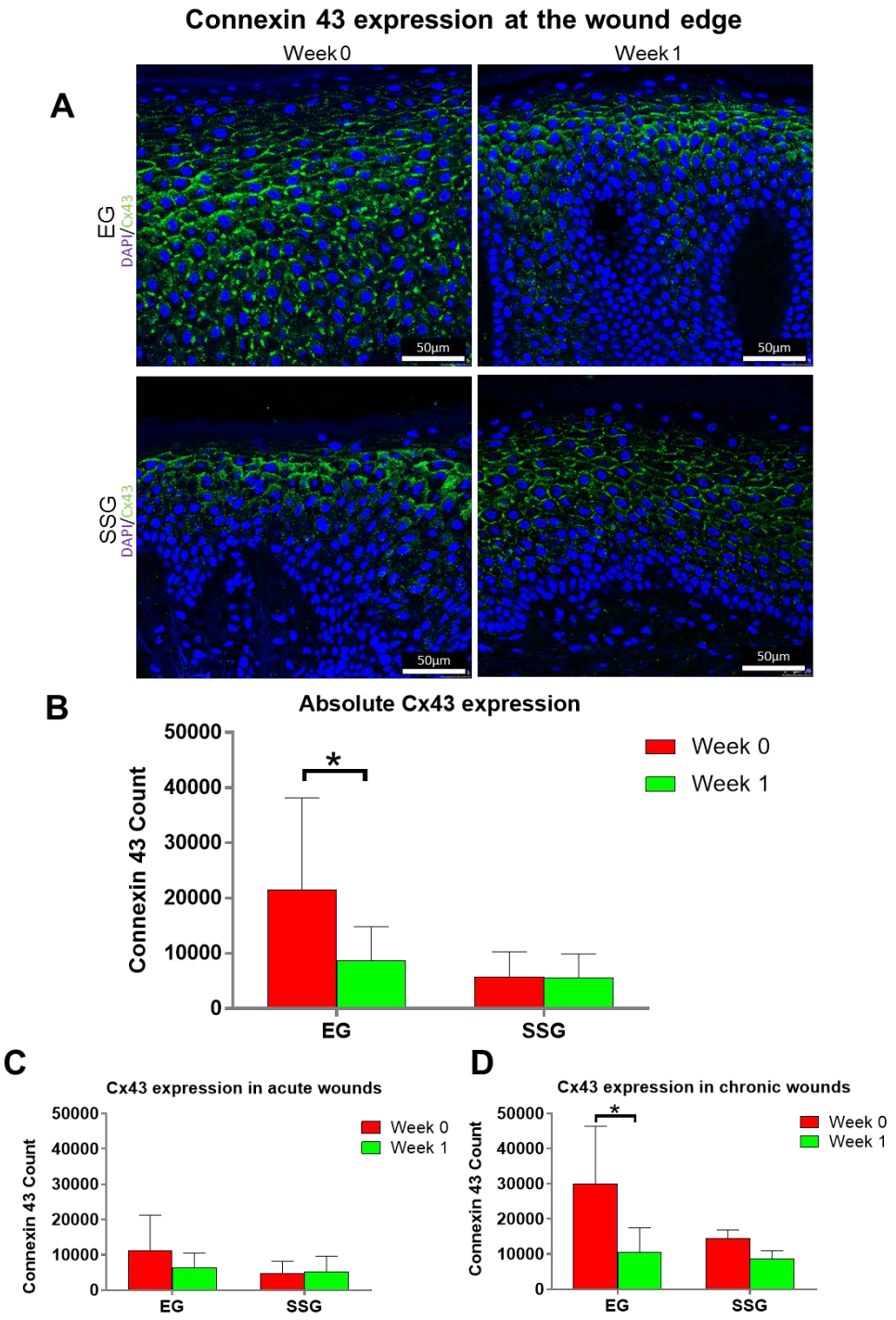


Figure 7.14 Connexin 43 expression at the wound edge

(A) Confocal images of Cx43 expression in each group at week 0 and 1. Scale bar = 50µm. Magnification 40x. (B) Mean absolute Cx43 expression. (C, D) Subgroup analysis of mean Cx expression in acute wounds and chronic wounds. Values expressed as mean ± standard deviation. *P<0.05 (paired t-test)

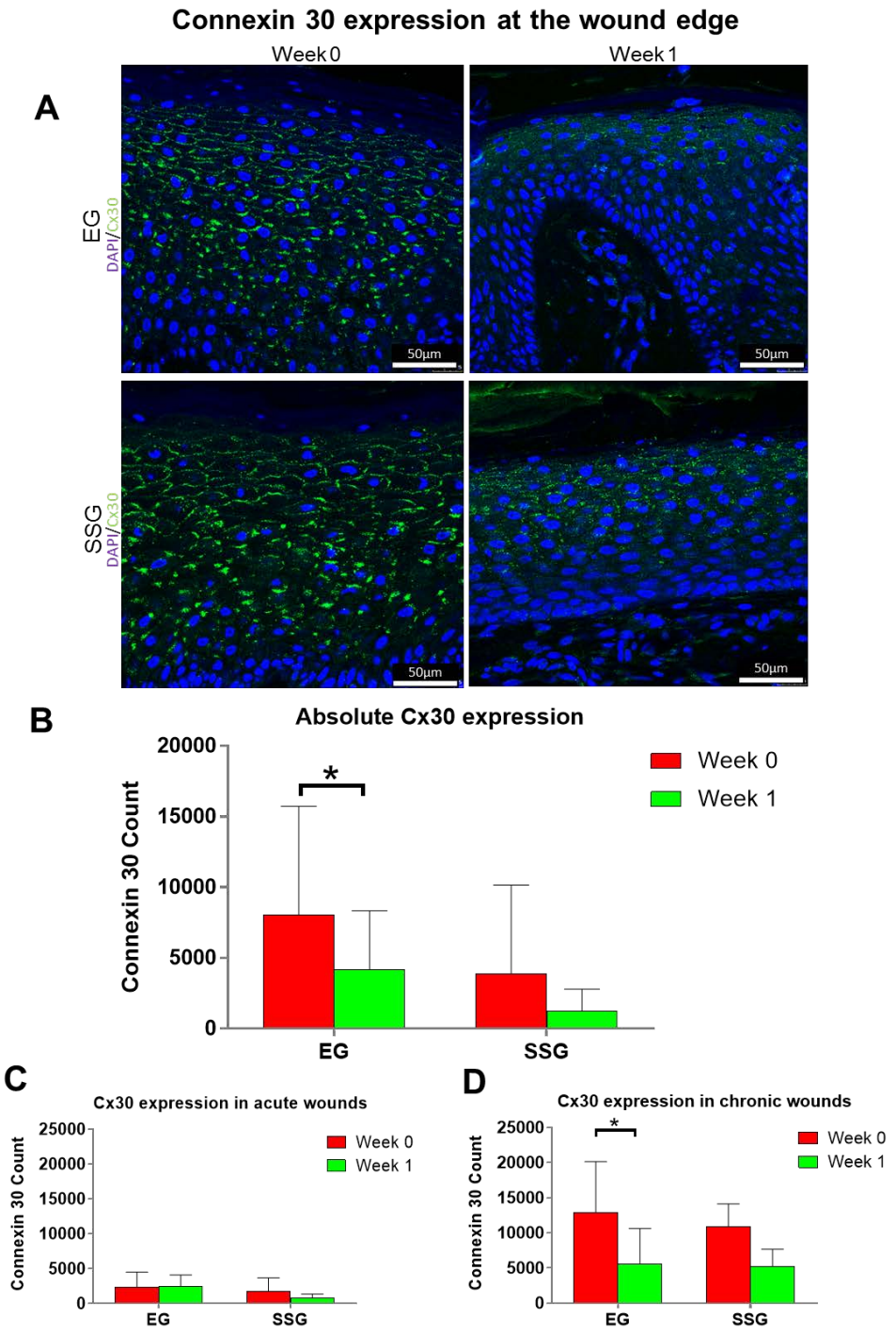


Figure 7.15 Connexin 30 expression at the wound edge

(A) Confocal images of Cx30 expression in each group at week 0 and 1. Scale bar = 50µm. Magnification 40x. (B) Mean absolute Cx30 expression. (C, D) Subgroup analysis of mean Cx expression in acute wounds and chronic wounds. Values expressed as mean ± standard deviation. *P<0.05 (paired t-test)

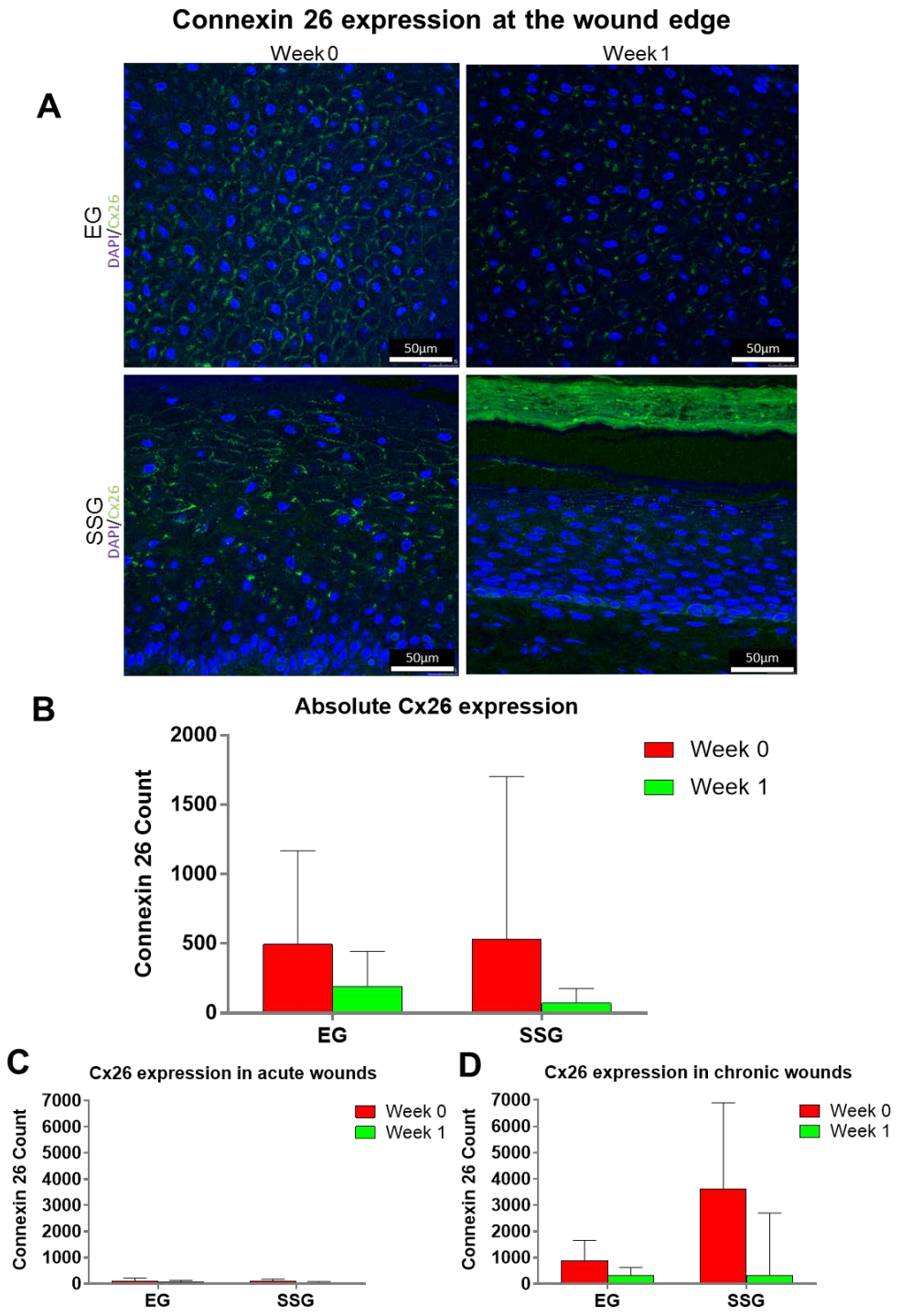


Figure 7.16 Connexin 26 expression at the wound edge

(A) Confocal images of Cx26 expression in each group at week 0 and 1. Scale bar = 50µm. Magnification 40x. (B) Mean absolute Cx26 expression. (C, D) Subgroup analysis of mean Cx expression in acute wounds and chronic wounds. Values expressed as mean ± standard deviation. *P<0.05 (paired t-test)

7.4.2 Analysis of wound bed biopsy

7.4.2.1 Histological feature of wound bed

The histological observation of the wound bed biopsies revealed distinct feature between groups. At week 1, increased inflammatory cells were seen after EG, both in acute and chronic wounds (Figure 7.17 A, B). Multinucleated cells, which are neutrophils, were observed to be increased after EG. In addition, chronic wound treated with EG demonstrated a change in the density of extracellular matrix, whereby it was noted to be more compact and dense.

In the wounds treated with SSG, the newly integrated skin graft was visible as a clear layer of epidermis (Figure 7.17 C). A reduction in expression of inflammatory cells was observed.

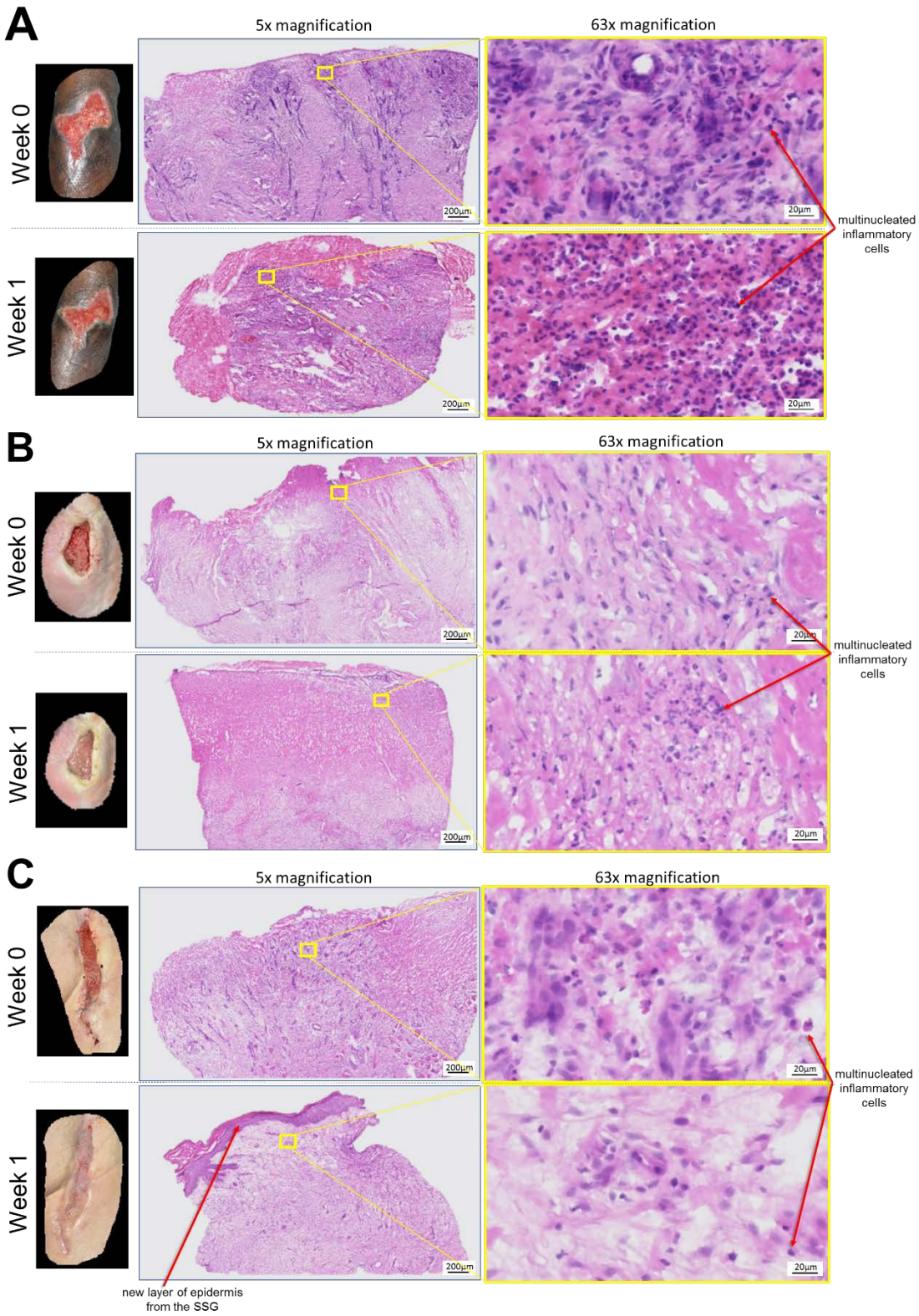


Figure 7.17 Histology of the wound bed of wounds treated with EG and SSG.

(A) H&E stained section of the acute wound treated with EG and the corresponding clinical photograph of the wound at week 0 and week 1 at 5x and 63x magnification. Increased

expression of inflammatory cells was observed after treatment. (B) H&E stained section of the chronic wound bed treated with EG and the corresponding clinical photograph of the wound at week 0 and week 1 at 5x and 63x magnification. Increased expression of inflammatory cells was observed after treatment. The ECM of the wound bed after treatment appears more compact and dense compared to before treatment. (C) H&E stained section of the wound bed treated with SSG and the corresponding clinical photograph of the wound at week 0 and week 1 at 5x and 63x magnification. A clear layer of epidermis is seen at week 1 along with decreased expression of inflammatory cells.

7.4.2.2 Ki67 expression in wound bed biopsy

A general increase in the nuclear expression of Ki67 antigen was observed, which was significant in the EG group (p=0.002, paired t-test) (Figure 7.18). Subgroup analysis revealed significantly increased expression of Ki67 in both the acute (p=0.017, paired t-test) and chronic (p=0.048, paired t-test) wounds after EG. Despite an increased expression of Ki67 after treatment in the SSG group, statistical significance was not achieved (absolute Ki67 expression: p=0.124, paired t-test; acute wound: p=0.124, paired t-test; chronic wound: no statistical test performed due to single sample in this group)

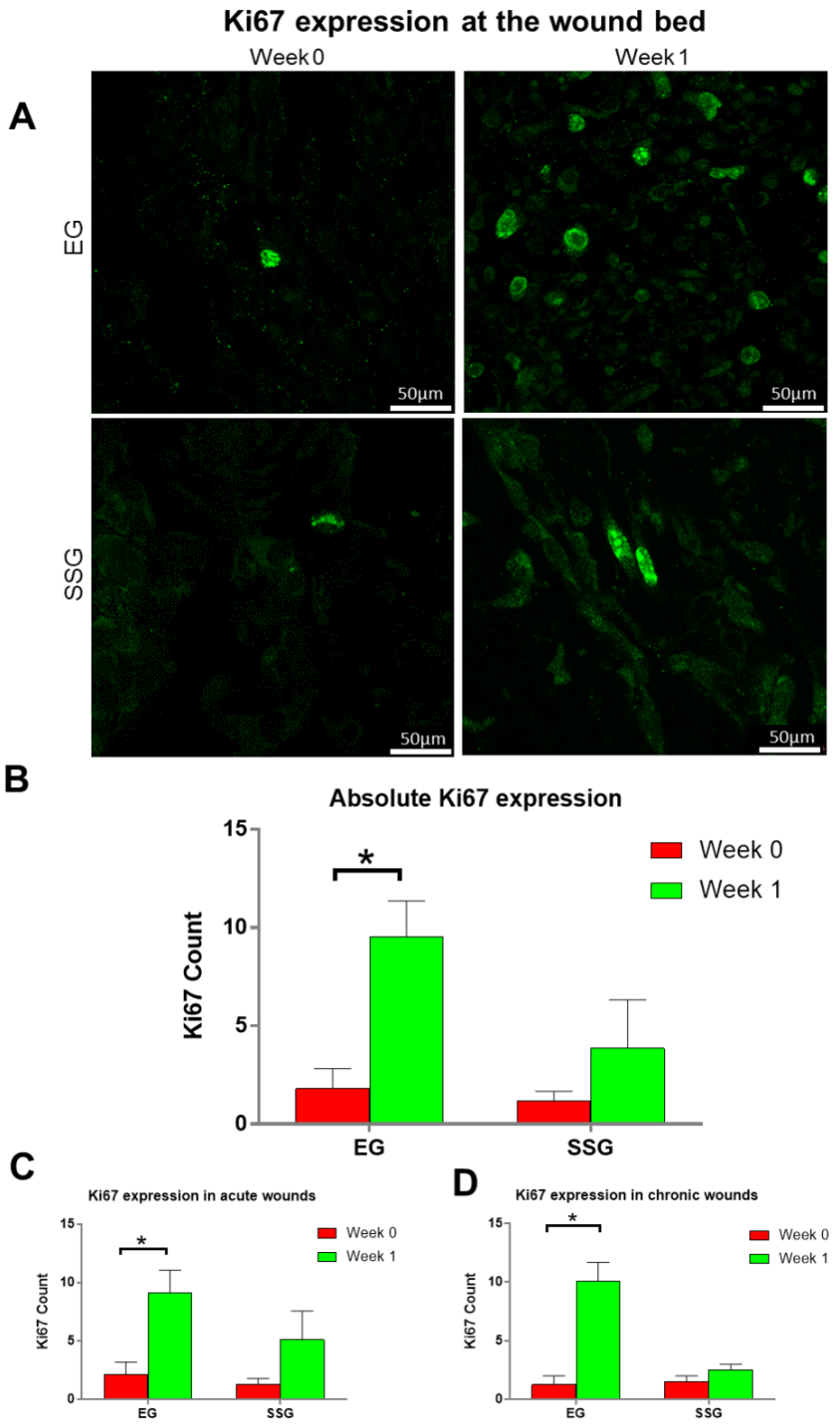


Figure 7.18 Ki67 expression at the wound bed

(**a**) Confocal images of Ki67 expression in each group at week 0 and 1. Ki67 is stained green (excited with 488nm laser). Scale bar = 50µm. Magnification 40x. (**b**) Mean absolute Ki67 expression. (**c, d**) Subgroup analysis of mean Cx expression in acute wounds and chronic wounds. Values expressed as mean ± standard deviation. *P<0.05 (paired t-test)

7.4.2.3 Cytokeratin 14 expression in wound bed biopsy

Cytokeratin 14 expression in the wound bed biopsies was observed in all wounds treated with SSG (Figure 7.19). No positive staining was observed in any of the wounds treated with EG at week 1 post grafting.

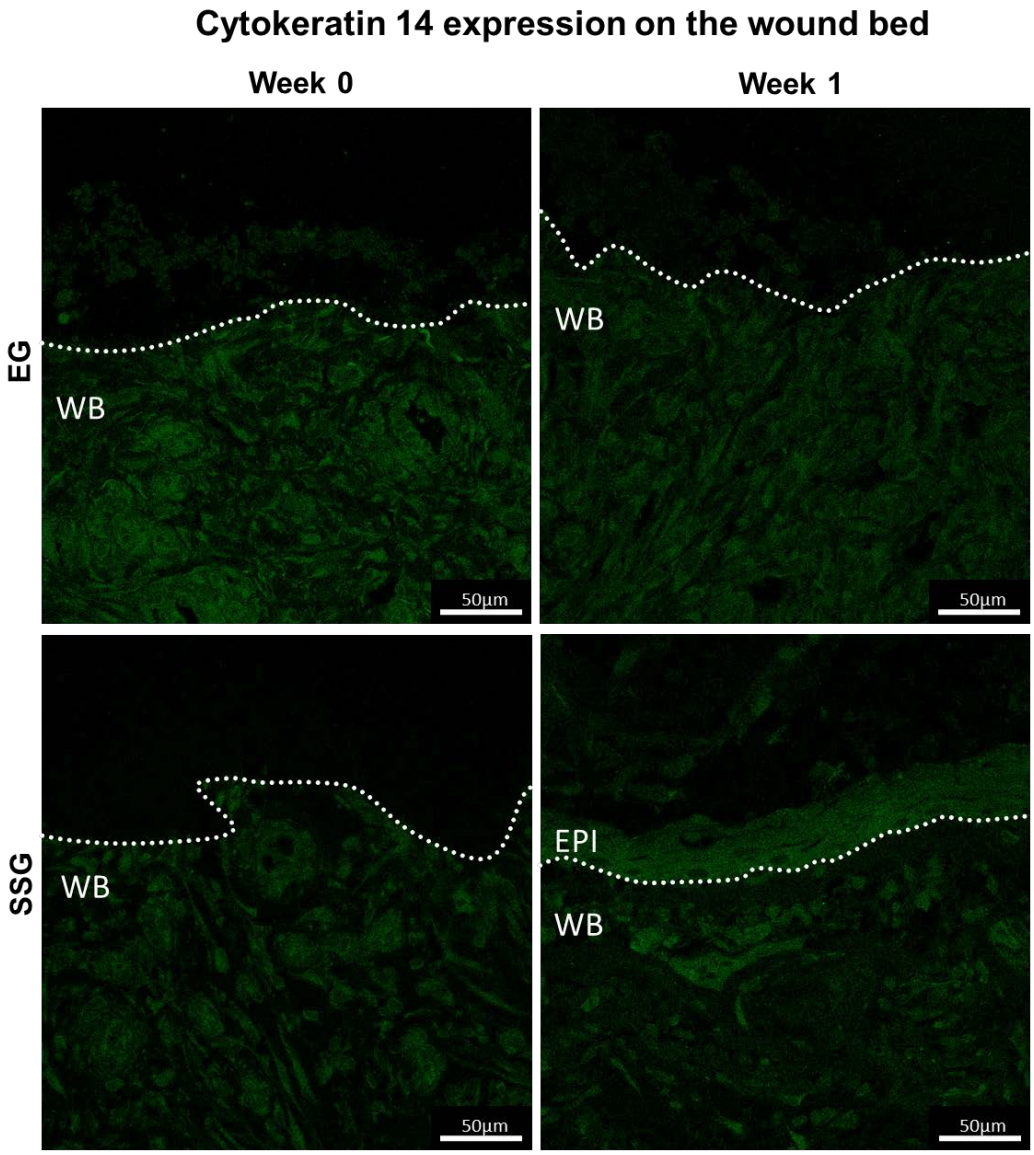


Figure 7.19 Cytokeratin 14 expression on the wound bed

Confocal images of cytokeratin 14 expression in each group at week 0 and 1. Cytokeratin 14 staining was seen at week 1 in the SSG group. Epi = epidermis, WB = wound bed. Dotted line represents the surface of the wound bed. Cytokeratin 14 is stained green (excited with 488nm laser). Scale bar = 50µm. Magnification 40x.

7.4.2.4 Cytokeratin 6 expression in wound bed biopsy

Cytokeratin 6 expression in the wound bed biopsies was observed in all wounds treated with SSG (Figure 7.20). No positive staining was observed in any of the wounds treated with EG at week 1 post grafting.

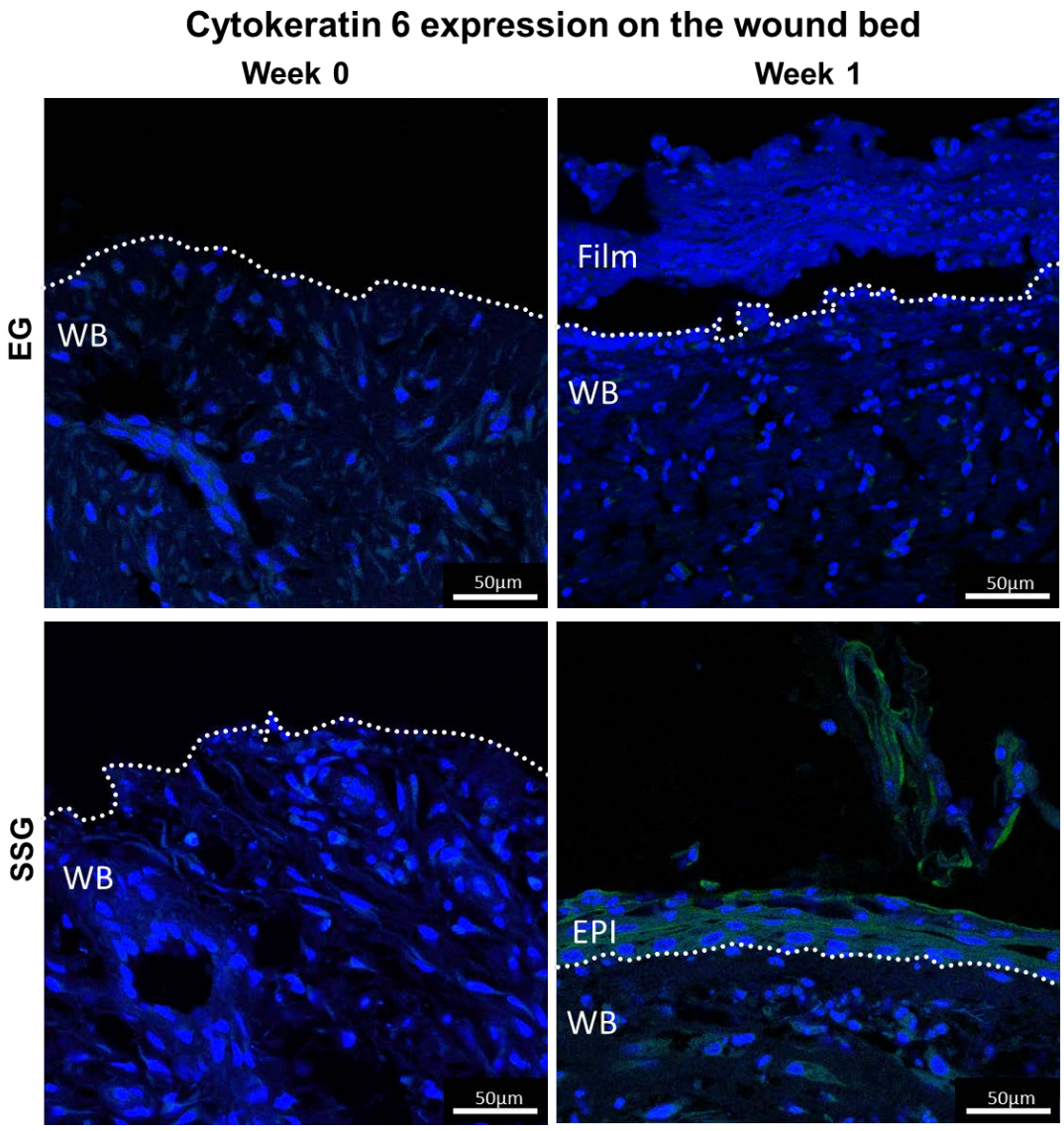


Figure 7.20 Cytokeratin 6 expression on the wound bed

Confocal images of cytokeratin 6 expression in each group at week 0 and 1. Cytokeratin 6 staining was seen at week 1 in the SSG group. Epi = epidermis, WB = wound bed, Film = clear layer of film/wound fluid overlying wound. Dotted line represents the surface of the wound bed. Cytokeratin 6 is stained green (excited with 488nm laser) while the nuclei is stained blue (excited with 405nm laser). Scale bar = 50µm. Magnification 40x.

7.5 Discussion

This study examined the clinical efficacy of EG and SSG and the mechanism of healing between these two autologous skin grafts. This is the first RCT to compare EG against SSG and is the first time that the in vivo mechanism of healing of EG is being outlined and compared against SSG.

The clinical results proved the non-inferiority of EG against SSG for wound healing. EG, however, demonstrated superior outcome in the donor site with shorted healing time, lower morbidity, and higher patient satisfaction. The donor site of EG displayed similar pigmentation, pliability and vascularity to the surrounding skin, with consistently short healing time in all patients. Hence, the key advantage of EG is the faster donor site healing and lower donor site morbidity which is associated with better patient satisfaction. This likely has a major positive implication to clinical practise as SSG, the current primary choice of autologous skin graft, often results in donor site which is painful, prone to infection and requires meticulous donor site management [162]. Furthermore, the optimal donor site dressing for SSG is yet to be determined and a systematic review of donor site dressings found no clear evidence to support the choice of a particular dressing [163]. Hence, the rapid donor site healing of EG does not only improve patient satisfaction, but likely reduces cost and healthcare resources as it only requires a single application of a simple film dressing.

Although the proportion of wound healing at 6 weeks and 12 weeks was slightly higher in the SSG group, this difference was not statistically significant. This study was not powered to detect the difference in wound healing outcome, instead it was powered to detect the difference in the donor site morbidity, as the wound healing outcome was assumed to be comparable between both treatment groups based on pilot data and historical control. However, despite the difference in the mean time for complete wound healing of about 1 week, there was no reported difference in patient satisfaction for the wound site between both treatment groups. Although the cost was not calculated for the purpose of this thesis, the lower

cost of EG with CelluTome (as reported in Chapter 6) and faster donor site healing reduces the number of dressing changes and clinic visits, hence is expected to have a lower overall cost.

The mechanistic study revealed that both the autologous skin grafts have different mechanism of healing. Significant downregulation of Cx43 and Cx30 was seen at the wound edge after EG, especially in chronic wounds. In this study, the Cx proteins were used as a marker of keratinocyte migration from the wound edge. The downregulation of Cx proteins by antisense have previously been shown to accelerate keratinocyte and fibroblast migration from the wound edge (reviewed in Chapter 1). The downregulation of Cx proteins in this study suggests the increased migration of keratinocytes from the wound edge into the wound bed in chronic wounds after EG. Despite demonstrating a reduced expression of Cx43 and Cx30 in acute wounds, it was not statistical significant as these proteins were known to be only minimally overexpressed in acute wounds as compared to chronic wounds. Hence, the low Cx levels still suggests that keratinocytes were actively migrating into the wound. This correlates with our previous clinical observation whereby an acute wound treated with EG demonstrated accelerated healing compared to another untreated acute wound in the same patient[164].

A change in the distribution of Cxs at the wound edge was also observed, whereby the Cxs were observed to be confined to the superior part of the epidermis and less expressed in the basal layer after treatment, emulating the expression pattern of an acute wound. As Cxs were known to act as a nexus interacting with other adhesion molecules and cytoskeletal components, the decreased expression in the basal layer suggests reduced adhesion and increased cytoskeletal dynamics, hence increased migration of the actively proliferating basal cells into the wound bed after treatment.

The wound bed, on the other hand, demonstrated increased inflammatory cells and proliferative marker (Ki67) expression after EG, suggesting the activation of the wound bed after grafting. Similar activation was not seen after SSG as the grafted skin forms an epithelial coverage for the wound, hence progressing towards the phenotype of a healed wound[165].

In chronic wounds, the wound bed activation after EG was accompanied by re-organisation of ECM, more compact and dense after treatment. In normal wound healing, the first step of the healing cascade is inflammation which involves recruitment of neutrophils and macrophages that secretes cytokines, chemokines and growth factors, which then initiates the production of collagen, seen clinically as granulation tissue[35, 121]. This process of increased inflammatory cells and collagen deposition were observed in the histology of chronic wounds after EG, confirming the activation of wound bed after EG. The increased collagen content in the ECM is a change which is normally observed in the proliferative phase of wound healing, whereby the growth and deposition of granulation tissue is critical for an inductive and supportive role for re-epithelialisation from the wound edge [166]. Therefore, the transplanted keratinocytes in the form of EG stimulates cell proliferation and creates a conducive micro-environment for healing.

Wound bed activation was seen despite the absence of keratinocytes on the wound bed from the transplanted graft at week 1 after EG, as demonstrated by the absence of cytokeratin 6 and 14. This suggests that EG does not integrate to the wound bed as seen with SSG. A SSG becomes incorporated to the host bed through the process of graft “take”. The success of a graft take depends on the extent and speed at which vascular perfusion is restored. The skin graft take occurs in three phases[165, 167]. The first phase is known as “plasmatic imbibition”, lasting about 24-48 h, which involves movement of fluid, carrying nutrients and dissolved oxygen, from wound bed to the graft. This is followed by “inosculation”, a process of fine network of capillary and fibrin layer ingrowth into the transplanted graft, which is then followed by active invasion by host vessels to produce definitive vasculature for the graft within the fifth or sixth day post-graft day.

The difference in the graft take phenomenon between EG and SSG is likely due to the structural difference between them. A SSG consists of epidermis and a thin layer of dermis, while an EG comprises of the epidermis only. The failure of skin graft take of EG is likely due to the lack of dermis in the EG, which prevents capillary ingrowth into the graft as the epidermis

is an avascular layer. The absence of dermis, however, enables direct interaction between the actively proliferating basal keratinocytes and the wound bed. This interaction enables wound bed activation as the basal keratinocytes carry keratinocyte stem cells and at the same time expresses a cocktail of cytokines and growth factors onto the wound bed (reviewed extensively in Chapter 4.2). Hence, the EG behaves like a bioactive dressing instead of providing an instant wound coverage as a SSG does. Figure 7.21 below summarises the proposed mechanism of healing by EG based on previous reports and our current finding.

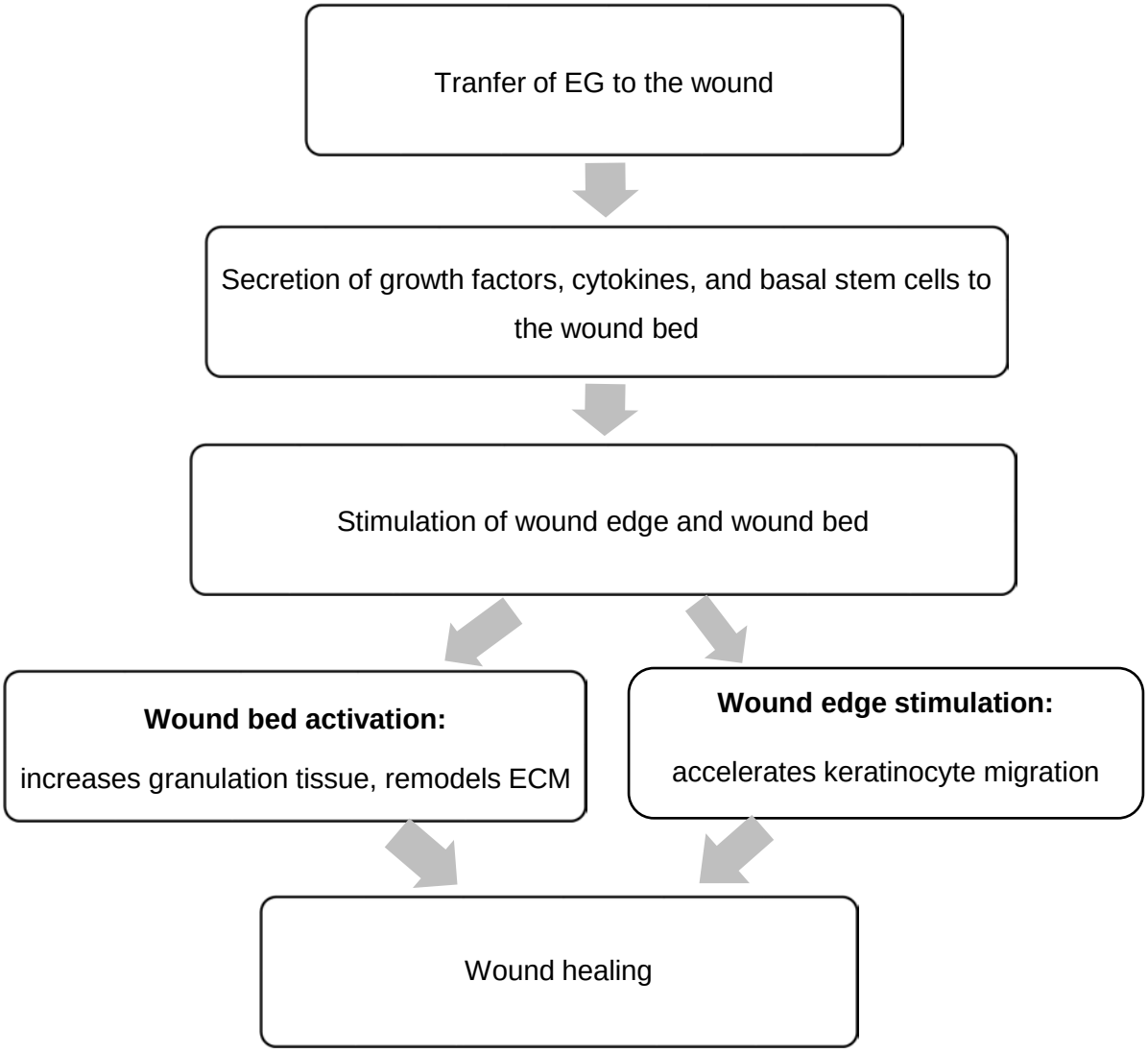


Figure 7.21 Proposed mechanism of wound healing by EG

This novel finding on the mechanism of healing of EG changes our perception towards EG as well as the clinical protocol of wound management after EG. Prior to this, EG was treated as a very fragile skin graft and the wound was usually left untouched for three to four weeks after grafting. Furthermore, there have been mixed reporting on the visibility of the grafts on the wound bed after grafting in several observational clinical study [92, 93]. Our finding provides a robust evidence that EG does not integrate to the wound bed. The increased keratinocyte migration from the wound edge and wound bed activation suggests that patients may benefit from multiple EG, repeating the procedure every 2 or 3 weekly. The repeated stimulation of the wound bed could provide autologous growth factors and cytokines to continuously encourage and promote healing of chronic, hard to heal wounds. Similarly, this may also benefit acute wounds - accelerate healing and reduce the number of dressing changes, hence improving patient's quality of life. However, despite the potential advantage of recurrent stimulation to the wound bed, the repeated grafting would likely reduce the cost effectiveness of this technique. Hence further evaluation is required to assess the advantage of repeated grafting and its cost implication compared to the single application of EG.

There were several limitations to this study. Despite no significant difference in the proportion of wound healing and the wound healing time between both groups, a difference in mean was observed. To determine the accuracy of the difference, a larger sample size that is powered to detect this difference is required. Besides that, despite the dressings used in the first week were standardised for both groups, the dressings used in the subsequent weeks were not kept constant as they had to be tailored to the wound exudate level at that time. However, the variation between dressings were limited. On the other hand, the wound biopsies taken from the patients could be subjected to interpatient variability and some histological features could have undergone minor alteration during the vigorous laboratory process. To limit tissue damage or histological alteration, all laboratory work was only performed after obtaining sufficient training with other samples that were not related to this trial.

7.6 Conclusion

This study suggests that treating wounds with EG has similar efficacy in terms of wound healing as SSG, but hold several advantages including quicker donor site healing, lower donor site morbidity, and superior long-term aesthetic appearance. The two autologous skin grafts have unique healing mechanism due to their structural construct. This study proved the feasibility of setting-up a translation RCT in wound healing and its benefit to patient care. Larger study is necessary to further define the treatment algorithm of EG and to compare the healing outcome on different types of wound.

Part 3: Overall Conclusion and Future Work

Chapter 8

8.0 Overall Conclusion

The main aim of this thesis was to explore the role of GJP in wound healing in two clinical settings: venous disease and epidermal grafting. Various study designs were involved in an attempt to achieve this. The work done in the initial few chapters (Chapter 1-6) generated pilot data in order to design a translational RCT with sound clinical and laboratory methodology (Chapter 7). All the initial studies were invaluable learning steps for me to methodologically progress into designing and executing a clinical trial. Surgical RCTs are often difficult to undertake successfully, and pose practical and methodological challenges. However, RCTs are essential in the assessment of a surgical innovation to inform best practise. Translational trials, such as this, are required to not only evaluate a new surgical technology, but to understand the underlying mechanism, which is an absolute necessity to further advancing surgical practise and improving patient's care.

The initial work in this thesis involves a review on the role of GJP in venous disease followed by an observational study on GJP expression in the early stages of venous disease. The review outlined the current understanding on the pathophysiology underlying skin changes that takes place in venous disease and the role of Cx proteins in poor healing of VLU. Cx proteins were previously known to play an important role in chronic, non-healing wounds, whereby they are overexpressed in these wounds and perturbs re-epithelialisation. Here, the upregulation of Cx in the early stages of venous disease (prior to wound formation) was proposed as both venous disease and upregulation of Cx proteins in chronic wounds are linked to ischaemia-reperfusion cycle and chronic inflammation. Following that, we carried out an observational study involving patients across different stages of venous disease to test our hypothesis that the epidermal Cx proteins were upregulated in the early stages of the disease. In this study, for the first time, we showed that Cx proteins were upregulated prior to wound formation, as early as in patients with varicose veins. This finding demonstrated that varicose veins are associated with Cx upregulation, likely suggesting that the skin in patient with venous disease is preconditioned for poor wound healing even prior to ulceration. This finding raises

a very important and interesting question on whether early treatment of varicose veins could prevent upregulation of Cx proteins and hence reduce the risk of disease progression and ulcer formation. Following that, the next question that needs to be address will the underlying mechanism behind the upregulation of the epidermal Cx proteins in patient with venous disease as it remains unclear if the ischaemia-reperfusion cycle secondary to venous hypertension is the causative factor of Cx upregulation. These questions will be addressed in the future work outlined in Chapter 9.

The next phase of the thesis involves a series of clinical studies to evaluate a novel surgical technology in harvesting EG in the outpatient setting. These studies were designed to generate pilot data for a RCT which was aimed at evaluating the efficacy and mechanism of wound healing of EG. The expression pattern of GJP was used here to evaluate the healing mechanism of EG. This section started with a systematic review to synthesis current evidence on EG for wound healing. I found that current studies lacked level 1 evidence, suggesting a need for a robust RCT to evaluate the efficacy of this technique in clinical practise. I then performed a review on the current understanding of the mechanism of wound healing by EG and outlined the possible mechanism based on the various in-vitro and in-vivo studies. This was then followed by a prospective study to evaluate the feasibility of EG in the outpatient setting as well as a cost analysis and patient reported outcome measure studies. These studies demonstrated that the use of EG in the outpatient setting likely results in improved wound healing and potentially saves healthcare resources and have desirable patient satisfaction. This then raised the question if the outcome of this technique is comparable with the current standard of care which is the SSG. This was addressed by performing the EPIGRAAFT trial to evaluate the efficacy of EG against SSG. Based on our pilot data and historical control, the outcome of both treatments at week 6 were noted to be comparable, hence the wound healing and donor site outcome were evaluated at this time point within the trial. The trial interestingly demonstrated that EG is not inferior to SSG in term of wound healing and is superior compared to SSG in terms of donor site outcomes. EG was noted to have

faster donor site healing with lower donor site morbidity due to the superficial nature of the harvest. This desirable outcome was further coupled with higher patient satisfaction. However, despite no significant difference in the proportion of wound healing and the wound healing time between both groups, a difference in mean healing time was observed. As this study was powered to detect the difference in the donor site morbidity instead of the proportion of wound healing between the two treatments, a larger sample size that is powered to detect this difference is required. This is the basis of the future work outlined in Chapter 10, which aims to determine the difference in healing outcome based on wound aetiology.

This trial also outlined the mechanism of wound healing by EG which was previously not well understood. Prior to this, the mechanism of healing by EG was just studies in several in-vitro and animal studies which involves well controlled environment. In this study, the histological findings were directly correlated with patient information and clinical outcome to further substantiate the clinical and laboratory findings. The finding of this study changed our understanding on the mechanism of healing of EG as we now know that it does not integrate to the wound bed as previously expected, hence behaves like a bioactive dressing instead of a skin substitute. This changes the way these wounds are managed post treatment and suggests the possibility of performing multiple EG or repeated EG. This study was designed to outline the general principles of wound healing by EG with the intention of improving current understanding on wound management involving EG for a more evidence based practise. The precise mechanism of EG, however, remains to be explored. In this study, the histological changes were observed and several protein markers were used to provide a broad understanding on the effect of the EG to the wound. However, the changes in the microenvironment of the wounds such as the changes in expression of growth factor, cytokines, MMPs and molecular regulators needs to be explored. It is important to further understand this mechanism in order to guide improvement of clinical practise. The future work described in Chapter 10 will explore the molecular mechanism of wound healing by EG.

Chapter 9

9.0 Future work for Part 1

The effect of varicose vein treatment on the connexin-43 protein expression in patients with venous disease.

9.1 Chapter summary

The aim of this study is to determine whether treatment of superficial varicose veins reduces Cx43 expression, hence preventing disease progression. In Chapter 3, we identified a step-wise increase of Cx43 with venous disease progression, suggesting that skin is predisposed to poor wound healing and venous ulceration from early stages of the disease. We hypothesise that early treatment of varicose vein could downregulate Cx43, hence prevents disease progression. If the downregulation of Cx43 with the treatment of varicose vein is observed, this would suggest that early treatment prevents future ulceration and further confirms that Cx43 is a biomarker of venous ulceration.

9.2 Aim

We aim to determine whether patients, at CEAP C2 onwards, will demonstrate a reduction of Cx43 expression following treatment of superficial varicose veins. If Cx43 expression responds to treatment, this could firmly suggest that early treatment of varicose veins could prevent future ulceration and re-affirm our finding that Cx is a biomarker of venous ulceration, which could help guide treatment in the future.

9.3 Study Objectives

9.3.1 Primary Objective

To determine the changes in the expression pattern of Cx43 GJP levels in the skin of patients with venous disease with treatment of varicose vein.

9.3.2 Secondary Objective

To determine the role of Cx43 as a biomarker to identify patients who need treatment of superficial veins to prevent leg ulceration.

9.4 Study design

This study is an observational study that involves taking skin biopsies from patients undergoing routine elective surgical care for varicose veins.

A minimum of 8 and maximum 20 patients in the C2 and C4 group of the CEAP classification will be enrolled in this study. The maximum sample size will be 40 patients. The biopsy will be part of a normal surgical incision within the clinical care pathway. A follow up biopsy will be taken at 3 months following treatment. Table 9.1 summarises the inclusion and exclusion

criteria while Figure 9.1 illustrates the patient's journey throughout the study. There are no further therapeutic interventions, changes to patient care, or patient treatment options.

Follow-up protocol

Following the varicose vein surgery, patients will be followed up as per routine clinical care. At 3 months following the initial treatment, patients will be reviewed in the outpatient clinic to assess the clinical outcome. During this follow-up, venous duplex ultrasound will be performed to confirm the absence of venous reflux. Patients will then undergo 4mm punch biopsies at two locations: one above the knee and one between the knee and ankle.

Inclusion Criteria	Exclusion Criteria
1. Male or female 2. Age >18 at time of consent 3. Patients with a venous disease, either C2 or C4 4. Patients undergoing planned surgery 5. Patients with venous reflux as documented by venous duplex ultrasound **6.** Patient understands and is willing to participate and can comply with follow-up regime	1. Previous history of excessive bleeding associated with surgical biopsies or trauma 2. Allergies to local anaesthesia 3. Known uncontrolled Diabetes Mellitus, as measured by HbA1c > 10%. 4. Presence of one or more medical conditions, including renal, hepatic, hematologic, active auto-immune or immune diseases that, would make the subject an inappropriate candidate 5. Any relevant medical problems causing concern

Table 9.1 Inclusion and Exclusion Criteria for Study Participants

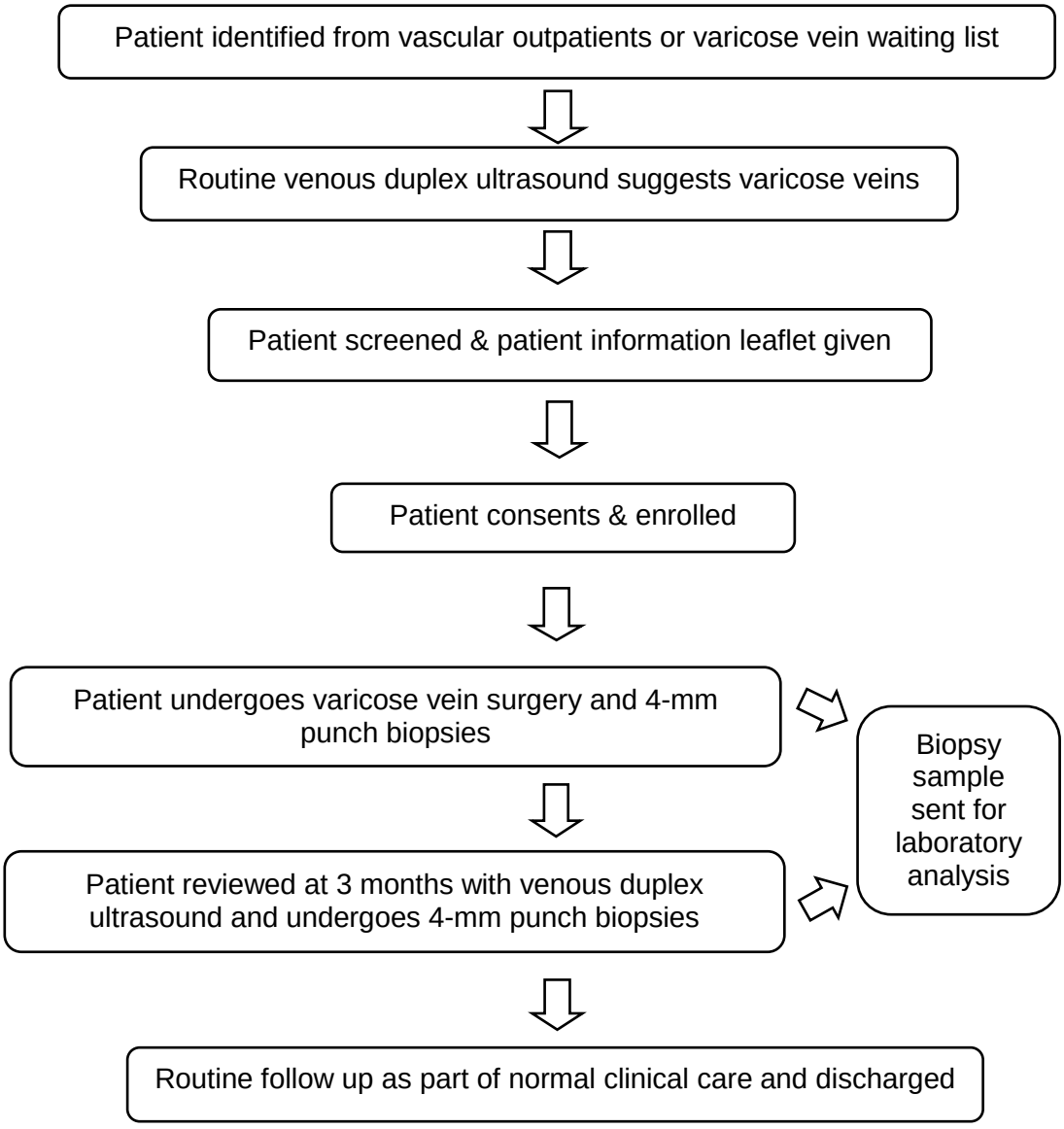


Figure 9.1 Flow chart illustrating patient journey throughout the study

9.5 Biopsy acquisition

Punch wound biopsies (4mm) will be taken from the surgical incision sites during surgery for varicose vein. Biopsies will be taken at the venous catheter insertion site at 2 locations: 5-10cm above the knee and 5-10cm above the ankle.

All biopsies will be taken after written informed consent is obtained from the patients. This study will be executed in accordance with the principles of the Declaration of Helsinki and the recommendations of Good Clinical Practice.

9.6 Laboratory studies methodology summary

Biopsy samples will be sectioned and stained for Cx43 as per previous protocol in Chapter 3. The confocal images of the tissue sections will be used for quantitative analysis of Cx43 levels in the epidermis as per previous protocol to determine the changes in expression with treatment. Supportive tests and analysis will also be performed using RT-PCT and gene expression assay.

9.7 Study setting

Participants will be recruited at two centres: University College London Hospital, London and the Royal Free Hospital, London.

9.8 Statistical Considerations

Sample size

We plan to investigate a minimum of eight and maximum of twenty patients from CEAP C2 and C4. A maximum of 40 patients will be included.

The sample size was determined through discussion with academic clinical and laboratory supervisors. Formal sample calculation is not possible at this time point as the proportion of difference in the Cx43 expression between groups with treatment is not known. We anticipate it would take 12 months to 24 months to recruit patients.

Baseline demographics of the groups will be recorded. The changes in the Cx43 expression with treatment will be recorded using paired t-test (normally distributed data) or Wilcoxon signed-ranked test (non-normally distributed data). Normality test will be performed using Shapiro-Wilk test. Significance will be taken at value $p<0.05$. All statistical analyses will be performed using IBM SPSS Statistics 24 software.

Chapter 10

10.0 Future work for Part 2

A platform adaptive randomised controlled trial on the role of epidermal grafting for wound healing (EPIGRAFT)

10.1 Chapter summary

EPIGRAFT aims to define the treatment algorithm for EG. We will use a novel Multi-Arm Multi-Stage (MAMS) platform protocol that incorporates different types of wounds, both acute wounds and chronic wounds. Further by undertaking early interim analysis we can assess those treatments that are most likely to work. This means as the trial progresses, patients are most likely to receive the best treatment for them (the trial adapts and learns).

In patients with acute wounds we will assess whether EG compared to dressings result in improved wound healing by 8 weeks.

In patients with chronic wounds (more than 3 months duration) we will compare EG, SSG and dressings. The study will look at how well wounds are healing over 8 weeks. If EG works well in chronic wounds at this point, we will offer multiple EG (MEG) where the procedure is performed many times to try and encourage the wound to heal.

We will also undertake a detailed laboratory study to look at how and why EG works by looking at healing wound at the molecular level. Because EG is still a new procedure, the study will need to train nurses and doctors on how to perform the procedure and look after the wound afterwards. We will use new ways of training by using virtual reality technology to watch and advice from afar.

10.2 Study Aim

In Chapter 8 we identified that EG has several advantageous in the donor site outcome, and not inferior to SSG in terms of wound healing. However, a difference in the mean healing time and proportion of wound healing was observed despite not statistically significant. This study aims to define treatment algorithm for EG by outlining the healing outcome based on wound type and correlating this to the molecular mechanism of healing.

10.3 Study design

EPIGRAFT is a novel Multi-Arm Multi-Stage (MAMS) platform protocol, which uses an efficient design to answer multiple questions in a single protocol.
The multi-arm part enables two trials to run concurrently: acute wounds (EPIGRAFT ACUTE) and chronic wounds (EPIGRAFT CHRONIC). They will start as simple randomisations. Following interim analysis, the EPIGRAFT CHRONIC trial will adapt to include new comparisons.

The multi-stage part enables the possibility to discontinue randomisation to arms not showing sufficient activity, based on pre-planned, interim, lack of-benefit analyses (at week 8); further to adapt and add new arms as new questions arise. This Platform protocol reduces the total number of patients needed to answer each question and time needed for trial completion.

10.3.1 EPIGRAFT pathway

Patients will be identified from the standard wound care clinical pathways. The trial protocol will be based in the outpatient dressing clinic. All patients will undergo routine clinical care using a standardised dressing protocol. Following screening, patients will be randomised. All

treatment arms will be based in the dressing clinic. Follow up in the study will be undertaken weekly till wound healing or 8 weeks and assessments made as part of these clinic visits.

EPIGRAFT ACUTE:

Patients will be randomised to either dressings (control arm) or EG group and followed up for 8 weeks. Figure 10.1 illustrates the study design for EPIGRAFT ACUTE.

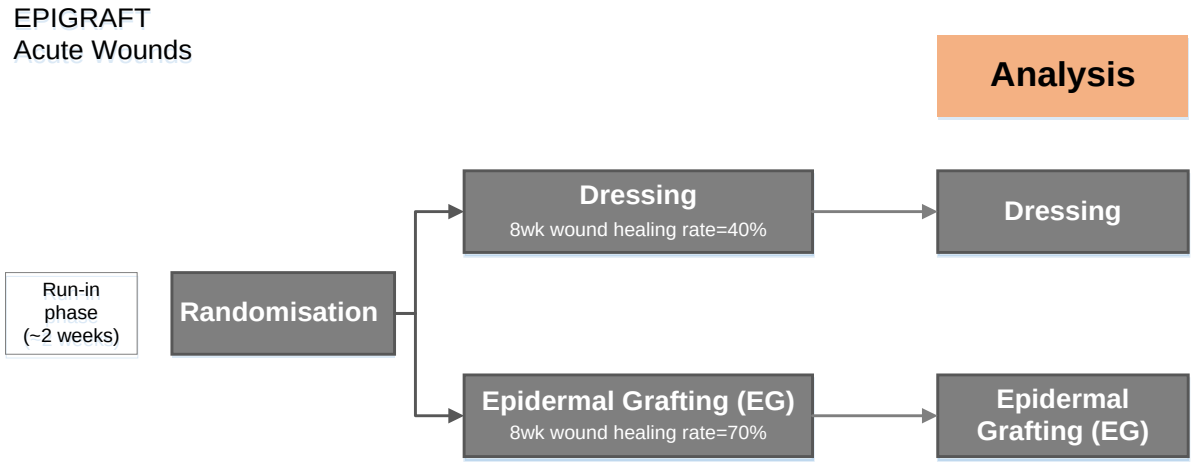


Figure 10.1 Study design for EPIGRAFT ACUTE

EPIGRAFT CHRONIC:

Patients will be randomised to either dressings, EG or SSG. MEG arm will be introduced after Stage 1 analysis. Following interim analysis, treatments that deemed beneficial will proceed to Stage 2(final analysis stage). Treatments that are deemed lack-of-benefit will be excluded from Stage 2. Figure 10.2 illustrates the study design for EPIGRAAFT CHRONIC.

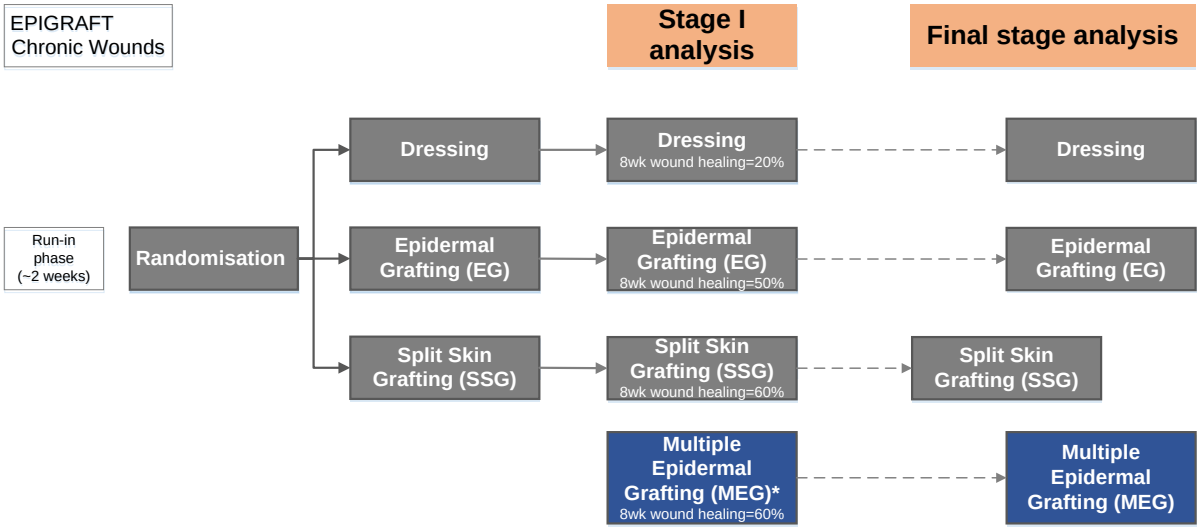


Figure 10.2 Study design for EPIGRAAFT CHRONIC

10.3.2 Mechanistic components of the study

Epidermal grafts are expected to behave like biologically active dressings, modulating wound healing response and are able to stimulate endogenous process of wound healing. Therefore, we further propose that the overarching mechanism of action of EG will be more as a bioactive wound dressing than traditional skin coverage (as seen in SSG) (hence the MEG arm of the trial).

All patients within the trial will undergo 4mm punch biopsy at week 0 and week 1 after treatment, as per previous protocol (described in Chapter 7.1.7). The tissue will be stored in RNAlater (Sigma-Aldrich) and immediately stored at -80^{o}C to preserve the RNA. Molecular analysis will be performed in collaboration with Blizard Institute, Queens Mary University London. Molecular analysis will be performing using RNA sequencing and ATAC sequencing. These techniques will allow the quantification of growth factors, cytokines, inflammatory markers, MMPs as well as other regulators of wound healing. The expression pattern of these factors before and after treatment will be determined.

Wound exudate sampling will be performed by applying a filter paper onto the wound for 2 minutes until it is moist. The filter paper will then be stored in a sterile vial and transferred to the laboratory. We will perform ELISA to determine the type and concentration of growth factors expressed by the EG and its response to the wound, both pre- and post-grafting, comparing it with SSG, MEG and control (dressings only).

10.3.3 Training and education

The technique of EG, protocol of wound care, dressing changes and wound appearances requires a 'learning curve'. Consequently, to ensure consistency of training, the EPIGRAFT lead center will provide centralised training masterclasses with advanced practice guidelines. This will be supported by augmented reality technology to enable onsite supervision and training at all centers. Fidelity of implementation will be formally assessed in Stage 2 of EPIGRAFT CHRONIC by the nested educational sub-study.

10.3.4 Trial team and collaboration

EPIGRAFT will be led by a multidisciplinary team of clinical trialists & academics (MRC & UCL), clinicians (consultants, trainees and nurses) who developed the technique in the NHS. This study will be carried out with industry partnership (Acelity).

10.4 Study Setting

Royal Free Hospital London will be the leading centre. The trial will be extended to 6 other plastic surgery units within London before expending to other centres within UK via trial networks.

10.5 Study population

Two populations of patients with significant wounds will be enrolled concurrently:
EPIGRAFT Acute wound trial: wounds less than 3 months in duration
EPIGRAFT Chronic wound trial: wounds greater than 3 months in duration

A wound is defined as, an epidermal deficit of greater than the size of a 50pence coin and less than the size of a palm. All patients will be those referred, following assessment and review, by a Consultant Plastic Surgeon, where SSG would be a reasonable option. There is no clear cut scientific definition of the ideal wound for this study but this clinical definition has been validated in our observational study (Chapter 5) and pilot RCT (Chapter 7 and 8).

10.6 Eligibility criteria

Inclusion criteria are as follows:

1. Age ≥ 18 years

2. Wound measuring more than 1 cm x 1 cm and less than 6 cm x 6 cm (1 % total body surface area)

3. Clean, healthy granulating bed

4. Patients will be required to understand and be willing to participate in the trial and be able to comply with the weekly visits and follow-up regime

Exclusion criteria are as follows:

1. Infected wound

2. Wound at the plantar of the foot

3. Unsuitable for SSG

4. Previous history of excessive bleeding associated with surgical biopsies or trauma

5. Uncontrolled diabetes mellitus, as measured by HbA1c ≥ 10 percent

6. Presence of one or more medical conditions including renal, hepatic, hematologic, active auto-immune or immune diseases

7. Use of systemic steroid or immunosuppressant

8. Not fit for surgery (ASA classification ≥ 4)

10.7 Interventions

10.7.1 Run-in-phase

All patients will undergo a 'run in phase' that will follow the routine dressing clinic protocol, to ensure appropriate debridement for wound bed preparation, that is free from infection

(clinically clean and two clear wound swabs). Following trial enrolment & informed consent, baseline 3D wound photographs will be taken for audit control and validation.

10.7.2 Split skin grafting

Patients will undergo this procedure in the operating theatre or in minor operations designated clinic room under general or local anaesthetic. SSG will be harvested from the thigh using an air dermatome, set to cut at the thickness of 5-10/1000 inch and meshed by 1:1.5. The wound will be dressed with Adaptic Touch (Systagenix), gauze and a Mefix or wool and crepe bandage depending on the site of the graft. The donor site will be dressed with Kaltostat (Alginate dressing) with a 2.5cm overlay beyond the wound margins and secured with Mefix. As per standard clinical practise, the graft will be checked at day 7 ± 2, and then on a weekly interval.

10.7.3 Epidermal grafting

Prior to grafting, the wound and donor sites are cleaned by the operating surgeon. The harvesting device will be applied to the donor site (thigh) for 3-40 min to harvest the EG as per existing clinical practice. The harvested epidermal grafts will then be transferred onto the wound using a non-adhesive silicone dressing (Adaptic Touch, Systagenix). The wound will then be dressed with a secondary dressing based on the wound type and exudate amount as deemed appropriate by the treating clinician. The dressings will be secured with a crepe bandage or a Mefix dressing (Mölnlycke Health). The donor site will be dressed with Tegaderm (3M). The wound and donor site will be reviewed on day 7 ± 2 post-grafting, and then on a weekly interval.

10.7.4 Multiple epidermal grafting (MEG)

The procedure will be identical to a single epidermal grafting. However, in line with mechanistic hypothesis that EG acts as a 'biological dressing' the procedure will be repeated at each fortnightly follow-up and dressing change. EG will be repeated with sequential application of fresh grafts to the wound, for 6 weeks or until the wound has achieved > 75% wound healing whichever is sooner.

10.7.5 Standard of care by dressings (Control)

Patients will receive standard of care by dressing management in a specialist dressing clinic. For the purpose of EPIGRAFT the dressing used will be standardised and mirror those dressings use in the intervention arms, namely Adaptic touch, Aquacel and then an outer dressing as described.

10.8 Outcomes

All patients will be followed at 7 days (+/-2days) following randomisation then weekly for 8 weeks or trial termination. On every follow-up visit, 3D photographs and measurements of the wound and donor site will be taken. The dressings will be changed and the wound gently cleaned. Fresh dressings will be reapplied.

10.8.1 Primary endpoint

Wound healing, defined as complete epithelialisation of the wound with no need for further dressings, as deemed clinically appropriate by two trial clinicians. 3D wound photographs will be taken for audit control and validation.

10.8.2 Secondary endpoints

- Rate of wound healing, as assessed by weekly 3D photography.
- Time for donor site healing
- Pain scores during dressing changes, reported by patients using Numerical Rating Scale, for donor site and wound dressing.
- Patient satisfaction, by PROMS from randomisation and at 8 weeks.
- Cost analysis of the interventions.
- Mechanistic and Education sub-studies.

10.9 Randomisation and power calculation

Power calculation and randomisation technique was discussed and designed along with MRC UCL Clinical Trials Unit, led by Professor Max Palmer. Sample size calculation was performed by Dr Gordana Jovic (statistician at MRC UCL Clinical Trials Unit).

EPIGRAFT Acute:

Patients with acute wounds, following the 'run in phase', will undergo informed consent then randomised on a 1:1 basis to either standard of care by dressings (control) or intervention by EG. Primary outcome measure is the proportion of patients whose wounds have healed at 8 week after randomisation, predicted to be 40% in control and 70% in EG arm. The comparison between EG and control arms will be done by testing the difference (EG vs. control) in proportion of patients whose wounds have healed at 8 weeks after randomisation. Using targeted power 90% and one-sided significance level 0.05, 90 patients are needed for the comparison of EG vs control (45 control, 45 EG). Table 10.1 outlines the sample size for EPIGRAFT ACUTE.

Arm	**Allocation ratio, Research arm vs Dressing**	**Total number of patients**	**Nominal one-sided significance level [power]**
Dressing	n/a, comparator arm	45	n/a, comparator arm
Epidermal Grafting	1:1	45	0.05 [90%]
Total	n/a	**90**	n/a

Table 10.1 EPIGRAFT Acute Wound randomisation, sample size

EPIGRAFT CHRONIC:

Following the 'run in phase', patients with chronic wounds will undergo informed consent and randomised to either standard of care by dressings(control), EG, SSG or MEG. It was predicted that the 8 weeks wound healing rates are: control 20%, EG 50%, SSG 60%, MEG 60% (lower than acute as 'hard to heal wounds'). EPIGRAFT CHRONIC is a two-stage trial; an early interim analysis will exclude 'lack of effect' intervention and trial feasibility followed by adaption in stage 2 to include a MEG arm. Table 10.2 outlines the sample size for EPIGRAFT CHRONIC.

Stage 1:

Initial randomisation will start between; control, EG, SSG on a 1:2:1 basis. Interim analysis will be performed; EG arm will be compared to control and SSG arm compared to control. The comparison will be done by testing the difference in proportion of patients whose wounds have healed at 8 weeks after randomisation (EG-control and SSG-control) using z-core; EG arm will be tested at 0.28 significance level and SSG at 0.27 with targeted power at 90%. A minimum of 71 patients in total are needed for Stage 1 interim analysis (18 control, 36 EG, 17 SSG).

Stage 2:

Assuming both EG and SSG will continue randomisation to Stage 2 and that MEG arm will be added, the overall allocation ratios would be; control 1, EG 2, SSG 1, MEG 2. To preserve overall type I error at 0.05 and due to the three comparisons being made, significance level of 0.05/3=0.016 and targeted power of 90% was used for the trial design. Sample size was calculated using Multi -Arm Multi-Stage methodology, as described by Bratton et al and implemented in Stata 14.1, -nstagebin- and - nstagebinopt- commands.

An additional 132 patients are required in stage 2.

The total maximum sample size for EPIGRAFT CHRONIC is 203 patients. Final comparison will include a maximum of: 45 control arm patients, 82 EG, 32 SSG, and 44 MEG arm patients. All calculations include 5% of patients expected to be lost to follow-up.

Arm	Allocation ratio, Research arm vs Dressing	STAGE 1 Number of patients to recruit for analysis	STAGE 1 Nominal one-sided significance level [power]	STAGE 2 Total number of patients to recruit¥	Pairwise$^{\pi}$ significance level [power]
Dressing	n/a, comparator arm	18	n/a, comparator arm	45	n/a, comparator arm
Epidermal Grafting	2:1	36	0.28 [96%]	82	0.0164 [90%]
Split Skin Grafting	1:1	17	0.27 [97%]	32	0.0162 [90%]
Multiple Epidermal Grafting	2:1*	n/a	n/a one stage only	44	0.016 [90%]
Total	n/a	**71**	n/a	**203**	n/a

*from Stage 2
¥assuming that EG and SSG will continue recruitment to Stage 2 and that MEG arm will be added. It also accounts for the rate of lost to follow-up
$^{\pi}$one-sided significance level and power for each comparison, EG vs Dressing, SSG vs Dressing, MEG vs Dressing

Table 10.2 EPIGRAFT Chronic Wound randomisation, sample size

10.10 Impact

If EG does not prove to be beneficial over standard of care by dressings in either acute or chronic wounds, we anticipate this technique will cease practice.

If EG does prove beneficial in patients with acute wounds, it is anticipated that that this technique will be proposed for wound management in A&E department or dressing clinics.

If EG is proven beneficial for patients with chronic wounds, it is anticipated that the platform of the EPIGRAFT could be expanded to include other comparators, perhaps as part of a HTA application of industry funded research program.

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List of Awards for PhD

Best Poster Award,

5th Congress of the World Union of Wound Healing Societies, 2016.

2nd Place Oral Presentation for European Venous Prize,

17th Meeting of the European Venous Forum, 2016.

Best Poster Communication Award,

Early Career Physiologists' Symposium, 2016.

Rob Clarke Abstract Award,

The Physiological Society, 2016.

Certificate of Merit for Excellent Abstract Presentation,

Charing Cross International Symposium, 2016.

PLASTA/ASiT Prize for Best Plastic Surgery Poster,

ASiT Surgical Conference, 2016.

IMPACT UCL Ph.D. Studentship Award 2014 – 2017

University College London

List of Funds and Grant Awards for PhD

British Association of Plastic Reconstructive and Aesthetic Surgeons (BAPRAS) Paton-Masser Memorial Fund 2017,

Principal applicant and Research Fellow, "Cellular mechanism of wound healing by autologous skin grafts" at **£5,000.00**

Robert Brown Travel Award 2017, at **£300**

To attend the Symposium on Advanced Wound Care Spring | Wound Healing Society meeting (SAWC Spring | WHS), San Diego, 2017

Rob Clarke Award Travel Grant 2016, at **£200**

To attend the Joint Meeting of the American Physiological Society and The Physiological Society, Dublin, 2016

School of Life and Medical Science Graduate Conference Fund 2016, at **£530.95**

To attend the Vascular Societies Annual Scientific Meeting, Bournemouth, 2015

Acelity Educational Research Grant 2015,

Co-applicant and Research Fellow, "*A Randomised Controlled Trial to Compare Epidermal Grafting with Split Skin Grafting for Wound Healing* (EPIGRAAFT)", at **£80,512.00.**

Covidien IMPACT UCL 2014,

Research Fellow, *"An observational study of connexin-43 protein expression in patients with venous disease"*, at **£65,345.50**.

List of Presentations from PhD

Conference presentations

1. **Cellular mechanism of wound healing by autologous skin grafts**. 30th Annual Symposium on Advanced Wound Care Spring/Wound Healing Society (SAWC Spring/WHS), San Diego, California, April 2017. (Poster Presentation)
2. **Cellular mechanism of wound healing by autologous skin grafts**. SARS Research & Academic Prize Session. ASiT International Surgical Conference, Bournemouth, April 2017. (Oral Presentation)
3. **A Randomised Controlled Trial to Compare Epidermal Grafting with Split Skin Grafting for Wound Healing (EPIGRAAFT Trial).**

   Future Project Prize Session, Society of Academic & Research Surgery (SARS) Annual Meeting, Royal College of Surgeons of Ireland, Dublin, January 2017. (Oral Presentation)

4. **EPIGRAAFT: A Randomised Controlled Trial to Compare Epidermal Grafting with Split Skin Grafting for Wound Healing**.

   Eleanor Davies-Colley Prize PhD Presentation, Division of Surgery UCL Away Day 2016, London, December 2016. (Oral Presentation)

5. **Novel biomarker in venous disease: the role of Connexin 43 to predict poor wound healing**.

   President's Prize Session, BAPRAS Winter Scientific Meeting, London, November 2016. (Oral Presentation)

6. **Connexin as a Biomarker of Poor Wound Healing**.

   Wounds Australia Conference 2016, Melbourne, November 2016. (Oral Presentation)

7. **Cellular mechanism of wound healing by autologous skin grafts.**

   5th Congress of the World Union of Wound Healing Societies, Florence, September 2016. (Oral Presentation)

8. **Connexin expression in venous disease progression: A biomarker for venous ulceration.**

   5th Congress of the World Union of Wound Healing Societies, Florence, September 2016. (Poster Presentation)

9. **Connexin 43 Overexpression in Venous Leg Ulcers and Chronic Venous Disease: A Possible Biomarker for Early Diagnosis**.

   Joint Meeting of the American Physiological Society and The Physiological Society, Dublin, July 2016. (Poster Presentation)

10. **Connexin as a Biomarker of Poor Wound Healing**.

    17th Meeting of the European Venous Forum, London, July 2016. (Oral Presentation)

11. **The CelluTome epidermal graft harvesting system: A patient reported outcome measure and cost evaluation study.**

    BAPRAS Summer Meeting, Bristol, June 2016. (Oral Presentation)

12. **Connexin as a Marker of Venous Ulceration**.

    European Wound Management Association, Bremen, Germany, May 2016. (Oral Presentation)

13. **Systematic Review and Meta-analysis of the Efficacy of Epidermal Grafting for Wound Healing.**

    European Wound Management Association, Bremen, Germany, May 2016. (Oral Presentation)

14. **The Use of a Novel Epidermal Graft Device to Optimise Outpatient Wound Management**.

ASGBI Belfast Surgical Week, May 2016. (Oral Presentation)

15. **The CelluTome epidermal graft harvesting system: A patient reported outcome measure and cost evaluation study**.

    ASGBI Belfast Surgical Week, May 2016. (Oral Presentation)

16. **Connexin as a Biomarker for Venous Ulceration.**

    European Society of Vascular Surgery Spring Meeting, London, May 2016. (Oral Presentation)

17. **Connexin Expression in Venous Disease Progression: A Biomarker for Venous Ulceration.**

    Charing Cross International Symposium, London, April 2016. (Oral Presentation)

18. **To CelluTome or not to CelluTome? A Patient Reported Outcome Measure and Cost Evaluation Study.**

    Association of Surgeons in Training (ASiT) Conference, Liverpool, March 2016. (Poster Presentation)

19. **Connexin as a Biomarker for Venous Ulceration.**

    SARS Research & Academic Prizes Session, Association of Surgeons in Training (ASiT) Conference, Liverpool, March 2016. (Oral Presentation)

20. **Systematic Review and Meta-analysis of the Efficacy of Epidermal Grafting for Wound Healing.**

    Association of Surgeons in Training (ASiT) Conference, Liverpool, March 2016. (Poster Presentation)

21. **A Prospective, Multicentre Study on Epidermal Grafting to Optimise Outpatient Wound Management.**

    Society of Academic & Research Surgery (SARS) Annual Meeting, Royal College of Surgeons of England, London, January 2016. (Oral Presentation)

22. **A Prospective, Multicentre Study on Epidermal Grafting to Optimise Outpatient Wound Management.**

    The Vascular Societies Annual Scientific Meeting, Bournemouth, November 2015. (Oral Presentation)

23. **Connexin Expression in Venous Disease Progression: A Biomarker for Venous Ulceration.**

    The Vascular Societies Annual Scientific Meeting, Bournemouth, November 2015. (Oral Presentation)

24. **Connexin Expression in Venous Disease Progression**.

    Eleanor Davies-Colley Prize Session, Division of Surgery UCL Away Day 2015, Cecil Sharp House, London, September 2015. (Oral Presentation)

25. **A Prospective Study on the Use of a Novel Epidermal Grafting Machine to Optimise Outpatient Wound Management.**

    Combined Summer Scientific Meeting BAPRAS and Royal Belgian Society for Plastic Surgery, Belgium, June 2015. (Oral Presentation)

26. **The use of Epidermal Grafts to Optimise Outpatient Wound Management**.

    European Wound Management Association, London, May 2015. (Poster Presentation)

27. **Epidermal Grafts: The Use of CelluTome to Optimise Outpatient Wound Healing, A Trainee's Experience.**

    Make Better Summit international, Rome, November 2014. (Oral Presentation)

Invited lectures

1. **Biological strategies for wound healing.**

   Charing Cross International Symposium, London, April 2017.

2. **Vascular Needs of the At-Risk Foot and its Management.**

   1st Integrated Vascular Staff Development Day Symposium, Central and North West London Podiatry Department, London, January 2017

3. **Stem cells for chronic wound**.

   11th National Wound Congress 2016, Antalya, Turkey, December 2016.

4. **Lymphoedema in conjunction with diabetic foot ulcer**.

   11th National Wound Congress 2016, Antalya, Turkey, December 2016.

5. **Epidermal grafting for wound healing**.

   11th National Wound Congress 2016, Antalya, Turkey, December 2016.

6. **Achieving wound closure through epidermal grafting in an outpatient setting**.

   Wounds Australia Conference 2016, Melbourne, Australia, November 2016.

7. **Venous leg ulceration and its associated skin changes**.

   Tissue Viability Symposium 2016, University College London Hospital, November 2016.

8. **The mechanism of healing of epidermal graft.**

   A*celity™ and the Royal Free Hospital COE Masterclass on Epidermal Grafting*, Royal Free Hospital London, April 2016.

9. **Designing Clinical Research to Introduce New Technology in Surgery.**

iBSc Surgical Sciences, University College London, November 2015.

List of Publications from PhD

Peer-reviewed manuscripts

1. M Kanapathy, O Smith, N Hachach-Haram, N Bystrzonowski, A Mosahebi, T Richards. **Systematic review and meta-analysis of the efficacy of epidermal grafting for wound healing**. *International Wound Journal*, Jan 2017

2. N Bystrzonowski, N Hachach-Haram, M Kanapathy, T Richards, A Mosahebi. **Epidermal graft accelerates the healing of acute wound: A self-controlled case report**. *Plastic and Reconstructive Surgery Global Open*, Nov 2016

3. M Kanapathy, N Hachach-Haram, N Bystrzonowski, J Connelly, E O'Toole, D Becker, A Mosahebi, T Richards. **Epidermal grafting for wound healing: A review on the harvesting systems, the ultrastructure of the graft and mechanism of wound healing**. *International Wound Journal*, October 2016

4. OJ Smith, SJ Edmonson, N Bystrzonowski, N Hachach-Haram, M Kanapathy, T Richards, A Mosahebi. **The CelluTome epidermal graft harvesting system: A patient reported outcome measure and cost evaluation study.** *International Wound Journal*, August 2016

5. M Kanapathy, O Smith, N Hachach-Haram, N Bystrzonowski, A Mosahebi, T Richards. **Protocol for a systematic review of the efficacy of epidermal grafting for wound healing.** *Systematic Reviews*, June 2016.

6. M Kanapathy, N Hachach-Haram, N Bystrzonowski, K Harding, A Mosahebi, T Richards. **EPIGRAAFT: Epidermal grafting versus split thickness skin grafting for wound healing: study protocol for a randomised controlled trial**. *Trials Journal*, May 2016

7. N Hachach-Haram, N Bystrzonowski, M Kanapathy, O Smith, K Harding, A Mosahebi, T Richards. **A prospective, multicentre study on the use of epidermal grafts to optimise outpatient wound management.** *International Wound Journal*. Feb 2017;14(1):241-249. Epub 2016 Mar 20

8. N Hachach-Haram, N Bystrzonowski, M Kanapathy, S Edmonson, L Twyman, T Richards, A Mosahebi. **The Use of Epidermal Grafting for the Management of Acute Wounds in the Outpatient Setting**. *Journal of Plastic, Reconstructive and Aesthetic Surgery*, Sep 2015;68(9):1317-8.

9. M Kanapathy, M Portou, J Tsui, T Richards. **Diabetic foot ulcers in conjunction with lower limb lymphedema: pathophysiology and treatment procedures**. *Chronic Wound Care Management and Research*, August 2015, Volume 2015:2;129—136.

Book Chapter

1. M Kanapathy, J Hunckler, A Mosahebi, T Richards. **Biological strategies for wound healing.** *CX Vascular and Endovascular Consensus Update – 39th edition*. BIBA Publishing, 2017. London, UK.

Appendix A: Part 1

Supplementary Information

A1: Ethical Boards Approvals

Health Research Authority

NRES Committee London - South East

Bristol Research Ethics Committee Centre

Level 3, Block B Whitefriars, Lewins Mead,

Bristol BS1 2NT

Tel: (0117) 3421382

05 June

2014

Mr Toby Richard
Senior Lecturer in Surgery, Honorary Consultant in Vascular & Endovascular Surgery
University College Hospital
UCL Division Surgery and
Interventional Science Fourth floor,
74 Huntley Street
London WC1E 6AU

Dear Mr Richard

Study title:	**An observational study determining and comparing connexin 43 protein-expression in adult chronic foot wounds, with further laboratory study of isolated fibroblast migration rates and gap junctional communication**
REC reference:	**11/LO/1483**
Amendment number:	**1**
Amendment date:	**14 May 2014**
IRAS project ID:	**79542**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper [Summary of changes]		

GP/consultant information sheets or letters	1.3	01 April 2014
Notice of Substantial Amendment (non-CTIMP) [First amendment]		14 May 2014
Other [Email with submission attached]		15 May 2014
Other [Muholan(CV) February 2014]		01 February 2014
Participant consent form	1.3	01 April 2014
Participant information sheet (PIS)	1.3	01 April 2014
Research protocol or project proposal [amended protocol 01042014N]	1.3	01 April 2014

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

11/LO/1483: Please quote this number on all correspondence

Yours sincerely

Wai Yeung

Mr Wai Yeung
Research Ethics Committee (REC) Assistant

pp Professor David
Caplin Chair

E-mail: nrescommittee.london-southeast@nhs.net

Copy to: *Ms Shahina Begum-Meah , University College London*
Ms Anna Jones,

NANYANG
TECHNOIOGICAL
UNIVERSITY

Research Support Office

Reg. NO. 200604393R

IRB-2015-05-003

25 May 2015

Professor David Laurence Becker
Lee Kong Chian School of Medicine

NTU INSTITUTIONAL REVIEW BOARD APPROVAL
Project Title: Investigations into wound/disease tissues
(Amount Approved: SGD$132,651.4; to be funded by University College London and Lee Kong Chian School of Medicine. NTU)
I refer to your application for ethics approval with respect to the above project.
The Board has deliberated on your application and noted from your application that your research involves collecting biological data from participants through punch biopsies

You have also confirmed that informed consent will be obtained from the participants and you have guaranteed the confidentiality of your participants' biodata obtained from them,

The documents reviewed are:

a) NTIJ IRB application form dated **04 May 2015**
b) Participant information sheet and consent form: version 1 dated **04 May 2015**
c) Data collection form: version 1 dated **04 May 2015**

The Board is therefore satisfied with the bioethical consideration for the project and approves the ethics application under Expedited review. The approval period is from 25 May 2015 to 05 January 2018. The NTU IRB reference number for this study is IRB-2015-05-003. Please use this reference number for all future correspondence.

The following protocol and compliances are to be observed upon NTU IRB approval

1. All research involving procedures greater than minimal risk on minors (individuals who are less than the legal age of 21 years old) requires IRB approved written Parental Consent and assent from the participant to be obtained before any research protocols can be administered. Minimal risk refers to an anticipated level of harm and discomfort that is no greater than that ordinarily encountered in daily life, or during the performance of routine educational, physical, or psychological examination.

2. Only the approved Participants Information Sheet and Consent Form should be used, it must be signed by each subject prior to initiation of any protocol procedures. In addition, each subject should be given a copy of the signed consent form.

3. Consent forms are important documents therefore they should be stored in the strictest arrangement. Loss of consent form would result in disciplinary action.

4. No deviation from, or changes of, the protocol should be initiated without prior written NTU IRB approval of an appropriate amendment.

5. The Principal Investigator should report promptly to NTU IRB regarding:
   a. Deviation from, or changes to the protocol,
   b. Changes increasing the risk to the subjects and/or affecting significantly the conduct of the trial
   c. All serious adverse events (SAEs) which are both serious and unexpected.
   d. New information that may affect adversely the safety of the subjects of the conduct of the trial.
   e. Completion of the study.

6. Continuing Review Request/ Notice of Study completion form should be submitted to NTU IRB for the following:
   a. Annual review: Status of the study should be reported to the NTU IRB at least annually using the Continuing Review Request/ Notice of Study completion form.
   b. Study completion or termination: Continuing Review Request/ Notice of Study completion form is to be submitted within 4 to 6 weeks of study completion or termination.

7. All Principal Investigators should comply with existing legislation that would have an impact on the domain of their research.

Professor Lee Sing Kong,
Chair, NTU Institutional Review
Board encl.

cc Dean, Lee Kong Chian School of Medicine
Members, NTU Institutional Review Board

A2: Patient Information Sheet

Consultant Vascular Surgeons

Mr O Agu MS FRCS FRCS (Gen)
Mr Toby Richards BSc MBBS FRCS MD
Mr Daryll Baker BSc, PhD, FRCS

Department of Vascular Services
University College Hospital
250 Euston Road
London
NW1 2PG

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PA: Tomisin Olarewaju
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Surgical Admission Enquiries:
Carina Nobrega
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Fax: 0203 447 9217
carina.nogrega@uclh.nhs.uk

Switchboard: 0845 155 5000
Outpatient Clinic Appointments: 0203 447 9393

15/08/2014
TR1/PRN/OOO

UCLH Project ID number: Form version: April 1st 2014 Version 1.3

PATIENT INFORMATION LEAFLET

Study Title: **Connexin-43 protein expression in venous disease**

Principal Researcher: **Dr Muholan Kanapathy**
muholan.kanapathy.13@ucl.ac.uk
07459029770

As a patient of UCLH or RFH NHS Trust you are being invited to take part in a research study. Before you decide whether you would like to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy or it may be obtained from CERES, PO Box 1365, London N16 0BW.

The purpose of the study People with venous disease are prone to poor wound healing and may develop leg ulceration. Leg Ulceration is a serious problem and causes patients to suffer considerable pain and discomfort. Poor wound healing is usually caused by a particular medical condition. These medical conditions include diabetes, infection, varicose veins, or chronic ischaemia (reduced blood supply). The purpose of this study is to understand the reasons why patients may develop poor wound healing. We would like to determine the levels of a particular protein called Connexin-43. It is known that high levels of Connexin-43 protein can interfere with the movement of certain cells necessary for skin to close over and heal the wound. This research will further our knowledge about the human wound healing process and it may help development of beneficial treatments in the future.

Why have I been chosen? We have chosen you because you have venous disease and undergoing routine clinical care and treatment. We plan to study up to 84 patients in total.

Do I have to take part? It is up to you to decide whether or not to take part. If you decide to take part, you will be given a copy of this information sheet and a signed copy of the consent form to

keep and be asked to sign a consent form. If you wish to withdraw from the study you may do so at any time and without giving a reason. If you decide not to take part in the study, this will not affect the standard of care you receive.

What will happen to me if I take part?
You will undergo three small 4-mm biopsies, during routine care at your operation. The biopsies will be taken from two locations on the leg undergoing treatment. The first will be at the level of the knee where the normal catheter to treat your varicose vein is placed and the second will be between the knee and ankle where you have varicose veins (or ulcer). A third biopsy will be taken from the other (normal) leg. This will be done by an experienced surgeon and a doctor will be present throughout. The sample will be carefully transferred and stored in the laboratory for further analysis. The sample will be 'gifted' by you for purposes of this research study. Following the laboratory studies required for this research study, the sample will be stored by the research team at UCL, in accordance to the Human Tissue Authority Code of Practice. The samples will then be transferred to our laboratory in Lee Kong Chian School of Medicine, Singapore. Ethical approval will be sought for additional projects in the future, should other researchers wish to use your sample.

Will taking part change my treatment? Here at UCLH, we would be undertaking a surgical incision as part of your operation. The small biopsy will be from the edge of this incision and not affect your care plan discussed between you and your consultant.

What data will be collected?
As mentioned above, a small 4-mm biopsy will be taken from you. Laboratory studies will be undertaken to determine the levels of Connexin-43 protein and further analyse cell movement.
You may also have a clinical photograph taken of your leg before your biopsies are taken, for purposes of the study. Should there be wider publication of the photograph in clinical journals or publications, you will be contacted and informed and further consent and permission to do this will be sought. All clinical photographs will remain anonymous and it will not be possible to identify you from the photograph.
The research team will also collect the data in the 'Varicose Veins Questionnaire' which you will be completing as part of your normal clinical pathway.

What are the benefits of taking part? You will not benefit directly from the study however it may help patients in the future.

What are the disadvantages or risks of taking part? The disadvantages or risks of taking part are minor. The biopsy should not affect the healing of your wound, and should you become concerned about your wound at any time, a further outpatient follow-up appointment can be arranged.

It is unlikely that the addition of a small biopsy to the surgical wound would result in any additional discomfort or pain at the time of biopsy. Local anaesthesia may be used to minimize this risk; this involves using a fine needle to deliver the anaesthetic just below the skin surface to numb the skin. Patients may experience some minor bleeding (a few drops of blood, similar to when a patient has a needle finger-prick blood glucose 'BM' test). If this happened it would be necessary to apply a small gauze dressing temporarily until the bleeding stops. Finally, there is small risk that the biopsy taken from you will not be suitable for use or in other words 'insufficient'. This means that the biopsy procedure has been unsuccessful due to technical problems at the time of taking biopsy, and it is inappropriate for the sample to be sent to the laboratory. If this occurs, you will be informed. It would be up to you to decide whether you wish to undergo further biopsy or withdraw from the research study.

What if new information becomes available? Sometimes during the course of a research project, new information becomes available about the subject being studied. If this happens your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form. It is possible that on receiving new information your research doctor might consider it to be in your best interests to withdraw from the study. He/she will explain the reasons and arrange for care to continue.

What happens if something goes wrong? All biopsies will be taken by an experienced surgeon and a qualified doctor will be present throughout the test with immediate access to emergency

equipment should it be required. We will take every care in the course of this study. If through our negligence any harm results you will be compensated. However, a claim may need to be pursued through legal action. The NHS Trusts are not permitted to carry indemnity for non-negligent (no-fault) harm.

Will my taking part in this study be kept confidential? All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. We are required to keep the data for a minimum of 5 years after the study has been completed. The biopsy samples will be stored in such a way that your details will linked to the stored tissue but not identifiable to researchers. The biopsy sample will be stored in the tissue bank for 5 years.

What will happen to the results of the research study? We plan to run the study for 2 years, after which the research team will analyse the results. The results will be published as soon as possible after study completion. You will not be identified in any report or publication.

Who is organising and funding the research? The Research fellow is organizing the research as part of an PhD thesis, supervised by Mr Toby Richards, Senior Lecturer and Consultant Vascular & Endovascular Surgeon. The laboratory studies will be undertaken in collaboration with Professor David Becker. The research is sponsored by the NHS.

Ethics Committee review All research using human subjects are reviewed before an ethics committee before they can proceed. This proposal was reviewed by UCLH Joint Research Ethics Committee.

Contact details for further information

Mr Toby Richards, Senior Lecturer & Consultant Vascular and Endovascular Surgeon
UCL Division of Surgery and Interventional Science, Fourth Floor
74, Huntley Street,
London,
WC1E 6AU
Email: Toby.Richards@ucl.ac.uk
Tel: 0207 6796454

A3: Patient Consent Form

Consultant Vascular Surgeons

Mr O Agu MS FRCS FRCS (Gen)
Mr Toby Richards BSc MBBS FRCS MD
Mr Daryll Baker BSc, PhD, FRCS

Department of Vascular Services
University College Hospital
250 Euston Road
London
NW1 2PG

Administrative Enquiries
PA: Tomisin Olarewaju
Tel: 020 3447 5173
Fax: 020 3447 9217
oluwatomisin.olarewaju@uclh.nhs.uk

Surgical Admission Enquiries:
Carina Nobrega
Tel: 0203 447 9112
Fax: 0203 447 9217
carina.nogrega@uclh.nhs.uk

Switchboard: 0845 155 5000
Outpatient Clinic Appointments: 0203 447 9393

15/08/2014
TR1/PRN/OOO

CONSENT FORM

Study Title: **Connexin 43 protein expression in venous disease**

Principal Researcher: Dr Muholan Kanapathy

Form version: April 1st 2014 Version 1.3

Centre Number:

UCLH Project ID number:

Patient identification number for this study: _____

To be completed by the volunteer

Please initial each box

1) I confirm that I have read and understood the information sheet dated ***April 1st 2014*** version 1.3 for the above study, and have had the opportunity to ask questions.	**Yes** ☐
2) I confirm that I have had sufficient time to consider whether or not I want to be included in the study.	**Yes** ☐
3) I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	**Yes** ☐
4) I understand that sections of my medical notes may be looked at by responsible individuals from list below or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	**Yes** ☐
5) I agree to take part in the above study.	**Yes** ☐
6) I agree to biopsies to be taken at:	**Yes** ☐
a) The level of the knee on the leg undergoing venous treatment	

b) Between the ankle and knee on the leg undergoing venous treatment **Yes** ☐

c) Between the ankle and knee on the leg which is not undergoing venous treatment **Yes** ☐

7) I agree that my GP can be informed of my involvement in this study. **Yes** ☐

8) I agree that an anonymous clinical photograph may be taken of my wound during this study. **Yes** ☐

_____	_____	_____
Name of Patient	Signature	Date

_____	_____	_____
Name of Person taking consent (if different from researcher)	Signature	Date

_____	_____	_____
Researcher	Signature	Date

Comments or concerns during the study

If you have any comments or concerns you may discuss these with the investigator. If you wish to go further and complain about any aspect of the way you have been approached or treated during the course of the study, you should write or get in touch with the Complaints Manager, UCL hospitals. Please quote the UCLH project number at the top this consent form.

Principal Researcher: Dr Muholan Kanapathy April 1st 2014: version 1.3

final page/

1 form for Patient,

1 to be kept as part of the study documentation,

1 to be kept with hospital notes.

A4: GP Letter

Consultant Vascular Surgeons

Mr O Agu MS FRCS FRCS (Gen)
Mr Toby Richards BSc MBBS FRCS MD
Mr Daryll Baker BSc, PhD, FRCS

Department of Vascular Services
University College Hospital
250 Euston Road
London
NW1 2PG

Administrative Enquiries
PA: Tomisin Olarewaju
Tel: 020 3447 5173
Fax: 020 3447 9217
oluwatomisin.olarewaju@uclh.nhs.uk

Surgical Admission Enquiries:
Carina Nobrega
Tel: 0203 447 9112
Fax: 0203 447 9217
carina.nogrega@uclh.nhs.uk

Switchboard: 0845 155 5000
Outpatient Clinic Appointments: 0203 447 9393

15/08/2014
TR1/PRN/OOO

UCLH Project ID number:

Form version: April 1st 2014 Version 1.3

Dear Dr.

Title of project: **Connexin 43 protein expression in venous disease**

Patient Name:

Patient d.o.b:

Patient NHS Number:

Your patient is taking part in a study by the Vascular Surgical Research Unit at University College London.

The study aims at investigating the levels of a protein called Connexin-43 in patients with venous disease.

Your patient will undergo small 4-mm wound edge biopsies, during routine care at their planned operation.

The biospies should not have an adverse effect on your patients' wound healing and your patient will continue their routine podiatry and dressing care. Please do not hesitate to contact the Vascular Research Team should you have any concerns or require any further information. I can be contacted either by email (muholan.kanapathy.13@ucl.ac.uk)or telephone (UCL 0207 6796454 or 07459029770).

Yours sincerely,

Dr Muholan Kanapathy

Research Fellow to Mr Toby Richards

A5: Clinical Wound Assessment Form

Wound assessment & biopsy form Version: April 1st 2014 Version 1.3

WOUND ASSESSMENT & WOUND EDGE BIOPSY FORM

Study Title: Connexin 43 protein expression in venous disease

Centre Number: UCLH Project ID number:

<table>
<tr><td colspan="2">Date</td><td>Patient identification number</td><td></td></tr>
<tr><td colspan="2">Location of Ulcer (please circle and tick as appropriate for all ulcers apart from abdominal)
Left / Right</td><td>☐ Forefoot
☐ Heel
☐ Planter aspect</td><td>☐ Lateral aspect
☐ 1st MTP
☐ Toes (indicate which number)
_____</td></tr>
<tr><td colspan="2">Length (in cm)</td><td>Width (in cm)</td><td>Depth (in cm)</td></tr>
<tr><td colspan="2">Appearance (please tick as appropriate)</td><td>Wound Base
☐ Pink (epithelializing)
☐ Red (granulating)
☐ Yellow (sloughy)
☐ Black (necrotic)</td><td>Surrounding Skin
☐ Healthy/intact
☐ Erythema
☐ Dry/Scaly
☐ Oedematous
☐ Fragile
☐ Macerated</td></tr>
<tr><td colspan="2">Other features (please tick as appropriate)</td><td rowspan="2">Pain
☐ Continuous
☐ On touch or movement
☐ None

Light touch sensation
☐ Present ☐ Absent</td><td rowspan="2">Infection ☐ Yes ☐ No

Exudate ☐ Yes ☐ No
(if yes please tick boxes below)
Quantity ☐ high ☐ medium ☐ low
Type ☐ blood ☐ serous ☐ pus
Odour ☐ offensive ☐ none</td></tr>
<tr><td colspan="2">Limb pulses present
☐ DP ☐ popliteal
☐ PT ☐ femoral</td></tr>
</table>

Wound illustration *(please indicate location of wound edge biopsies)*

Clinical photograph taken? ☐ Yes ☐ No

Complications of wound edge biopsies? ☐ Yes ☐ No *(please state free text below)*

Appendix B: Part 2

Supplementary Information

B1: PROSPERO registration

UNIVERSITY of York
Centre for Reviews and Dissemination

NHS
National Institute for Health Research

PROSPERO International prospective register of systematic reviews

Systematic review and meta-analysis of the efficacy of epidermal grafting for wound healing

Muholan Kanapathy, Oliver Smith, Nadine Hachach-Haram, Nicola Bystrzonowski, Toby Richards, Afshin Mosahebi

Citation
Muholan Kanapathy, Oliver Smith, Nadine Hachach-Haram, Nicola Bystrzonowski, Toby Richards, Afshin Mosahebi. Systematic review and meta-analysis of the efficacy of epidermal grafting for wound healing. PROSPERO 2016:CRD42016033051 Available from
http://www.crd.york.ac.uk/PROSPERO_REBRANDING/display_record.asp?ID=CRD42016033051

Review question(s)
This systematic review is intended to evaluate the current evidence on epidermal grafting for wound healing to establish the efficacy of this technique in clinical setting by measuring the proportion of wounds healed and the mean wound healing time

Searches
The following electronic databases will be searched:

• MEDLINE via OvidSP

• EMBASE via OvidSP

• Cochrane Central Register of Controlled Trials (CENTRAL)

Restrictions/limitations:

No language or publication restrictions will be applied.

Types of study to be included
Retrospective and prospective observation and interventional studies will be included.

Condition or domain being studied
The feasibility of performing epidermal grafting in outpatient setting with minimal donor site morbidity permits patient to maintain independence throughout the treatment besides reducing healthcare resources in wound management. There are several reports on successful wound healing with epidermal grafting, however, to-date the evidence on the efficacy of this technique has not been reviewed. We propose reviewing the current evidence on epidermal grafting for acute and chronic wound healing to establish the efficacy of this technique.

Participants/ population
Inclusion criteria

The following study inclusion criteria will be used:

(i) studies involving adult patients between 18-90 years old;

(ii) epidermal grafting for wound healing;

(iii) available information containing at least the following: number of subjects, method of epidermal graft harvesting, and healing time.

Exclusion criteria

The exclusion criteria were: case series of less than three cases; studies describing the use of epidermal grafting in skin pigmentation disorder such as vitiligo; and studies describing only the harvest technique without treatment outcome.

Intervention(s), exposure(s)
Epidermal grafting for treatment of acute and chronic wound.

Comparator(s)/ control
Split thickness skin grafting for treatment of acute and chronic wound.

Outcome(s)
Primary outcomes
The efficacy of epidermal grafting for wound healing in the clinical setting.

The efficacy will be measured by wound healing time (time for complete re-epithelialisation or change in wound area) and the proportion of wounds healed during trial period.

Secondary outcomes
The secondary outcomes are the donor site healing time, the type of wounds that benefits best from this treatment, the need for anaesthesia, economic evaluation, health related quality of life, and adverse events.

Data extraction, (selection and coding)
The following data will be extracted: first author, year of publication, country, study design, number of studied subjects, sex, mean age, comorbidity, number of wounds treated, wound aetiology, mean wound duration, mean wound size, pre-grafting wound quality, epidermal graft harvesting method, use of anaesthesia, donor site dressing, wound dressing, wound healing time, number of wounds with complete healing, number of wounds with 50-99 percent healing, number of failures, donor site healing time, and complications.

Data from all full-text articles accepted for final analysis will be independently retrieved by two authors using a data extraction form. Any differences will be resolved by discussion.

Risk of bias (quality) assessment
The Cochrane collaboration risk of bias assessment tool will be applied to assess the randomised control trials.

Strategy for data synthesis
The number of events and the total number of patients in each group will be collected for the outcome parameters. The pooled proportion and 95% confidence intervals will be calculated as the summary estimate. Fixed effect or random effects model will be applied.

Analysis of subgroups or subsets
Assuming that sufficiently robust patient groups can be identified from the studies, the different harvesting methods and wound aetiologies will be considered separately.

Dissemination plans
Conference presentation and publication in a peer-review journal.

Contact details for further information
Mr Kanapathy

Division of Surgery and Interventional Science,

Level 9, Royal Free Hospital,

Pond Street, London, United Kingdom, NW3 2QG

muholan.kanapathy.13@ucl.ac.uk

Organisational affiliation of the review
Division of Surgery and Interventional Science, University College London

Review team
Mr Muholan Kanapathy, Division of Surgery and Interventional Science, University College London
Dr Oliver Smith, Department of Plastic Surgery, Royal Free Hospital London
Miss Nadine Hachach-Haram, Department of Plastic Surgery, Royal Free Hospital London
Miss Nicola Bystrzonowski, Department of Plastic Surgery, Royal Free Hospital London
Professor Toby Richards, Division of Surgery and Interventional Science, University College London
Mr Afshin Mosahebi, Department of Plastic Surgery, Royal Free Hospital London

Anticipated or actual start date
01 October 2015

Anticipated completion date
31 May 2016

Funding sources/sponsors
University College London

Conflicts of interest
None known

Language
English

Country
England

Subject index terms status
Subject indexing assigned by CRD

Subject index terms
Epidermis; Humans; Transplants; Wound Healing

Stage of review
Ongoing

Date of registration in PROSPERO
12 January 2016

Date of publication of this revision
12 January 2016

Stage of review at time of this submission	Started	Completed
Preliminary searches	Yes	Yes
Piloting of the study selection process	Yes	Yes
Formal screening of search results against eligibility criteria	Yes	Yes
Data extraction	Yes	No
Risk of bias (quality) assessment	No	No
Data analysis	No	No

B2: Patient Satisfaction Questionnaire

Study Title: **A Multicentre Randomised Controlled Trial to Evaluate the Efficacy of Epidermal Grafting in Wound Healing (EPIGRAAFT)**

Subject ID: _____ **Follow-up week:** ☐ Week 6 ☐ 3 months

Patient skin graft satisfaction questionnaire

1. How noticeable do you find your donor site?

Very noticeable (5)	Somewhat noticeable (4)	Slightly noticeable (3)	Not noticeable at all (2)	No particular feeling (1)

2. Do you worry about your donor site appearance?

I worry very much (5)	I worry somewhat (4)	I worry a little (3)	I do not worry (2)	No particular feeling (1)

3. Did you suffer any problems with your donor site?

Very problematic (5)	Somewhat problematic (4)	Slightly problematic(3)	Not problematic at all(2)	No particular feeling (1)

4. Overall how would you rate the outcome of your donor site?

Very satisfied (7)	Somewhat satisfied (6)	Slightly satisfied (5)	No particular feeling (4)	Slightly unsatisfied (3)	Somewhat unsatisfied (2)	Very unsatisfied (1)

5. How noticeable do you find your graft site?

Very noticeable (5)	Somewhat noticeable (4)	Slightly noticeable (3)	Not noticeable at all (2)	No particular feeling (1)

6. Do you worry about your graft site appearance?

I worry very much (5)	I worry somewhat (4)	I worry a little (3)	I do not worry (2)	No particular feeling (1)

7. Did you suffer any problems with your graft site?

Very problematic (5)	Somewhat problematic (4)	Slightly problematic(3)	Not problematic at all(2)	No particular feeling (1)

8. Overall how would you rate the outcome of your graft site?

Very satisfied (7)	Somewhat satisfied (6)	Slightly satisfied (5)	No particular feeling (4)	Slightly unsatisfied (3)	Somewhat unsatisfied (2)	Very unsatisfied (1)

B3: Ethical Board Approvals

Health Research Authority
National Research Ethics Service

NRES Committee London - Fulham
Barlow House
3rd Floor, 4 Minshull Street
Manchester
M1 3DZ

14 May 2015

Mr Toby Richards
Senior Lecturer and Consultant in Vascular & Endovascular Surgery
University College London
UCL Division Surgery and Interventional Science,
Fourth floor, 74 Huntley Street, London,
United Kingdom
WC1E 6AU

Dear Mr Richards

Study title:	**A Multicentre Randomised Controlled Trial to Compare Epidermal Grafting with Split Skin Grafting for Wound Healing**
REC reference:	**15/LO/0556**
IRAS project ID:	**174318**

Thank you for your submission, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager, Anna Bannister, nrescommittee.london-fulham@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.
Management permission or approval must be obtained from each host organisation prior to the

start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from NRES. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet completed any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as an SSA application(s) has been reviewed. In the meantime no study procedures should be initiated at non-NHS sites.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance Confirmation Letter from UCL]		04 March 2015
GP/consultant information sheets or letters [Letter to GP]	1.0	10 January 2015
IRAS Checklist XML [Checklist_10032015]		10 March 2015
Participant consent form [Consent form]	1.0	10 January 2015
Participant information sheet (PIS) [Patient information sheet]	1.0	10 January 2015
Participant information sheet (PIS)	1.1- Tracked	11 May 2015
REC Application Form [REC_Form_10032015]		10 March 2015
Research protocol or project proposal [Research Protocol]	1.0	10 January 2015
Summary CV for Chief Investigator (CI) [CV Toby Richards]		09 March 2015
Summary CV for student [CV Muholan]		09 March 2015
Summary CV for supervisor (student research) [CV Afshin Mosahebi]		09 March 2015
Validated questionnaire [Patient skin graft satisfaction questionnaire]		10 January 2015

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

15/LO/0556	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely

pp M. Southgate

On behalf of Dr Charles Mackworth-Young
Chair

Email: nrescommittee.london-fulham@nhs.net

Enclosures: After ethical review – guidance for Researchers

Copy to: *Mr Dave Wilson*

Liba Stone, Royal Free Hospital

Royal Free London NHS
NHS Foundation Trust

Research & Development Department
Royal Free Hospital
Pond Street
Ground Floor, Room 649
London
NW3 2QG

www.royalfree.nhs.uk
Switchboard: 020 7794 0500 EXT: 33211
Fax: 020 7830 2468
Direct line: 020 7317 7558

FINAL R&D APPROVAL – NHS PERMISSION **01/10/2015**

Chief Investigator: Dr Toby Richards
Local Collaborator: Mr Afshin Mosahebi

Dear Dr Richards,

Project ID: 9417 (Please quote in all correspondence)
REC Ref: 15/LO/0556
Title: Epidermal grafting in wound healing

Thank you for registering the above study with the Royal Free R&D office. I am pleased to inform you that your study now has local NHS Permission (R&D approval) to proceed and recruit participants at Royal Free London NHS Foundation Trust subject to Sponsor confirmation.

Please note that all documents received have been reviewed and this approval is granted on the basis of the key documents provided which are ethically approved by the Research Ethics Committee:

Document	Date
REC approval and REC approved documents	14/05/2015
MHRA Approval (if applicable)	N/A
Agreement between sponsor and RFL (if applicable)	N/A
ARSAC licence (if applicable)	N/A

As Principal Investigator you are required to ensure that your study is conducted in accordance with the requirements on the attached sheet. These include the conditions of your NHS Permission.

Do not hesitate to contact a member of the team should you have any queries.

Yours sincerely

P.P Dr Adele Fielding
Director of Research and Development
Royal Free London NHS Foundation Trust

B4: Patient Information Sheet

Royal Free Hampstead NHS
NHS Trust

Project ID number: Form version: April 26th 2016, Version 1.2

PATIENT INFORMATION LEAFLET

Study Title: **A Multicentre Randomised Controlled Trial to Evaluate the Efficacy of Epidermal Grafting in Wound Healing (EPIGRAAFT)**

Chief Investigator: **Mr Afshin Mosahebi / Professor Toby Richards**

As a patient of RFH NHS Trust you are being invited to take part in a research study. Before you decide whether you would like to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy or it may be obtained from CERES, PO Box 1365, London N16 0BW.

The purpose of the study: Split thickness skin grafting is the normal standard of care for wound closure. However, this is an invasive procedure and associated with pain also there can be additional donor site morbidity. Epidermal grafting is an emerging clinical alternative that is gaining clinical practise. Epidermal grafting (EG) is an alternative method of autologous skin grafting that 'harvests' a finer layer of skin than traditional Split Skin grafting (SSG). This potentially results in less pain and reduced donor site morbidity but only delivers several cell layers to the wound so may be less effective at healing a wound. It is not known if EG is an effective alternative to SSG.

We wish to compare these two clinical practises; epidermal grafting and split thickness skin grafting in wound healing. Further to undertake a translational study to investigate the mechanism by which each technique achieves wound healing.

Further the mechanism to achieve wound healing may be different. EG promotes wound healing by expressing growth factors that accelerates wound healing and encourages cell migration. Whereas SSG is a transplant of several skin layers that integrated to the existing wound bed as a formal skin covering. Specifically, EG is expected to accelerate wound healing by expressing favourable growth factors and regulating a particular protein called Connexin-43. It is known that high levels of Connexin-43 protein can interfere with the movement of certain cells necessary for wound closure. This research will further our knowledge about the human wound healing process and it may help development of beneficial treatments in the future

Why have I been chosen? We have chosen you because you have wound that your consultant plastic surgeon considers would benefit from skin grafting. You have been selected as appropriate criteria and undergoing routine clinical care and treatment. We plan to study up to 40 patients in total.

Do I have to take part? It is up to you to decide whether or not to take part. If you decide to take part you will be given a copy of this information sheet and a signed copy of the consent form to keep and be asked to sign a consent form. If you wish to withdraw from the study you may do so at any time and without giving a reason. If you decide not to take part in the study, this will not affect the standard of care you receive.

What will happen to me if I take part?

You will be provided with this Patient Information Sheet and given appropriate time to consider the trail and ask any questions. Following Informed Consent, you will undergo a wound fluid sampling and two small 4-mm biopsies. The wound fluid sampling will be performed by simply applying a filter paper onto your wound for about 2 minutes until it is moist. The biopsies will be taken from two locations on the wound undergoing treatment. The first will be at the center of the wound and the second will be at the wound edge. This will be done by an experienced surgeon and a nurse will be present throughout. The sample will be carefully transferred and stored in the laboratory for further analysis. The sample will be 'gifted' by you for purposes of this research study. Following the laboratory studies required for this research study, the sample will be stored by the research team at UCL, in accordance to the Human Tissue Authority Code of Practice. Ethical approval will be sought for additional projects in the future, should other researchers wish to use your sample.

Upon completing the biopsies, you will be randomised into either the epidermal graft group or the split thickness skin graft group. You will then receive the designated treatment. You will be reviewed in outpatient specialist clinic weekly for up to 6 weeks or until your wound heals. If your wound fails to heal at week 6 ± 2, the treating clinician will discuss with you regarding the need for re-grafting along with repeat biopsies as per protocol.

What data will be collected?

Some parts of your medical record will be included into this study. As mentioned above, two 4-mm biopsies will be taken from you. Laboratory studies will be undertaken to determine the levels of growth factors, cytokeratin and Connexin-43 protein.

Clinical photographs of your wound will be taken before and after the biopsies, for purposes of the study. These photographs will be stored in our photo diary and used to analyse the size and the wound healing rate. These photographs might be used in clinical journals or publications. All clinical photographs will remain anonymous and it will not be possible to identify you from the photograph.

You will be asked about the pain at the wound and the donor site at day 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 35, and 42. You will also be required to complete a Patient skin graft satisfaction questionnaire as part of your normal clinical pathway.

What are the benefits of taking part? It is possible that there may be no direct benefits from taking part in this study, however the treatment may improve your wound healing. The information obtained from this study may likely benefit the future patients.

What are the disadvantages or risks of taking part? The disadvantages or risks of taking part are minor. There is possibility that the epidermal graft will not benefit your wound healing. There are several reasons this could take place; namely, infection and your general medical condition.

The biopsy should not affect the healing of your wound, and should you become concerned about your wound at any time, a further outpatient follow-up appointment can be arranged.

It is unlikely that the addition of a small biopsy to the surgical wound would result in any additional discomfort or pain at the time of biopsy. Local anaesthesia may be used to minimize this risk; this involves using a fine needle to deliver the anaesthetic just below the skin surface to numb the skin. Patients may experience some minor bleeding (a few drops of blood, similar to when a patient has a needle finger-prick blood glucose 'BM' test). If this happened it would be necessary to apply a small gauze dressing temporarily until the bleeding stops. Finally, there is small risk that the biopsy taken from you will not be suitable for use or in other words 'insufficient'. This means that the biopsy procedure has been unsuccessful due to technical problems at the time of taking biopsy, and it is inappropriate for the sample to be sent to the laboratory. If this occurs, you will be informed. It would be up to you to decide whether you wish to undergo further biopsy or withdraw from the research study.

What if new information becomes available? Sometimes during the course of a research project, new information becomes available about the subject being studied. If this happens your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form. It is possible that on receiving new information your research doctor might consider it to be in your best interests to withdraw from the study. He/she will explain the reasons and arrange for care to continue.

What happens if something goes wrong? If you experience any problem from the treatment, you should contact us straight away. All biopsies will be taken by an experienced surgeon and a nurse will be present throughout the test with immediate access to emergency equipment should it be required. We will take every care in the course of this study. If through our negligence any harm results, you will be compensated. However, a claim may need to be pursued through legal action. The NHS Trusts are not permitted to carry indemnity for non-negligent (no-fault) harm.

Will my taking part in this study be kept confidential? All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. We are required to keep the data for a minimum of 5 years after the study has been completed. The biopsy samples will be stored in such a way that your details will linked to the stored tissue but not identifiable to researchers. The biopsy sample will be stored in the tissue bank for 5 years.

What will happen to the results of the research study? We plan to run the study for 2 years, after which the research team will analyse the results. The results will be published as soon as possible after study completion. You will not be identified in any report or publication.

Who is organising and funding the research? The Research fellow is organizing the research as part of a PhD thesis, supervised by Mr Toby Richards, Senior Lecturer and Consultant Vascular & Endovascular Surgeon and Mr Afshin Mosahebi, Consultant Plastic Surgeon. The research is sponsored by the NHS.

Ethics Committee review All research using human subjects are reviewed before an ethics committee before they can proceed. This proposal was reviewed by Research Ethics Committee.

Contact details for further information

Professor Toby Richards, Professor of Surgery & Consultant Vascular and Endovascular Surgeon, UCL Division of Surgery and Interventional Science, Fourth Floor 74, Huntley Street, London, WC1E 6AU Email: toby.richards@ucl.ac.uk Tel: 0207 6796454

Mr Afshin Mosahebi, Consultant Plastic Surgeon and Honorary Senior Lecturer,
Department of Plastic and Reconstructive Surgery, Royal Free Hampstead NHS Trust Hospital, Pond Street, London, NW3 2QG Email: a.mosahebi@ucl.ac.uk Tel: 020 77940500 ext 35556

B5: Patient Consent Form

Royal Free Hampstead NHS
NHS Trust

CONSENT FORM

Study Title: **A Multicentre Randomised Controlled Trial to Evaluate the Efficacy of Epidermal Grafting in Wound Healing (EPIGRAAFT)**

Form version: May 11th 2015, Version 1.1

Project ID number:

Chief Investigator : **Mr Afshin Mosahebi / Professor Toby Richards**

To be completed by the volunteer **Please initial each box**

1) I confirm that I have read and understood the information sheet dated May 11th 2015, version 1.1 for the above study, and have had the opportunity to ask questions. ☐

2) I confirm that I have had sufficient time to consider whether or not I want to be included in the study. ☐

3) I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

4) I understand that relevant sections of my medical notes and data collected during the study may be looked at by investigators of the trial, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records (which may include them being sent a copy of this consent form). ☐

5) I agree to biopsies to be taken at the wound bed and the wound edge at the start of the treatment and at day 7. ☐

6) I understand that I am gifting my tissue to the investigators and in doing so I give up all future claims to its use that may include further research. ☐

7) I agree that my GP can be informed of my involvement in this study. ☐

8) I agree that anonymous clinical photographs may be taken of my wound during this study follow-up. ☐

9) I agree to take part in the above study. ☐

_____	_____	_____
Name of patient	Signature	Date
_____	_____	_____
Name of person taking consent (if different from researcher)	Signature	Date
_____	_____	_____
Researcher	Signature	Date

When completed: 1 form for patient, 1 to be kept as part of the researcher site file, 1 to be kept with hospital notes.

Contact details for further information

Professor Toby Richards, Professor of Surgery & Consultant Vascular and Endovascular Surgeon,
Email: toby.richards@ucl.ac.uk Tel: 0207 6796454

Mr Afshin Mosahebi, Consultant Plastic Surgeon and Honorary Senior Lecturer,
Email: a.mosahebi@ucl.ac.uk Tel: 020 77940500 ext 35556

B5: GP Letter

Royal Free Hampstead NHS
NHS Trust

Project ID number: Form version: January 1st 2015 Version 1.0

Dear Dr.

Title of project: **A Multicentre Randomised Controlled Trial to Evaluate the Efficacy of Epidermal Grafting in Wound Healing**

Patient Name: Patient d.o.b:

Patient NHS Number:

Your patient is taking part in above research study, by the Department of Plastic Surgery at the Royal Free Hospital. We are performing a clinical trial to determine the efficacy of epidermal graft in wound healing against split thickness skin graft.

In this clinical trail, we wish to determine the wound healing at 6 weeks as well as the donor site healing rate. Patients will be randomise to receive the epidermal graft or split thickness skin graft. Your patient will undergo two small 4-mm biopsies, one at the centre of the wound and one at the wound edge, followed by skin grafting (either epidermal graft or split thickness skin graft) over the wound. We will review your patient weekly for the duration of 6 weeks or until wound heals.

The biospies should not have an adverse effect on your patients' wound healing and your patient will continue their dressing care. Please do not hesitate to contact the Department of Plastic Surgery at the Royal Free Hospital should you have any concerns or require any further information. I can be contacted either by email (toby.richards@ucl.ac.uk) or telephone (UCL 0207 6796454).

Yours sincerely,

B6: Clinical Wound Assessment Form

Form version: July 27th 2015 Version 1.1

WOUND ASSESSMENT FORM

Study Title: A Multicentre Randomised Controlled Trial to Evaluate the Efficacy of Epidermal Grafting in Wound Healing (EPIGRAAFT)

Research ID Number :
Date of Birth :
Patient Name :
Hospital Number/MRN :
Phone number :

PATIENT INFORMATION

Age : _____
Sex ☐ Male ☐ Female
Weight : _____ kg
Height : _____ cm

MEDICAL HISTORY

Diabetes	☐ Yes	☐ No	If yes, specify type:	☐ Type 1 ☐ Type 2
Hypertension	☐ Yes	☐ No		
Peripheral Arterial Disease	☐ Yes	☐ No		
Chronic Venous Disease (Varicose veins/DVT)	☐ Yes	☐ No		
Lymphoedema	☐ Yes	☐ No		
Cancer	☐ Yes	☐ No	If yes, specify type:	☐ Skin ☐ Others
Renal insufficiency (*exclusion criteria*)	☐ Yes	☐ No		
Liver Disease (*exclusion criteria*)	☐ Yes	☐ No		
Haematological condition (*exclusion criteria*)	☐ Yes	☐ No		
Active auto-immune disease (*exclusion criteria*)	☐ Yes	☐ No		

Oral/topical steroid ☐ Yes ☐ No. If yes, specify treatment: _____
Anticoagulant/Antiplatelet ☐ Yes ☐ No. If yes, specify treatment: _____

Smoker ☐ Current smoker ☐ Never smoked ☐ Previous smoker

Has the patient meet the inclusion and exclusion criteria? ☐ Yes ☐ No

WOUND INFORMATION

Type of wound ☐ Acute wound (<3 months) ☐ Chronic wound (>3months)

Duration of wound : _____ weeks

Wound aetiology ☐ Venous ulcer ☐ Arterial Ulcer ☐ Diabetic ulcer
☐ Pressure ulcer ☐ Trauma ☐ Wound dehiscence
☐ Burns ☐ Pyogenic Granuloma ☐ Split skin graft donor site
☐ Others, specify: _____

Location of wound: ☐ Foot ☐ Ankle ☐ Leg ☐ Knee ☐ Thigh
☐ Hand ☐ Arm ☐ Forearm
☐ Abdomen ☐ Chest ☐ Back

For limbs, specify site: ☐ Left ☐ Right

Size of wound: Length : _____ cm Total wound area: _____ cm^2
Width : _____ cm
Depth : _____ cm

Wound base appearance: ☐ Pink (epithelializing) ☐ Red (granulating)
☐ Yellow (sloughy) ☐ Black (necrotic)

Surrounding skin appearance: ☐ Healthy/intact ☐ Erythema ☐ Dry/Scaly
☐ Oedematous ☐ Fragile ☐ Macerated

Exudate: ☐ Yes ☐ No
If yes, specify: *Quantity* ☐ high ☐ medium ☐ low
Type ☐ blood ☐ serous ☐ pus
Odour ☐ offensive ☐ none

Pain: ☐ Continuous ☐ On touch or movement ☐ None
Pain score (0 to 10): _____

Wound bed preparation: ☐ NPWT ☐ Dressing, specify: _____
Microbiological investigation: Date of test: _____ Result: ☐ No bacterial growth
☐ Positive bacterial growth
If positive, specify: _____

Clinical photograph taken? ☐ Yes ☐ No

Biopsy taken? ☐ Yes ☐ No

Wound fluid taken? ☐ Yes ☐ No

PUSH Tool Score of wound:

Length x Width (in cm^2)	**0** 0	**1** < 0.3	**2** 0.3-0.6	**3** 0.7-1.0	**4** 1.1-2.0	**5** 2.1-3.0	**Sub-score**
		6 3.1-4.0	**7** 4.1-8.0	**8** 8.1-12.0	**9** 12.1-24.0	**10** >24.0	
Exudate Amount	**0** None	**1** Light	**2** Moderate	**3** Heavy			**Sub-score**
Tissue Type	**0** Closed	**1** Epithelial Tissue	**2** Granulation Tissue	**3** Slough	**4** Necrotic Tissue		**Sub-score**
							Total Score

TREATMENT/PROCEDURE

Date of treatment: _____

Type of treatment: ☐ Epidermal graft (CelluTome) ☐ Split thickness skin graft (SSG)

Site of graft harvest: ☐ Left thigh ☐ Right thigh ☐ others, specify: _____

Pain score (0 to 10): _____

Dressing Wound: _____ Donor site: _____

Procedure setting: ☐ Inpatient ☐ Outpatient

If inpatient, Duration of immobilisation: _____ days

Length of hospitalisation: _____ days

Complication: ☐ Hematoma ☐ Graft infection ☐ Others: _____

☐ None

Completed by:

Name: _____ Signature: _____

WEEKLY REVIEW FORM

Date of review :

Research ID Number : Date of Birth :

Patient Name : Hospital Number/MRN :

Follow-up week : ☐ Week 1 ☐ Week 2 ☐ Week 3 ☐ Week 4
☐ Week 5 ☐ Week 6 ☐ 3 months ☐ Others: _____

Pain score (0 to 10) : _____

Size of wound: Length : _____ cm Total wound area: _____ cm^2
Width : _____ cm
Depth : _____ cm

Wound base appearance: ☐ Pink (epithelializing) ☐ Red (granulating)
☐ Yellow (sloughy) ☐ Black (necrotic)

Surrounding skin appearance: ☐ Healthy/intact ☐ Erythema ☐ Dry/Scaly
☐ Oedematous ☐ Fragile ☐ Macerated

Exudate: ☐ Yes ☐ No
If yes, specify: *Quantity* ☐ high ☐ medium ☐ low
Type ☐ blood ☐ serous ☐ pus
Odour ☐ offensive ☐ none

PUSH Tool Score for **wound**:

Length x Width (in cm^2)	**0** 0	**1** < 0.3	**2** 0.3-0.6	**3** 0.7-1.0	**4** 1.1-2.0	**5** 2.1-3.0	**Sub-score**
		6 3.1-4.0	**7** 4.1-8.0	**8** 8.1-12.0	**9** 12.1-24.0	**10** >24.0	
Exudate Amount	**0** None	**1** Light	**2** Moderate	**3** Heavy			**Sub-score**
Tissue Type	**0** Closed	**1** Epithelial Tissue	**2** Granulation Tissue	**3** Slough	**4** Necrotic Tissue		**Sub-score**
							Total Score

Donor site healed? ☐ Yes ☐ No If no, specify percentage of healed area: ____%

Donor site appearance ☐ Scab ☐ Hypopigmentation ☐ Hyperpigmentation ☐ Unnoticeable

PUSH Tool Score for **donor site**:

Length x Width (in cm^2)	**0** 0	**1** < 0.3	**2** 0.3-0.6	**3** 0.7-1.0	**4** 1.1-2.0	**5** 2.1-3.0	**Sub-score**
		6 3.1-4.0	**7** 4.1-8.0	**8** 8.1-12.0	**9** 12.1-24.0	**10** >24.0	
Exudate Amount	**0** None	**1** Light	**2** Moderate	**3** Heavy			**Sub-score**
Tissue Type	**0** Closed	**1** Epithelial Tissue	**2** Granulation Tissue	**3** Slough	**4** Necrotic Tissue		**Sub-score**
							Total Score

Biopsy taken (*only for week 1 post grafting*)? ☐ Yes ☐ No

Wound fluid taken? ☐ Yes ☐ No

Clinical photograph taken? ☐ Yes ☐ No

Dressing Wound: _____ Donor site: _____

Comment:

Completed by:

Name: _____ Signature: _____

STUDY EXIT

☐ Study completed

Date of completion: _____

☐ Subject withdrew consent prior to completing all required visits

If checked, please describe: _____

☐Investigator withdrew the subjects

If checked, please describe: _____

☐ Subject lost to follow-up

Date of last contact: _____

☐ Death

Completed by:

Name: _____ Signature: _____