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Upregulation of epidermal gap junctional proteins in patients with venous disease

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Background: Leg ulceration is a feared complication of venous insufficiency. It is not known whether varicose veins predispose skin to poor wound healing. The expression pattern of gap junctional protein connexin, a known marker of poor wound healing, was investigated across various stages of venous disease.

Methods: Patients undergoing intervention for varicose veins were assessed according to the Clinical Etiologic Anatomic Pathophysiologic (CEAP) classification of varicose veins. Paired 4-mm punch biopsies were taken from above the ankle (pathological) and above the knee (control). Tissues were stained with haematoxylin and eosin, and for connexin 43, connexin 30 and connexin 26.

Results: Forty-eight paired biopsies were taken (12 each for CEAP class C0, C2, C4 and C6). The pathological skin showed progressive epithelial hyperthickening, an 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 connexin expression and mean connexin expression per cell in the pathological skin similarly increased across the CEAP classes from as early as C2. Increasing levels of connexin in control skin were also noted, indicating progression of the disease proximally. Connexin 43 expression showed the strongest positive correlation between pathological and control skin.

Conclusion: Connexins were overexpressed in patients with simple varicose veins, with a stepwise increased expression through venous eczema to ulceration. Connexin 43 is a potential biomarker for venous disease. This finding suggests that varicose veins predispose skin to poor wound healing.

Surgical relevance

The overexpression of connexins, a family of gap junctional proteins, is known to cause poor healing in venous leg ulceration. It is not known whether there is any association with superficial venous disease. Here, connexin proteins were overexpressed in patients with uncomplicated varicose veins, before histological skin changes. Connexin could be biomarker of venous disease progression.

About one-third of adults have varicose veins (VVs), with over 35 000 treatments done in the UK annually[1,2]. VVs commonly occur owing to venous valve incompetence; one-third of patients may develop skin changes, such as venous eczema, and the lifetime risk of venous ulceration is 3–6 per cent[1,3–5]. Venous ulcers are the commonest type of leg ulcer, with a prevalence of 0.3–0.5 per cent, costing about £2–3 billion (€2.2–3.4 billion, exchange rate 26 July 2017) and 2 million lost work days per year[6–10]. The progression of venous disease can be classified by clinical manifestation (C), aetiological factors (E), the anatomical distribution of disease (A) and underlying pathophysiological findings (P). The CEAP classification according to disease severity is: C0, no visible venous disease; C1, spider veins; C2, VVs; C3, oedema; C4, lipodermatosclerosis; C5, healed ulcer; and C6, active ulcer[11]. A cohort study carried out over 13 years highlighted that VVs are a major risk factor, but the mechanism influencing venous disease progression from CEAP class C2 to C6 remains unclear[1,12]. Furthermore, it is not known whether uncomplicated VVs predispose to poor skin healing.

The limited knowledge on the progression of venous disease has resulted in few advances being made in identifying patients who might benefit from early surgical treatment. Early intervention might render patients less prone to ulceration[5]. Investigating the histological and cellular characteristics of the skin of patients across the CEAP classification may establish whether VVs predispose to poor wound healing and ulceration.

A factor known to cause poor wound healing in venous ulcers is the gap junctional protein connexin[13]. Connexin proteins are specialized clusters of plasma membrane channels, which facilitate communication and exchange of ions and metabolites less than 1 kDa in size

between adjacent cells[14]. The intercellular communication mediated by the gap junctional proteins is important during cellular development, and in the maintenance of tissue homeostasis[14–16]. Connexin proteins also have multiprotein interactions, which influence both cellular adhesion and cytoskeletal dynamics, and therefore cellular migration in wound healing[17]. Precise communication via connexin proteins is integral to normal wound healing[17,18]. Of the nine different connexins expressed in human epidermis, connexin 43, connexin 30 and connexin 26 are the most abundant, connexin 43 being the most ubiquitous[14].

Overexpression of connexin proteins in the skin of patients with venous ulcers has been shown to delay keratinocyte migration, resulting in poor wound healing. Downregulation of connexin 43 using connexin 43 antisense in rodents and humans accelerates wound healing[13,17]. Here, the expression pattern of the principal epidermal connexin proteins was studied across the CEAP classification in patients with venous disease to explore the changes and the expression of these proteins in undamaged skin.

+A: Methods

Patients from the four main stages of the CEAP classification, namely C0, C2, C4 and C6, were enrolled. Patients were eligible for inclusion in the study if they were aged over 18 years and fulfilled the CEAP classification criteria. Exclusion criteria were the presence of arterial disease, connective tissue disorders, systemic inflammatory disease, diabetic mellitus, cancer, concurrent skin disease and allergies to local anaesthesia.

All patients with C2, C4 and C6 disease underwent duplex ultrasonography to confirm the presence of venous reflux and to exclude mixed arteriovenous disease. Patients in C0 were assessed clinically to exclude symptoms and signs of venous reflux. Paired 4-mm punch biopsies of the skin were taken from each patient with C2, C4 and C6 disease at the

time of VV surgery. Biopsies were paired; one above the knee and defined as control skin, and one below the knee (15–20 cm above the ankle) and defined as pathological skin. These locations corresponded to the insertion site of the endovenous catheter (control) or the VV avulsion site (pathological) in normal clinical practice. In the C4 group, the below-knee biopsies were taken in close proximity to a patch of hardened skin, where the avulsion was performed. In patients with ulceration (C6), biopsies were taken 1 mm away from the wound margin to obtain the highest level of connexin expression, according to a published protocol[13]. For the C0 group, similar biopsies were taken from patients undergoing total knee replacement surgery.

All biopsies were taken after written informed consent had been obtained from the patient. 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 (11/LO/1483) and Nanyang Technological University Institutional Review Board (IRB-2015-05-003). All biopsies were obtained at the University College London Hospital and Royal Free Hospital, London, UK. Preliminary laboratory analysis was undertaken at the Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, and the final analysis carried out at University College London under similar laboratory conditions.

+B: Biopsy preservation and cryosectioning

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

haematoxylin and eosin using standard methods. Imaging was performed using a Zeiss AxioScan Z1 slide scanner (Zeiss) at 20 × magnification.

+B: Histological analysis

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

The number of epidermal rete ridges per millimetre (downward projection of epidermis at dermoepidermal junction) was calculated using a selected section (1 mm) of the epidermis that best represented the skin section. The mean 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 the stratum corneum and the rete ridge trough.

+B: Immunohistochemistry

Tissue sections were thawed, immersed in PBS to dissolve excess OCT medium, permeabilized for 15 min in 0.2 per cent Triton X-100 and blocked using PBS (0.1 mol/l) for 30 min. Primary antibodies were prepared in PBS: connexin 43 (1 : 4000; Sigma, Poole, UK), connexin 26 (1 : 200; Fisher Scientific, UK) and connexin 30 (1 : 200; Fisher Scientific). For connexin 43 staining, the tissues were incubated with the primary antibody for 1 h at room temperature; those for connexin 30 and connexin 26 staining were incubated overnight with the primary antibody at 4°C. For negative controls, the primary antibody was omitted from the preparation. The tissue was washed with PBS three times, each for 5 min, and stained with secondary antibody (Alexa Fluor 488 goat antirabbit 1 : 400; Fisher Scientific) and incubated at room temperature for 1 h. Nuclei were stained using Hoechst (1 : 10 000; Fisher

Scientific) for 5 min followed by three 5-min PBS washes. Coverslips were mounted using Citifluor (glycerol/PBS solution; Citifluor, London, UK) and sealed with nail varnish.

+B: Confocal microscopy

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

+B: Connexin quantification

ImageJ was used for quantification. Images were converted to binary images using an identical threshold. The epidermal threshold was kept constant between all images, being set at 80, with a recognized pixel threshold size of 2 to infinity used for all images[13]. Regions of interest were marked manually to include the epidermis only, excluding any areas of autofluorescence in the stratum corneum.

The connexin levels of the six confocal images from each tissue section were used to quantify the absolute mean connexin expression. Fold increase in connexin expression for each individual was calculated by comparing expression levels in the pathological and matched control skin sections.

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

+B: Statistical analysis

Normality testing was done using the Kolmogorov–Smirnoff test, which confirmed that connexin expression was distributed normally in each class. All data are presented as mean(s.d.). Statistical differences were determined using paired t test for paired samples and Student's t test for two unpaired groups. For more than two groups, one-way ANOVA, followed by Bonferroni test for multiple comparisons, was applied. The relationship between connexin protein expression in the pathological and control skin was tested by Pearson's correlation analysis. $P < 0.050$ was considered statistically significant. All statistical analyses were performed using SPSS® version 22 (IBM, Armonk, New York, USA).

+A: Results

A total of forty-eight patients were enrolled in this study. Paired samples were taken from 12 patients each with C0, C2, C4 and C6 venous disease. There were 22 men and 26 women. The overall mean(s.d.) age was 66.1(21.1) (range 32–89) years. Duplex ultrasound imaging confirmed the presence of superficial venous reflux in all patients from C2 onwards, and four of 12 patients with C6 disease also had segmental deep venous reflux. A pair of samples from C4, and another from C6, were damaged during the collection process and were not included in the analysis. Samples from 12 patients each in C0 and C2, and 11 each in C4 and C6 were included in the final analysis.

+B: 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 was seen with increasing disease severity: progressive epithelial hyperthickening, an increase in the depth and number of epidermal rete ridges, an increase in inflammatory cells, and loss of dermal architecture in the upper dermis (*Fig. 1*). The most prominent change observed was the increase in epithelial thickness at C6. The number of rete ridges per millimetre of epidermis was, however, significantly increased

in pathological skin as early as C2 and the depth was increased significantly from C4 onwards. This was accompanied by the loss of dermal architecture.

+B: Epidermal connexin protein overexpression

The overall absolute expression of connexin 43, connexin 30 and connexin 26 in pathological skin was similarly increased across the CEAP classes (*Fig. 2*). The overexpression of connexins in a separate cohort with C6 venous disease has been described previously[13]; however, in the present samples, overexpression started as early as C2 and C4. No overexpression was noted at C0. Connexin 43 had the highest level of expression in each class. Connexin 30 had lower expression in C0, C2 and C4 but it increased significantly in C6, as did expression of connexin 26.

The pattern of mean connexin expression per cell in the epidermis corresponded to the trend in absolute connexin expression across the CEAP classes. Significant overexpression in terms of mean connexin per cell was observed as early as C2 for all three connexins. No significant difference was noted in the connexin expression per cell between the control and pathological skin in C0.

An increasing trend in connexin expression was also noted in control skin across the CEAP classes, suggesting the progression of changes in normal skin proximally. A significant increase in connexin 43 expression in control skin was seen from C4 onwards (*P* < 0.001 for C4 *versus* C0 and for C6 *versus* C0). No significant difference was noted between C4 and C6. For connexin 30, a significant difference was observed only between C4 and C0 (*P* = 0.003), whereas for connexin 26 a significant difference was observed only between C6 and C0 (*P* < 0.001).

Connexin proteins were greatly overexpressed in pathological skin compared with control skin (*Table 1*). Connexin 26 and connexin 30 had a greater mean fold increase than

connexin 43 as they were expressed at relatively lower levels in control skin. There was a 432- and 38-fold increase in connexin 30 and connexin 26 expression respectively in pathological compared with control samples from patients with C6 venous disease. In contrast, connexin 43 expression was increased by a mean of seven times.

+B: Distribution pattern of connexin proteins with disease progression

Connexin 43 was generally expressed in all layers of the epidermis, with the highest intensity in the stratum spinosum and lowest intensity in the stratum basale (*Fig. 2*). The expression pattern changed with disease progression. In C2 and C0, the highest expression was seen along the upper portion of the stratum spinosum. In C4 disease, connexin 43 was expressed further down the stratum spinosum, approaching the stratum basale, and in C6 it was expressed throughout the epidermis, producing a fish-scale pattern.

Similar to connexin 43, connexin 30 was expressed throughout the epidermis in C6. The expression of connexin 30 in C0, C2, C4 and control skin was, however, very weak and sporadic. Although expressed at low intensity, it was visible along the stratum spinosum and granulosum. Despite no noticeable difference in the distribution pattern in undamaged skin, the intensity was higher in C4. The temporal and spatial expression pattern of connexin 26 was similar to that of connexin 30 across the four classes.

+B: Correlation of protein expression between pathological and control skin

Of all the connexin proteins, connexin 43 had the strongest positive correlation between expression levels in pathological skin and control skin ($r = 0.63$, $P = 0.001$) (*Fig. 3*). This suggests that connexin 43 expression increases steadily with disease progression.

+A: Discussion

Connexin proteins were previously known to be upregulated in diabetic, pressure and venous leg ulcers[13]. Here, the expression pattern of the principal epidermal connexin proteins was

evaluated across the stages of venous disease. The study demonstrated stepwise sequential increased expression of the principal epidermal connexin proteins as early as C2 venous disease. This finding suggests that VVs may predispose skin to poor wound healing and increase the risk of future ulceration. In addition, connexin 43 appears to be a sensitive biomarker of venous disease progression.

Connexin 43 upregulation has been implicated in impaired keratinocyte migration and poor wound healing[17]. The negative effect of connexin protein overexpression on cellular migration is mediated by both gap junctional intercellular communication and non-junctional-mediated effects. Connexin proteins act as a nexus, interacting with adhesion molecules, tight junctions and cytoskeletal components via the long cytoplasmic C-terminal tail, either directly or via adaptors[17,19,20]. A doubling of connexin 43 expression was shown to halve cellular migration[17]. The striking order of magnitude increase observed here in C6 venous disease could have a profound negative effect on healing. Despite increased absolute connexin levels in C2 and C4 venous disease, the fold increases were comparable to that in C0. This was due to increased connexin levels in control skin at C2 and C4, signifying clinical progression of the disease from the distal to the proximal part of the leg. These skin changes, possibly secondary to venous hypertension, were not previously known to extend proximally as the clinical signs of venous disease are usually confined to the medial aspect of the leg. The connexin upregulation identified here suggests that skin is preconditioned to poor wound healing and this extends proximally with disease progression.

Connexin 30 and connexin 26 were previously known to be overexpressed only at wound margins. Persistent connexin 26 overexpression maintains a hyperproliferative state, slowing down healing and stalling the transition to the remodelling stage, and leads to immune cell infiltration[21]. In the present study, connexin 30 and connexin 26 were expressed at low levels in undamaged skin, but were significantly overexpressed when ulceration

occurred. The observed upregulation at C2 and C4, which was also related to epidermal hyperthickening, suggests that the overexpression takes place before ulceration develops.

Early histological changes were also seen in C2 and C4 venous disease. The increase in the number and depth of rete ridges indicates that perfusion of the epidermis is compromised. The avascular epidermis is entirely dependent on the highly vascularized dermis for perfusion. 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[22]. The increase in epidermal thickness and worsening perfusion could ultimately result in skin breakdown.

The chronic inflammation seen in C4 venous disease has been reported previously in several studies, which documented the presence of inflammatory cells in the skin of patients with lipodermatosclerosis and venous ulcers[12]. The exact mechanism remains unclear; however, it has been hypothesized to occur as a result of leucocyte trapping and neutrophil activation secondary to ischaemia–reperfusion cycles, a consequence of venous hypertension[4,12]. This also leads to leucocyte sequestration, and on reperfusion the leucocytes are activated and release reactive species causing further oxidative damage to the ischaemic tissue[23,24]. It is not known whether prolonged hypoxia is the trigger for this sterile inflammation.

This study has several limitations. Duplex ultrasound assessment was not done in patients with no venous disease (C0), although the prevalence of venous reflux in the general population is estimated to be about 20 per cent[25]. Patients were, however, assessed clinically to ensure they has no signs of venous disease, and those with a history of superficial or deep venous reflux were excluded. Four of 12 patients with leg ulceration (C6) had segmental deep venous reflux; however, there was no difference in the distribution pattern or expression

intensity of connexin between these and the other patients with ulcers. Additionally, a formal sample size calculation was not done as the difference in connexin expression between the CEAP classes was not known previously. The study enabled a power calculation to identify the numbers needed for a future longitudinal study to compare connexin levels before and after treatment of VVs.

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Fig. 1 Epithelial thickness. **a** Representative haematoxylin and eosin-stained section of pathological and control skin samples for each Clinical Etiologic Anatomic Pathophysiologic (CEAP) class (original magnification × 20). **b** Epithelial thickness, **c** number of epidermal rete ridges and **d** depth of epidermal rete ridges in each CEAP class. Values are mean(s.d.). *$P < 0.050$ (paired t test)

Fig. 2 Connexin expression across the Clinical Etiologic Anatomic Pathophysiologic (CEAP) classification. **a** Confocal images of skin sections stained for connexin 43 (green) and counterstained with Hoescht for nuclei (blue) (original magnification × 40). Increased connexin expression is seen with disease progression in pathological skin samples across CEAP classes, but not in control skin. **b–d** Absolute connexin expression and **e–g** connexin expression per cell in each CEAP class for connexin 43 (**b,e**), connexin 30 (**c,f**) and connexin 26 (**d,g**). Values are mean(s.d.). *$P < 0.050$ (paired t test)

Fig. 3 Correlation between absolute connexin protein expression in pathological *versus* control skin: **a** connexin 43 ($r = 0.63$, $P = 0.001$), **b** connexin 30 ($r = 0.07$, $P = 0.67$) and **c** connexin 26 ($r = 0.49$, $P = 0.002$)

Table 1 Fold increase in connexin level between control and pathological skin according to CEAP class

	CEAP class			
	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

Values are mean(s.d.). Clinical Etiologic Anatomic Pathophysiologic.