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Varicose vein predisposes skin to poor wound healing by early upregulation of gap junctional proteins

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Title page

Title:

Upregulation of epidermal gap junctional proteins in patients with venous disease

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Short title

Connexin expression in venous disease

Key words

Varicose vein, venous disease, gap junctional protein, connexin, wound healing

Abstract

Background: Venous leg ulceration is the most feared complication of venous insufficiency. However, it is not known if 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 stages of venous disease.

Methods: Patients undergoing intervention for varicose veins 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, Connexin 43, Connexin 30, and Connexin 26.

Results: 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 connexin expression and mean connexin expression per cell in the pathological skin similarly increased across the CEAP from as early as C2. Increasing levels of connexin in the control skin was also seen, indicating the progression of the disease proximally. Elevated Connexin 43 expression had the strongest positive correlation between the pathological and control skin.

Conclusion: Connexins were overexpressed in patients with simple varicose veins, with a stepwise increased expression through venous eczema to ulceration, and support the role of Connexin 43 as a biomarker for poor wound healing and ulceration. This finding suggests that varicose veins predispose patients skin to poor wound healing, supporting a need for early surgical intervention to prevent ulceration.

Surgical relevance

The overexpression of connexin family of gap junctional protein is known to cause poor healing in venous leg ulceration but is not known if there is any association in patients with superficial venous disease. Here, the connexin proteins were observed to be overexpressed as early as in patients with varicose veins, even prior to histological changes. This is the first time it has been shown that superficial venous disease likely predispose skin to poor wound healing and increase the risk of future ulceration, and that connexin is a potential biomarker of venous disease progression.

Introduction

About one third of adults have varicose veins (VVs) with over 35,000 VVs surgeries performed in the UK annually[1, 2]. VVs, which commonly occur due to valve incompetence, could lead to severe complications: one third may develop skin changes, such as pigmentation and venous eczema; while 3-6% have a lifetime risk of venous ulceration[1, 3-5]. This makes venous leg ulcer(VLU) the commonest type of lower limb ulcer, with a prevalence of 0.3-0.5%, comprising 70-80% of all ulcers, and costing about £2-3 billion and 2 million lost workdays per year[6-10]. The progression of venous disease can be classified by clinical manifestation(C), etiologic factor(E), anatomic distribution of disease(A), and underlying pathophysiologic finding(P), as per the CEAP classification according to the disease severity: C0=no visible venous disease, C1=spider veins, C2=varicose veins, C3=edema, C4=lipodermatosclerosis, C5=healed VLU, and C6=active VLU[11]. A cohort study carried out over 13 years highlighted that VVs are a major risk factor of venous disease progression, however, the mechanism that influences the progression from the CEAP class C2 to C6 remains unclear[1, 12]. Furthermore, it is not known if VVs predispose patients to poor wound healing.

The current limited knowledge on the natural progression of the disease has resulted in few advances being made in identifying patients who might benefit from early surgical intervention. Owing to the high incidence of disease progression amongst patients with VVs, early intervention may render patients less prone to ulceration[5]. Investigating the histological and cellular characteristics of the skin of patients across the CEAP classification may establish if VVs predispose patients to poor wound healing and ulceration.

A particular factor which is known to cause poor wound healing in VLUs is the gap junctional proteins, Connexin[13]. The connexin proteins are specialised clusters of plasma membrane channels, which facilitate communication and exchange of ions and metabolites less than 1kDa 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 are integral to the normal wound reparatory process[17, 18]. Of the 9 different connexins expressed in the human epidermis, Connexin 43, Connexin 30 and Connexin 26 are the most abundant connexins expressed in the human epidermis, with Connexin 43 being the most ubiquitous[14].

The overexpression of connexin proteins in VLUs was previously shown to delay keratinocyte migration, resulting in poor wound healing, whilst 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 across the CEAP classification were observed in patients with venous disease to better understand the early skin changes and the expression of these proteins in the pre-wounded skin.

Methods

Patient selection and biopsy acquisition

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. Patients were eligible for study inclusion if they were aged >18 years and fulfilled the CEAP classification criteria. The exclusion criteria were the presence of arterial disease, connective tissue disorders, systemic

inflammatory disorder, diabetic mellitus, cancer, concurrent skin disease and allergies to local anaesthesia.

All patients in C2, C4, and C6 were scanned with duplex ultrasonography to confirm the presence of venous reflux and to exclude mixed arteriovenous disease. Patients in C0 were clinically assessed to exclude signs and history of venous reflux. Paired 4mm punch biopsies of the skin were taken from each patient at the time they underwent operation for treatment of varicose veins. Biopsies were paired; one above the knee and defined as control skin and one below the knee (15-20cm above the ankle) and defined as pathological skin. These locations corresponded to the endovenous catheter insertion site (control) or varicose vein avulsions site (pathological) as per 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 biopsy was taken at 1mm away from the wound margin to obtain the highest connexin expression, as per our previous protocol[13]. For a separate control (C0) group biopsies were taken from patients undergoing total knee replacement surgery.

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). 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.

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°C until processing. Prior to cryosectioning, tissues were embedded in optimal cutting temperature (OCT) medium (BDH-Poole, UK) and stored at -20°C for 24 hours. 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).

Haematoxylin and eosin (H&E) staining

All sections were stained with H&E using standard methods. Imaging was performed using a Zeiss AxioScan Z1 slide scanner at 20x magnification.

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 (downward projection of epidermis at dermo-epidermal junction) 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.

Immunohistochemistry

Tissue sections were thawed, immersed in PBS to dissolve excess OCT, permeabilized for 15 minutes in 0.2% Triton X-100 and blocked using PBS (0.1 mol L^{-1}) for 30 minutes. Primary antibodies were prepared in PBS: Connexin 43 (1:4000; C6219, Sigma - Poole, UK), Connexin 26 (1:200; 10202093, Fisher Scientific, UK), and Connexin 30 (1:200; 10795723, Fisher Scientific, UK). The tissues stained for Connexin 43 were incubated with the primary antibody for 1 hour at room temperature, while tissues stained for Connexin 30 and Connexin 26 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 minutes and stained with secondary antibody (Alexa Fluor 488 goat anti-rabbit, 10729174, 1:400; Fisher Scientific, UK) and incubated at room temperature for 1 hour. Nuclei were stained using Hoechst (1:10000; 10150888, Fisher Scientific, UK) for 5 minutes followed by 3 x 5 minute PBS washes. Coverslips were mounted using Citifluor (Glycerol/PBS solution, Citifluor Ltd, London, UK) and sealed with nail varnish.

Confocal microscopy

A Leica TCS SP8 confocal microscope (Leica, Mannheim, Germany) 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 S1)~~. All parameters were kept constant between the patient's control and pathological skin sections to allow direct comparison.

Connexin quantification

ImageJ was used for quantification. Images were converted to binary images 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[13]. Regions of

interest were manually marked to selectively include the epidermis only, excluding any areas of auto-fluorescence in the stratum corneum.

The connexin levels of the six confocal images from each tissue section were used to quantify the mean connexin expression. This data was presented as the 'absolute connexin expression', which was used for the statistical analysis and is presented in the graphs. The corresponding fold-increase data, comparing the connexin expression in the pathological and control skin, were presented in the tables 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 connexin expression per cell was calculated by the ratio of the overall connexin expression to the corresponding number of nuclei present in each tissue section. The average connexin expression per cell was compared between groups.

Statistical analysis

All data were presented as the mean ± standard deviation. Statistical differences were determined using paired t-test for paired group and Student's t-test for two unpaired groups. For more than two groups, one-way analysis of variance (ANOVA) test, followed by Bonferroni test for multiple comparisons, was applied. The relationship between the connexin protein expression in the pathological and control skin was tested by Pearson's correlation. Significance was taken at values $p<0.05$. Normality testing was performed using Kolmogorov-Smirnoff test; the connexin expression was normally distributed in each class. All statistical analyses were performed using IBM SPSS Statistics 22 software.

Results

Patient demography

A total of forty-eight patients were enrolled into this study, with 22 males (45.8%) and 26 females (54.1%). The overall mean age was 66.1 ± 21.1 years (range: 32-89 years). Duplex ultrasound confirmed the presence of superficial venous reflux in all patients from C2 onwards, while 4 out of 12 patients in C6 had segmental deep venous reflux.

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 1). 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.

Epidermal Connexin proteins overexpression

The overall absolute connexin expression for Connexin 43, Connexin 30 and Connexin 26 in the pathological skin were similarly increased across the CEAP class (Figure 2). The overexpression of the connexins in C6 has been previously described[13]. Interestingly, the overexpression takes place as early as C2 and C4. No significant overexpression was noted at C0. Connexin 43 had the highest expression in each class. Connexin 30 had lesser expression in C0, C2 and C4 but increased significantly in C6, as did Connexin 26.

The mean connexin expression per cell in the epidermis corresponds to the trend of the absolute connexin expression across the CEAP class. A significant overexpression of the

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 of connexin expression was also observed in the control skin across the CEAP class, suggesting the progression of the disease proximally. Significant increase of Connexin 43 expression in the control skin was seen from C4 onwards: C4 vs C0 ($p<0.001$) and C6 vs C0 ($p<0.001$). Interestingly, no significant difference was noted between C4 vs C6. For Connexin 30, significant difference was only observed between C4 vs C0 ($p=0.003$); while for Connexin 26, significant difference was only observed between C6 vs C0 ($p<0.001$).

Compared to the control skin, connexin proteins were overexpressed multiple fold higher in the pathological skin (Table 1). Connexin 26 and Connexin 30 had a greater mean fold increase compared to Connexin 43 as they were expressed at relatively lower levels in the control skin compared to Connexin 43. There was a striking and significant 431-fold and 38-fold increase in Connexin 30 and Connexin 26 at C6. In contrast, Connexin 43 was elevated by an average of 6-fold at C6.

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 (Figure 2). 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, Connexin 43 was expressed further down the stratum spinosum, approaching the stratum basale, and in C6, Connexin 43 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 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 Connexin 26 was similar to Connexin 30 throughout the four classes.

Correlation of the Cx proteins expression between the pathological and control skin

Compared to the expression pattern of all the connexin proteins, Connexin 43 had the strongest positive correlation between the expression in the pathological skin and control skin ($r=0.63$, $p=0.001$) (Figure 3). This suggests that Connexin 43 expression increases steadily with the disease progression.

Discussion

Connexin proteins were previously known to be upregulated in diabetic, pressure and VLUs[13]. Here, the expression pattern of the principal epidermal connexin proteins across the stages of venous disease, especially in pre-wounded skin, was evaluated. This study demonstrated that there is a stepwise sequential increased expression in the principal epidermal connexin proteins as early as C2. This finding 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 Connexin 43 is a sensitive biomarker of venous disease progression. These findings support a conclusion that treating VVs early could help prevent future ulceration.

Connexin 43 upregulation in VLUs has been implicated in impaired keratinocyte migration and poor wound healing[17]. The negative effect of the 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]. An increase of Connexin 43 by one-fold was shown to halve cellular migration[17]. The striking multiple-fold increase that was observed here in C6 could have a profound negative effect on healing. Despite increased absolute connexin levels at C2 and C4, the fold-increases are comparable to that of C0. This is due to increased connexin 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 connexin 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 connexin overexpression, which, in the context of VLUs, was previously thought only to be a feature of wound chronicity.

Connexin 30 and Connexin 26 were previously only known to be overexpressed at the wound edge. The persistent Connexin 26 overexpression maintains a hyper-proliferative state, slowing down healing, stalling the transition to the remodelling stage, and leads to immune cell infiltration[21]. This study found that Connexin 30 and Connexin 26 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.

This study 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[22]. 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 has 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[23, 24]. This cycle 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[25]. Patients were, however, clinically assessed to ensure absence of signs of venous disease and patients with history of superficial or deep venous reflux were excluded. 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 connexin observed within these patients. Additionally, a

formal sample calculation was not performed as the difference in connexin expression between the CEAP classes was previously not known, and this is the first time that it has been established that connexins are overexpression prior to wounding. This, however, enabled us to perform post-study power calculations to identify the numbers needed for the future longitudinal study to compare connexin levels before and after treatment of VVs. Further work is needed to assess whether intervention and treatment of superficial venous disease in patients with varicose veins could reverse the elevation of connexin s and thereby improve the skin's ability to heal, thus reducing the risk of developing venous leg ulcers.

Conclusion

This study showed that the principal epidermal connexins were overexpressed in patients with simple varicose veins, with a stepwise increased expression through venous eczema to ulceration. The cellular and structural changes correlate with the clinical stage of the disease, supporting the role of Connexin 43 as a potential biomarker for venous ulceration. This finding could further suggest that the skin in patients with venous disease is preconditioned for poor wound healing prior to ulceration.

Conflict of interest

The authors have no conflict of interest to declare.

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List of tables

Table 1: Mean fold increase

	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 shows the mean fold increase in each CEAP class for Connexin 43, Connexin 30 and Connexin 26. Values represent mean ± standard deviation, corrected to second decimal place.

List of Figures

Figure 1: Epithelial thickness

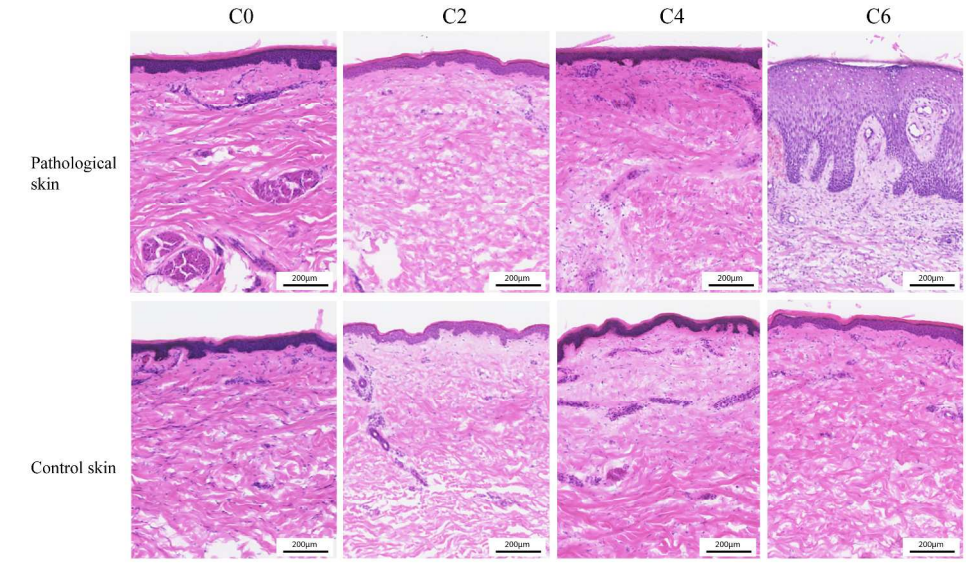
a Haematoxylin and eosin-stained section of the skin section for each CEAP class. The mean epithelial thickness of each CEAP class is indicated at the bottom of the image. Scale bar = 200µm. Magnification 20x. Bar charts show the (**b**) mean epithelial thickness, (**c**) number of epidermal rete ridges,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

Figure 2: Connexin expression across the CEAP classification

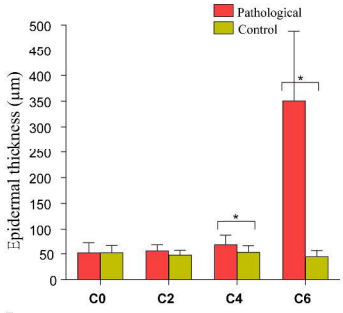
(A, B, C) Confocal images of Connexin 43, Connexin 30 and Connexin 26 expression in each group. Skin sections were stained green for Connexin (green) and counterstained with Hoescht for nuclei (blue). Increase of Connexin expression is seen with the disease progression in the pathological skin. No obvious increase in Connexin expression is seen in the control skin across the CEAP. Scale bar = 50µm. Magnification 40x. (D, E, F) depicts the mean absolute Connexin expression and (G, H, I) depicts the mean Connexin expression per cell for each CEAP class. Values expressed as mean ± standard deviation. *P<0.05 (paired t-test)

Figure 3: Correlation of absolute Connexin proteins expression

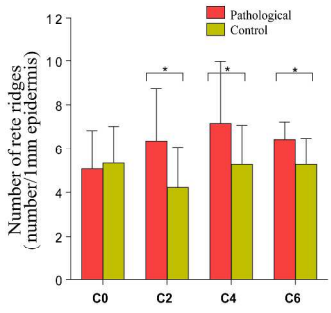
Pearson's correlation of the absolute Connexin proteins expression in the pathological skin versus control skin.



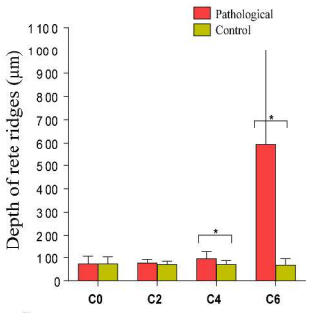
a Histology of the skin



b Mean epidermal thickness



c Number of rete ridges



d Depth of rete ridges

Figure 1

492x486mm (300 x 300 DPI)

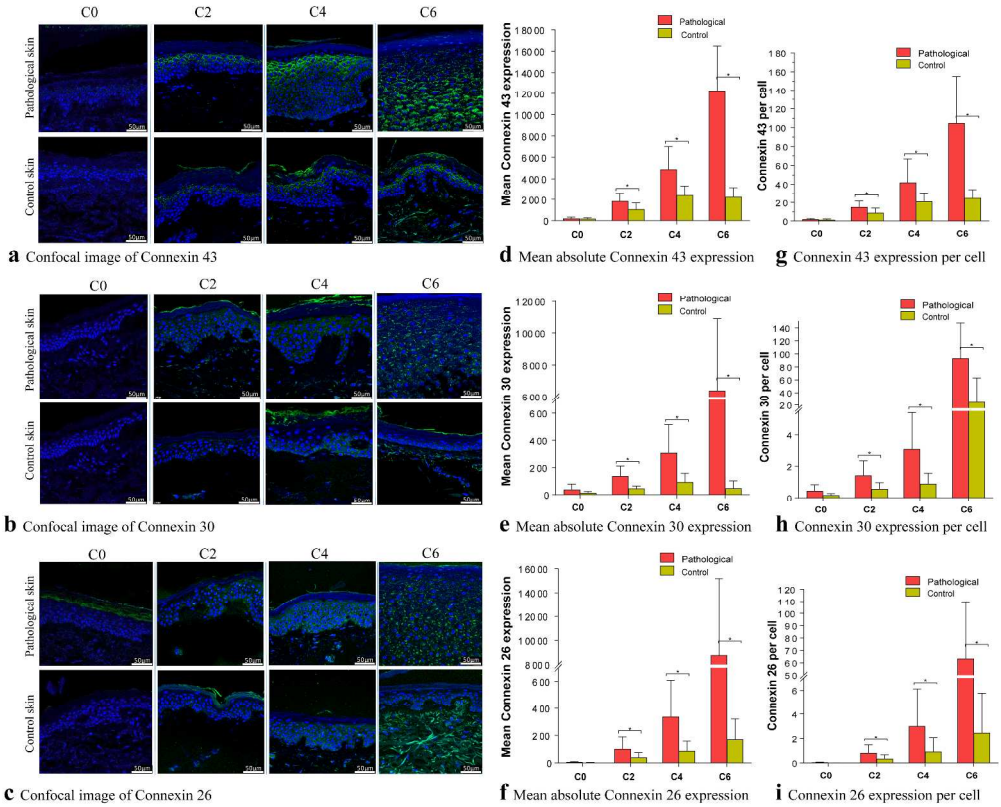
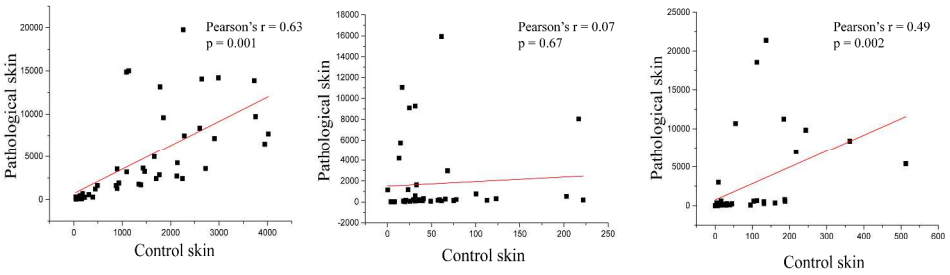


Figure 2

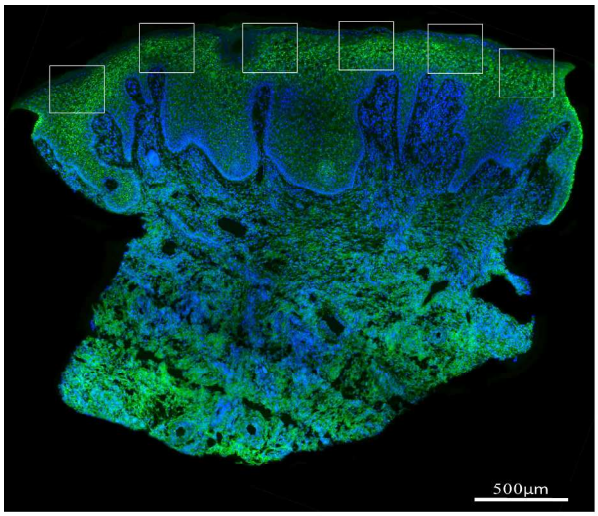
574x469mm (300 x 300 DPI)



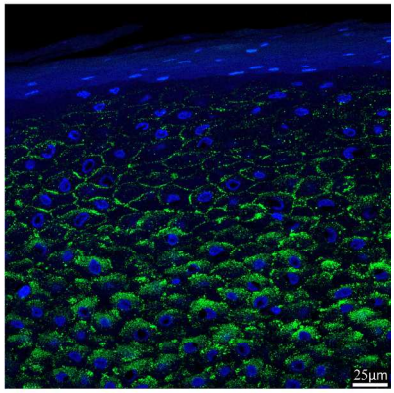
a Connexin 43 **b** Connexin 30 **c** Connexin 26

Figure 3

527x187mm (300 x 300 DPI)



a Confocal image of an entire skin section



b High magnification image

324x178mm (300 x 300 DPI)