Objective assessment of dermal fibrosis in cutaneous scarring: using optical coherence tomography, high frequency ultrasound and immunohistomorphometry of human skin

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Objective assessment of dermal fibrosis in cutaneous scarring: using optical coherence
tomography, high frequency ultrasound and immuno-histo-morphometry of human skin

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Running head: Assessment of dermal fibrosis
What’s already known about this topic?

- Objective studies of the progression of scar formation and the properties of mature scars are necessary in order to evaluate clinical treatment as well as research focused on developing novel methods for management of dermal fibrosis.
- Optical coherence tomography (OCT) and high-frequency ultrasound (HFUS) are two known non-invasive techniques that are used effectively for measuring structural and physiological changes in cutaneous tissue.

What does this study add?

- OCT and HFUS are useful tools for non-invasive monitoring of cutaneous fibrosis enabling quantitative sequential temporal measurements of cutaneous thickness similar to histology.
- OCT attenuation coefficient (better in resolution) and HFUS intensity (better in depth) provide an indication of collagen deposition in skin over the course of healing supported by immunohistochemical analysis.
- Choice of device is dependent upon wound/scar type, the parameters to be measured and morphological detail required.
Summary

Background Non-invasive quantitative assessment of dermal fibrosis remains a challenge. Optical coherence tomography (OCT) and high-frequency ultrasound (HFUS) can accurately measure structural and physiological changes in skin.

Objectives To perform quantitative analysis of cutaneous fibrosis.

Methods 62 healthy volunteers underwent multiple sequential skin biopsies (day 0 and 1-8 weekly thereafter) with OCT and HFUS measurements at each time-point supported with immuno-histo-morphometry analysis.

Results HFUS and OCT provided quantitative measurements of skin thickness, which increased from uninjured skin (1.18mm, 1.2mm respectively) to week 1 (1.28mm; p=0.01, 1.27mm; p=0.02) and compared favourably with H&E. Spearman’s correlation showed good agreement between techniques (p<0.001). HFUS intensity, corresponded to dermal density, with reduction from uninjured skin (42%) to week 8 (29%) (p=0.02). OCT attenuation coefficient linked with collagen density and reduced at week 8 (1.43mm) (p<0.001). Herovici analysis showed that mature collagen was highest in uninjured skin (72%) compared to week 8 (42%) (p=0.04). Elastin was highest in uninjured skin (49.1%) and lowest at week 4 (23.0%, p=0.01), fibronectin was greatest at week 4 (0.72Au) and reduced at week 8 (0.56Au) and α-SMA increased from uninjured skin (11.5%) to week 8 (67%) (p=0.003).

Conclusions Time-matched comparison images between H&E, OCT and HFUS demonstrated that epidermal/dermal structures were better distinguished by OCT. HFUS enabled deeper visualisation of the dermis including the subcutaneous tissue. Choice of device was relevant upon the depth of scar type, parameters to be measured and morphological detail required in order to provide better objective quantitative indices of the quality and extent of dermal fibrosis.
Introduction

Cutaneous healing is a dynamic process during which there is a resolution of angiogenesis, wound contraction, and tissue remodelling.\textsuperscript{1,2} Skin healing process results in the formation of a scar, which can be defined as a dermal fibrous tissue that replaces normal cutaneous tissue following injury.\textsuperscript{3} Scars often demonstrate a significantly reduced or a total loss of several essential skin functions, including barrier function, mechanical and physical properties, and important physiological parameters.\textsuperscript{4} A multitude of scar treatment options are available but despite this, none can totally erase a scar and many can result in unsatisfactory or inconsistent outcome, with no single treatment method having been universally adopted.\textsuperscript{5} Therefore, in order to evaluate current clinical treatment modalities as well as research focused on developing novel methods for scar management, objective quantitative methods of skin scarring are required.\textsuperscript{6}

Histological analysis of biopsied scar tissue remains the gold standard for assessment and diagnosis of normal and pathological scarring as it this enables visualisation of tissue architecture down to the cellular level.\textsuperscript{3,7} Nevertheless, tissue sections take time to be processed, are invasive, can only provide snapshots of the scarring process, biopsies cannot be repeated at the same site and may create a further wound or scar and can be associated with patient-related complications including delayed healing or infection.\textsuperscript{4} Therefore, there is an unmet need for urgent development of non-invasive devices for quantitative assessment of dermal fibrosis (as an index of cutaneous scarring) for the purposes of objective evaluation of response to scarring therapies and outcomes.\textsuperscript{6}

A number of technologies have been used including magnetic resonance imaging (MRI),\textsuperscript{7} confocal laser microscopy (CLM),\textsuperscript{8} ultrasound (US)\textsuperscript{9} and optical coherence tomography
Nevertheless, there is generally an inverse relationship between penetration depth and resolution. A number of studies have used CLM, which has a high resolution but the limited penetration depth hinders the ability of this modality to study collagen density alterations in the dermis.\textsuperscript{12-14} 20MHz frequency US systems have also been used which have a good penetration depth but a rather low resolution which limits their ability to evaluate finer dermal tissue variations.\textsuperscript{9,15} Although possessing excellent penetration depth, magnetic resonance imaging (MRI) has restricted resolution and only enables assessment of gross architectural skin changes.\textsuperscript{16} OCT can image deeper structures than confocal microscopy while maintaining a resolution that exceeds that of US although high frequency US (HFUS) provides deep dermal penetration with higher resolution.\textsuperscript{17} For the purpose of imaging cutaneous fibrosis, OCT provides an optimal balance between penetration depth and resolution.\textsuperscript{18} Although these tools have the potential to offer distinct advantages in wound and scar evaluation, their appropriate application and limitations remain to be determined. Furthermore, there is a lack of validation in the use of certain devices in wound repair, where objective measurements taken by non-invasive devices have been corroborated by immunohistochemical analysis.

We have previously compared OCT to histological assessment of acute wound healing and showed that characteristically architectural changes that correlate with histological phases of cutaneous wound healing could be identified with OCT.\textsuperscript{10} Additionally, a useful novel measure of fibroplasia and remodelling referred to as the mean grey value (MGV) was identified.\textsuperscript{10}

In view of the above, for the purpose of this study we compared OCT with an equivalent form of HFUS. Thus, a 50MHz HFUS was used as this allowed a similar penetration depth
to OCT. Therefore, our aim was to generate normative data of dermal fibrosis following sequential temporal cutaneous biopsies created in healthy volunteers using OCT and HFUS with the intention of comparing both devices and to have these findings validated by histological and immunohistochemical analysis.
Materials and methods

Healthy volunteer participants were enrolled into the study at the Manchester University NHS Foundation Trust, England, UK. University of Manchester Research Ethics Committee and Trust Research and Development department approval were granted (Reference: UoM 14333). All participants provided written consent to take part in the study.

Demographics

Demographic data is displayed in Table 1. Sixty-two healthy volunteers participated, most of which were female (55%). The majority of subjects were between 21-30 years of age (68%) and most were of Caucasian ethnicity (94%).

Study Design

On day 0, all 62 participants had a 5mm diameter full thickness skin biopsy performed under local anaesthetic (Lidocaine 1%) to both their upper inner arms, 5cm from the axillary hairline and parallel to the medial epicondyle. The participants were split into 7 groups (weeks 1, 2, 3, 4, 5, 6 and 8) in order to evaluate multiple time points where 6mm re-biopsies were performed at their respective time points (Figure 1). This would not have been possible in one cohort due to the large number of punch biopsies that would need to be performed in each participant. All wounds healed successfully in each group. The biopsy wound sites were dressed with Kaltostat (ConvaTec, Middlesex, UK), gauze and Tegaderm + pad dressing (3M, Minnesota, USA). Objective non-invasive imaging modalities were used at each time point for all groups prior to any intervention to monitor the progression of wound healing with measurements taken in triplicate with average values used in final analysis. The same area was located at each time point for each of the devices by the use of the small probe which was positioned over the biopsy sites in the centre of the probe area. Once positioned,
the area could be easily visualised in the software to ensure the biopsy site/scar was positioned centrally and incorporated the scar edges prior to image acquisition. The measurements were taken blinded to the previous images acquired. All clinical measurements and analyses were performed by the same researcher.

Optical coherence tomography (OCT)

The OCT device (Vivosight, Michaelson, UK) uses low-coherence near infrared light to produce an image of optical scattering from tissues, effectively creating an “optical ultrasound” at a resolution close to histopathology (Figure 2a)i). The frequency domain OCT device used offers a lateral optical resolution of <7.5 μm and an axial resolution of 10 μm. The penetration depth lies at approximately 1.2–1.8mm due to scattering effects and a scan area of 6 mm x 6 mm. Every scar was measured by setting the handheld imaging probe onto the lesion. Then its position was corrected by monitoring the trace of the aiming laser and the real time OCT image of the scar until a good cross-sectional image was displayed on the screen. The device enabled quantitative measurements for 1) the attenuation coefficient - which is the amount by which the optical signal reduces with distance travelled into the skin and has been related to collagen density and organization and 2) the epidermal plus dermal skin thickness. We manually measured on five predefined places in the OCT image from the skin surface reflection or entrance signal to the first well-demarcated change of reflectance intensity as expressed in a more signal-poor zone indicating base of dermis (Figure 2a)ii). The Image J software of the system provided the calculated distance between the two measurement lines.
50MHz High Frequency Ultrasound (HFUS)

The HFUS device (Dermascan C, Cortex, Denmark) uses sound waves, and the ultrasonic waves are partially reflected at the boundary between adjacent structures and produce echoes of different amplitudes (Figure 2b)i). The 50MHz probe has a lower resolution of 30 x 60um than OCT and a greater penetration depth of 3mm, with a scan area of 2.7 x 6mm. Water was employed as a coupling agent between the skin surface and the probe. Prior to each measurement a thin layer of conducting ultrasound gel was applied to the transducer. Minimal pressure was applied to preserve the thickness and echogenicity of the lesions. HFUS enabled quantitative measurements in the B-mode software for 1) the intensity of reflections in the skin, which can be quantified as echodensity and 2) the epidermal plus dermal skin thickness. HFUS skin thickness measurements were performed automatically in the software by detection of the border between the epidermis and dermis based on the A-scan. Average thickness values were automatically for the maximum distance between outer edge of epidermis and base of dermis and the minimum distance (Figure 2b)ii).

Laboratory Techniques

Histology

Formalin-fixed paraffin-embedded tissue sections at a thickness of 5µm were prepared on glass slides. Haemotoxylin and eosin staining (H&E) was performed to assess morphological and histological changes in the punch biopsies over time.

Immunohistochemical staining

Immunohistochemical staining methodology provided in Supplementary material 1. Details of antibodies used are listed in Table 2.
Statistics and data analysis

Statistical information provided in Supplementary material 1.
Results

Dermal fibrosis can display an overexpression of a number of growth factors which can directly stimulate the proliferation of fibroblasts, their differentiation into myofibroblasts, and production of excessive extracellular matrix including collagen.\textsuperscript{4} Accurate assessment of dermal fibrosis is important as it is a good indicator of pathological scarring and mirrors an excessive amount of collagen synthesis. The results will now be presented in relation to scar thickness, HFUS intensity or dermal density and OCT attenuation coefficient relating to collagen density and morphological features including histological and immunohistochemical analysis of collagen, elastin, fibronectin and alpha-smooth muscle actin (alpha-SMA) which play an important role in fibrogenesis.

Scar thickness

HFUS images of uninjured skin showed that there are three layers of different echogenicity. The outermost layer known as an epidermal entrance echo was highly echogenic. Underneath, there was a dermal layer, which is less echogenic than the entrance echo and the third layer related to the non-echogenic hypodermis (Figure 3a). Scar images presented as more hypoechoic as they were more homogenic and thus appeared darker than uninjured skin beneath the strong hyperechoic entrance echo. Quantitative measurements demonstrated that there was an increase in skin thickness from day 0 (uninjured skin) (1.18mm) to week 1 (1.28mm) (p=0.01), with a slight reduction to week 6 (1.23mm) and subsequent increase to week 8 (1.28mm) (Figure 3b).

OCT images showed that the layered structure of uninjured skin was clear with differentiation between the epidermis, papillary dermis and reticular dermis and skin appendages, including hair shafts and sebaceous glands evident (Figure 4a). Blood vessels
appeared as transverse hyporeflective areas. Scarred skin showed the newly deposited extracellular matrix from 3 weeks after injury and then more ordered fibrosis with a thickened epidermis and a lack of defining features such as rete ridges thereafter.

Measurements demonstrated an increase in skin thickness from day 0 (uninjured skin) (1.2mm) to all other time points. This increase was significant at week 1 (1.27mm) (p=0.02) and week 8 (1.28mm) (p=0.03) (Figure 4b). Spearman’s correlation was performed which demonstrated good agreement between HFUS and OCT skin thickness measurements, this was significant at day 0 to week 5 (p<0.001) (Figure 5).

H&E staining also corroborated these findings (Figure 6a), as measurements demonstrated increased skin thickness from day 0 uninjured skin (1.18mm) to all time points, with a significant increase at week 1(1.26mm) (p=0.01) (Figure 6b). Taking the histopathological skin thickness as “gold standard”, OCT and HFUS tended to overestimate skin thickness compared to H&E analysis (Figure 6c).

**HFUS intensity**

The total intensity was measured by HFUS, which corresponds to the dermal density or echogenicity of the skin. Lower numbers or thinner collagen fibres can reflect a weaker echo compared to thicker collagen fibres providing a stronger echo. Measurements showed that there was an initial drop at week 1 continuing to week 3, after which echogenicity readings slowly built back up. There was a significant reduction in echogenicity from day 0 uninjured skin (42%) to week 8 (29%) (p=0.02) (Figure 7a).
OCT attenuation coefficient

OCT attenuation coefficient is the amount by which the optical signal reduces with distance travelled into the skin and has been related to collagen density and organisation. The results showed that the attenuation coefficient significantly reduced from uninjured skin (2.2Au) to week 8 (1.35Au) (p<0.001) (Figure 7b).

Morphological features

Time matched images with H&E demonstrated that re-epithelialisation was evident with both OCT and HFUS one week after injury (Figure 8). H&E and OCT both displayed a haemostatic crust but this was not detected by HFUS. All subjects had complete epithelial cover after 2 weeks. By week 8, OCT and H&E showed that the healed epithelium was thickened and homogeneous. This was also shown by the strong hyperechoic band of the epidermis in the HFUS images. After 2 weeks post initial biopsies, OCT, HFUS and H&E showed tissue remodelling and fibrosis until 8 weeks.

Herovici staining demonstrated that mature collagen was greatest in uninjured skin (72%) compared to week 1 (19%) (p=0.001), week 2 (21%) (p=0.02) and week 3 (20%) (p=0.02) (Figure 9a). Immature collagen was greater at all subsequent time points in scar skin compared to uninjured skin (19%) particularly at week 1 (68%) (p=0.01), week 2 (52%) (p=0.04) and week 3 (48%) (p=0.02). Collagen I and III intensity measurements corroborated this trend. Collagen I intensity was higher in uninjured tissues compared to scar tissues with the greatest intensity from beneath and at the wound edges over time (Figure 9b), whilst Collagen III intensity was greater in scar tissues compared to normal skin (Figure 9c). HFUS intensity and OCT attenuation coefficient demonstrated a similar trend as mature collagen
shown by herovici staining and Collagen I intensity, with higher levels in uninjured skin reducing to week 1 and a gradual increase over time (Figure 10).

Elastin was significantly greater in uninjured skin (49.1%) compared to scar tissue with lowest values at week 4 (23.0%, p=0.01) and a gradual return to values similar to baseline by week 8 (41.4%) (Figure 11a). Fibronectin intensity was greatest at week 4 (0.72Au) and reduced to week 8 (0.56Au) (Figure 11b). Furthermore, alpha-SMA percentage marker area gradually increased from baseline in uninjured skin (11.5%) to week 8 (67%) (p=0.003) (Figure 11c).
Discussion

In this unique human clinical study of 62 healthy volunteers, we have demonstrated that OCT and HFUS are useful tools for non-invasive monitoring of cutaneous fibrosis that enable quantitative sequential temporal measurements of cutaneous thickness similar to those of histology. OCT attenuation coefficient values and HFUS intensity provided an indication of the intensity of collagen deposition in the skin over the course of healing as supported by immunohistochemical analysis. Additionally, OCT enabled greater visualisation of morphological detail compared to HFUS, whilst, HFUS provided deeper penetration in comparison to OCT.

The total intensity measured by HFUS corresponding to the dermal density or echogenicity of the skin showed a significant reduction from uninjured skin to week 8 indicating lower numbers or thinner collagen fibres reflecting a weaker echo compared to thicker collagen fibres providing a stronger echo evidenced in uninjured skin. There was an initial drop at the early time points, after which echogenicity readings slowly built back up. OCT attenuation coefficient, which is the amount by which the optical signal reduces with distance travelled into the skin and has been related to collagen density and organisation was also significantly reduced from uninjured skin to week 8. Again this showed a reduction at the earlier time points and a subsequent increase thereafter. HFUS intensity and OCT attenuation coefficient showed a similar trend to mature collagen as evidenced by Herovici and collagen I analyses. Time-matched comparison images between H&E, OCT and HFUS demonstrated that layers such as the epidermis, dermis, structures and blood vessels could be well distinguished by OCT allowing description of architectural details. However, in contrast to histopathology, single cells cannot be visualised. HFUS enabled visualisation of the epidermis, dermis and subcutaneous tissue and indication of a hypoechoic area at the wound site but did not show
haemostatic crust or other specific structural details. Taking the histopathological measurement of skin thickness as “gold standard”, OCT and HFUS both showed a tendency towards overestimation of skin thickness compared to H&E analysis.

OCT attenuation coefficient and HFUS intensity showed significantly reduced reflectivity at week 1 related to loss of dermal tissue secondary to injury and reflected the lower intensity of subcutaneous fat due to the high water content. Values subsequently increased, which indicate fibroblast recruitment to the wound bed seen histologically, resulting in dermal regeneration and thus tissue fibrosis leading to mature scar tissue formation.

Skin thickness measurements were similar across OCT, HFUS and H&E, however, OCT and HFUS tended to overestimate skin thickness compared to histological measurements. This may be due to tissue shrinkage after formalin fixation and embedding in paraffin. This phenomenon has been investigated in a study evaluating HFUS and histological thickness measurements of tumour thickness, which showed that histological measurements were slightly lower than HFUS. It was suggested that the tissue expanded after excision then subsequently contracted post formalin fixation. Despite the small discrepancies between OCT, HFUS and histological measurements, the modalities show there is a distinct trend between time points which are clear with both devices. We performed Spearman’s correlation which demonstrated good agreement between HFUS and OCT skin thickness measurements, this was significant at day 0 (uninjured skin) to week 5. Correlations were reduced at weeks 6, 7 and 8 and this may have been due to a lower number of subjects in these groups (n=12, n=6, n=6 respectively) compared to the earlier time points which could have led to more variation. A number of studies have used HFUS as a measure of skin thickness and found this to be capable of producing valid and reproducible results in both, healthy and scarred
tissue. Both inter-observer and reproducibility of the device have been assessed and was found to have a strong intraclass correlation coefficient of 0.94. However, a major limitation of these studies was that they evaluated the consistency, and not accuracy, of measurements on post-burn scars. In addition, despite a number of studies evaluating HFUS there has been a lack of studies using a device greater than 20MHz as we have used a 50MHz.

We evaluated a number of fibrotic markers including; fibronectin, elastin and alpha-SMA, to identify trends in normal cutaneous healing. The ability to characterize the extent and rate of progression of dermal fibrosis is critically important in determining degree of fibrotic severity, healing potential, and determining response to therapies across all forms of cutaneous fibrosis. In normal acute wound healing, fibronectin plays a critical role in ECM organisation and stability and this role is visible in all phases. Accumulation of fibronectin-rich fibrillar matrix will stimulate further matrix deposition. When a scar matures, fibronectin is broken down to create place for collagen deposition, which gives strength to the final scar. This mirrors our results which showed that fibronectin was greatest at week 4 and reduced thereafter to week 8. Elastin plays a vital role in the structure and function of the skin. Elastin fibres contribute to recoil and resilience in tissues with its role closely linked with collagen. An intact elastic fibre network is absent after cutaneous injury, which contributes to the diminished physical properties of scars compared with uninjured skin. Here, we showed that elastin was significantly greater in uninjured skin compared to scar tissue with lowest values at week 4 and a gradual return to values similar but lower than uninjured skin by week 8. Furthermore, alpha-SMA gradually increased from baseline in uninjured skin to week 8. The gradual increase in mature collagen I from week 1 to week 8, links with increased wound contraction evidenced by alpha-SMA.
The correct choice of device is paramount for a particular wound or scar type, as deeper wounds or thicker scars such as hypertrophic and keloid scars may not be detected as accurately with OCT compared to HFUS. Preferably, both modalities could be combined for maximum theranostic value. Each modality allowed the detection and monitoring of cutaneous healing. In contrast to HFUS, OCT enabled a greater resolution of the morphology and architecture, although cellular morphology could not be identified with both modalities. Although it was possible to identify morphological aspects, assessment of these characteristics should be performed with caution due to the subjective nature, thus, our primary focus was on the quantitative abilities of these devices. Data regarding the direct comparisons of HFUS, OCT and histopathology is limited, nevertheless, one study investigated the accuracy of HFUS and OCT for tumour thickness measurements of basal cell carcinomas and actinic keratosis and compared their results with histological measurements. They demonstrated more accurate results with OCT due to better resolution and contrast from the infrared radiation compared to the contrast of acoustic signals from the HFUS.

The advantages of both devices are that they are non-invasive and side effect free. Furthermore, they can serially assess a wound or scar over time without interfering in the disease or healing process. The scanning process for both modalities are fast and images are ready for analysis within seconds which can enable rapid diagnosis. OCT and HFUS have been shown to be precise in terms of repeatability and reproducibility with low variation coefficients and high resolution. In contrast with HFUS, OCT does not require any use of gels. Both devices are able to give high resolution images and the analyses algorithms are automatic, not involving any operator interpretation in relation to quantitative aspects.
Limitations are that OCT maximum scanning depth is 2 mm but, in practice, resolution over 1.25 mm from the surface is poor. Significant epidermal thickening or haemostatic crust can hinder the scan by producing shadowing and thus reducing the resolution. For diagnostic purposes, OCT may be most useful for epidermal, papillary dermal and upper reticular dermal pathologies. Furthermore, unlike histological analysis, OCT cannot identify individual cells. OCT and HFUS may be useful as complementary imaging biomarkers. For both devices, patient cooperation is essential as movement results in low quality scan images, therefore, certain patient cohorts may be unsuitable.

Our study used a large number of subjects (n=62) to assess healing over an 8 week period with a predominantly homogenous cohort with respect to age and ethnicity. Limitations of this study are longer term follow-up and a more heterogenous cohort. It would have been beneficial to perform these analyses in other ethnic groups as substantial differences in scarring have been noted in dark pigmented skins. Based on our data, we conclude that both OCT and HFUS are suitable tools for non-invasive monitoring of cutaneous healing and may be useful in choosing the optimal therapeutic regimen. Despite slightly overestimating skin thickness compared to histopathological measurements, OCT and HFUS demonstrated the same trend over time. OCT attenuation coefficient values and HFUS intensity provided an indication of collagen levels in the skin over the progression of healing. OCT enabled greater visualisation in terms of resolution of morphological detail of the dermis compared to HFUS. On the other hand, HFUS enables deeper penetration in comparison to OCT. These findings have demonstrated that the choice
of device is important and dependent upon wound or scar type, the parameters to be measured and the morphological detail required.
References


Figure Legends

Figure 1: Flowcharts demonstrating the clinical and experimental methodology.

Clinical: Sixty-two healthy volunteers were recruited and had objective measurements performed at multiple sequential time points. Objective non-invasive devices were used including optical coherence tomography (OCT), which provided measurements of attenuation coefficient and skin thickness and high frequency ultrasound (HFUS), which measured intensity and skin thickness.

Experimental: Punch biopsies were performed on day 0 and weekly from weeks 1 to 8. Tissues were placed in formalin and underwent tissue processing for histological and immunohistochemical analysis.

Figure 2: Comparison of device parameters.

a) i) Optical coherence tomography (OCT) device. ii) OCT uses low-coherence near infrared light to produce an image of optical scattering from tissues, effectively creating an “optical ultrasound” at a resolution close to histopathology. iii) The device has a lateral optical resolution of <7.5 μm and an axial resolution of 10 μm. The penetration depth lies at approximately 1.2–1.8mm due to scattering effects and a scan area of 6 mm × 6 mm.

b) i) High frequency ultrasound (HFUS) device. ii) HFUS uses sound waves, and the ultrasonic waves are partially reflected at the boundary between adjacent structures and produce echoes of different amplitudes. The epidermis, dermis and subcutis are displayed in the image. iii) We chose to use a 50MHZ probe which has a lower resolution of 30 x 60um than OCT and a greater penetration depth of 3mm with a scan area of 2.7 x 6mm.
**Figure 3:** High frequency ultrasound (HFUS) images show that scars present as more hypoechoic than normal skin beneath a strong hyperechoic entrance echo. They also demonstrate an increase in skin thickness from day 0 uninjured skin to week 1 with a slight reduction over time and subsequent increase to week 8. Furthermore, quantitative measurements of skin thickness provided by the device showed a significant increase from baseline to week 1 (p=0.01).

**Figure 4:** a) Optical coherence tomography (OCT) images demonstrate an increase in skin thickness from day 0 to all other time points. b) The graph shows that this increase was significant at week 1 (p=0.02) and week 8 (p=0.03).

**Figure 5:** Spearman’s correlation analysis demonstrated good agreement between HFUS and OCT skin thickness measurements. The graphs show that this was significant at day 0 (p<0.001), week 1 (p<0.001), week 2 (p<0.001), week 3 (p<0.001), week 4 (p<0.001), week 5 (p<0.001).

**Figure 6:** a) Haematoxylin & Eosin (H&E) staining demonstrated increased skin thickness b) from day 0 uninjured skin (1.18mm) to all time points, with a significant increase at week 1 (1.26mm) (p=0.01). c) Taking the histopathological skin thickness as “gold standard”, OCT and HFUS tended to overestimate skin thickness compared to H&E analysis.

**Figure 7:** a) i) The total intensity was measured by high frequency ultrasound (HFUS) which corresponds to the dermal density or echogenicity of the skin. Lower numbers or thinner collagen fibres can reflect a weaker echo compared to thicker collagen fibres providing a
stronger echo. ii) Measurements showed a significant reduction in echogenicity from day 0 uninjured skin (42%) to week 8 (29%) (p=0.02).

b) i) Optical coherence tomography (OCT) attenuation coefficient is the amount by which the optical signal reduces with distance travelled into the skin and has been related to collagen density and organisation. ii) The results showed that the attenuation coefficient significantly reduced from uninjured skin (2.2Au) to week 8 (1.35Au) (p<0.001).

**Figure 8:** Time matched images with haematoxylin & eosin (H&E) demonstrated that re-epithelialisation was evident with both optical coherence tomography (OCT) and high frequency ultrasound (HFUS) one week after injury. H&E and OCT both displayed a haemostatic crust but this was not detected by HFUS. All subjects had complete epithelial cover after 2 weeks. By week 8, OCT and H&E showed that the healed epithelium was thickened and homogeneous. This was also shown by the strong hyperechoic band of the epidermis in the HFUS images. After 2 weeks post initial biopsies, OCT, HFUS and H&E showed tissue remodelling and fibrosis until 8 weeks.

**Figure 9:** Collagen analysis. a) i) Herovici staining demonstrated that a)ii) mature collagen was greatest in uninjured skin (72%) compared to week 1 (19%) (p=0.001), week 2 (21%) (p=0.02) and week 3 (20%) (p=0.02). Immature collagen was greater at all subsequent time points in scar skin compared to uninjured skin (19%) particularly at week 1 (68%) (p=0.01), week 2 (52%) (p=0.04) and week 3 (48%) (p=0.02). b) i) Collagen I images. b)ii) Collagen I intensity was higher in uninjured tissues compared to scar tissues with the greatest intensity from beneath and at the wound edges over time. c) i) Collagen III images. c)ii) Collagen III intensity was greater in scar tissues compared to normal skin.
**Figure 10:** Graphical representation of the trends between high-frequency ultrasound (HFUS) intensity, optical coherence tomography (OCT) attenuation coefficient, Herovici collagen analyses and immunohistochemical analysis of collagen I and III. The trends show that HFUS intensity linked most closely with herovici mature collagen and collagen I whilst OCT attenuation coefficient showed a similar trend except for week 2 and week 8.

**Figure 11:** a)i) Elastin images. a)ii) Elastin was significantly greater in uninjured skin (49.1%) compared to scar tissue with lowest values at week 4 (23.0%, p=0.01) and a gradual return to values similar to baseline by week 8 (41.4%). b)i) Fibronectin images. b)ii) Fibronectin intensity was greatest at week 4 (0.72Au) and reduced to week 8 (0.56Au). c)i) Alpha-smooth muscle actin images. c)ii) Alpha-smooth muscle actin percentage marker area gradually increased from baseline in uninjured skin (11.5%) to week 8 (67%) (p=0.003).
**Table 1:** A table displaying the demographic data of the healthy volunteers.

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**Table 2:** Details of primary antibodies used, incubation details, secondary antibodies used and detection methods.

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<th>Primary antibody name, product code and company</th>
<th>Primary antibody raised species, isotype and concentration</th>
<th>Primary antibody incubation details</th>
<th>Secondary antibody, company, concentration, incubation details</th>
<th>Detection method</th>
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<td>Overnight, 4°C</td>
<td>Universal antibody by Novolink TM Leica Biosystems Newcastle ltd, Newcastle Upon Tyne, UK cat. RE7150-K (1h room temp)</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>Collagen III Ab6310, Abcam, UK</td>
<td>Mouse (monoclonal) IgG1, 1:1000 dilution</td>
<td>Overnight, 4°C</td>
<td>Universal antibody by Novolink TM Leica Biosystems Newcastle ltd, Newcastle Upon Tyne, UK cat. RE7150-K (1h room temp)</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>Fibronectin Ab2413, Abcam, UK</td>
<td>Rabbit (polyclonal), IgG, 1:650 dilution</td>
<td>Overnight, 4°C</td>
<td>Universal antibody by Novolink TM Leica Biosystems Newcastle ltd, Newcastle Upon Tyne, UK cat. RE7150-K (1h room temp)</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>Elastin Ab23747, Abcam, UK</td>
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<td>Universal antibody by Novolink TM Leica Biosystems Newcastle ltd, Newcastle Upon Tyne, UK cat. RE7150-K (1h room temp)</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>Alpha smooth muscle actin IR611, Dako</td>
<td>Mouse (monoclonal), IgG2a, kappa, 1:200 dilution</td>
<td>Overnight, 4°C</td>
<td>Universal antibody by Novolink TM Leica Biosystems Newcastle ltd, Newcastle Upon Tyne, UK cat. RE7150-K (1h room temp)</td>
<td>Peroxidase</td>
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Figure 1: Flowcharts demonstrating the clinical and experimental methodology. Clinical: Sixty-two healthy volunteers were recruited and had objective measurements performed at multiple sequential time points. Objective non-invasive devices were used including optical coherence tomography (OCT), which provided measurements of attenuation coefficient and skin thickness and high frequency ultrasound (HFUS), which measured intensity and skin thickness. Experimental: Punch biopsies were performed on day 0 and weekly from weeks 1 to 8. Tissues were placed in formalin and underwent tissue processing for histological and immunohistochemical analysis.
Figure 2: Figure 2: Comparison of device parameters.

a) i) Optical coherence tomography (OCT) device. ii) OCT uses low-coherence near infrared light to produce an image of optical scattering from tissues, effectively creating an "optical ultrasound" at a resolution close to histopathology. iii) The device has a lateral optical resolution of $<7.5\mu m$ and an axial resolution of $10\mu m$. The penetration depth lies at approximately 1.2–1.8mm due to scattering effects and a scan area of 6 mm $\times$ 6 mm.

b) i) High frequency ultrasound (HFUS) device. ii) HFUS uses sound waves, and the ultrasonic waves are partially reflected at the boundary between adjacent structures and produce echoes of different amplitudes. The epidermis, dermis and subcutis are displayed in the image. iii) We chose to use a 50MHZ probe which has a lower resolution of 30 x 60μm than OCT and a greater penetration depth of 3mm with a scan area of 2.7 x 6mm.
Figure 3: High frequency ultrasound (HFUS) images show that scars present as more hypoechoic than normal skin beneath a strong hyperechoic entrance echo. They also demonstrate an increase in skin thickness from day 0 uninjured skin to week 1 with a slight reduction over time and subsequent increase to week 8. Furthermore, quantitative measurements of skin thickness provided by the device showed a significant increase from baseline to week 1 (p=0.01).
Figure 4: a) Optical coherence tomography (OCT) images demonstrate an increase in skin thickness from day 0 to all other time points. b) The graph shows that this increase was significant at week 1 (p=0.02) and week 8 (p=0.03).
Figure 5: Spearman’s correlation analysis demonstrated good agreement between HFUS and OCT skin thickness measurements. The graphs show that this was significant at day 0 ($p<0.001$), week 1 ($p<0.001$), week 2 ($p<0.001$), week 3 ($p<0.001$), week 4 ($p<0.001$), week 5 ($p<0.001$).
Figure 6: a) Haematoxylin & Eosin (H&E) staining demonstrated increased skin thickness b) from day 0 uninjured skin (1.18mm) to all time points, with a significant increase at week 1(1.26mm) (p=0.01). c) Taking the histopathological skin thickness as “gold standard”, OCT and HFUS tended to overestimate skin thickness compared to H&E analysis.
Figure 7: a) i) The total intensity was measured by high frequency ultrasound (HFUS) which corresponds to the dermal density or echogenicity of the skin. Lower numbers or thinner collagen fibres can reflect a weaker echo compared to thicker collagen fibres providing a stronger echo. ii) Measurements showed a significant reduction in echogenicity from day 0 uninjured skin (42%) to week 8 (29%) (p=0.02).

b) i) Optical coherence tomography (OCT) attenuation coefficient is the amount by which the optical signal reduces with distance travelled into the skin and has been related to collagen density and organisation. ii) The results showed that the attenuation coefficient significantly reduced from uninjured skin (2.2Au) to week 8 (1.35Au) (p<0.001).
Figure 8: Time matched images with haematoxylin & eosin (H&E) demonstrated that re-epithelialisation was evident with both optical coherence tomography (OCT) and high frequency ultrasound (HFUS) one week after injury. H&E and OCT both displayed a haemostatic crust but this was not detected by HFUS. All subjects had complete epithelial cover after 2 weeks. By week 8, OCT and H&E showed that the healed epithelium was thickened and homogeneous. This was also shown by the strong hyperechoic band of the epidermis in the HFUS images. After 2 weeks post initial biopsies, OCT, HFUS and H&E showed tissue remodelling and fibrosis until 8 weeks.
Figure 9: Collagen analysis. a)i) Herovici staining demonstrated that a)ii) mature collagen was greatest in uninjured skin (72%) compared to week 1 (19%) (p=0.001), week 2 (21%) (p=0.02) and week 3 (20%) (p=0.02). Immature collagen was greater at all subsequent time points in scar skin compared to uninjured skin (19%) particularly at week 1 (68%) (p=0.01), week 2 (52%) (p=0.04) and week 3 (48%) (p=0.02). b)i) Collagen I images. b)ii) Collagen I intensity was higher in uninjured tissues compared to scar tissues with the greatest intensity from beneath and at the wound edges over time. c)i) Collagen III images. c)ii) Collagen III intensity was greater in scar tissues compared to normal skin.

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<th>Immature Collagen</th>
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Figure 9: Collagen analysis. a)i) Herovici staining demonstrated that a)ii) mature collagen was greatest in uninjured skin (72%) compared to week 1 (19%) (p=0.001), week 2 (21%) (p=0.02) and week 3 (20%) (p=0.02). Immature collagen was greater at all subsequent time points in scar skin compared to uninjured skin (19%) particularly at week 1 (68%) (p=0.01), week 2 (52%) (p=0.04) and week 3 (48%) (p=0.02). b)i) Collagen I images. b)ii) Collagen I intensity was higher in uninjured tissues compared to scar tissues with the greatest intensity from beneath and at the wound edges over time. c)i) Collagen III images. c)ii) Collagen III intensity was greater in scar tissues compared to normal skin.
Figure 10: Graphical representation of the trends between high-frequency ultrasound (HFUS) intensity, optical coherence tomography (OCT) attenuation coefficient, Herovici collagen analyses and immunohistochemical analysis of collagen I and III. The trends show that HFUS intensity linked most closely with herovici mature collagen and collagen I whilst OCT attenuation coefficient showed a similar trend except for week 2 and week 8.
Figure 11: a)i) Elastin images. a)ii) Elastin was significantly greater in uninjured skin (49.1%) compared to scar tissue with lowest values at week 4 (23.0%, p=0.01) and a gradual return to values similar to baseline by week 8 (41.4%). b)i) Fibronectin images. b)ii) Fibronectin intensity was greatest at week 4 (0.72Au) and reduced to week 8 (0.56Au). c)i) Alpha-smooth muscle actin images. c)ii) Alpha-smooth muscle actin percentage marker area gradually increased from baseline in uninjured skin (11.5%) to week 8 (67%) (p=0.003).
Supplementary Material 1

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A table displaying the group allocation N numbers for the participant clinical measurement acquisition
Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue sections at a thickness of 5µm were mounted onto positively-charged glass slides with consecutive tissue sample in duplicate on each slide. Slides were dewaxed with xylene and ethanol and rehydrated. Antigen retrieval was performed using citrate buffer, pH 6. Immunohistological slides were prepared according to the Leica Novolink peroxidise staining kit protocol (Milton Keynes, UK), with overnight incubation of primary antibody at 4 °C, and development of peroxidase staining with DAB chromogen reagent for 5 minutes. All tissue sections were stained and prepared in the same batch in order to reduce variability. Details of antibodies used are listed in Table 2.

Statistics and data analysis

Image acquisition was performed in triplicates for all objective non-invasive device measurements. A scan was performed at the biopsy site three times at each time point ensuring the same area each time. Analyses were then performed for each of the three images and an average value taken as the final result. Clinical data was analysed by independent statisticians using paired t tests. Histological slides for Herovici and immunohistochemical slides for alpha smooth muscle actin, fibronectin, elastin, collagen I and collagen III were quantitatively analysed by Definiens Tissue Studio software version 64.4.0 (Definiens, Munich, Germany). Three participant samples per group were analysed. For each batch of microscope slides, whole sections of stained tissue in duplicate were scanned, with exposure settings standardised in order to eliminate variability. Initially, in the cellular analysis module, 12 subsets were selected in the tissue specimen for site recognition. Nuclear detection, nuclear area detection and nucleus classification were also done on the subsets and haematoxylin threshold was adjusted for cell detection. Analyses were performed in 20X.
magnification. Two independent pathologists also verified histological analysis of haematoxylin and eosin. Data was analysed using paired t tests (significance at p<0.05).