Perspectives on burn scar evaluation and artificial skin
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Collagen morphology in human skin and scar tissue: no adaptations in response to mechanical loading at joints

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Although collagen is the most prevalent protein of the skin and is considered to have a major role in strength and function of the human integument, many details concerning the collagen structure remain elusive. During wound healing, the random orientation of the destroyed collagen network is lost and replaced by smaller collagen bundles that are predominantly of parallel orientation. It is hypothesised that polarisation of collagen bundles is caused by the direction of mechanical tension, especially at joints.

For this study, collagen orientation and bundle packing were quantified by means of Fourier analysis in both scar tissue and normal skin. We hypothesised that collagen organisation at joint areas differs from adjacent areas due to mechanical tension. Biopsies of scar tissue and normal skin were taken from nine patients at joints and at locations adjacent to the joint, which served as control. Biopsies were processed in sections parallel and perpendicular to the epidermis to observe the dermal architecture from different perspectives.

Collagen bundles of scar tissue were orientated in a more parallel fashion and more densely packed than bundles of normal skin (p<0.001). Collagen seemed more randomly orientated in deep dermis compared to superficial dermis, especially in normal skin (p=0.06). Also, normal skin had a more random organisation in those sections that were cut parallel compared to those that were cut perpendicular to the epidermis (p=0.02). In contrast to our hypothesis, we could not show significant differences in collagen morphology between joints and control areas of scar tissue (p=0.98) or of normal skin (p=0.30).

This study gives new quantitative insights in the structure of the human skin as it shows that collagen morphology differs with respect to superficial and deep dermal layers and parallel and perpendicular planes, but not in response to mechanical tension.
Introduction

The importance of the collagen structure for the strength and function of the skin is widely recognised. The changes in collagen organisation during connective tissue disorders, such as Systemic Sclerosis\textsuperscript{1,2} and Ehlers-Danlos Syndrome\textsuperscript{3,4}, are extensively studied by light and electron microscopy. Similar studies have been performed to evaluate the collagen structure in scar tissue. Scars often become hypertrophic, firm and initially show reduced tensile strength. These consequences have been related to the poor quality of the restored collagen network\textsuperscript{5,6}. Most studies on wound healing and scar formation address attention to qualitative aspects of the structure of this protein\textsuperscript{7-9}. Microscopic analysis reveals that in normal skin, collagen bundles show a basket-weave-like pattern with a random orientation, whereas scar tissue consists of smaller bundles aligned in a parallel fashion to the epidermis\textsuperscript{5}. Mechanical tension has been proposed to play a major role in the orientation of the bundles in scar tissue\textsuperscript{5,10}. Nevertheless, clinical studies that provide evidence for or against the influence of mechanical tension on bundle orientation are absent except for one study\textsuperscript{11}, which was published already in 1961. In that study, Holmstrand and colleagues used X-ray diffraction to objectify anisotropy of the tissue, nevertheless they were not able to show ‘a clear-cut correlation between the orientation of the fibrils and the direction of tension on the scars’.

For the present study we use a convenient and objective technique for the evaluation of collagen orientation and collagen bundle packing: Fourier analysis\textsuperscript{12,13}. This mathematical technique was shown to be reliable, accurate, and superior to conventional ratings by observers using light microscopy\textsuperscript{12,13}.

A study was initiated to quantify collagen orientation and bundle packing of scar tissue and normal skin by means of the Fourier analysis. Our hypothesis was that mechanical tension causes polarisation of the collagen bundles in the direction of the stress. Furthermore, we studied orientation and packing of collagen bundles of scar tissue and normal skin (from tension and control areas) for changes in depth and differences in morphology in the plane parallel and perpendicular to the epidermis.
During clinical studies it is difficult to record accurately to what extent scars have endured mechanical stress for the long period (months to years). This is in contrast with experimental studies. Here, devices can be used for a long period of time that allow controlled traction on a wound or a scar\(^{14-15}\). Despite these controllable practical experimental studies, there is definitely a need for clinical studies on this subject because wound healing and scar formation in humans show considerable differences with tissue restoration in animals\(^{16}\). For practical purposes we selected flexion surface areas of joints as ‘tension-areas’ since they endure much mechanical stress due to joint mobility, and furthermore they are notorious locations with respect to scar formation\(^{17,18}\). Locations adjacent to the joint served as control areas.

**Materials and methods**

The ethics committee from the Red Cross Hospital approved the protocol before the start of the study. Two inclusion criteria were employed. Firstly, location of the burn scars had to allow biopsies of normal skin and scar tissue at an area of mechanical tension (flexion area of joint) and at an area adjacent to this joint that served as control. Control biopsies were taken between this joint and the proximal or distal joint. If not all biopsies could be harvested from the same extremity, locations at the contra-lateral side were also used for the evaluation. If it was impossible to harvest four biopsies, at least two biopsies were obtained that allowed paired analysis of tension or control areas. Due to the number of biopsies and their size, we only included patients who required general anaesthesia for a reconstructive procedure of a burn scar. General anaesthesia was therefore the second inclusion criteria. Nine patients enrolled this study (5 males and 4 females) after giving written informed consent. The mean age of the population was 40.7 with a standard deviation (SD) of 14.1 years. The scars were biopsied at an average of 2.5 years after the burn injury (range: 4 months - 8 years).
Harvesting and processing of the biopsies

Biopsies were obtained with an elliptical form of approximately 1 by 2 cm. The longitudinal axis of the biopsy paralleled the longitudinal axis of the extremity that is the direction of most muscular strength and activity and thus mechanical tension. The wounds that remained after removal of the biopsy were sutured.

By cutting the biopsies in the longitudinal axis, two symmetric parts were created. The tissue was fixed in buffered 4% formalin. The first half of each biopsy was processed into histological slides (5 μm) in a routine fashion with the section perpendicular to the epidermis of the biopsy. The second half was cut with the section parallel to the epidermis. The counting of sections started at the epidermal/dermal border. From there, the complete dermis was processed. Each forty-fifth section was preserved and processed like the slides of the first half (the number of slides was chosen because of practical considerations). Sections were processed parallel to the epidermal plane and allowed collagen morphometry at different depths of the scar and skin. Levels 1 to 6 were approximately at 225, 450, 675, 900, 1125 and 1350 μm distance from the epidermal/dermal border. Sections were stained with hematoxylin-eosin. Figure 1 illustrates how both halves of the biopsy were processed, as well as the levels of measurements.
Confocal laser-scanning microscopy

The sections were scanned with a confocal laser-scanning microscope (Leica Micro Optics, Heidelberg, Germany) with a 6.3 x 0.2 objective and a 50µm pinhole making use of the fluorescent properties of eosin (excitation: 488nm, detection: LP 610nm). In this way the interference of the hematoxylin stained nuclei in standard brightfield image was avoided. Images were adapted to the full dynamic range of the system (8 bit) to standardise the contrast/brightness ratio.

Images of 794µm x 794µm were created from the sections of the superficial dermal layer and the deep dermis that were processed perpendicular to the epidermis. These sections were scanned with the epidermis orientated in a horizontal plane. The centre of the sections that were processed parallel to the epidermis were scanned in a similar manner.

Fourier analysis

Image analysis was done with the Fast Fourier Transform module of the Qwin Pro image analysis software (version 2.2, Leica Imaging Systems LTD, Cambridge, UK). The width/height ratio of the zeroth-order maximum in the generated power plot of the image was an estimate of the orientation: the collagen orientation index. Fourier analysis of an image with parallel collagen orientation yields an elongated power spectrum that leads to a small orientation index. Perfectly random tissue results in an orientation index that approximates one. The distance between the centres of gravity (d) of the first-order maxima was used to calculate the distance between the centres of the collagen bundles (λ) by using the formula $\lambda = 794 \mu m \times (1/(0.5xd))$. The distance $\lambda$ was regarded as indicator of averaged collagen bundle packing. Figure 2 shows the superficial and deep dermal layer of normal skin and scar tissue together with the zeroth and first-order maxima of Fourier analysis.

Statistical analysis

Data were analysed by the statistical program SPSS for Windows 8.0 (SPSS Inc. Chicago IL USA). A paired sample $t$ test was applied for all paired data of the Fourier analysis (collagen orientation and bundle packing). The independent sample $t$ test was used for the compari-
son of the data from the perpendicular and parallel sections. Analysis was done on separate groups containing only sections of a certain level of the biopsy, and also after pooling the sections per biopsy. As suggested by literature, both the 95% confidence interval of the difference (CI) and p-value are given. The significance criterion was set at 0.05.

**Results**

Tension and control areas were analysed in a paired design for normal skin and scar tissue. The organisation of scar tissue differed considerably from normal skin. We calculated the ‘overall’ collagen orientation index and collagen bundle packing by evaluating all perpendicular and parallel orientated sections pairs (n=70) of the group of normal skin and scar tissue. Normal skin was found to have a significantly higher collagen orientation index (0.74, SD: 0.11) than scar tissue (0.56, SD: 0.16), which shows that normal tissue is organised in a more random fashion. The difference was highly significant (CI: 0.13 - 0.22, p<0.001). In addition, a statistically significant difference (CI: 3.1 - 8.2, p<0.001) was found for bundle packing, as the distance between the centre of collagen bundles of normal skin (\(\lambda=23.7, \text{SD: 8.2}\)) was larger compared to those found in scar tissue (\(\lambda=18.1, \text{SD: 6.4}\)). The aspect of normal skin and scar tissue by confocal laser-scanning microscopy is shown in Figure 2. A paired sample t test was performed to indicate differences between tension and control areas with respect to collagen orientation index and collagen bundle packing. These data, given in Table 1, show that the orientation index and bundle packing do not differ significantly between tension and control areas.

Further analysis was directed at the differences in collagen organisation between: 1) superficial and deeper layers of the skin and scar; and 2) the parallel and perpendicular planes of the skin. From here, data of tension and control areas were pooled as we demonstrated no differences concerning collagen orientation index and collagen bundle packing between biopsies taken at joints or their control areas. Mechanical tension is therefore not a confounder for this analysis. Table 2 shows detailed information on the collagen orien-
Figure 2: Collagen structure: confocal laser scanning microscopy and Fourier analysis.

Figure 2 shows the superficial and deep dermal layer of sections of a biopsy in a plane perpendicular to the epidermis taken from normal skin at an unburned left knee (Figure 2a and 2b) and from a six months old scar tissue after treatment of a full thickness burn wound of the right knee of the same patient (2c and 2d) respectively. Power spectra of the Fourier analysis are inserted in the right upper section of each image: the zeroth-order spectrum is placed above the first order spectrum. Normal skin shows thicker bundles in a more random fashion. The superficial layers of both normal skin and scar tissue are aligned in a more parallel fashion compared to the deeper layers. This is also shown by the zeroth order spectra that are less elongated for the deeper layers compared to the superficial layers. The collagen bundle packing, defined as the distance between the centres of the bundles, is represented by the first order spectra; the distance between the maxima of the spectra is inversely related to the distance between the bundle centres. Scale bar = 100 μm.
Perspectives on burn scar evaluation and artificial skin
Section 2: Clinical and microscopic aspects of scar tissue

<table>
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<tr>
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<tr>
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<td>17.4</td>
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Table 1 Collagen orientation index and collagen bundle packing: tension versus control areas

tation index, collagen bundle packing, and number of available sections per level, for normal skin and scar tissue.

The data of the superficial and deep dermal layers of the perpendicular sections, which are given in Table 2, were compared. The difference between collagen orientation index of the superficial and deep dermal layers of normal skin approached statistical significance (CI: -0.13 to 0.00, p=0.062). The difference was smaller between the superficial and deep dermal layers of scar tissue (CI: -0.15 to 0.06, p=0.348). For collagen bundle packing no statistically significant differences were found between superficial and deep dermal layers for scar tissue (CI: -3.9 to 1.4, p=0.336) and normal skin (CI: -4.9 to 3.6, p=0.735). The confocal laser-scanning microscopy images of Figure 2 show that the superficial layers of both normal skin and scar tissue are aligned in a more parallel fashion compared to the deeper layers. Note that the zeroth order spectra of Fourier analysis are less elongated for the deeper layers compared to the superficial layers, which also indicates a more random orientation of deeper layers.

The average collagen orientation index and collagen bundle packing of each level of the parallel orientated sections, which is given in Table 2, is plotted in Figure 3a and 3b. Figure 3a shows that the average collagen orientation index for normal skin and scar tissue was relatively low in the superficial layers and increased gradually in
depth, which means that collagen shows a more random organisation in deeper parallel layers. Despite the few sections at the deepest level 6 (n=6), a statistically significant difference was found for the orientation index of normal skin between the most superficial layer at approximately 225 µm and the deepest layer at approximately 1350 µm (Wilcoxon signed ranks test, p=0.028).

A similar phenomenon occurs for collagen bundle packing in normal skin as the average λ increased gradually (Figure 3b). In contrast, collagen bundle packing in scar tissue seemed constant at all depths.

Our data indicated differences in collagen characteristics between the plane perpendicular and parallel to the epidermis. In general, the collagen orientation index of normal skin was larger for the averaged data of the parallel sections (0.76, SD: 0.11) in comparison to the averaged data of the perpendicular sections (0.70, SD: 0.10), this difference was statistically significant in an independent-samples t test (CI: -0.11 - -0.01, p=0.017). Collagen bundle packing approached a statistically significant difference between perpendicular sections (22.0, SD: 5.6) and parallel sections (24.9, SD: 8.7) of normal skin (CI: -6.5 - 0.7, p=0.108). For scar tissue smaller differences were found that were not statistically significant different for collagen orientation index (CI: -0.11 - 0.04, p=0.392) and collagen bundle packing (CI: -3.7 - 0.7, p=0.173).
Perspectives on burn scar evaluation and artificial skin
Section 2: Clinical and microscopic aspects of scar tissue

<table>
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<td><strong>Scar tissue</strong></td>
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<td>Superficial layer</td>
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*Table 2a* Sections cut perpendicular to epidermis

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<td>Level 6 (1300 μm)</td>
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<td>0.62</td>
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*Table 2b* Sections cut parallel to epidermis
Collagen morphology in human skin and scar tissue: no adaptations in response to mechanical loading at joints.

Figure 3a  Collagen orientation index at different surface levels

Figure 3b  Collagen bundle packing at different surface levels
Discussion

Although considerable attention has been focussed on collagen and its structure by subjective evaluations like conventional light microscopy, quantitative analyses on dermal collagen structure is required to gain a better understanding of the normal and pathological conditions. This study provides statistical evidence for a generally accepted idea postulated in descriptive studies that collagen bundles are orientated in a more parallel fashion in scar tissue compared to normal skin.

It has been hypothesised that collagen bundles are aligned in the direction of mechanical tension during scar formation. In more recent studies on the relation between mechanical tension and collagen organisation, attention shifted from collagen bundles to fibroblasts and myofibroblast, which are the cells that produce collagen. These cells were found to respond to mechanical tension with respect to their organisation, and by apoptosis. Our data, however, do not support the hypothesis that mechanical tension over joints polarises collagen bundle orientation in the direction of stress, as no difference could be found between collagen orientation index for joints and control areas for scar tissue (orientation index 0.58 and 0.57 respectively, p=0.98) and normal skin (0.74 and 0.71 respectively, p=0.30). Laufer and colleagues came to the same conclusion in an animal model where incisions were made in different directions on the back of a pig. Their data suggested that mechanical tension was not likely to be a major factor in determining the orientation of fibroblasts and collagen fibres. Table 1 shows that if mechanical tension at joints would cause polarisation of collagen at all it would be found in normal skin as biopsies from normal skin showed a smaller collagen orientation index at joints (0.71) compared to control areas (0.74). This difference was not statistically significant (p=0.30). The absence of a statistically significant difference could be related to the relatively small sample size of this study. A power-analysis calculation (α=0.05, Power=80%) showed that a study of 200 pairs of sections may detect a statistically significant difference for the collagen orientation index of normal skin. After an interim evaluation we did not consider further inclusion of patients, as we believed
that the results so far sufficiently answered the aims and hypotheses of this study.

All sections of scar tissue, cut parallel and perpendicular to the epidermal plane, showed predominantly small collagen bundles running in the same horizontal direction (Figure 2). Parts of biopsies of two patients were additionally cut in the plane perpendicular to the direction of the mechanical tension. Therefore sections were available in three dimensions. If all bundles run in the direction of mechanical tension, collagen bundles would have appeared as small dots in these sections. Since this was not the case, we postulated that these bundles may be part of ‘collagen planes’.

In contrast to collagen bundle orientation, few published studies exist regarding collagen bundle packing. Collagen bundle packing differed significantly between scar tissue and normal skin as distances between centres of the bundles were much smaller in scar tissue compared to normal skin. The electron microscopy images of normal skin and scar tissue of Figure 4 show that the collagen bundles of scar tissue are more packed than the collagen bundles of normal skin. In agreement with results for the collagen orientation, no influence could be established for mechanical tension on bundle packing during this study.

We concluded that mechanical tension caused by joint mobility does not affect collagen structure. We question therefore whether tensile load is a causative factor for complications which occur typically at flexion areas of joints, such as contracture. Others proposed a theory that mucopolysaccharides function as glue that bind the buckled collagen bundles when the joint is in flexed position. This theory requires a more scientific basis by experimental and clinical studies yet.

In normal skin, the collagen orientation index of the superficial versus deeper dermal layer (cut perpendicular to the epidermis) approached a statistically significant difference ($p=0.062$). Others mentioned this finding without a quantitative analysis. Sections cut in a plane parallel to the epidermis also showed an increase of the collagen orientation index for deeper layers, as shown in Figure 3a.

Our data showed differences between collagen structure of normal skin in the plane perpendicular and parallel to the epidermis. Orien-
Figure 4  Electron microscopy images of collagen bundle packing in normal skin and scar tissue.

Figure 4 shows collagen fibres by means of transmission electron microscopy. Collagen of scar tissue, as shown in Figure 4a, is more densely packed than collagen of normal skin (C) of Figure 4b (F=Fibroblast). Scale bar = 10 μm.
tation indexes were generally larger for collagen in parallel planes in comparison with perpendicular planes. This suggested that bundles run in a more random fashion in the plane that parallels the epidermis. We confirmed this impression by comparing the sections of both cutting directions with an independent-samples t test, where a significant difference was established for the collagen orientation index in normal skin between both planes but not for scar tissue.

Why does normal skin have a predominantly random organisation, especially at the deeper levels? The nature of this organisation must be related to the functions of the skin as integument. The skin endures considerable shearing and pulling forces. It seems therefore evident that the structure of collagen, which is considered to be the most important functional component, is organised to resist these forces. It is plausible to assume that superficial tissue has to endure most of the forces that are orientated predominantly parallel to the surface, whereas collagen in the deeper layers requires the capacity of intercepting forces both parallel and perpendicular to the surface area. This proposition is tenable from a morphological point of view, because deeper layers transfer forces by bundles that run perpendicularly to the underlying tissues.

Collagen morphology of scar tissue differed significantly from that of normal skin, as collagen bundles of scar tissue were more parallel orientated and more densely packed. We established that collagen has a more random organisation in the plane parallel to the epidermis compared to the perpendicular plane. This organisation becomes gradually more random for deeper layers. In contrast to generally accepted ideas, mechanical tension on joints did not influence collagen morphology of scar tissue, nor that of normal skin.

**Acknowledgements**

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References

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