Adaptation of the dermal collagen structure of human skin and scar tissue in response to stretch: An experimental study

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ABSTRACT

Surgeons are often faced with large defects that are difficult to close. Stretching adjacent skin can facilitate wound closure. In clinical practice, intraoperative stretching is performed in a cyclical or continuous fashion. However, exact mechanisms of tissue adaptation to stretch remain unclear. Therefore, we investigated collagen and elastin orientation and morphology of stretched and unstretched healthy skin and scars. Tissue samples were stretched, fixed in stretched-out position, and processed for histology. Objective methods were used to quantify the collagen orientation index (COI), bundle thickness, and bundle spacing. Also sections were analyzed for elastin orientation and quantity. Significantly more parallel aligned collagen bundles were found after cyclical (COI = 0.57) and continuous stretch (COI = 0.57) compared with nonstretched skin (COI = 0.40). Similarly, more parallel aligned elastin was found after stretch. Also, significantly thicker collagen bundles and more bundle spacing were found after stretch. For stretched scars, significantly more parallel aligned collagen was found (COI = 0.61) compared with nonstretched scars (COI = 0.49). In conclusion, both elastin and collagen realign in a parallel fashion in response to stretch. For healthy skin, thicker bundles and more space between the bundles were found. Rapid changes in extension, alignment, and collagen morphology appear to be the underlying mechanisms of adaptation to stretching.

In clinical practice, surgeons are often faced with large skin defects that are difficult to close primarily. To close these wounds, techniques with poor cosmetic outcome, such as split-skin grafting of the defect as well as complex reconstructions, such as (free) flaps, can be performed. However, the need for these (complex) techniques can sometimes be circumvented by stretching adjacent healthy skin, followed by primary closure. The skin can be relatively simply stretched by exerting tension along the wound margins using a skin-stretching device, which was introduced by Hirshowitz et al.1 This device allows intraoperative stretching in approximately 30 minutes by exerting stretch onto the skin for 4 minutes, alternated by 1 minute of rest. This stretch regimen was showed to be effective in various experimental and clinical trials.1–4 This way of cyclical skin stretching is feasible due to mechanical creep (i.e., elongation of skin with a constant load over time).5 Although mechanical creep has been frequently described, the underlying mechanisms have not been fully elucidated. Besides cyclical skin stretching, other stretching systems are used that apply continuous stretch onto the skin.6–9 To elucidate the adaptation mechanisms of cyclically and continuously stretched healthy skin, comparison with stretched scar tissue may provide valuable additional data. Moreover, knowledge on the adaptation of scar tissue in response to stretch is clinically relevant: scar tissue is stretched not only in splinting to correct burn scar contraction, but also in tissue expansion or in skin-stretching. Up to now, collagen morphology of stretched scar tissue has never been investigated.

The larger part of the skin is the dermis and 85% of this dermis consists of collagen.10 Collagen is a major component of the dermis and plays a key role in the strength and elasticity of skin. Therefore, for investigating the adaptation of healthy skin and scar tissue in response to stretch, collagen morphology may play an important role. In this study, we focus on the adaptation of collagen morphology after stretch and thereby measure the collagen orientation, collagen bundle thickness (BTh), and collagen bundle spacing (BSp). We already demonstrated differences in collagen morphology between healthy skin and different types of scars.11 Besides collagen,
the viscoelastic properties of healthy skin and scar tissue may be affected by the quantity and direction of elastic fibers. Therefore, also the percentage and orientation of elastic fibers were analyzed.

Up to now, results of previously conducted research on adaptation of collagen and elastin in response to stretch were not conclusive. Various studies on porcine skin described histological changes after stretch: qualitative histological analysis showed no alignment of the collagen bundles after tissue expansion. A drawback of this study is that only 4-mm punch biopsies were taken and biopsies were fixed in non-stretched position. On the other hand, collagen orientation after stretching was described as being parallel aligned to the skin surface. In other studies, as opposed to qualitative assessment, the collagen orientation after cyclical stretch was quantitatively assessed, which showed alignment of the collagen bundles in the stretch direction. However, both studies at that time did not fix the porcine skin in stretched-out position, which may have had influence on alignment of the collagen bundles. Therefore, based on this previously published literature, alignment of collagen in response to stretch remains a controversial concept. This study is an attempt to solve this controversy. We applied cyclical and continuous stretch on human healthy skin and continuous stretch on scar tissue and compared these with nonstretched skin and scar tissue, respectively. Cyclical stretch was applied because it was previously shown that more extension was reached through repeating skin stretch several times. This was compared with a continuously exerted high amount of stretch, another stretch mechanism that is regularly used for wound closure. We hypothesized that by exerting a continuous high stretching force, no additional extension of healthy skin or scar tissue would be reached. Unique for this study is the combination of three factors: healthy skin and scar tissue were stretched and compared with each other, all samples were fixated in stretched-out position, and objective quantitative analyses were performed using Fourier analysis and Distance Mapping. These methods were shown to be reliable and valid for the quantification of the collagen orientation and collagen morphology. Lastly, also the role of elastin with respect to stretching tissue was critically viewed. Further knowledge on adaptation of healthy skin and scar tissue in response to stretch may contribute to improvement of current therapies: this study may further elucidate which method of stretching may prove best for closure of large wounds. Moreover, our experiments may explain potential adaptation of scar tissue when splitting is performed to correct burn scar contractures.

Materials and Methods

Patients

Patient material was collected from July 2009 until February 2010. Healthy skin was obtained from 20 patients (19 female) undergoing surgery where healthy skin with a minimum size of 7 × 7 cm became available. Patients were excluded when stretch marks were present on the area of interest. The mean patient age was 38 years (23–51 years) and the mean body mass index at the time of surgery was 26 (20–33).

Scars tissue was obtained from 10 patients (five female) undergoing reconstructive surgery. Those patients were included when the scar was at least 1 year old and had a minimum size of 7 × 7 cm. The mean patient age was 35 years (6–54 years) with a mean age of the scar of 11 years (1–32 years). Nine out of 10 scars were burn scars and one scar remained after split-skin grafting of a wound. From these 10 scars, seven were hypertrophic and three were nortrophic scars.

All patients were healthy with an insignificant medical history. All patient material used was residual tissue, which is normally discarded after surgery. The protocols of the Federation of Dutch Medical Scientific Societies, which are adapted by the coordinating Ethics Committee in the Netherlands, permit the use of anonymized residual tissue. All patients or legal representatives gave written informed consent.

Stretch procedure

For healthy skin, a preoperative design was made to allow for determination of the skin sample dimensions in vivo. After harvesting, the stretch procedure was performed within 24 hours. If the stretch procedure could not be performed immediately postoperatively, the skin was kept refrigerated at approximately 7 °C in a sterile sodium chloride solution. Three strips of 3.5 × 7.0 cm were cut. Subsequently, strips were fixed on a workbench with a skin-stretching device that allowed controlled regulation of mechanical load (Figure 1). During the stretch sessions, the skin was moistened regularly using a sodium chloride solution to prevent dehydration. Extension of the skin strip was monitored using a caliper. The strips were clamped in situ in stretched-out position at the end of each procedure by specially designed clips. Three stretch procedures were performed on the healthy skin strips: the first strip being a control strip, where no stretching was performed (Ctrl). The second strip was cyclically stretched: six times stretching for 4 minutes using 30 N, alternated by 1 minute of relaxation between every cycle (Str.6×30N). This stretch regimen was used because it was proven to be effective in both experimental and clinical experiments. The third strip was continuously stretched for 30 minutes using 55 N (Str.55N). Fifty-five newton can be considered as a very high load onto the skin. Exercising more stretch onto the tissue became impracticable in this experimental setup. This continuous stretch regimen, which is also used in clinical practice, was chosen for two reasons: first, to compare whether a
higher force of 55 N compared with 30 N that was exerted with cyclical stretching would have an effect on the extension of the skin strips and, second, to investigate whether a high stretching force would result in more permanent tissue damage (e.g., stretch marks).

For scar tissue, two strips of 3.5 × 7.0 cm were harvested: a control strip (Ctrl-Scar) and a strip undergoing continuous stretch of 55 N during 30 minutes (Str.55N-Scar). Fixing the strips was similar to the strips of healthy skin.

Histology

Once the strips were fixed in the clips, the tissue was immediately fixed in 4% freshly prepared formaldehyde for at least 24 hours. Subsequently, biopsies were dehydrated and embedded in paraffin. Sections (5 μm) were mounted on glass slides and cut parallel with the direction of the stretch. All sections were stained using hematoxylin-eosin and elastin was stained according to von Giesson.

Confocal laser scanning microscopy

We used the fluorescent properties of eosin to avoid dominance of hematoxylin-stained nuclei in a classical bright-field image. Sections were imaged using a Leica SP2-AOBS confocal microscope (Leica Microsystems, Mannheim, Germany). Excitation and emission of eosin were performed at 561 and 580–640 nm, respectively. A 10 ×/numerical aperture (NA) 0.4 objective was used with an additional zoom of 1.89, resulting in a scanned area of 794 × 794 μm and a pixel size of 0.78 μm. Images had a 1024 × 1024 format. A pinhole setting of one Airy was used, resulting in an optical section with an approximate thickness of 5 μm. All acquired images were adapted to the full dynamic range of the system (8 bits). The same protocol was used for all images and each image was taken in the middle of the section, directly underneath the epidermis.

Fourier analysis

All images were analyzed with the Fourier analysis using Qwin Pro software (Leica Imaging Systems, Cambridge, UK). A zeroth-order maximum power plot was generated. The [1 – (width/length)] ratio, which is generated from this zeroth-order maximum power plot, stands for the collagen orientation index (COI) of the image: zero corresponds to a perfectly random orientation and one to a perfectly parallel alignment of the collagen bundles. The Fourier analysis is reliable and has been validated for this purpose.

Distance Mapping

The thickness of collagen bundles and the distance between collagen bundles were measured with Qwin Pro software (Leica Imaging Systems). After segmentation of either collagen bundles or the space between bundles, the thickness of the bundles and the BSp, respectively, were determined using the combination of a distance function and a skeleton function. A distance function creates a gray image from a binary image by setting the gray level at each point to a value that represents its distance to the nearest edge. A skeleton function reduces a binary image to pixel-wide lines by subsequent erosion steps. Using the skeleton as a mask to measure the densities in the distance plot gives an average value of the width of the structures involved. Two outcome values were generated: the average thickness of the collagen bundles or 

Elastic fibers

The quantity and orientation of the elastic fibers were analyzed by two experienced observers who scored all von Giesson-stained sections independently from each other. The sections were blinded to prevent bias. A five-step scale was used for scoring the orientation: 1 = extremely random, 2 = predominantly random, 3 = mixed organization, 4 = predominantly parallel, and 5 = extremely parallel. This five-step scale has been previously used to score the collagen orientation and was found to be reliable and valid. A five-step scale was used for scoring the quantity of elastic fibers: 1 = none (0%), 2 = lower than average (0–4%), 3 = average (4–6%), 4 = above average (6–10%), and 5 = excessive (>10%). These percentages were based on studies demonstrating that the average amount of elastin in the dermis ranges between 4% and 6%.

Statistical analysis

Data were analyzed using PASW 18.0 (SPSS, Chicago, IL). Normal distribution was tested by applying the Kolmogorov–Smirnov test, by analyzing the histograms, and by calculating the skewness and kurtosis. If the population was normally distributed, paired data were tested using the paired t test and independent data were tested using the independent t test. If the population was not normally distributed, the Mann–Whitney U (MWU) test or, in case of paired data, the Wilcoxon signed-rank test was used to identify significant differences. The range and p-value are given where appropriate. The two-tailed significance criterion was set at 0.05.

RESULTS

Stretching of healthy skin and scar tissue

Stretching the healthy skin resulted in an average augmentation in length from 2.5 to 3.8 cm (+53%, 36–64%, n = 18) for the Str.6 × 30N group. The same augmentation from 2.5 to 3.8 cm (+53%, 20–72%, n = 18) was found in the Str.55N group. For stretched scar tissue, length of the strips increased from 2.5 to 3.1 cm (+23%, 12–36%, n = 10). A significant difference was found between the extensibility of cyclically stretched healthy skin and continuously stretched scar tissue (+53% vs. +23%, MWU test, p < 0.001) and between continuously stretched healthy skin and continuously stretched scar tissue (+53% vs. +23%, MWU test, p < 0.001).

Collagen orientation index (COI)

Representative images of healthy skin in the Ctrl, Str.6 × 30N, and Str.55N groups are shown in Figure 2A–C. For the non-stretched and stretched scar tissue, representative images are shown in Figure 3A and B, respectively.
The results for the COI of healthy skin that was subjected to different stretch regimens are displayed in Figure 4: a significantly higher COI was found for the Str.55N and Str.6 × 30N compared with the Ctrl group (Table 1). No differences were found between the Str.55N and Str.6 × 30N groups. In Figure 5, the results are displayed for the COI of nonstretched and stretched scar tissue. A significantly higher COI was found for the Str.55N-Scar compared with the Ctrl-Scar group (Table 1).

**Collagen BTh and BSp**

The BTh and BSp of healthy skin biopsies were significantly higher in the Str.6 × 30N and the Str.55N than the Ctrl group.

**Table 1.** Collagen orientation and morphology of healthy skin and scar tissue

<table>
<thead>
<tr>
<th></th>
<th>Healthy skin</th>
<th>Scar tissue</th>
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<td></td>
<td>n</td>
<td>Ctrl</td>
<td>Str.6 × 30N</td>
<td>Str.55N</td>
<td>n</td>
<td>Ctrl-Scar</td>
<td>Str.55N-Scar</td>
<td>p-value</td>
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<tr>
<td>COI</td>
<td>20</td>
<td>0.40 (0.17–0.56)</td>
<td>0.57 (0.33–0.82)</td>
<td>0.57 (0.25–0.78)</td>
<td>10</td>
<td>0.49 (0.17–0.81)</td>
<td>0.61 (0.38–0.86)</td>
<td>0.047</td>
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<tr>
<td>BTh (µm)</td>
<td>20</td>
<td>5.39 (4.31–6.63)</td>
<td>5.98 (5.17–6.88)</td>
<td>5.94 (4.70–7.19)</td>
<td>10</td>
<td>5.77 (4.87–6.97)</td>
<td>5.75 (4.31–7.53)</td>
<td>0.767</td>
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<tr>
<td>BSp (µm)</td>
<td>20</td>
<td>10.37 (7.57–16.64)</td>
<td>12.87 (9.95–20.49)</td>
<td>11.81 (8.58–14.84)</td>
<td>10</td>
<td>8.53 (7.47–10.06)</td>
<td>8.92 (7.67–10.29)</td>
<td>0.285</td>
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Mean values and range of the COI, bundle thickness, and bundle spacing of healthy skin and scar tissue. Statistical testing for healthy skin is represented in Figures 4 and 6A and B. Statistical testing of scar tissue was performed using the Wilcoxon signed-rank test.

BSp, bundle spacing; BTh, bundle thickness; COI, collagen orientation index.
whereas for scar tissue no significant differences between the Ctrl-Scar and the Str.55N-Scar groups were found (Table 1).

Elastic fibers

The scores of both observers for quantity and orientation of the elastic fibers were averaged. In healthy skin, analysis showed significantly more parallel-orientated elastic fibers in the Str.6×30N and the Str.55N groups compared with the Ctrl group. Between the Str.6×30N and the Str.55N groups, no significant difference was found (Table 2). The quantity of elastic fibers did not differ between these three groups. For scar tissue, no significant differences in orientation and in quantity of the elastic fibers between the Ctrl-Scar and Str.55N-Scar groups were found (Table 2).

Concerning the quantity of the elastic fibers in both stretched and unstretched healthy skin (n=59), in none of the samples more than 10% of elastic fibers was scored and only in five out of the 59 cases (8.5%) the elastic fibers’ percentage was scored as being higher than 6%. The average quantity of elastic fibers in healthy skin was significantly higher in the Str.6×30N and the Str.55N groups compared with the Ctrl group. Between the Str.6×30N and the Str.55N groups, no significant difference was found (Table 2).

Mean values and range of the elastin orientation and quantity of healthy skin and scar tissue. Statistical testing for healthy skin was performed using the paired t test and statistical testing for scar tissue was due to the small numbers in the group performed by the MWU test. For the paired t test, the p-values are as following: Ctrl—Str.6×30N, p<0.001; Ctrl—Str.55N, p<0.001; Str.6×30N—Str.55N, p=0.99; Ctrl—Str.6×30N, p=0.35; Ctrl—Str.55N, p=0.12; Str.6×30N—Str.55N, p=0.45. *p≤0.05. In some of the cases, the von Giesson-stained slide could not be analyzed because of many artifacts. Also, in a small number of the cases, the von Giesson-stained slides were lacking. This explains for the incomplete treatment groups.

MWU, Mann–Whitney U; SD, standard deviation.

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DISCUSSION

In the present study, it was objectively shown that when human healthy skin and scar tissue are stretched, the collagen morphology adapts rapidly and significantly. For the first time, stretched tissue was instantly fixed chemically in stretched-out position and analyzed using objective, reliable, and valid techniques. First, it was found that when healthy skin is stretched, collagen bundles realign in a parallel fashion in the direction of stretch. Second, the collagen bundles are thicker and there is more space between the collagen bundles in the stretched vs. the nonstretched healthy skin. Third, no differences in collagen orientation and collagen bundle morphology were found when comparing cyclically and continuously stretched healthy skin. Fourth, in scar tissue, stretch resulted in more parallel-orientated collagen bundles, whereas the BTh and spacing were not affected. Last, for healthy skin, alignment of the elastic fibers after stretching was noted.

It was showed that scar tissue had significantly less potential to increase in length compared with cyclically and continuously stretched healthy skin (+23% compared with +53 and +53%, respectively). We were able to correlate these extensibility results to histological changes in collagen structure: before stretch, scar tissue already shows a more parallel alignment of the collagen bundles than healthy skin (COI\textsubscript{scar tissue} = 0.49 vs. COI\textsubscript{healthy skin} = 0.40, \(p = 0.082\), MWU test). This is in agreement with previously published literature\textsuperscript{11,24–27} Thus, in scar tissue, collagen bundles may have less potential to extend in response to stretch. Also, collagen bundles in scar tissue were more densely packed than in healthy skin (BS\textsubscript{scar tissue} = 8.5 vs. BS\textsubscript{healthy skin} = 10, \(p = 0.005\), MWU test). When relatively more collagen bundles are present, which is the case in scar tissue, extension is limited. The difference in extensibility and adaptation of the collagen bundles is visualized and explained in two abstract models (Figure 7A and B).

To our knowledge, this is the first time that collagen and elastin morphology of healthy skin and scar tissue was investigated in response to stretch in a human model, with comparison between cyclical and continuous stretch, and using objective, reliable, and valid analysis techniques. In addition, the stretched tissue was instantly fixed chemically in its stretched-out position. By using this study methodology, we have attempted to mimic the clinical stretching situation as much as possible. Previously published (porcine) studies did not fix their stretched tissue in stretched-out position.\textsuperscript{5,15,16} Moreover, qualitative analysis was performed or a quantitative analysis was performed, using an analysis technique that had not been tested on its reliability and validity. In our opinion, based on the current study, a straightforward conclusion can be drawn: collagen fibers show a parallel alignment in response to stretch in both healthy skin and scars.

Extension of healthy skin after stretch was similar after cyclical and continuous stretch. This similar amount of stretching, which included 153% of the original length, means in our opinion that stretching has occurred to the limits in both cases. We may conclude, as previously hypothesized, that a continuous high tension (55 N) and thus a higher degree of force on skin does not lead to more extension as compared with cyclical stretch with lower tension applied. When we translate this to clinical practice, this implicates that there is no need to exert a mechanical load onto the skin above 30 N: it can be concluded that cyclical stretch with lower tension applied should be preferred over continuous stretch with high tension to prevent unnecessary tissue damage and to reduce the potential chance on stretch marks.

We found thicker collagen bundles and more space between the bundles after stretching of healthy skin. It has always been assumed that stretching results in a shift of extra-

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**Figure 6.** Box plots representing the bundle thickness (A) and bundle spacing (B) of nonstretched, cyclically, and continuously stretched healthy skin. The box displays the interquartile range (IQR), with the line in the box indicating the median value. The mean values are indicated at the left side of the box. Error bars indicate the range (minimum and maximum values). When the values exceed 1.5 \(\times\) IQR, they are indicated as an outlier. Statistical testing was performed using the Wilcoxon signed-rank test.
cellular fluid out of the dermis.\textsuperscript{18,28,29} Therefore, we expected that stretching of healthy skin would result in thinner rather than thicker collagen bundles. A possible explanation for our results is that the thicker bundles found after stretch are in fact (optically misleading) closely packed smaller collagen fibers, where the fluid in between these fibers was displaced. Or vice versa, it may be so that the true thickness of collagen bundles can only be observed correctly when stretched. If bundles may untwine in relaxed condition, we may normally not be looking at the true bundles, but we are observing subunits of the bundles instead. A possible explanation for the increased BSp is influx of fluid, most likely from blood vessels, as edema formation in response to stretching of the tissue. However, it cannot be completely excluded that the increased BSp is an artifact caused by the use of chemical fixation for tissue preservation after skin stretching. The fixative is a solution with high osmolarity, and due to binding of the formaldehyde molecules to tissue components it may cause influx of water and thus swelling of the dermis. On the other hand, this is an unlikely process because the volume of the solution of formaldehyde was in our experiments by far larger than the tissue volume and the usual effect of the hyperosmolarity of the fixative is tissue shrinkage rather than tissue swelling. In conclusion, the thicker collagen bundles and the enlarged

Figure 7. (A) Abstract model on the adaptation of collagen in healthy skin in response to stretch. When mild stretch (extension A) (which can be caused by bending of the knee) is exerted, skin extension is reached by little alignment of the collagen bundles. When large cyclical or continuous stretching forces are applied (extension B), the healthy skin is stretched beyond its intrinsic extensibility. Besides evident alignment of the collagen bundles, more space between collagen bundles results in additional extension of the healthy skin (extension B). (B) Abstract model of the adaptation of collagen in scar tissue in response to stretch. When mild stretch is exerted onto scar tissue, no extension of the scar tissue is reached. The extension of the scar tissue that is necessary to bend the knee is often reached by stretching the adjacent healthy skin next to the scar tissue or when no adjacent healthy skin is present, simply no extension is possible (scar contracture). When large continuous stretching forces are exerted (extension C), scar tissue does have the ability to stretch: alignment of the collagen bundles takes place. However, this extension is less in comparison with healthy skin: no further stretching of scar tissue is possible beyond its intrinsic extensibility — designed by Maartje Kunen.
space in between collagen bundles are unexpected phenomena that occurred in our skin-stretching experiments that need further investigations.

The effect of stretching on elastic fibers in the present study is largely in agreement with descriptive data of Gibson et al., where our data showed that when healthy skin was stretched, either cyclically or continuously, alignment of the elastic fibers in the stretch direction was noted. On the other hand, our analysis of the concentration of elastic fibers showed that the percentage of elastic fibers in healthy skin, in the majority of the samples, ranged between 0 and 4% and was only in 8.5% (5/59) of the cases scored above 6%. This relatively low concentration of elastic fibers in the dermis together with the large percentage of collagen bundles (85%), which very clearly align in response to stretch, is in our opinion in contrast with the generally accepted idea that viscoelastic properties of the skin highly rely on the elastic fibers. We feel that, as previously suggested by Silver et al., the viscoelastic behavior of skin in response to stretch is probably for the greater part explained by the capacity of the collagen network instead of the elastic fibers. This is further supported by previously found histological findings that show that the elastic fibers form a secondary network, which is looped around the collagen bundles. Instead of being responsible for the viscoelastic behavior of skin in response to stretch, it seems that these elastic fibers play a supportive role for the collagen bundles. Given these histological findings and given the relatively low percentage of elastic fibers that we found in human healthy skin and scars, this implicates that most of the stretch that is applied onto skin in clinical practice must be supported by collagen bundles instead of elastic fibers.

In conclusion, we have shown that stretch of healthy skin and scar tissue results in significant differences in collagen orientation and morphology. For the first time, this was shown for human tissue, which was processed and fixed in stretched-out position and which was analyzed using objective, reliable, and valid image analysis techniques. The changes in collagen structure after stretch may explain the differences in viscoelasticity that are clinically observed changes in collagen structure after stretch may explain the differences in viscoelasticity that are clinically observed.


