


## SCIENTIFIC ARTICLE

# Histomorphological Alterations of Human Anterior Cruciate Ligament Grafts During Mid-Term and Long-Term Remodeling

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**Objective:** The aim of the present paper is to analyze mid-term and long-term alterations of human anterior cruciate ligament (ACL) grafts during the remodeling process with special regards to cellularity,  $\alpha$ -smooth muscle protein ( $\alpha$ SMP) expression, and crimp length in comparison to the native ACL.

**Methods:** A total of 34 patients were included (23 male and 11 female). Biopsies of 13 semitendinosus tendon and 14 patellar tendon autografts were obtained during surgical revision secondary to an ACL reconstruction. According to the interval between the index procedure and sample collection, the patients were divided into four groups: 4–12 months, 13–60 months, 61–108 months, and >108 months. Seven samples of native ruptured ACL tissue obtained during surgical intervention served as control. All biopsies were taken from the intraligamentous part of the ACL or the graft. Histomorphological and immunohistochemical analyses were conducted after samples were stained using hematoxylin–eosin, Giemsa, and  $\alpha$ SMP enzyme-labeled antibodies. The total cell density, the numbers of fibroblasts and fibrocytes, the fibroblast/fibrocyte ratio, the number of  $\alpha$ SMP+ cell nuclei, and the percentage of  $\alpha$ SMP+ cells per fibroblast as well as the crimp lengths were determined using light microscopy.

**Results:** In the early phase of remodeling, the grafts featured extensively high total cell counts ( $1021.2 \pm 327.8$ ,  $P = 0.001$ ), with high numbers of fibroblasts ( $841.4 \pm 245.2$ ,  $P = 0.002$ ), fibrocytes ( $174.5 \pm 113.0$ ,  $P = 0.04$ ), and  $\alpha$ SMP+ cells ( $78.3 \pm 95.0$ ,  $P = 0.02$ ) compared to controls ( $390.1 \pm 141.7$ ,  $304.5 \pm 160.8$ ,  $65.6 \pm 31.4$  and  $2.3 \pm 2.6$ , respectively). Thereafter, the numbers of all cell entities decreased. After more than 108 months, the percentage of  $\alpha$ SMP+ cells per fibroblast reached physiological values (ratio  $1.3 \pm 1.0$ ,  $P = 0.41$ ; control  $0.8 \pm 0.8$ ), while the total cell count ( $834.3 \pm 183.7$ ,  $P = 0.001$ ) as well as the numbers of fibroblasts ( $663.5 \pm 192.6$ ,  $P = 0.006$ ) and fibrocytes ( $134.1 \pm 73.0$ ,  $P = 0.049$ ) remained significantly high. The fibroblast/fibrocyte ratio showed no significant alterations over the course of time compared to the controls. The collagen crimp lengths were elongated by tendency in the early phase ( $28.8 \pm 12.9$  mm,  $P = 0.15$ ; control  $20.7 \pm 2.2$  mm) and significantly shortened over time, with the lowest values in the long term ( $14.8 \pm 2.0$  mm,  $P = 0.001$ ). The comparison of biopsies from semitendinosus tendon and patellar tendon autografts revealed no significant differences for any of the histomorphological parameters investigated.

**Conclusion:** This study reveals distinctive mid-term and long-term immunomorphological alterations during human ACL graft remodeling. These data clearly indicate that the remodeling is a process that continues for 9 years or more.

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Furthermore, it seems to be a process of adaptation rather than full restoration. Even in the long run, several biological properties of the native ACL are not completely reestablished.

**Key words:** ACL; Anterior cruciate ligament; Ligamentization; Reconstruction; Remodeling

## Introduction

Anterior cruciate ligament (ACL) grafts undergo a distinct remodeling process in response to the exposure to the synovial fluid and the altered biomechanical forces. This “phenomenon of ligamentization” was first described by Amiel *et al*<sup>1</sup>. Over the course of time, the graft more closely resembles the structure and features of the native ACL. Until now, no clear consensus on the time-dependent differences in remodeling stages and the ending of changes in remodeling capacities has been reached<sup>2–6</sup>.

However, given the fact that patients return to sports 6 to 12 months after the ACL reconstruction, precisely understanding the morphological alterations and properties of the grafts over time is of utmost importance<sup>7</sup>. In this context, it is particularly important to emphasize the role of human studies. The results of animal studies can only be transferred to humans to a certain extent. The remodeling processes in animals show an extended phase of necrosis in the beginning, which is significantly less pronounced in human graft tissues, and overall progress more quickly<sup>8–11</sup>. The current literature on the remodeling of human ACL grafts is mainly based on the findings of Rougraff *et al*.<sup>6</sup>, who differentiated four distinct stages of the ligamentization process. The stage of repopulation covers the first 2 months after ACL reconstruction followed by the stage of rapid remodeling, which lasts for up to 10 months. Thereafter, the stage of graft maturation goes on for another 2 years and the final stage of ligamentous graft maturation is completed approximately 3 years after surgery. Different stages and deviating timelines have been reported by several other groups<sup>12–15</sup>. There is strong dissent across the different studies concerning the overall duration of the remodeling process. The specifications range from 15 to 48 or more months. Besides the heterogeneity, the number of human biopsy studies is limited and the available studies predominately concentrate on cell count or the collagenous structure over a short assessment period (of up to 2 years)<sup>3–6</sup>. Furthermore, precise and detailed information on cell distribution and immunomorphological alteration of the ACL graft tissues is limited.

The physiological ACL, in contrast, is characterized by the specific distribution and density of fibroblasts and  $\alpha$ -smooth muscle protein ( $\alpha$ SMP)-expressing myofibroblasts<sup>16</sup>. The myofibroblasts, with their contractile potency, contribute to the collagen crimp and the viscoelastic properties of the healthy ACL<sup>17</sup>. Nevertheless, it has not been investigated in detail how density and distribution of fibroblasts, fibrocytes, and  $\alpha$ SMP+ myofibroblasts as well as collagen crimp length alter over time during the ligamentization

process of ACL grafts. We hypothesized that the process of ligamentization is accompanied by histomorphological alterations of the ACL grafts over a significantly longer period of time than has been accounted for in the literature to date. Thus, the aim of the current study was to analyze the mid-term and long-term alterations of human ACL grafts during the remodeling process with special regards to cellularity and collagen crimp characteristics in comparison to the native ACL. The purpose of the study was the determination of the following parameters over the course of time: (i) the total cell density as well as the numbers of fibroblasts and fibrocytes; (ii) the fibroblast/fibrocyte ratio, the numbers of  $\alpha$ SMP+ cell nuclei, and the percentage of  $\alpha$ SMP+ cells per fibroblast; and (iii) the collagen crimp lengths.

## Methods

### Inclusion and Exclusion Criteria

The inclusion criteria were: (i) acute ACL rupture or ACL re-rupture due to an adequate trauma for the control and the intervention group, respectively; (ii) biopsy obtained from the intact mid-substance of the ACL or ACL graft; and (iii) sufficient amounts of tissue. The exclusion criteria were: (i) chronic ACL graft failure; (ii) clinical signs of arthrofibrosis; and (iii) specimens with obvious signs of hematoma or necrosis.

### Sample Collection

The current fundamental research study included 34 patients (23 male and 11 female). Patient demographics are presented in Table 1. Sample collection was carried out within 2 years and from consecutive patients by the same surgeon. During surgical revision secondary to an ACL reconstruction, 13 specimens of semitendinosus tendon (STG) and 14 specimens of patellar tendon autografts (PTG) were obtained as biopsies. According to the interval between the index procedure and sample collection, the patients were divided into four groups: 4–12 months, 13–60 months, 61–108 months, and >108 months. Seven samples of native ruptured ACL tissue were obtained during surgical intervention within a mean of 16.9 days after trauma and served as control. All samples were taken from the intraligamentous part of the ACL or the graft. In this study, differences between the grafts in comparison to the native ACL were investigated over time. In addition, the potential differences between the two graft types were evaluated within the groups. Quantitative and qualitative analyses were conducted by means of histomorphological and immunohistochemical examination under light microscopy. Specimens with clear signs of

inflammation or necrosis were excluded in advance. The study was approved through the local ethics committee.

### Preparation of Samples for Histomorphological Examination

Samples were fixed in 4% buffered formalin, embedded in paraffin, and sliced into 5–7- $\mu\text{m}$ -thick longitudinal sections. After deparaffinization and rehydration, conventional staining with hematoxylin–eosin (HE) and Giemsa was performed.

The detection of  $\alpha\text{SMP}$  was carried out by immunohistochemical staining with enzyme-labeled antibodies using a dilution of 1:200 (mouse anti-human actin  $\alpha$ -smooth muscle monoclonal antibody; Sigma-Aldrich, Darmstadt, Germany). In the beginning, the samples were deparaffinized and rehydrated, followed by the application of 3%  $\text{H}_2\text{O}_2$  to block any endogenous peroxidase. Afterwards, the samples were passed through a process of blocking phase, primary antibody incubation, post block, secondary antibody incubation, and color reaction with HRP-Polymer (ZytoChem-Plus HRP Polymer-Kit; Zytomed Systems) and chromogen AEC (AEC Substrate Kit; Zytomed Systems, Berlin, Germany). Finally, they were counterstained with hematoxylin.  $\alpha\text{SMP}$  staining of blood vessels served as an internal control (Fig. 1).

Analysis of the cellularity and crimp length were performed using light microscopy.

### Outcome Measures

#### Cellularity

The total cell density (cells/ $\text{mm}^2$ ), the number of fibroblasts (cells/ $\text{mm}^2$ ), the number of fibrocytes (cells/ $\text{mm}^2$ ), the fibroblast/fibrocyte ratio, the number of  $\alpha\text{SMP}+$  cell nuclei (cells/ $\text{mm}^2$ ), and the percentage of  $\alpha\text{SMP}+$  cells per fibroblast were determined. Between 7 and 12 regions of interest (ROI) were selected for every specimen using light microscopy with  $524 \times 393 \mu\text{m}$  (20 $\times$  magnification, Olympus SC30). Digital photographs (Stream Motion 1.9, Olympus) of every ROI in the exact intraligamentous localization were taken for all

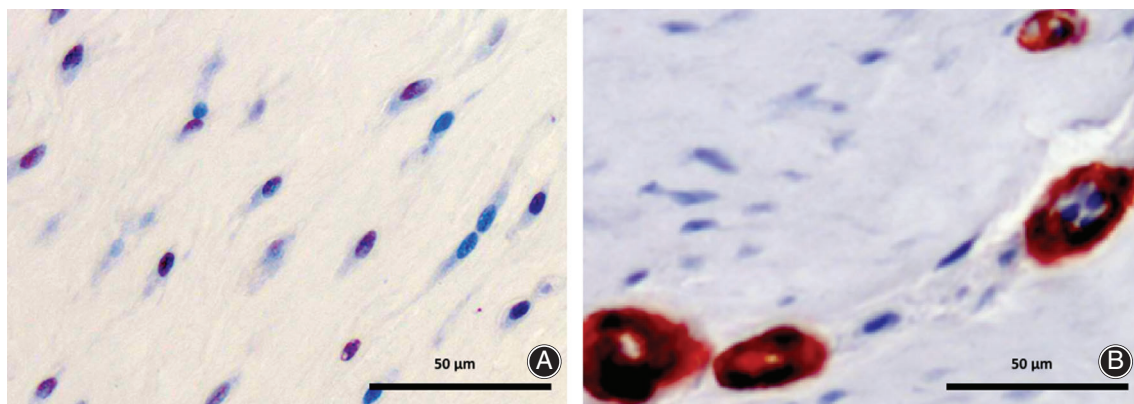
staining and then evaluated. The number of fibroblasts (also known as round fibroblasts) and the number of fibrocytes (also known as spindle-shaped or fusiform fibroblasts) were determined using HE or Giemsa staining (Fig. 2A,B).

#### Crimp length

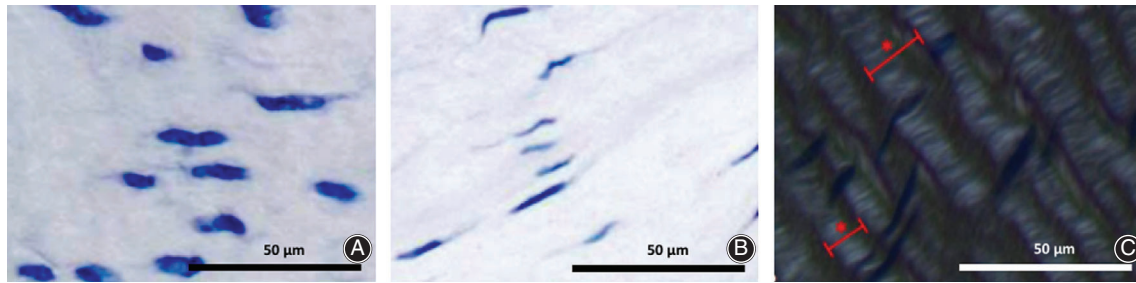
The crimp contributes significantly to the adaptation of the ligament to biomechanically acting forces<sup>18</sup> and can be detected very well in polarization (Fig. 2C). The quantitative evaluation of the crimp length ( $\mu\text{m}$ ) was performed manually by two examiners (HM and KH) and double-checked at a different time point. Interrater reliability was 0.87. Ten individual measurements of crimp length per ROI were performed on polarized microscopic images (Olympus Simple Polarizing attachment BX-Pol, Tokyo, Japan; Giemsa staining). Counting was performed with the Adobe Photoshop counting tool (Adobe Photoshop CS 4 Extended, Version 11.0, Adobe Systems, San Jose, CA, USA). Length measurement was performed with the Stream Motion 1.9 Software by Olympus. Mean values and standard deviations were determined.

#### Statistical Evaluation

Data are given as mean  $\pm$  standard deviation. The IBM SPSS statistics program (version 24, IBM) was used for statistical evaluation. The results of all parameters investigated were checked for normal distribution. Statistical analyses were performed to compare the independent samples using the Student *t*-test for parametric and the Mann–Whitney *U*-test for nonparametric data. A  $P < 0.05$  was considered statistically significant. Representing a marker of differentiation, the number of fibroblasts (cells/ $\text{mm}^2$ ) compared to the native ACL was set as the primary endpoint. The total cell density, fibrocytes (cells/ $\text{mm}^2$ ), the fibroblast/fibrocyte ratio,  $\alpha\text{SMP}+$  cell nuclei (cells/ $\text{mm}^2$ ), the percentage of  $\alpha\text{SMP}+$  cells per fibroblast, and the crimp length were determined as secondary endpoints.



**Fig 1** Immunohistochemical staining of anterior cruciate ligament graft tissue, showing the typical red-brown coloring of  $\alpha$ -smooth muscle protein ( $\alpha\text{SMP}$ )-positively stained cell nuclei (A).  $\alpha\text{SMP}$ -staining of blood vessels served as an internal control (B).



**Fig 2** The different morphologic cell types: The more common fibroblasts with round to ovoid nuclei (A) and spindle-shaped or fusiform fibrocytes (B, both Giemsa staining). The detection of crimp length (\*) was performed by measuring the wavelength in  $\mu\text{m}$  (C, Giemsa staining with polarization microscopy).

## Results

### Cellularity

#### Total Cell Density and Numbers of Fibroblasts and Fibrocytes

The total cell density was increased in the ACL graft tissues across all time points compared to the mean density of  $390.1 (\pm 141.7)$  cells/ $\text{mm}^2$  in native ACL tissues. Significant differences were revealed in the 4–12 months group ( $1021.2 \pm 327.8$  cells/ $\text{mm}^2$ ;  $P = 0.001$ ), the 13–60 months group ( $813.9 \pm 265.6$  cells/ $\text{mm}^2$ ;  $P = 0.002$ ), and the >108 months group ( $834.3 \pm 183.7$  cells/ $\text{mm}^2$ ;  $P = 0.001$ ).

Accordingly, the numbers of fibroblasts were increased in the ACL graft tissues across all timepoints compared to the mean cell count of  $304.5 (\pm 160.8)$  cells/ $\text{mm}^2$  in native ACL tissues. Significant differences were revealed in the 4–12 months group ( $841.4 \pm 245.2$  cells/ $\text{mm}^2$ ;  $p = 0.002$ ), the 13–60 months group ( $719.0 \pm 269.7$  cells/ $\text{mm}^2$ ;  $P = 0.004$ ), and the >108 months group ( $663.5 \pm 192.9$  cells/ $\text{mm}^2$ ;  $P = 0.006$ ).

Furthermore, the numbers of fibrocytes were increased in the ACL grafts at all timepoints investigated compared to the mean cell count of  $65.6 \pm 31.4$  cells/ $\text{mm}^2$  in the controls. Significant differences were revealed only in the 4–12 months group ( $174.5 \pm 113.0$  cells/ $\text{mm}^2$ ;  $P = 0.04$ ) and the >108 months group ( $134.1 \pm 73.0$  cells/ $\text{mm}^2$ ;  $P = 0.049$ ).

#### Fibroblast/Fibrocyte Ratio, Numbers of $\alpha\text{SMP}^+$ Cells, and Percentage of $\alpha\text{SMP}^+$ Cells/Fibroblasts

There were no significant differences in the fibroblast/fibrocyte ratio in ACL graft tissues at any timepoint compared to the mean ratio of  $7.6 \pm 8.1$  detected in the native ACL tissues.

The number of  $\alpha\text{SMP}^+$  cells was significantly increased in the 4–12 months group, which showed a mean cell count of  $78.3 \pm 95.0$  cells/ $\text{mm}^2$  compared to  $2.3 \pm 2.6$  cells/ $\text{mm}^2$  in the control ( $P = 0.02$ ). The elevated mean values decreased over time in the 13–60 months group ( $52.8 \pm 79.7$  cells/ $\text{mm}^2$ ), the 61–108 months group ( $24.1 \pm 33.5$  cells/ $\text{mm}^2$ )

and the >108 months group ( $8.7 \pm 7.7$  cells/ $\text{mm}^2$ ) but not statistically significantly.

The percentage of  $\alpha\text{SMP}^+$  cells per fibroblast was significantly increased in the 4–12 months group ( $13.0 \pm 14.9\%$ ;  $P = 0.045$ ) and the 61–108 months group ( $3.3 \pm 2.3\%$ ;  $P = 0.02$ ) compared to the control ( $0.8 \pm 0.8\%$ ). The highest values were detected at the earliest timepoint and were subsequently decreasing over time. The percentage of  $\alpha\text{SMP}^+$  cells per fibroblast finally reached comparable values to the control ( $1.3 \pm 1.0\%$ ). At this timepoint, no significant differences were revealed (Table 1).

#### Semitendinosus Tendon and Patellar Tendon Grafts

According to the distribution of the two different graft types within the groups, comparative analyses of the potential differences were feasible for the 13–60 (semitendinosus tendon grafts:  $n = 5$ ; patellar tendon grafts:  $n = 3$ ) and the 61–108 months groups (semitendinosus tendon grafts:  $n = 3$ ; patellar tendon grafts:  $n = 5$ ). In both groups, no significant differences were revealed for any of the cellularity parameters investigated (Table 2).

#### Crimp Length

The mean crimp length found in the native ACL tissues was  $20.7 \pm 2.2 \mu\text{m}$ . The crimp length increased by tendency in the 4–12 months groups. Over time, the crimp lengths in the graft tissues significantly decreased compared to the native ACL tissue, with lowest values in the >108 months group (Table 1). The analysis of potential crimp length differences between the two different graft types revealed no significant differences in the 13–60 and the 61–108 months groups (Table 2).

#### Discussion

This is the first biopsy study providing detailed mid-term and long-term immunomorphological findings in human ACL graft remodeling. In the early phase, the grafts feature extensively high total cell counts with high numbers of fibroblasts, fibrocytes, and  $\alpha\text{SMP}^+$  cells. Over time, the numbers of all cell entities slowly decrease. After more than 108 months, the percentage of  $\alpha\text{SMP}^+$  cells per fibroblast



**TABLE 1** Sample distribution, patient demographics, and results of control and intervention groups

|                                    | Control       | 4–12 months                | 13–60 months              | 61–108 months            | >108 months               |
|------------------------------------|---------------|----------------------------|---------------------------|--------------------------|---------------------------|
| <i>n</i>                           | 7 (STG = 7)   | 5 (STG = 5)                | 8 (STG = 5; PTG = 3)      | 8 (STG = 3; PT = 5)      | 6 (PTG = 6)               |
| Age (years)                        | 33.9 ± 17.0   | 22.3 ± 3.8                 | 30.5 ± 10.8               | 35.7 ± 8.5               | 40.8 ± 13.2               |
| Interval                           | 16.9 ± 16.0*  | 8.8 ± 4.5 <sup>†</sup>     | 36.3 ± 12.6 <sup>†</sup>  | 90.7 ± 9.1 <sup>†</sup>  | 132.1 ± 19.7 <sup>†</sup> |
| Total cell density/mm <sup>2</sup> | 390.1 ± 141.7 | 1021.2 ± 327.8 (P = 0.001) | 813.9 ± 265.6 (P = 0.002) | 744.7 ± 529.2 (P = 0.11) | 834.3 ± 183.7 (P = 0.001) |
| Fibroblasts/mm <sup>2</sup>        | 304.5 ± 160.8 | 841.4 ± 245.2 (P = 0.002)  | 719.0 ± 269.7 (P = 0.004) | 603.3 ± 450.3 (P = 0.12) | 663.5 ± 192.9 (P = 0.006) |
| Fibrocytes/mm <sup>2</sup>         | 65.6 ± 31.4   | 174.5 ± 113.0 (P = 0.04)   | 90.4 ± 50.6 (P = 0.28)    | 130.0 ± 75.4 (P = 0.06)  | 134.1 ± 73.0 (P = 0.049)  |
| Fibroblast/fibrocyte ratio         | 7.6 ± 8.1     | 6.7 ± 4.3 (P = 0.85)       | 8.6 ± 5.1 (P = 0.77)      | 5.1 ± 1.6 (P = 0.44)     | 6.6 ± 4.4 (P = 0.82)      |
| αSMP+ cells/mm <sup>2</sup>        | 2.3 ± 2.6     | 78.3 ± 95.0 (P = 0.02)     | 52.8 ± 79.7 (P = 0.12)    | 24.1 ± 33.5 (P = 0.11)   | 8.7 ± 7.7 (P = 0.07)      |
| αSMP+ cells/fibroblast (%)         | 0.8 ± 0.8     | 13.0 ± 14.9 (P = 0.045)    | 8.4 ± 12.9 (P = 0.15)     | 3.3 ± 2.3 (P = 0.02)     | 1.3 ± 1.0 (P = 0.41)      |
| Crimp length (μm)                  | 20.7 ± 2.2    | 28.8 ± 12.9 (P = 0.15)     | 15.8 ± 3.3 (P = 0.008)    | 16.6 ± 4.6 (P = 0.07)    | 14.8 ± 2.0 (P = 0.001)    |

Results are presented as mean values ± standard deviation.; ACL, anterior cruciate ligament; αSMP, α-smooth muscle protein; PTG, patellar tendon grafts; STG, Semitendinosus tendon. Statistical analyses were performed comparing each group to control.; \*ACL rupture: ACL reconstruction/biopsy (days); <sup>†</sup>ACL reconstruction: revision/biopsy (months).

**TABLE 2** Results of subgroup analyses comparing mid-term and long-term alteration in semitendinosus tendon (STG) and patellar tendon grafts (PTG)

| Graft ( <i>n</i> )                 | 13–60 months  |               |                 | 61–108 months |               |                 |
|------------------------------------|---------------|---------------|-----------------|---------------|---------------|-----------------|
|                                    | STG (5)       | PTG (3)       | <i>P</i> value  | STG (3)       | PTG (5)       | <i>P</i> -value |
| Total cell density/mm <sup>2</sup> | 833.4 ± 241.9 | 781.3 ± 356.9 | <i>P</i> = 0.81 | 533.0 ± 72.4  | 871.7 ± 658.5 | <i>P</i> = 0.42 |
| Fibroblasts/mm <sup>2</sup>        | 742.9 ± 273.3 | 679.1 ± 318.3 | <i>P</i> = 0.77 | 404.6 ± 66.7  | 722.5 ± 552.5 | <i>P</i> = 0.37 |
| Fibrocytes/mm <sup>2</sup>         | 89.3 ± 47.7   | 92.2 ± 66.5   | <i>P</i> = 0.94 | 120.8 ± 47.0  | 135.6 ± 93.4  | <i>P</i> = 0.81 |
| Fibroblasts/ Fibrocytes ratio      | 7.9 ± 5.9     | 9.6 ± 4.7     | <i>P</i> = 0.71 | 4.5 ± 1.4     | 5.3 ± 1.9     | <i>P</i> = 0.59 |
| αSMP+ cells/mm <sup>2</sup>        | 58.9 ± 95.1   | 42.6 ± 62.7   | <i>P</i> = 0.80 | 14.9 ± 13.9   | 29.6 ± 42.0   | <i>P</i> = 0.59 |
| αSMP+ cells/fibroblast (%)         | 6.2 ± 9.4     | 12.1 ± 19.4   | <i>P</i> = 0.58 | 3.5 ± 3.0     | 3.2 ± 2.3     | <i>P</i> = 0.89 |
| Crimp length (μm)                  | 15.7 ± 3.5    | 15.9 ± 3.6    | <i>P</i> = 0.95 | 15.2 ± 6.0    | 17.2 ± 4.6    | <i>P</i> = 0.67 |

reaches physiological values while the total cell count as well as the numbers of fibroblasts and fibrocytes remain significantly high. The collagen crimp lengths are elongated by tendency in the early phase and significantly shortened over time compared to controls, with the lowest values in the long term. These data clearly reveal that the remodeling of ACL grafts is a process that continues for 9 years or more. Furthermore, it seems to be a process of adaptation rather than full restoration. Several biological properties of the physiological ACL are not being fully restored, even in the long run.

Regarding the cellularity of the native human ACL, Murray and Spector<sup>7</sup>, Murray *et al.*<sup>19</sup>, and Janssen *et al.*<sup>2</sup> all described similar total cell densities that concur with the present findings. Across all studies, the total cell density of the native ACL ranges between 395 and 536 cells/mm<sup>2</sup>. Janssen *et al.* further investigated the total cell density in a time-dependent manner. Besides a significant increase after 6 to 12 months, the authors also observed a tendency for an even higher density after 13 to 24 months and a moderate decrease after more than 24 months. The significant increase in short-

term and mid-term total cell density is confirmed in the present study. Furthermore, the present findings reveal that the graft tissue features a very slowly decreasing cell density that in the end is still highly increased, with 834.3 ± 183.7 cells/mm<sup>2</sup> after 9 or more years. The increase in total cell density is attributed to the proliferative fibroblast response and the differentiation into fibrocytes. The present findings reveal a relatively stable fibroblast/fibrocyte ratio across all timepoints investigated. In contrast, the ratio of αSMP+ myofibroblasts per fibroblast changes significantly over the course of time. Myofibroblasts have contractile properties and the ability to exert isometric tension on their extra-cellular matrix. They are responsible for the restoration of the crimping structure of the collagen fibers and the *in-situ* tension of the ACL graft<sup>12</sup>. According to the present findings in native ACL, Janssen *et al.* observed 5.7 ± 8.9 αSMP+ cells/mm<sup>2</sup> and Murray *et al.* detected 0.8%–1.0% αSMP+ cells/total cell density<sup>19</sup>. Janssen *et al.* observed a peak number of αSMP+ cells/mm<sup>2</sup> in the group 13 to 24 months after the ACL reconstruction, with a decrease in the group after 24 months. According to Weiler

*et al.*, the presence of these cells during the early remodeling is involved in the fiber bundle formation<sup>20</sup>. In the present study, the ACL graft tissues feature a significant increase in the number of  $\alpha$ SMP+ cells/mm<sup>2</sup> and the  $\alpha$ SMP+ cells/fibroblast ratio after 4 to 12 months. Both parameters then decline stepwise over time. After 9 or more years, the percentage of  $\alpha$ SMP+ cells per fibroblast shows values comparable to the control. Nevertheless, the prolonged increased numbers of  $\alpha$ SMP+ cells may have an influence on the crimping structure of the collagen fibers of the ACL graft.

The detected crimp length of native ACL tissue of  $20.7 \pm 2.2 \mu\text{m}$  in this study is comparable to the results of Murray and Spector<sup>16</sup> and well as Weiss *et al.*<sup>17</sup> However, even though alterations of the crimp pattern are implemented in the ligament maturity index introduced by Murray *et al.*<sup>21</sup>, no study has investigated the quantitative changes of the human ACL graft crimp pattern during the ligamentization process. Abe *et al.* only semi-quantitatively distinguished low and high frequency crimp pattern and described a comparable pattern of graft and native ACL tissue after 1 year.<sup>4</sup> The present study reveals an elongated crimp length in the initial phase and significantly decreased lengths over time. The long-term results after 9 or more years show the lowest values compared to controls.

While the substitutes in animal studies resemble native ACL in morphological aspects as early as 1 year after ACL reconstruction<sup>8, 22</sup>, human studies show extremely heterogeneous results. To date, no consensus has been reached on the actual duration of the ligamentization process of human graft tissue. Based on the histological evaluation for vascularity, cellularity, fiber pattern, and metaplasia, Falconiero *et al.* concluded that after 12 months, the graft maturity resembles that of a normal ACL<sup>5</sup>. The biochemical findings by Marumo *et al.* suggest that regarding the amount of collagen crosslinks and their architecture, the graft tissue resembles those of the native ACL within 1 year<sup>23</sup>. The ultrastructural study results of Abe *et al.* suggested that human grafts were still immature even at 1 year postoperatively<sup>4</sup>. According to Sanchez *et al.*, the process of ligamentization undergoes distinct stages over a period of 2 years<sup>3</sup>. In addition, Janssen *et al.* described human graft tissues showing typical stages of remodeling, which is not complete up to 2 years after ACL reconstruction<sup>2</sup>. Based on their observations, Rougraff *et al.* concluded that the process of ligamentization takes as long as 3 years to complete<sup>11</sup>. Dong *et al.* divided their patients into two groups: a mid-term group of 13 to 30 months and a long-term group of 31 to 62 months. Indicating a slowdown of

graft maturation, no significant difference with respect to large-diameter collagen fibrils, bimodal distribution, graft vascularity, cellularity, metaplasia, or cellular metabolic status were found between the two groups<sup>24</sup>. The transmission electron microscopy studies by Zaffagnini *et al.* revealed that ACL grafts undergo a transformation process but do not match the ultrastructure pattern of a normal ACL up to 10 years<sup>25, 26</sup>. Regarding this controversy, the present study corroborates the belief that the process of ligamentization takes place for 9 years or more and that it is, in fact, a process of adaptation rather than full restoration.

The analysis of potential differences in remodeling capacities between STG and PTG grafts revealed no significant differences for any parameter investigated. These findings support the observations made by other groups that the remodeling processes in STG and PTG take place in a comparable manner<sup>23, 25, 26</sup>.

In general, and when interpreting studies concerning ACL morphology and graft remodeling, different internal and external influences should be taken into account. Human ACLs themselves differ inter-individually by age and gender<sup>16, 19</sup>. The intraarticular environment and remodeling processes are influenced by inflammatory cytokines that are released depending on concomitant injuries, meniscal and chondral damage, type of intervention, biomechanical conditions like tunnel location or graft tensioning, and the rehabilitation program<sup>27-29</sup>. Patients with an ACL graft return to sports approximately 1 year after ACL reconstruction. At this point in time, highly active remodeling processes continue, as stated in many human studies and shown in this study<sup>5-7, 13, 14</sup>. Over time, these processes seem to slow down.

The present study has some limitations. The number of samples is limited, and two different graft tissues were included. Furthermore, tissue samples from acute ACL injuries served as control and no healthy native ACL tissue samples were included. These limitations are distinctive and common for human biopsy studies after primary ACL reconstruction.

### Conclusion

The remodeling process leads to assimilation of the graft tissue to the native ACL. Excessive cell counts and elongation of the crimp length in the beginning decrease over time. The structural changes take place for 9 years or more. Even after this long period, the grafts do not completely restore the morphological structure of the native ACL.

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