

## IMPROVED TENDON HEALING USING bFGF, BMP-12 AND TGF $\beta_1$ IN A RAT MODEL

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### Abstract

Several growth factors (GFs) are expressed as tendons heal, but it remains unknown whether their combined application enhances the healing process. This matter was addressed by applying a combination of basic fibroblast growth factor (bFGF), bone morphogenetic protein 12 (BMP-12) and transforming growth factor beta 1 (TGF $\beta_1$ ) in a rat Achilles tendon transection model. GFs were applied in one of the three following ways: i) direct application of all three factors at the time of surgery; ii) sequential, tiered percutaneous injection of individual factors immediately after surgery, 48 h and 96 h later; iii) load of all three factors onto a collagen sponge implanted at the time of surgery. After 1, 2, 4 and 8 weeks, healing was assessed based on tendon length and thickness, mechanical strength, stiffness and histology. Best results were achieved when GFs were loaded onto a collagen sponge – with a rapid increase in mechanical strength (load to failure, 71.2 N *vs.* 7.7 N in controls), consistent tendon length over time (9.9 mm *vs.* 16.2 mm in controls) and faster tendon remodelling, as measured by histology – followed by tiered injection therapy over 96 h.

In conclusion, implantation of a GF-loaded collagen sponge could provide a promising treatment, especially in high-performance athletes and revision cases prone to re-rupture. For conservative treatment, tiered percutaneous GF application could be an option for improving clinical outcome.

**Keywords:** Achilles tendon, tendon healing, tendon engineering, rat, growth factors.

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### Introduction

Tendon ruptures are common sporting injuries (Schepesis *et al.*, 2002), with an increasing incidence (Fox *et al.*, 2016; Ganestam *et al.*, 2016; Huttunen *et al.*, 2014; Jacobsson *et al.*, 2012; Lantto *et al.*, 2015; Mattila *et al.*, 2015; Sheth *et al.*, 2017; Wertz *et al.*, 2013). Treatment options include both conservative and operative approaches. However, up to 12 % of Achilles tendons re-rupture during healing (Soroceanu *et al.*, 2012; van der Eng *et al.*, 2013), with the rate rising to 16.6 % in young athletes (< 30 years) (Rettig *et al.*, 2005). Re-ruptures can result in prolonged rehabilitation, reduced quality of life and compromised subsequent re-healing, as seen for the rotator cuff (Shamsudin *et al.*, 2015). For instance,

for professional American football players, an acute Achilles tendon tear leads to a career ending in one third of the cases (Mai *et al.*, 2016; Parekh *et al.*, 2009). An optimised tendon healing protocol should produce quick and reliable healing, with a rapid return of mechanical strength, to minimise re-rupture, and maintenance of correct tendon length, to ensure appropriate load transmission.

Tendon healing is promoted and guided by a variety of growth factors (GFs) produced locally that orchestrate the complex biology of tissue healing. Numerous GFs are identified, including connective tissue growth factor (CTGF), insulin-like growth factor (IGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), bone morphogenetic

proteins (BMPs) and transforming growth factor beta (TGF $\beta$ ) (Docheva *et al.*, 2015; Evans, 1999; Freedman *et al.*, 2014; Heisterbach *et al.*, 2012; Kaux *et al.*, 2014; Molloy *et al.*, 2003; Müller *et al.*, 2015; Ohba *et al.*, 2012; Sharma and Maffulli, 2006; Würgler-Hauri *et al.*, 2007; Yang *et al.*, 2013).

Many studies investigate the influence of individual GFs on tendon healing (Docheva *et al.*, 2015; Müller *et al.*, 2015). GFs can be applied percutaneously, during open surgery or through carriers, such as scaffolds or coated suture materials, which are supposed to reduce the rate of local GF clearance (Hamada *et al.*, 2006; Rickert *et al.*, 2001; Uggen *et al.*, 2010). However, none of these GFs has advanced to clinical use because of a lack of convincing pre-clinical results (Docheva *et al.*, 2015).

In contrast, autologous blood products containing multiple GFs – including the ones mentioned above – are widely used in clinics. Platelet rich plasma (PRP) and autologous conditioned serum (ACS) are known to improve tendon healing (Aspenberg and Virchenko, 2004; Bosch *et al.*, 2010; de Almeida *et al.*, 2012; Gosens *et al.*, 2012; Majewski *et al.*, 2009; Parafioriti *et al.*, 2011; Schnabel *et al.*, 2007; Seijas *et al.*, 2013). However, depending on production protocols, PRP can contain not only GFs, but also a variety of additional agents – including interleukins, chemokines, proteinases, inhibitors of proteinases, adhesion molecules, sphingolipids, thromboxanes, purine nucleotides, serotonin, calcium, pyrogens and cells (Evans, 2013; Serhan and Savill, 2005) – thus, constituting a variable and uncharacterised “cocktail” of different molecules and cells. Currently, it remains unclear which autologous blood product is most effective in promoting tendon regeneration (Docheva *et al.*, 2015) and, although it is appreciated that tendon healing is mediated by several GFs, which is the best formulation or combination for clinical application.

*In vitro* data are available on different combinations of GFs, with sequential or simultaneous administration (Gaspar *et al.*, 2015; Spanoudes *et al.*, 2014). Continuous exposure of human tenocytes to PDGF-BB and bFGF for 2 weeks and to TGF $\beta_3$  for another 2 weeks results in survival of tenocytes for 28 d *in vitro* (Qiu *et al.*, 2016). Proliferation and collagen production of canine flexor tendon fibroblasts in the presence of both PDGF-BB and bFGF is higher than for each GF alone (Thomopoulos *et al.*, 2005). Similarly, proliferation of rabbit flexor tendon tenocytes increases more in response to a combination of IGF1, PDGF-BB and bFGF as compared to individual GF application (Costa *et al.*, 2006). A combination of VEGF, TGF $\beta_3$  and BMP-14 enhances the tenogenic differentiation of rabbit bone-marrow-derived mesenchymal stem cells (Bottagisio and Lovati, 2017). While a combined administration of IGF1 and BMP-14 increases proliferation and collagen synthesis in equine tenocytes (Caliari and Harley, 2013). Overall, simultaneous and/or sequential supplementation with more than one GF during tendon healing seems to be beneficial. However,

based upon the effects observed with incompletely characterised blood products, such as PRP or ACS, the correct, specific combination of GFs for tendon healing remains unknown (Spanoudes *et al.*, 2014).

Previous studies on the expression of GFs during the spontaneous healing of supraspinatus and Achilles tendons in rats (Heisterbach *et al.*, 2012; Würgler-Hauri *et al.*, 2007) suggest that bFGF, BMP-12 and TGF $\beta_1$  are particularly important in this regard. These three GFs are elevated during healing over an 8-week period. Higher expression rates are found for BMP-12 than for BMP-13 and -14 (Würgler-Hauri *et al.*, 2007). Interestingly, Heisterbach *et al.* (2012) do not detect increased VEGF expression in their model, therefore, the present study focused on bFGF, BMP-12 and TGF $\beta_1$ . bFGF is elevated early during tendon healing (Heisterbach *et al.*, 2012; Würgler-Hauri *et al.*, 2007) and, thus, is well positioned to promote early events in the healing process (Müller *et al.*, 2015). Elevated bFGF mRNA expression is found in tenocytes, fibroblasts and inflammatory cells in the surrounding tendon sheath of healing flexor tendons in rabbits (Chang *et al.*, 1998). bFGF plays an important role in angiogenesis, which is needed to provide a blood supply to the healing tendon. It is also a mitogen and a chemotactic agent in addition to improving tendon healing. For example, administration of bFGF in a rat patellar tendon model within the first week after injury leads to increased cellularity and collagen type III deposition within the defect (Chan *et al.*, 2000). Moreover, bFGF gene transfer increases expression of type I and type III collagen *in vitro* (Feng *et al.*, 2007; Wang *et al.*, 2005b). However, promising results demonstrating improved tendon healing by bFGF gene transfer (Tang *et al.*, 2008) are not reproducible (Kraus *et al.*, 2016; Kraus *et al.*, 2014). In a small series of 8 rabbits, Achilles tendons heal better than controls if treated with bFGF (Najafbeygi *et al.*, 2017). Furthermore, bFGF reduces adhesions in an Achilles tendon model in rats (Sha *et al.*, 2004). Improved Achilles tendon healing is reported using bFGF together with VEGF in a rabbit model where both GFs are coated onto a stabilising mineral nanostructure (Yu *et al.*, 2017). However, in a larger study using 40 rabbits, repeated treatment with VEGF, bFGF and rPDGF (day of surgery and day 3, 5 and 7) does not improve healing under conservative or operative conditions (Konerding *et al.*, 2010).

BMP-12, BMP-13 and BMP-14 are morphogens that induce the tenogenic differentiation of mesenchymal progenitor cells (Shen *et al.*, 2013). During embryogenesis, BMP-12 – also known as growth differentiation factor 7 (GDF7) or cartilage-derived morphogenetic protein 3 (CDMP3) – and BMP-13 (GDF6, CDMP2) induce expression of elastin and collagen I, resulting in stronger tendons (Forslund and Aspenberg, 2001). They both are present in healthy human Achilles tendons (Chuen *et al.*, 2004; Fu *et al.*, 2003). BMP-14 (GDF5, CDMP1) increases tendon resistance and stiffness, but also induces cartilage formation (Rickert *et al.*, 2001).

BMP-12, together with BMP-2 and BMP-7 – also known as osteogenic protein 1 (OP1) – regulates ingrowth at the enthesis, the anatomical junction of tendon and ligament with bone (Chen *et al.*, 2011; Hashimoto *et al.*, 2007; Kovacevic and Rodeo, 2008; Rodeo *et al.*, 1999). During tendon healing, elevated levels of BMP-12, BMP-13 and BMP-14 are found within the first week of healing, gradually decreasing thereafter (Heisterbach *et al.*, 2012; Würzler-Hauri *et al.*, 2007). As well as promoting tenogenic differentiation of precursors, BMP-12 can accelerate tendon remodelling by increasing the production of type I collagen (Lou *et al.*, 2001) and shifting the cell population from fibroblasts to fibrocytes (Majewski *et al.*, 2008).

TGF $\beta$  stimulates tenocyte migration, mitogenesis and production of extracellular matrix, including collagen types I and III (Kashiwagi *et al.*, 2004; Klein *et al.*, 2002), favouring an early switch from type III to type I collagen (Majewski *et al.*, 2012). TGF $\beta$  is highly activated throughout the whole healing process (Chang *et al.*, 2000; Natsu-ume *et al.*, 1997), with early and late peaks of expression (Heisterbach *et al.*, 2012; Juneja *et al.*, 2013).

Although the effects of single GFs on tendon healing have been investigated, no specific combination of GFs has been studied in detail. In the current study, a combination of bFGF, BMP-12 and TGF $\beta_1$  was explored. This selection combines GFs that collectively are mitogenic, chemotactic, angiogenic and morphogenetic, while stimulating matrix synthesis. All of the three GFs are already tested individually, resulting in modest improvement in tendon repair (Chan *et al.*, 2000; Feng *et al.*, 2007; Forslund and Aspenberg, 2001; Kashiwagi *et al.*, 2004; Klein *et al.*, 2002; Kraus *et al.*, 2016; Lou *et al.*, 2001; Majewski *et al.*, 2008; Majewski *et al.*, 2012; Tang *et al.*, 2008; Wang *et al.*, 2005b).

These GFs were introduced into transected rat Achilles tendons in three different ways. A combined, local injection at the time of surgery was compared to a tiered, sequential, local application and to a type I collagen sponge loaded with the 3 GFs. The sponge was expected to prolong GF-release, as soluble GFs are known for their short half-life and their rapid clearance (Edelman *et al.*, 1993; Wakefield *et al.*, 1990; Wang *et al.*, 2005a). Controls did not receive GFs or were treated with an unloaded collagen sponge, as appropriate. The former group reflected the circumstances of conservative clinical treatment.

## Materials and Methods

### Study design

The study was approved by the institutional Animal Committee (Kantonales Veterinäramt Basel, Switzerland. Number 2378). 200 adult male Sprague Dawley rats, weighing 400–425 g (Harlan, Horst, the Netherlands), were randomly assigned to one of

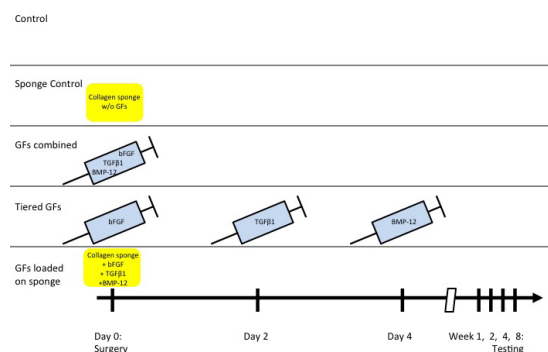
the 5 study and control groups and to one of the 4 time points, as detailed below (Fig. 1). 40 rats were used for each group and 10 tendons were tested 1, 2, 4 and 8 weeks after surgical transection of the right Achilles tendon. 7 tendons were used for biomechanical testing and 3 for histology at each time point. An *a priori* power analysis of the ANOVA for biomechanical testing using G\*Power 3.1 software (Faul *et al.*, 2007) showed a power of 0.78, assuming a large effect size of 0.4 and a significance level  $\alpha = 0.05$ .

### Animal model

Under general anaesthesia (isoflurane), rats were placed prone onto a heated surgery table. Preoperatively, 0.05 mg/kg buprenorphine for analgesia and 20 mg/kg cefazolin for antibiotic prophylaxis were administered subcutaneously. The right leg was shaved, disinfected and covered with surgical drapes, leaving the right limb exposed. For all groups, the skin and, subsequently, the paratenon were incised longitudinally and all fascicles of the Achilles tendon (Szaro *et al.*, 2012) were completely transected perpendicular to the collagen fibres, 5 mm proximal to the calcaneal insertion. The plantar tendon was transected as well, to prevent internal splinting (Fig. 2).

Rats were randomised into the following groups (Fig. 1):

- 1) untreated control;
- 2) sponge control: a type I collagen sponge was placed between the transected tendon ends and the paratenon, secured in place by closing the paratenon with sutures (Müller *et al.*, 2016) (Fig. 2);
- 3) combined GF group: bFGF, BMP-12 and TGF $\beta_1$  were directly applied between the transected tendon ends within the paratenon sheath during surgery (Fig. 2). 100 ng of each GF were used



**Fig. 1.** Experimental design. 200 rats underwent transection of the right Achilles tendon and were randomly assigned to five groups of 40 animals, as indicated. Details of each treatment group are given in the methods section. Animals were euthanised 1, 2, 4 and 8 weeks after surgery and healing assessed by morphometry, mechanical testing and histology.



(Chan *et al.*, 1997; Fu *et al.*, 2003; Kashiwagi *et al.*, 2004) in a volume of 10  $\mu$ L of buffer (see Growth factors);

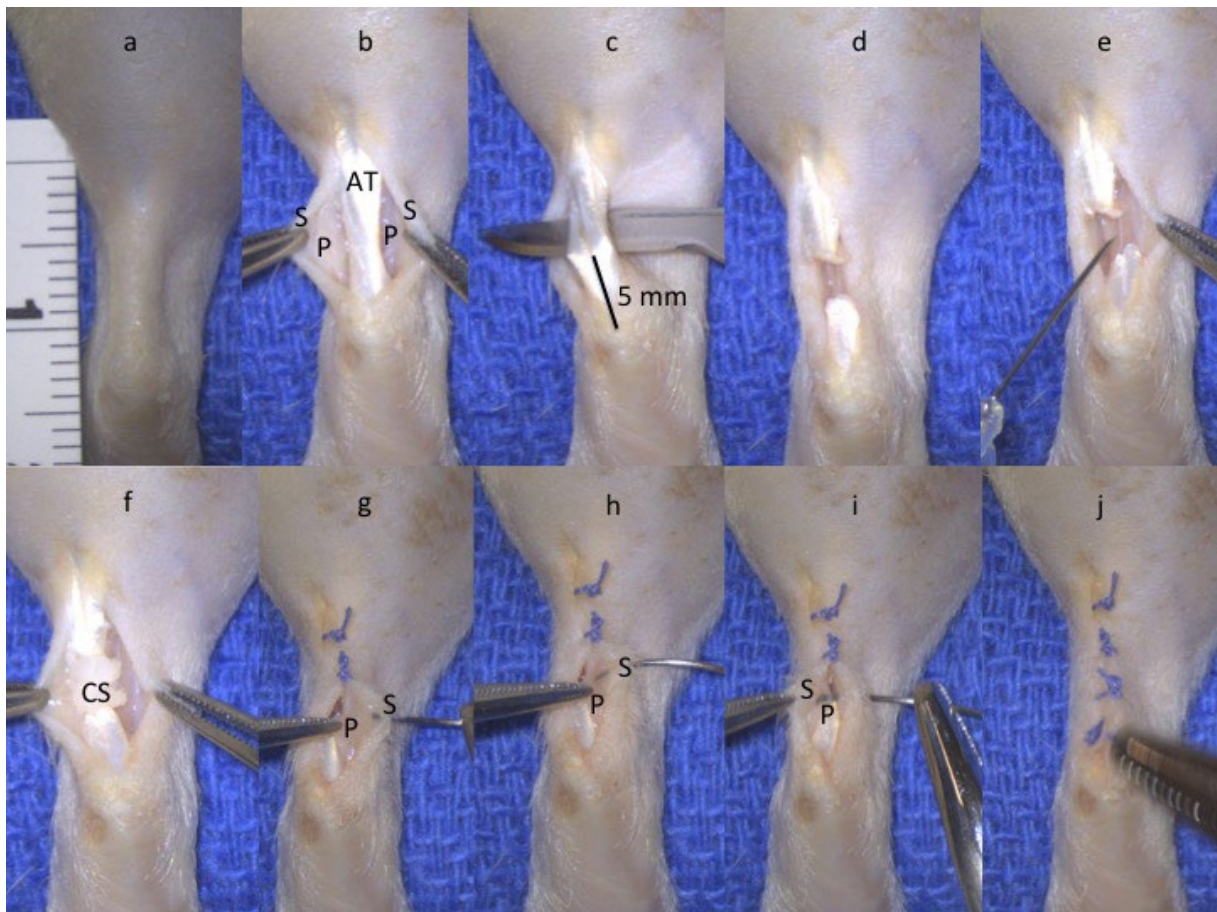
4) tiered GF group: 100 ng of bFGF were applied during surgery into the tendon defect, as bFGF increases cellularity and it is augmented early during natural tendon healing (Freedman *et al.*, 2014; Heisterbach *et al.*, 2012; Würgler-Hauri *et al.*, 2007). 48 h postoperatively, 100 ng of TGF $\beta$ <sub>1</sub> were introduced directly into the tendon gap – marked by the middle skin suture, to facilitate accuracy – by percutaneous injection, to promote an early switch from type III to type I collagen (Majewski *et al.*, 2012). Finally, 100 ng of BMP-12 were applied in the same manner 96 h postoperatively, to accelerate tendon remodelling by increasing type I collagen production (Lou *et al.*, 2001) and shifting the cell population from fibroblasts to fibrocytes (Majewski *et al.*, 2008);

5) sponge GF group: sponges were loaded with 100 ng each of bFGF, BMP-12 and TGF $\beta$ <sub>1</sub>. The

sponge was placed into the tendon defect in the same manner as in the sponge control group.

After tendon treatment, the paratenon, which was not dissected off the skin, was closed together with the skin using Vicryl 4-0 single stitches (Müller *et al.*, 2018) (Fig. 2). The rats recovered on a heated pad postoperatively. No cast immobilisation was applied to the operated legs and the animals were allowed to move within their cages to simulate early functional therapy (Krapf *et al.*, 2012) and to support endogenous GF production (Bring *et al.*, 2010). Animals received 5 more doses of 0.05 mg/kg buprenorphine subcutaneously every 12 h. In the tiered GF group, animals were anaesthetised twice again with isoflurane for percutaneous GF application at 48 and 96 h.

Rats were euthanised with CO<sub>2</sub> after isoflurane anaesthesia. Dorsally, the skin was incised longitudinally along the whole thigh below the knee to the heel and whole muscle-tendon-bone units harvested for testing. Random contralateral Achilles



**Fig. 2.** Surgical procedure. (a) The right leg was shaved and disinfected. (b) About 15 mm of the skin and paratenon were incised longitudinally, whereas the paratenon (P) was not dissected from the skin (S) and the Achilles tendon (AT) was exposed. (c,d) The entire Achilles tendon, including the plantar tendon, was transected perpendicularly, 5 mm proximal to its insertion. Depending on treatment group, either (e) GFs were injected into the defect or (f) the collagen sponge (CS) was placed into the defect. (g-j) Afterwards, the paratenon and the skin were closed with 4 Vicryl 4-0 single stitches. (g) The needle was first passed through the skin (S), (h) then through the paratenon (P) of the same side. (i) Next, the paratenon (P) and skin (S) of the other side were grabbed with the needle and the knot tied. The wound was closed with 4 knots. (j) Knot 2 and 3 marked the centre of tendon defect for later GF injection of the tiered GF group.

tendons were harvested as normal controls (native tendons). Samples for biomechanical testing were immediately wrapped in gauze soaked with saline and frozen at  $-20^{\circ}\text{C}$ . Samples for histology were immediately fixed in 4 % formalin.

### Growth factors

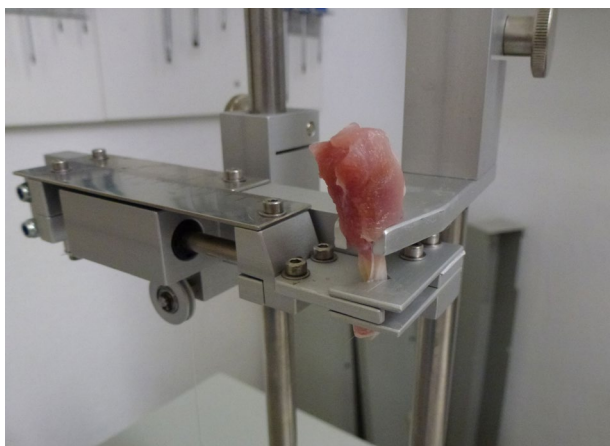
100 ng of each GF (bFGF, BMP-12, TGF $\beta_1$ ; R&D Systems, Abingdon, Oxon, UK), dissolved in 10  $\mu\text{L}$  phosphate buffered saline (PBS) containing 4 mM HCl and 1 mg/mL human serum albumin (Chan *et al.*, 1997; Fu *et al.*, 2003; Kashiwagi *et al.*, 2004), were applied to all treatment groups, either combined, tiered or loaded onto a sponge.

### Sponge preparation

The equine type I collagen sponge (Tachtop, Takeda, Osaka, Japan) used by Müller *et al.* (2016) was aseptically cut into equal pieces of  $5 \times 4 \times 4$  mm, immediately prior to implantation. For the sponge group without GFs, no further preparation was necessary. For sponges loaded with GFs, 100 ng of each GF dissolved in 10  $\mu\text{L}$  of buffer were pipetted onto the sponge.

### Biomechanical testing

All tests were carried out at room temperature ( $23^{\circ}\text{C}$ ). On the day of testing, samples were thawed at room temperature for 4 h and kept moist with saline solution. Before testing, tendon length was measured at the anterior portion of the tendon from the calcaneal insertion to the soleus muscle using a precision calliper (Digital Calliper, Tesa, Lausanne, Switzerland) with an accuracy of 0.01 mm. The cross-sectional area was determined at the healing site using a custom designed gauge, gently creating a square cross section under a defined load of 0.2 N. The diagonal ( $d$ ) of the square was measured at an



**Fig. 3.** Measurement of Achilles tendon cross sectional area. The muscle-tendon-bone unit was held by the muscle so that the healing site of the tendon could be gently formed into a square cross section under a defined load of 0.2 N; the diagonal was measured.

accuracy of 0.05 mm (Fig. 3). The cross-sectional area was calculated as  $d^2/2$ . For biomechanical testing, the specimens were fastened with the calcaneus in a clamp and the muscle in a cryoclamp (Wieloch *et al.*, 2004). Both clamps were attached to a static testing machine (Z010; Zwick, Ulm, Germany). To prevent dehydration, specimens were moistened with Ringer's solution (Braun Medical, Sempach, Switzerland). Just before testing, the muscle was frozen in a cryoclamp with liquid nitrogen. Tendons were not cyclically preconditioned prior to testing because the cryoclamp maintained the testing temperature only for a short time. As soon as the muscle, but not the tendon was frozen, as confirmed manually with a metal needle, testing was started (Majewski *et al.*, 2009). Physiologically, the Achilles tendon was loaded dynamically. Hence, a displacement rate of 1000 mm/min was chosen, which corresponded to a relative strain rate of 100-200 %/s, depending on the tendon length. This variation in strain rate was unlikely to affect the results as a prior study shows that the elastic modulus does not vary at different high strain rates (Ng *et al.*, 2004). Force-displacement curves were constantly recorded for subsequent data analysis. Stiffness in N/mm was calculated from the linear part of the load-elongation and stress-strain curves, respectively. Load to failure in N was defined as the maximum load at failure.

### Histological examination

Histological examination was performed according to an established protocol (Majewski *et al.*, 2009). Tendons were immediately fixed in 4 % buffered formalin (pH 7.4) for 24 h, dehydrated and embedded in paraffin wax. 5  $\mu\text{m}$ -thick longitudinal sections at the mid-substance of the tendon were stained with haematoxylin and eosin (H&E).

Histological analysis was performed by the semi-quantitative score of Bonar, as published by Maffulli *et al.* (2008). Collagen ordering, vascularity, ground substance and tenocyte appearance were evaluated by 2 blinded investigators on a scale from 0 to 3, where 0 indicated normal, 1 slightly abnormal, 2 abnormal and 3 markedly abnormal appearance.

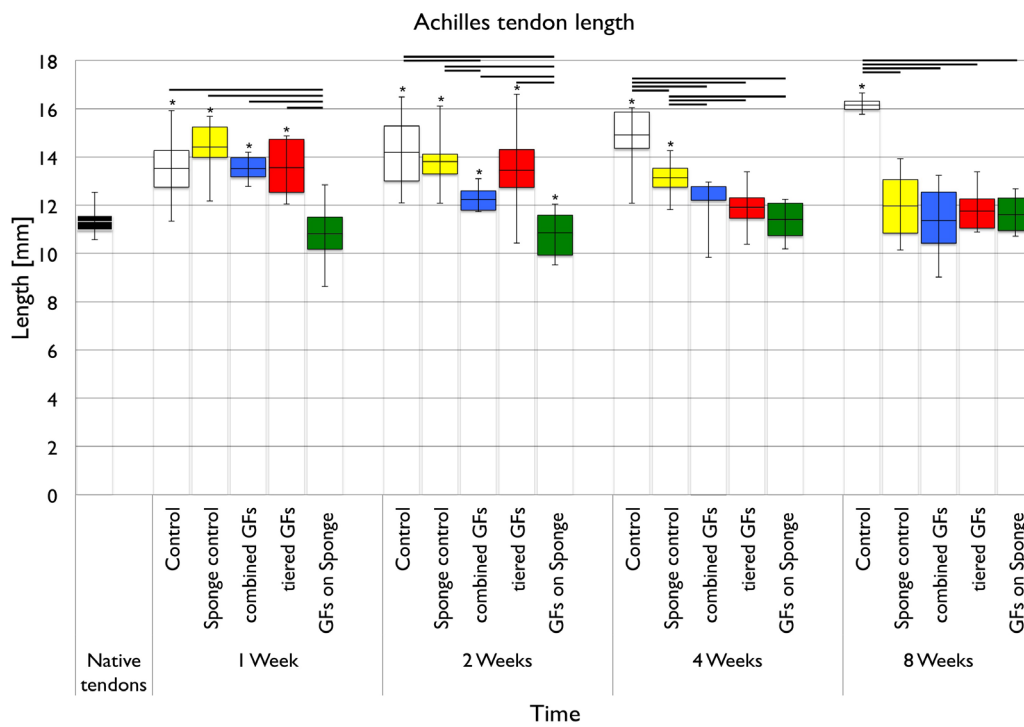
### Statistics

Data concerning tendon length, width and biomechanical properties were used without normalisation. Two-way ANOVA with a Tukey honest significant difference *post-hoc* analysis was performed using the GraphPad Prism 6.0c.205 software. The level of significance was set to  $p \leq 0.05$ .

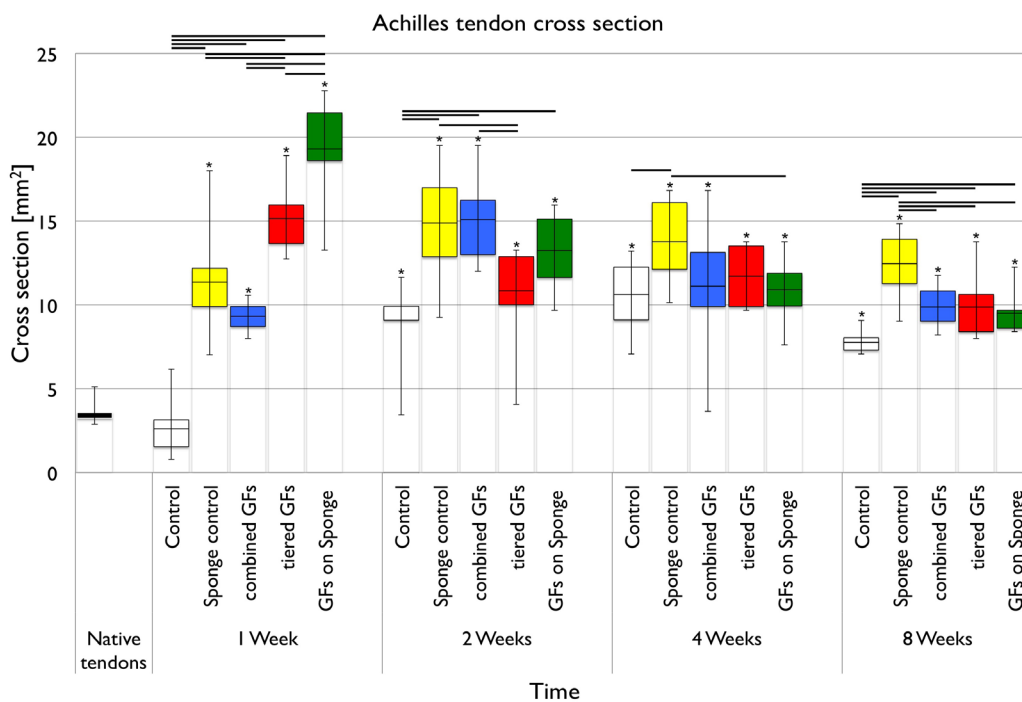
## Results

### Tendon length

After 1 week, tendons showed significant elongation in the control ( $13.5 \pm 1.5$  mm), sponge control ( $14.4 \pm 1.2$  mm), combined GFs ( $13.5 \pm 0.6$  mm) and



**Fig. 4.** Effect of treatments on Achilles tendon length. Values are given in mm (X-axis) according to the treatment group over the healing course of 1, 2, 4 and 8 weeks after tenotomy (Y-axis); boxes range from 1st to 3rd quartile, with whiskers showing minimum and maximum values. The mean value is indicated within each box. The black box on the left shows corresponding native tendons without tenotomy. Statistical significance ( $p \leq 0.05$ ) between experimental groups and native tendons is indicated with an asterisk (\*) and between each experimental group with a horizontal line (—).



**Fig. 5.** Effect of treatments on Achilles tendon cross sectional area. Values are given in mm<sup>2</sup> (X-axis) according to the treatment group over the healing course of 1, 2, 4 and 8 weeks after tenotomy (Y-axis); boxes range from 1st to 3rd quartile, with whiskers showing minimum and maximum values. The mean value is indicated within each box. The black box on the left shows minimum, maximum and mean value of corresponding native tendons without tenotomy. Statistical significance ( $p \leq 0.05$ ) between experimental groups and native tendons is indicated with an asterisk (\*) and between each experimental group with a horizontal line (—).



tiered GFs ( $13.6 \pm 1.2$  mm) groups when compared to native tendons ( $11.3 \pm 0.7$  mm) and GFs on sponge tendons ( $10.8 \pm 1.4$  mm). Uniquely, tendons receiving GFs on collagen sponges were the same length as native tendons for the entire study (Fig. 4).

After 2 weeks, control ( $14.2 \pm 1.7$  mm), sponge control ( $13.8 \pm 1.3$  mm) and tiered GFs tendons ( $13.5 \pm 1.9$  mm) remained significantly longer than native and GFs on sponge tendons ( $10.7 \pm 1.0$  mm). The tendons of the combined GFs group were significantly shorter ( $12.2 \pm 0.6$  mm) than control, sponge control and tiered GFs groups, but still significantly longer than native and GFs on sponge groups.

After 4 weeks, control tendons ( $14.9 \pm 1.7$  mm) were significantly longer than all other groups. Sponge control tendons ( $13.1 \pm 0.8$  mm) were significantly longer than combined GFs ( $12.1 \pm 1.1$  mm), tiered GFs ( $11.9 \pm 0.9$  mm) and GFs on sponge ( $11.4 \pm 0.8$  mm) tendons, which were all in the range of native tendons.

After 8 weeks, all treatment tendons (combined GFs,  $11.4 \pm 1.6$  mm; tiered GFs,  $11.7 \pm 1.0$  mm; GFs on sponge,  $11.6 \pm 0.8$  mm), including sponge control tendons ( $12.0 \pm 1.5$  mm), were in the range of native tendons. However, control tendons continued to elongate with time, reaching  $16.2 \pm 0.3$  mm in length by 8 weeks.

### Tendon cross section

Control tendons ( $2.6 \pm 1.8$  mm<sup>2</sup>) were not significantly thinner than native tendons ( $3.6 \pm 0.7$  mm<sup>2</sup>) after 1 week. However, all the treatment groups showed a significant increase in cross sectional area as compared to native tendons and untreated controls. Sponge control ( $11.4 \pm 3.4$  mm<sup>2</sup>) and combined GFs group ( $9.3 \pm 0.9$  mm<sup>2</sup>) were moderately increased in thickness. However, tendons receiving tiered GFs ( $15.2 \pm 2.1$  mm<sup>2</sup>) or GFs on sponge ( $19.3 \pm 3.2$  mm<sup>2</sup>) were strikingly thicker than the other groups, being approximately five times thicker than native tendons (Fig. 5).

After 2 weeks, all groups were significantly thicker than native tendons. Tendons receiving control sponge ( $14.9 \pm 3.8$  mm<sup>2</sup>), combined GFs ( $15.1 \pm 2.6$  mm<sup>2</sup>) and GFs on sponge ( $13.2 \pm 2.3$  mm<sup>2</sup>) showed significantly larger cross-sectional area than tendons receiving tiered GFs ( $10.9 \pm 3.3$  mm<sup>2</sup>) and control group ( $8.8 \pm 2.6$  mm<sup>2</sup>) (Fig. 5).

After 4 weeks, significantly thicker tendons were found in the sponge control group ( $13.8 \pm 2.6$  mm<sup>2</sup>) when compared with native tendons, control group ( $10.6 \pm 2.3$  mm<sup>2</sup>) and GFs on sponge group ( $10.9 \pm 2.1$  mm<sup>2</sup>). Tendons receiving combined GFs ( $11.1 \pm 4.1$  mm<sup>2</sup>) and tiered GFs ( $11.7 \pm 1.9$  mm<sup>2</sup>) were comparably as thick as the sponge control group, but without statistically significant differences from the other groups (Fig. 5).

After 8 weeks, control tendons were significantly thicker ( $7.8 \pm 0.7$  mm<sup>2</sup>) than native tendons, but significantly thinner than the other groups [sponge

control ( $12.5 \pm 2.1$  mm<sup>2</sup>), combined GFs ( $9.9 \pm 1.3$  mm<sup>2</sup>), tiered GFs ( $9.9 \pm 2.2$  mm<sup>2</sup>) and GFs on sponge ( $9.5 \pm 1.4$  mm<sup>2</sup>)]. Tendons from the sponge control group were significantly thicker than the other groups after 8 weeks, when the thinnest tendons were found in the control group (Fig. 5).

### Tendon load to failure

After 1 week, all treatment groups, including the sponge control group, had significantly higher load to failure values as compared to the control group ( $7.7 \pm 5.0$  N). The greatest load to failure was found for tendons receiving GFs on sponge ( $71.2 \pm 15.0$  N), with values suggesting that the early repair tissue was already approximately 50 % stronger than native tendons ( $52.8 \pm 12.3$  N). Decreasing strength was measured for tendons receiving tiered GFs ( $42.6 \pm 9.3$  N), sponge controls ( $31.3 \pm 6.9$  N) and combined GFs ( $31 \pm 8.9$  N), respectively (Fig. 6).

After 2 weeks, tendons recovered from all groups, including the sponge control group, were significantly stronger than control tendons ( $43.6 \pm 4.3$  N): combined GFs,  $65.6 \pm 7.4$  N; tiered GFs,  $64.0 \pm 18.1$  N; GFs on sponge,  $76.6 \pm 11.2$  N; sponge control,  $62.3 \pm 19.7$  N. Tendons from the combined GFs and GFs on sponge groups were significantly stronger than native tendons, whereas the control tendons were significantly weaker (Fig. 6).

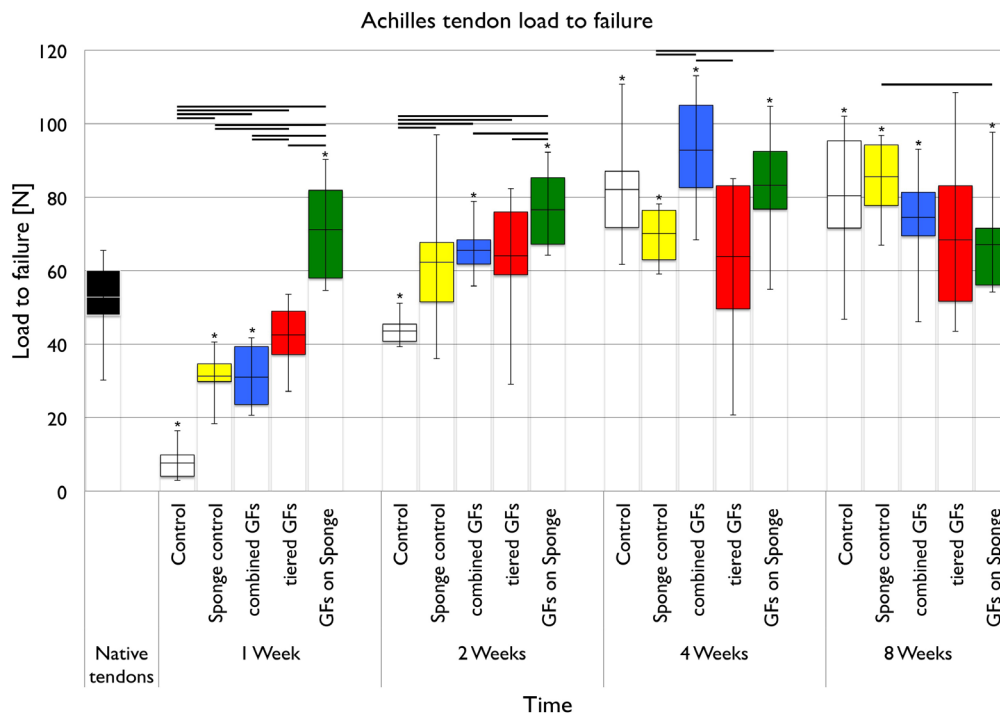
After 4 weeks, tendons from all groups showed higher load to failure than native tendons, which was statistically significant, except for the tiered GFs group ( $63.9 \pm 25.2$  N). No statistical difference was found between the control ( $82.1 \pm 16$  N) and the other groups. Tendons receiving combined GFs ( $92.8 \pm 16.6$  N) were significantly stronger than those receiving sponge controls ( $70.2 \pm 8.1$  N) and tiered GFs. Tendons receiving GFs on sponge ( $83.3 \pm 16.6$  N) were also significantly stronger than those of the sponge control group (Fig. 6).

After 8 weeks, all treated tendons were significantly stronger than native tendons, apart from those receiving tiered GFs ( $68.4 \pm 24.4$  N). Tendons from the sponge control group ( $85.6 \pm 13$  N) were significantly stronger than those from the GFs on sponge group ( $67.1 \pm 15.8$  N). By 8 weeks, all operated tendons, including those of the controls, had higher load to failure values than native tendons (Fig. 6).

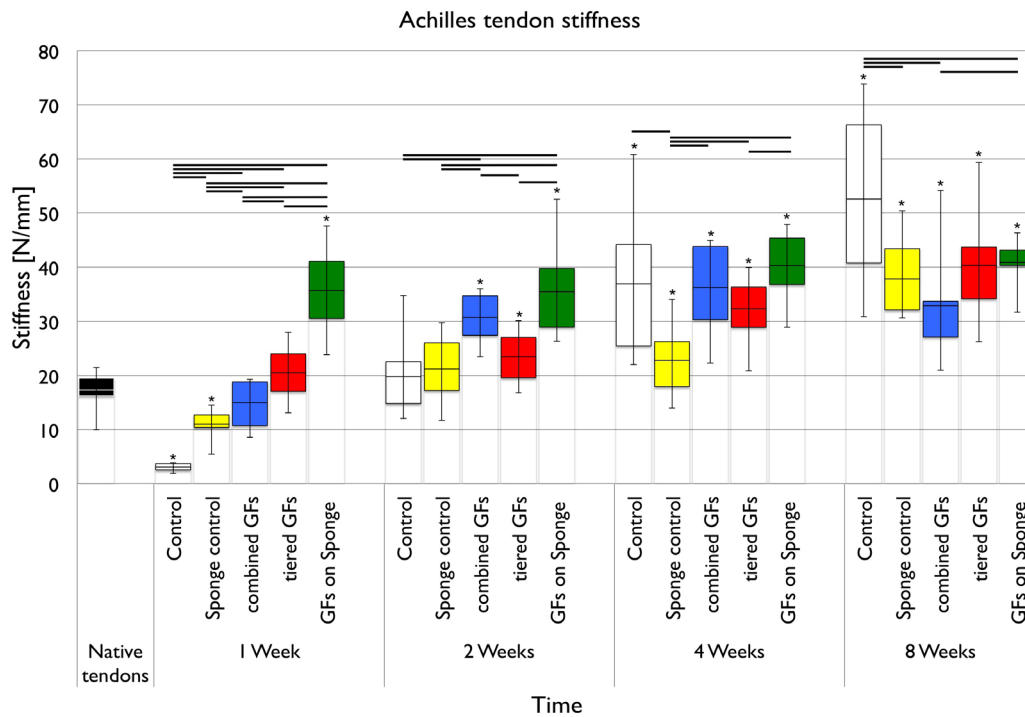
Interestingly, at no time point did defects receiving GFs on sponge break at the repair site. Instead, they failed distally at the transition of the middle to the distal third of the tendon. In contrast, control and sponge control tendons failed at the repair site after 1 and 2 weeks, as did tendons receiving the combined and tiered GFs after 1 week. This finding was remarkable as early failure stress was comparable for all groups, indicating the strong tendon callus of the GFs on sponge group.

### Tendon stiffness

A comparable pattern to tendon load to failure was found for tendon stiffness. After 1 week, the highest

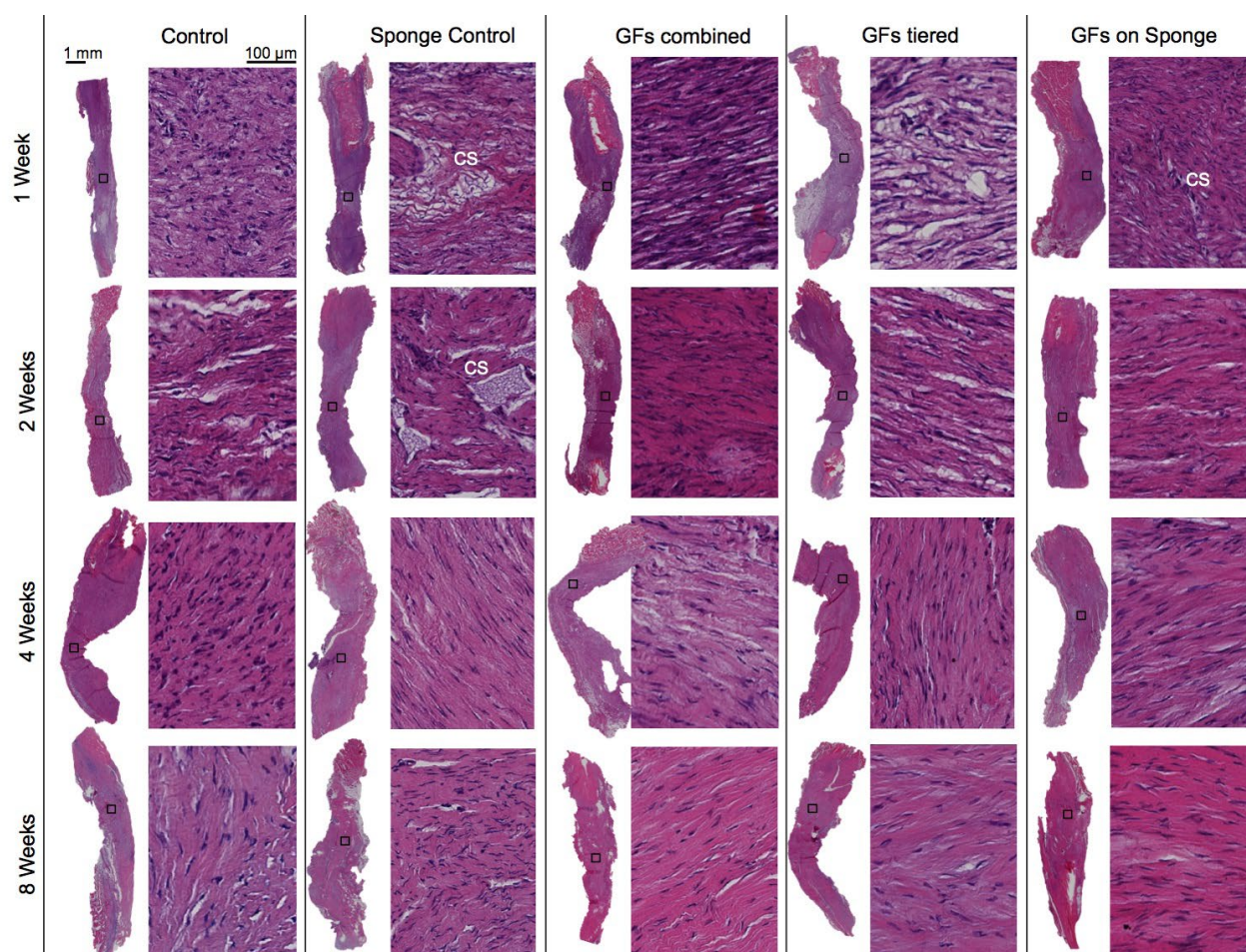


**Fig. 6.** Effect of treatments on Achilles tendon strength. Load to failure [N] (X-axis) is shown according to the treatment group over the healing course of 1, 2, 4 and 8 weeks after tenotomy (Y-axis); boxes range from 1st to 3rd quartile, with whiskers showing minimum and maximum values. The mean value is indicated within each box. The black box on the left shows minimum, maximum and mean value of corresponding native tendons without tenotomy. Statistical significance ( $p \leq 0.05$ ) between experimental groups and native tendons is indicated with an asterisk (\*) and between each experimental group with a horizontal line (—).



**Fig. 7.** Effect of treatments on Achilles tendon stiffness. Values are given in N/mm (X-axis) according to the treatment group over the healing course of 1, 2, 4, and 8 weeks after tenotomy (Y-axis); boxes range from 1st to 3rd quartile, with whiskers showing minimum and maximum values. The mean value is indicated within each box. The black box on the left shows minimum, maximum and mean value of corresponding native tendons without tenotomy. Statistical significance ( $p \leq 0.05$ ) between experimental groups and native tendons is indicated with an asterisk (\*) and between each experimental group with a horizontal line (—).





**Fig. 8.** Histological assessment. Haematoxylin and eosin staining is shown for all groups at 1, 2, 4 and 8 weeks. For each group, a longitudinal section (4×) of an entire tendon is shown on the left-hand side. On the right hand-side, a representative 20× image of the healing tissue is illustrated. For each condition, the detail image corresponds to the black box in the entire tendon image. Remnants of the collagen sponge are indicated with CS for collagen sponge groups at 1 and 2 weeks and GFs on sponge group at 1 week. Thereafter no collagen sponge was found on histology.

stiffness was measured for tendons receiving GFs on sponge ( $35.7 \pm 8.5$  N/mm) and the lowest for the control group ( $3.1 \pm 0.8$  N/mm) (Fig. 7).

After 2 weeks, the highest values were found for tendons receiving GFs on sponge ( $35.5 \pm 9.5$  N/mm) and the lowest for the control tendons ( $19.8 \pm 8.1$  N/mm). However, the stiffness increased for control tendons over time up to the highest values ( $57.2 \pm 16.3$  N/mm) of any group after 8 weeks. At this time, the stiffness of tendons receiving GFs loaded on sponge had remained constant ( $40.9 \pm 4.7$  N/mm). Tendons of all other treatment groups, including the sponge control group, remained less stiff than those receiving GFs on sponge (Fig. 7).

### Histology

Histological examination of tendons showed heterogeneous patterns of collagen fibres in all groups at all time points, with earlier fibre growth, bigger collagen bundles and higher degree of collagen crimp after 1, 2 and 4 weeks in tendons receiving GFs (Fig. 8). Moreover, an earlier shift

from fibroblasts to fibrocytes was observed within the tendons receiving the GFs. Collagen appearance was the highest after 8 weeks and all groups showed a similar histological examination with a slightly more tendinous appearance, thicker collagen bundles and more fibre-oriented cells in tendons receiving GFs loaded on sponge. However, some fibro-cartilaginous and endochondral formations were found in each group at this time. No signs of inflammation were seen within any group at any time point.

Overall, the mean histological Bonar score showed slightly better results in tendons from the GF loaded sponge group after 1, 2 and 8 weeks, compared to all the other groups. After 4 weeks, the Bonar score was slightly better for tendons from the tiered GFs group. Interestingly, the Bonar score for tendons recovered from the collagen sponge group was in the range of the treatment groups after 4 and 8 weeks, differently from the control group. The injectable GF treatment resulted in a higher vascularity after 1 week and early tenocyte appearance was better in the GF groups (Tables 1-5).

**Table 1.** Overall Bonar score for healing tendons in each group. Mean Bonar score and standard deviation (SD) for collagen ordering, vascularity, ground substance and tenocyte appearance from 0-3, whereas 0 indicates normal, 1 slightly abnormal, 2 abnormal and 3 markedly abnormal appearance.

Bonar score	1 week	2 weeks	4 weeks	8 weeks
Control	2.5 ± 0.4	2.3 ± 0.5	2.0 ± 0.5	1.7 ± 0.4
Sponge control without GFs	2.3 ± 0.2	2.2 ± 0.5	1.7 ± 0.4	1.5 ± 0.3
Combined GFs (day 0)	2.3 ± 0.5	2.1 ± 0.5	1.8 ± 0.4	1.5 ± 0.5
Tiered GFs (day 0, 2, 4)	2.2 ± 0.4	2.1 ± 0.4	1.5 ± 0.5	1.5 ± 0.5
GFs loaded on sponge	2.1 ± 0.4	2.0 ± 0.4	1.6 ± 0.6	1.4 ± 0.5

**Table 2.** Bonar score for collagen ordering of healing tendons in each group. Mean Bonar score and SD for collagen ordering of healing tendons from 0-3, whereas 0 indicates normal, 1 slightly abnormal, 2 abnormal and 3 markedly abnormal appearance.

Bonar score	1 week	2 weeks	4 weeks	8 weeks
Control	3.0 ± 0	2.7 ± 0.5	2.3 ± 0.5	2.0 ± 0
Sponge control without GFs	3.0 ± 0	2.3 ± 0.5	2.0 ± 0	2.0 ± 0
Combined GFs (day 0)	2.7 ± 0.5	2.3 ± 0.5	1.8 ± 0.4	1.5 ± 0.5
Tiered GFs (day 0, 2, 4)	2.7 ± 0.5	2.2 ± 0.5	1.7 ± 0.4	1.5 ± 0.5
GFs loaded on sponge	2.7 ± 0.5	2.3 ± 0.5	1.8 ± 0.4	1.5 ± 0.5

**Table 3.** Bonar score for vascularity of healing tendons in each group. Mean Bonar score and SD for vascularity of healing tendons from 0-3, whereas 0 indicates normal, 1 slightly abnormal, 2 abnormal and 3 markedly abnormal appearance.

Bonar score	1 week	2 weeks	4 weeks	8 weeks
Control	1.7 ± 0.5	1.7 ± 0.5	1.7 ± 0.5	1.7 ± 0.5
Sponge control without GFs	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.5
Combined GFs (day 0)	2.3 ± 0.5	1.7 ± 0.5	1.7 ± 0.5	1.7 ± 0.5
Tiered GFs (day 0, 2, 4)	2.3 ± 0.5	2.0 ± 0.0	1.7 ± 0.5	1.7 ± 0.5
GFs loaded on sponge	1.7 ± 0.5	1.7 ± 0.5	1.3 ± 0.5	1.3 ± 0.5

**Table 4.** Bonar score for ground substance of healing tendons in each group. Mean Bonar score and SD for ground substance of healing tendons from 0-3, whereas 0 indicates normal, 1 slightly abnormal, 2 abnormal and 3 markedly abnormal appearance.

Bonar score	1 week	2 weeks	4 weeks	8 weeks
Control	2.5 ± 0.5	2.3 ± 0.5	2.0 ± 0.6	1.5 ± 0.5
Sponge control without GFs	2.0 ± 0	2.3 ± 0.5	1.7 ± 0.7	1.2 ± 0.4
Combined GFs (day 0)	2.2 ± 0.4	2.3 ± 0.5	1.7 ± 0.5	1.2 ± 0.7
Tiered GFs (day 0, 2, 4)	1.7 ± 0.5	2.2 ± 0.4	1.3 ± 0.5	1.3 ± 0.5
GFs loaded on sponge	1.8 ± 0.4	2.0 ± 0	1.3 ± 0.7	1.3 ± 0.5

**Table 5.** Bonar score for tenocyte appearance of healing tendons in each group. Mean Bonar score and SD for tenocyte appearance of healing tendons from 0-3, whereas 0 indicates normal, 1 slightly abnormal, 2 abnormal and 3 markedly abnormal appearance.

Bonar score	1 week	2 weeks	4 weeks	8 weeks
Control	2.7 ± 0.5	2.7 ± 0.5	1.8 ± 0.5	1.5 ± 0.5
Sponge control without GFs	2.7 ± 0.5	2.7 ± 0.5	1.7 ± 0.5	1.3 ± 0.5
Combined GFs (day 0)	1.8 ± 0.7	2.0 ± 0.6	1.8 ± 0.4	1.7 ± 0.5
Tiered GFs (day 0, 2, 4)	2.2 ± 0.4	2.2 ± 0.4	1.5 ± 0.5	1.5 ± 0.5
GFs loaded on sponge	2.3 ± 0.4	2.0 ± 0.4	1.7 ± 0.5	1.5 ± 0.5



## Discussion

Much evidence supports the use of GFs to enhance tendon healing (Docheva *et al.*, 2015; Evans, 1999; Molloy *et al.*, 2003; Sharma and Maffulli, 2006), but numerous issues remain to be resolved, including which GFs to use and when and how to deliver them. Single GFs provide little benefit or only incremental improvement to tendon healing *in vitro* and in animal models (Chan *et al.*, 2000; Feng *et al.*, 2007; Forslund and Aspenberg, 2001; Gelberman *et al.*, 2016; Kashiwagi *et al.*, 2004; Klein *et al.*, 2002; Kraus *et al.*, 2016; Kraus *et al.*, 2014; Majewski *et al.*, 2008; Majewski *et al.*, 2012; Tang *et al.*, 2008; Wang *et al.*, 2005b), suggesting that mixtures of different GFs may be necessary to modulate the complex process of tendon healing in a substantial manner. This may explain why ACS and PRP show some ability to improve tendon healing (Aspenberg and Virchenko, 2004; Bosch *et al.*, 2010; de Almeida *et al.*, 2012; Gosens *et al.*, 2012; Majewski *et al.*, 2009; Parafioriti *et al.*, 2011; Schnabel *et al.*, 2007; Seijas *et al.*, 2013). However, a single blood product administration may not be as effective as repetitive injections (Parafioriti *et al.*, 2011). In the current study three factors were evaluated: bFGF, BMP-12 and TGF $\beta_1$  promote different stages of tendon healing and show promising results in animal models when delivered singly (Chan *et al.*, 2000; Feng *et al.*, 2007; Majewski *et al.*, 2008; Majewski *et al.*, 2012; Tang *et al.*, 2008). These GFs were delivered in a single dose immediately after transection of the Achilles tendon or in a sequential, tiered fashion at times corresponding to predicted physiological needs. Injection of GFs into the defect in solution or associated with a collagen sponge was also compared.

In the current rat model, the transected Achilles tendon healed spontaneously, similarly to most human Achilles tendon ruptures. However, conservative treatment in humans, unlike rats, requires adequate immobilisation, physical therapy and appropriate loading of the healing tissue according to the phase of healing (Kadokia *et al.*, 2017). The goals of any therapeutic intervention are to accelerate the recovery of full mechanical strength and provide a regenerate that is as close as possible to the original, uninjured tendon. The latter includes not only restoration of biological structure, but also restoration of the original tendon length: if the repaired tendon is too long, it cannot transmit load efficiently. Tendon elongation is a clinical problem when treating Achilles tendon ruptures conservatively (Cetti *et al.*, 1994; Krapf *et al.*, 2012; Metz *et al.*, 2008).

Bearing these outcome measures in mind, the present results showed convincingly that delivering the 3 GFs on a collagen sponge gave superior results. Even though the animal model used lacked the early functional therapy administered to human patients and conservatively treated rat Achilles tendons tend to elongate (Krapf *et al.*, 2012), it is remarkable that

treating the tendons with the 3 GFs loaded on a sponge ensured normal tendon length throughout the entire healing process; something no other treatment achieved. However, Achilles tendons of the other GF groups and the sponge control group remodelled as healing progressed, eventually achieving the same length as the GF on sponge group and native tendons.

Within 1 week, defects receiving the GFs delivered on a collagen sponge were already stronger and stiffer than normal tendons, whereas those of all other groups, except tiered delivery, were substantially weaker. This rapid recovery of mechanical strength was accompanied by a dramatic, 5-fold increase in cross sectional area, exceeding that of all other groups. Part of this could have been due to the presence of the sponge itself in the groups receiving sponges. Nevertheless, most of the increase was probably related more to the increase in the formation of healing tissue than the collagen sponge itself, as the sponge was resorbed after about 2 weeks in both sponge groups. However, it needs to be pointed out that the aim of tendon engineering is to come as close as possible to normal tissue and a 5-fold increase in cross sectional area is abnormal. However, this massive tendon callus formed in the GFs on sponge group remodelled during subsequent healing and by 8 weeks was comparable to the other GF groups, apart from the sponge control group, but still larger than control tendons.

The superior properties of the GFs applied to the collagen sponge could reflect binding and slow release of the GFs by the sponge. In addition, the sponge alone can stimulate a certain amount of regeneration (Müller *et al.*, 2016), suggesting a possible biological synergy between the sponge and the GFs. Further research is necessary to tease out these potential interactions.

Other approaches use nanostructural coatings to prolong the release of bFGF and VEGF for several weeks (Yu *et al.*, 2017) or exploit new protocols to decellularise bovine Achilles tendons for use as scaffolds retaining high concentrations of bFGF and TGF $\beta_1$  (Ning *et al.*, 2017). These developments indicate current interest in combining GFs and extending their release profiles.

Because implantation of a collagen sponge requires surgery, there is interest in delivering agents by percutaneous injection. Of relevance to this mode of delivery, the soluble GFs also improved tendon healing, although less dramatically than when delivered on a collagen sponge. They reversed the elongation of the regenerating tendon, improved its mechanical strength and stiffness, increased its cross-sectional area and improved its histological appearance. Only minor differences in efficacy were noted between tiered and simultaneous delivery of the GFs. The ability of GFs to improve healing when delivered percutaneously is important, given the high re-rupture rates of Achilles tendons treated conservatively (Bhandari *et al.*, 2002; Ingvar *et al.*,



2005; Khan and Carey Smith, 2010; Majewski *et al.*, 2002; Pajala *et al.*, 2002).

Clinically, the rapid increase in mechanical strength of tendons receiving GFs on collagen sponges could translate into earlier load-bearing and rehabilitation. This, in turn, could accelerate maturation and remodelling of the regenerate that, by 8 weeks, was still histologically immature.

Future experiments will determine whether normal dimensions, mechanics and histology would be restored in rats maintained beyond the 8-week duration of this study. Additional studies are required to determine whether the approaches studied here for tendon-to-tendon healing also apply to tendon-to-bone healing.

This study had the following limitations. In the tiered group, the second and third injections were made percutaneously in between the two centre stitches in the line of the Achilles tendon. However, it was impossible to guarantee a correct placement of the tip of the needle into the healing zone, for which another incision and surgical dissection or ultrasound guidance would have been needed. The fact that the results for the tiered group were better than those for the combined GF group suggested that correct positioning of the needle was achieved.

Another limitation was that no release profiles of the GFs were obtained and it is possible that the GFs were quickly released into the surrounding tissues. However, given the improved healing resulting from the GF administration, it is likely that sufficient GF persisted to influence healing. The choice of the GFs was based upon the results of previous studies (Heisterbach *et al.*, 2012; Würzler-Hauri *et al.*, 2007) and the doses of the GFs used were chosen from the literature (Chan *et al.*, 1997; Fu *et al.*, 2003; Kashiwagi *et al.*, 2004). This left open the possibility that there might be better combinations and dosages of GFs than the one tested in this study.

The formation of adhesions was not addressed, although this is a major aspect to consider for intra-synovial tendon repair. Adhesions of the healing tendon with the surrounding tissue were noted for all groups, typically at 1 week, after which they disappeared. Overall, by the end of the study at 8 weeks, the adhesions had remodelled in all groups into a smooth adjacent soft tissue. This may also be due to the fact that the animals were allowed to move around, simulating functional therapy (Krapf *et al.*, 2012), to reduce the formation of adhesions (Cetti *et al.*, 1994; Dugan and Hobler, 1994; Mandelbaum *et al.*, 1995; Rettig *et al.*, 2005; Soma and Mandelbaum, 1995; van der Eng *et al.*, 2013).

The main outcome parameters of this study were morphometry, mechanical testing and histology. Molecular or biochemical characterisation of the repair tissue was not attempted, although this would be an interesting goal for a future study.

The GF treatment used did not prevent the formation of endochondral ossification, which was

seen in all groups after 8 weeks. Ossification is a well-known phenomenon during rat Achilles tendon healing (Hsieh *et al.*, 2016a; Hsieh *et al.*, 2016b; Rooney *et al.*, 1992); preventing it was not a primary goal of this study.

## Conclusion

Combined treatment with the three GFs (bFGF, BMP-12 and TGF $\beta_1$ ) accelerated and improved tendon healing, leading to regenerates that were shorter, stronger and histologically superior to those not receiving GFs. The most consistent results were obtained when GFs were loaded onto a collagen sponge that was inserted into the defect, possibly because of sustained GF release. The most impressive improvement of tendon properties was found during the early tendon healing (1 and 2 weeks), which can be interpreted as the early rehabilitation phase of a patient. This is highly important clinically, because most re-tears occur during this period. Thus, a collagen sponge loaded with these GFs could be a promising device in the operative treatment of high-performance athletes, high-risk patients with tendon defects or revision cases, where it is particularly necessary to prevent re-ruptures.

In cases of conservative treatment, a percutaneous GF application could be a reliable option to improve the clinical outcome. Despite the advantages of the tiered GFs group, a single combined GF application with bFGF, BMP-12 and TGF $\beta_1$  could be considered to reduce the number of injections, with only slightly inferior results regarding tendon properties. Further clinical studies will be needed to determine the best modality of administration.

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## Discussion with Reviewers

**Denitsa Docheva:** What are the next steps for improving the combined delivery of growth factors? What other combination of growth factors might be superior to the one evaluated in this model?

**Authors:** The next step could be the characterisation of the mechanisms of action of these GFs in combination with the collagen sponge. A variety of molecular, immunohistochemical and cellular techniques could be brought to bear on this matter. Because this study only used one dose of each GF, there is scope for dose-optimisation. Then, a well-characterised, optimised system could advance for testing in large animals prior to clinical trials.

**Denitsa Docheva:** Frequent research and clinical problems during the repair process of an injured tendon are endochondral ossification and vascular sprouting. Previous research based on rat models of tendon injury, as well as this manuscript, demonstrate in-tendinous ossification. Was the rate of ossification different among the experimental groups in this study? Could the authors consider further optimisation of the collagen sponge with relevant inhibitors?

**Authors:** The rate of ossifications was comparable for all groups after 8 weeks. New strategies using stem cells (periodontal ligament cells or scleraxis-programmed tendon progenitor cells) show reduced ossifications during rat Achilles tendon healing (Hsieh *et al.*, 2016a; Hsieh *et al.*, 2016b). Hence, further studies testing stem cells in combination with the presented model would be promising.

**Arianna Lovati:** Regarding the surgical procedure, in the absence of any cast or internal splint, why did the authors not perform a tenorrhaphy? What is the rationale for this choice?

**Authors:** After repairing the Achilles tendon transection with a Kessler-Kirchmeyer stitch, the biomechanical properties are dominated by the sutures used for tendon repair rather than the properties of the repair tissue (Majewski *et al.*, 2008; Majewski *et al.*, 2009; Majewski *et al.*, 2012). Therefore, surgical and non-surgical treatment were compared in this model (Krapf *et al.*, 2012), showing superior mechanical and histological findings for the operated and conservative groups, respectively, within the first 2 weeks. However, significant tendon elongation was found for the conservative group. To test the effect of the GFs alone, without the confounding intervention of surgical repair, the tendon transection was not repaired. This strategy is used in a previous study as to treating the defect with a collagen sponge only (Müller *et al.*, 2016).

**Arianna Lovati:** Without any stabilisation, it is intuitive that the load-bearing onto the operated limb was affected, thus altering the biomechanical response. Did the authors consider this important issue in the biomechanical data?

**Authors:** This is an interesting point since mechanical load is needed for healing. In a comparable transection model in rats, Korntner *et al.* (2017; additional reference) show that there is indeed decreased load on the operated foot after 1 week. Interestingly, weight-bearing onto the operated foot increases markedly by 2 weeks. This agrees with our own observations of the animals, which only limped for a limited time.

**Arianna Lovati:** A concern related to the proposed surgical model is the unavoidable sliding of the tendon stumps, thus determining the elongation of

the structure itself. What was the authors' strategy to prevent these occurrences?

**Authors:** Sliding of the tendon stumps was definitively a disadvantage of the model and needs to be avoided in the treatment of patients. In a study comparing surgical treatment and conservative treatment of transected rat Achilles tendons, significant elongation of tendons is found in the conservatively treated group (Krapf *et al.*, 2012). Very interestingly, transected tendons in the conservative control group of the current study increased in length over time, as in Krapf *et al.* (2012), and were significantly longer than those of all other groups after 8 weeks. In contrast, all tendon treatment groups, including the sponge control group, were of normal length by 8 weeks.

**Arianna Lovati:** Did the authors consider a potential migration of the collagen sponge in the absence of a fixation system?

**Authors:** No migration of the collagen sponge was observed in this study or in a previous study testing the effects of the collagen sponge only (Müller *et al.*, 2016).

#### Additional Reference

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**Editor's note:** The Scientific Editor responsible for this paper was Juerg Gasser.