Local variations in mechanical properties of human hamstring tendon autografts for anterior cruciate ligament reconstruction do not translate to a mechanically inferior strand

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Local variations in mechanical properties of human hamstring tendon autografts for anterior cruciate ligament reconstruction do not translate to a mechanically inferior strand

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ABSTRACT

A ruptured anterior cruciate ligament (ACL) is often reconstructed with a multiple-strand autograft of a semitendinosus tendon alone or combined with a gracilis tendon. Up to 10% of patients experience graft rupture. This potentially results from excessive local tissue strains under physiological loading which could either result in direct mechanical failure of the graft or induce mechanobiological weakening. Since the original location in the hamstring tendon cannot be traced back from an autograft rupture site, this study explored whether clinical outcome could be further improved by avoiding specific locations or regions of human semitendinosus and/or gracilis tendons in ACL grafts due to potential mechanical or biochemical inferiority. Additionally, it examined numerically which clinically relevant graft configurations experience the lowest strains – and therefore the lowest rupture risk – when loaded with equal force.

Remnant full-length gracilis tendons from human ACL reconstructions and full-length semitendinosus- and ipsilateral gracilis tendons of human cadaveric specimens were subjected to a stress-relaxation test. Locations at high risk of mechanical failure were identified using particle tracking to calculate local axial strains. As biochemical properties, the water-, collagen-, glycosaminoglycan- and DNA content per tissue region (representing graft strands) were determined. A viscoelastic lumped parameter model per tendon region was calculated. These models were applied in clinically relevant virtual graft configurations, which were exposed to physiological loading. Configurations that provided lower stiffness – i.e., experiencing higher strains under equal force – were assumed to be at higher risk of failure. Suitability of the gracilis tendon proper to replace semitendinosus tendon-muscle-tendon junction strands was examined.

Deviations in local axial strains from the globally applied strain were of similar magnitude as the applied strain. Locations of maximum strains were uniformly distributed over tendon lengths. Biochemical compositions varied between tissue regions, but no trends were detected. Viscoelastic parameters were not significantly different between regions within a tendon, although semitendinosus tendons were stiffer than gracilis tendons. Virtual grafts with a full-length semitendinosus tendon alone or combined with a gracilis tendon displayed the lowest strains, whereas strains increased when gracilis tendon strands were tested for their suitability to replace semitendinosus muscle-tendon junction strands.

Locations experiencing high local axial strains - which could increase risk of rupture - were present, but no specific region within any of the investigated graft configurations was found to be mechanically or biochemically deviant. Consequently, no specific tendon region could be indicated to provide a higher risk of rupture for mechanical or biochemical reasons. The semitendinosus tendon provided superior stiffness to a graft compared to...
1. Introduction

With an annual occurrence of approximately 7 per 10,000 people, rupture of the anterior cruciate ligament (ACL) is one of the most frequent knee injuries [Sanders et al., 2016]. Due to the non-self-healing properties of the ACL [Vakken and Murray, 2011], a surgical ACL reconstruction is often required to regain knee stability and return to sports [Lai et al., 2018]. The most commonly used graft types in this procedure are the patellar tendon graft - which was outside the scope of this research - and the hamstring tendon autograft, consisting of a semitendinosus tendon (ST) either solely or in combination with a gracilis tendon (GR) [Cerulli et al., 2013]. The tendons are characterized by a highly hierarchical tissue structure: they are composed of fascicles, which contain fibroblast-like cells and collagen fibrils. The fibrils in turn consist of assembled collagen molecules, and are predominantly oriented in the main loading direction [Snedeker and Foolen, 2017]. Within a full-length tendon, the muscle-tendon junction (MTJ), tendon proper and bone-tendon junction (BTJ) regions are distinguished [Snedeker and Foolen, 2017]. Maximum length of the MTJ and tendon proper - as close to the BTJ as possible - are included in the graft used for ACL reconstruction, in order to obtain the required graft length and diameter [Abramowitch et al., 2010]. Graft length should be sufficient to span the intra-articular space and enable stable fixation [Goyal et al., 2010], while graft diameters of 8 mm or higher decrease the failure rate after ACL reconstruction [Conte et al., 2014]. To achieve these suitable graft dimensions, constructs can be looped in various ways. This results in grafts with three to eight strands, consisting of ST or a combination of ST and GR tissue [Vinagre et al., 2017]. The primary demand of ACL grafts is sufficient stiffness to withstand the physiological tensile forces the native ACL is exposed to, without rupturing or over-straining [Escamilla et al., 2012; Shelburne et al., 2004]. However, despite meeting these requirements in the widespread use of hamstring tendons for ACL reconstruction and leading to overall acceptable clinical results, the re-rupture rate after ACL reconstruction is up to 10% [Löcherbach et al., 2010].

Autograft rupture after reconstruction is most frequently reported near the femoral insertion site, possibly as a result of intercondylar notch impingement [Magnussen et al., 2012; Van Eck et al., 2011]. However, much is still unknown about the graft failure mechanism and its relation to the tendon tissue properties, also considering that the rupture site cannot easily be traced back to the original hamstring tendon region or location [Dustmann et al., 2008]. Various factors in the tendon structure have been proposed to be involved in autograft failure. From a mechanical perspective, failure of single collagen fibrils in tendon tissue can occur when tissue loading induces local axial strains exceeding 4% [Robi et al., 2013]. Consequently, this can accumulate into plastic deformation and ultimately complete tissue rupture at local axial strains of 8–10% [Robi et al., 2013]. However, from a mechanobiological perspective, the failure of single collagen fibrils can already increase the load experienced by resident cells, alter cellular properties, and promote local degeneration of the extracellular matrix [Robi et al., 2013]. Specifically for the ACL autograft, this process - initiated by the high local axial strains - may compromise ligamentization and adaptation of the tendon tissue to its new environment, and consequently increase the risk of graft rupture [Janssen et al., 2011; Janssen and Scheffer, 2018]. Although in both mechanisms the local axial strain under tissue loading is a key component, available literature regarding variation in local axial strains within (hamstring) tendons and whether high axial strains can be localized to a specific tissue region is scarce [Khodabakhshi et al., 2013; Screen et al., 2004]. However, such a region could be the weakest link in an ACL autograft and thereby result in increased risk of graft rupture. Low-stiffness tissue locations will strain more compared to other locations within the same strain, are therefore more likely to experience the critical strains aforementioned under mechanical loading, and contribute less to withstanding the load applied to the entire graft. Consequently, the load on the higher-stiffness strands increases, as well as (local) tissue strains throughout the entire graft, and the risk of graft rupture. Besides that, a large plastic deformation component in the (local) axial strains amplifies the risk of excessive joint laxity and instability [Siebold et al., 2011]. Consequently, altered joint loading can increase the risk of osteoarthritis development [Friel and Chu, 2013]. Altogether, local and regional mechanical properties of ST and GR tissue are relevant factors for the short- and long-term clinical outcome of surgical ACL reconstruction with a hamstring tendon autograft.

Regional variability in viscoelastic mechanical properties within one tendon and between the ST and GR have been reported [Abramowitch et al., 2010; Arruda et al., 2006; Smeets et al., 2017], where the MTJ is mostly considered to have the lowest stiffness of the distinct tissue regions [Arruda et al., 2006]. Particularly from the mechanobiological perspective, the biochemical composition of a tissue region is of interest, since the types of extracellular matrix components affect how globally applied loads are sensed locally by resident cells [Ghosh and Ingber, 2007; Ladoux and Nicolas, 2012]. Thus far, regional differences in biochemical composition have not been thoroughly investigated and quantified. With availability of the GR alongside with the ST comes the opportunity to combine the mechanically most suitable tissue regions of both tendons into a graft, such that it can withstand the physiological tensile forces on the native ACL without over-straining or rupturing [Escamilla et al., 2012; Shelburne et al., 2004]. Despite the reported regional mechanical variations within tendons [Abramowitch et al., 2010; Arruda et al., 2006; Smeets et al., 2017], this has not been translated to a thorough investigation on whether the global mechanical properties of an ACL graft can be improved by only combining the potentially mechanically most suitable ST and GR regions. The graft strands containing MTJ tissue were expected to display different viscoelastic behavior – and possibly different biochemical composition – compared with strands consisting of tendon proper tissue, potentially making the MTJ-side less suitable for its new mechanical function. This is based on the MTJ tissue to have a different morphology compared to the tendon proper, i.e., it appears as more sheet like [Snedeker and Foolen, 2017]. Second, in processing a tendon into a graft, residual muscle tissue is physically scraped off, a procedure that potentially weakens the MTJ tissue. In case the MTJ is mechanically inferior, this may explain the higher re-ruptures rates of growing juveniles, as they have shorter tendons and thus require more of the MTJ-tissue to achieve the desired length and width of the graft. Consequently, graft configurations consisting of the ST and GR tendon proper were hypothesized to be mechanically superior - i.e. experiencing lower axial strains under the same loading - to configurations containing MTJ strands, and thus potentially further improving graft failure rate.

The global aim of this research was to evaluate commonly used hamstring tendon graft configurations and whether these could be related to graft failure mechanisms, in order to search for options to further decrease the risk of graft rupture. This was explored by examining whether specific graft configurations combining various regions of ST and GR tendons are mechanically or mechanobiologically favorable over others. The aim was subdivided into three research questions. 1) Do human full-length ST and GR contain tissue locations or regions at higher risk of mechanical failure, and if so, can any locations or regions consistently at higher risk of failure be identified? To answer this, the
local strain variations within remnant full-length GR from ACL reconstructions were quantified in a tensile test. Cadaveric full-length ST and GR (obtained from the same leg) were also examined to be able to extrapolate cadaveric ST and patient GR to the ST source material used in clinical practice. 2) Do biochemical compositions of human ST and GR vary between tissue regions such that the (mechanobiological) cell environment may deviate throughout the graft? This question was answered by assessing regional biochemical compositions for the tendons examined in the tensile tests. 3) Which specific graft configurations combining various regions of ST and GR tendons experience lower strains and 6% global strain was determined, and averaged over the tendon length. Besides that, minimal and maximal local strains were determined at the end of stress-relaxation, and expressed as relative

2. Materials and methods

2.1. Tendon tissue

Table 1 displays used tendon types and patient characteristics. Nine remnant full-length GR which were harvested during ACL reconstructive surgeries between September 2018 and October 2019 were obtained and stored at −30 °C until further use. These tendons comprised redundantly harvested and otherwise normal and intact GR tissue, where ST alone provided sufficient graft length and diameter. The Medical Ethical Committee at Máxima Medical Center (Veldhoven, the Netherlands) declared that this study did not meet the criteria as stated by the Medical Research Involving Human Subjects Acts (WMO), and the local committee approved use of the remnant tendon tissue for the present study (METC N16.148).

In order to compare the ipsilateral ST and GR, six full-length ST and GR from the same legs were dissected from four fresh frozen cadavers (Department of Anatomy, Maastricht University, the Netherlands). Table 1 shows the specimen characteristics. Muscle tissue was resected from the dissected tendons as much as possible before storage at −80 °C. Tendons were thawed at room temperature before testing, and kept in phosphate buffered saline at room temperature, except during handling.

2.2. Mechanical testing setup and loading protocol

See Fig. 1 for a schematic overview of the experimental setup used to determine the local and regional viscoelastic properties of the hamstring tendon samples. A random speckle pattern of graphite (Kontakt Chemie GRAPHIT 33, sprayed into a petri dish for easier manipulation) was applied to each tendon before mechanical testing using a cotton swab. This strategy was applied in order to track the pattern particles for post-testing local strain measurements, while preventing aliasing in the particle tracking. A mechanical test system (MTS Criterion® Series 42) was used in combination with custom made steel brush clamps [Peters, 1987] and a liquid tank (see Fig. A1). After mounting the tendon in the setup in unstrained state, the tank was filled with phosphate buffered saline – covering the entire sample – in order to reduce possible altering of the mechanical properties due to tissue dehydration.

After 3–5 min for equilibration, the tendons were tensioned at 15 mm/min until a load of 2 N, defining the sample’s starting length l₀ for the tensile test. Subsequently a strain of 6% was applied at a constant rate of l₀/min, reflecting physiological tendon- and ACL strain [Bates et al., 2015; Robi et al., 2013; Schilaty et al., 2018] and exposing elastic tissue deformations. After the ramp test, stress-relaxation of 20 min was applied, exposing viscous deformations, which relate to the risk of joint laxity. After the mechanical test, the part of the tendons that was in between the clamps was dissected into four equal parts – representing the clinically most common four-way folding – and a biopsy was taken per tendon quarter for biochemical analysis. The biopsies were cut into small pieces, where the tendon surface and thereby applied graphite was scraped off as much as possible. The biopsies were snap-frozen and stored at −30 °C until further use in biochemical assays.

2.3. Local strain analysis

In order to assess potential mechanically weaker points in the tendons, local strains were determined over the course of the mechanical test [Raghupathy and Barocas, 2010; Raghupathy et al., 2011; Soetens et al., 2018]. Therefore, during mechanical testing, samples were recorded with a Nikon D7000 digital photo camera at a resolution of 1920 × 1080 pixels and a rate of 24 fps. Digital recordings of the mechanical test were processed in Matlab (Mathworks, version R2019a). To prevent oversampling and reduce computational cost, the images corresponding to the ramp test were converted to 8 fps, and the subsequent frame rate for the stress-relaxation images was exponentially descending over time. This decreasing frame rate was defined to follow the exponential force decay during stress-relaxation.

Applied graphite at the tendon surface was used to determine local displacements with the strain tracking code provided by Victor Barocas’ Group (University of Minnesota [Raghupathy and Barocas, 2010]). Local displacement output data - with the end of the pre-load phase as the reference frame - was converted to local axial strains εₓ(X, Y), which were visualized in heat maps (Fig. 1), as well as local shear strains εᵧ(X, Y):

\[
εₓ(X, Y + ΔY / 2) = \frac{1}{2} \left( \frac{uₓ(X, Y + ΔY) - uₓ(X, Y)}{ΔY} + \frac{uₓ(X + ΔX, Y) - uₓ(X, Y)}{ΔX} \right)
\]

(1)

\[
εᵧ(X, Y + ΔY / 2) = \frac{uᵧ(X, Y + ΔY) - uᵧ(X, Y)}{ΔY}
\]

(2)

where uₓ and uᵧ are the displacements in lateral and axial direction, and ΔX and ΔY are the distances between tracked nodes in lateral and axial direction in the reference frame, respectively.

Subsequently, in order to assess the degree of local axial strain variation within tendons, the absolute difference between the local axial strains and 6% global strain was determined, and averaged over the tendon length. To prevent oversampling and reduce computational cost, the images corresponding to the ramp test were converted to 8 fps, and the subsequent frame rate for the stress-relaxation images was exponentially descending over time. This decreasing frame rate was defined to follow the exponential force decay during stress-relaxation.

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(1)

\[
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Table 1

| Tendon type               | left/right/unknown | age (years) mean ± std. dev. | male/female |
|---------------------------|--------------------|------------------------------|-------------|
| gracilis patient (GR)     | 5/2/2              | 26 ± 9                       | 4/5         |
| gracilis cadaver (C, GR)  | 3/3/0              | 75 ± 16                      | 2/4         |
| semitendinosus cadaver (C, ST) |                |                              |             |

* One sample was sliding between clamps during mechanical test.
Fig. 1. Experimental setup. Full-length ST and GR were subjected to a ramp test (6% strain), followed by 20 min stress-relaxation. Using graphite speckle pattern tracking, local displacements and strains were determined. Tendons were virtually subdivided into two, three and four equal parts, representing different ways of folding, and lumped parameter models were created per region. Biochemical compositions were determined from a biopsy per tendon quarter. A-F) schematic virtual graft configurations: black spring-dashpot and thick outlines schemes represent ST, colored schemes and thin outlines represent GR. Dark tones represent the BTJ-side, light tones the MTJ-side. A) four-strand ST (four-way folding); B) four-strand ST + GR (two-way folding); C) four-strand ST + GR without one strand MTJ (three-way folding); D) four-strand ST + GR without two strands MTJ (four-way folding); E) six-strand ST + GR (three-way folding); F) eight-strand ST + GR (four-way folding). These virtual grafts were subjected to a physiological loading protocol: preload of 80 N and 10 cycles (1 Hz) of simulated dynamic early rehabilitation with peak forces of additional 300 N.
positions along the full-length tendon (0% = BTJ-side; 100% = MTJ-side).

2.4. Biochemical assays

The biochemical tissue composition per tendon region was determined by means of the water-, collagen-, GAG- and DNA content. To this end tissue biopsies were weighed before and after lyophilization, and sample water content was calculated from the difference between weights. Lyophilized samples were digested overnight at 60 °C in 140 μg/ml papain (Sigma-Aldrich P5306) solution in 100 mM phosphate buffer in MilliQ with 5 mM L-cysteine (Sigma-Aldrich C1276) and 5 mM ethylenediaminetetraacetic acid (Sigma-Aldrich ED2SS) [Kim et al., 1988]. Digested samples were centrifuged for 10 min at 12,000 rpm. As a measure for cell content, DNA concentration in the supernatant was determined with a plate reader using the absorbance at 550 nm, with 40.5 mM glycine (Sigma-Aldrich G8898) and 40.5 mM NaCl (Merck 100244) [Farndale et al., 1986]. The GAG content was calculated using 13.5% of collagen weight to dry weight.

For glycosaminoglycan (GAG) quantification with shark cartilage chondroitin sulfate (Sigma-Aldrich C4384) as reference, 40 μl of the samples’ supernatant after centrifugation was mixed with 250 μl of 1.9-dimethylmethyle blue (Sigma-Aldrich 341088) solution in MilliQ with 40.5 mM glycine (Sigma-Aldrich G8898) and 40.5 mM NaCl (Merck 106404) [Fardanethal., 1986]. The GAG content was calculated using the difference between sample absorbance at 540 nm and 595 nm in a BioTek Synergy HTX plate reader, and normalized to the biopsies’ dry weight.

Collagen content was determined by means of hydroxyproline content, using the Chloramine-T assay with trans-4-hydroxy-L-proline (Sigma 402893), and incubated for 20 min. 250 C9887) in 80% acetate-citrate buffer (pH 6.0), 10% MilliQ and 10% 100244) in MilliQ, and centrifuged for 5 min at 12,000 rpm. 25 μl supernatant after centrifugation was mixed with 150 μl Chloramine-T (Sigma-Aldrich H54409) as reference [Huszar et al., 1980]. Digested samples were digested overnight at 60 °C in 140 mg/ml papain (Sigma-Aldrich P5306) solution in 100 mM phosphate buffer in MilliQ with 5 mM L-cysteine (Sigma-Aldrich C1276) and 5 mM ethylenediaminetetraacetic acid (Sigma-Aldrich ED2SS) [Kim et al., 1988]. Digested samples were centrifuged for 10 min at 12,000 rpm. As a measure for cell content, DNA concentration in the supernatant was determined with a plate reader using the absorbance at 550 nm, with 40.5 mM glycine (Sigma-Aldrich G8898) and 40.5 mM NaCl (Merck 100244) [Farndale et al., 1986]. The GAG content was calculated using 13.5% of collagen weight to dry weight.

The calculated parameter values corresponding to the tendon regions for all included samples provided ranges of values for the spring- and viscous constants k_E, k_VE and μ_m per tendon type (GR and ST). In order to cover these ranges, the median (Q2) as well as the first (Q1) and third quartile (Q3) of each of the three parameters per tendon region and tendon type was considered, as indicated in Table A.2. All combinations of Q1, Q2 and Q3 for the spring- and viscous constants were included, providing twenty-seven combinations of parameter values per strand (all combinations are indicated in Table A.3). These combinations were used to build six virtual grafts (Fig. 1A–F). Four configurations are commonly used in clinical practice: quadruple ST (Fig. 1A), classic four-strand ST + GR (Fig. 1B), six-strand ST + GR (Fig. 1E) and eight-strand ST + GR (Fig. 1F). The other two configurations consisted of the expected strongest strands only: the two distal strands of ST + GR combined (i.e., six-/eight-strand ST + GR where one/two MTJ-side strands per tendon were omitted; Fig. 1C and D). Since all four, six or eight parallel strands n = {1, …, N} for N = {4, 6, 8} experience equal strain at every time point t (ε_t = ε), and the sum of the forces on the parallel strands equals the total graft force, the virtual graft strain over time (ε_t) with time step Δt was calculated using [Oomens et al., 2009]:

\[ \varepsilon_{t+\Delta t} = \varepsilon_t + \frac{\sum_{n=1}^{N}(F_{t_1} - k_{\text{E}} \varepsilon_t) \Delta t}{k_{\text{E}} + k_{\text{VE}}}, \quad \varepsilon_{t} = \sum_{n=1}^{N}(k_{\text{E}} + k_{\text{VE}}) \varepsilon_t \]

where ε_t is the force in the tendon parts m = {1, …, M} for M = {2, 3, 4}.

The standard linear viscoelastic model consisted of an elastic component (spring) in parallel to a viscoelastic component (spring and dashpot in series). At the start of stress-relaxation, the behavior of the dashpot could be described as a rigid connection, such that both springs contributed to the stiffness [Oomens et al., 2009]. At the end of stress-relaxation, the dashpot had fully relaxed, and only the elastic spring contributed to the stiffness [Oomens et al., 2009]. Therefore, the force at the beginning (F_0) and end (F_end) of the tensile test for every tendon part m was:

\[ F_{0,m} = (k_{E,m} + k_{VE,m}) \varepsilon_{0,m} \quad (4) \]
\[ F_{end,m} = k_{E,m} \varepsilon_{end,m} \quad (5) \]

where k_E,m and k_VE,m are the coefficients representing the elastic and viscoelastic spring constant, respectively, and ε_0,m and ε_end,m are the strains in part m at the start and end of the stress-relaxation. The typical time constant τ_m for force decay during stress-relaxation was derived from the relaxation of the dashpot, and was defined as:

\[ \tau_m = \frac{\eta_m}{k_{VE,m}} \quad (6) \]

with η_m being the coefficient representing the viscous constant of the dashpot in the viscoelastic component.

The virtual grafts were exposed to a physiologically relevant loading protocol [Amis and Jakob, 1998; Escamilla et al., 2012; Shelburne et al., 2004; Shelburne et al., 2005], consisting of a surgically applied static fixation force followed by walking, which is generally requested from patients shortly after surgery [Janssen et al., 2018]. The static fixation force (pre-tension; s = 1) was set to 80 N [Amis and Jakob, 1998]. After 300 s, strain creep due to this pre-tension had reached equilibrium. Subsequently, 10 loading-unloading cycles (s = {2, …, 11}) of 1 Hz with force steps of 300 N were applied, matching values in the native anterior cruciate ligament during normal level walking [Escamilla et al., 2012; Shelburne et al., 2004; Shelburne et al., 2005]. Mechanical behavior of the virtual graft constructs was compared by means of the resulting
strain over time. Since the highest strains would provide the largest risk of graft laxity and failure, peak strains of relevant graft combinations were statistically compared. In that perspective, the grafts without MTJ-side strands (C-D) were compared with grafts of matching ways of tendon folding (E-F, respectively). Besides that, the most frequently used graft in clinical practice (quadruple ST; A) was compared with all other configurations (B–F).

2.6. Statistical analysis

Statistical calculations were performed using SPSS Statistics (IBM®, version 27). Shapiro-Wilk tests were used to check for data normality.

From the local strain data, the absolute difference of local versus global axial strain and relative minimum and maximum strain positions were compared between patient- and cadaveric GR using Independent T-test in case of data normality or Mann-Whitney U test in case of non-normality. The same measures were compared between cadaveric GR and ST within legs using Wilcoxon Signed Ranks Test, due to violation of data normality. Uniform distribution of relative minimum and maximum strain positions within tendons was explored using Kolmogorov-Smirnov test.

Biochemical compositions of the tissue regions per tendon type (cadaveric ST, cadaveric GR and patient GR) were compared using a Friedman test, due to non-normally distributed data. Dunn-Bonferroni post hoc tests (legacy method) were carried out for post-hoc comparison of individual regions in case of a significant difference in the Friedman test.

Estimated lumped parameter values were converted log(x+1) in order to visualize data in box plots. For three- and four-way folding, all lumped parameter values were compared between regions m using Friedman test, and Wilcoxon signed rank test for two-way folding (due to non-normally distributed data).

Virtual graft peak strain data was compared between the graft configurations using Kruskal-Wallis test, and post-hoc Mann Whitney tests were used to further explore differences between graft configurations of equal number of strands and/or equal tendon folding.

For all tests, significance was set at α = 0.05, and a Bonferroni correction was applied for all post-hoc multi-comparison analyses.

3. Results

3.1. Large variations in local axial strains are uniformly distributed within and between tendons

Global force profiles over time of the ramp tests of 6% global strain and subsequent stress-relaxation (Fig. 2) showed generally lower peak forces at 6% strain in GR compared to ST, which could be ascribed to the anatomically larger diameter of ST compared to GR. The alternately ascending and descending force curves in Fig. 2 during the ramp tests can indicate local plastic deformation and/or damage. Approximately half of the peak force dissipated during stress-relaxation in all tendons, with force dissipation representing the viscous component in the mechanical behavior.

Local axial strains were similar along the tendon length from BTJ to MTJ-side during the ramp test, which exposed elastic behavior, and variability along the tendon length appeared mainly during stress-relaxation, which exposed viscous behavior (example of heat map visualization shown in Fig. 1, and a movie for every sample in online supplements). In the starting frame the displacement tracking points were as close to the tissue clamps as possible. However, in certain experiments there seemed to appear some tissue from between the clamps during the ramp test – mainly on the MTJ side – introducing a space where local strains could not be tracked between the clamps and the outer tracking points. In order to distinguish between fascicle sliding and complete sample slip between the clamps, the clamped tissue length before and after testing was checked, and no difference in clamped tissue length was observed.

The absolute difference between the local axial strain at the end of stress-relaxation and the globally applied strain, averaged over the tendon length, indicated the strain variability along the tendon length (Fig. 3A, Table A1). The variability along the length was found to be of the same order of magnitude as the 6% global strain. At some locations, even negative axial strains were observed (movies in online supplements: Video A1-A.20, with corresponding legend in Fig. A3). In order to explore whether these negative local strains might have been related to fascicle sliding and lower longitudinal fascicle strain, the local shear strains at those locations determined using formula (2) were examined, and were found to be larger than the globally applied axial strain ($\epsilon_{xy} = 0.1 – 1.0$).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.jmbbm.2021.105010

Regarding the relative positions of minimal and maximal local strain along the tendon length (BTJ-side set to 0% and MTJ-side set to 100%), no consistent pattern of low and high local strains with regard to tendon position was observed within and between tendons (Fig. 3B and C, complete overview in Table A1). Only for the minimal local strain in the cadaveric GR, the performed uniformity test was statistically significant (p = 0.04), with an apparent expected value towards the MTJ-side. All other statistical tests for uniformity were not statistically significant, and did not link these minimal and maximal strains to a particular location of the GR and ST.

Statistical comparison of patient GR to cadaveric GR as well as cadaveric ST to cadaveric GR provided no significant differences for any of the quantities described above (Table A1). Therefore, patient GR and cadaver GR calculations were combined for further mechanical and computational analyses, and cadaver ST data was applied to represent patient ST for virtual graft calculations.

3.2. Biochemical composition differs not consistently between tendon regions

From the biochemical composition per tendon quarter (Fig. 4, complete overview per sample in Fig. A2), several statistically significant differences between the region closest to the MTJ-side and other regions were found, particularly in water- and DNA content. However, no region with a consistently deviant composition from all other regions was detected, similarly to the local axial strain data.

3.3. No hamstring tendon region provides consistently deviant lumped parameter values

Log-transformed lumped parameters from tendons computationally split in two, three or four equal parts in series are displayed in Figs. 5-6 (complete overview in Table A.2). Lumped parameter values corresponding to $k_{m,n}, k_{ST,m}$ and $\eta_m$ varied within and between strands. Spring constants were generally lower for GR than for ST, but no statistically significant difference was detected between different tendon parts from the BTJ-side towards the MTJ-side within the same tendon in case of two-, three- or four-way folding.

3.4. Four-strand ST + GR constructs without MTJ are mechanically inferior to quadruple ST

The resulting strain behavior of virtual graft constructs consisting of full-length tendons or tendons without MTJ (Fig. 1A-F) exposed to physiologically relevant forces (80 N pre-load and additional 300 N dynamic loading) is displayed in Fig. 7. An overview of the calculated maximum peak strains for the various combinations of parameter values (capturing Q1, Q2 and Q3 for the three parameters) per construct is shown in Table A.3. Maximum peak strains of the virtual grafts differed significantly (Kruskal-Wallis test, p < 0.001) between the graft constructs. Post-hoc pairwise comparison (Mann-Whitney U test with
Fig. 2. ST samples provide higher stiffnesses at 6% global strain compared to patient and cadaver GR samples. Global (applied) strain over time during ramp- and stress relaxation test, as well as global output force per tendon over time, depicted for cadaveric ST, cadaveric GR and patient GR. Note the different scales for the two phases (ramp strain and stress relaxation) on the time axes.
Bonferroni correction) of constructs of equal number of strands or equal tendon folding showed a statistically significant difference between construct D and F ($p < 0.001$; favors F) and between A and D ($p = 0.010$; favors A). Difference between A and C ($p = 0.019$; favors A) and between C and E ($p = 0.011$; favors E) approached statistical significance, considering the Bonferroni correction. No statistically significant difference was found between constructs A and B ($p = 0.40$), between A and E ($p = 0.65$), and between A and F ($p = 0.11$). Average peak strains (black line in Fig. 7) for constructs C and D were around 8–10%, thereby matching the values for graft failure via the mechanical mechanism described in 1. Introduction. Average peak strains for all other configurations did not exceed 6%, the value considered for physiological (global) tendon- and ACL strain. Furthermore, no mechanical difference between the quadruple ST graft and combined full-length GR and ST grafts could be detected.

**4. Discussion**

Considering the mechanical and mechanobiological mechanisms that could evoke ACL graft rupture, it was clinically relevant to know whether specific ST or GR regions were more prone to failure via one of these mechanisms. Therefore, the purpose of this study was to examine whether specific graft configurations combining various regions of ST and GR tendons are mechanically or mechanobiologically favorable over others, in order to further improve clinical results of ACL reconstruction. Firstly, human hamstring tendons were mechanically tested, and local axial strains were determined to examine local or regional variations in mechanical properties. The results indicated a large variation in local and regional tendon strains in human ST and GR, but no clear pattern from BTJ-side to MTJ-side was detected. Secondly, it was investigated whether variations in biochemical properties within full-length ST and GR resulted in consistently mechanobiologically inferior tissue regions. Despite the presence of variations in biochemical properties - possibly relating to structural and functional differences between the tendon regions [Snedeker and Foolen, 2017] - no biochemically deviating tissue region could be identified. Lastly, it was determined numerically whether combining various regions of ST and GR tendons could result in mechanically superior or inferior graft configurations. Viscoelastic lumped parameter models showed that mechanical properties of virtual graft configurations where the GR was added to the full-length ST were not significantly different to the standard quadruple ST - i.e., experiencing similar strains under the same loads: average peak strains did exceed the physiological 6% strain. However, four-strand configurations where the ST MTJ-side was removed and replaced by GR tendon proper strained more (average peak strains around 8–10%) under the same loading. The four-strand ST + GR configurations without MTJ-side strands were therefore considered to be mechanically inferior to the investigated full-length tendon graft configurations of equal number of strands or equal tendon folding.

The investigated ST and GR samples showed a large variation in local axial strains within and between tendons after application of a ramp test. The present study reported a variable strain distribution along the
Fig. 4. Despite variations in biochemical composition between regions, no consistently biochemically distinct tendon region was found. A) Water, B) collagen-, C) GAG, and D) DNA-content averaged over tissue groups per region. GR, patient gracilis tendon; C_GR, cadaver gracilis tendon; C_ST, cadaver semitendinosus tendon. *p < 0.05, with black lines indicating the significantly different regions.

Fig. 5. None of the GR regions provides mechanical parameters deviating from the other regions, in any of the tendon folding configurations. Boxplots of determined lumped parameter values per tissue region m (representing strands) for patient- and cadaveric gracilis tendons combined, calculated using formula 4-6. Colors match two-, three- and four-way splitting of the region of interest from displacement tracking - representing tendon folding into strands - corresponding with colors in Fig. 1. $k_{E,m}$: elastic spring constant region m; $k_{VE,m}$: viscoelastic spring constant region m; $\eta_m$: viscous constant in the viscoelastic component region m; BTJ: bone-tendon junction side; MTJ: muscle-tendon junction side. p-values correspond to statistical comparison of tendon regions within one graph.
were found. Therefore, we speculated local micro-damage and/or particularly on the MTJ side - during the ramp test. Complete sample slip - certain videos that some tissue appeared from between the clamps - differences in clamped tissue length before and after mechanical testing - points visible at the start of the tensile test. However, it seemed in mowitch et al. highlighted another scale with the regional strains.

Maximal local strains in the range where mechanobiological (4% axial strain – 10% strain) and four-way splitting of the region of interest from displacement tracking - representing tendon folding intro strands - corresponding with colors in Fig. 1. k_Em: elastic spring constant region m; k_VE m: viscoelastic spring constant region m; η_m: viscous constant in the viscoelastic component region m; BTJ: bone-tendon junction side; MTJ: muscle-tendon junction side. p-values correspond to statistical comparison of tendon regions m within one graph.

Fig. 6. None of the ST regions provides mechanical parameters deviating from the other regions, in any of the tendon folding configurations. Boxplots of determined lumped parameter values per tissue region m (representing strands) for cadaveric semitendinosus tendons, calculated using formula 4-6. Colors match two-, three- and four-way splitting of the region of interest from displacement tracking - representing tendon folding intro strands - corresponding with colors in Fig. 1. k_Em: elastic spring constant region m; k_VE m: viscoelastic spring constant region m; η_m: viscous constant in the viscoelastic component region m; BTJ: bone-tendon junction side. MTJ: muscle-tendon junction side. p-values correspond to statistical comparison of tendon regions m within one graph.

Hamstring tendon length as a result of the globally applied strains, with maximal local strains in the range where mechanobiological (4% axial strain [Robi et al., 2013]) and even mechanical failure (8–10% strain [Luque-Seron and Medina-Porqueres, 2016; Robi et al., 2013]) could occur. Therefore, it could be stated that local risk of graft rupture was found to be present in every tendon, but could not be attributed to a particular tendon location or region that could be included in a graft. We speculate that this indicates inter-patient variability in tendon tissue properties, as well as the potential added value of determining patient-specific tendon tissue properties [Van Vijven et al., 2020] in order to create the optimal autograft.

Previously, various studies were performed involving the regional - but not local - variation in tendon strain. In rat tibialis anterior tendons, the BTJ-side was the stiffest, while the extensibility was highest at the MTJ-side [Arruda et al., 2006]. Abramowitch et al. (2010) found gracilis tendons to be stiffer than semitendinosus tendons, and distal halves (BTJ-side) to be stiffer than proximal halves (MTJ-side) for strains up to 6%. The absence of such clear patterns in our data could be related to the fact that our research focused on the local axial strains - in order to link fascicle strain to the risk of fascicle and tissue rupture - whereas Abramowitch et al. highlighted another scale with the regional strains. Therefore, in our analyses, the tendons were split into distinct regions virtually rather than physically: at any time point the displacement tracking software and subsequent local strain analysis only captured points visible at the start of the tensile test. However, it seemed in certain videos that some tissue appeared from between the clamps - particularly on the MTJ side - during the ramp test. Complete sample slip seemed an unlikely explanation for this phenomenon, because no differences in clamped tissue length before and after mechanical testing were found. Therefore, we speculated local micro-damage and/or tendon fascicle sliding [Thorpe et al., 2013] could have been the underlying mechanism. This was possibly also reflected in the local negative axial strains, which coincided with high shear strains, implying significant fascicle sliding. Fascicle sliding also might have implied slightly more viscous behavior on the MTJ side of the tendon, although this was not detected for the adjacent points that were tracked. Due to the local rather than regional variation in axial strains, it seemed acceptable to assume that this larger viscosity was restricted to that particular location. Therefore, similar mechanical properties for each region within a tendon, representing a graft strand, seemed to be plausible. Here, the reported mechanical tissue properties were similar to the properties described in literature [Cicone et al., 2006; Karathanasopoulos and Ganghoffer, 2019]. The lumped parameter model used in this research was a simplified model compared to the finite element models created in previous research [Papachristou et al., 2007; Peña et al., 2005; Westermann et al., 2013]. However, the similarity in mechanical loading between the tensile tests and virtual graft configurations, combined with the global rather than local mechanical behavior of the entire graft that was computed, would have made a finite element model redundant for the intended purpose and supported the simpler lumped parameter model.

High local axial strains could increase graft rupture risk not only via mechanical failure, but also variations in mechanobiological stimulation of resident cells could induce local variations in ligamentization of the tissue [Janssen and Scheffler, 2014]. Excessive external loading could lead to disproportional tissue remodeling [Van Vijven et al., 2020], which could over the long term negatively affect graft integrity [Janssen and Scheffler, 2014]. It was described that cells perceive biophysical cues derived from extracellular matrix composition – affecting local transmission of globally applied forces – as well as biological cues from...
Fig. 7. Virtual graft configurations where one or multiple strands on the MTJ-side are omitted provide higher peak strains compared to the configurations with full-length tendons. Resulting strains of virtual grafts subjected to a preload of 80 N and simulated dynamic early rehabilitation with peak forces of 300 N. Figures show average resulting strains (black line) together with strain value ranges (grey), calculated based on the median and first and third quartile lumped parameter values. Red lines indicate 4% strain, the lowest threshold value potentially providing tissue damage. Schematic graft configurations: thick lines represent ST, thin lines GR. Dark tones represent the BTJ-side, light tones the MTJ-side. Colors represent ways of folding corresponding with colors in Fig. 1. A) four-strand ST (four-way folding); B) four-strand ST + GR (two-way folding); C) four-strand ST + GR, each without one strand on the MTJ-side (three-way folding); D) four-strand ST + GR, each without two strands on the MTJ-side (four-way folding); E) six-strand ST + GR (three-way folding); F) eight-strand ST + GR (four-way folding).
the required graft dimensions after both ST and GR harvesting [Lee et al., 2019], despite GR regeneration was reported before. Regional biochemical tissue properties found in this research were similar to compositions described in literature [Aparecida de Aro et al., 2012; Snedeker and Foolen, 2017], although collagen contents were somewhat lower, especially in certain MTJ regions. This could indicate a biochemical difference between the MTJ and other tendon regions, but it may also have been related to the presence of remnant muscle tissue in these particular samples, which was present during the mechanical test and therefore also included in the biochemical analysis.

Thus far, when considering various ACL graft configurations of the same hamstring tendon, no differences in complete graft mechanical properties [Broadhead et al., 2017; Snow et al., 2012] and clinical outcome [Krishna et al., 2018] were detected. Besides that, functional clinical outcome was not found to be associated with the use of ST alone or inclusion of the GR in the graft [Suyung et al., 2015; Niki et al., 2011; Salmon et al., 2005]. However, most commonly ST and/or GR autografts were considered as one experimental group, regardless of the specific tendon(s) used. The experimental group comprising all hamstring tendon autografts was then compared to another graft source [Maletis et al., 2007; Marder et al., 1991; Sajovic et al., 2006; Sajovic et al., 2011; Shaieb et al., 2002; Wilson et al., 1999]. Previously, a higher stiffness and failure load were found ex vivo for a four-strand ST graft (similar to configuration A) when compared to a four-strand ST + GR (similar to configuration B) or a four-strand GR, although the failure strain was higher in the four-strand ST + GR [Paulhe et al., 2015]. In our virtual graft data, we did not detect differences in peak strains between these configurations. The clinical decision on whether to include a GR or not was dependent on the required graft length and diameter [Boniello et al., 2015; Conte et al., 2014; Vinagre et al., 2017] and achievability with the estimated ST dimensions [Conte et al., 2014]. The mechanical consequences of inclusion of the GR and its potential to replace mechanically inferior regions of the ST were not investigated before. Our virtual graft data indicated that the mechanical properties of the GR were insufficient to replace ST strands. This replacement was initially hypothesized to be a suitable solution if specific ST strands would turn out to be mechanically inferior to others, but also could have been a solution in case ST regions appear macroscopically damaged, e.g., when damaged during the harvesting procedure [Charalambous et al., 2009]. In such cases, the GR could still be used to reinforce the graft by increasing total diameter, but not to replace damaged ST areas. An additional reason to exclude the GR in a graft – if possible considering the required graft dimensions – would be donor site morbidity. Overall leg functionality after only ST harvesting was found to be better than after both ST and GR harvesting [Lee et al., 2019], despite GR regeneration after having superior to ST regeneration [Janssen et al., 2013].

For the virtual graft analyses, cadaveric ST properties were extrapolated to patient ST - even though the latter was generally younger. With a mean age of 26 years and a similar prevalence of male and female patients, the included patient GR samples in this research originated from a population reflecting the demographics of people undergoing ACL reconstruction [Magnussen et al., 2016]. Since the decision whether to include the GR in a graft is based on the dimensions of a graft that can be created of the ST alone rather than on the GR properties [Vanhoenacker et al., 2017], the cadaveric GR samples - which were suitable to be included in the graft if required - were representative for clinically applied graft tissue. No significant difference was detected between cadaveric GR and patient GR in this research, but inter-sample variations were large. Besides that, age-related differences in biochemical and structural properties in human hamstring tendons were reported before to be absent [Gagliano et al., 2018], supporting the assumption of cadaveric ST to be representative for patient ST properties, as applied in the virtual graft configurations. Follow-up research on these graft configurations could involve mechanical tests with actual folded tendon constructs, which could be compared to the mechanical model. However, tracing local strains - and therefore local risk of rupture - inside those constructs would be more challenging.

A few limitations and side notes to this study should be considered. In all clinically performed ACL reconstructions, the ST was included in the hamstring tendon autograft. Consequently, the full-length ST was the most relevant tissue to examine, but rarely available as remnant tissue after surgery. Patient ST properties for the lumped parameter models and virtual graft configurations were therefore derived from combined data corresponding to more abundantly available patient GR and cadaveric GR and ST. Since no difference was observed between the patient and cadaveric GR, we assumed that the cadaveric ST tissues were also representative for the patient ST. The negative axial strains provided by the local strain tracking at some locations in the tissues seemed erroneous for tensile experiments. Besides that, the quadrilateral elements connecting the corresponding tracking points generally turned out to decrease in surface area. This uncovered a limitation of displacement tracking imaging from only one angle, allowing only in-plane projections of points to be tracked. Since the negative axial strains coincided with high in-plane shear strains, we speculated that also large out-of-plane deformations and shear strains were present at those locations, thereby decreasing the surface areas of in-plane element projections and providing negative axial strains. However, both described fascicle failure mechanisms depend on the axial strain rather than the shear strain, where the reported axial strain values would not provide fascicle damage. If the split point between strands coincided with these negative axial strain values, the resulting strain in the entire strand also decreased, and provided lower lumped parameter values. As can be seen in Figs. 5 and 6, some of the calculations using formulas 4 and 5 consequently provided (near) zero values for the lumped parameters. These parameter values also resulted in high strains in the virtual grafts, and thereby maybe in overestimation of the maximal strain values in Fig. 7 and Table A.3, but probably the average and minimal strains were not affected by this.

5. Conclusions

Despite variations in both local/regional strains upon loading and regional biochemical compositions, no consistently deviant strand within a hamstring tendon autograft – that should be avoided in the graft for mechanical or mechanobiological reasons – were found. The superior stiffness of the semitendinosus tendon compared to the gracilis tendon potentially provides a lower risk of experiencing high local strains under the same loading. Thus, it is recommended that the semitendinosus tendon is used, whereas the gracilis tendon can be used to increase graft length and/or diameter to desired levels - and therefore reinforce the graft - but should not be used to replace semitendinosus tendon regions. As our data does not indicate the presence of a possible ‘weak strand’, other possible reasons for graft rupture should be investigated.

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Data statement

Our data is available upon reasonable request, directed to the corresponding author.

Author statement

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