Supraspinatus and deltoid muscle fiber diversity in rotator cuff tear conditions

Morten Kjaer Ravn
"University of Southern Denmark"
Trine Ivarsen Ostergaard
Syddansk Universitet
Henrik Daa Schroeder
Odense Universitetshospital
Jens Randel Nyengaard
Aarhus Universitet Health
Kate Lykke Lambertsen
Syddansk Universitet
Lars Henrik Frich (lars.henrik.frich@rsyd.dk)
Odense Universitetshospital https://orcid.org/0000-0002-0147-4211

Research article

Keywords: Muscle fiber type, Type 1; Type 2, Stereology, Fiber diameter, Atrophy factors

Posted Date: December 3rd, 2019

DOI: https://doi.org/10.21203/rs.2.18116/v1

Running title: Muscle fiber profiles

Supraspinatus and deltoid muscle fiber diversity in rotator cuff tear conditions

Morten Kjaer Ravn, [1]Department of Orthopaedics, Odense University Hospital, Sdr. Boulevard 29, DK-5000 Odense, Denmark; [2]The Orthopaedic Research Unit, Department of Clinical Research, University of Southern Denmark, J.B. Winsloewsvej 19, DK-5000 Odense, Denmark. mortenkravn@gmail.com

Trine Ivarsen Ostergaard, [1]Department of Orthopaedics, Odense University Hospital, Sdr. Boulevard 29, DK-5000 Odense, Denmark; [2]The Orthopaedic Research Unit, Department of Clinical Research, University of Southern Denmark, J.B. Winsloewsvej 19, DK-5000 Odense, Denmark. Trineivarsen92@gmail.com

Henrik Daa Schroeder, [3]Department of Pathology, Odense University Hospital, J.B. Winsloewsvej 15, DK-5000 Odense, Denmark. Henrik.daa.schroeder@rsyd.dk

Jens Randel Nyengaard, [4]Department of Clinical Medicine – Core Centre for Molecular Morphology, Section for Stereology and Microscopy, Center for Stochastic Geometry and Advanced Bioimaging, Aarhus University, Palle Juul Jensens Boulevard 82, DK-8200 Aarhus N, Denmark. jrnyengaaard@clin.au.dk

Kate Lykke Lambertsen, [5]Department of Neurobiology Research, Institute of Molecular Medicine, University of Southern Denmark, J.B. Winsloewsvej 21, DK-5000 Odense, Denmark; [6]Department of Neurology, Odense University Hospital, Sdr. Boulevard 29, DK-5000 Odense, Denmark; [7]BRIDGE – Brain Research – Inter-Disciplinary Guided Excellence, Department of Clinical Research, University of Southern Denmark, J.B. Winsloewsvej 19, DK-5000 Odense, Denmark. klambertsen@health.sdu.dk

Lars Henrik Frich, [1]Department of Orthopaedics, Odense University Hospital, Sdr. Boulevard 29, DK-5000 Odense, Denmark; [2]The Orthopaedic Research Unit, Department of Clinical Research, University of Southern Denmark, J.B. Winsloewsvej 19, DK-5000 Odense, Denmark. Lars.henrik.frich@rsyd.dk

***Corresponding author:** Lars Henrik Frich, J.B. Winsloewsvej 4, 5000 Odense C, Denmark. E-mail: lars.henrik.frich@rsyd.dk

Abstract

Background: Rotator cuff (RC) tears are associated with RC muscle atrophy and changes in composition that are crucial to the prognosis of RC repair. The aim of this study was to characterize muscle fiber composition in the supraspinatus (SS) muscle under tear conditions.

Methods: Muscle biopsies were obtained from 21 patients undergoing surgery for RC tendon tear. Biopsies were obtained from the musculotendinous junction of the SS muscle and control biopsies were harvested from the deltoid muscle (DT). Biopsies were immunohistochemically processed for detection of type 1 (slow type) and type 2 (fast type) fibers and analyzed using unbiased, stereological principles. We counted the total numbers of type 1 and 2 muscle fibers/mm^2 and fiber diameter was used to estimate muscle fiber atrophy and hypertrophy.

Results: We found significantly more type 2 cells/mm^2 in the SS compared to the DT ($p<0.01$). In addition, we found a significantly higher fraction of type 1 fibers than type 2 fibers in the DT ($p<0.01$), whereas both fiber types were equally present in the SS. The diameters of SS cells were generally smaller than those of DT cells. Atrophy of especially SS type 2 fibers was also demonstrated. Fiber atrophy was more pronounced in men than women.

Conclusion: The changes in the composition of SS muscle cell types suggest a shift from type 1 to type 2 muscle fibers and atrophy of both type 1 and 2 fibers. This composition indicates loss of endurance and rapid fatigue of the SS muscle under RC tear conditions.

Keywords: Muscle fiber type; Type 1; Type 2; Stereology; Fiber diameter; Atrophy factors

Background:

The 4 muscles in the rotator cuff (RC) coordinate shoulder movements as well as provide structural stability to the shoulder joint [1]. The RC is vulnerable to considerable morbidity [2, 3] and RC tears are common with a life prevalence of 10-20% [4, 5]. The musculotendinous unit of the supraspinatus (SS) is especially prone to lesions [6] but only sparse information is available regarding the pathophysiological response of the SS muscle to traumatic tear conditions.

Although it is generally accepted that timing of RC tendon repair is extremely important to prevent atrophy and fatty infiltration of muscles [7, 8], patients who are found eligible to reattachment of lesioned RC tendons are often offered delayed surgical intervention. One major reason is a surgical failure rate of more than 30% [9] that occurs despite ongoing improvements and perfection of the surgical intervention. The importance of the compositional and degenerative changes in the RC muscles as causes to surgical failure have become increasingly apparent [7, 10].

Skeletal muscle consists of different fiber types characterized by their specific myosin heavy chain (MHC) isoforms. Skeletal muscles in humans are basically composed of a mixture of MHC type 1 (slow twitch) and MHC type 2 (fast twitch) fibers. The two types differ in their function. In humans, myosin ATPase hydrolysis rates for fast twitch fibers are 2-3 times greater than those for slow fibers [11, 12] and the growth capacity of type 2 fibers is approximately 50% greater than that of type 1 fibers [13]. Additionally, type 1 fibers mainly depend on aerobic/oxidative energy metabolism, whereas type 2 fibers mainly depend on anaerobic/glycolytic metabolism [14, 15]. This is significant for movement of the joints, where type 1 fibers enable slow contractions and endurance, whereas type 2 fibers are responsible for fine motor control and power with the ability to perform rapid contractions [16].

A non-pathological SS muscle in cadaveric 65-year old (± 12 years) men and women was shown to consists of approximately 54% type 1 fibers [12], but physical activity and tear of the SS tendon may alter the fiber type composition [10, 17, 18]. Fiber type composition is also influenced by training activity of the individual and muscle disuse or denervation, that may be associated with a type 1 to type 2 shift [19], reviewed in [20]. Also age is suggested to affect muscle fiber composition, as older men have smaller type 2 muscle fibers than younger men [21], though other studies show no age-dependent difference in fiber type [22, 23]. Gender appears to affect muscle fiber size, as in men type 2 muscle fibers tend to be slightly larger than type 1 fibers, whereas in women the diameter of the two fiber types are identical [24]. This gender dependent difference in thickness is believed to be caused by greater muscle strength in men [25] and muscle strength is correlated to the size of type 2 muscle fibers [26]. Several of these factors, such as fiber atrophy, loss of muscle fibers and fatty infiltration are also main predictors of RC function. Therefore, a comprehensive characterization of SS muscle composition and degree of atrophy may lead to a better understanding of the pathogenesis of muscle wasting/pathology in relation to RC tear. This is a prerequisite tool in the design of therapeutic interventions appropriate for healing of RC tears. The aim of the present study was therefore to analyze muscle fiber profiles of the SS muscle in tear conditions using design-unbiased stereology [27, 28]. Furthermore, fiber atrophy of the SS muscle was assessed by measuring fiber diameter and gender dependent differences in fiber type composition were established.

Methods

Patients

Twenty-one (21) consecutive patients (mean age 60.3 ± 4.0 years, range 45-73 years) suffering lesion of 1 or more RC tendon tears involving the SS tendon in all cases. Fourten (14) males and 7 females, with SS tendon tear had biopsies taken from the SS musculotendinous junction during surgery. In addition, for comparison biopsies from assumed healthy, ipsilateral DT muscles were taken as well. Inclusion criteria were shoulder trauma, MRI-confirmed RC lesion and willingness to undergo surgery. All tears were confirmed at surgery. Twenty (20) biopsies from DT and 21 biopsies from SS were obtained using 3x3 mm biopsy punch through the lateral portal during arthroscopic RC tendon repair. Biopsies from the deltoid muscle were obtained from deltoid during artroscopical approach or mini-open surgery. Exclusion criteria were severe retraction of tendons, fatty infiltration higher than grade 2 [29], diabetes, autoimmune diseases, previous shoulder surgery, fractures or a dislocated shoulder.

Informed written consent was obtained from all patients. Ethical approval was granted by the local research ethics committee (The Regional Committees on Health Research Ethics for Southern Denmark, J. No. S-20160037) and the study was reported to the Danish Data Protection Agency.

Immunohistochemistry, fiber typing and histology

Immediately after removal, biopsies were immersion fixed in 10% phosphate-buffered formaldehyde, embedded in paraffin and cut into 4 μm thick microtome sections. For immunohistochemical staining, sections were deparaffinized, demasked in cell conditioning 1 (CC1, Roche) buffer and blocked in 1.5% H_2O_2 in tris-buffered saline (TBS, pH 7.4), after which they were loaded onto a Bench-Mark ULTRA IHC/ISH staining module (Ventana Medical Systems, Tuscon, Arizona). Sections were initially stained for myosin fast type

heavy chain (type 2 fibers) using a monoclonal mouse anti-myosin (skeletal, fast) antibody (1:8,000, NovoCastra, clone MY32), followed by heat inactivation of the immune complex and then stained for myosin slow type heavy chain (type 1 fibers) using a monoclonal mouse anti-myosin (skeletal, slow) antibody (1:50, NovoCastra, clone WB-MHCs). The OptiView DAB IHC Detection Kit (Roche Tissue Diagnostics) was used for development of myosin fast type heavy chain fibers and the ultraView Universal Alkaline Phosphatase Red Detection Kit (Roche Tissue Diagnostics) for development of myosin slow type heavy chain fibers. Hematoxylin was used for identification of nuclei using standard protocols at the Department of Pathology, Odense University Hospital.

Stereology

The 41 biopsies were analyzed blinded twice by two independent raters. All biopsies were analyzed using an Olympus BX50 microscope equipped with an Olympus DP70 digital camera attached to a PC with newCAST software (Visiopharm Integrator System, Hoersholm, Denmark).

Type 1 and type 2 cell profiles sampled by the 2D unbiased counting frame within the delineation along with corners hitting tissue were quantified using the counting tool [30]. In practice, a masking tool was used to delineate biopsies at a 4x lens, followed by analysis of type 1 and type 2 cells using a 20x lens (Figure 1). Each biopsy was analyzed using approximately 30 fields of views obtained by systematic uniformly random sampling throughout the biopsy cross-section. The measurement tool, set on the diameter setting, was used to measure the greatest diameter perpendicular to the longest axis of the cross-sectioned cell profiles inside the frame.

Estimation of the total number of type 1 and type 2 cell profiles per area

The number of type 1 and type 2 cell profiles per area, Q_A(cells), was estimated as:

145 $$Q_A(\text{cells}) = \left(\frac{\sum Q(cells)}{\frac{a(frame)}{4} \cdot \sum P(biopsy)} \right)$$

146 $Q(cells)$ was the total number of either type 1 or type 2 cell profiles sampled by the counting
147 frame, $a(frame)$ was the size of the counting frame (55,000 μm^2), and $P(biopsy)$ was the
148 number of count frame corners (=4) hitting biopsy tissue [30].
149 Fractions of the counted fiber types were calculated in both rating one and rating two. For
150 each rating, a mean fraction was found for each fiber type in the two muscles.
151 **Estimation of fiber Diameter**
152 To determine the changes in fiber size, the greatest diameter perpendicular to the longest axis
153 of the sampled cell profiles was measured [31], because it is not altered by either oblique
154 sectioning or kinking of the fibers. Normal adult muscle fibers are between 40-80 μm in
155 diameter in males, and between 30-70 μm in diameter in females [24]. A mean diameter was
156 found for both fiber types in each biopsy. One biopsy was eliminated, due to technical
157 artefacts.
158 **Estimation of hypertrophy and atrophy factors in SS and DT muscles**
159 To quantify the degree of atrophy and hypertrophy, Feret's diameter was used to calculate
160 atrophy and hypertrophy factors. Using these calculations, it was possible to detect hidden
161 and/or selective atrophy or hypertrophy in both fiber types. It is based on the theory that more
162 extreme size changes, are more impactful [24]. Thus, they are arranged into intervals based on
163 the fiber diameter. The intervals are then multiplied by a factor based on their size, e.g. fibers
164 between 1-10 μm are multiplied by 4, and fibers sized 20-30 μm are only multiplied by 2.
165 Hypertrophy factors are multiplied likewise. When all the atrophied and hypertrophied fibers
166 are found, the factors can be calculated by the following equation:

167 $$\frac{The\ products\ of\ all\ the\ atrophied\ fibers\ multiplied}{the\ total\ number\ of\ cell\ profiles} \cdot 1{,}000$$

168 The result can then be compared to an upper limit, determining whether there is atrophy of the
169 cells. The upper limits were compared to the upper limits found in m. biceps brachii (BB)
170 [24]. The upper limits for atrophy were 150 in both type 1 and 2 cell profiles for men. The
171 upper limits for women were 100 in type 1 cell profiles and 150 in type 2 cell profiles.
172 Hypertrophy upper limits for men were 300 for type 1 and 500 for type 2, and for women 200
173 and 150, respectively [24]. While analyzing this data, one biological outlier was identified and
174 as a result a single biopsy was eliminated from this analysis.

175 **ELISA for fatty acid binding protein 4**

176 SS (n=16) and DT (n=16) muscle samples were homogenized at 4°C in Mesoscale Lysis
177 buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100; pH 7.5)
178 containing Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich) and Complete Mini
179 EDTA-free Protease Inhibitor (Roche). Lysates were rotated end-over-end for 1 hour at 4°C
180 and cleared by centrifugation at 13,000 g at 4°C for 20 min. Protein content in the lysates was
181 measured by the bicinchoninic acid method using the Thermo Scientific Micro BCA^{TM}
182 Protein assay Kit (Pierce Chemical Co). ELISA for fatty acid binding protein 4 (FABP4) was
183 performed using a FABP4 ELISA kit (Biosite) according to the manufacturer's instructions.

184 **Statistical analysis**

185 Data are reported as mean (standard deviation (SD)) and $p<0.05$ was used to indicate
186 significant differences. Comparisons were done using two-way ANOVA followed by the
187 relevant *post hoc* test and student's t-test. Rout test was used to detect outliers. A simple
188 correlation analysis was performed to assess the inter- and intra-rater correlation.
189 Additionally, an intra-rater correlation was examined using the coefficient of variance (CV)
190 and coefficient of error (CE) and a ratio between these two coefficients. While analyzing the
191 data, a single biopsy from rater 1's first rating, was identified as a technical outlier due to the

192 tissue quality and therefore eliminated from this analysis. Removal of one outlier did not
193 affect results

194 CV expresses SD as a percentage of the sample mean. This is useful when estimating the size
195 of the variation relative to the size of the observation. Also, it has the advantage that it is
196 independent of the units of the observation. In this study, CV was calculated from the two
197 raters´ total number of type 1 and type 2 cells/mm². CV was used to make an intra-variance
198 correlation of both raters, for the type 1 and type 2 cell counts. The equation used was:

199 $$CV = \frac{SD}{mean}$$

200 Thereafter the CVs from the first and second rating were used to make a CV_mean for type 1
201 cells/mm² and a CV_mean for type 2 cells/mm² using the following equation:

202 $$CV_{mean} = \left(\frac{CV_1^2 + CV_2^2}{2}\right)^{0.5}$$

203 In normal muscle the CV is less than 0.25. Any measure greater than this is considered
204 abnormal variability. SD was calculated from the number of cells that had their diameter
205 measured [32].

206 CE was used to determine the precision of the estimates from the double estimates [33].

Results

Based on unbiased stereological estimation of the total number of type 1 and type 2 fibers in SS musculotendinous unit after RC tendon tear, we found a total number of 54.6 (21.6) type 1 cells/mm^2 and a total number of 53.5 (19.1) type 2 cells/mm^2 (Figure 2A). In the DT muscle, we found a total number of 47.1 (18.1) type 1 cells/mm^2 and a total number of 33.7 (13.8) type 2 cells/mm^2 (Figure 2A). When comparing the SS and the DT muscles, we found significantly more type 2 fibers/mm^2 in the SS muscle compared to the DT muscle (two-way ANOVA: muscle type **$p<0.01$, $F_{1.78} = 11.2$), whereas the number of type 1 fibers/mm^2 was comparable (Figure 2A).

In the SS muscle, 49.6 (13.0) % of the cells were type 1 fibers and 50.4 (13.0) % were type 2 fibers (Figure 2B). In the DT muscle, 57.5 (13.7) % were type 1 fibers and 42.5 (13.7) % were type 2 fibers (Figure 2B). When comparing SS and DT muscle tissue, we found significantly different type 1 and 2 fractions in the DT muscle (two-way ANOVA: Interaction **$p<0.01$, $F_{1.78} = 7.25$, fiber type *$p<0.05$, $F_{1.78} = 5.8$). In contrast, the fractions were comparable in the SS muscle (Figure 2B).

In total, fiber diameter was estimated on 1,366 SS fibers and 907 DT fibers (Figure 3). Means were calculated for each biopsy. Afterwards an overall mean and SD was found for each fiber type subdivided in muscle types and gender. In the SS muscle, the mean diameter of type 1 fibers in women were 42.2 (13.1) μm and in men 48.9 (16.6) μm. In the SS muscle, the mean diameter of type 2 fibers in women were 36.3 (13.3) μm and in men 48.3 (18.3) μm. In the DT muscle, the mean diameter of type 1 fibers in women were 53.0 (12.5) μm and in men 60.4 (17.7) μm. In the DT muscle, the mean diameter of type 2 fibers in women were 42.3 (12.3) μm and in men 55.9 (20.8) μm. When comparing SS and DT muscle diameters, we found a significant difference in diameter between type 1 fibers in both men ($p< 0.001$,

231 Student's t-test) and women ($p<0.001$) and between type 2 fibers in both men ($p<0.001$) and
232 women ($p<0.001$) with larger fiber diameters in DT compared to SS. In general, fiber
233 diameter appeared to be smaller in SS compared to DT (please compare the displacement of
234 the SS histogram to the left compared to the histogram for DT in Figure 3A-D). As atrophy
235 was apparent in most SS biopsies (Figure 4), fiber diameters measured were used to find
236 atrophy and hypertrophy factors (Table 1). The results for men showed atrophy for both type
237 1 and type 2 muscle fibers in SS (Figure 3A,B and Table 1) and atrophy for type 2 fibers in
238 women (Figure 3C,D and Table 1). Hypertrophy was not apparent in any fiber type in women
239 and men (Figure 3A-D and Table 1). Fatty infiltration, assessed by fatty acid binding protein
240 4 (FABP4), revealed comparable and low levels in the SS (753.6 ± 384.7 pg/mg) and deltoid
241 (660.3 ± 412.3 pg/mg) muscles ($p=0.53$)(Figure 5).

242 **Inter-rater and intra-rater reliability**

243 Results from the simple correlation analysis showed decent and comparable intra-rater R^2
244 values for type 1 and 2 fibers for both raters. For rater 1, the intra-rater value for type 1 fibers
245 was 0.56 and for type 2 fibers 0.48. For rater 2, the intra-rater value for type 1 fibers was 0.69
246 and for type 2 fibers 0.59. Inter-rater R^2 values were subsequently good for both type 1 (0.65)
247 and type 2 (0.65) [34].

248 Mean CE^2/CV^2 intra-rater analysis for rater 1 was 0.16 for type 1 fibers, and 0.22 for type 2
249 fibers. For rater 2 the mean results were 0.16 for type 1 fibers, and 0.20 for type 2 fibers.

Discussion

Rotator cuff (RC) tears affect many patients' daily lives, and treatment can be challenging, as the lesions in turn lead to atrophy and fatty infiltration of the RC muscles [6]. The pennatial anatomy of RC muscles is consistent within species [35] but muscle fiber composition is influenced by physical activity of the individual [36] and can change due to functional impairment and tendon tear [10, 37]. Since tear-induced muscle changes can influence the outcome of tendon surgery, the aim of this study was to map the extent of muscle damage through histochemical evaluation of the micro-architectural properties of the SS musculature in tear conditions. This study demonstrated only minor changes in SS muscle fiber type composition but atrophy of both type 1 and 2 fiber profiles in RC tear conditions.

In cadaveric RC muscles without long-term diseases or known myopathies, type 1 SS muscle fiber content has been shown to be 54% [12]. This result is based on a small number of samples but represents the best normative data on the fraction of muscle fiber types within the SS muscle. In comparison, our results revealed a decreased fraction (50%) of type 1 cells in SS muscle following RC tendon tear, suggesting a minor change in muscle type composition. This is in line with a previous study demonstrating reduced MHC1 content in SS muscle fibers obtained from patients with full-thickness RC tears compared to patients with partial-thickness RC tears [37]. In contrast to previous studies showing a reduction in the fraction of type 1 fibers in the lesioned SS muscle compared to the DT muscle [10], we observed comparable fractions of type 1 or type 2 fibers between SS and DT muscles. We did, however, observe a significant difference in the fraction of type 1 and type 2 fibers in the presumably healthy DT, a difference that was not present in the SS muscle.

To our knowledge, this is the first study to take advantage of design-unbiased stereology in order to count the total number of type 1 and type 2 muscle fiber cell profiles per area in the SS muscle and in the ipsilateral DT muscle after RC tendon tear. When comparing the

number of type 1 cells/mm² between SS and DT muscles, we did not find any significant differences. When comparing the number of type 2 fibers/mm^2, however, our results revealed a significantly increased number of type 2 cells/mm² in the SS compared to the DT muscle. This suggests that following SS tendon tear there is either an increase in type 2 fibers or a loss in type 1 fibers. Our data thus support the suggestions by Gigliotti et al [10], Lundgreen et al [37] and Butt et al [17] that there is a decrease in the number of type 1 fibers. Whether this is caused by a loss of muscle fiber or a conversion of type 1 to type 2 fibers remains a matter of debate.

Previous studies have demonstrated a decrease in fiber diameter and shift towards smaller-diameter fibers in the lesioned SS muscle compared to the DT muscle [10, 38]. In the present study, we demonstrated that in both women and men, there were significant differences in fiber diameter in both type 1 and type 2 fibers derived from SS muscle compared to DT muscle. Irlenbusch et al [16] found a greater reduction in type 2 muscle fiber diameter, compared to type 1 muscle fibers, when looking at biopsies taken at SS tendon surgery. They suggested that a disturbance of muscular coordination in patients with SS tendon rupture is responsible for the atrophy occurring. They also implied that type 2 fibers, responsible for fine motor control of the joint, often are impaired in these patients due to atrophy.

In line with our findings, Lundgreen et al [37] found statistically significant smaller average diameter of both type 1 and type 2 fibers in full-thickness tears, compared to partial-thickness tears. Despite findings of decreased fiber diameter in SS muscle following RC tears, Butt and colleagues recently showed that the mean myofiber cross sectional area had significantly increased in the SS muscle one year after SS tendon surgery [17]. Repairing the SS tendon thus seems to induce regeneration of the muscle, thereby reversing the shift in muscle fiber type composition [17]. These findings indicate that a SS tendon rupture with denervation and lack of activation of the muscle also induces a shift from type 1 muscle fiber isoforms to type

2 muscle fiber isoforms, as seen in our study, as well as suggested by Gigliotti et al [10] and Lundgreen et al [37].

From a clinical standpoint, early tendon repair, restoring the muscle to its original length, is the best method to avoid pathological changes in the corresponding muscle, as suggested by Gibbons et al. [39]. With this ongoing research, it is becoming increasingly apparent that long-term atrophy and architectural changes can be averted by early restoration of a point of attachment. Difficulties in treating RC tears and poor outcomes after surgical re-attachment [40, 41], however, call for additional treatment of the muscle itself. Meyer et al [42], from their animal studies, suggested that skeletal muscle progenitor cells are capable of contributing to muscle hypertrophy and regeneration regardless of tear severity. This, however, may not happen in patients, but can be a focus for future research in muscle regeneration following RC repair and could pave the way for better outcomes after RC tear surgery.

The measured diameters are influenced by shrinkage due to paraffin embedding. To discover hidden fiber atrophy or hypertrophy among the two types of fiber cells, we calculated atrophy and hypertrophy factors based on the measured diameters of the cell profiles. Since the atrophy factor of healthy SS muscle is unknown, the atrophy factors' upper limits were compared to the anatomically similar *biceps brachii* (BB) muscle in order to determine whether hidden atrophy was present. SS and BB muscles are somewhat similar in relation to fiber type 1 composition with BB having a proportion of 52% in elderly adults [21]. In the present study, the calculated factors showed clear atrophy of both type 1 and type 2 SS muscle fibers in men, and for type 2 fibers in women, when compared to BB. Hypertrophy was not apparent in any fiber types.

Aging is associated with reduced muscle strength and atrophy of type 2 muscle fibers [43]. Recent data in humans suggest that mRNA abundance and MHC isoform protein composition

would favor a fast-to-slow isoform shift with aging and in response to endurance exercise training [44]. Recent research shows that the growth capacity of fast twitch fibers is approximately 50% greater than that of slow-twitch fibers [13]. This calls for care in the planning of rehabilitation following muscle waste in elderly.

With an average timespan of 11.2 months from lesion to surgery, our patients had experienced disuse of the RC for different lengths of time. Studies have shown that disuse of muscles can lead to a shift in expression from type 1 fibers towards faster type 2 MHC isoforms, making it difficult to identify atrophy in fibers that were originally slow [45, 46]. This might explain the lesser degree of type 1 atrophy found in this study. Furthermore, it raises questions as to whether the larger fractions of type 2 cells found in all SS estimates (50%) when compared to the fraction of type 2 cells found in normal muscle (46%) [12] might be caused by the shift from type 1 to type 2 cells, or simply a loss of type 1 cells [10]. This question cannot be answered with a biopsy but will require a full transect of the muscle.

In a study of animal muscle fibers, it was suggested that specifically stretch and mechanical loading may induce a shift from fast twitch muscle fibers to slow twitch muscle fibers [47]. Increased activity or exercise of specific muscles are also associated with a fast-to-slow transition, i.e. an overall shift from more fatigable to less fatigable isoforms [19, 44].

In this study several limitations require consideration. The ipsilateral DT has been used as a standard of reference in a number of studies [48, 49] justifying the use of paired statistics and increasing the power of the analyses. The rationale for using the DT muscle for comparison could, however, be challenged, as it may be asymptomatically affected.

Analysis of the degree of fatty infiltration, which is indicative of muscle weakness, was estimated for both the SS and DT muscle. In this study, fatty infiltration was determined based on the content FABP4 protein levels in the SS and DT muscles, which we found were comparable between the 2 muscles. This in contrast to a recent study by Lee et al., which used

a different and semi-quantittaive technique to assess FABP4 and demonstrated lower FABP4 levels, representative of absence of fatty infiltration, in the DT muscle compaired to the SS muscle [50]. These findings are in favor of the use of DT as a reference muscle.

In athletes, the DT is predominantly composed of type 1 fibers [51] and the report from Meyer et al [42] found that muscles adjacent to the affected SS had fewer skeletal muscle progenitor cells. This indicates that the observed muscle profiles adjacent to a lesioned SS muscle may be affected with altered regenerative properties.

Another limitation inherent in muscle biopsy studies is that changes in muscle composition may not be uniformly distributed throughout the muscle. Our priority was to harvest all biopsies from the musculotendinous junction of the SS muscle during an arthroscopic approach from the subacromial space, thereby ensuring uniformity of the biopsy procedure.

The biopsies from SS and DT were analyzed using design-unbiased stereology giving very precise estimates [27, 28]. In addition, in this study analysis of the samples was done blinded twice by two independent raters. In contrast, previous studies used less precise methods such as photographically enlarged paper prints of the samples and semi-automated image analyses [10, 16, 37, 38]. Our results showed a large degree of coherency between the two raters, as well as individually for each rater.

Ratios of CE^2/CV^2 were made for both types of cells for both raters. To reach an acceptable level of precision when using stereology, the CE^2 should be half or lower than CV^2 [28]. The results indicate this was the case.

Conclusion

In this study we found evidence of atrophy of both type 1 and 2 muscle cells and changes in the composition of SS muscle cell types indicative of a shift from type 1 to type 2 muscle fibers. The fiber atrophy and compositional change also indicate loss of endurance and rapid fatigue in the SS muscle following RC tendon tear. Furthermore, we found that a SS tendon tear affects the composition of adjacent muscles such as the DT.

List of abbreviations

BB, biceps brachii; CV, coefficient of variance; CE, coefficient of error; FABP4, fatty acid binding protein 4; DT, deltoid; MHC, myosin heavy chain; RC, rotator cuff; SD, standard deviation; SS, supraspinatus.

Declarations:

Ethichs approval and consent to participate

Written, informed consent was obtained from all participants. This trial has been approved by the Regional Committees on Health Research Ethics for Southern Denmark and Odense University Hospital (S-20160037).

Consent for publication

Not applicable

Availability of data and materials

All data are hosted at OPEN (Odense Patient Explorative Network), which allows data sharing upon request.

https://www.sdu.dk/da/om_sdu/institutter_centre/klinisk_institut/forskning/forskningsenheder/open.Aspx

Funding

This work was supported by research grants from the Danish Rheumatism Association (A5347). The funding body had no influence on study design, data collection, interpretation of the results, or the final manuscript.

Competing interests

All authors declare no competing interests.

Author's contributions

LHF conceived the study.

HDS performed immunohistochemical staining of MHC type 1 and 2 fibers.

MKR and TIO performed stereological analyses with the assistance of JRN.

KLL performed FABP4 ELISA.

LHF and KLL wrote the manuscript with the assistance of MKR and TIO. All authors have read and approved the final draft.

Acknowledgements

We thank Claire Gudex for proofreading the manuscript.

Author's information

Not applicable

References

1. Eovaldi BJ, Varacallo M: **Anatomy, Shoulder and Upper Limb, Shoulder Muscles**. In: *StatPearls.* edn. Treasure Island (FL); 2018.
2. Nho SJ, Yadav H, Shindle MK, Macgillivray JD: **Rotator cuff degeneration: etiology and pathogenesis**. *Am J Sports Med* 2008, **36**(5):987-993.
3. Laron D, Samagh SP, Liu X, Kim HT, Feeley BT: **Muscle degeneration in rotator cuff tears**. *J Shoulder Elbow Surg* 2012, **21**(2):164-174.
4. Yamaguchi K, Ditsios K, Middleton WD, Hildebolt CF, Galatz LM, Teefey SA: **The demographic and morphological features of rotator cuff disease. A comparison of asymptomatic and symptomatic shoulders**. *J Bone Joint Surg Am* 2006, **88**(8):1699-1704.
5. Yamamoto A, Takagishi K, Osawa T, Yanagawa T, Nakajima D, Shitara H, Kobayashi T: **Prevalence and risk factors of a rotator cuff tear in the general population**. *J Shoulder Elbow Surg* 2010, **19**(1):116-120.
6. Barry JJ, Lansdown DA, Cheung S, Feeley BT, Ma CB: **The relationship between tear severity, fatty infiltration, and muscle atrophy in the supraspinatus**. *J Shoulder Elbow Surg* 2013, **22**(1):18-25.
7. Melis B, Nemoz C, Walch G: **Muscle fatty infiltration in rotator cuff tears: descriptive analysis of 1688 cases**. *Orthop Traumatol Surg Res* 2009, **95**(5):319-324.
8. Jamali AA, Afshar P, Abrams RA, Lieber RL: **Skeletal muscle response to tenotomy**. *Muscle & nerve* 2000, **23**(6):851-862.
9. Isaac C, Gharaibeh B, Witt M, Wright VJ, Huard J: **Biologic approaches to enhance rotator cuff healing after injury**. *J Shoulder Elbow Surg* 2012, **21**(2):181-190.
10. Gigliotti D, Xu MC, Davidson MJ, Macdonald PB, Leiter JRS, Anderson JE: **Fibrosis, low vascularity, and fewer slow fibers after rotator-cuff injury**. *Muscle & nerve* 2017, **55**(5):715-726.
11. Taylor AW, Essen B, Saltin B: **Myosin ATPase in skeletal muscle of healthy men**. *Acta Physiol Scand* 1974, **91**(4):568-570.
12. Lovering RM, Russ DW: **Fiber type composition of cadaveric human rotator cuff muscles**. *J Orthop Sports Phys Ther* 2008, **38**(11):674-680.
13. Adams GR, Bamman MM: **Characterization and regulation of mechanical loading-induced compensatory muscle hypertrophy**. *Compr Physiol* 2012, **2**(4):2829-2870.
14. Pette D, Staron RS: **Mammalian skeletal muscle fiber type transitions**. *Int Rev Cytol* 1997, **170**:143-223.

15. Pette D, Peuker H, Staron RS: **The impact of biochemical methods for single muscle fibre analysis**. *Acta Physiol Scand* 1999, **166**(4):261-277.
16. Irlenbusch U, Gansen HK: **Muscle biopsy investigations on neuromuscular insufficiency of the rotator cuff: a contribution to the functional impingement of the shoulder joint**. *J Shoulder Elbow Surg* 2003, **12**(5):422-426.
17. Butt U, Rashid MS, Temperley D, Crank S, Birch A, Freemont AJ, Trail IA: **Muscle regeneration following repair of the rotator cuff**. *Bone Joint J* 2016, **98-B**(10):1389-1394.
18. Schoenfeld BJ: **The mechanisms of muscle hypertrophy and their application to resistance training**. *J Strength Cond Res* 2010, **24**(10):2857-2872.
19. Shepstone TN, Tang JE, Dallaire S, Schuenke MD, Staron RS, Phillips SM: **Short-term high- vs. low-velocity isokinetic lengthening training results in greater hypertrophy of the elbow flexors in young men**. *Journal of applied physiology* 2005, **98**(5):1768-1776.
20. Ciciliot S, Rossi AC, Dyar KA, Blaauw B, Schiaffino S: **Muscle type and fiber type specificity in muscle wasting**. *Int J Biochem Cell Biol* 2013, **45**(10):2191-2199.
21. Klitgaard H, Mantoni M, Schiaffino S, Ausoni S, Gorza L, Laurent-Winter C, Schnohr P, Saltin B: **Function, morphology and protein expression of ageing skeletal muscle: a cross-sectional study of elderly men with different training backgrounds**. *Acta Physiol Scand* 1990, **140**(1):41-54.
22. Marx JO, Kraemer WJ, Nindl BC, Larsson L: **Effects of aging on human skeletal muscle myosin heavy-chain mRNA content and protein isoform expression**. *J Gerontol A Biol Sci Med Sci* 2002, **57**(6):B232-238.
23. Welle S, Brooks AI, Delehanty JM, Needler N, Thornton CA: **Gene expression profile of aging in human muscle**. *Physiological genomics* 2003, **14**(2):149-159.
24. Dubowitz V, Sewry CA, Oldfors A, Lane RJM: **Muscle biopsy : a practical approach**; 2013.
25. Kim K, Kim HG, Song D, Yoon JY, Chung ME: **Ultrasound Dimensions of the Rotator Cuff and Other Associated Structures in Korean Healthy Adults**. *J Korean Med Sci* 2016, **31**(9):1472-1478.
26. Lassche S, Stienen GJ, Irving TC, van der Maarel SM, Voermans NC, Padberg GW, Granzier H, van Engelen BG, Ottenheijm CA: **Sarcomeric dysfunction contributes to muscle weakness in facioscapulohumeral muscular dystrophy**. *Neurology* 2013, **80**(8):733-737.
27. Gundersen HJ, Jensen EB: **The efficiency of systematic sampling in stereology and its prediction**. *J Microsc* 1987, **147**(Pt 3):229-263.

28. Gundersen HJ: **Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson**. *J Microsc* 1986, **143**(Pt 1):3-45.
29. Goutallier D, Postel JM, Bernageau J, Lavau L, Voisin MC: **Fatty muscle degeneration in cuff ruptures. Pre- and postoperative evaluation by CT scan**. *Clin Orthop Relat Res* 1994(304):78-83.
30. Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A *et al*: **Some new, simple and efficient stereological methods and their use in pathological research and diagnosis**. *Apmis* 1988, **96**(5):379-394.
31. Briguet A, Courdier-Fruh I, Foster M, Meier T, Magyar JP: **Histological parameters for the quantitative assessment of muscular dystrophy in the mdx-mouse**. *Neuromuscular disorders : NMD* 2004, **14**(10):675-682.
32. Larsen KB: **Using the Optical Fractionator to Estimate Total Cell Numbers in the Normal and Abnormal Developing Human Forebrain**. *Front Neuroanat* 2017, **11**:112.
33. Gundersen HJ, Jensen EB, Kieu K, Nielsen J: **The efficiency of systematic sampling in stereology--reconsidered**. *J Microsc* 1999, **193**(Pt 3):199-211.
34. Oremus M, Oremus C, Hall GB, McKinnon MC, Ect, Cognition Systematic Review T: **Inter-rater and test-retest reliability of quality assessments by novice student raters using the Jadad and Newcastle-Ottawa Scales**. *BMJ Open* 2012, **2**(4).
35. Lieber RL, Friden J: **Functional and clinical significance of skeletal muscle architecture**. *Muscle & nerve* 2000, **23**(11):1647-1666.
36. Roberts MD, Haun CT, Mobley CB, Mumford PW, Romero MA, Roberson PA, Vann CG, McCarthy JJ: **Physiological Differences Between Low Versus High Skeletal Muscle Hypertrophic Responders to Resistance Exercise Training: Current Perspectives and Future Research Directions**. *Front Physiol* 2018, **9**:834.
37. Lundgreen K, Lian OB, Engebretsen L, Scott A: **Lower muscle regenerative potential in full-thickness supraspinatus tears compared to partial-thickness tears**. *Acta Orthop* 2013, **84**(6):565-570.
38. Gigliotti D, Leiter JR, Macek B, Davidson MJ, MacDonald PB, Anderson JE: **Atrophy, inducible satellite cell activation, and possible denervation of supraspinatus muscle in injured human rotator-cuff muscle**. *Am J Physiol Cell Physiol* 2015, **309**(6):C383-391.
39. Gibbons MC, Sato EJ, Bachasson D, Cheng T, Azimi H, Schenk S, Engler AJ, Singh A, Ward SR: **Muscle architectural changes after massive human rotator cuff tear**. *J Orthop Res* 2016, **34**(12):2089-2095.

40. Greenspoon JA, Petri M, Warth RJ, Millett PJ: **Massive rotator cuff tears: pathomechanics, current treatment options, and clinical outcomes**. *J Shoulder Elbow Surg* 2015, **24**(9):1493-1505.
41. Godeneche A, Elia F, Kempf JF, Nich C, Berhouet J, Saffarini M, Collin P, Sofcot: **Fatty infiltration of stage 1 or higher significantly compromises long-term healing of supraspinatus repairs**. *J Shoulder Elbow Surg* 2017, **26**(10):1818-1825.
42. Meyer GA, Farris AL, Sato E, Gibbons M, Lane JG, Ward SR, Engler AJ: **Muscle progenitor cell regenerative capacity in the torn rotator cuff**. *J Orthop Res* 2015, **33**(3):421-429.
43. Lexell J: **Human aging, muscle mass, and fiber type composition**. *J Gerontol A Biol Sci Med Sci* 1995, **50 Spec No**:11-16.
44. Short KR, Vittone JL, Bigelow ML, Proctor DN, Coenen-Schimke JM, Rys P, Nair KS: **Changes in myosin heavy chain mRNA and protein expression in human skeletal muscle with age and endurance exercise training**. *Journal of applied physiology* 2005, **99**(1):95-102.
45. Blaauw B, Schiaffino S, Reggiani C: **Mechanisms modulating skeletal muscle phenotype**. *Compr Physiol* 2013, **3**(4):1645-1687.
46. Baldwin KM, Haddad F, Pandorf CE, Roy RR, Edgerton VR: **Alterations in muscle mass and contractile phenotype in response to unloading models: role of transcriptional/pretranslational mechanisms**. *Front Physiol* 2013, **4**:284.
47. Pette D, Staron RS: **Transitions of muscle fiber phenotypic profiles**. *Histochem Cell Biol* 2001, **115**(5):359-372.
48. Ashry R, Schweitzer ME, Cunningham P, Cohen J, Babb J, Cantos A: **Muscle atrophy as a consequence of rotator cuff tears: should we compare the muscles of the rotator cuff with those of the deltoid?** *Skeletal Radiol* 2007, **36**(9):841-845.
49. de Witte PB, Werner S, ter Braak LM, Veeger HE, Nelissen RG, de Groot JH: **The Supraspinatus and the Deltoid - not just two arm elevators**. *Hum Mov Sci* 2014, **33**:273-283.
50. Lee YS, Kim JY, Kim KI, Ki SY, Chung SW: **Effect of Fatty Acid-Binding Protein 4 Inhibition on Rotator Cuff Muscle Quality: Histological, Biomechanical, and Biomolecular Analysis**. *Am J Sports Med* 2019, **47**(13):3089-3099.
51. Mandroukas A, Metaxas T, Kesidis N, Christoulas K, Vamvakoudis E, Stefanidis P, Heller J, Ekblom B, Mandroukas K: **Deltoid muscle fiber characteristics in adolescent and adult wrestlers**. *J Sports Med Phys Fitness* 2010, **50**(2):113-120.

549 **Figure legends**

550 **Figure 1. Stereological setup.** A typical step in analyzing a biopsy, showing the counting
551 frame with exclusion (red) and inclusion (green) guards, muscle cells and the delineation
552 (yellow) of the biopsy.

553 **Figure 2. Muscle fiber composition after RC tendon tear.** (A) Estimation of the total
554 number of muscle fiber cells/mm² demonstrating significantly more type 2 cells in SS
555 compared to DT. (B) Fractions of type 1 cells and type 2 cells in SS and DT demonstrating a
556 significantly increased percentage of type 1 fibers compared to type 2 fibers in the DT
557 muscle. Values are mean (%) ± SD, **p<0.01.

558 **Figure 3. Histograms representing fiber diameter distribution in SS and DT muscle**
559 **after RC tendon tear.** (A-D) Distribution of type 1 (A) and type 2 (B) fibers in men and type
560 1 (C) and type 2 (D) fibers in women. Lines indicate limits for atrophy and hypertrophy.
561 Please note the shift towards the left of the muscle fiber diameter distribution in SS muscle
562 compared to the distribution of DT muscle fiber diameter.

563 **Figure 4. Myosin heavy chain staining of SS muscle tissue.** Representative
564 immunohistochemical staining of type 1 (red) and type 2 (brown) myosin heavy chain
565 positive muscle fibers in a SS muscle from a female with a 28-months old RC tendon tear.
566 Dotted rectangle demarcates an area with normal-sized muscle fibers, whereas the solid
567 rectangle demarcates an area dominated by atrophied type 1 and type 2 muscle fibers. Note
568 the massive fatty infiltration in this SS muscle 28 months after RC tear. Scale bar: 200 μm.

569 **Figure 5. MABP4 protein levels in SS and DT muscle tissue.** ELISA for MABP4 protein
570 levels demonstrating comparable MABP4 levels in SS and DT muscle after RC tendon tear.
571 Values are mean (pg/mg) ± SD, n=16/group.

572

Figures

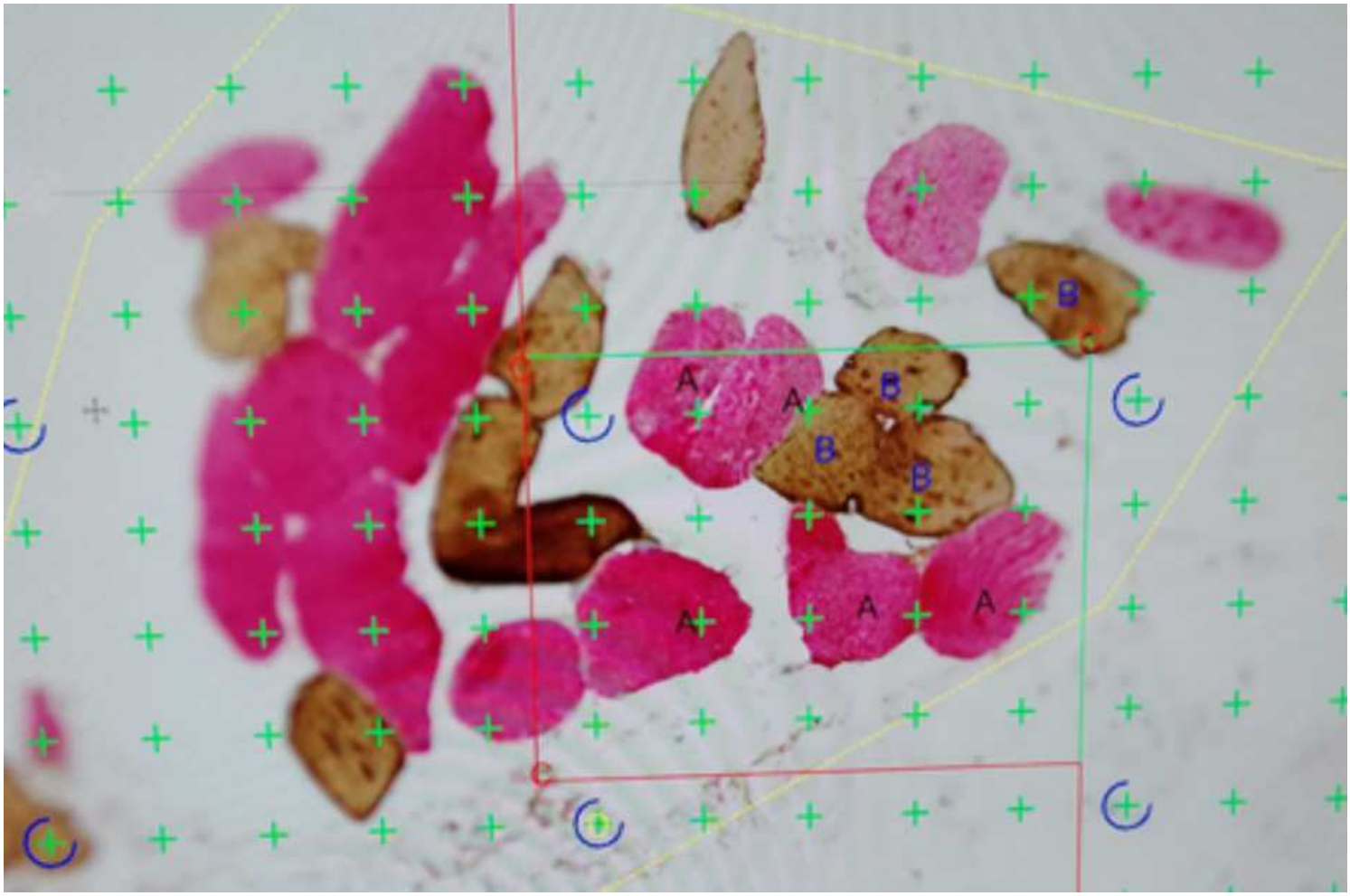

Figure 1

Stereological setup. A typical step in analyzing a biopsy, showing the counting frame with exclusion (red) and inclusion (green) guards, muscle cells and the delineation (yellow) of the biopsy.

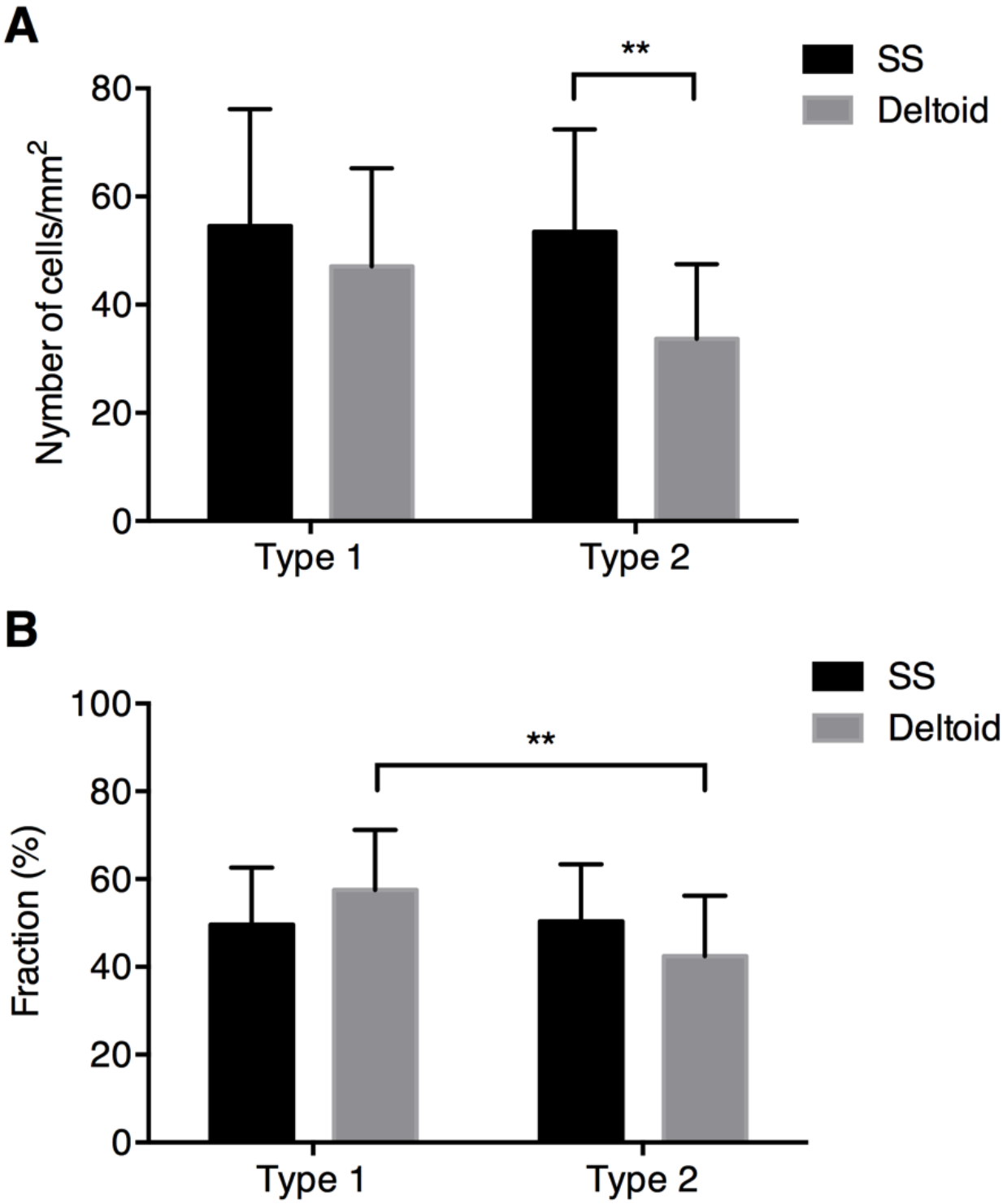

Figure 2

Muscle fiber composition after RC tendon tear. (A) Estimation of the total number of muscle fiber cells/mm² demonstrating significantly more type 2 cells in SS compared to DT. (B) Fractions of type 1 cells and type 2 cells in SS and DT demonstrating a significantly increased percentage of type 1 fibers compared to type 2 fibers in the DT muscle. Values are mean (%) ± SD, **p<0.01.

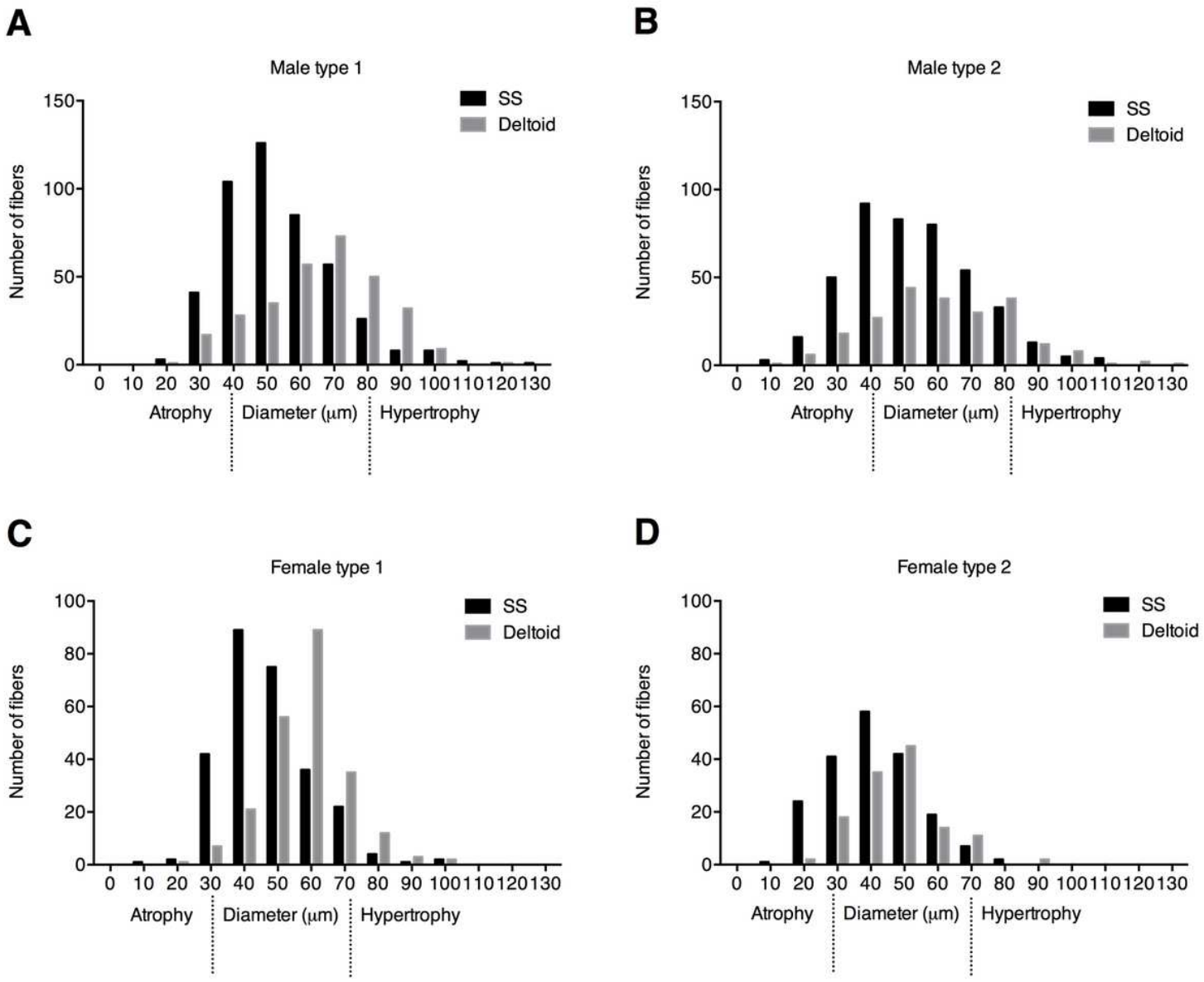

Figure 3

Histograms representing fiber diameter distribution in SS and DT muscle after RC tendon tear. (A-D) Distribution of type 1 (A) and type 2 (B) fibers in men and type 1 (C) and type 2 (D) fibers in women. Lines indicate limits for atrophy and hypertrophy. Please note the shift towards the left of the muscle fiber diameter distribution in SS muscle compared to the distribution of DT muscle fiber diameter.

Figure 4

Myosin heavy chain staining of SS muscle tissue. Representative immunohistochemical staining of type 1 (red) and type 2 (brown) myosin heavy chain positive muscle fibers in a SS muscle from a female with a 28-months old RC tendon tear. Dotted rectangle demarcates an area with normal-sized muscle fibers,

whereas the solid rectangle demarcates an area dominated by atrophied type 1 and type 2 muscle fibers. Note the massive fatty infiltration in this SS muscle 28 months after RC tear. Scale bar: 200 µm.

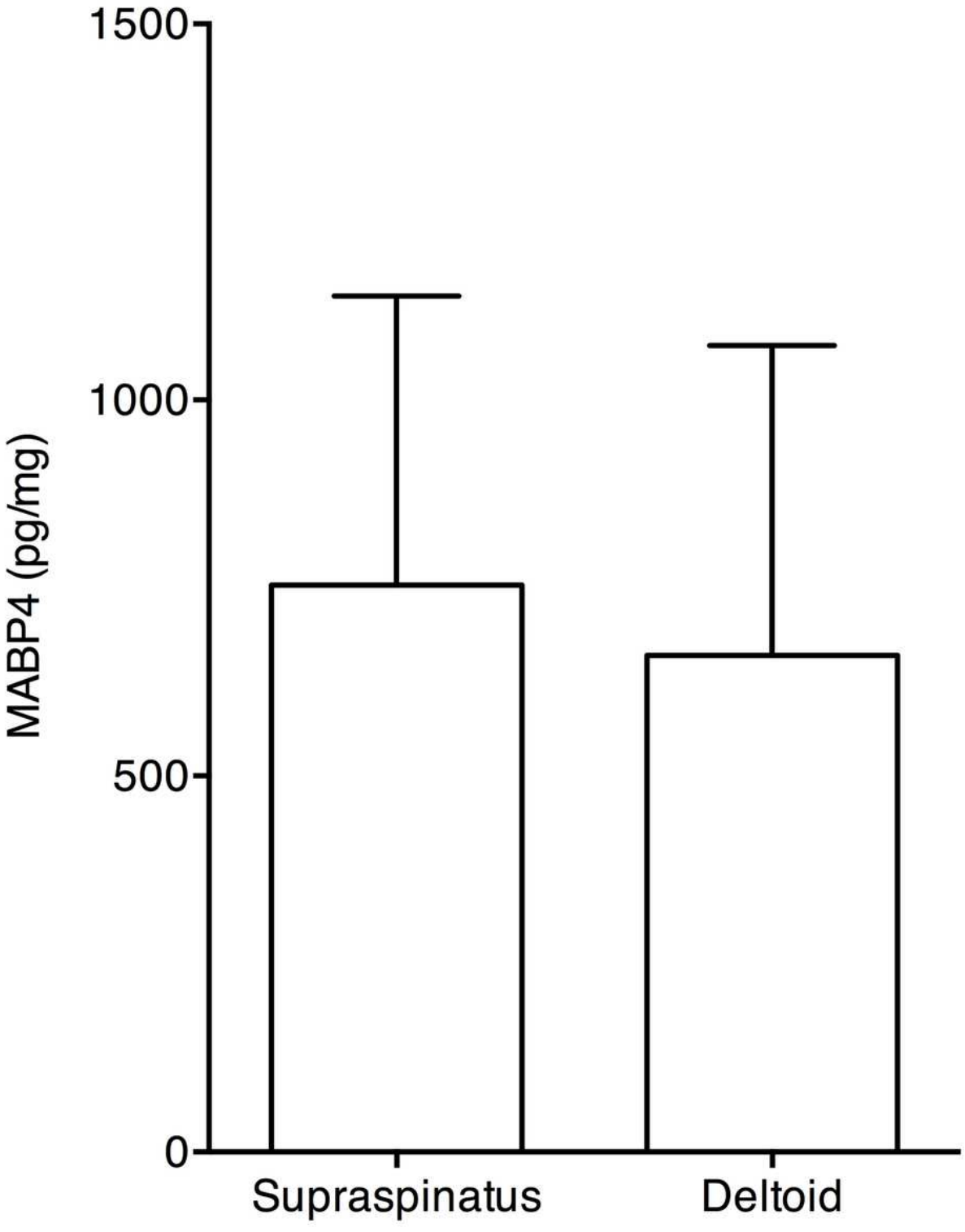

Figure 5

MABP4 protein levels in SS and DT muscle tissue. ELISA for MABP4 protein levels demonstrating comparable MABP4 levels in SS and DT muscle after RC tendon tear. Values are mean (pg/mg) ± SD, n=16/group.