



## The inflammatory response of the supraspinatus muscle in rotator cuff tear conditions

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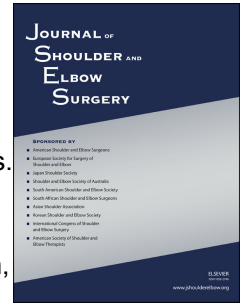
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**The inflammatory response of the supraspinatus muscle in rotator cuff tear conditions.**

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**Running title:** Muscle inflammation and rotator cuff

**Conflicts of interest:** None

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## **Ethics**

Ethical approval was granted by The Regional Committees on Health Research Ethics for Southern Denmark, J. No. S-20160037, and the study reported to The Danish Data Protection Agency (16/9714). The project was approved by the Orthopedic Research Board, Odense University Hospital.

# 1        **The inflammatory response of the supraspinatus muscle in rotator cuff tear conditions.**

## 2                                **Running title: Muscle inflammation and rotator cuff**

### 3 4        **Abstract**

5        **Background:** Rotator cuff (RC) disorders involve a spectrum of shoulder conditions from early  
6        tendinopathy to full-thickness tears leading to impaired shoulder function and pain. The pathology  
7        of RC disorder is, nonetheless, still largely unknown. It is our hypothesis that supraspinatus (SS)  
8        tendon tear leads to sustained inflammatory changes of the SS muscle along with fatty infiltration  
9        and muscle degeneration, which are threshold markers for poor RC muscle function. The aim of this  
10       study was to determine the extent of this muscle inflammation in conjunction with lipid  
11       accumulation and fibrosis in RC tear conditions.

12       **Methods:** We used proteomics, histology, electrochemiluminescence immunoassay, and qPCR  
13       analyses to evaluate inflammatory and degenerative markers and fatty infiltration in biopsies from  
14       22 patients undergoing surgery with repair of a full- thickness supraspinatus (SS) tendon tear.

15       **Results:** Bioinformatic analysis showed that proteins involved in innate immunity, extracellular  
16       matrix organization, and lipid metabolism were among the most upregulated whereas mitochondrial  
17       electronic transport chain along with muscle fiber function were among the most downregulated.  
18       Histological analysis confirmed changes in muscle fiber organization and the presence of  
19       inflammation and fatty infiltration. Inflammation appeared to be driven by a high number of  
20       infiltrating macrophages, accompanied by elevated matrix metalloprotease levels and changes in  
21       transforming growth factor- $\beta$  and cytokine levels in the SS compared to the deltoid muscle.

22       **Conclusions:** We demonstrated massive SS muscle inflammation after tendon tear combined with  
23       fatty infiltration and degeneration. The regulation of tissue repair is thus extremely complex, and it  
24       may have opposite effects at different time points of healing. Inhibition or stimulation of muscle  
25       inflammation may be a potential target to enhance outcome of the repaired torn RC.

26 **Level of evidence:** Basic Science Study; Histology, Molecular and Cell Biology

27 **Keywords:** Shoulder disorder, muscle damage, proteomics, protein changes, extracellular matrix  
28 degeneration, fatty infiltration

29

### 30 **Introduction**

31 Rotator cuff (RC) lesions are some of the most common shoulder conditions in humans and can  
32 lead to weakness, pain, and limited/reduced mobility. The prevalence of RC tears is age-dependent,  
33 and both partial and full-thickness RC tears increase markedly after 50 years of age<sup>48</sup>. The etiology  
34 of RC diseases is multifactorial with frequent involvement of the supraspinatus (SS) tendon<sup>5</sup>. Full-  
35 thickness SS tears do not heal spontaneously, and surgically repaired RC tears tend to heal poorly  
36<sup>15; 33</sup>. A recent Cochrane review questioned whether repair of RC tears provides meaningful benefit  
37 to patients with symptomatic RC tears<sup>24</sup>. There is, therefore, a pressing need to better understand  
38 the pathophysiology behind RC tear conditions in order to improve results after surgical repair of  
39 RC tendon tears.

40 In full-thickness RC tears, increased numbers of immune cells have been demonstrated in the  
41 synovial tissue adjacent to the SS tendon<sup>1</sup> and an increase in tear size correlated with a greater pro-  
42 inflammatory response in the synovium<sup>6; 45</sup>. Recent data suggest that the RC muscles also become  
43 inflamed in the presence of an RC tear<sup>14</sup>, and results from experimental models suggest that acute  
44 inflammation plays a detrimental role in the onset of chronic muscle damage following RC tears<sup>16;</sup>  
45<sup>29</sup>. It is also generally agreed that chronic RC tendon lesion leads to degenerative muscle changes  
46 in the form of fatty infiltration and fibrosis<sup>10</sup>.

47 Several animal studies have provided evidence of significant increases in inflammatory cytokines,  
48 growth factors, and matrix metalloproteases (MMPs), indicating muscle inflammation following  
49 experimental RC tendon tear<sup>16; 29</sup>. Changes in the biology of human RC muscles in tear conditions

50 remain poorly defined at the cellular level, however, and RC muscle as a target for inflammation  
51 following RC tear is only sparsely understood<sup>14;23</sup>.  
52 The aim of this study was to provide a more robust understanding of the inflammatory environment  
53 of human SS muscle in RC tear conditions. It is our hypothesis that inflammatory conditions  
54 precede disturbances of the muscle architecture, and eventually lead to RC muscle fatty infiltration  
55 and degeneration. To investigate this, we applied quantitative proteomics followed by histological,  
56 multiplex chemiluminescence, and qPCR analyses of inflammation in SS and deltoid muscle  
57 biopsies that were harvested from patients undergoing surgery for RC tears.

58

## 59 **Materials and methods**

### 60 **Patient cohort**

61 Patients (n=22) with a relevant shoulder trauma and clinical signs of an RC lesion were recruited  
62 (Supplementary Table 1). Median age of lesions was 3.3 months (IQR 2-14 months). Patients  
63 underwent preoperative magnetic resonance imaging scan revealing SS tendon tear, and all tears  
64 were confirmed at surgery. Informed written consent was obtained from all patients. The workflow  
65 of the project is presented in Figure 1.

66

### 67 **Human tissue**

68 The RC and musculotendinous junction of the SS muscle were gently débrided from fascia and  
69 bursal tissue using a blunt shaver. SS tendon and muscle biopsies were harvested from the edge of  
70 the tendon and approximately one centimeter medial to the tendon, respectively, under direct  
71 visualization from the arthroscope. Comparative biopsies were taken from assumed healthy,  
72 ipsilateral deltoid muscles. Biopsies were snap-frozen on dry ice and stored at -80°C or fixed in  
73 10% neutral buffered formalin and embedded in paraffin.

74

75 **Blood samples**

76 Blood samples were obtained in EDTA coated test tubes immediately prior to surgery to estimate  
77 preoperative peripheral inflammation. Hemoglobin, C-reactive protein and white blood cell counts  
78 were analyzed. The patient cohort was also tested for presence of rheumatic factors (anti-nuclear,  
79 anti-cyclic citrullinated, and mitochondrial antibodies and rheumafactor).

80

81 **Histology and Immunohistochemistry**

82 SS or deltoid muscle tissue was sectioned into 2 $\mu$ m thick sections on a microtome.

83 Immunohistochemistry for CD68 was performed on the OMNIS platform (Dako/Agilent, Denmark)  
84 using mouse anti-CD68 (1:50, Clone PG-M1) antibody and EnVision<sup>TM</sup> FLEX as detection system,  
85 while immunohistochemistry for FOXP3, CD3, and adipophilin was performed on a BenchMark  
86 Ultra immunostainer (Ventana Medical Systems, AZ, USA) using mouse anti-FOXP3 (1:40, clone  
87 236A/E7), rabbit anti-CD3 (“*ready-to-use*”, clone 2GV6), and mouse anti-adipophilin (1:50, clone  
88 AP125) antibodies and the OptiView-DAB detection system. Parallel sections were stained with  
89 hematoxylin and eosin (HE).

90 Slides were scanned on a NanoZoomer 2.0 RS scanner (Hamamatsu Photonics, Visiopharm,

91 Denmark). To produce merged images, NDP view (Hamamatsu Photonics, version 2.6.17) was

92 applied to find identical regions on neighboring cells stained for FOXP3 and CD3, respectively.

93 The images were processed and merged using Photoshop C6 (Adobe Systems, CA, USA) and

94 ImageJ/Fiji. The area with muscle tissue in each biopsy was determined using the freehand region

95 function and the number of CD3 and FOXP3 within this area was manually counted at 20x

96 magnification.

97

98 **Proteomics**99 *Homogenization of tissue biopsies*

100 Approximately 1mm<sup>3</sup> was cut from the frozen tendon and muscle biopsies and was homogenized by  
101 bead beating using ss 0.9-2.0mm beads (Bullet blender Gold, Next Advance Inc, USA) in reducing  
102 lysis buffer (5% sodium deoxycholate (SDC; Sigma Aldrich, USA), protease inhibitors (cOmplete,  
103 Roche) in 50mM TEAB (Sigma Aldrich). Protein concentration in lysates was determined by  
104 protein A280 and lysates stored at -80°C (DeNoviX, USA).

105 Samples were further processed using an optimized SDC filter-aided sample preparation protein  
106 digestion (SDC-FASP) essentially as <sup>4</sup> using 100µg protein starting material in 10kDa molecular  
107 weight cutoff spin-filters (Merch Millipore, Singapore). The lysates were reduced (10mM TCEP)  
108 and alkylated (50mM Chloroacetamide) each for 15min in digestion buffer (5% SDC in 50mM  
109 TEAB; Sigma-Aldrich). Overnight trypsin digestion at 37°C was performed by addition of 200µl  
110 0.5% digestion buffer containing Trypsin 1:50 (v:v; Pierce, USA). Peptides were extracted by  
111 acidification and phase-separation, where 3:1 (v/v) ethyl acetate of sample volume was added and  
112 acidified by adding Trifluoroacetic acid (Sigma-Aldrich) to a final concentration of 1% (pH<2)  
113 followed by centrifugation. The collected lower aqueous phase containing the peptides was dried in  
114 a vacuum centrifuge and dissolved in 0.1M TEAB. Peptide concentration in lysates was determined  
115 by protein A280 and lysates stored at -80°C (DeNoviX, USA).

116 *iTRAQ labeling*

117 A total of 5µg of each sample was labeled with a 4-plex iTRAQ kit (AB Sciex, USA) according to  
118 manufacturer's instructions. Briefly, samples were re-dissolved to total volume of 25µL 0.1M  
119 TEAB, pH 8.5 while 290µL 96% ethanol were added to iTRAQ reagents. Next, 50µL of the  
120 iTRAQ reagents was transferred to the samples, which were then labelled, mixed after 1h of

121 incubation at room temperature dried, and resuspended in 2% acetonitrile (AcN), 0.1% TFA  
122 (Sigma-Aldrich).

### 123 *iTRAQ sample analysis*

124 Samples were analyzed per UPLC-TandemMS in technical duplicates. Labeled peptides were  
125 separated by a nanoUPLC system (Thermo Scientific, USA) coupled online to a Q Exactive HF MS  
126 (Thermo Scientific) using a reverse phase C18 trapping column setup with 75cm main column  
127 (Thermo Scientific) with loading in 2% solvent B (0.1% FA in AcN) gradient and separated by  
128 176min gradient from 11%B to 30%B with a constant flow rate at 250nL/min. MS was operated in  
129 positive mode using Top10 data-dependent acquisition (MS1 m/z 375-1,500 at R 120,000) and  
130 tandems sequencing using fixed m/z range at 110 and a MS2 resolution of 15,000.

### 131 *Database searches*

132 Raw data were processed using Proteome Discoverer v2.3.0.523 (Thermo Scientific). Sequest HT  
133 was set as the search engine against the reviewed Uniprot Homo sapiens reference protein database  
134 (09/2017). iTRAQ 4-plex labeling of N-terminal and lysine and carbamidomethylation (C) were set  
135 as fixed modifications while oxidation (M), deamidation (N/Q) and protein N-terminal acetylation  
136 were included as variable modifications. Precursor mass tolerance and fragment mass tolerance  
137 were set at 10ppm and 0.05Da, respectively. PSMs were filtered using percolator with a strict false  
138 discovery rate (FDR) of 1% and a relaxed FDR of 5%. Unique and razor peptides were used for  
139 quantification, and iTRAQ channels were normalized to total peptide amount. Master proteins were  
140 filtered for high protein FDR confidence. MS data have been deposited to the ProteomeXchange  
141 Consortium via the PRIDE<sup>39</sup> partner repository with the dataset identifier PXD014037.

### 142 *Bioinformatics analyses and functional annotation of regulated proteins*

143 Normalized abundances were used for further analyses of proteins identified with two or more  
144 unique peptides. Data distribution was assessed with Perseus v.1.5.3.2 software and differentially

145 regulated features were selected using t-test with a post-hoc background-based adjusted p-  
146 value < 0.05<sup>35</sup>. Venny 2.1 (<http://bioinfogp.cnb.csic.es/tools/venny>) was used to compare the  
147 regulated proteins among the different comparisons. ToppGene Suite<sup>8</sup> was used for functional  
148 enrichment of regulated proteins according to Gene Ontology (GO) terms and pathway analysis.  
149 Enriched lists were further accessed by String app on Cytoscape v3.6.1<sup>44</sup>.

150

151 **Reverse transcription quantitative PCR (RT-qPCR) analysis of *FOXP3*, *MYOG*, and *MMP13***  
152 **in SS and deltoid muscle tissue**

153 *RNA extraction*

154 Muscle biopsies (n=18/group) were isolated using TRIzol<sup>®</sup> Reagent. Phase separation was  
155 performed using chloroform and isopropyl alcohol was used to precipitate RNA. The RNA  
156 concentrations and purities were determined using a Nanodrop Spectrophotometer (Thermo  
157 Scientific).

158 *cDNA synthesis*

159 RNA samples were diluted to obtain a concentration of 250ng/μL, and reverse transcription was  
160 performed using an Applied Biosystem kit according to the manufacturer's instructions. The  
161 synthesis was performed using an MJ Research PTC-225 Gradient Thermal Cycler (Marshall  
162 Scientific). cDNA samples were diluted to lower the concentrations to ~50ng/μL.

163 *RT-qPCR*

164 RT-qPCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad) and  
165 analyzed using SYBR green. Samples were run against standard curves generated from serial  
166 dilutions from a pool of all samples. Values were normalized to *ACTB* ( $\beta$ -actin) as the reference  
167 gene and calibrated to a pool of cDNA obtained from one pectoralis and one subscapularis muscle  
168 biopsy. Triplicates of all samples, standards, and negative controls were conducted. To ensure no

169 sign of primer dimer formation or contamination, a no amplification control (NAC), a no template  
170 control (NTC), and a no reverse transcriptase (NRT) were included as controls.

171 RT-qPCR cycling conditions were as follows: 10min at 95°C, followed by 40 cycles of denaturing  
172 at 95°C for 15 seconds, 30 seconds at annealing temperature, and extension at 72°C at 30 seconds.

173 Primer sequences were: *ACTB*, sense 5'-GGCCACGGCTGCTTC-3' and anti-sense 5'-

174 GTTGGCGTACAGGTCTTTGC-3' ( $T_a$  52°C and  $T_m$  84°C), *FOXP3*, sense 5'-

175 CCCGGATGTGAGAAGGTCTT-3' and anti-sense 5'-TTCTCCTTCTCCAGCACCCAG-3' ( $T_a$

176 57°C and  $T_m$  82°C), *MYOG*, sense 5'-GCCCTGATGCTAGGAAGCC-3' and anti-sense 5'-

177 CTGAATGAGGGCGTCCAGTC-3' ( $T_a$  70°C and  $T_m$  85°C), and *MMP13*, sense 5'-CGC CAG

178 ACA AAT GTG ACC CT-3' and anti-sense 5'-CAG GCG CCA GAA GAA TCT GT-3' ( $T_a$  55°C

179 and  $T_m$  77°C). Primer specificity was ensured by generation and evaluation of melting curves.

180 Primers were purchased from TAG Copenhagen.

181

## 182 **Electrochemiluminescence analysis**

### 183 *Protein purification*

184 Tendon and muscle samples were homogenized at 4°C in Mesoscale Lysis buffer containing

185 Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich) and Complete Mini EDTA-free Protease

186 Inhibitor (Roche). Protein content was measured by the bicinchoninic acid method using the

187 Thermo Scientific Micro BCA<sup>TM</sup> Protein assay Kit (Pierce Chemical Co)<sup>40</sup>.

### 188 *Multiplex analysis*

189 Protein concentrations in tendon and muscle samples were measured using an MSD human U-Plex

190 Biomarker Multiplex Kit, a U-PLEX human TGF- $\beta$  Combo Kit, a human MMP 3-Plex Ultra-

191 Sensitive Kit, and human TNF-RI and TNF-RII Ultra-Sensitive Kits (all from Mesoscale), using the

192 MSD QuickPlex (SQ120) Plate Reader (Mesoscale) according to the manufacturer's instructions.

193 ICAM-1 and VCAM-1 analyses were performed on SS and deltoid muscle tissue using V-PLEX  
194 Vascular Injury Panel 2 (Mesoscale). Samples were run in duplex and diluted 2- or 4-fold in Diluent  
195 41 prior to measurement. Data were analyzed using MSD Discovery Workbench software. The  
196 lower limit of detection was a calculated concentration based on a signal 2.5 standard deviations  
197 (SD) above the blank (zero) calibrator and coefficient of variation (CV) values below 25% were  
198 accepted.

199

## 200 **Statistical analysis**

201 To examine differences in protein expression between SS and deltoid muscle tissue, paired  
202 Student's t-test was used. Correlation analyses between cytokine, MMPs, and growth factors versus  
203 age of lesion used Spearman's test. To examine the correlation between the relative expression of  
204 *FOXP3*, *MYOG*, and *MMP13* in SS and deltoid muscles, a paired Wilcoxon test was carried out.  
205 The outlier test ROUT was used to identify and remove outliers more than 2 SD from the dataset.  
206 All statistical analyses were carried out using GraphPad Prism. P-values  $\leq 0.05$  were considered  
207 statistically significant. Data are presented as mean  $\pm$  SD or median (25, 75 interquartile range,  
208 IQR).

209

## 210 **Results**

### 211 **Mass spectrometry-based proteomics analysis shows protein regulation upon RC lesion**

212 Using quantitative mass spectrometry (MS) proteomics, a total of 2,463 proteins were identified, of  
213 which 1,895 had two or more unique peptides (Supplementary Table S2). Moreover, 417 quantified  
214 proteins were shared by all tissues of all patients and could be assessed by principal component  
215 analysis (PCA) (Supplementary Figure 1A), which showed a clear distribution pattern even in the  
216 absence of well-delimited clusters.

217 To better understand protein regulation underlying RC pathology, SS muscle protein expression  
218 pattern was compared to deltoid as a non-RC shoulder muscle control. A total of 239 proteins were  
219 regulated. Of these, 114 were more highly expressed in the SS muscle (Figure 2A, Supplementary  
220 Table S3). Gene ontology analysis showed 'extracellular matrix organization' and 'neutrophil  
221 degranulation' among the most enriched biological processes (Supplemental Figure 2, Table S4)  
222 while 'degradation of extracellular matrix', 'innate immune system' and 'neutrophil degranulation'  
223 were among the enriched pathways of upregulated proteins (Figure 2B, Supplementary Figure 2,  
224 and Supplementary Table S4). Interestingly, 'mitochondrial cellular localization', and 'muscle  
225 system process' were among the enriched annotations of downregulated proteins (Figure 2B,  
226 Supplementary Figure 3, and Table S4). A detailed list of regulated proteins involved in the above-  
227 mentioned processes, along with experimental ratio, are provided in Figure 2C-K.

228 To address the molecular response of different RC tissues, the comparison between SS muscle and  
229 SS tendon showed 139 differentially expressed proteins (Supplementary Table S5). Pathways or  
230 gene ontologies related to immune response or inflammatory processes were not enriched among  
231 these regulated proteins.

232 In contrast, pointing towards a common molecular signature between SS muscle and SS tendon in  
233 RC disease, 38 proteins were regulated in both tissues when compared to deltoid muscle  
234 (Supplementary Figure 1B, Supplementary Tables S6 and S7). Several of these proteins were  
235 related to catalytic activity, with mitochondrial protein complex being the main cellular component  
236 (Supplementary Figure 1C).

237

### 238 **RC lesion results in atrophy and lipid accumulation**

239 Staining with hematoxylin & eosin revealed muscle fiber changes already 1½ months after tendon  
240 lesion represented by internalization of nuclei and muscle fibers of varying size after the tendon tear

241 (Figure 3A). In addition, pathological infiltrating adipocytes were detected, as substantial fat  
242 infiltration was seen together with arrays of intracellular myonuclei and varying fiber size,  
243 suggestive of degeneration (Figure 3B,C).

244 Inflammatory changes appeared to be intensified from 1½ months to 6 months with abundant  
245 stromal inflammatory phagocytic cells represented by the presence of CD68<sup>+</sup> macrophages (Figure  
246 3D and 3E). At 24 months, degeneration was clearly seen with a substantial proportion of muscle  
247 cells replaced by fat cells indicative of muscle cell degeneration (Figure 3F). At 24 months, absent  
248 or few CD68<sup>+</sup> positive cells indicated decreased inflammation. The deltoid muscle did not  
249 demonstrate similar inflammatory and degenerative changes (Figure 3G-L).

250 Adipophilin/perilipin-2 immunohistochemistry for detection of lipids in myofibers demonstrated  
251 that adipophilin was localized to the surface of intra-cellular lipid droplets (Figure 4). The  
252 expression varied both between patients (please compare Figure 4A and Figure 4C) and between  
253 muscles from the same patient (please compare Figure 4A with Figure 4B). Regional differences  
254 within muscles were also seen (Figure 4), and some individual fibers presented increased lipid  
255 accumulation both as number and size of droplets.

256

### 257 **RC tendon tear leads to changes in inflammatory mediators in SS muscle**

258 None of the patients showed any signs of peripheral inflammation as mean blood leukocyte counts,  
259 hemoglobin, and C-reactive protein values were within normal ranges and all patients were negative  
260 for rheumatic factors. Given the findings of significant changes in the proteome of SS compared to  
261 deltoid muscle after RC tear, we investigated changes in a variety of pro- and anti-inflammatory  
262 cytokines, chemokines, receptors, and growth factors (Figure 5 and Table 1). We found lower  
263 CCL19 levels (Figure 5A) but higher CXCL5 levels (Figure 5B) in the SS compared to the deltoid  
264 muscle. IL-1 $\beta$  (Figure 5C), IL-6 (Figure 5D), IL-8 (Figure 5E), and IL-33 levels (Figure 5F) were

265 higher in SS compared to deltoid muscle. IL-7 levels (Figure 5G), IL-15 (Figure 5H), IL-17A  
266 (Figure 5I), and IFN- $\alpha$ 2a levels (Figure 5J) were in SS compared to deltoid muscle. Despite  
267 comparable levels of TNF (Figure 5K), TNFR1 (Figure 5L) and TNFR2 (Figure 5M) levels were  
268 changed in the SS muscle compared to the deltoid after RC tendon tear. G-CSF levels were lower in  
269 SS muscle compared to the deltoid (Figure 5N).

270 We observed no significant correlation between age of lesion and CCL19 ( $r=-0.05$ ,  $p=1$ ), CXCL5  
271 ( $r=-0.26$ ,  $p=0.31$ ), IL-1 $\beta$  ( $r=-0.30$ ,  $p=0.3$ ), IL-6 ( $r=-0.48$ ,  $p=0.07$ ), IL-8 ( $r=-0.43$ ,  $p=0.11$ ), IL-33  
272 ( $r=0.16$ ,  $p=0.54$ ), IL-7 ( $r=0.35$ ,  $p=0.39$ ), IL-15 ( $r=0.07$ ,  $p=0.79$ ), IL-17A ( $r=0.09$ ,  $p=0.76$ ), IFN-  
273  $\alpha$ 2a ( $r=0.39$ ,  $p=0.3$ ), TNF ( $r=-0.39$ ,  $p=0.15$ ), TNFR1 ( $r=-0.4$ ,  $p=0.29$ ), TNFR2 ( $r=-0.16$ ,  $p=0.66$ ),  
274 or G-CSF ( $r=0.16$ ,  $p=0.71$ ).

275 Finally, several cytokine levels were similar in SS and deltoid muscle (Table 1). The concentration  
276 of most cytokines, chemokines, growth factors, and MMPs was high in the SS tendon  
277 (Supplementary Table 8).

278

### 279 **The regenerative potential appears to be impaired in the SS muscle after RC tendon tear**

280 IL-17 production characterizes pro-inflammatory T helper 17 lymphocytes (Th17) and innate  
281 immune cells<sup>11</sup>. Th17 has been shown to have opposing effects in the immune response from  
282 regulatory T cells (Treg)<sup>49</sup>, which is important in muscle regeneration (reviewed in<sup>7</sup>) and whose  
283 master gene is the transcription factor Forkhead box P3 (FOXP3). We found decreased IL-17A  
284 levels in SS muscle compared to deltoid muscle (Figure 5G). Therefore, we next investigated  
285 *FOXP3* mRNA expression and found that *FOXP3* mRNA levels were significantly lower in SS  
286 muscle compared to deltoid muscle (Figure 6A). However, when we counted FOXP3<sup>+</sup> cells (Figure  
287 6B) and CD3<sup>+</sup> T cells (Figure 6C) in SS and deltoid muscle tissue sections (Figure 6D), we did not  
288 observe any significant differences in the number of FOXP3<sup>+</sup> cells/mm<sup>2</sup> or CD3<sup>+</sup> T cells/mm<sup>2</sup>.

289 Approximately 7.5% of all T cells in the SS muscle were FOXP3<sup>+</sup> Treg cells (overlay in Figure 6D,  
290 upper panel), and approximately 13% of all T cells in the deltoid muscle were FOXP3<sup>+</sup> Treg cells  
291 (overlay in Figure 6D, middle panel). The number of T cells/mm<sup>2</sup> were, however, quite variable  
292 (Figure 6D).

293 To investigate gene expression levels involved in myogenesis, we estimated *MYOG* expression and  
294 found the relative mRNA levels to be significantly decreased in the SS compared to the deltoid  
295 muscle (Figure 6E).

296

### 297 **Changes in matrix metalloproteinases and transforming growth factors after RC tendon tear**

298 As gene ontology analysis showed changes in proteins involved in ‘extracellular matrix  
299 organization’, we investigated changes in the levels of 4 matrix metalloproteinases (MMPs) known  
300 to be involved in the degradation of the extracellular matrix<sup>38</sup>.

301 We did not observe any differences in MMP1 levels between SS and deltoid muscle (Figure 7A).

302 However, levels of MMP3 (that is known to degrade collagen types II-IV, IX, and X and to have  
303 important regulatory functions such as activation of other MMPs) were significantly higher in SS  
304 compared to deltoid muscle (Figure 7B). Furthermore, levels of MMP-9 (known to degrade  
305 collagen fragments IV and V) were changed (Figure 7C) suggesting decreased MMP-9 levels in  
306 deltoid compared to SS muscle.

307 *MMP13* (known to degrade primarily collagen fragments II) mRNA gene expression was absent in  
308 SS ( $0.005 \pm 0.006$ , n=12) and deltoid ( $0.002 \pm 0.002$ , n=16) muscle tissue, whereas *MMP13* mRNA  
309 gene expression was present in SS tendon tissue ( $4.95 \pm 5.33$ , n=4) in RC tear conditions.

310 A significant negative correlation was found between age of lesion and MMP9 levels ( $r=-0.61$ ,

311  $p=0.03$ ). We found no significant correlation between age of lesion and MMP1 levels ( $r=0.05$ ,

312  $p=0.87$ ) and a tendency of a correlation with MMP3 levels ( $r=-0.46$ ,  $p=0.08$ ).

313 In line with previous findings in the SS enthesis (reviewed in <sup>22</sup>), we found that protein levels of  
314 MMP-1 (known to specifically break down most subtypes of collagen, providing mechanical  
315 strength to tissues) were higher in SS tendon than in muscle tissue (Supplementary Table 8). Also,  
316 MMP-3 was high in the SS tendon (Supplementary Table 8).  
317 We also investigated changes in transforming growth factors (TGF), known to be affected in SS  
318 enthesis following RC tear (reviewed in <sup>22</sup>) (Figure 7D-F). Levels of TGF $\beta$ 1 (Figure 7D) and  
319 TGF $\beta$ 3 (Figure 7F) were higher in SS muscle, suggesting increased TGF $\beta$ 1 and TGF $\beta$ 3 levels in SS  
320 muscle compared to deltoid muscle. We found no correlation between age of lesion and TGF $\beta$ 1  
321 ( $r=0.12$ ,  $p=0.7$ ), TGF $\beta$ 2 ( $r=-0.07$ ,  $p=0.83$ ), or TGF $\beta$ 3 ( $r=-0.32$ ,  $p=0.34$ ).

## 322 Discussion 323

324 In this study, we demonstrated massive SS muscle inflammation after tendon tear combined with  
325 fatty infiltration and degeneration. Simultaneous changes in the innate immune response, cytokines,  
326 and proteins related to extracellular matrix reorganization and mediation of fibrosis in the SS  
327 musculature were also seen.

328 In line with previous experimental studies using mice <sup>28</sup> and rats <sup>12; 17; 18</sup> we observed high numbers  
329 of infiltrating monocytes/macrophages in SS muscle in early cases of tendon tear; this tendency  
330 ceased after 24 months, however. Inflammation was (initially) driven by high numbers of  
331 infiltrating CD68<sup>+</sup> macrophages, which are thought to be key sources of TGF- $\beta$ 1 linked to fibrosis  
332 in chronically injured muscle <sup>30</sup>.

333 Upregulated proteins in SS muscle compared to deltoid also showed 'neutrophil degranulation'  
334 among the most enriched processes. Neutrophils have been identified as the main cells infiltrating  
335 the muscle after injury <sup>46</sup>, and neutrophil-derived oxidants extended tissue damage in a rabbit model  
336 of stretch skeletal muscle injury <sup>47</sup>. On the other hand, blocking cell infiltration compromised the  
337 initial regenerative response, suggesting a role for neutrophils in muscle growth and repair by

338 removal of tissue debris and activation of satellite cells<sup>47</sup>. Moreover, a recent study has shown that  
339 neutrophil-secreted proteases can also have an immunoregulatory role by activating IL-33<sup>9</sup>. IL-33  
340 has been shown to be produced by fibro-adipogenic progenitor (FAP) cells, which are uniformly  
341 present in the interstitial space in skeletal muscle and respond to muscle damage<sup>20</sup>. Our  
342 observation of higher IL-33 levels in SS muscle compared to deltoid muscle indicate an activation  
343 of FAPs, which are the source of adipocytes in muscle fatty infiltration. Muscle Foxp3<sup>+</sup> regulatory  
344 T cells (Tregs) are characterized by high levels of expression of the IL-33 receptor, ST2, and are  
345 known to potentiate regeneration in acute and chronic injury models<sup>7</sup>. Despite comparable numbers  
346 of Foxp3<sup>+</sup> Tregs/mm<sup>2</sup>, we saw a significant decrease in *FOXP3* mRNA levels in SS muscle  
347 compared to deltoid muscle following RC tear, suggestive of repressed gene expression in Tregs  
348 located in SS muscle. Many cytokines and factors can negatively regulate *FOXP3* gene expression,  
349 including IL-6, IL-7, TGF- $\beta$ , and G-CSF,<sup>21; 32; 42</sup> all of which we observed to be different between  
350 SS and deltoid muscle.

351 The expression of IL-15 has been shown to inhibit fatty infiltration and facilitate muscle  
352 regeneration through regulation of FAP cells<sup>23</sup>. In the present study, IL-15 levels were  
353 significantly lower in the SS compared to the deltoid muscle, supporting our findings that  
354 adipocytes appear in the SS and that the regenerative potential appears to be reduced in SS muscle  
355 after RC tendon tear.

356 IL-17 is a pro-inflammatory cytokine secreted by activated CD4<sup>+</sup> T-helper cells (Th17), which are  
357 highly pro-inflammatory and induce severe autoimmunity (reviewed in<sup>27</sup>). IL-17 levels are  
358 increased in early human tendinopathy, mediating inflammatory and tissue remodeling events<sup>34</sup>,  
359 and IL-17 inhibits myoblast differentiation<sup>26</sup>. In the present study, IL-17 levels were significantly  
360 lower in SS than in deltoid muscle, but the exact relevance of decreased IL-17 levels remains to be  
361 elucidated.

362 Our gene ontology analysis showed changes in ‘extracellular matrix organization’ and ‘degradation  
363 of extracellular matrix’ especially due to the upregulation of CMA1, COL5A3, CTSS, ELN, and  
364 MMP19 in SS compared to deltoid muscle. To our knowledge, no one has investigated MMP levels  
365 in human SS muscle under tear conditions. MMPs are a large group of proteolytic enzymes  
366 responsible for tissue remodeling and degradation of extracellular matrix. In our study, MMP-3 and  
367 MMP-9 levels were significantly increased in SS compared to deltoid muscle. MMP-3 is one of the  
368 primary activators of MMP-9 from its inactive proenzyme form<sup>36</sup>. MMP-9 is produced by a variety  
369 of cells, including fibroblasts. MMP-9 appears to be a regulatory factor in neutrophil migration  
370 across the basement membrane<sup>13</sup>, and it also plays several important functions within neutrophil  
371 action<sup>27</sup> such as degrading extracellular matrix, activation of IL-1 $\beta$ , and cleavage of several  
372 chemokines<sup>37</sup>. In vitro studies have demonstrated that inhibition of MMP-9 reduced the levels of  
373 active TGF- $\beta$ 1 and reduced several TGF- $\beta$ 1-driven responses such as fibroblast stimulation<sup>25</sup>. In  
374 this context, MMP-9 appears to activate or stimulate the release of a number of cytokines and  
375 growth factors, including TGF- $\beta$ 1<sup>31</sup>, which we found to be elevated in SS compared to deltoid  
376 muscle. MMP-9 activity positively correlated to skeletal muscle atrophy in immobilized rats<sup>41</sup>,  
377 supporting a role in muscle atrophy. This is in line with our present findings of a positive  
378 correlation between MMP-9 levels and age of RC lesion. Altogether, this suggests that MMP-9  
379 plays an important role in SS muscle remodeling.

380 While only a few studies have applied a large-scale proteomics approach to address RC  
381 pathophysiology, these studies combined found several markers indicative of tissue remodeling and  
382 suggested an untapped potential for proteomics in tendon research (reviewed in<sup>43</sup>). A multi-omics  
383 methodology applied to a rat RC injury model to study myosteatosis identified disrupted  
384 mitochondrial function as one of the underlying mechanisms of lipid accumulation in muscle fibers  
385<sup>19</sup>. In our study, we identified 32 downregulated mitochondrial proteins, 15 of which are associated

386 with mitochondrial electron transport chain. Mitochondrial dysfunction reduces energy production  
387 and the dysfunction of these organelles has been connected to the myosteatorsis that is commonly  
388 reported following RC tendon injury<sup>3</sup>. Our finding of steatotic adipophilin positive muscle fibers  
389 and changes in lipid metabolism and mitochondrial function supports this connection.

390

391 This study has certain inherent limitations related to the variability of disease severity and duration  
392 and the sample size, and the results are biased towards patients with RC tears who chose to undergo  
393 surgery. The enrolled patients comprised four smokers and seven patients who received pain killers  
394 on a daily basis. The adverse consequences of smoking and mild anti-inflammatory drug intake on  
395 the inflammatory response was not assessed due to lack of statistical power. Another limitation  
396 inherent in muscle biopsy studies is the difficulty of ensuring uniform biopsy procedures, which  
397 are important to secure reproducibility and to account for regional variations in protein  
398 composition. In this study, biopsies were obtained close to the musculotendinous junction of the SS  
399 muscle in all patients using an all-arthroscopic approach from the bursal side, limiting possible  
400 location-dependent variations.

401 The rationale for using the deltoid muscle for comparison could be challenged as it may be  
402 asymptotically affected. The ipsilateral deltoid has been used as a standard of reference in a  
403 number of studies<sup>2; 13</sup>, also justifying the use of paired statistics and increasing the power of the  
404 analyses.

405

## 406 **Conclusions**

407 This study demonstrated massive muscle changes after SS tendon tear characterized by high  
408 numbers of inflammatory macrophages in lesions less than three months old and overall changes in  
409 cytokine levels, MMP levels, and growth factors. Our proteome analysis demonstrated that proteins

410 involved in inflammation, extracellular matrix reorganization, and lipid metabolism were among the  
 411 most enriched. Knowing that massive inflammation with infiltration of immune cells into the RC  
 412 musculotendinous lesion disrupt normal muscle regeneration, this implies that intervention with  
 413 repair of the tendon lesion and concomitant target-specific adjuvant treatment of the inflammatory  
 414 state of the SS muscle could be key to improving RC muscle recovery.

415

## 416 **Figure legends**

417

418 **Figure 1. Schematic workflow applied to the study of molecular pathways involved in the RC**  
 419 **lesion.** Biopsies of supraspinatus tendon and supraspinatus and deltoid muscle biopsies from ten  
 420 patients undergoing surgery for partial or full-thickness RC were evaluated.

421

422 **Figure 2. Proteomics of muscle biopsies.** (A) Differentially regulated protein pattern between SS  
 423 and deltoid muscles from patients with RC lesion (FDR 5%). Downregulated proteins are  
 424 represented in blue and upregulated in red. Dotted lines highlight proteins at least 2 times  
 425 overrepresented in each tissue. (B) Protein-protein network of SS vs deltoid muscle-regulated  
 426 proteins grouped according functional enrichment. Underrepresented proteins in SS muscle are  
 427 shown in blue while overrepresented are shown in red. (C-K) Levels of upregulated (red) or  
 428 downregulated (blue) proteins involved in Leukocyte mediated immunity (C), Immune effector  
 429 process (D), Immune system (E), Positive regulation of lipid metabolic processes (F), Metabolism  
 430 of lipids (G), Electron transport chain (H), Muscle structure development (J), Extracellular matrix  
 431 organization (I), and Striated muscle contraction (K) as measured by proteomics (n=10 muscles per  
 432 group). Abbreviations: ADAR, double-stranded RNA-specific adenosine deaminase; ADIPOQ,  
 433 adiponectin; ALDH5A1, succinate-semialdehyde dehydrogenase, mitochondrial; ANKRD1,  
 434 ankyrin repeat domain-containing protein 1; ANKRD2, ankyrin repeat domain-containing protein 2;  
 435 AP1B1, AP-complex subunit beta-1; APOA1, apolipoprotein A-1; APOA2, apolipoprotein A-2;  
 436 APOA4, apolipoprotein A-4; APOE, apolipoprotein E; CMA1, chymase; COL5A3, collagen alpha-  
 437 3(V) chain; COX6A1, cytochrome c oxidase subunit 6A1; COX6A2, cytochrome c oxidase subunit  
 438 6A2; COX6B1, cytochrome c oxidase subunit 6B1; CTSA, cathepsin A; CTSC, cathepsin C; CTSS,  
 439 cathepsin S; DNM1, dynamin-1; DNM2, dynamin-2; EEF1A2, elongation factor-1 alpha; ELN,

440 elastin; ENG, endoglin; F2, prothrombin; FABP4, fatty acid-binding protein; FASN, fatty acid  
 441 synthase; FBN1, fibrillin-1; FDXR, NADPH:adrenodoxin oxidoreductase, mitochondrial; FITM2,  
 442 fat storage-inducing transmembrane protein 2; GGH, gamma-glutamyl hydrolase; GLRX5,  
 443 glutaredoxin-related protein 5, mitochondrial; GNS, N-acetylglucosamine-6-sulfatase; GSR,  
 444 glutathione reductase, mitochondrial; HEXA, beta-hexosaminidase subunit alpha; HLA-A, HLA  
 445 class I histocompatibility antigen, A-31 alpha chain; HPGDS, hematopoietic prostaglandin D  
 446 synthase; HUWE1, E3 ubiquitin-protein ligase; ICAM1, intercellular adhesion molecule 2;  
 447 LGALS1, galectin-1; MFAP4, microfibril-associated glycoprotein 4; MMP19, matrix  
 448 metalloproteinase-19; MYH3, myosin-3; MYH7, myosin-7; MYL2, myosin light chain 2; MYL3,  
 449 myosin light chain 3; MYL6B, myosin light chain 6B; MYOZ2, myozenin-2; ND4, NADH-  
 450 ubiquinone oxidoreductase chain 4; NDUFA4, cytochrome c oxidase subunit NDFUA4; NDUFA9,  
 451 NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial; NDUFAB1,  
 452 acyl carrier protein, mitochondrial; NDUFB3, NADH dehydrogenase [ubiquinone] 1 beta  
 453 subcomplex subunit 3; NDUFC2, NADH dehydrogenase [ubiquinone] 1 subunit C2; NDUFS5,  
 454 NADH dehydrogenase [ubiquinone] iron-sulfur protein 5; NDUFS7, NADH dehydrogenase  
 455 [ubiquinone] iron-sulfur protein 7, mitochondrial; NUP62, nuclear pore glycoprotein p62; OSTF1,  
 456 osteoclast-stimulating factor 1; PLIN1, perilipin-1; PON2, serum paraoxonase/arylesterase 2; PPT1,  
 457 palmitoyl-protein thioesterase 1; PRDX4, peroxiredoxin-4; PTPRC, receptor-type tyrosine-protein  
 458 phosphatase C; PYCARD, apoptosis-associated speck-like protein containing a CARD; SACM1L,  
 459 phosphatidylinositide phosphatase SAC1; SERPINB12, serpin B12; SLC25A1, tricarboxylate  
 460 transport protein, mitochondrial; SNRPA1, U2 small nuclear ribonucleoprotein A; SORBS1, sorbin  
 461 and SH3 domain-containing protein 1; TNNI1, troponin I, slow skeletal muscle; TNNT1, troponin  
 462 T, slow skeletal muscle; UCHL1, ubiquitin carboxyl-terminal hydrolase isozyme L1; UQCR11,  
 463 cytochrome b-c1 complex subunit 10; VCAM1, vascular cell adhesion protein 1.

464

465 **Figure 3. Histological analysis of SS muscle.** (A-C) H&E-stained tissue sections of SS muscle  
 466 biopsies from representative patients at 1½ months (A), 6 months (B), and 28 months (C) after RC  
 467 tendon tear demonstrating the presence of muscle fibers with internal nuclei (arrows) and nuclear  
 468 chains (arrow heads). (D-F) Immunohistochemical staining of SS muscle biopsies from  
 469 representative patients at 2½ months (D), 6 months (E), and 28 months (F) demonstrating the  
 470 presence of a high number of CD68<sup>+</sup> macrophages (arrows) within the first 6 months after RC  
 471 tendon tear. Please note the presence of massive fatty cell infiltration (\*, asterisks) between muscle

472 fibers already early after RC tendon tear. (G-I) H&E-stained tissue sections of deltoid biopsies from  
 473 representative patients at 1½ months (G), 6 months (H), and 24 months (I) after RC tendon tear. (J-  
 474 L) CD68 immunohistochemical staining of deltoid muscle biopsies from representative  
 475 patients at 1½ months (J), 6 months (K), and 24 months (L) after RC tendon tear. Scale bar: 100µm.  
 476

477 **Figure 4. Immunohistochemical staining for adipophilin.** (A,B) Adipophilin expression in SS  
 478 (A) and deltoid (B) muscle fibers in a patient with a 6-month-old RC lesion, showing higher  
 479 adipophilin expression in the SS muscle compared to the deltoid. Adipophilin is seen as a granular  
 480 staining in the muscle fiber cytoplasm and due to its localization to the surface of lipid vacuoles, it  
 481 visualizes the distribution of intracellular lipid. (C,D) Adipophilin expression in SS and deltoid  
 482 muscle fibers from patient with a >72-month-old RC tear (C) and another patient with a 7-month-  
 483 old RC lesion (D). Please note that the distribution of adipophilin can be uneven with different  
 484 expression in neighboring fascicles in both the SS and deltoid muscles. Scale bar: 100µm.  
 485

486 **Figure 5. Cytokine and TNF receptor protein expression in SS and deltoid muscle tissue in**  
 487 **RC tear conditions.** (A-N) Electrochemiluminescence immunoassay analysis of CCL19 (A),  
 488 CXCL5 (B), IL-1β (C), IL-6 (D), IL-8 (E), IL-33 (F), IL-7 (G), IL-15 (H), IL-17A (I), INF-α2a (J),  
 489 TNF (K), TNFR1 (L), TNFR2 (M), and G-CSG (N) protein levels in SS and deltoid muscle  
 490 biopsies from patients with RC tendon tear. \*\*\*p<0.001, \*\*p<0.01. \*p<0.05, Student's t-test (n =  
 491 22/group). Samples with CV values above 25% were excluded in individual analyses.  
 492

493 **Figure 6. FOXP3 and MYOG mRNA expression is lower in SS muscle than in deltoid muscle**  
 494 **tissue in RC tear conditions.** (A) *FOXP3* mRNA levels in SS and deltoid muscle biopsies  
 495 demonstrated significantly lower expression levels in SS compared to deltoid muscle. \*\*p<0.01,  
 496 paired Student's t-test (n=18/group). Two outliers in the SS muscle and two outliers in the deltoid  
 497 muscle group were removed according to ROUT's outlier test. (B-C) The number of FOXP3<sup>+</sup> Treg  
 498 cells/mm<sup>2</sup> (B) and the total number of CD3<sup>+</sup> T cells (C) were comparable between SS and deltoid  
 499 muscle in RC tear conditions. (D) Representative FOXP3 and CD3 immunohistochemically stained  
 500 tissue sections from SS and deltoid muscle demonstrating overlay between subsets of FOXP3<sup>+</sup> and  
 501 CD3<sup>+</sup> T cells, representing the presence of Treg cells (arrows) in both SS and deltoid muscle in RC  
 502 tear conditions. Scale bars: 50µm (top and middle panels) and 100µm (bottom panel). (E) *MYOG*

503 mRNA levels in SS and deltoid muscle biopsies demonstrated significantly lower expression levels  
 504 in SS compared to deltoid muscle.  $**p<0.01$ , paired Student's t-test (n=18/group).

505

506 **Figure 7. Matrix metalloprotease and transforming growth factor- $\beta$  protein expression in SS**  
 507 **and deltoid muscle tissue under RC tear conditions.** (A-F) Electrochemiluminescence  
 508 immunoassay analysis of MMP-1 (A), MMP-3 (B), MMP-9 (C), TGF $\beta$ 1 (D), TGF $\beta$ 2 (E), and  
 509 TGF $\beta$ 3 (F) protein levels in SS and deltoid muscle biopsies from patients with RC tendon tear.  
 510  $*p<0.05$ , Student's test (n = 22/group). Samples with CV values above 25% were excluded in  
 511 individual analyses.

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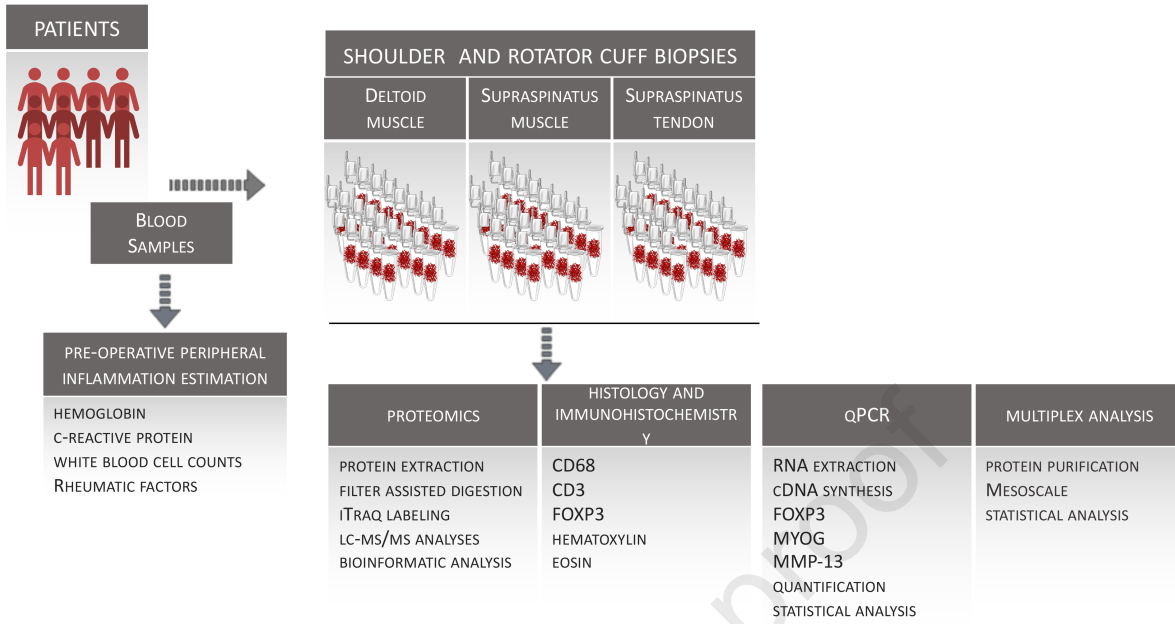
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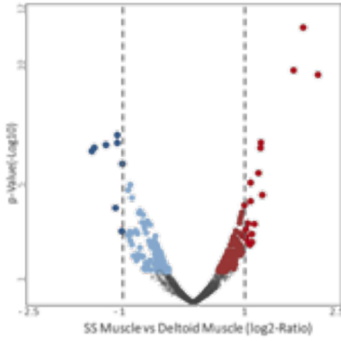
Table 1. Cytokine analysis in patients with RC tear.

	SS muscle (pg/mg)	Deltoid muscle (pg/mg)	P-value
IL-1 $\alpha$	2.44 $\pm$ 1.38 (n=13)	2.21 $\pm$ 1.72 (n=15)	0.83
IL-1Ra	46.83 $\pm$ 20.24 (n=14)	34.40 $\pm$ 12.94 (n=15)	0.33
IL-2	0.64 $\pm$ 0.47 (n=4)	0.41 $\pm$ 0.24 (n=5)	0.50
IL-2Ra	93.08 $\pm$ 56.28 (n=8)	99.11 $\pm$ 76.81 (n=7)	0.87
IL-4	0.04 $\pm$ 0.07 (n=6)	0.07 $\pm$ 0.08 (n=6)	0.53
IL-9	1.12 $\pm$ 0.72 (n=16)	1.10 $\pm$ 0.62 (n=17)	0.98
IL-12/IL-23p40	14.35 $\pm$ 29.01 (n=15)	14.64 $\pm$ 7.71 (n=18)	0.78
IL-12p70	0.23 $\pm$ 0.07 (n=6)	0.39 $\pm$ 0.32 (n=7)	0.55
IL-13	2.76 $\pm$ 2.25 (n=3)	4.35 $\pm$ 3.68 (n=3)	0.31
MIF	28,656 $\pm$ 18,419 (n=9)	26,617 $\pm$ 19,295 (n=8)	0.36
IFN- $\beta$	46.08 $\pm$ 27.05 (n=8)	34.05 $\pm$ 37.80 (n=7)	0.56
IFN- $\gamma$	2.23 $\pm$ 3.48 (n=5)	2.92 $\pm$ 2.11 (n=4)	0.95
FLT3L	14.29 $\pm$ 5.13 (n=5)	15.01 $\pm$ 8.78 (n=4)	0.93
TRAIL	54.41 $\pm$ 23.81 (n=17)	47.00 $\pm$ 17.70 (n=19)	0.50
CXCL1/GRO $\alpha$	2.63 $\pm$ 2.05 (n=9)	3.92 $\pm$ 3.37 (n=8)	0.16
CXCL10/IP-10	8.73 $\pm$ 4.00 (n=7)	8.69 $\pm$ 2.94 (n=9)	0.86
CXCL11/I-TAC	6.74 $\pm$ 2.02 (n=15)	7.78 $\pm$ 3.00 (n=19)	0.28
CCL2/MCP1	4.03 $\pm$ 2.38 (n=8)	4.83 $\pm$ 2.17 (n=9)	0.33
CCL3/MIP-1 $\alpha$	4.58 $\pm$ 2.35 (n=5)	4.01 $\pm$ 4.05 (n=2)	-
CCL4/MIP-1 $\beta$	5.17 $\pm$ 1.62 (n=9)	6.35 $\pm$ 1.54 (n=8)	0.29
CCL7/MCP-3	4.92 $\pm$ 2.59 (n=8)	4.03 $\pm$ 3.41 (N=6)	0.33
CCL8/MCP-2	1.52 $\pm$ 0.76 (n=8)	1.71 $\pm$ 1.11 (n=4)	0.48
CCL13/MCP4	15.64 $\pm$ 11.80 (n=8)	9.57 $\pm$ 7.33 (n=7)	0.24
CCL17/TARC	3.05 $\pm$ 1.01 (n=7)	3.40 $\pm$ 0.59 (n=8)	0.41
M-CSF	5.45 $\pm$ 3.46 (n=9)	6.27 $\pm$ 3.39 (n=6)	0.84
GM-CSF	0.13 $\pm$ 0.14 (n=3)	0.10 $\pm$ 0.11 (n=7)	0.33
LT- $\alpha$	1.60 $\pm$ 0.79 (n=6)	1.67 $\pm$ 0.61 (n=9)	0.41
YKL-40/CHI3L1	121.40 $\pm$ 37.64 (n=5)	340.80 $\pm$ 274.40 (n=9)	0.18
VEGF-A	93.36 $\pm$ 31.19 (n=7)	134.40 $\pm$ 53.22 (n=9)	0.19

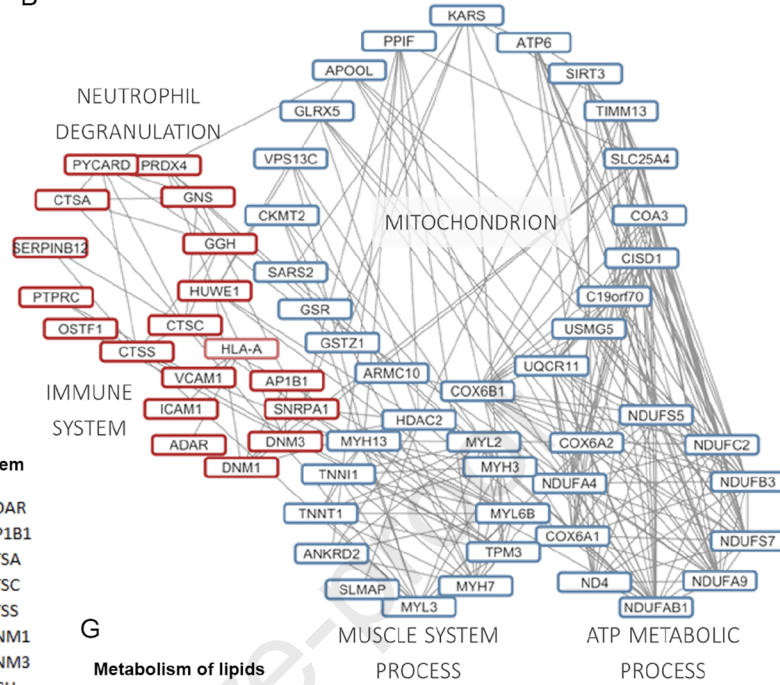
Paired Student's t-test



A



B



C

Leukocyte mediated immunity

1.62	CTSA
1.83	CTSC
1.74	CTSS
2.06	GGH
1.84	GNS
1.89	HLA-A
1.70	HUWE1
1.73	ICAM1
4.61	OSTF1
1.50	PRDX4
1.68	PTPRC
2.41	PYCARD
1.66	SERPINB12

E

Immune system

1.69	ADAR
1.53	AP1B1
1.62	CTSA
1.83	CTSC
1.74	CTSS
1.87	DNM1
1.99	DNM3
2.06	GGH
1.84	GNS
1.89	HLA-A
1.70	HUWE1
1.73	ICAM1
1.64	NUP62
4.61	OSTF1
1.50	PRDX4
1.68	PTPRC
2.41	PYCARD
1.66	SERPINB12
1.90	SNRPA1
1.87	VCAM1

D

Immune effector process

1.69	ADAR
1.62	CTSA
1.83	CTSC
1.74	CTSS
2.06	GGH
1.84	GNS
1.89	HLA-A
1.70	HUWE1
1.73	ICAM1
1.49	LGALS1
4.61	OSTF1
1.50	PRDX4
1.68	PTPRC
2.41	PYCARD
1.66	SERPINB12

F

Positive regulation of lipid metabolic process

1.39	ADIPOQ
1.05	APOA1
0.83	APOA2
1.12	APOA4
1.11	APOE
1.10	EEF1A2
1.02	F2
1.28	SORBS1

G

Metabolism of lipids

1.62	CTSA
1.59	FABP4
1.62	FASN
4.89	FDXR
1.70	FITM2
2.00	HEXA
2.02	HPGDS
1.52	PLIN1
1.89	PON2
1.79	PPT1
1.81	SACM1L
1.78	SLC25A1

J

Muscle structure development

0.47	ANKRD1
0.68	ANKRD2
0.72	MYH3
0.79	MYH7
0.78	MYL2
0.76	MYL3
0.76	MYL6B
0.70	MYOZ2
0.69	TNNI1
0.41	UCHL1

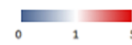
MUSCLE SYSTEM PROCESS

ATP METABOLIC PROCESS

H

Electron transport chain

0.78	ALDH5A1
0.77	COX6A1
0.47	COX6A2
0.82	COX6B1
0.80	GLRX5
0.78	GSR
0.81	ND4
0.77	NDUFA4
0.75	NDUFA9
0.79	NDUFAB1
0.84	NDUFB3
0.50	NDUFC2
0.74	NDUFS5
0.83	NDUFS7
0.72	UQCRL1



I

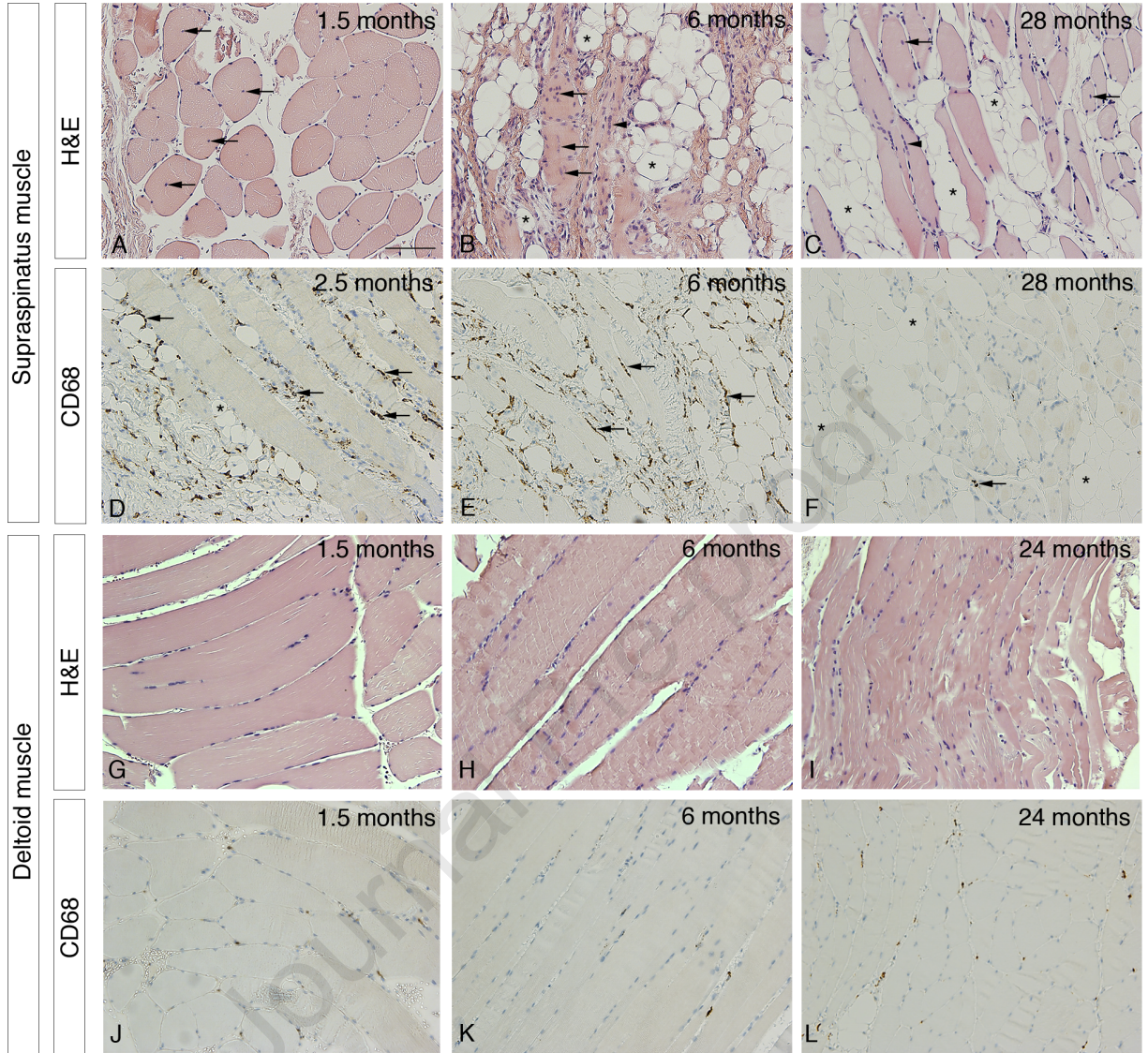
Extracellular matrix organization

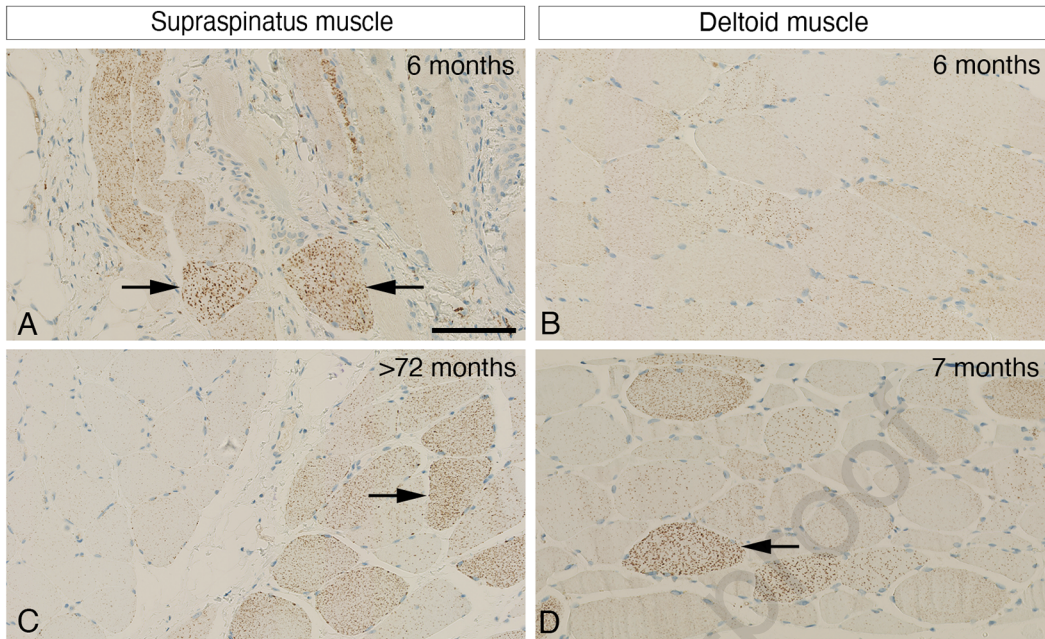
2.03	CMA1
3.49	COL5A3
1.74	CTSS
2.40	ELN
1.94	ENG
1.55	FBN1
1.73	ICAM1
1.91	MFAP4
2.17	MMP19
1.50	PRDX4
1.87	VCAM1

K

Striated muscle contraction

0.72	MYH3
0.79	MYH7
0.78	MYL2
0.76	MYL3
0.69	TNNI1
0.75	TNNT1





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