

# Muscle Gene Expression Patterns in Human Rotator Cuff Pathology

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**Background:** Rotator cuff pathology is a common source of shoulder pain with variable etiology and pathoanatomical characteristics. Pathological processes of fatty infiltration, muscle atrophy, and fibrosis have all been invoked as causes for poor outcomes after rotator cuff tear repair. The aims of this study were to measure the expression of key genes associated with adipogenesis, myogenesis, and fibrosis in human rotator cuff muscle after injury and to compare the expression among groups of patients with varied severities of rotator cuff pathology.

**Methods:** Biopsies of the supraspinatus muscle were obtained arthroscopically from twenty-seven patients in the following operative groups: bursitis (n = 10), tendinopathy (n = 7), full-thickness rotator cuff tear (n = 8), and massive rotator cuff tear (n = 2). Quantitative polymerase chain reaction (qPCR) was performed to characterize gene expression pathways involved in myogenesis, adipogenesis, and fibrosis.

**Results:** Patients with a massive tear demonstrated downregulation of the fibrogenic, adipogenic, and myogenic genes, indicating that the muscle was not in a state of active change and may have difficulty responding to stimuli. Patients with a full-thickness tear showed upregulation of fibrotic and adipogenic genes; at the tissue level, these correspond to the pathologies most detrimental to outcomes of surgical repair. Patients with bursitis or tendinopathy still expressed myogenic genes, indicating that the muscle may be attempting to accommodate the mechanical deficiencies induced by the tendon tear.

**Conclusions:** Gene expression in human rotator cuff muscles varied according to tendon injury severity. Patients with bursitis and tendinopathy appeared to be expressing pro-myogenic genes, whereas patients with a full-thickness tear were expressing genes associated with fatty atrophy and fibrosis. In contrast, patients with a massive tear appeared to have downregulation of all gene programs except inhibition of myogenesis.

**Clinical Relevance:** These data highlight the difficulty in treating massive tears and suggest that the timing of treatment may be important for muscle recovery. Specifically, earlier interventions to address tendon injury may allow muscles to respond more appropriately to mechanical stimuli.

**Peer Review:** This article was reviewed by the Editor-in-Chief and one Deputy Editor, and it underwent blinded review by two or more outside experts. The Deputy Editor reviewed each revision of the article, and it underwent a final review by the Editor-in-Chief prior to publication. Final corrections and clarifications occurred during one or more exchanges between the author(s) and copyeditors.

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**Disclosure:** One or more of the authors received payments or services, either directly or indirectly (i.e., via his or her institution), from a third party in support of an aspect of this work. In addition, one or more of the authors, or his or her institution, has had a financial relationship, in the thirty-six months prior to submission of this work, with an entity in the biomedical arena that could be perceived to influence or have the potential to influence what is written in this work. No author has had any other relationships, or has engaged in any other activities, that could be perceived to influence or have the potential to influence what is written in this work. The complete **Disclosures of Potential Conflicts of Interest** submitted by authors are always provided with the online version of the article.



A commentary by Theodore A. Blaine, MD, is linked to the online version of this article at [jbj.s.org](http://jbj.s.org).

**R**otator cuff pathology is a common clinical problem. Approximately ten per 1000 patients seen by primary care physicians present with shoulder pain<sup>1</sup>; of these patients, 74% display signs of impingement, and 85% present with painful or torn rotator cuff muscle(s). In addition, a recent cadaveric study showed that the prevalence of full-thickness tears is approximately 30% in those over sixty years of age and is correlated with age<sup>2</sup>. Importantly, rotator cuff tears may lead to irreversible changes in the structure and physiological properties of the muscles<sup>3-5</sup>. Although repair of small tears may be successful in relieving pain and improving muscle biomechanics, repair of large and massive tears remains a challenge<sup>6,7</sup>. Several reasons likely underlie poor surgical outcomes, including the development of fatty infiltration of the muscle<sup>8,9</sup>, increased fibrosis<sup>7,10</sup>, and muscle atrophy<sup>8,11</sup>. Understanding the molecular basis of these muscular changes would help us to understand rotator cuff pathology, and it may subsequently lead to treatment strategies to prevent its initiation and/or progression.

There are many transcriptional pathways that control various aspects of the adipogenic, fibrogenic, and myogenic programs, and there is cross-talk between these pathways. In the adipogenesis program, CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor  $\gamma$  (PPARG) coordinate the generation of mature adipocytes as well as triglyceride synthesis<sup>12-14</sup>. C/EBPs and PPARG can also regulate muscle differentiation<sup>15,16</sup> and fibrosis<sup>17-19</sup>. In the myogenesis program, myogenic differentiation 1 (MYOD1) and myogenic factor 5 (MYF5) are important for myogenic differentiation of satellite cells, myoblast commitment, and induction of myogenin (MYOG, which helps control the formation of myotubes and normal muscle development)<sup>20</sup>. On the other hand, myostatin (MSTN), which is secreted by muscle, inhibits myogenesis<sup>21</sup> and muscle hypertrophy<sup>22-24</sup> (via regulation of mammalian target of rapamycin [MTOR]) but promotes fibrosis<sup>25,26</sup> and adipogenesis<sup>21</sup>. An animal model of rotator cuff injury has also shown that MTOR modulates fatty infiltration<sup>27</sup>, and it regulates fibrosis<sup>28-30</sup>. Growth factors such as transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) and connective tissue growth factor (CTGF) are considered master regulators of fibrosis, which in simple terms represents an imbalance between the breakdown of extracellular matrix proteins by matrix metalloproteinases (MMPs) and the activity of tissue inhibitors of metalloproteinase (TIMPs), which inhibit MMPs<sup>31</sup>. Although there have been some studies of gene expression during muscle degeneration in animal models of the

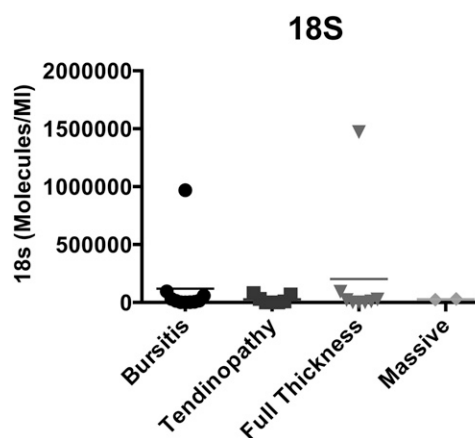


Fig. 1

Expression of the 18S rRNA housekeeping gene in the biopsy sample from each patient, showing that two of the samples had values an order of magnitude greater than those in the other samples. The horizontal lines represent the means in the four rotator cuff pathology groups.

rotator cuff<sup>27,32-34</sup>, this process is poorly understood in humans; we are aware of only one study investigating the role of atrophy-related gene expression in human rotator cuff muscle<sup>35</sup>.

In the present study, the expression levels of five myogenic, six adipogenic, and nine fibrogenic genes were analyzed in the supraspinatus muscles of patients with bursitis, tendinopathy, a full-thickness tear, or a massive tear to understand the expression patterns reflecting the regulatory mechanisms of the factors that affect muscle mass and health in these patients. We broadly hypothesized that these families of genes are differentially expressed among patients who differ with respect to rotator cuff tendon tear severity. Specifically, we hypothesized that expression of myogenic genes would be lower in patients with a more severe rotator cuff tear than in patients with a less severe tear, whereas the reverse would be true for expression of adipogenic and fibrogenic genes. Overall, an understanding of these gene expression patterns and complex muscular adaptations to a tear may lead to the development of new human therapeutics that may alter current treatment.

## Materials and Methods

### Subjects

Under an institutional review board-approved protocol, biopsies of the supraspinatus were obtained arthroscopically from twenty-seven patients

TABLE I Subject Demographics

	Age* (yr)	M/F	Body Mass Index* (kg/m <sup>2</sup> )	Diabetes, Y/N
Bursitis, n = 10	50.00 ± 7.39	5/5	30.13 ± 5.10	2/8
Tendinopathy, n = 7	54.14 ± 9.00	3/4	28.72 ± 5.00	2/5
Full-thickness tear, n = 8	61.29 ± 6.24	4/4	28.37 ± 3.72	4/4
Massive tear, n = 2	55.00 ± 3.54	2/0	33.02 ± 7.14	0/2

\*The values are given as the mean and the standard deviation.

(fourteen male and thirteen female; mean age [and standard deviation],  $55 \pm 9$  years) by one surgeon (J.G.L.). This muscle was selected because it is the most commonly injured rotator cuff muscle. Rotator cuff pathology was categorized on the basis of intraoperative observations and MRI (magnetic resonance imaging) findings<sup>36</sup> as bursitis in ten patients, tendinopathy in seven, a full-thickness tear in eight, and a massive tear in two (Table I). Patients in the bursitis group were identified on the basis of the presence of inflamed tissue and gross synovitis. Patients in the tendinopathy group exhibited rotator cuff tendon fraying, thinning, or delamination, none of which required surgical repair. Patients in the full-thickness and massive tear groups were identified on the basis of a complete tendon detachment from the bone that required surgical repair, with a massive tear resulting in retraction of the musculotendinous unit to the level of the glenoid.

### Muscle Sampling

Muscle biopsy samples were obtained with an arthroscopic rongeur under direct observation, and post-biopsy muscle bleeding confirmed that the sample was from viable muscle. Muscle samples were immediately placed in RNAlater (QIAGEN, Valencia, California) on ice, then stored at  $-80^{\circ}\text{C}$  until further processing.

### qPCR (Quantitative Polymerase Chain Reaction)

Total RNA was isolated from muscle with use of an RNeasy kit (QIAGEN). Briefly, approximately 10 to 30 mg of tissue was pulverized in the presence of liquid nitrogen with use of a Cryopress (MicroTech-Nichion, Funabashi, Chiba, Japan). One milliliter of QIAzol lysis reagent and 200  $\mu\text{L}$  of chloroform were added, and the sample was vortexed for five minutes and incubated on ice for thirty minutes. The sample was centrifuged for fifteen minutes at 10,000 rpm,

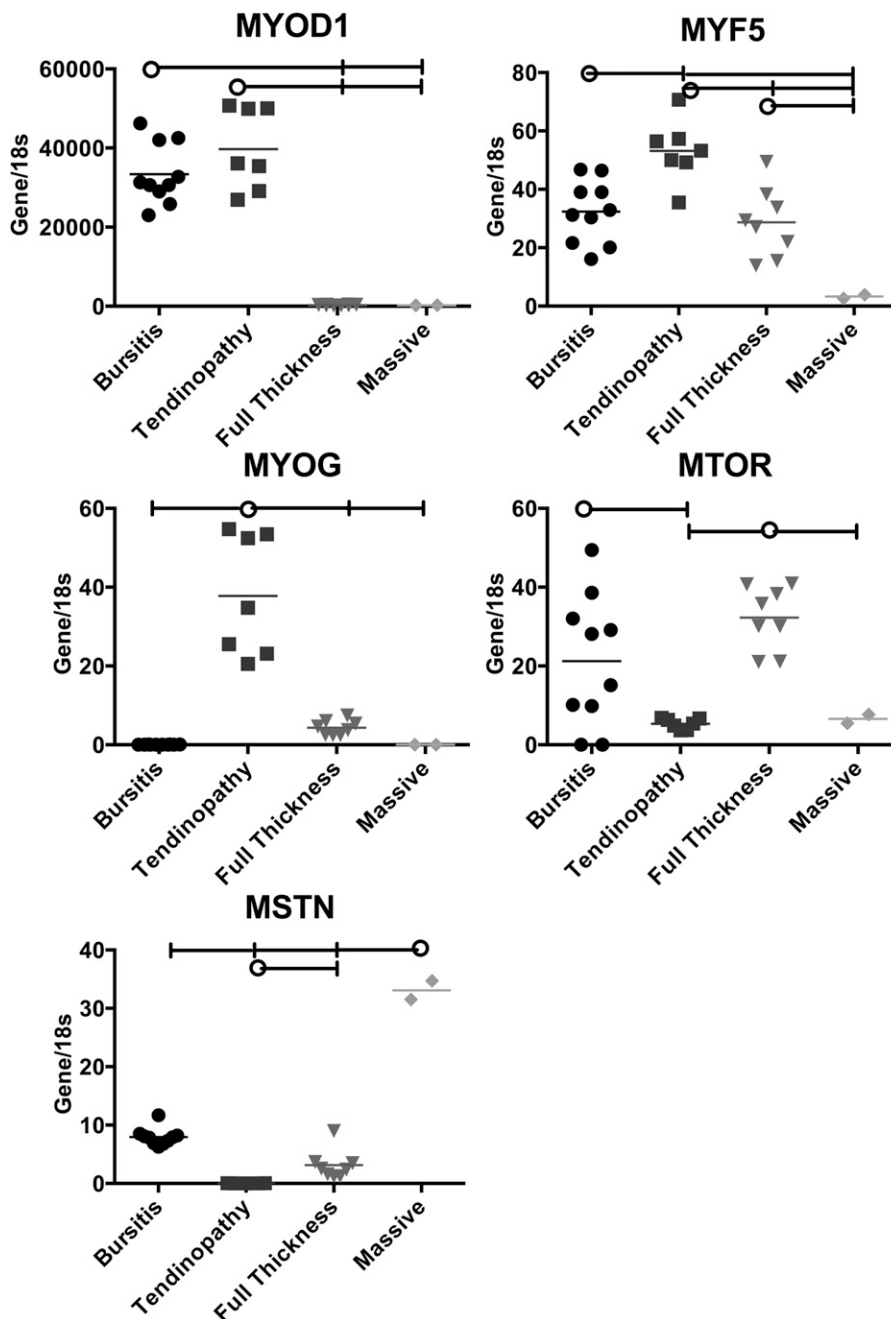


Fig. 2

Expression of myogenesis genes relative to 18S rRNA in the samples from each rotator cuff pathology group. The horizontal lines at the tops of the panels indicate significant differences between the group identified with the circle and the groups identified with a vertical tick mark. The horizontal lines within the data points represent the means in the four rotator cuff pathology groups. MYOD1, MYF5, MYOG, and MTOR upregulate the myogenesis pathway. MSTN downregulates myogenesis.

the aqueous phase was removed, and an equal volume of 70% ethanol was added. The sample was immediately loaded onto an RNeasy column and centrifuged for thirty seconds at 9000 rpm, followed by two washing steps (with the buffers provided with the kit). RNA was eluted with 30  $\mu$ L of RNase and DNase-free water. The RNA concentration was measured with use of a NanoDrop ND-1000 spectrophotometer (Wilmington, Delaware). Complementary DNA (cDNA) was prepared by reverse-transcribing 5  $\mu$ g of total RNA to cDNA with use of a QuantiTect Reverse Transcription Kit (QIAGEN), and 2  $\mu$ L of the cDNA was used for qPCR utilizing validated, gene-specific primers (QIAGEN) and the SYBR Green (QIAGEN) method. Standards were made by cloning the PCR products into the pDrive vector with use of a PCR cloning kit (QIAGEN). The results were expressed as molecules/mL and were normalized to the molecular concentration of 18S rRNA (ribosomal RNA).

Selected genes (see Appendix) related to myogenesis (MYOD1, MYF5, MYOG, MTOR, and MSTN) were quantified in each sample and compared among the groups. Similarly, selected genes related to adipogenesis (PPARG, PPARG [PPAR delta], CEBPA [C/EBP alpha], ADIPOQ [adiponectin], LEP

[leptin], and WNT10B) and to fibrosis (TGFB1, CTGF, COL1A1 [collagen type 1 A1], COL3A1 [collagen type 3 A1], TIMP1, TIMP3, MMP1, MMP3, and MMP9) were quantified and compared. The selection of these genes was based on previous reports of relevant gene expression in animal models of rotator cuff tears<sup>27,34,35,37</sup> and a review of the literature related to muscle adipogenesis and muscle fibrosis<sup>31</sup>. Although measurement of expression of a larger number of genes would have been ideal, the size of the muscle sample collected, and therefore the amount of RNA that could be extracted, prohibited a broader assessment of the transcriptional profile. In preliminary experiments, expression of the 18S transcript was more stable than that of GAPDH (glyceraldehyde 3-phosphate dehydrogenase), so we chose to perform the normalization relative to this housekeeping gene. However, there was some variability in 18S expression among the sample groups (Fig. 1).

### Data Analysis

Normalized gene expression was compared among groups with use of one-way ANOVA (analysis of variance) with post hoc Tukey testing. A p value of <0.05

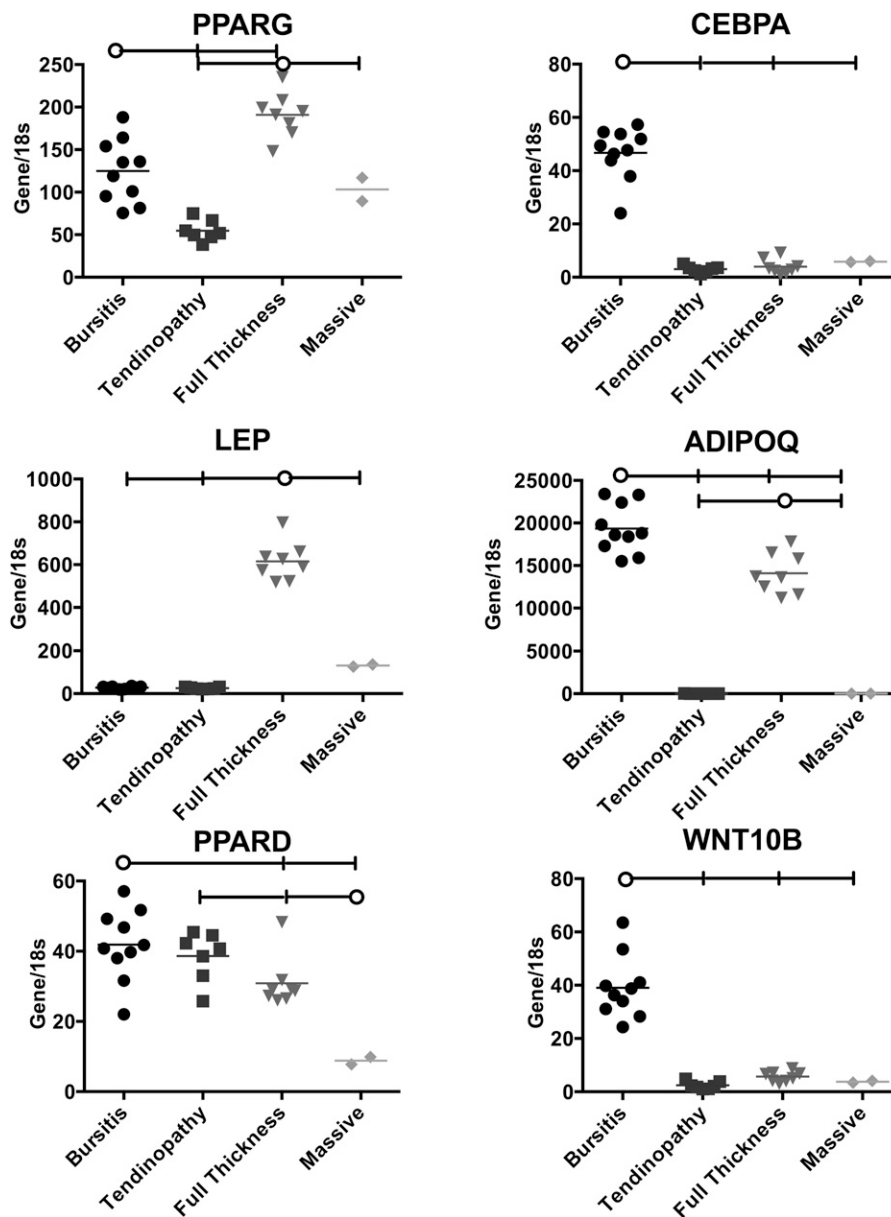


Fig. 3

Expression of adipogenesis genes relative to 18S rRNA in the samples from each rotator cuff pathology group. The horizontal lines at the tops of the panels indicate significant differences between the group identified with the circle and the groups identified with a vertical tick mark. The horizontal lines within the data points represent the means in the four rotator cuff pathology groups. PPARG, CEBPA, ADIPOQ, and LEP upregulate the adipogenesis pathway. WNT10B and PPARG downregulate adipogenesis.

was considered significant. All analyses were performed with SPSS software (version 21.0; IBM, Armonk, New York).

### Source of Funding

Financial support for this work was provided by the Orthopaedic Research and Education Foundation and NIH (National Institutes of Health) grants R24 HD050837 and R01 HD073180.

### Results

The myogenic gene expression patterns varied among the groups (Fig. 2). Expression of a gene involved with early determination (MYOD1) was greater in the bursitis group compared with the full-thickness and massive tear groups, and expression of a gene involved with differentiation (MYF5) was also greater in the bursitis group compared with the massive tear group. Interestingly, the bursitis group also exhibited greater expression of a gene related to muscle hyper-

trophy (MTOR) compared with the tendinopathy group. The tendinopathy group showed greater expression of genes related to myogenic determination (MYOD1) and myogenic differentiation (MYF5 and MYOG) compared with the full-thickness and massive tear groups. The full-thickness tear group showed greater expression of a gene related to muscle hypertrophy (MTOR) compared with the tendinopathy and massive tear groups, but the level was similar to that in the bursitis group. Perhaps most strikingly, the massive tear group demonstrated the lowest expression of all genes except MSTN (which was three to twentyfold greater than that in all other groups).

The bursitis group had greater expression of two adipogenic genes, CEBPA (a promoter of adipogenesis) and WNT10B (a potent inhibitor of adipogenesis), compared with the other groups (Fig. 3). The tendinopathy group expressed relatively low levels

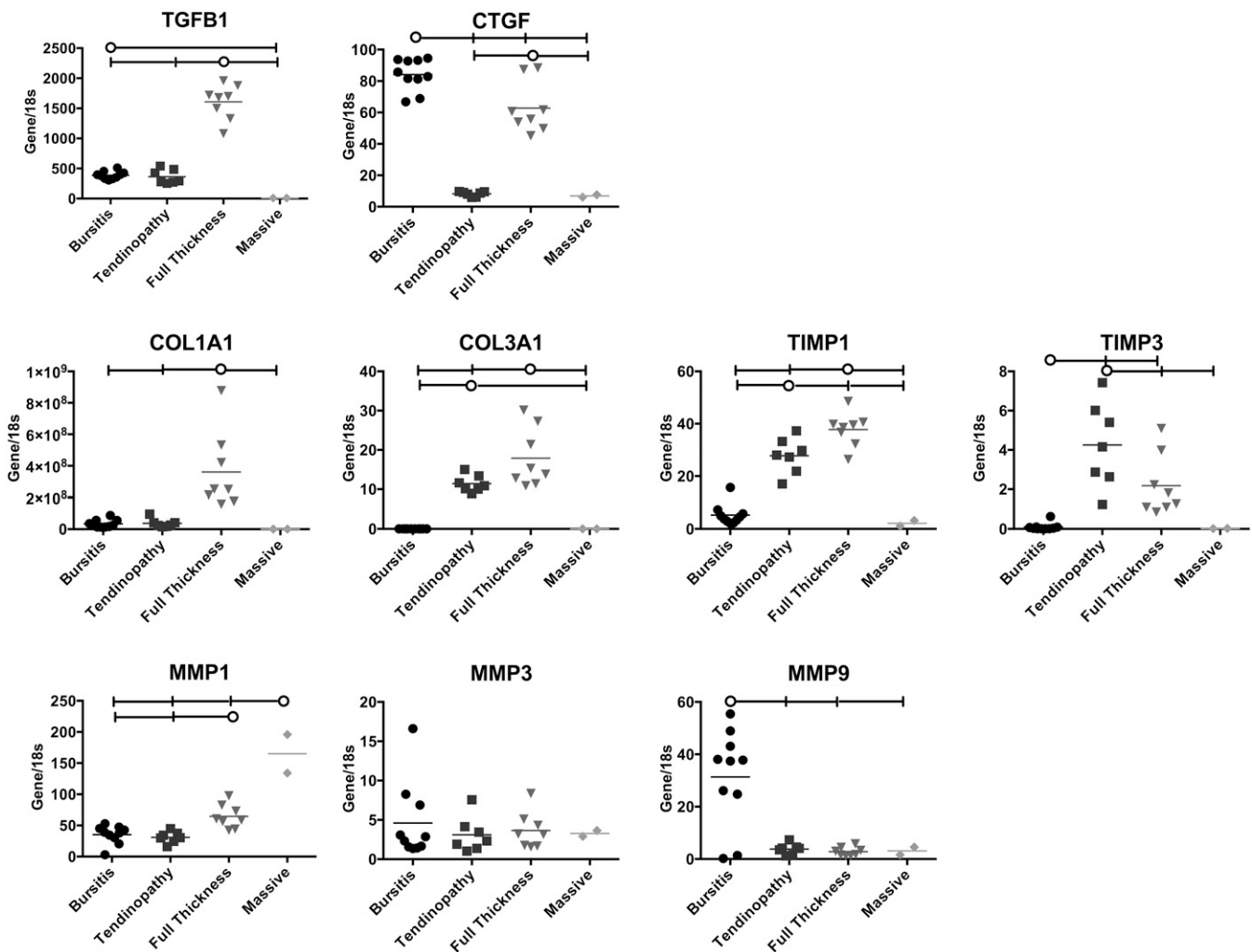


Fig. 4  
Expression of fibrosis genes relative to 18S rRNA in the samples from each rotator cuff pathology group. The horizontal lines at the tops of the panels indicate significant differences between the group identified with the circle and the groups identified with a vertical tic mark. The horizontal lines within the data points represent the means in the four rotator cuff pathology groups. TGFB1, CTGF, COL1A1, and COL3A1 upregulate the fibrosis pathway. MMP1, MMP3, and MMP9 break down fibrotic tissue (therefore downregulating fibrosis) and are inhibited by TIMP1 and TIMP3 (which therefore upregulate fibrosis).

of all adipogenic genes except PPARG, which is associated with adipogenic inhibition. The full-thickness tear group showed slightly higher expression of PPARG and a large increase in LEP compared with other groups. Again, the massive tear group demonstrated low gene expression compared with the other groups except in the case of PPARG.

The gene expression patterns related to fibrosis were also interesting (Fig. 4). The bursitis group had greater expression of CTGF and MMP9 (involved in collagen breakdown) compared with all other groups. The tendinopathy and full-thickness tear groups demonstrated greater expression of COL3A1, TIMP1, and TIMP3 compared with the bursitis and massive tear groups. However, COL1A1, COL3A1, and TIMP1 were greater in the full-thickness tear group than in the tendinopathy group, and TIMP3 was greater in the tendinopathy group compared with the full-thickness tear group. The full-thickness tear group also showed greater expression of TGFB1 and CTGF, suggesting an ongoing inflammatory process in this group but not in the tendinopathy group. Finally, the massive tear group again showed lower expression of all genes except MMP1.

## Discussion

The purpose of this study was to understand the gene expression patterns in human supraspinatus muscles with various degrees of tendon injury. The data demonstrated that myogenic, adipogenic, and fibrogenic gene expression patterns are related to rotator cuff disease severity. First, the bursitis group demonstrated increased expression of genes involved in early myogenic determination (MYOD1), differentiation from satellite cells to myoblasts (MYF5), and muscle growth due to terminally differentiated myocytes (MTOR). Similarly, the tendinopathy group demonstrated increased expression of genes involved in early differentiation from satellite cells to myoblasts (MYOD1 and MYF5) and intermediate differentiation from myoblasts to terminally differentiated myocytes (MYOG). These data indicate that muscles with the milder types of rotator cuff injury are capable of, and in the process of, generating a repair or recovery process beginning from the earliest stages of muscle development, probably by recruitment of satellite cells. The full-thickness tear group showed increased expression of MTOR, which is related to muscle hypertrophy from terminally differentiated myocytes, and this may indicate a recovery response that is less robust than that in milder injuries. Perhaps most importantly, the massive tear group showed decreased expression of all genes related to myogenesis except MSTN, which is a potent inhibitor of myogenesis. These data indicate that severely (and perhaps more chronically) injured muscles are in an active state of muscle inhibition. This has been observed previously in human subjects with rotator cuff tears<sup>35</sup>, although the expression data presented here indicate a much greater ratio between muscles with a massive tear and less severe injury states as well as a more obvious effect when massive tears are compared with full-thickness tears. Similarly, upregulation of MSTN has been observed previously in animal models of rotator cuff injury, although the relative change compared with controls was extremely small<sup>38</sup>.

In the context of adipogenesis, the bursitis group had elevated gene expression of CEBPA and ADIPOQ (proadipogenic) but also a very large increase in WNT10B, which is a potent inhibitor of adipogenesis. These data indicate that there may be competing gene expression patterns that mitigate fatty deposition in these muscles. This is in contrast to the full-thickness tear group, which had increased expression of PPARG, LEP, and ADIPOQ compared with the other groups and no concomitant increase in WNT10B to suppress fat deposition. These data indicate that the full-thickness tear group is perhaps actively depositing fat in the muscle. Again, the massive tear group had relatively low levels of expression of this family of genes, indicating that fat deposition is no longer an active process in the muscle. Although we are aware of no human rotator cuff data regarding gene expression markers of adipogenesis, some data are available in animals. These data suggest similar trends to those observed in our study, although the relative differences between injured and control animals were much smaller and appear to be related to the addition of neurotomy<sup>38</sup>.

The family of genes related to fibrosis showed evidence of profibrotic activity in the tendinopathy group (COL3A1, TIMP1, and TIMP3). However, the full-thickness tear group showed increased gene expression of TGFB1 and CTGF, which are involved in inflammation, and COL1A1, COL3A1, TIMP1, and TIMP3, which are related to collagen synthesis and suppression of collagen breakdown. These data indicate that muscle fibrosis may begin as early as clinical tendinopathy and is likely fully underway in full-thickness tears. Again, the massive tear group demonstrated relatively low levels of expression of the genes in this family with the exception of MSTN, which has been shown to directly promote fibrosis and, as mentioned previously, inhibit myogenesis. This may indicate that the process of fibrosis has been completed in muscles with such an injury. We are aware of no previous data involving human rotator cuff muscles with which these values can be compared; however, the expression levels of TGFB1 and COL1A1 do parallel those observed in rodent models of complete tears<sup>34</sup>. Importantly, the values that we measured in massive tears are much lower than those observed in any of the animal models.


In order to relate gene expression to the clinical presentation of the individual patients, we examined all available MRI studies and quantified muscle and fat volumes and fractions in the suprascapular fossa with use of previously defined methods<sup>39</sup>. As all of our patients fell into the Goutallier stage-I and II categories despite the variability in tendon tear size, there were no significant correlations between gene expression and MRI-based measures of fatty infiltration. However, the failure to relate the gene expression and clinical results is most likely the result of an inadequate sample size relative to the small range in the amounts of muscle and fat. Many more subjects with a much wider range of structural adaptations will be needed to identify such correlations.

There are a number of limitations to this study. First, there was no control group. We explored the idea of obtaining

muscle biopsy samples from patients without rotator cuff injury but ultimately decided against it because (1) it was considered unethical to explore the subacromial space without indication, and (2) we suspected that these patients would likely have pain that limited mobility and may therefore have altered gene expression patterns. We also chose to avoid using a rotator cuff muscle other than the supraspinatus as a control because we believe that there are inherent differences in gene expression among muscles<sup>40</sup> and that the rotator cuff muscles have unique architectural features that influence their mechanosensitivity<sup>41</sup>. Second, there were relatively few patients in the massive tear group. We explored the idea of obtaining biopsies from patients scheduled for total shoulder arthroplasty but decided that such patients would not be representative of patients with a massive tear that was to undergo arthroscopic reconstruction. Third, we do not know the relative fractions of the various cell types in our biopsy samples. Although we know that each biopsy sample was obtained from viable muscle, it is unclear whether the patients with a more severe tear had greater fractions of fat or fibroblast cell types. This is a topic of much experimental work in our laboratory at this time. We are exploring the use of cell sorting and flow cytometry to address this question experimentally, but multinucleated muscle cells are excluded with each technique and there are no generally accepted fibroblast markers. Thus, although we obtained expression data in the present study, we were unable to ascertain the cellular source(s) of the individual transcripts. It is possible that an immunohistochemical approach will help answer this important question in the future. Fourth, similar to the situation in any gene expression study, the relative importance of each gene in a family is different. For example, a fivefold increase in one gene is not necessarily less important than a tenfold increase in another gene of the same program because the relative influence of each gene is unknown. Finally, the gene transcripts explored in this study represent an incomplete list of those known to be important in muscle plasticity, and information on their temporal regulation cannot be gleaned from patients with an unknown tear duration. Also, in future studies it will be important to measure protein levels and activation state (e.g., phosphorylation level) to support our gene expression data.

In conclusion, to our knowledge this is the first study to quantify myogenic, adipogenic, and fibrogenic gene expression in human rotator cuff muscles across a range of pathology severities, and it revealed varied gene expression patterns among these different groups. Specifically, patients with a massive tear demonstrated downregulation of adipogenic and myogenic genes, indicating that the muscle is not in a state of active change and may have difficulty responding to growth stimuli. Patients with a full-thickness tear showed upregulation of fibrotic and adipogenic genes, which, at the tissue level, are associated with the pathologies most detrimental to outcomes. Patients with bursitis or tendinopathy were still expressing myogenic genes, indicating that the muscle may be in a state of hypertrophy to accommodate the mechanical deficiencies induced by the tendon injury. Clinically, these data may shed some light on the difficulty in treating massive tears and suggest that the timing of treatment may be important for muscle recovery. Cases of severe or chronic injury may require a pharmacologic, biologic, or biomechanical treatment to the muscle to “jump-start” the hypertrophy or regeneration process.

### Appendix

 A table showing the genes and PCR primers used in the study is available with the online version of this article as a data supplement at [jbjs.org](http://jbjs.org). ■

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