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BASIC SCIENCE

Increased Fibrogenic Gene Expression in Multifidus Muscles of Patients With Chronic Versus Acute Lumbar Spine Pathology

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Study Design. Prospective observational study-basic science (Level 1).

Objective. The aim of this study was to compare expression of functional groups of genes within the atrophic, myogenic, fibrogenic, adipogenic, and inflammatory pathways between paraspinal muscle biopsies from individuals with acute and chronic lumbar spine pathology.

Summary of Background Data. Low back pain is a complex and multifactorial condition that affects a majority of the general population annually. Changes in muscle tissue composition (*i.e.*, fatty and fibrotic infiltration) are a common feature in individuals with lumbar spine pathology associated with low back pain, which often results in functional loss. Understanding the molecular underpinnings of these degenerative changes in different phases of disease progression may improve disease prevention and treatment specificity.

Methods. Intraoperative biopsies of the multifidus muscle were obtained from individuals undergoing surgery for acute (<6-month duration) or chronic (>6-month duration) lumbar spine pathology. Expression of 42 genes related to myogenesis, atrophy, adipogenesis, metabolism, inflammation, and fibrosis were measured in 33 samples (eight acute, 25 chronic) using qPCR, and tissue composition of fat, muscle, and fibrosis was quantified using histology.

Results. We found that tissue composition of the biopsies was heterogeneous, resulting in a trend toward lower RNA yields in biopsies with higher proportions of fat ($r < -0.39$, $P < 0.1$). There were no significant differences in gene expression patterns for atrophy ($P > 0.635$), adipogenesis ($P > 0.317$), myogenesis ($P > 0.320$), or inflammatory ($P > 0.413$) genes after adjusting for the proportion of muscle, fat, and connective tissue. However, in the fibrogenesis pathway, we found significant upregulation of CTGF ($P = 0.046$), and trends for upregulation of COL1A1 ($P = 0.061$), and downregulation of MMP1 and MMP9 ($P = 0.061$) in the chronic group.

Conclusion. There is increased fibrogenic gene expression in individuals with chronic disease when compared to acute disease, without significant differences in atrophic, myogenic, adipogenic, or inflammatory pathways, suggesting increased efforts should be made to prevent or reverse fibrogenesis to improve patient function in this population.

Key words: gene expression, histology, human, low back pain, lumbar spine, multifidus, muscle, spine.

Level of Evidence: N/A

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Low back pain is a common condition that affects 65% to 85% of the general population at some point in their lifetime.[1] Although the causes of low back pain are complex and multifactorial, individuals with this condition demonstrate degenerative changes in the paraspinal muscles.[2,3] Most of these changes have been characterized on a whole-muscle level using imaging methods such as magnetic resonance imaging (MRI), or computed tomography (CT), and have described both atrophy of the paraspinal muscles in the form of reduced cross-sectional area (CSA), as well as increased fatty infiltration throughout the muscles.[3–6] On a microstructural scale, histological analyses of paraspinal muscle biopsies in both animal and human studies of lumbar spine degeneration also corroborate these findings,[7,8] with the addition of observations of muscle fiber

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degeneration,[2,7] fibrotic deposition,[2,9] inflammation,[2,10] and reduced vascularity.[2,11] It is not unusual to see >50% of the muscle compartment or sample replaced by fat and/or fibrotic tissue in these studies,[2,3] which is thought to have important implications regarding paraspinal muscle function in its role as a spinal stabilizer. Importantly, the presence of these muscle-specific changes has been associated with reduced patient function, higher levels of disability, higher likelihood of symptom recurrence, and poor postsurgical prognosis.[8,12–16]

These observations have led to additional questions about what is driving the tissue compositional changes of fat and fibrotic infiltration in this population. Some hypothesize that these compositional changes are a secondary consequence of disuse-related atrophy, in which non-muscle tissue fills in the area that used to be occupied by muscle. Alternatively, there is evidence that muscle cells actively degenerate as opposed to simple atrophy, a process that may be facilitated by inflammation or denervation.[2,17] As such, information on the molecular pathways that drives tissue compositional change may provide key information on the mechanisms of muscle loss in this population, and ultimately guide more effective treatment strategies.

For example, with the clinical assumption that muscle-specific changes are driven by disuse-related atrophy, the primary approach toward mitigating these processes has been to reverse this using treatment such as targeted rehabilitation to facilitate muscle hypertrophy and ultimately restore strength and function. However, if the processes that are driving changes in muscle structure and function change and become more complex during the course of disease progression, then identifying and incorporating treatments that target these specific rate-limiting factors are important in restoring muscle function and ultimately improving clinical prognosis in this patient population.

One of the tools that has been used to investigate molecular drivers of chronic degenerative changes in tissues from a variety of musculoskeletal conditions is gene expression.[18] As a tool, gene expression allows for an investigation of individual genes within functional pathways that are thought to contribute to a pathological phenotype. However, expression of a single gene in isolation often oversimplifies a complex process that likely involves several regulatory and feedback pathways. As such, investigations of single genes in the absence of their regulators often provide incomplete information regarding the effect of a single gene on a pathological phenotype. An additional challenge to performing gene expression in human muscle tissue is that profound tissue heterogeneity exists in samples, which can confound transcript abundance, making interpretation difficult.[18,19] Additionally, these tissue compositional changes are thought to progress with chronicity of disease, introducing an additional component of variability to human studies.[2]

Currently, most literature utilizing gene expression as a tool for understanding muscle degenerative changes in the spine has utilized animal models such as rabbit and sheep,[9,10,20] and only one other study exists investigating gene expression of paraspinal muscle in adults with lumbar spine pathology.[21] In the aforementioned human study, gene expression from individuals with and without lumbar kyphosis was compared, and was limited to three genes primarily related to inflammation.

In light of the paucity of literature investigating molecular pathways of muscle degeneration during the course of human lumbar spine disease, the goal of this study was to investigate gene expression of functional groups of genes that were empirically consistent with the observed morphological phenotypes commonly observed in lumbar spine muscle degeneration. Therefore, multiple genes within the atrophic, myogenic, fibrogenic, adipogenic, and inflammatory pathways were compared between paraspinal muscle biopsies from individuals with acute and chronic lumbar spine pathology. We hypothesized that atrophic, fibrogenic, and adipogenic pathways would be upregulated, and inflammatory and myogenic pathways would be downregulated in individuals with chronic pathology as compared to acute pathology. The clinical implication of these findings would help identify molecular pathways that are rate-limiting to recovery in this population to more appropriately prevent maladaptive changes in muscle as disease progresses.

METHODS

This was a cross-sectional prospective observational study (Level I) of 33 muscle samples (eight acute, and 25 chronic) obtained from patients undergoing posterior approach lumbar spine surgery for degenerative lumbar spine pathology. Patients were categorized into the acute group if their duration of symptoms was <6 months, and they were categorized into the chronic group if their duration of symptoms was >6 months. All patients underwent an informed consent process to obtain an intraoperative biopsy of the multifidus muscle in the lumbar spine and were included if they were undergoing surgery including decompressions, laminoforaminotomies, discectomies, or fusions. Patients with any diagnosed myopathy or systemic neurological condition were excluded. This study was performed in accordance with Declaration of Helsinki and under approval of the UC San Diego Institutional Review Board (IRB111647). Samples were obtained from a standardized location as previously described,[2] split in half upon retrieval, and immediately flash frozen in liquid nitrogen cooled isopentane and stored at -80° until processing. From half of the sample, 10-micron sections were obtained from optimal cutting temperature-embedded frozen sample using a Leica (CM3050S, Buffalo Grove) cryostat. Gomori Trichrome stains were used to visualize gross muscle morphology and quantify tissue content using ImageJ (http://imagej.nih.gov/ij) as previously described.[2] The other half of the tissue sample was homogenized and used for RNA extraction and subsequent gene expression analyses.

RNA Isolation and Quantitative PCR

For gene expression analysis, approximately 30 to 50 mg of the muscle biopsy was homogenized in bead tubes (Navy, NextAdvance) with TRIZOL (Ambion). RNeasy spin columns (Qiagen) were used to extract RNA using the manufacturer's protocol. One microgram of complimentary DNA (cDNA) was reverse transcribed with iScript cDNA Synthesis kits (Biorad). Quantitative PCR was carried out on custom plates on a BioRad CFX384 Touch qPCR analyzer for a panel of 42 genes associated with myogenic, atrophic, adipogenic, fibrogenic, and inflammatory pathways (Supplementary Table 1, http://links.lww.com/BRS/B468), with a cycle threshold determined using a SYBR green fluorophore. On-plate quality assessment was performed to assess gDNA contamination and RNA quality.

Statistical Analysis

To determine the influence of tissue composition on RNA yield, concentration values (nanograms per microliters) for yield were regressed against proportions of muscle, fat, and connective tissue from each biopsy sample. Raw cycle-threshold values (Ct values) were obtained from all samples and read into a qPCR expression set using the R Bioconductor package HTqPCR,[22] and were quantile normalized to the mean Ct value to obtain gene expression values. A maximum Ct value of 39 was applied to all genes of interest to allow for statistical comparison, with lower values indicating higher expression in this method.[22] Tissue composition was included in a linear regression model with normalized gene expression values to account for the potential influence of different tissue types on the genes of interest. Unsupervised hierarchical clustering using Euclidean distance was applied to the normalized expression values to determine the ability of gene expression patterns to differentiate between acute and chronic patient groups, and to identify potential sub-clustering within groups. Differential expression values (delta-delta-Ct) were calculated with the limmaCtData wrapper in HTqPCR for the Bioconductor package limma using a moderated t-test. All raw p-values were adjusted for within-gene group multiple comparisons using the Benjamini & Hochberg method.[23] Significance was set at an adjusted P value threshold of <0.05, and trends were defined as adjusted P values of < 0.08.

RESULTS

Tissue composition of the muscle biopsies obtained was heterogeneous, with muscle making up a mean (SD) of approximately 49.6 (18.0)%, adipose tissue making up 14.3 (12.3)%, and connective tissue (fibrosis) making up 21.2 (12.7)% of the tissue based on cross-sectional analyses. There were no significant differences in tissue composition or RNA yield between acute and chronic biopsies (Table 1). Similarly, there was no relationship between tissue composition and gene expression values ($P > 0.134$)

Unsupervised hierarchical clustering of normalized expression values resulted in two primary clusters that did not differentiate acute samples from chronic samples. There was no obvious subclustering segregating functional gene groups (Figure 1). There were no significant differences in gene expression patterns for genes within the atrophic ($P > 0.635$), adipogenic ($P > 0.317$), inflammatory ($P > 0.413$), or myogenic ($P > 0.320$) pathways; however, within the fibrosis gene group, CTGF was significantly upregulated ($P = 0.046$) in the chronic patients as compared to the acute patients. Similarly, the *COL1A1* gene trended toward upregulation ($P = 0.061$), whereas MMP1 and MMP9 both trended toward downregulation in the chronic patients as compared to the acute patients ($P = 0.061$) (Figure 2).

TABLE 1. Demographic and Biopsy Characteristics

	Acute	Chronic
Age, y	62.5 (5.6)	61.1 (16.9)
Sex (M:F)	4:4	16:9
Fat (%)	16.3 (16.2)	14.2 (11.7)
Muscle (%)	46.2 (22.5)	50.9 (17.4)
Collagen (%)	21.2 (14.1)	20.1 (11.6)
RNA yield, ng/μL	124.1 (57.6)	122.9 (55.3)

DISCUSSION

The broad aim of this study was to investigate the gene expression patterns for functional gene groups related to histomorphologic changes in muscle observed in acute and chronic lumbar spine pathology. Secondarily, we wanted to ensure that these patterns were not confounded by tissue compositional heterogeneity within muscle samples, given that this is a common feature in this patient population, and has been shown to influence gene expression patterns in other musculoskeletal conditions.[18] Although gene expression patterns for the majority of gene groups did not differentiate patients with acute versus chronic disease, several genes in the fibrosis category were significant or trended toward significance, suggesting that fibrosis remains an active biological process in the chronic disease state in contrast to adipogenesis, atrophy, or inflammation. Specifically, CTGF and COL1A1 were both upregulated in the chronic group as compared to the acute group, whereas MMP1 and MMP9 were downregulated. CTGF and COL1A1 are both related to activation and distribution of collagen networks. CTGF is a matricellular protein and is involved in extracellular matrix production,[24,25] and *COL1A1* is a gene related to production of type I collagen. MMP1 and MMP9 are both matrix metalloproteinases, which are involved in the breakdown of extracellular matrix in normal physiological processes of tissue remodeling. MMP1 has the ability to cleave interstitial collagen types I, II, and II, and MMP9 is a gelatinase that degrades denatured collagen types IV, VII, and X in many tissues.[26] Together, the upregulation of collagen deposition and downregulation of extracellular matrix breakdown is consistent with the fibrotic phenotype seen in chronically diseased paraspinal muscle.

Contrary to our hypothesis, we did not see any differential expression in the other gene pathways of atrophy,

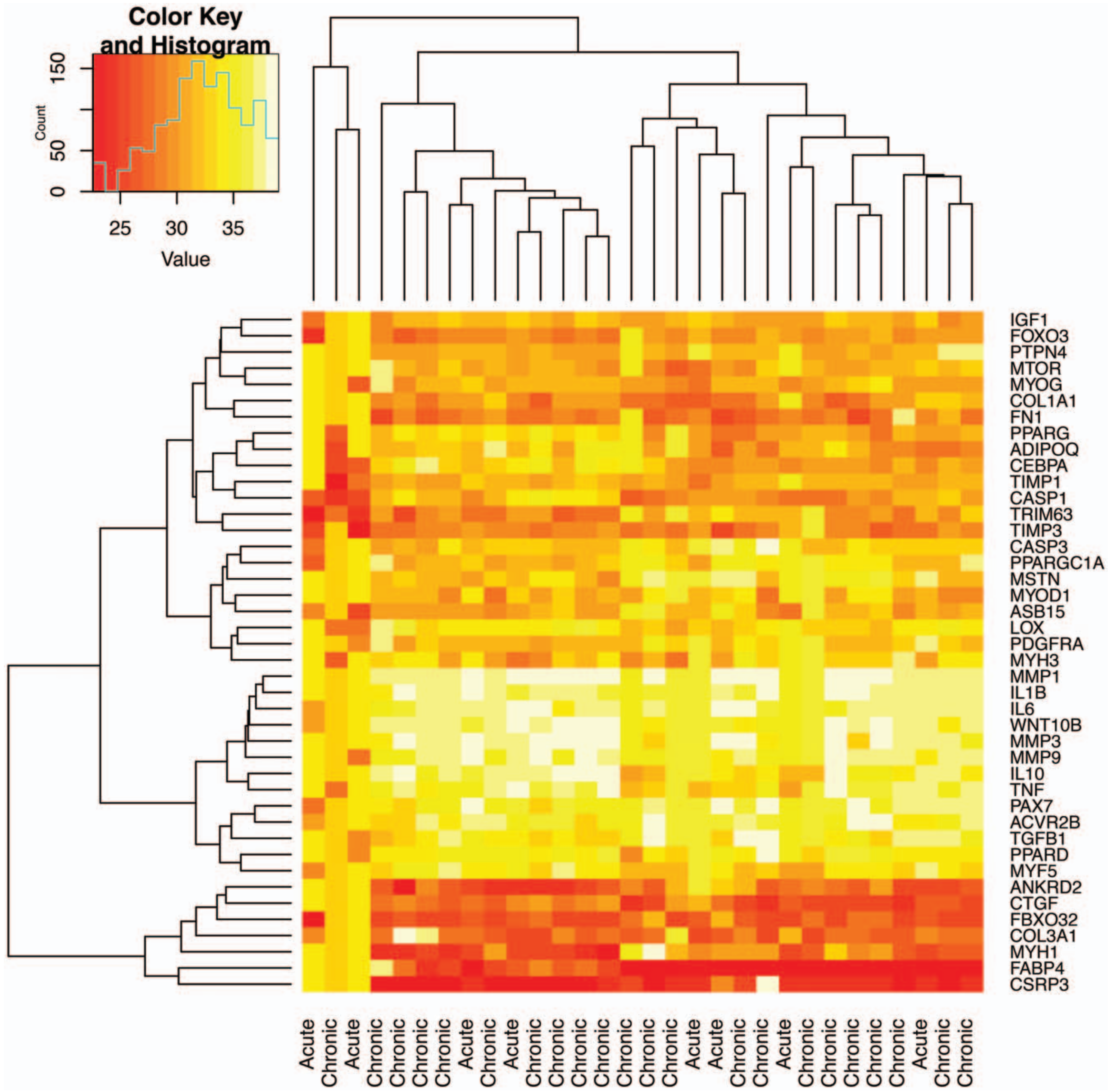

Figure 1. Hierarchical cluster analysis of multifidus muscle biopsies from acute and chronic patients. The patient group (acute or chronic) is indicated on the bottom axis of the heatmap, and gene abbreviations are indicated on the right side. Highly expressed genes are denoted by red coloring, and low-expressed genes are denoted by yellow coloring.

adipogenesis, myogenesis, or inflammation. These findings are interesting given that previous literature has suggested that atrophy and fatty infiltration are the primary phenotypic indications of declining muscle health in this population.[5,27–29] However, these observations have historically been informed by observations of decreased muscle CSA and increased fatty deposition in magnetic resonance imaging (MRI),[5,30,31] where fibrosis or connective tissue would not be distinguishable from muscle in most clinically utilized imaging protocols (T1- or T2-weighted images), and therefore may not have been recognized as an obvious feature of disease. Together, along with previous literature, these findings suggest that the relationship between atrophy and fatty infiltration with chronicity could be overestimated, whereas the fibrogenic processes may have been underestimated. This may also indicate that the current treatment paradigm of reducing inflammation and reversing atrophy may have reduced efficacy in chronic stages of disease because these pathways are no longer active, as compared to fibrogenic processes.

The lack of differential expression in the inflammatory gene group is also in contradiction to the only other investigation of gene expression in the lumbar paraspinal muscle of individuals with lumbar spine pathology. However, the small number of genes studied, the small sample size, and the group of patients compared in this study may explain

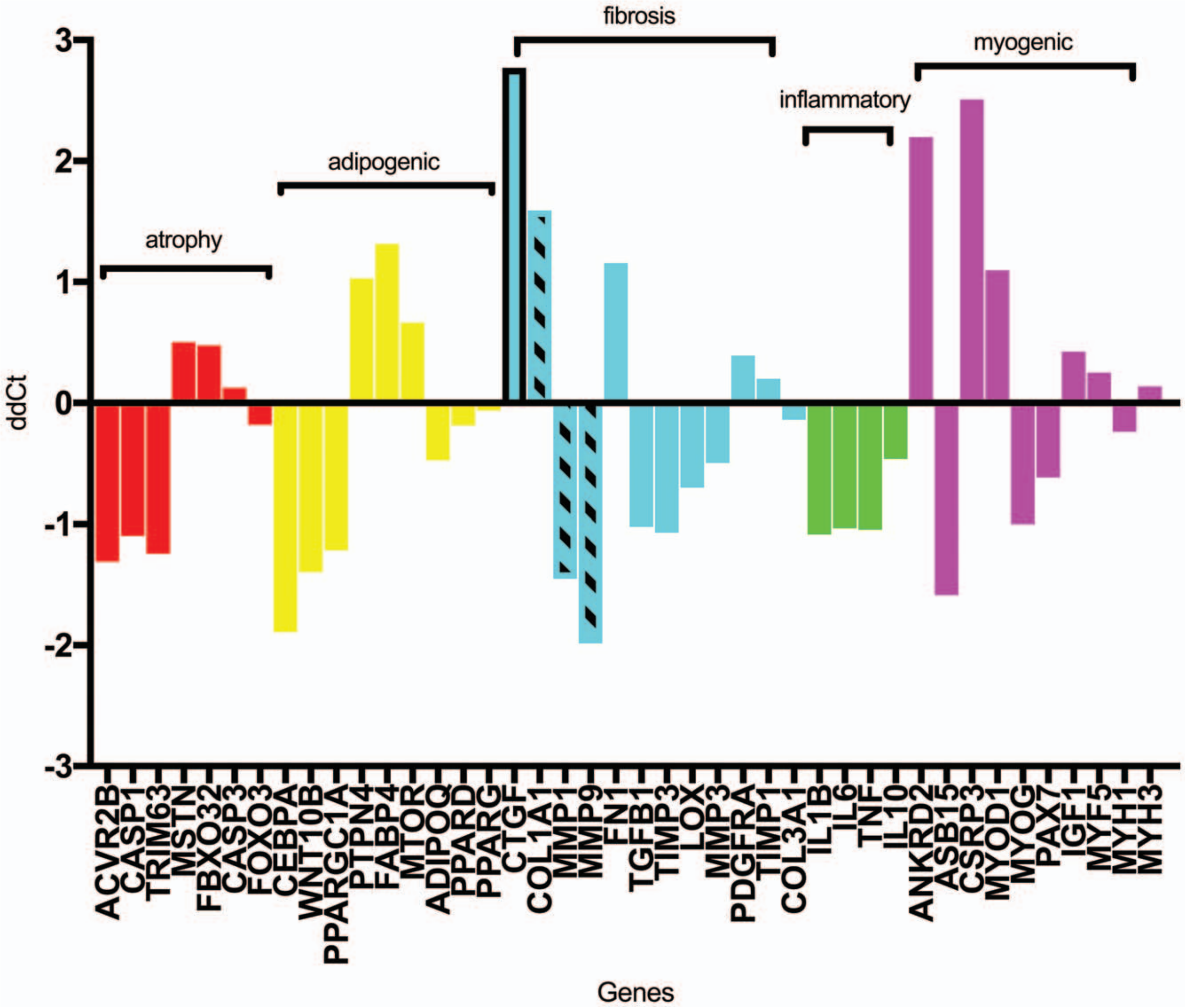

Figure 2. Delta-delta Ct ratios of expression between chronic and acute patient groups (acute group is the reference group). Bars with a solid outline indicate a significant comparison ($P < 0.05$), whereas hatched bars indicate a trend ($P < 0.08$). Solid bars with no outline are not significantly different.

this discrepancy. In the aforementioned study, gene expression was compared between individuals with low back pain who had lumbar kyphosis (N = 7) and those who had normal lumbar alignment (N = 5). Their analysis was limited to tumor necrosis factor (TNF)α, and interleukin-6 (IL-6) as inflammatory biomarkers, and peroxisome proliferator-activated receptor coactivator 1a as a marker of mitochondrial biogenesis. Although they reported roughly a two-fold increase in TNFα and a four-fold increase in IL-6 in the patient group with kyphotic posture as compared to the normal posture group, they reported *P* values of 0.048 for both genes with no reported statistical corrections for multiple comparisons.[21] Duration of symptoms was also not described in this study. These analytical strategies may have resulted in an overestimation of differential expression in comparison with the methods used in the present study, in which no significant differential expression was observed between acute and chronic patients. This also highlights the potential for overinterpretation of results when investigating a single gene as opposed to a cluster of genes within a pathway of interest.

The upregulation of fibrogenic genes has also been reported in muscle samples from patients with adolescent idiopathic scoliosis. A study investigating *TGFβ* gene expression between concave and convex sides of the scoliotic curve found that TGFβ was upregulated on the side of the concavity, which may suggest that chronic unloading plays a role in fibrogenic pathways in paraspinal muscle, although *TGFβ* is a gene that is implicated in multiple physiological processes.[32] In a sheep model of intervertebral disc degeneration in which gene expression and histology were performed on the multifidus muscle pre- and post-injury, the multifidus muscle demonstrated no histologic signs of muscle atrophy, but exhibited increased connective tissue and fat proportions 6 months post injury as compared to pre-injury. In parallel, collagen-1 was upregulated after injury.[9] Interestingly, gene expression for inflammatory cytokines, such as TNF-β, and IL-1β was also upregulated in this study, whereas TGFβ was not significantly changed relative to baseline. Additionally, genes related to atrophy were also unchanged relative to baseline.

This study had some limitations. Primarily, our group definitions may have contributed to the lack of differential expression in the hypothesized pathways. In our study, we defined individuals with lumbar spine pathology of <6-month duration as being "acute," and those with >6-month symptom duration as being "chronic." However, clinical definitions of acute and chronic often use a more conservative threshold of 3 months because of the assumption that most musculoskeletal tissues (including muscle, bone, ligament, and tendon) should have had sufficient time to heal from an injury within this timeframe.[33] However, it is reasonable that gene expression changes in muscle tissue may occur on an even shorter timeline given the high adaptive capacity of muscle relative to other musculoskeletal tissues.[34–36] This is supported by the fact that even at the 6-month timepoint, changes in tissue composition (high levels of fat and fibrosis) have already manifested, suggesting that the majority of gene expression in the pathways of interest have already occurred. Although we recognize this limitation, redefining our "acute" group with a more conservative timeline was not feasible because of the infrequency of surgically managed acute low back pain. As standard of care for treatment of low back pain often includes weeks or months of conservative management in the form of pain medications, physical therapy, and injections, obtaining muscle samples from individuals undergoing surgical treatment with symptom durations of <3 months that have not been exposed to an acute trauma is rare. Given this limitation, the differential expression from these analyses may be more appropriately interpreted as a representation of processes associated with late-stage disease progression.

CONCLUSION

This study is the first to evaluate a comprehensive set of atrophic, myogenic, fibrogenic, inflammatory, and adipogenic gene groups in paraspinal muscle of individuals with acute compared to chronic lumbar spine pathology. Our results demonstrate that an increase in fibrogenic gene expression is observed in individuals with late stage disease, whereas atrophic, adipogenic, and inflammatory programs are less predominant. These results provide important information in understanding muscle degeneration in human musculoskeletal disease and highlights the importance of studying functional groups of genes associated with phenotypes of interest. The lack of atrophic and inflammatory processes and upregulation of fibrogenic processes in the chronic stage of disease suggests that prevention or reversal of fibrogenesis may be a feasible treatment target for future research toward the goal of improved patient function and prognosis in this population.

➢ Key Points

- ☐ Tissue composition of the multifidus muscle is highly heterogeneous and contains high proportions of fat and fibrosis.
- ☐ Gene expression comparisons across multiple gene programs indicate that fibrogenesis is upregulated in chronic disease compared to acute disease
- ☐ Atrophic, adipogenic, and inflammatory programs are not predominant after the first 6 months of disease.
- ☐ Identifying ways of preventing or reversing active fibrogenesis may improve treatment efficacy in patients with chronic lumbar spine pathology.

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