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Regional and bilateral MRI and gene signatures in facioscapulohumeral dystrophy: implications for clinical trial design and mechanisms of disease progression

Chao-Jen Wong¹, Seth D. Friedman², Lauren Snider¹, Sean R. Bennett¹, Takako I. Jones³, Peter L. Jones³, Dennis W.W. Shaw², Silvia S. Blemker⁴, Lara Riem⁴, Olivia DuCharme⁴, Richard J.F.L. Lemmers⁵, Silvere M. van der Maarel⁵, Leo H. Wang⁶, Rabi Tawil⁷, Jeffrey M. Statland⁸, Stephen J. Tapscott^{1,6,*}

¹Division of Human Biology, Fred Hutchinson Cancer Center, 1100 Fairview Ave N, Seattle, WA 98109, United States

²Department of Radiology, Seattle Children's Hospital, 4540 Sandpoint Way, Seattle, WA 98105, United States

³Department of Pharmacology, University of Nevada, Reno School of Medicine, 1664 North Virginia Street, Reno, NV 89557, United States

⁴Springbok Analytics, 100 W South St, Charlottesville, VA 22902, United States

⁵Department of Human Genetics, Leiden University Medical Center, Einthovenweg 20, 2333 ZC Leiden, The Netherlands

⁶Department of Neurology, University of Washington, 1959 NE Pacific St, Seattle, WA 98105, United States

⁷Department of Neurology, University of Rochester Medical Center, 601 Elm St, Rochester, NY 14642, United States

⁸Department of Neurology, University of Kansas Medical Center, 3901 Rainbow Blvd, Kansas City, KA 66160, United States

*Corresponding author. Division of Human Biology, Fred Hutchinson Cancer Center, 1100 Fairview Ave N, Seattle, WA 98109, United States.

E-mail: stapscot@fredhutch.org

Abstract

Identifying the aberrant expression of DUX4 in skeletal muscle as the cause of facioscapulohumeral dystrophy (FSHD) has led to rational therapeutic development and clinical trials. Several studies support the use of MRI characteristics and the expression of DUX4-regulated genes in muscle biopsies as biomarkers of FSHD disease activity and progression. We performed lower-extremity MRI and muscle biopsies in the mid-portion of the tibialis anterior (TA) muscles bilaterally in FSHD subjects and validated our prior reports of the strong association between MRI characteristics and expression of genes regulated by DUX4 and other gene categories associated with FSHD disease activity. We further show that measurements of normalized fat content in the entire TA muscle strongly predict molecular signatures in the mid-portion of the TA, indicating that regional biopsies can accurately measure progression in the whole muscle and providing a strong basis for inclusion of MRI and molecular biomarkers in clinical trial design. An unanticipated finding was the strong correlations of molecular signatures in the bilateral comparisons, including markers of B-cells and other immune cell populations, suggesting that a systemic immune cell infiltration of skeletal muscle might have a role in disease progression.

Keywords: facioscapulohumeral dystrophy; DUX4; MRI; complement

Introduction

Facioscapulohumeral dystrophy (FSHD) is the third most prevalent muscular dystrophy in adults affecting between 1/8000–1/20 000 individuals world-wide [1]. Genetic and molecular biology studies determined that FSHD is caused by the aberrant expression of the transcription factor DUX4 in skeletal muscle [2–4]. DUX4 is a retrogene embedded in the D4Z4 macrosatellite repeat array in the subtelomeric region of chromosome 4 and 10 [5, 6]. DUX4 is normally expressed in the early human embryo and regulates a portion of the first wave of zygotic gene activation (ZGA) [7–9]. Following its brief expression in the embryo, DUX4 is epigenetically silenced in most somatic tissues including skeletal muscle. Mutations that decrease epigenetic silencing of the D4Z4 repeat result in aberrant DUX4 expression in skeletal muscle where it activates expression of the early embryonic transcription program and causes FSHD [10, 11].

Although the facial and upper extremity muscles are often affected first in FSHD, it progresses to involve nearly all skeletal

muscles of the body [12, 13]. However, unlike some other muscular dystrophies, FSHD is characterized by asymmetric muscle involvement, particularly in the initial phases of disease progression. The asymmetric clinical presentation correlate with changes in muscle MRI characteristics. Many MRI studies support a model of disease progression that initiates with T2/STIR MRI signal hyperintensity (STIR+), consistent with inflammation or edema, followed by T1 MRI signal hyperintensity resulting from fat infiltration and subsequent loss of the STIR+ signal after fat replaces the muscle tissue [14–20]. A recent study evaluated MRI-determined fat infiltration along the whole length of an affected muscle over time and showed that fat infiltration starts at the distal end of the muscle and progresses centrally [21], possibly modifying the model of progression to include early fat infiltration distally before more central muscle STIR hyperintensity and fat infiltration.

Our previous studies [22, 23] showed that the changes in MRI correlate with changes in muscle histopathology, ranging from

mild changes of increased extracellular matrix (ECM) deposition and complement activation through stages of inflammation with signs of muscle damage and regeneration, culminating in fat replacement of the muscle. The gene expression signature as measured by high throughput sequencing of RNA from muscle biopsies also showed elevation of gene sets representing ECM, complement, and inflammation that correlated with increased disease activity and showed the eventual loss of skeletal muscle gene expression in the later stages associated with fat replacement. It is likely that these progressive changes are caused by the aberrant expression of DUX4 in the muscle because DUX4 regulated genes were expressed at very low levels in the early stages of muscle pathology and progressively increase as the other features of the disease pathology increase. Specifically, using an arbitrary cut-off of DUX4 target gene expression based on RNA sequencing to assign a biopsy as DUX4+ or DUX4- showed a high correlation of STIR+ signal with DUX4+ biopsies [22, 23]. A similar study also showed an association between a higher expression of DUX4-target genes and STIR+ muscle and that fat fraction measures increased the sensitivity of identifying muscles with higher levels of DUX4-target gene expression [24].

Our current study was designed to validate the correlation between STIR signal and the DUX4 signature, and to determine whether the disease activity observed in each muscle represented muscle-specific disease progression or a systemic progression with asynchronous manifestation of clinical features, MRI, or muscle markers of disease progression. We enrolled 34 FSHD subjects and performed functional studies, lower extremity MRI, and bilateral biopsy of the tibialis anterior (TA) muscles. The findings validate our prior study by confirming a strong correlation between MRI status and the DUX4-signature of gene expression and extend the prior study by showing the value of fat infiltration measured across the whole muscle as a predictor of local muscle disease progression. An unanticipated finding of the current study is the correlation of MRI and the gene expression signature between the TA muscles in each subject, particularly the signature of immune cell genes, suggesting a systemic component of disease progression.

Results

Study cohort

The study cohort included 34 FSHD subjects, 16 female and 18 male, aged 21 to 69 (mean $47.1 \pm 14SD$) recruited at three sites (University of Washington Medical Center, University of Rochester Medical Center, and Kansas University Medical Center) of the Seattle Paul D. Wellstone Muscular Dystrophy Specialized Research Center. All participants underwent initial functional testing that included lower extremity dorsiflexor strength measurement, lower extremity MRI, and needle muscle biopsy of both tibialis anterior muscles. Fiducial markers on the mid-portion of the TA muscle were used to identify the muscle biopsy site; however, muscle STIR score was based on the STIR signal for the entire muscle. Muscle biopsies were processed for histology, RNA extraction and sequencing with the composite data shown in [Supplementary Table 1](#). DNA was extracted for bisulfite sequencing (BSS) of two regions, one being the DUX4 exons 2–3 on the distal most repeat unit of 4qA permissive allele(s), and the second being upstream of the DUX4 coding sequence present in all 4q and 10q D4Z4 repeats. Of the 68 attempted biopsies in the 34 subjects, four biopsy samples (01-0019L, 01-0020R, 01-0029R, 13-0006R) were not adequate for histopathology scoring; three biopsies (13-0006R, 13-0008R/L) in two subjects yielded insufficient RNA for sequencing

and the RNA from a fourth biopsy (32-0028L) showed degradation (RNA Integrity number (RIN) = 4.0), excluding these four biopsies from further analysis.

Transcript-based quality assessment of muscle tissue

Although the intent of the needle muscle biopsy is to determine gene expression in muscle tissue, some biopsies do not capture sufficient muscle tissue to be informative, either because of replacement of the muscle by fat, contamination by blood, or biopsy of fibrotic regions. In this study we used sets of genes to estimate the relative representations of blood (HBA1, HBA2, HBB), fat (FASN, LEP, SCD) and skeletal muscle (ACTA1, TNNT3, MYH1), each of which was characterized by a score of cumulated scaled transcripts per million (TPM)— $\sum_{i=1}^n \log_{10}(TPM_i + 1)$. In this bilateral cohort, samples 13-0009R and 13-0007R exhibited low muscle content (below 3% quantile) and elevated fat content (above 97% quantile) ([Supplementary Fig. 1a](#)). The principal component analysis ([Supplementary Fig. 1b](#)) also indicated low-muscle characteristics of these two samples: the loading variables of the first two components revealed low expression in the muscle genes of 13-0009R and 13-0007R. It is reasonable to suspect that these two muscle-low and fat-high content biopsies did not accurately reflect skeletal muscle gene expression. As a result, we excluded them from the analyses of the association of RNA expression levels (e.g. DUX4 signature) to MRI characteristics and other clinical measures. Based on data from our prior longitudinal study [22] and the current study, we propose applying assessment of muscle, fat, and blood content as a criteria to limit analyses to biopsies that have a sufficient representation of skeletal muscle ([Supplementary Fig. 1c](#)); e.g. identifying samples with a low muscle score (cumulated scaled TPM) or high fat score as having inadequate muscle tissue for analysis of certain gene categories like DUX4-target genes or the other gene baskets described below. It is interesting to note that the muscle-low samples in this study and our prior longitudinal study [22] mostly exhibited elevated extracellular matrix, inflammatory, and complement activation signatures, possibly reflecting biopsy of an area of advanced disease with significant muscle loss.

Baskets of DUX4-regulated genes distinguish mild-moderate FSHD from control muscle

Yao et al. [25] identified 67 DUX4-regulated genes significantly up-regulated in DUX4-target-positive FSHD biopsy samples, FSHD myotubes, and DUX4-transduced muscle cells. The RNA expression level of this set of 67 genes or a subset of four genes (LEUTX, PRAMEF2, KHDC1L, TRIM43) equally distinguished the more affected FSHD samples from controls and were proposed as candidate FSHD molecular biomarkers. Our subsequent studies determined that mildly or moderately affected FSHD muscles (based on normal MRI and near-normal pathology scores) had modest elevations of some of these DUX4-regulated genes [22, 23]. Therefore, we aimed to identify subsets of the original 67 DUX4-regulated genes that most reliably distinguished mild-to-moderate FSHD muscle from control, in contrast to the prior optimization for distinguishing the more affected biopsies from controls. We started with the set of 67 genes and first excluded duplicated or deleted genes in the GRCh38 genome build (instead of previously used hg19), as well as variants in the same gene family showing highly-correlated expression levels to their primary genes (see details in Methods; [Supplementary Fig. 2a](#)). Using the RNA-seq dataset from the prior longitudinal study, we applied both DESeq2 and Receiver Operator Characteristic (ROC)

curves to rank and select 29 out of the original 67 candidates as robust and reliable DUX4-target genes for discriminating mild-moderate FSHD from control samples (Supplementary Table 2).

We further refined this to a basket of six (ZSCAN4, CCNA1, PRAMEF5, KHDC1L, MBD3L2, H3Y1) or twelve (PRAMEF15, PRAMEF4, TRIM49, MBD3L3, HNRNPCL2, TRIM43, in addition to the six) of the best discriminating genes. The random Forest algorithm and leave-one-out cross-validation evaluated the classification performance of all three baskets—with 6, 12, and 29 DUX4-regulated genes. The resulting accuracy rate in classifying mild-to-moderate FSHD samples in the prior longitudinal study data, used to discover these baskets, demonstrated that these baskets outperformed the previously used four genes (LEUTX, PRAMEF2, TRIM43, KHDC1L) (Supplementary Fig. 2b), likely because the prior set was optimized based on comparisons of more affected biopsies to controls. Unless otherwise indicated, this study uses the basket of six genes (DUX4 M6 score) as the DUX4 score (determined by $\sum_i \log_{10}(TPM_i + 1)$).

MRI measures of regional fat fraction and whole muscle fat infiltration

The local fat fraction at the region of the biopsy ranged from 0.09% to 93.4% (mean $15.7\% \pm 25.8\%$ SD) in the 68 TA MRI images; most TA muscles (54/68) had a regional fat fraction of less than 10%. An alternative method used an artificial intelligence-based approach to generate whole muscle measures of normalized fat content for each TA muscle, termed whole muscle fat percent to distinguish it from regional fat fraction for clarity in this study (see Methods). TA whole muscle fat percent ranged from 3.3% to 82.2% (mean $23.5\% \pm 24.6\%$ SD). These two measures, regional fat fraction and whole muscle fat percent, showed strong correlation in cases where regional fat fraction was greater than 10% (Pearson = 0.89; Fig. 1a), which corresponded to a whole muscle fat percent of ~40%. In contrast, muscles with a regional fat fraction below 10% were not well discriminated, whereas the whole muscle fat percent showed a greater linear discrimination in this group with fat percentages ranging between 0% to 40%. These findings are consistent with a prior study showing that whole muscle fat analysis identifies initial changes in fat content at the distal muscle ends prior to the central region of the muscle [21]. Indeed, with the exception of muscles that have progressed to very high fat infiltration and lost their STIR+ signal, the STIR– muscles showed distal and, to a lesser extent, proximal fat infiltration with little central fat replacement; whereas most muscles with increased central fat were also STIR+ (Fig. 1b). The RNA-seq fat content (based on transcripts of the three fat marker genes (FASN, LEP, SCD, see above) was moderately correlated to the regional fat fraction (Pearson = 0.65) and slightly better correlated with the whole muscle fat percent (Pearson = 0.69) (Fig. 1c). Because of the better discrimination in the low-fat range, we used the whole muscle fat percent measure for the remainder of the analyses.

STIR, fat infiltration, and DUX4 molecular signature

Of 68 muscles in the 34 subjects, 43 had a STIR rating = 0 (STIR–) and 25 had elevated STIR signal (STIR+). Although STIR signal was rated on a four-point scale (6, 3, 4, and 12 muscles were rated STIR 1, 2, 3, and 4, respectively), our analyses will consider a binary rating of STIR– (rating = 0) or STIR+ (rating = 1–4). STIR+ muscles showed higher levels of whole muscle fat percent (median = 42%) compared to STIR– muscles (median = 9%) (Fig. 1d, $p = 2e-8$), however, a subset of muscles with very high whole muscle fat percent (>73%) were rated STIR– consistent with prior studies

showing loss of STIR signal with advanced fat infiltration (note that the biopsies from these two muscles did not yield sufficient RNA for sequencing).

Similar to our prior study showing the association of STIR signal with the DUX4 molecular signature [22, 23], the STIR+ muscles had significantly higher DUX4 scores (mean = 3.2) than the STIR– muscles (mean = 0.41), validating a strong association between STIR status and the DUX4 score (Wilcoxon p -value = $7e-10$). Indeed, muscles with greater than 20% whole muscle fat percent were all STIR+ and all had a relatively high DUX4-score (Fig. 2a, DUX4-score plotted on a log scale); however, note that the two STIR– muscles with high fat content (see Fig. 1b) did not yield sufficient RNA for sequencing and were not included in this analysis. The same data plotted with a linear scale for the DUX4 score better shows the decline of the DUX4 score at the higher levels of fat infiltration (Fig. 2b).

The strong correlation between the STIR status and DUX4 score allowed us to build a logistic regression model (see Methods) to predict whether a muscle would have a DUX4 score greater than a specific threshold (Fig. 2c and Supplementary Table 3). For example, if the muscle was STIR– with 20% fat infiltration, the probability of the muscle meeting the threshold of a DUX4 score >0.5 was 41% (Fig. 2c, blue line), whereas a STIR+ muscle with 20% whole muscle fat infiltration had a 93% probability of a DUX4-score above this threshold (Fig. 2c, red line).

Association of the DUX4 score to histopathological scores and clinical data

The quantitative myometry muscle strength of the TA ranged from 1.8 to 43 kg (mean 17.2 ± 10.8 SD) and demonstrated an inverse correlation with DUX4 scores (Pearson = -0.5 ; Fig. 2d). The clinical severity score (CSS) showed a moderate positive correlation with the DUX4 score (Pearson = 0.49, Fig. 2e). The histopathology score (a rating between 0 to 12 based on variability in fiber size, interstitial fibrosis, central nucleation, and necrosis/regeneration, each ranging from 0–3) also showed a moderate correlation (Pearson = 0.5; Fig. 2f) with the DUX4 score, similar to our prior study [22].

Inflammatory, ECM, complement, and immunoglobulin signatures

In addition to DUX4-regulated genes, our prior studies showed that many other genes had elevated expression in FSHD muscle, including genes related to inflammation, complement, immune cells, and extracellular matrix (ECM) [22, 25, 26]. Using a similar approach to the generation of the DUX4-regulated gene baskets, we used the datasets from our prior longitudinal study [22] to identify subsets of genes in each of these categories that most robustly distinguished mild-moderate FSHD from controls (see Methods). By these methods we established baskets of six genes representing signatures for four additional categories: inflammatory (TNC, COL19A1, COMP, THBS1, SFRP2, ADAM12); ECM (PRG4, RUNX1, CCL19, PLA2G2A, CCL18, CDKN1A); immunoglobulin (IGHG1, IGHG2, IGHG3, IGHG4, IGKC, FCGR2B); and complement (C1QA, C1QB, C1QC, C1R, C1S, C3).

Applying these additional gene baskets to the current dataset of TA muscle biopsies validated that the scaled cumulated score ($\sum_i \log_{10}(TPM_i + 1)$) of each basket distinguished both STIR– and STIR+ FSHD muscle from the historical control biopsies (Fig. 3a) and showed greater elevation in muscles that were STIR+ and had greater than 20% whole muscle fat percent (Supplementary Fig. 3a and b). The expression level of each basket, including

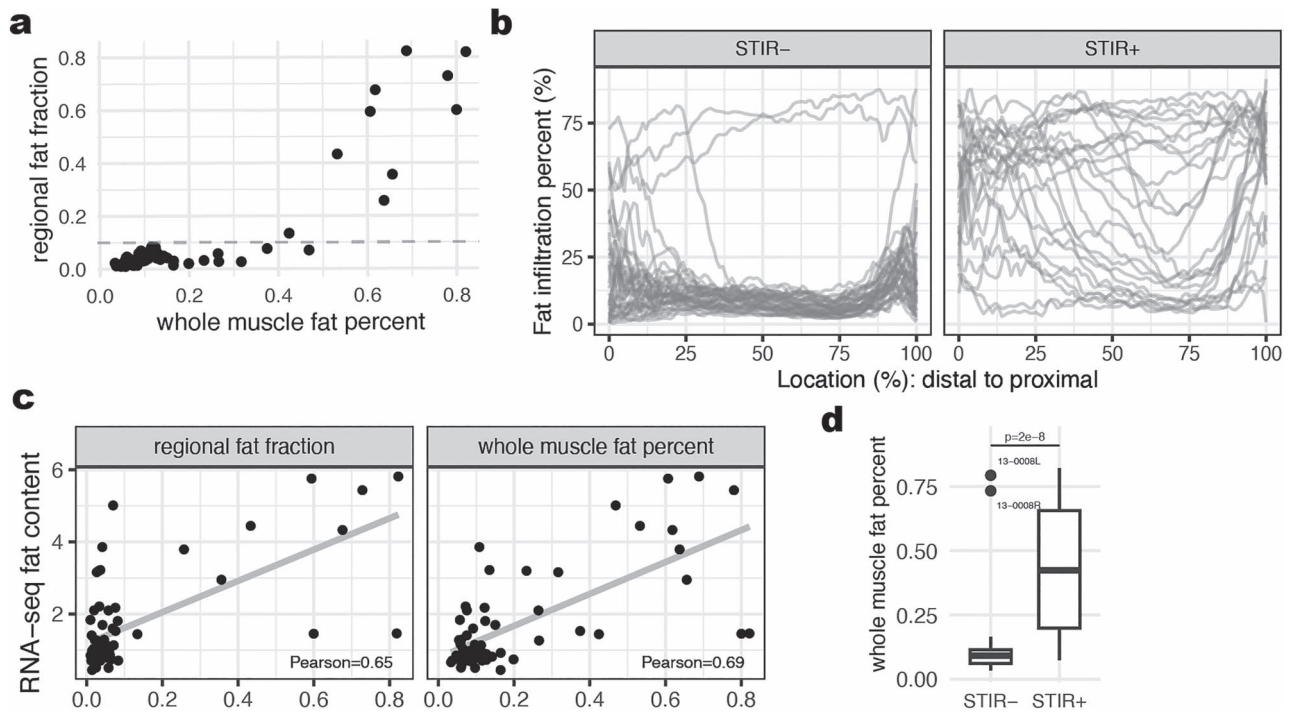


Figure 1. Comparison of regional fat fraction and total muscle fat percentage. (a) At the lower levels of fat infiltration, whole muscle fat percent shows increased values relative to regional fat fraction, possibly due to earlier fat infiltration in the distal and proximal ends of the muscle compared to the region of the biopsy that was generally near the middle of the TA muscle. (b) Fat infiltration percent over the length of each TA muscle from distal (0) to proximal (100) for STIR- and STIR+ muscles. STIR- muscles show fat infiltration in the distal and proximal regions with the exception of muscles with very high fat infiltration that have likely lost prior STIR+ signal, whereas STIR+ muscles show fat infiltration progressing into the central portion of the muscle. (c) Correlation between RNA sequencing inference of fat content with regional or whole muscle MRI measurements of fat content. (d) STIR+ muscles show higher whole muscle fat content with the exception of muscles that have progressed to very high fat percentages.

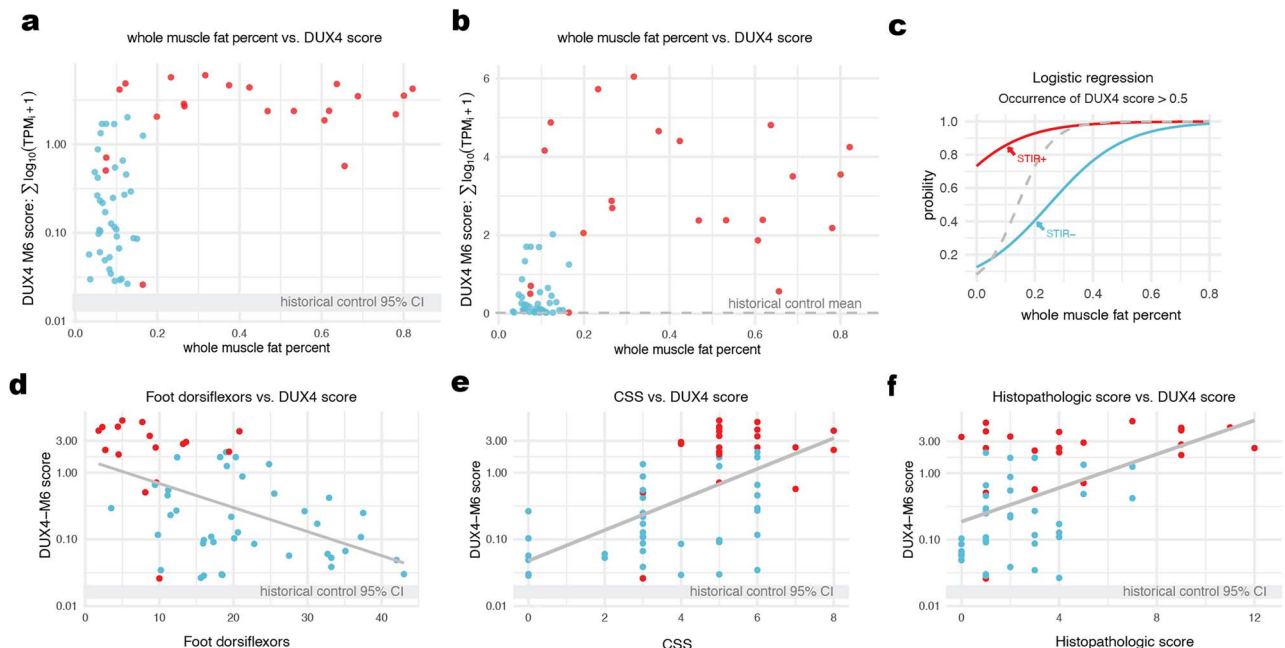


Figure 2. Muscles with whole muscle fat percent greater than 20% and MRI+ muscles have high DUX4 scores that correlate with functional and histopathological measures. (a) Scatter plot showing that MRI+ muscles mostly have elevated DUX4 scores, and that MRI+ muscles with greater than whole muscle fat infiltration of greater than 20% have uniformly high DUX4 scores. DUX4 scores plotted on a log scale emphasizes the difference between the levels in the historical control muscle biopsies (shaded area indicates the 95% confidence interval (CI) for the distribution of the DUX4 scores in the control biopsies). (b) Same as in (a) but plotted on a linear scale. (c) Logistic regression predicting the occurrence of a DUX4 score > 0.5. The predictors include the whole muscle fat percent and STIR status of the muscle biopsies and outcome is the occurrence of DUX4 score > 0.5. STIR- muscles indicated in blue, STIR+ in red. Dashed gray line indicates all muscles regardless of STIR status. (d) TA muscle strength in kg and the DUX4 score; (e) the clinical severity score (CSS) and the DUX4 score; (f) the histopathology score and the DUX4 score. STIR- muscles indicated in blue, STIR+ in red.

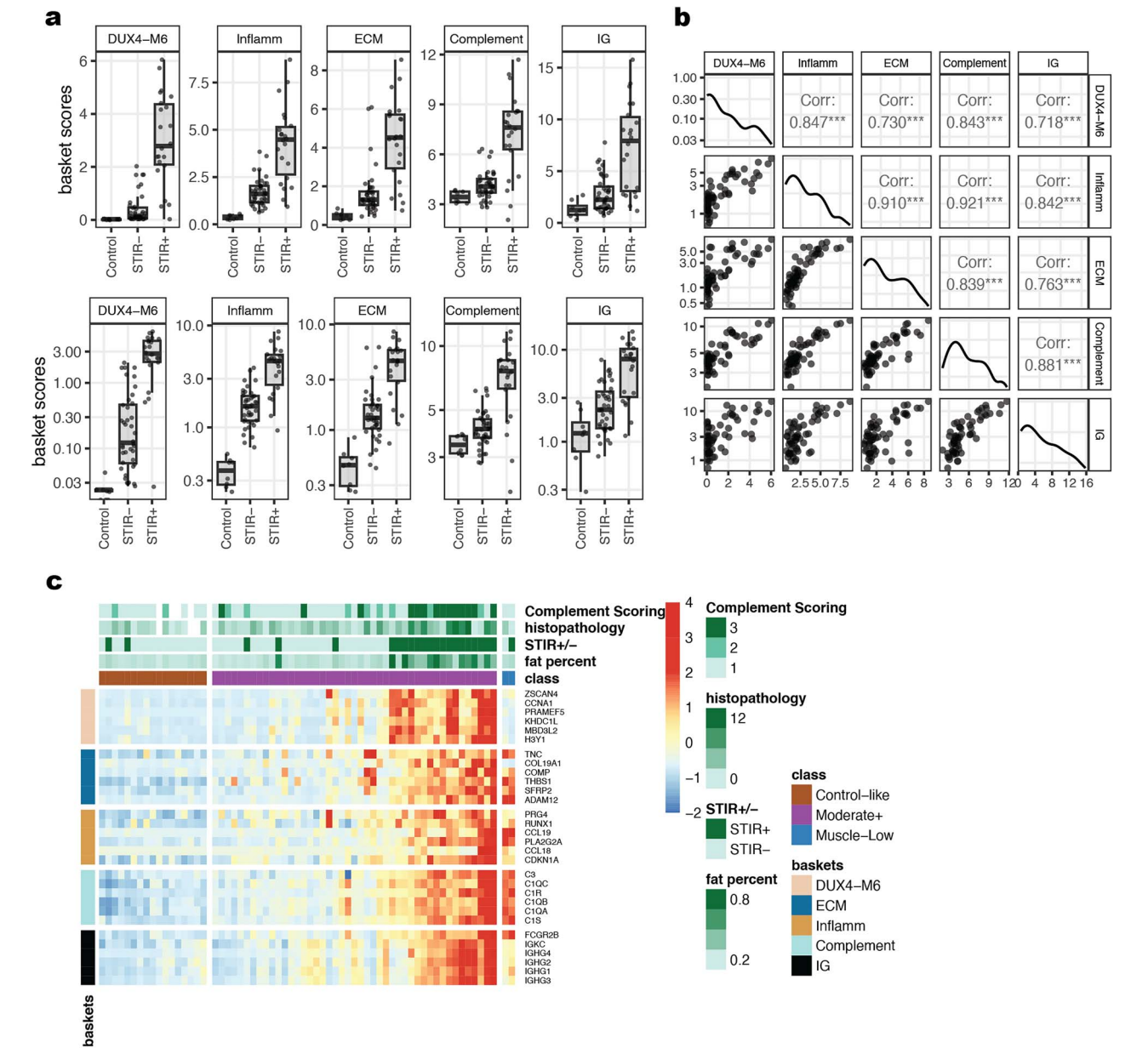


Figure 3. Baskets of genes in different categories distinguish between STIR⁻ and STIR⁺ muscles. (a) Distribution of the basket scores in FSHD STIR⁺ compared to STIR⁻ muscle, and also compared to historic controls (quadriceps biopsies from non-FSHD, unaffected subjects (see Methods)). Top panel plotted on a linear scale and bottom panel on a log scale to show separation from control samples. (b) Correlation between the basket scores in each biopsy. (c) Heatmap illustrating row-wise z-score of expression levels for the indicated basket gene. STIR⁺ muscle exhibit elevated expression compared to STIR⁻. The muscle-low samples are included here to demonstrate their distinct attributes characterized by low-level DUX4 signatures but elevated in ECM, inflammatory, and complement activation.

the DUX4 basket, was highly correlated with the other baskets across muscle biopsies (Fig. 3b). Correlations with other parameters, such as whole muscle fat percent, tibialis anterior strength, sub-categories of histopathologic scores, and CSS are shown in Supplementary Fig. 3c.

Using the gene expression levels of the five baskets representing DUX4, ECM, Inflammatory, Complement Activation, and IG molecular signatures, we applied a supervised machine learning algorithm (random forest) to classify the current bilateral biopsied muscles into Control-like and Moderate+ groups. The training model used the prior longitudinal RNA-seq dataset, as described in the Methods section. Our analysis revealed that samples classified as Moderate+ demonstrated increased expression levels in each of the five gene signature baskets (Fig. 3c). On the other

hand, the Muscle-Low samples, characterized by low expression of muscle markers, exhibited minimal to undetectable DUX4 signature but showed elevated levels in ECM, Inflammatory, and Complement Activation signatures.

Bilateral comparisons reveal symmetric trends

Comparisons of the MRI and molecular characteristics of the left and right TA biopsies in the same individual revealed relatively strong correlations. Ten of the subjects were rated STIR⁺ in both TA muscles, 19 STIR⁻ bilaterally, and only five discordant for STIR, which differs significantly from the expected values of 4.5 STIR⁺/+, 13.5 STIR⁺/-, and 16 STIR⁻/+ (Supplementary Fig. 4a), determined by random process simulation on pairing 43 STIR⁺ and 25 STIR⁻ samples (see Methods). Similarly, there is a strong

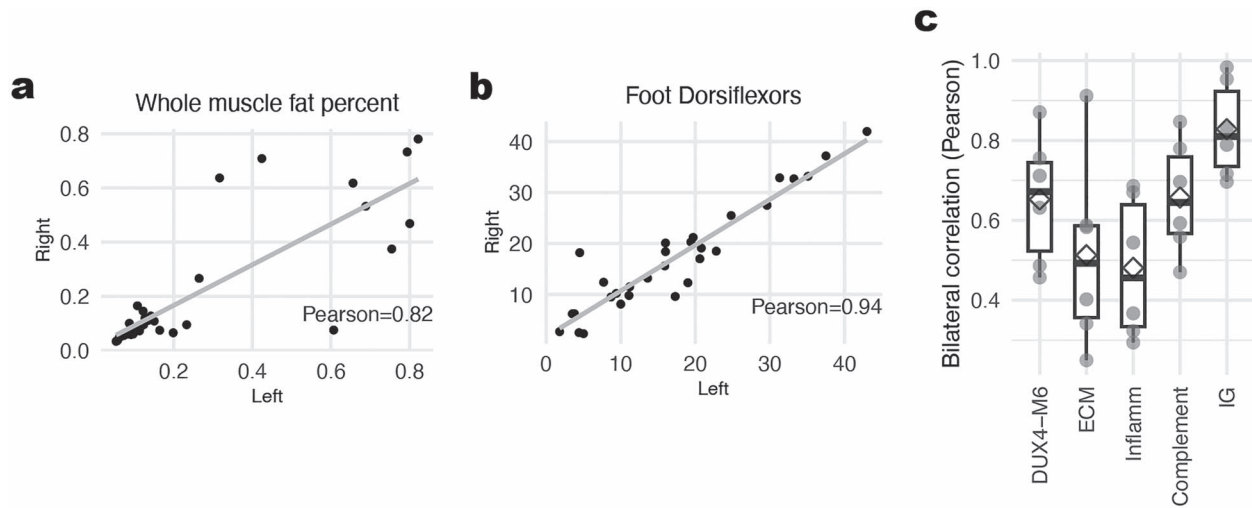


Figure 4. Bilateral comparisons of fat infiltration, TA strength, and gene baskets. Scatter plots show correlation between R and L TA for whole muscle fat infiltration (a) and TA strength (b). (c) Whisker plots showing the correlation between the R and L TA for each basket of genes indicated. Each dot represents the Pearson correlation for each gene in the basket and was calculated based on the gene expression level in TPM. The diamond represents the average of the basket.

correlation in whole muscle fat percent bilaterally (Pearson = 0.82; Fig. 4a) and in muscle strength (Pearson = 0.94; Fig. 4b). Expression of the different baskets of FSHD-elevated genes also showed moderate-to-strong correlation bilaterally with the average Pearson correlations ranging from 0.48 (Inflammation) to 0.82 (IG) (Fig. 4c and Supplementary Fig. 4b). In contrast, histopathologic scores were not highly correlated (Supplementary Fig. 4c).

DNA methylation of the FSHD permissive FSHD D4Z4 allele

Of the 68 muscle biopsies collected from 34 subjects, one DNA extraction was inadequate for bisulfite sequencing (13-0006L) and subject 01-0022 had high DNA methylation associated with a duplication of the D4Z4 repeat region and was not included in this analysis. The bisulfite sequencing of subjects with a 4qA161S allele showed a median CpG methylation for the region sequenced of between 0%–24% (mean = 6.8%); whereas subjects with a 4qA161L allele had a higher range of 5%–43% (mean = 16.2%) (Fig. 5a and Supplementary Table 4). The correlation between the left and right TA biopsies was high (Pearson = 0.85; Fig. 5b). Yet, the methylation levels did not show an association with the STIR status (Fig. 6c) nor demonstrate correlation with other variables such as mRNA levels and clinical scores (Fig. 5d).

Complement protein deposition in FSHD muscle

Our prior study showed complement deposition in FSHD biopsies by immunodetection with an antibody to the C5b-9 component of the membrane attack complex (MAC) [23]. In our current study, we graded sections from each biopsy for immunodetection of C5b-9 based on a scale of 1–3: 1, little-to-no C5b-9 staining and no positive capillaries; 2, mild C5b-9 staining with 1–2 positive capillaries per 100 fibers; 3, mild-to-moderate C5b-9 staining with greater than 3 positive capillaries per 100 fibers (Supplementary Fig. 5 and Supplementary Table 1). Of the 63 samples that could be evaluated, 27 were graded 1 (43%), 11 graded 2 (17%) and 15 graded 3 (24%). There were moderate correlations between the complement grade and each of the gene basket scores for the same biopsy (Spearman correlation: DUX4 = 0.54, IG = 0.51, Complement = 0.45, Inflammation = 0.45, ECM = 0.45) (Fig. 6).

Immune cell infiltration in FSHD muscle

In the prior longitudinal cohort, we observed enrichment of immune response genes in the affected biopsies that included indications of complement activation, chemokine signaling, and indications of T and B cell gene expression [22]. To extend this prior analysis, we focused on the 860 immune cell-type specific genes annotated in the IRIS (Immune Response In Silico) dataset [27] and determined that 63 were differentially expressed (adjusted P-values <0.05 corresponding to null hypothesis in which the log change is equal to 1) in the DUX4-high group compared to the controls in the prior longitudinal study (Supplementary Table 5). Heat maps showed robust expression of this immune cell gene set both in the prior longitudinal dataset used to identify this gene set and in the current bilateral study dataset as validation (Supplementary Figs 6 and 7). Similar to the strong right/left correlations of the genes in the IG basket (see Supplementary Fig. 4b), the genes attributed to B cells and plasma cells also show moderate-to-strong right/left correlations (Supplementary Fig. 8a and Supplementary Table 5).

To further explore the composition of the immune cell infiltration, we employed the PLIER (Pathway-Level Information Extractor) [28] algorithm for RNA contribution analysis to infer the immune cell-type enrichment. In the longitudinal dataset, this identified IgG and IgA memory B cells, CD4 T helper 1 (Th1), neutrophils (resting stage), and dendritic cells as enriched in the more affected muscles, particularly in samples classified as IG-High, High, and especially Muscle-Low (Supplementary Fig. 8b). Similarly, in the current bilateral dataset, the more affected muscles, classified as Moderate+, also demonstrated enriched contributions of IgG and IgA memory B cells, neutrophils, and dendritic cells (Supplementary Fig. 8c).

Discussion

In our previous clinical studies, we used MRI to select a muscle for biopsy and showed a correlation between STIR+ status and exceeding a threshold of DUX4 target gene expression [22, 23]. Considerable effort was made to biopsy the region of the muscle with a specific MRI signal characteristic under the assumption that disease progression in the muscle might be focal or regional

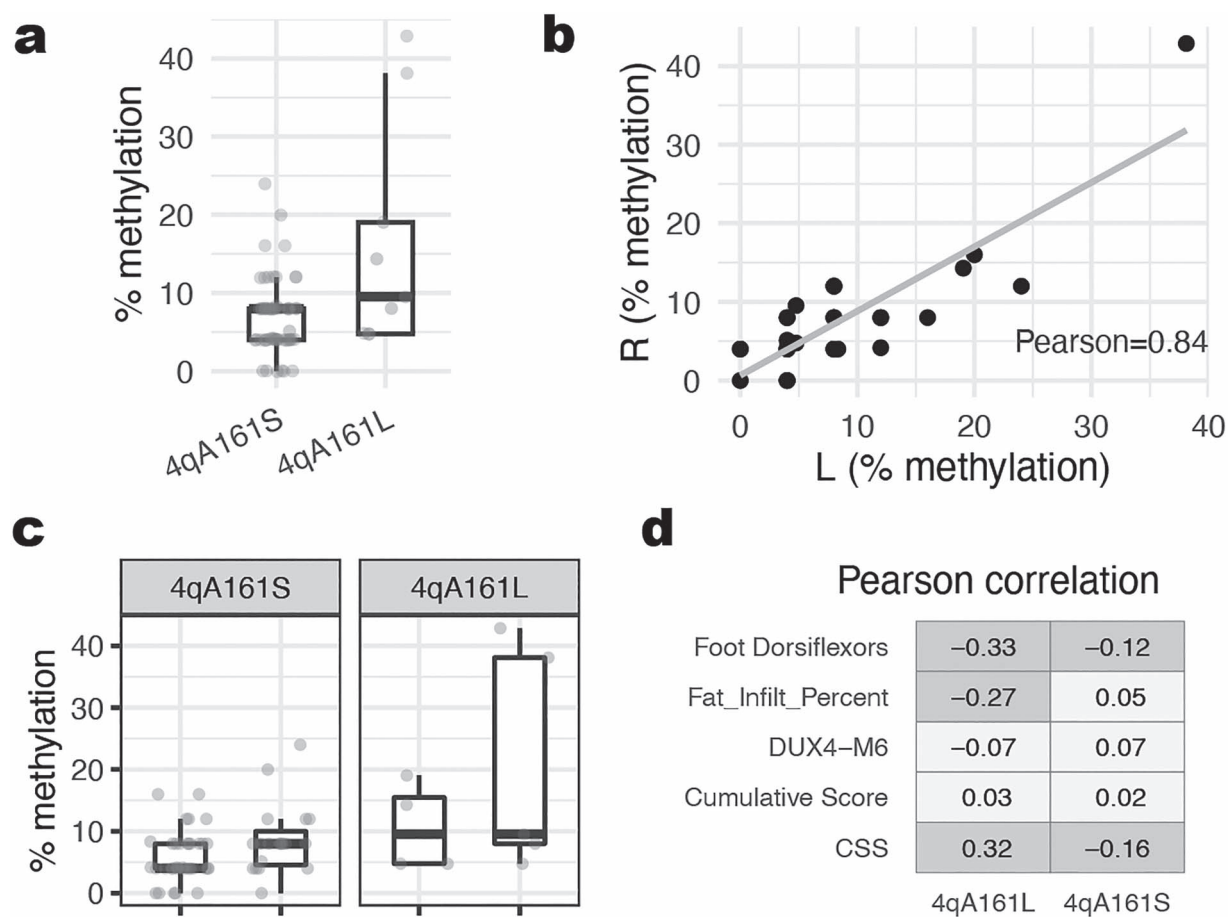


Figure 5. Methylation levels show intra-subject correlation bilaterally not with other parameters. (a) Methylation levels in pathologic 4qA-short and 4qA-long alleles. (b) Bilateral correlation of methylation levels. (c) Methylation levels are not associated with MRI STIR signal. (d) Correlations between methylation levels and the indicated parameter do not show any moderate or strong correlations.

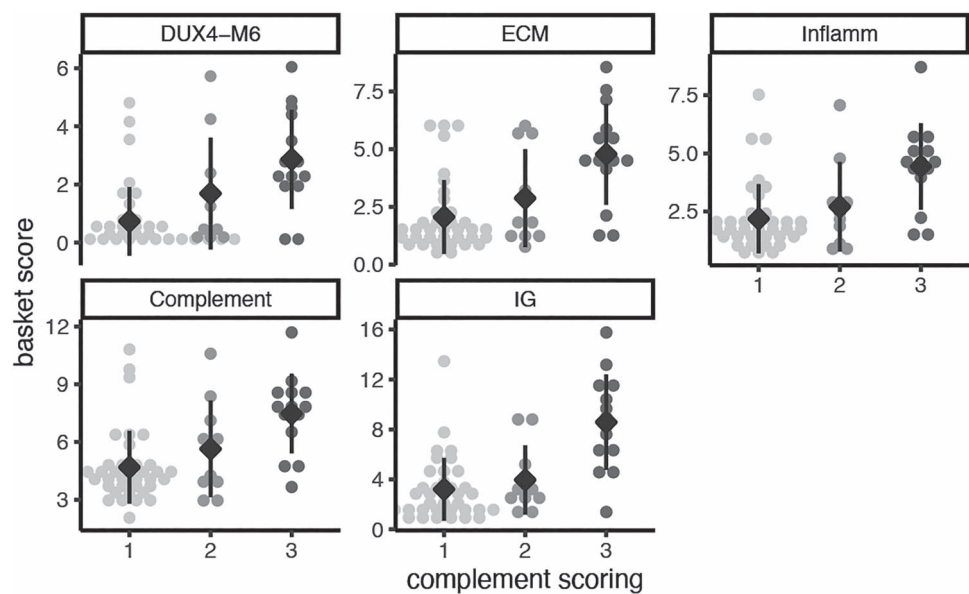


Figure 6. Dot plots illustrating the relationship between basket scores of bilateral muscle biopsies and complement scoring graded on the scale of 1, 2, or 3.

rather than whole muscle. In the current study, biopsies were performed in the mid-portion of the TA muscles and fiducial targets were used to localize the biopsy region for correlation with regional MRI characteristics. The strong correlation between

the molecular signatures and whole muscle MRI characteristics indicates that disease progression is not an entirely focal process, but suggests that the molecular signatures of disease progression spread through the entire muscle even at early stages. This might

have significant implications for the design of clinical trials measuring response to therapies because it suggests that a biopsy in a central region of a muscle can accurately reflect the stage of disease progression in that muscle as a whole.

This conclusion is further strengthened by the calculations of whole muscle fat infiltration. Whole muscle fat infiltration showed a stronger association with the molecular signature than the similarly calculated regional muscle fat infiltration in the area of the muscle biopsy. Because fat infiltration begins in the distal regions of the muscle [21] and the biopsy was performed in the central portion of the muscle, this finding also indicates that the molecular signature reflects a whole muscle level of disease activity rather than only focal or regional inflammation or disease. If confirmed by additional studies, this suggests that stringent MRI-designation of a specific biopsy site is not necessary to capture disease activity and will significantly simplify clinical trial design.

In addition, the moderate-to-strong correlations between measurements of disease activity in the bilateral TA muscles also suggests that FSHD progression is not only driven by focal events in an individual skeletal muscle but might also reflect a systemic progression of the disease. Although speculative at this time, the high R/L correlation of mRNAs that characterize specific immune cell populations, particularly B cells, in the bilateral TA muscle biopsies raises the possibility that a systemic immune response to FSHD antigens might have a role in coordinating disease progression in different muscles of an individual.

In summary, this study validated the previously identified strong correlation of an elevated DUX4-signature with STIR+ muscles and revealed unanticipated findings that support a model of entire muscle and even systemic disease progression. First, the DUX4-signature at a biopsy site in the mid-portion of the muscle correlates with the entire muscle MRI signal, indicating that progression is not entirely regional or focal. Second, the moderate-to-strong correlations between bilateral TA muscle molecular signatures support a model that incorporates an element of systemic disease progression, perhaps mediated by circulating factors such as B or T cells. Our current study supports using MRI determinations of STIR and whole muscle fat infiltration together with molecular signatures as measures of disease progression in FSHD in future clinical trials.

Materials and methods

The study was conducted jointly at the University of Washington, the University of Rochester, and University of Kansas through the Seattle Paul D. Wellstone Muscular Dystrophy Cooperative Research Center. The study was approved by the Human Subjects Committee at each institution, with written informed consent obtained for all participants. Patients were examined and given a 10-point Clinical Severity Score (CSS). The muscle biopsy was generally performed within a few days of the MRI and strength testing, but the interval was as long as four weeks for some subjects.

Historical control biopsies

Figure 3A includes re-analysis of previously described RNA seq data from control muscle biopsies [23], included as a historical control comparison. For this dataset, quadriceps biopsies were obtained using similar procedures from nine unaffected subjects (six female, three male) with a mean age of 35 [19–35], who were relatives of FSHD subjects evaluated and genetically confirmed at the University of Rochester. The RNA sequencing reads from the prior study were re-analyzed in parallel to the current study,

however, a different muscle (quadriceps) was biopsied in contrast to the current study (TA).

Magnetic resonance imaging (MRI)

All MRI examinations were performed on a 3T Siemens scanners running E11C. Fiducial stickers placed at the time of MRI were used for biopsy targeting of the TA's and localizing muscle features. T1/T2 Dixon and STIR sequences were centered around the tibial spine (upper/lower station) and acquired using flexible array coils. Sequence parameters were as follows: T1_Dixon: TE1 = 1.35 ms, TE2 = 2.58 ms, TR = 4.12 ms, matrix = 448 × 266, voxel size = 1.1 × 1.1 × 4.0 mm, 104 slices; T2_Dixon (2 echoes): TE = 97, TR = 5570 ms, matrix = 448 × 364, voxel size = 1.1 × 1.1 × 8.0 mm, 40 slices; STIR: TE = 38 ms, TR = 3150 ms, matrix = 320 × 220, voxel size = 1.1 × 1.1 × 5.0, 40 slices).

MRI analytics

Data for each subject were converted into slicer and regions-of-interest created to approximate the punctate biopsy regions ($\sim 0.1 \pm 0.03$ cm) using fiducial landmarks visible on the scans to generate regional fat estimates and UTE measures. STIR hyperintensity were rated qualitatively (by DS and SF) on a four-point scale: 0, normal appearance; 1, very mild diffuse elevation/may be artifact, close to zero; 2, mild diffuse elevation; 3, moderate areas of increased signal intensity; 4, severe involvement of entire muscle. STIR+ features were first scored independently by two raters. Discordant ratings (approximately 10%) were then evaluated for consensus.

T1-weighted Dixon scans were processed using a combination of an artificially intelligent (AI)-based algorithm and manual vetting (Springbok Analytics, Charlottesville, VA). In brief, the AI algorithm segmented the TA from all MRI axial slices [29]. The AI-based segmentation was then vetted by a team of trained segmentation engineers and evaluated by a single researcher (OD) to ensure consistency of segmentation and provide a final 3D label of the TA. The TA volume was calculated by summing the voxel volumes from the segmented TA labels on all slices. To reduce the effects of body size on muscle size across patients, muscle volume was normalized by the product of the patient's height and mass. Lower extremity muscle volumes have been previously shown to vary with the product of height and body mass in healthy, active subjects [30]. To minimize the effect of varying coverage ranges on total TA volume captured; the TA volume was further normalized by the anatomical muscle length of the TA. The anatomical length for the TA was calculated by taking the sum of the 3D Euclidean distances between adjacent centroids of the TA in axial slices [30]. Fat infiltration % was found for each pixel and was calculated as the pixel's fat series intensity divided by the sum of the pixel's fat series intensity and water series intensity [31]. Whole TA fat infiltration was found by taking the average fat infiltration across all pixels corresponding to the labeled TA. A vectorized version of fat infiltration along muscle length was found by computing fat infiltration of the TA at each axial slice as a function of axial slice progression along the TA (from distal to proximal).

As described in the text, values of fat within the regional sample, entire muscle, and STIR features were reduced for comparison to RNA data.

Muscle biopsy for pathologic grading and biomarker studies

Subjects underwent bilateral tibialis anterior muscle biopsy. Fiducial targets on TA were used to localized confluent STIR+ regions

in the mid-portion of the TA if present. Biopsies were obtained under sterile condition. Modified Bergstrom needles were used at all three sites. The average weight of the biopsies was 50 mg for biomarker studies and 50–100 mg for histopathology. Up to three insertions were performed at the same site to obtain adequate sample amount.

Muscle pathology grading

The histopathologic samples were graded by R. Tawil for the severity of the pathologic changes based on 10 μ m sections stained with Hematoxylin & Eosin and Trichrome. A pathologic severity score is determined for each biopsy based on a 12-point scale giving a 0–3 score to each of four major histologic features: variability in fiber size, percent of centrally located nuclei, interstitial fibrosis, and muscle fiber necrosis/regeneration/inflammation [32].

Muscle biopsy immunostaining

Serial sections of frozen tissue were cut at 10 microns and fixed in chilled acetone for 10 min and then air dried for 10 min. Sections were washed in Tris Buffered Saline (TBS). The sections were incubated in primary antibody in Dako Monoclonal Anti-Human C5b9 antibody (catalog number M0777) at a concentration of 1:25 and Vector Labs biotinylated *Ulex Europaeus* Agglutinin I (catalog number B1065) at a concentration of 1:1000 for 1 h. The C5b-9 stained sections were washed in TBS and then incubated for 20 min secondary antibody Vector labs biotinylated goat anti mouse (catalog number BA9200). The *Ulex* sections were washed in TBS and put directly into the ABC as the *Ulex* primary antibody is biotinylated. The C5b-9 sections were washed in TBS and then incubated for 20 min in Vector labs Standard ABC (catalog number PK6100) at 1:100. The sections were washed in TBS and then developed in Vector nova red (catalog number SK4800) for 10 min. The sections were washed in TBS and then counterstained in Mayer's hematoxylin for 3 min. The sections were dehydrated and then cover slipped with permount. For the C5b-9 staining, the sections graded 1 had no positive capillaries and little-to-no C5b-9 staining; Grade 2 had mild C5b-9 staining with 1–2 positive capillaries per 100 fibers; Grade 3 had mild-to-moderate C5b-9 staining with greater than 3 positive capillaries per 100 fibers.

Muscle strength testing

Quantitative muscle strength assessment of the tibialis anterior muscles was performed using a fixed myometry testing system with a force transducer attached by an inelastic strap to a metal frame and was measured in kilograms.

RNA-seq library preparation and sequencing

RNA was extracted from frozen crushed muscle biopsy material using TRIzol and single end 100 nt Illumina sequencing was performed by the Fred Hutchinson Cancer Center Genomics Core.

RNA-seq preprocessing and data analysis

The RNA-seq preprocessing pipeline started with filtering out unqualified raw reads and trimming the Illumina universal adapters by Trimmomatic, followed by alignment against genome built GRCh38(p13) by Rsubread. The gene features were collected from GENCODE, version 35 and the gene counts were profiled by using the GenomicAlignment::summarizedOverlap() function with 'IntersectionStrict' mode, counting reads that completely fall within the range of exons and ignoring ambiguous reads straddling different gene features. The normalization, regularized

log transformation and differential analysis were performed by DESeq2.

Curation for baskets of DUX4 and other FSHD-specific signatures

Here, our goal is to curate a subset of DUX4-targeted genes to represent the DUX4 signature with better performance in discriminating mild-to-moderately affected muscles on molecular levels. The workflow (Supplementary Fig. 2a) started with 67 DUX4-targeted genes that are robustly up-regulated in DUX4-target-positive FSHD biopsy samples, FSHD myotubes, and DUX4-transduced muscle cells [25], followed by [1] lifting the coordinates and annotation from the initially used hg19 to GRCh38 genome build; [2] removing duplicated or deleted DUX4 targeted genes in the GRCh38 genome build instead of previously used hg19; and [3] removing four TRIM 49 variants whose gene expressions were synchronized with TRIM49; the same applied to three TRIM53 variants and two TRIM43 variants.

This process retained 53 of the original 67 genes. To determine the subset(s) of these 53 genes that best discriminate mild-moderate FSHD muscle from control, we employed DESeq2 (adjusted p-value <0.05) and Receiver Operator Characteristic curves (with specificity set to 0.8) to rank the significance of the candidates. These analyses were conducted on the longitudinal study RNA-seq dataset, including the controls and samples that were previously categorized as Mild and Moderate. To ensure the reliability of the candidate genes, additional filtering was applied to confirm that the RNA expression levels of the candidates met two criteria: (1) a sufficient elevation in more severely affected muscles (TPM > 2 in samples categorized as High and IG-High samples) and (2) detectable levels in the current bilateral RNA-seq samples. Subsequently, we identified the 29 most effective and reliable discriminators for mild-moderate FSHD compared to control muscles (Supplementary Table 2). We further refined baskets of the top-ranked genes (by DESeq2 and ROC) include a basket of six (ZSCAN4, CCNA1, PRAMEF5, KHDC1L, MDB3L2, H3Y1) and twelve (PRAMEF15, PRAMEF4, TRIM49, MBD3L3, HNRNPCL2, TRIM43, in addition to the six). We evaluated the performance of these three baskets of discriminators using random forest along with leave-on-out cross-validation to estimate the accuracy rate in distinguishing mild-moderate FSHD from controls. All three baskets of DUX4 outperformed the original four robust genes (LEUTX, PRAMEF2, KHDC1L, TRIM43) that were previously used.

Next, we curated the inflammatory and extracellular matrix basket genes similar to the DUX4 basket. We started with the up-regulated genes (in the mild-moderate affected biopsies) associated with inflammatory response and extracellular matrix and ranked them by DESeq2 and ROC. In addition, we selected the top-ranked and best at showing stability between the initial and follow-up visits based on Pearson correlation. As a result, we curated TNC, COL19A1, COMP, THBS1, SFRP2, ADAM12 for the inflammatory basket and PRG4, RUNX1, CCL19, PLA2G2A, CCL18, CDKN1A for ECM.

Finally, for the complement pathway and immunoglobulin baskets, the curation started with the genes associated with complement classical/alternative activation pathway (GO:0006956, GO:0006957, and GO:0006958) and humoral immune response mediated by circulating immunoglobulin (GO:0002455). Since only a few of these genes were differentially expressed in the mild-moderate affected muscle, we selected the genes that are up-regulated (by DESeq2, adjusted p-value <0.05) in more affected biopsied muscles (previously categorized as IG-High and High

group). The final list preferred the more expressed and stable between the left and right TA muscles (by Pearson correlation). Hence, we obtained IGHG1, IGHG2, IGHG3, IGHG4, IGKC, and FCGR2B for the immunoglobulin basket and C1QA, C1QB, C1QC, C1R, C1S, C3 for the complement activation basket.

Classification of TA biopsies using FSHD molecular signatures and machine learning

Based on the gene expression profiling, we aimed to classify the TA biopsied muscles as Mild and Moderate+ classes using random forest and FSHD molecular signatures, represented by the DUX4, ECM, Inflammatory, Complement, and IG basket genes. To build the training mode, we employed the RNA-seq profiling in TPM from our longitudinal study [22] as the training set where the FSHD biopsies were previously classified (by k-means) into Mild, Moderate, IG-High, and High classes. (Note that the k-means clustered the Mild class with the control samples.) The training metric included two sets of samples: Control and Moderate+, composed of the Moderate, IG-High, and High groups. The leave-one-out cross-validation on the random forest training model yielded 90% accuracy in distinguishing the Moderate+ from the controls. We thus applied the training model to the bilateral TA biopsies, excluding two muscle-low samples, and resulted in 17 Mild (control-like) and 45 Moderate+ samples.

RNA deconvolution analysis to detect immune cells infiltration

We used the PLIER package [28] to perform RNA deconvolution analysis, which allow us to estimate the relative proportion of different cell type contributions to the transcriptome. To do this, we utilized a generic cell-type marker dataset from the immune response in silico [27]. PLIER functions by decomposing an RNA gene expression matrix to construct latent variables (LVs) that represent the weights of relevant subsets of cell type markers. The high-confident LVs depicted in [Supplementary Fig. 8b and c](#) were selected based on the criteria $AUC > 0.7$ and $FDR < 0.05$.

Logistic regression to predict the dichotomy outcomes using MRI characteristics

Using generalized linear models (GLM), we confirmed that the DUX4+/- dichotomous status of the biopsied muscles (partitioned by the threshold of DUX4 score at 1) is linearly associated with the STIR+/- status ($p = 1e-5$) and whole muscle fat percent ($p = 3e-3$). This linear relationship hinted that we could use a logistic regression model with MRI variables as predictors to predict the odds of DUX4+/- outcomes of a TA muscle. To test the stability of the prediction, we performed the same method on our longitudinal and bilateral TA studies using the STIR+/- and regional fat fraction as predictors. The predictions of the two studies yielded similar results ([Supplementary Fig. 3](#)).

Simulation on pairing samples from two pools of STIR status

We performed 1000 runs of simulation, in which each run, 34 pairs were randomly drawn from 43 STIR- and 25 STIR+ samples, yielding the numbers of STIR+/, STIR-/, and discordance STIR+/- pairs. This simulation built three distributions of the number of three types of pairs, and the expected values of each pair type are 4.5 pairs for STIR+/, 13.5 for STIR- and 16 and STIR+/- ([Supplementary Fig. 5a](#)).

DNA methylation analysis

The Trizol/chloroform extracts were frozen after RNA isolation and used for the isolation of genomic DNA from the same

biopsy samples. The SSLP haplotype was analyzed as described on genomic DNA samples to confirm their identities [33]. Genomic DNA (0.6–1.5 μ g) was bisulfite (BS)-converted and processed per manufactured instructions (EpiTect Bisulfite kit, Qiagen). The BS-converted genomic DNA was used for bisulfite sequencing (BSS) analysis using the 4qA (300 ng), 4qA-L (150 ng) and 4qA DUX4 5' (150 ng) amplicons, as described in [34, 35], with the following modifications: BS-PCR products were sequenced by next-generation sequencing (NGS) using the Ion Chef and Ion GeneStudio S5 System (Thermo Fisher Scientific). To accommodate the NGS, the forward and reverse BS-PCR primers used in the nested BS-PCR were fused with Ion Xpress barcode adaptor per manufacturer's instructions (Ion Amplicon Library Preparation User Guide, PN 4468326 Rev C). Sequences were analyzed to provide >3000 independent nonidentical sequencing reads per amplicon.

Code and data availability

The raw RNA-seq read files will be deposited to Gene Expression Omnibus with accession numbers GSE242912. Code scripts and a collection of processed data—gene counts, annotation, clinical scores and meta data—are available in R/Bioconductor formatted datasets from our GitHub repository: https://github.com/FredHutch/Wellstone_BiLateral_Biopsy. The repository also hosts a GitHub-page Book, with detailed narratives of analysis and reproducible R codes (https://fredhutch.github.io/Wellstone_BiLateral_Biopsy). Tabular data from MRI-derived measures are available on reasonable request.

Software

The statistics analysis and visualization were performed using R/4.2.2, Tidyverse packages, and Bioconductor v3.16 packages; the most frequently used packages are tibble, dplyr, ggplot2, DESeq2, ROC, caret, GenomicAlignments and bookdown. Bioinformatics tools used for preprocessing data include fastqc, Trimmomatic (0.32), SAMtools (1.10) and Rsubreads (2.8.1).

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Supplementary data

[Supplementary data](#) is available at *HMG Journal* online.

Conflict of interest statement: L.H.W., S.D.F., T.I.J., P.L.J., S.M.M., R.T., J.M.S. and S.J.T. consult and serve on boards for pharmaceutical companies interested in clinical trial design for FSHD. P.L.J., T.I.J., S.M.M., and S.J.T. serve of the Board of Directors for Renogenyx, a company developing therapeutics for FSHD. S.B., L.R. and O.D. are employed by Springbok Analytics and have stock and/or stock options.

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