Preserved single muscle fiber specific force in facioscapulohumeral muscular dystrophy

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ARTICLE

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Abstract

Objective

To investigate single muscle fiber contractile performance in muscle biopsies from patients with facioscapulohumeral muscular dystrophy (FSHD), one of the most common hereditary muscle disorders.

Methods

We collected 50 muscle biopsies (26 vastus lateralis, 24 tibialis anterior) from 14 patients with genetically confirmed FSHD and 12 healthy controls. Single muscle fibers (n = 547) were isolated for contractile measurements. Titin content and titin phosphorylation were examined in vastus lateralis muscle biopsies.

Results

Single muscle fiber specific force was intact at saturating and physiologic calcium concentrations in all FSHD biopsies, with ($FSHD_{FAT}$) and without ($FSHD_{NORMAL}$) fatty infiltration, compared to healthy controls. Myofilament calcium sensitivity of force is increased in single muscle fibers obtained from FSHD muscle biopsies with increased fatty infiltration, but not in FSHD muscle biopsies without fatty infiltration (pCa_{50}: 5.77–5.80 in healthy controls, 5.74–5.83 in $FSHD_{NORMAL}$, and 5.86–5.90 in $FSHD_{FAT}$ single muscle fibers). Cross-bridge cycling kinetics at saturating calcium concentrations and myofilament cooperativity did not differ from healthy controls. Development of single muscle fiber passive tension was changed in all FSHD vastus lateralis and in $FSHD_{FAT}$ tibialis anterior, resulting in increased fiber stiffness. Titin content was increased in FSHD vastus lateralis biopsies; however, titin phosphorylation did not differ from healthy controls.

Conclusion

Muscle weakness in patients with FSHD is not caused by reduced specific force of individual muscle fibers, even in severely affected tissue with marked fatty infiltration of muscle tissue.

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Glossary

AGE = agarose gel electrophoresis; **ANOVA** = analysis of variance; **BMI** = body mass index; **CSS** = clinical severity scale; **FSHD** = facioscapulohumeral muscular dystrophy; **MRC** = Medical Research Council; **MyHC** = myosin heavy chain; **SDS** = sodium dodecyl sulfate; **TIRM** = turbo inversion recovery sequences.

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common hereditary muscle disorders in adults, affecting 12/100,000 people.[1] It is characterized by asymmetrical muscle involvement, with prominent weakness of the face, shoulder girdle, and foot dorsiflexors. In later stages, weakness extends to the hamstrings, trunk, and pelvic girdle.[2] Disease progression is associated with atrophy and fatty infiltration of muscle tissue that can be visualized on MRI.[3]

The primary mediator of FSHD pathology is considered to be expression of *DUX4*, a gene epigenetically repressed in most somatic tissues located in each unit of the D4Z4 repeat array on chromosome 4.[4] In patients with FSHD, inappropriate DUX4 protein expression in skeletal muscle is facilitated by D4Z4 chromatin relaxation. This only occurs from the permissive 4qA haplotype, which contains a polymorphic polyadenylation signal that stabilizes the DUX4 transcript in somatic tissue.[4] In FSHD1, the most common form of FSHD (95% of all cases), chromatin relaxation is the result of shortening of the D4Z4 repeat to ≤10 units.[5] In FSHD2, chromatin relaxation is the result of a heterozygous mutation in D4Z4 chromatin regulators such as SMCHD1 or rarely DNMT3B, together with a relative shortening of the D4Z4 repeat to ≤20 units.[6,7]

Following the discovery of the genetic cause of FSHD, research has focused on the downstream effects of DUX4 expression. DUX4 is a transcription factor that is normally expressed in the human germline during early development, where it appears to play a role in zygotic genome activation.[8] When misexpressed in somatic cells, DUX4 activates a complex combination of coding and noncoding DNA elements.[9,10] The effects of DUX4 are dose-dependent and include induction of apoptotic cell death, inhibition of myogenesis and muscle regeneration, expression of stem cell genes, and suppression of the innate immune response.[9–14] The effect of DUX4 on muscle contractility is largely unknown. Increasing this knowledge is important as small molecule drugs are being developed that improve muscle fiber contractility.[15,16]

We have previously investigated the contractile properties of FSHD muscle fibers in a pilot study and demonstrated alterations in sarcomeric function, which included reduced specific force in type 2 muscle fibers and increased myofilament calcium sensitivity of force.[17] Single muscle fiber specific force, that is, the amount of force generated corrected for fiber size, is an important determinant of in vivo muscle strength. Myofilament calcium sensitivity of force reflects the ease of skeletal muscle contraction in response to calcium.

Our previous study included only 4 muscle biopsies, which precluded associations with disease severity and warranted confirmation in a larger cohort. In the current study, we included 14 patients with FSHD and 12 healthy controls. Each participant contributed 2 muscle biopsies: one from the vastus lateralis and one from the tibialis anterior. We investigated single muscle fiber contractile properties in 547 single muscle fibers obtained from these muscle biopsies and correlated our findings with FSHD disease severity using quantitative muscle MRI.

Methods

Participants

Patients with genetically confirmed FSHD were recruited from the Radboud University Medical Center. Healthy individuals without a history of neuromuscular disease were included as controls. Exclusion criteria were based on contraindications for participation as well as known factors that may influence skeletal muscle function as a whole or single muscle fiber contractility, specifically age <18 or ≥65 years, diabetes mellitus, chronic obstructive pulmonary disease, chronic heart failure, current malignancy, previous treatment with chemotherapy or radiation therapy, use of corticosteroids during more than 2 weeks in the last 5 years, current use of statins, wheelchair bound, or contraindications for MRI or muscle biopsy.[18] Age matching was applied on the group level, resulting in an adjustment of the lower age limit to ~40 years during patient recruitment. FSHD disease severity was assessed using the Ricci et al.[19] clinical severity scale (CSS). Muscle strength was tested using the Medical Research Council (MRC) grading scale.

Standard protocol approvals, registrations, and patient consents

The Medical Ethics Review Committee region Arnhem-Nijmegen approved this study (no. 2011/181). Informed consent was obtained from all individual participants included in the study.

Quantitative muscle MRI

Prior to MRI, muscle strength of the quadriceps and tibialis anterior were assessed with manual muscle testing using the MRC grading scale. The right leg was biopsied except in the presence of asymmetric weakness, in which case the weakest leg was biopsied. The prospective biopsy site was marked on the skin at about 1/3 of the distance between anterior superior iliac spine and patella for the upper leg and on the maximum muscle bulk of the tibialis anterior for the lower leg. Prior to MRI scanning, a fish oil marker was attached to the skin at the prospective muscle biopsy site. Next, transversal T1-weighted

and multiecho T2 images and turbo inversion recovery sequences (TIRM) of the upper and lower leg were acquired on a 3T MRI system (Tim TRIO; Siemens, Erlangen, Germany), centered on the fish oil marker to facilitate correlation between radiologic findings and tissue-based studies. MRI-guided FSHD vastus lateralis muscle biopsies (see below) were performed immediately after MRI scanning. All other biopsies (see below) were performed on the same day, targeting the marked biopsy site. The percentage of fatty infiltration of the vastus lateralis and tibialis anterior was quantified by manually tracing the outline of the muscle on the multiecho T2 images corresponding to the fish oil marker. For MRI-guided biopsies, an additional fat percentage was determined in a ~1 cm circular area drawn around the MRI-guided biopsy site.[20] FSHD biopsies obtained from a muscle or area with <15% of fatty infiltration are referred to as $FSHD_{NORMAL}$. FSHD biopsies obtained from a muscle or area with ≥15% of fatty infiltration are referred to as $FSHD_{FAT}$.

Muscle biopsy collection

One muscle of the vastus lateralis and one of the tibialis anterior was obtained from each participant. Bergström needle biopsies were performed by an experienced neurology resident (S.L.) taking routine antiseptic precautions.[21] In patients with FSHD, all vastus lateralis muscle biopsies except one were performed with MRI guidance by an intervention radiologist.[22] This was done to obtain a precise measure of the amount of fatty infiltration and TIRM hyperintensity at the muscle biopsy site. This was helpful because in patients with FSHD, fatty infiltration of the vastus lateralis can be patchy even on a transverse axial plane, and TIRM hyperintensity can be focal. The tibialis anterior was chosen because this muscle is affected early and severely in patients with FSHD. The vastus lateralis was included to obtain additional tissue from a less affected muscle in the lower limb. In all cases, biopsy specimens were split in sections and either snap-frozen in isopentane and stored at −80°C for morphometric and immunohistochemical analysis or deposited in a solution containing half glycerol and half relaxing solution and stored at −20°C for single fiber studies. The composition of these solutions is described elsewhere.[23]

Immunohistochemistry

Frozen sections underwent hematoxylin phloxine staining to evaluate variability in fiber size, extent of central nucleation, necrosis and regeneration, and interstitial fibrosis, which were graded as normal (0), mild (1), moderate (2), or severe (3). Severity scores of these 4 measures were then added to provide a cumulative histopathologic sum score between 0 and 12.[24] All histopathology sum scores were assigned by an experienced neuropathologist who was not aware whether a biopsy belonged to the FSHD or control group.

Single muscle fiber studies

Fiber preparation

Biopsy material was placed in a relaxing solution containing 1% Triton X-100 and kept at 4°C during isolation of single muscle fibers.[25] Triton is used to permeabilize the plasma membranes, resulting in "skinned" muscle fibers, which permits analysis of sarcomeric function. Protease inhibitors were added to the solution to prevent protein degradation. Single muscle fibers were isolated and fiber ends were attached to aluminum t-clips, which were mounted between a length motor on one end and a force transducer on the other. Sarcomere length was set at 2.5 μm for measurements of maximum and specific force, cross-bridge cycling kinetics, and calcium sensitivity of force. Fiber width and depth were measured to calculate the fiber cross-sectional area.

Maximum and specific force

We determined maximum force at a sarcomere length of 2.5 μm by activating the fibers with a saturating Ca^{2+} solution (pCa 4.5). Specific force was determined by dividing the maximum force by the fiber cross-sectional area, reflecting the force generated by the sarcomeres.

Cross-bridge cycling kinetics

After development of maximum force, rapid unloading and shortening of the muscle fiber was applied to determine the rate constant of force redevelopment (ktr). The ktr reflects the fraction of strongly bound cross-bridges. After redevelopment of maximum force, slight length perturbations of −0.9%, −0.6%, −0.3%, 0.3%, 0.6%, and 0.9% were imposed on the muscle fiber to determine active stiffness, which reflects the number of attached cross-bridges during activation. Specific force was divided by normalized active stiffness to calculate the tension/stiffness ratio, which reflects the amount of force generated per cross-bridge (assuming intact myofibrils).

Calcium sensitivity of force

After measurement of maximum force and cross-bridge cycling kinetics, the fiber was rested for 5 minutes. Sarcomere length was verified and adjusted if necessary before continuing with the measurements. Calcium sensitivity of force was determined by transferring the muscle fiber to solutions with incremental concentrations of Ca^{2+} (pCa 6.2–4.5). Fibers were excluded if maximum force at pCa 4.5 decreased ≥25% from the force obtained during measurement of maximum force and cross-bridge cycling kinetics. Force-pCa data were fitted to the Hill equation to provide the pCa_{50}, which is the pCa at which 50% of maximal active tension is reached. The steepness of the force-calcium sensitivity curve h (Hillslope) represents the degree of actin-myosin cross-bridge cooperativity.[26]

Passive force

After measurement of calcium sensitivity, the fiber was loosened and rested for 5 minutes. Fibers were set at their slack length (i.e., the fiber length at which passive force is zero) and from there were stretched with a constant velocity of 10% length change/second to a sarcomere length of 3.2 μm, held for 90 seconds, and then released back to slack length. Tension development during stretch was determined to assess passive force. Sarcomere length was assessed during the hold phase; fibers with inhomogeneous sarcomere length distribution (≥0.3 μm variation) were excluded from the analysis.

Table 1 Participants with facioscapulohumeral muscular dystrophy (FSHD) and muscle biopsies

Clinical features			Genetics	MRC		MRI fat, %			Histopathology[a]	
Sex	Age, y	CSS	D4Z4 repeat size (n) or methylation (%)	QF	TA	VL[b]	VL[c]	TA	VL	TA
FSHD1										
M	52	2	7	5	5	1	9	10	4	6
M	59	2	10	5	5	5	5	6	1	2
F	44	3	5	5	5	0	8	10	3	4
F	59	3	9	5	5	1	2	5	3	4
M	49	4	8	5	5	0	2	1	1	2
M	55	5	6	5	2	0	0	84	5	—[d]
M	56	6	8	5	2	3	12	80	3	9
F	52	7	8	5	0	58	39	20	6	7
F	49	8	2; Mosaic[e]	2.5	2	88	92	97	11	8
F	56	8	7	4	2	5	3	69	4	3
FSHD2										
F	48	6	SMCHD1, 8%	4.5	3.5	5	10	6	4	1
F	57	6	SMCHD1, 5%	5	5	28	21	6	2	4
M	61	6	SMCHD1, 24%	4.5	4.5	65	61	64	—[f]	7
M	50	8	SMCHD1, <10%	2.5	4.5	53	82	4	10	—[d]

Abbreviations: CSS = clinical severity score; MRC = Medical Research Council; QF = quadriceps femoris; TA = tibialis anterior; VL = vastus lateralis.
[a] Total histopathologic severity score.
[b] Fat percentage for the area directly surrounding the MRI-guided biopsy site.
[c] Fat percentage for the entire VL at the level of the muscle biopsy.
[d] Not enough tissue was obtained for histologic analysis; single fiber studies were prioritized.
[e] Two-unit D4Z4 repeat in 65% of leukocytes.
[f] Participant declined TA muscle biopsy.

Myosin heavy chain (MyHC) fiber typing

After contractile experiments, individual fibers were stored in 25 μL of sodium dodecyl sulfate (SDS) sample buffer until MyHC isoforms analysis. MyHC isoform composition and concentration of isolated single fibers was determined using SDS polyacrylamide gel electrophoresis.[27] Sample volumes of 8 μL were loaded per lane. Gels were run for 24 hours at 15°C and a constant voltage of 275 V. The composition of the sample buffer and stacking gel is described elsewhere.[23] In hybrid fibers (15% of FSHD and 9% of control fibers), fiber type was assigned as type 1 or type 2 based on the predominant MyHC isoform. Because there was only a limited amount of type 2X fibers (6 FSHD and 4 control fibers), these were analyzed together with type 2A fibers.

Titin content and PEVK phosphorylation

Flash frozen biopsies were ground to a fine powder and resuspended in an 8M urea buffer, with 50% glycerol and protease inhibitors. Tissue homogenates were run on a 1% SDS–agarose gel electrophoresis (AGE) gel to electrophorically separate titin from other proteins. Gels were run at 15 mA per gel for 3 hours and 15 minutes, then stained using Neuhoff Coomassie brilliant blue staining protocol and scanned using a commercial scanner (Epson 800; Epson Corporation, Long Beach, CA). For western blots, samples were run on 0.8% SDS-AGE and subsequently transferred onto Immobilon-P PVDF 0.45 μm membranes (Millipore, Billerica, MA) using a semi-dry transfer cell for 2.5 hours at 1.3 mA/cm^2. Membranes were incubated with primary antibodies against titin N-terminus (TTN monoclonal antibody [M09], clone 6H5; Abnova, Taipei, Taiwan), C-terminus (M8M9; Myomedix, Mannheim, Germany), PEVK region (clone 9D10; Developmental Studies Hybridoma Bank, Iowa City, IA), and phospho-specific antibodies against PEVK S11878 and S12022 (serine locations based on the cardiac isoform, sequence Q8WZ42-3; custom antibodies) at 4°C overnight.[28,29] Infrared western blots were analyzed using Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Statistics

Statistical analysis was performed with IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL). Continuous data were analyzed using one-way analysis of variance (ANOVA) with post hoc comparisons using Bonferroni correction for multiple comparisons. Ordinal data were analyzed using χ^2. Single fiber measurements were analyzed with linear mixed models. A

random intercept was modeled for individual biopsies and individual participants, using a variance components covariance structure. Post hoc comparisons were assessed using Bonferroni correction for multiple comparisons. Passive tension curve fits were analyzed with a 2-way ANOVA with repeated measures, followed by linear mixed models to assess differences between groups at different sarcomere lengths. Titin ratios were determined by taking the linear intercept of multiple increasing total protein sample loadings as a function of the concurrent MyHC intercept. Data are reported as mean ± SEM or median ± interquartile range unless otherwise specified.

Data availability

Anonymized data are available on request via the corresponding author. Supplemental tables are available at doi.org/10.5061/dryad.04gq02h.

Results

Participants and muscle biopsies

We included 14 patients with FSHD and 12 healthy controls who did not differ in age, sex distribution, or body mass index (BMI) (age: FSHD 53.4 ± 1.3 years, control 53.8 ± 1.7 years, p = 0.823; sex: 50% male in both groups, p = 1.000; BMI: FSHD 25.3 ± 1.0, control 27.3 ± 1.5, p = 0.267). Mean FSHD disease duration was 29.6 ± 3.4 years and median Ricci Clinical Severity Score was 6.0 ± 4.3. Detailed information about individual participants with FSHD is provided in table 1. From these participants, we obtained 26 vastus lateralis and 24 tibialis anterior muscle biopsies. Fatty infiltration of the vastus lateralis and tibialis anterior on MRI ranged from 1.1% to 97.4% in participants with FSHD (figure 1). Sixteen FSHD biopsies were obtained from a muscle or area with <15% of fatty infiltration on MRI and are referred to as $FSHD_{NORMAL}$. Ten FSHD biopsies were obtained from a muscle or area with ≥15% of fatty infiltration and are referred to as $FSHD_{FAT}$ (table 2). In vivo muscle strength measured with the MRC grading scale (0–5) was normal in healthy control and $FSHD_{NORMAL}$ muscles. Muscle strength was reduced in $FSHD_{FAT}$ quadriceps and tibialis anterior. Histopathologic sum scores did not significantly differ between healthy control and $FSHD_{NORMAL}$ muscle biopsies but were increased in $FSHD_{FAT}$ muscle biopsies (table 2).

Single muscle fiber contractile properties

Single muscle fiber studies: Maximum and specific force

Single muscle fiber studies were performed in all biopsies. For each muscle biopsy, 10–19 single muscle fibers were measured (547 fibers in total, table 3). In vastus lateralis type 1 (slow-twitch) single muscle fibers, we observed no significant differences in fiber cross-sectional area, maximum force, and specific force among $FSHD_{ALL}$, $FSHD_{NORMAL}$, and $FSHD_{FAT}$ fibers compared to healthy controls. Vastus lateralis type 2 (fast-twitch) single muscle fibers obtained from $FSHD_{NORMAL}$ and $FSHD_{FAT}$ biopsies had an increased cross-sectional area compared to healthy controls and showed increased maximum force generation. Specific force, that is, the amount of force corrected for fiber cross-sectional area, was unchanged in all FSHD vastus lateralis single muscle fibers. This indicates that sarcomeric contractile function is intact at saturating calcium concentrations. In tibialis anterior type 1 and type 2 single muscle fiber cross-sectional area, maximum force and specific force did not differ among $FSHD_{ALL}$, $FSHD_{NORMAL}$, and $FSHD_{FAT}$ tibialis anterior fibers compared to healthy controls.

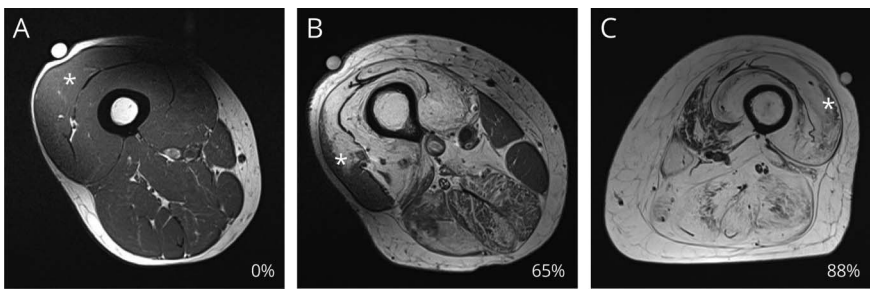
Single muscle fiber studies: Cross-bridge cycling kinetics

In line with our finding that FSHD single muscle fiber specific force is not different from healthy controls, we found no differences in cross-bridge cycling kinetics (table e-1, doi.org/10.5061/dryad.04gq02h).

Single fiber studies: Calcium sensitivity of force

The calcium sensitivity of force was measured in 221 healthy control and 194 FSHD single muscle fibers. The pCa_{50}, which represents the negative logarithm of the Ca^{2+} concentration at which single muscle fibers produce 50% of their maximum force, was significantly increased in all fibers obtained from $FSHD_{FAT}$ muscle biopsies compared to fibers obtained from healthy control and $FSHD_{NORMAL}$ muscle biopsies. This indicates that calcium sensitivity of force is increased in single muscle fibers obtained from $FSHD_{FAT}$ biopsies. There was no difference between fibers obtained from vastus lateralis compared to fibers obtained from tibialis anterior $FSHD_{FAT}$ muscle

Figure 1 Example T1 MRI



(A) MRI scan of the right upper leg of participant F8, a 49-year-old man with facioscapulohumeral muscular dystrophy (FSHD) with a clinical severity scale (CSS) score of 4/10. MRI-guided biopsy site with 0% fatty infiltration. (B) MRI scan of the right upper leg of participant F10, a 61-year-old man with FSHD2 and a CSS score of 6/10. MRI-guided biopsy site with 65% fatty infiltration. A more lateral biopsy site was chosen because only fat was present underneath the marker site. (C) MRI scan of the left upper leg of participant F5, a 49-year-old woman with FSHD with a CSS score of 8/10. MRI-guided biopsy site with 88% fatty infiltration. *MRI-guided muscle biopsy site in the vastus lateralis.

Table 2 Muscle biopsy characteristics

		FSHD total group vs control		FSHD subgroups vs control		
	Control	$FSHD_{ALL}$	*p* Value	$FSHD_{NORMAL}$	$FSHD_{FAT}$	*p* Value
Muscle biopsies, n						
All	24	26	—	16	10	—
VL	12	14	—	9	5	—
TA	12	12	—	7	5	—
MRC score (0–5)						
All	5.0 ± 0.0	5.0 ± 2.3[a]	0.021[a]	5.0 ± 0.0	2.5 ± 2.6[a–c]	<0.001[a]
Quadriceps	5.0 ± 0.0	5.0 ± 0.6	0.151	5.0 ± 0.3	4.5 ± 2.5[a,c,d]	0.031[a]
Tibialis anterior	5.0 ± 0.0	4.5 ± 3.0	0.042[a]	5.0 ± 0.0	2.0 ± 2.3[a,c,d]	0.001[a]
Fatty infiltration on MRI (%)						
All	5.4 ± 1.1	24.9 ± 6.4[a]	0.006[a]	4.0 ± 0.8	62.0 ± 8.6[a,e]	<0.001[a]
VL	6.9 ± 2.1[f]	19.0 ± 8.1	0.168	2.1 ± 0.6[g]	57.5 ± 7.8[a,e,g]	<0.001[a]
TA	3.9 ± 0.8[f]	31.2 ± 10.2[a]	0.022[a]	6.4 ± 2.1[h]	66.1 ± 12.9[a,e,h]	<0.001[a]
Histopathology sum score (0–12)						
All	2.0 ± 2.0	4.0 ± 4.0	0.064	3.5 ± 2.0	7.0 ± 5.0[a,d]	0.001[a]
VL	1.0 ± 1.0	4.0 ± 3.0	0.174	3.5 ± 2.0	8.0 ± 5.0[i]	0.012[a]
TA	2.0 ± 1.0	4.0 ± 5.0	0.224	4.0 ± 2.0	7.0 ± 5.0[a,j]	0.019[a]

Abbreviations: FSHD = facioscapulohumeral muscular dystrophy; MRC = Medical Research Council; TA = tibialis anterior; VL = vastus lateralis.
Number of muscle biopsies, MRC score from the corresponding muscle, amount of fatty infiltration on MRI at the approximate level of the routine Bergström needle muscle biopsy site or the exact area directly surrounding the MRI-guided muscle biopsy site, and histopathology sum score are shown. Because 2 biopsies were obtained from each participant, but biopsies were classified according to the amount of fatty infiltration, each participant can contribute 2 $FSHD_{NORMAL}$ biopsies, 2 $FSHD_{FAT}$ biopsies, or one of each. Data are reported as mean ± SEM for continuous data and median ± interquartile range for ordinal data.
[a] Significant.
[b] $p < 0.001$ Compared to control, not significant compared to $FSHD_{NORMAL}$.
[c] $p < 0.05$ Compared to $FSHD_{NORMAL}$, not significant compared to control.
[d] $p < 0.01$ Compared to control, not significant compared to $FSHD_{NORMAL}$.
[e] $p < 0.001$ Compared to control and $FSHD_{NORMAL}$.
[f] $p < 0.05$ Compared to control and $FSHD_{NORMAL}$.
[g] Fat percentage measured at MRI-guided biopsy site.
[h] Fat percentage for whole muscle at slice corresponding to intended muscle biopsy site.
[i] Not significant in post hoc analysis.
[j] $p < 0.05$ Compared to control, not significant compared to $FSHD_{NORMAL}$.

biopsies ($p = 0.858$, table 4 and figure 2), nor was there a difference between type 1 and type 2 $FSHD_{FAT}$ fibers ($p = 0.596$).

Single muscle fiber studies: Force at physiologic Ca^{2+} concentrations

During maximal contraction, intracellular Ca^{2+} may rise from resting levels of ~0.1 μM (pCa 7.0) to 10 μM (pCa 5.0; a 100-fold increase). However, in physiologic conditions, muscle fibers usually operate at submaximal calcium concentrations, typically resulting in a Ca^{2+} concentration of 1–3 μM (pCa 6.0–~5.5). Specific force at physiologic Ca^{2+} concentrations did not differ between single muscle fibers obtained from healthy control compared to fibers obtained from $FSHD_{NORMAL}$ and $FSHD_{FAT}$ biopsies, despite markedly increased calcium sensitivity in fibers obtained from $FSHD_{FAT}$ biopsies (figure 2). We conclude that increased calcium sensitivity in single muscle fibers obtained from $FSHD_{FAT}$ biopsies maintains specific force generation at physiologic Ca^{2+} concentrations.

Single muscle fiber studies: Cooperativity of activation

The steepness of the force-calcium sensitivity curve was significantly increased in $FSHD_{FAT}$ tibialis anterior type 1 fibers, but not in $FSHD_{FAT}$ vastus lateralis or $FSHD_{FAT}$ tibialis anterior type 2 fibers. This indicates that increased $FSHD_{FAT}$ muscle fiber calcium sensitivity we observed in all $FSHD_{FAT}$ single muscle fibers is not caused by increased cooperativity of activation (table e-2, doi.org/10.5061/dryad.04gq02h). In other words, the slope of the FSHD force–pCa curve is not changed compared to healthy controls but is shifted leftward on the X-axis.

Single muscle fiber studies: Passive force

Based on previous findings, we hypothesized that increased calcium sensitivity of force in $FSHD_{FAT}$ biopsies is caused by titin-based stiffening of muscle fibers and consequential facilitation of actin–myosin coupling.[17] Hence, we measured single muscle fiber passive force, which in skinned fibers reflects titin-based stiffness. Only a limited amount of FSHD

Table 3 Single muscle fiber studies: cross-sectional area and maximum force

		FSHD total group vs control		FSHD subgroups vs control		
	Control	$FSHD_{ALL}$	*p* Value	$FSHD_{NORMAL}$	$FSHD_{FAT}$	*p* Value
Single muscle fibers, n						
Type 1						
All	142	130	—	61	69	—
VL	81	49	—	19	30	—
TA	61	81	—	42	39	—
Type 2						
All	140	135	—	100	35	—
VL	70	108	—	80	28	—
TA	70	27	—	20	7	—
Single muscle fiber cross-sectional area, μm^2						
Type 1						
All	5,861 ± 185	6,156 ± 365	0.581	5,570 ± 303	6,675 ± 630	0.496
VL	5,545 ± 178	6,610 ± 779	0.236	5,271 ± 443	7,458 ± 122	0.056
TA	6,284 ± 356	5,882 ± 350	0.783	5,705 ± 393	6,074 ± 596	0.960
Type 2						
All	6,717 ± 261	7,693 ± 351	0.251	7,847 ± 414	72,536 ± 664	0.484
VL	4,721 ± 191[a]	7,723 ± 405[a]	0.013[a]	7,768 ± 486	7,594 ± 729	0.030[a,b]
TA	8,713 ± 350	7,575 ± 689	0.496	8,164 ± 726	5,891 ± 1,591	0.607
Single muscle fiber maximum force, mN						
Type 1						
All (n = 272)	1.1 ± 0.03	1.11 ± 0.07	0.414	1.06 ± 0.06	1.15 ± 0.12	0.694
VL (n = 130)	0.99 ± 0.03	1.21 ± 0.15	0.149	0.97 ± 0.08	1.37 ± 0.23[a,c,d]	0.021[a]
TA (n = 142)	1.05 ± 0.06	1.05 ± 0.06	0.949	1.11 ± 0.07	0.99 ± 0.10	0.534
Type 2						
All (n = 275)	1.41 ± 0.06	1.59 ± 0.07	0.260	1.41 ± 0.06	1.63 ± 0.08	0.518

Continued

Table 3 Single muscle fiber studies: cross-sectional area and maximum force *(continued)*

		FSHD total group vs control		FSHD subgroups vs control		
	Control	$FSHD_{ALL}$	*p* Value	$FSHD_{NORMAL}$	$FSHD_{FAT}$	*p* Value
VL (n = 178)	0.99 ± 0.05[a]	1.57 ± 0.08[a]	0.015[a]	1.57 ± 0.09	1.57 ± 0.16	0.035[a,b]
TA (n = 97)	1.83 ± 0.08	1.67 ± 0.17	0.867	1.11 ± 0.07	0.99 ± 0.10	0.699
Single muscle fiber specific force, mN/mm^2						
Type 1						
All	178.94 ± 3.43	180.03 ± 3.89	0.878	194.52 ± 4.80	167.22 ± 5.57	0.090
VL	181.86 ± 4.38	182.95 ± 6.48	0.912	189.40 ± 9.41	178.86 ± 8.79	0.756
TA	175.06 ± 5.47	178.26 ± 4.88	0.814	196.83 ± 5.57	158.27 ± 6.92[a,c,e]	0.046[a]
Type 2						
All	213.67 ± 4.24	210.68 ± 4.21	0.611	214.73 ± 4.78	199.09 ± 8.66	0.372
VL	215.32 ± 6.65	209.89 ± 4.55	0.507	210.54 ± 5.36	208.03 ± 8.72	0.787
TA	212.02 ± 5.31	213.81 ± 10.76	0.884	231.47 ± 9.82	163.33 ± 22.13[a,c,e]	0.023[a]

Abbreviations: FSHD = facioscapulohumeral muscular dystrophy; TA = tibialis anterior; VL = vastus lateralis.
Data are reported as mean ± SEM.
[a] Significant.
[b] Not significant in post hoc analysis.
[c] $p < 0.05$ Compared to control and $FSHD_{NORMAL}$.
[d] $p < 0.05$ Compared to control, not significant compared to $FSHD_{NORMAL}$.
[e] $p < 0.05$ Compared to $FSHD_{NORMAL}$, not significant compared to control.

Table 4 Single muscle fiber studies: calcium sensitivity of force

		FSHD total group vs control		FSHD subgroups vs control		
	Control	$FSHD_{ALL}$	*p* Value	$FSHD_{NORMAL}$	$FSHD_{FAT}$	*p* Value
Fibers, n						
Type 1						
All	121	108	—	51	57	—
VL	69	39	—	15	24	—
TA	52	69	—	36	33	—
Type 2						
All	100	86	—	66	20	—
VL	41	61	—	47	14	—
TA	59	25	—	19	6	—
pCa_{50}						
Type 1						
All	5.77 ± 0.01	5.84 ± 0.01[a]	0.028[a]	5.81 ± 0.01	5.88 ± 0.01[a,b]	0.009[a]
VL	5.77 ± 0.01	5.86 ± 0.02[a]	0.020[a]	5.83 ± 0.02	5.88 ± 0.02[a,c]	0.037[a]
TA	5.78 ± 0.01	5.83 ± 0.01	0.074	5.80 ± 0.01	5.87 ± 0.02[a,c]	0.013[a]
Type 2						
All	5.79 ± 0.01	5.80 ± 0.01	0.352	5.77 ± 0.01	5.87 ± 0.02[a,d]	0.002[a]
VL	5.80 ± 0.01	5.80 ± 0.01	0.572	5.79 ± 0.01	5.86 ± 0.03	0.157
TA	5.78 ± 0.01	5.78 ± 0.02	0.742	5.74 ± 0.02	5.90 ± 0.04[a,d]	0.002[a]

Abbreviations: FSHD = facioscapulohumeral muscular dystrophy; TA = tibialis anterior; VL = vastus lateralis.
Data are reported as mean ± SEM.
[a] Significant.
[b] $p < 0.01$ Compared to control, not significant compared to $FSHD_{NORMAL}$.
[c] $p < 0.05$ Compared to control, not significant compared to $FSHD_{NORMAL}$.
[d] $p < 0.01$ Compared to control and $FSHD_{NORMAL}$.

fibers were available for analysis of passive force (80 healthy control, 29 $FSHD_{NORMAL}$, and 15 $FSHD_{FAT}$ single muscle fibers). Hence, we were limited to comparison of healthy control, $FSHD_{NORMAL}$, and $FSHD_{FAT}$ subgroups without individual analyses per fiber type or muscle.

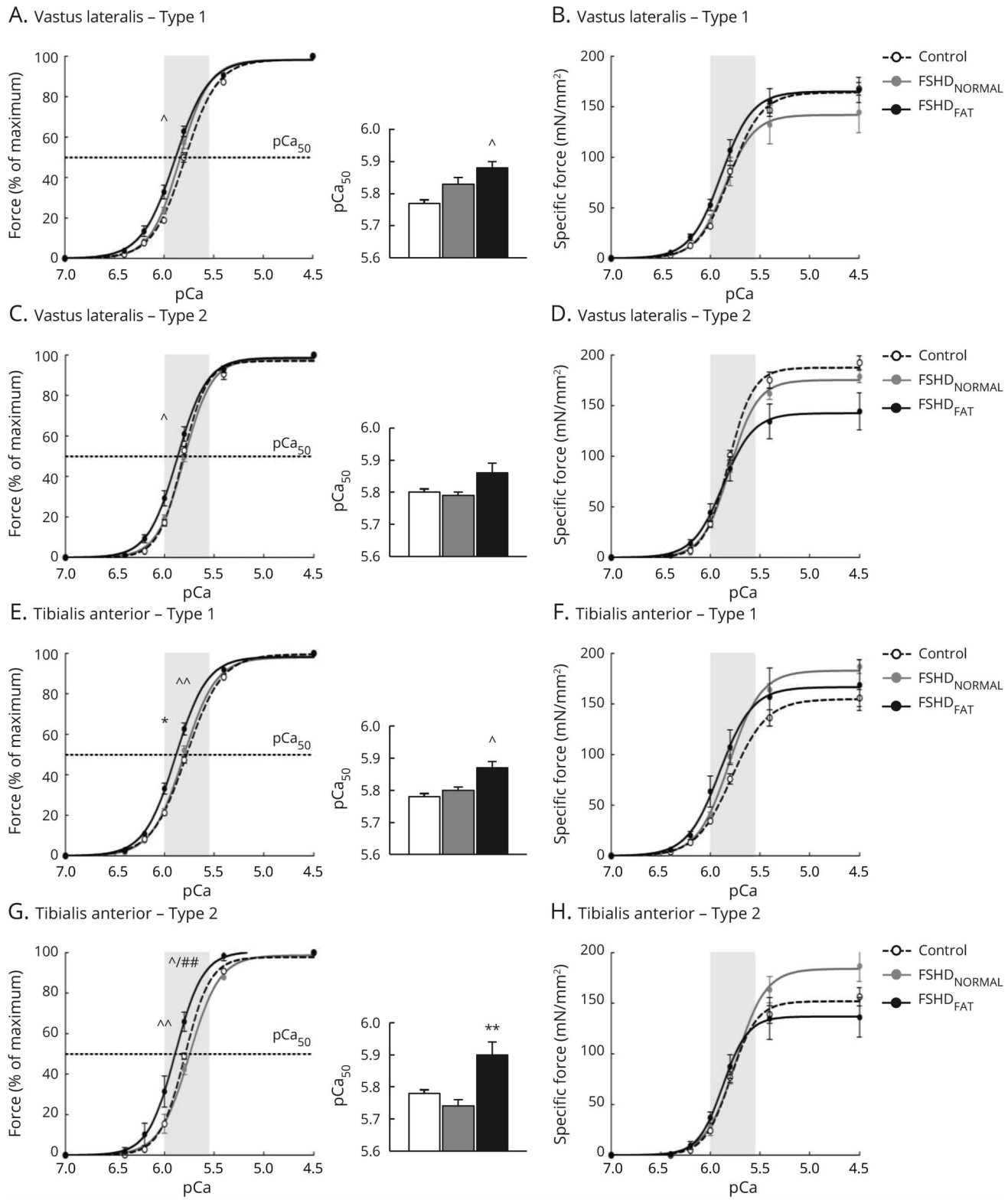
Passive force development was significantly higher in single muscle fibers obtained from FSHD muscle biopsies ($FSHD_{ALL}$) compared to healthy control muscle fibers (p = 0.021 for $FSHD_{ALL}$ vs healthy control), resulting in increased fiber stiffness. Passive force development was significantly increased in fibers obtained from $FSHD_{NORMAL}$ biopsies (p = 0.026 for $FSHD_{NORMAL}$ vs healthy control, figure 3A). Passive force development was also increased in fibers obtained from $FSHD_{FAT}$ biopsies, but this finding failed to reached significance, most likely due to low statistical power (p = 0.143 for $FSHD_{FAT}$ vs healthy control, p = 0.784 for $FSHD_{NORMAL}$ vs $FSHD_{FAT}$; figure 3A).

Post hoc comparisons of passive force at different sarcomere lengths showed no significant difference between $FSHD_{ALL}$ compared to healthy control, nor between $FSHD_{NORMAL}$ and $FSHD_{FAT}$ subgroups compared to healthy control muscle fibers. These findings indicate that passive force development is increased in all FSHD fibers, independent of the amount of fatty infiltration. Hence, the increased calcium sensitivity of force that was only observed in $FSHD_{FAT}$ fibers is not related to increased fiber stiffness in $FSHD_{FAT}$ single muscle fibers.

Muscle biopsy studies: titin content and phosphorylation

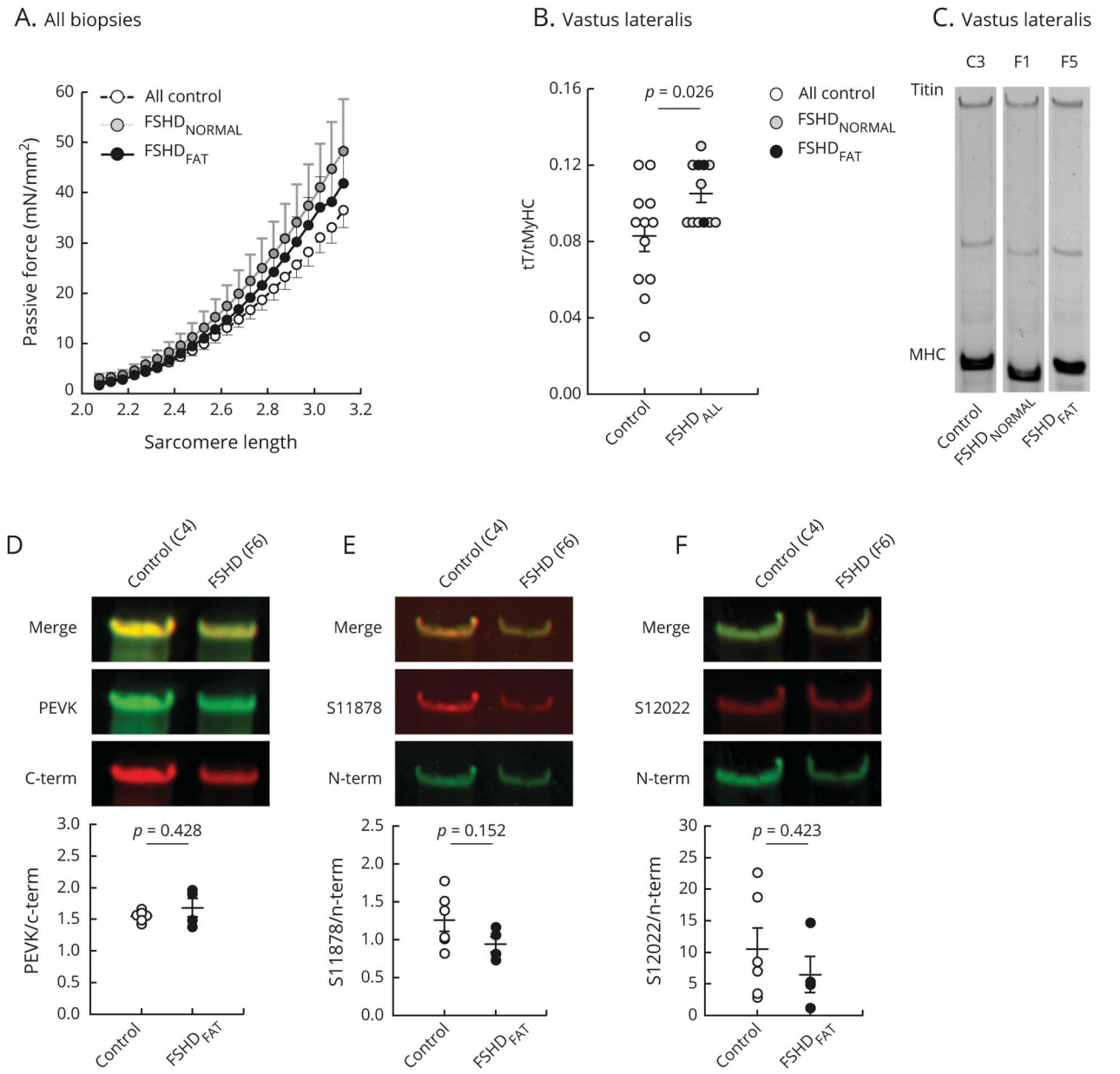
We analyzed titin content and phosphorylation of the titin PEVK segment in vastus lateralis biopsies. Compatible with our single muscle fiber results, titin content was increased in all FSHD biopsies compared to healthy controls but did not differ between $FSHD_{NORMAL}$ and $FSHD_{FAT}$ vastus lateralis muscle biopsies (p = 0.020 for $FSHD_{ALL}$ compared to healthy control, figure 3C). PEVK phosphorylation was only examined in $FSHD_{FAT}$ vastus lateralis muscle biopsies and did not differ from healthy control vastus lateralis biopsies (figure 3, D–F).

Figure 2 Increased calcium sensitivity of force in FSHDFAT muscle fibers maintains specific force



(A, B) Normalized force pCa and analysis of pCa_{50}—the pCa at which 50% of maximum force is reached—of vastus lateralis type 1 fibers demonstrates that calcium sensitivity of force is increased in facioscapulohumeral muscular dystrophy (FSHD) muscle fibers obtained from $FSHD_{FAT}$ biopsies, while specific force is preserved. Physiologic (Ca^{2+}) range during muscle contraction is marked by the gray area. (C, D) Normalized force pCa and analysis of pCa_{50} of vastus lateralis type 2 demonstrates increased normalized force generation at a physiologic pCa of 6.0, with preserved specific force generation. (E, F) Normalized force pCa and analysis of pCa_{50} of tibialis anterior type 1 fibers demonstrates that calcium sensitivity of force is increased in FSHD muscle fibers obtained from $FSHD_{FAT}$ biopsies, while specific force is preserved. (G, H) Normalized force pCa and analysis of pCa_{50} of tibialis anterior type 2 fibers demonstrates that calcium sensitivity is increased in FSHD muscle fibers obtained from $FSHD_{FAT}$ biopsies, while specific force is preserved.

Figure 3 Passive force, titin content, and titin phosphorylation



(A) Single muscle fibers are stretched from slack length to a sarcomere length of 3.2 μm. Resulting passive force development differs in single muscle fibers obtained from $FSHD_{NORMAL}$ and $FSHD_{FAT}$ muscle biopsies compared to healthy control fibers but does not differ between facioscapulohumeral muscular dystrophy (FSHD) subgroups. There are no significant differences between groups in post hoc comparisons at different sarcomere lengths. (B) Vastus lateralis titin content, measured as the ratio between total titin and total myosin heavy chain (MyHC), is significantly increased in $FSHD_{ALL}$ muscle biopsies, but does not differ between $FSHD_{NORMAL}$ and $FSHD_{FAT}$ subgroups. (C) Representative vastus lateralis titin content gels for healthy controls (shown is the vastus lateralis muscle biopsy from participant C3), $FSHD_{NORMAL}$ (participant F1), and $FSHD_{FAT}$ (participant F5) biopsies. (D) No change in total PEVK signal, suggesting splicing of titin is not affected. (E, F) No change in phosphorylation pS11878 and pS12022 within the titin PEVK segment, which suggests that increased passive force is not directly mediated by either alternative titin splicing or through PKCa phosphorylation of the c-terminal PEVK region.

Discussion

In this study, we investigated the contractile properties of 547 single muscle fibers isolated from 50 muscle biopsies from 14 patients with FSHD and 12 healthy controls. Single muscle fiber specific force measurements at physiologic and saturating Ca^{2+} concentrations did not differ from healthy controls, even in fibers obtained from fat infiltrated muscles. This indicates that sarcomeric function is intact and that clinical muscle weakness in patients with FSHD is not caused by reduced specific force of individual muscle fibers.

Single muscle fiber calcium sensitivity of force, which is an important measure that determines the ease of skeletal muscle contraction in response to calcium, was increased in single muscle fibers obtained from FSHD muscle biopsies with fatty infiltration ($FSHD_{FAT}$) compared to fibers obtained from healthy controls. However, increased calcium sensitivity of

force did not result in supramaximal specific force generation. This suggests that increased calcium sensitivity in $FSHD_{FAT}$ single muscle fibers is a compensatory mechanism to maintain specific force in muscles with fatty infiltration.

In other muscle diseases, alternations in single muscle fiber calcium sensitivity of force are usually the result of mutations that directly alter the structural elements of the sarcomere. For example, increased calcium sensitivity of force has been observed in muscle fibers of patients with *TPM2* and *TPM3* mutations.[30] Single muscle fiber calcium sensitivity of force was intact in muscle biopsies from patients with inclusion body myositis, an acquired muscle disease with marked inflammation and fatty infiltration.[31] However, these fibers also had reduced specific force due to myosin loss, which may prohibit compensatory changes in calcium sensitivity.

Single muscle fiber contractile function has previously been investigated in various other neuromuscular disorders. Specific force is generally reduced in myopathies that involve mutations of the force-generating structural components of the sarcomere, such as nemaline myopathy caused by mutations in *NEB*, *ACTA1*, *TPM2*, and *TPM3*.[30] Reduced muscle fiber specific force is also found in myotonic dystrophy, a multisystem disorder caused by a repeat expansion of the *DMPK* gene, and in inclusion body myositis, an inflammatory and degenerative acquired muscle disease.[31,32] In contrast, single muscle fiber specific force in FSHD is intact even in severely affected tissue with marked fatty infiltration. This indicates that muscle weakness in FSHD does not occur through a primary effect of DUX4 on muscle fiber contractility. Instead, muscle weakness in FSHD is most likely the result of DUX4-induced toxicity and consequential loss of muscle fibers resulting in atrophy and fatty infiltration, but not through a primary effect on muscle fiber contractility.

Our current findings contrast with previous results of a pilot study from our group that showed reduced specific force in type 2 FSHD single muscle fibers obtained from 4 FSHD muscle biopsies.[17] Aside from the smaller sample size, the divergent results can also be explained by differences in the healthy control groups. In the pilot study, the control group consisted of relatively young, physically active volunteers. In the current study, we deliberately selected healthy controls with similar age, sex, and lifestyle.

Based on previous findings in 4 FSHD muscle biopsies, we hypothesized that increased calcium sensitivity results from reduced myofilament lattice spacing induced by changes in muscle fiber passive tension.[17] However, our current findings in this larger cohort disprove this hypothesis. Although passive force development was increased in $FSHD_{NORMAL}$ and $FSHD_{FAT}$ single muscle fibers, changes in passive force did not explain the increased calcium sensitivity of force that was exclusive to $FSHD_{FAT}$ muscle fibers.

In skinned single muscle fibers, passive force is primarily mediated by titin, an elastic myofilament that functions as a molecular spring that extends and generates force when muscles are stretched.[33] In this study, the increased passive force in all FSHD single muscle fibers was associated with an increased titin/MyHC ratio. In the sarcomere, titin stoichiometry dictates a 6:1 ratio of titin molecules per half sarcomere.[34] Our findings might suggest a change in this ratio, due to incorporation of an increased number of titin molecules per half sarcomere—a reduced amount of myosin heavy chain molecules would have resulted in reduced single muscle fiber specific force. The presence of changes in passive force development even in mildly affected muscle biopsies without fatty infiltration suggests that this may be an early consequence of disease pathology. Whether DUX4 plays a role in the observed changes in passive force development warrants further investigation.

The strength of this study is the large size of the cohort of patients with FSHD and the inclusion of healthy control biopsies specifically collected for this study. Furthermore, we have used quantitative MRI and MRI-guided muscle biopsies to compare changes in early disease (associated with low levels of fatty infiltration <15%) with progressive disease in fat infiltrated muscle biopsies. The MRI-guided muscle biopsy technique allowed us to obtain an exact measurement of fatty infiltration in vastus lateralis muscle biopsies. Longitudinal studies with repeated muscle biopsies would provide additional information, but it is difficult to engage participants for repeated follow-up biopsies. In this study, almost all participants contributed 2 muscle biopsies on the same day.

Our study has several limitations. First, we used permeabilized single muscle fibers to investigate changes on the level of the sarcomere. This technique prohibits the measurement of upstream processes in excitation–contraction coupling, such as calcium handling or calcium signaling. A previous study found no difference in calcium handling or calcium signaling between FSHD and control myotubes, which suggests that excitation–contraction coupling and calcium handling are intact.[35] Furthermore, isolation of single muscle fibers may favor stronger fibers due to exclusion of fibers with reduced quality on visual inspection or fibers that break prior to initiation of the measurement protocol. Passive force measurements were limited by a small number of fibers.

This study demonstrates that clinical muscle weakness in patients with FSHD is not caused by reduced specific force of individual muscle fibers, even in severely affected tissue with marked fatty infiltration of muscle tissue on muscle MRI. Although specific force is intact at physiologic and saturating Ca^{2+} concentrations, other contractile measures are changed in FSHD single muscle fibers. Muscle fiber calcium sensitivity of force was increased in severely affected tissue and probably is a compensatory mechanism to maintain specific force. Development of passive force was increased in FSHD muscle with and without fatty infiltration, suggesting an early event in disease pathology. Our results increase our understanding of the development of clinical muscle weakness in patients with FSHD.

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Coen A.C. Ottenheijm, PhD	Department of Physiology, Amsterdam University Medical Center, the Netherlands	Author	Obtained funding, supervision of single muscle fiber and titin data collection, interpretation of the data, revising the manuscript for intellectual content
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