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Impaired oxygen demand during exercise is related to oxidative stress and muscle function in Facioscapulohumeral Muscular Dystrophy

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Abstract

Aims
Facioscapulohumeral muscular dystrophy (FSHD) causes progressive muscle weakness and loss. This study aims to compare changes in quadriceps oxygenation and hemodynamics during maximal voluntary quadriceps isometric contraction (MVCQ) and to determine the relationships between these parameters and systemic oxidative stress markers and muscle structural parameters and muscle volume in patients with FSHD and healthy controls.

Methods and results
17 patients with FSHD and 14 sedentary healthy controls were matched for age and physical activity level. Blood antioxidant status and stress markers were evaluated. The quadriceps tissue oxygenation index was evaluated by near infrared spectroscopy during MVCQ. Quadriceps volume was determined by magnetic resonance imaging. Quadriceps muscle samples were obtained to evaluate muscle fiber typology and mitochondria morphology by transmission electron microscopy (TEM). Groups were compared by the unpaired Student t-test or by the non-parametric Kruskal-Wallis test in case of skewed distributions with p<0.05. Associations were assessed by Spearman correlations. Compared to controls, patients with FSHD displayed a significantly lower local O2 consumption per second that was correlated with significantly lower MVCQ and systemic oxidative stress marker levels. Although no difference in muscle typology was observed between groups, TEM showed abnormal aggregation of mitochondria near blood capillaries in FSHD muscles.

Conclusions
These results suggest that patients with FSHD have a lower O2 demand during MVCQ and that TOI measured by NIRS during MVC could be useful to determine the muscle oxidative capacity. Furthermore, these adaptations are related to the muscle structure reorganization linked to the occurrence of oxidative stress.

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Introduction
Facioscapulohumeral muscular dystrophy (FSHD), one of the most prevalent forms of muscular dystrophy (about 4 individuals every 100,000 people) [1], is an autosomal dominant disorder that causes progressive muscle weakness and skeletal muscle loss. Muscle strength reduction contributes to chronic fatigue
through a decreased level of physical activity [2]. Despite major progress in understanding FSHD genetic basis, the specific pathogenic mechanisms of the disorder remain unclear and no curative treatment is available. In previous studies, we demonstrated that the reduced physical performance of patients with FSHD is associated with mitochondrial dysfunction and oxidative stress imbalance [3] and can be improved by antioxidant supplementation [4].

Recently, it has been reported that near infrared spectroscopy (NIRS) can be used to precisely assess muscle mitochondrial respiratory capacity in humans [5], thus allowing the non-invasive, real-time monitoring of muscle oxygenation at rest and during exercise (for a review, see [6,7]). In the field of neuromuscular diseases, NIRS has been used mainly to explore oxygen (O2) uptake impairment in patients with mitochondrial diseases [8–10]. To the best of our knowledge, there are only few reports on muscle oxygenation in patients with muscular dystrophy [11–13]. In these studies, NIRS was used to monitor muscle oxygenation during isokinetic contractions, treadmill exercise or continuous isometric contractions at low intensity. Different results concerning the level of muscle oxygenation during contraction were obtained in function of the dystrophy type and exercise workload [11,12,14].

As patients with FSHD present peripheral skeletal muscle dysfunction [3,4] and reduced cytochrome c oxidase activity and ATP synthesis [3], we hypothesized that NIRS signal during maximal muscle contraction (MVC) is altered in these patients compared with age-matched healthy sedentary controls. We also hypothesized that in patients with FSHD, variations in muscle mitochondrial respiratory capacity determined by NIRS could be related to the level of systemic oxidative stress markers, muscle volume parameters and muscle typology.

**Methods**

**Study design**

This study was not randomized. This was a transversal comparative study of a sub-group of patients recruited from both arms of a double-blind, placebo-controlled, randomized trial in patients with FSHD entitled “Effects Antioxidants Supplementation on Muscular Function Patients Facioscapulohumeral Dystrophy (FSHD)” at the Clinical Physiology Department, Montpellier University Hospital (France). Recruitment to the main study “Effects Antioxidants Supplementation on Muscular Function Patients Facioscapulohumeral Dystrophy (FSHD)” began in May 2010 and a protocol amendment to permit additional analyses and enrolment of healthy controls to the present study was approved 9 months later. The present study allowed to compare changes in quadriceps oxygenation and hemodynamics during maximal voluntary quadriceps isometric contraction and to determine the relationships between these parameters and both systemic oxidative stress markers and muscle function. The inclusion criteria for the main study were published in Passerieu et al., 2015 [4]. Briefly, patients with FSHD between four and nine D4Z4 repeat units and with positive family history for FSHD, aged from 18 to 60 years and without HIV and/or hepatitis were included. Controls were sedentary individuals (i.e., less than 1h of physical activity per week). The exclusion criteria for all subjects were: cardiovascular contraindications to exercise, peripheral arterial vascular disease, cardiac or pulmonary disease, diabetes or human immunodeficiency virus infection and presence of current drug treatments, including vitamins and/or antioxidants.

**Ethics committee approvals**

The original full study protocol was approved by the institutional ethics committee of the Montpellier University Hospitals (Comité de Protection des Personnes de Montpellier - ref. 09 01 01; ID-RB: 2008-A011582-53) and the French National Agency Medicine and Health Products Safety (ANSM - B900956-40), and was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments and the European guidelines for good clinical practice. This study was registered on clinicaltrials.gov (NCT01596803). The subgroup of patients with FSHD and healthy controls study was approved by the institutional ethics committee of the Montpellier University Hospital (Comité de Protection des Personnes de Montpellier - ref. 09 01 01; ID-RB: 2008-A011582-53) as a separate amendment. The trial objectives, study design, risks, and benefits were explained and written informed consent was obtained from all participants. This amendment allowed to compare changes in quadriceps oxygenation and hemodynamics during maximal voluntary quadriceps isometric contraction and to determine the relationships between these parameters and both systemic oxidative stress markers and muscle structural parameters (localization of mitochondria, typology and muscle volume determined by magnetic resonance imaging in patients with FSHD and healthy controls).

**Exercise protocol**

All subjects (FSHD and CONT) came twice to the laboratory at the INSERM U1046, Centre Hospitalier Regional Universitaire (CHRU) of Montpellier, France. During the first visit, the written informed consent and baseline clinical and physical activity data were collected. Moreover, a magnetic resonance imaging (MRI) examination was performed. Then, subjects were familiarized with the testing procedure and performed the maximal voluntary quadriceps isometric contraction (MVC40) test associated with muscle oxygenation.
measurement by NIRS in a well-ventilated room at a temperature of 20–22°C. During the second visit, all subjects underwent a skeletal muscle biopsy and blood sampling (Figure 1).

Figure 1 Study design and exercise protocol. Physical test consisted of 3 to 5 maximal voluntary quadriceps isometric contraction (MVC) for about 6s to evaluate the maximal peak torque and the oxygenation parameters with the near-infrared spectroscopy (NIRS). The text between the boxes indicates the rest periods.

**FIRST VISIT**
- Written informed consent
- Baseline clinical and MRI
- Physical test

**SECONgD VISIT**
- Skeletal muscle biopsy
- Blood sampling

**Data analysis**
- MVC
- NIRS
- Blood

**Force determination during maximal voluntary contraction**

The exercise protocol (MVC) was performed on an adapted exercise bench (Banc de Koch, Genin Medical, France) in seated position with knees and hips flexed at 90° and arms crossed over the chest, as routinely done in our laboratory [18]. All volunteers performed three maximum isometric knee extension trials to obtain at least two values with less than 10% variability and the best value was taken as the MVC, if they could not reach this variability, two others MVC were realized. Each participant was instructed to perform a maximum fast force rise and to maintain the maximum contraction force for about 6s during isometric contractions, with at least 1 min of rest between contractions (Figure 1).

**Determination of blood antioxidant status and stress markers**

The antioxidant status and stress markers were evaluated as previously described [4]. Reduced (GSH) and oxidized glutathione (GSSG), glutathione peroxidase (GSH-Px), copper-zinc superoxide dismutase (CuZn-SOD) and marker of lipid peroxidation (lipid peroxides) were measured in venous blood samples. The detection of urinary 8-hydroxyguanosine (8-OH-dG, a marker of DNA oxidation (oxidized DNA)) levels was normalized to creatinine levels in the urine. Each parameter was routinely determined at Liège University Hospital, Belgium, as previously described [3]. The reference intervals of the mean values of antioxidant and oxidative stress markers were obtained from a large healthy population [15–17].

**Determination of muscle oxygenation by near-infrared spectroscopy and data analysis**

The NIRS signal reflects the balance between O2 supply by the circulatory system and O2 consumption by the muscle and thus allows measuring the quantitative variations in muscle oxygenation, but also the kinetics of deoxygenation and reoxygenation (for a review, see [6,7]). For this study, a spectrometer NIRO 200 was used (Hamamatsu Photonics, Japan). This apparatus measures concentration changes in oxyhemoglobin (O2-Hb), deoxyhemoglobin (Hb), and total hemoglobin (Hb) that are expressed in µmol.1−1.cm. The tissue oxygenation index (TOI) = O2-Hb/tHb, expressed as a percentage, gives an absolute measure of O2-Hb saturation. The optical probe consisted of one emitter with three laser-emitting diodes (775, 810, 850nm) and one detector with two silicon photodiodes (4 mm between photodiodes) separated by a distance of 4 cm. The probe unit supported by a rubber shell was firmly attached to the skin of the distal region of the dominant leg, 15 cm above the knee joint, parallel to the major axis of the quadriceps muscle. Double-sided adhesive tape was used to prevent the probe from sliding on the skin during the exercise. A soft black cloth covered the rubber shell, and all wires were taped down to minimize their movement during exercise.
Prior to each testing session, the NIRO-200 initialization procedure was carried out to set each laser power automatically in order to establish the optimum measurement conditions, and all used procedures were previously described by [21,22]. NIRS signals were sampled at 2 Hz by the NIRO-200 apparatus and collected simultaneously with torque data using the Biopac Acqknowledge Data Acquisition software. They were saved using the NIRO-200 Online software (Version 1.20.03) and stored on the computer for later analysis.

**NIRS data analysis**

The ΔTOI deoxygenation slope (ΔTOI_slope), which represents muscle O₂ consumption per unit of time, was determined as the negative slope of the least square regression line of ΔTOI during the contraction phase. The minimum ΔTOI amplitude (ΔTOI_min) was the difference between the minimum TOI value reached at the end of the contraction phase and the mean 30s baseline TOI value. To provide an estimation of efficiency, ΔTOI_slope values were normalized to the force peak (i.e., ΔTOI_slope/MVC) in both groups. The ΔTOI reoxygenation slope (ΔTOI_slope), which represents the muscle oxidative capacity and/or O₂ supply, was the average positive slope of the least square regression line of ΔTOI during the 6s after reaching ΔTOI_min [21,22]. The maximum ΔTOI (ΔTOI_max) was the difference between the maximum TOI value reached during the 15s relaxation phase and the corresponding mean 30s baseline TOI value. The mean ΔtHb amplitude (ΔtHb_mean) was the difference between the mean tHb value during the contraction phase and the corresponding mean 30s tHb. The t_s was the half time of oxygenated hemoglobin (O₂Hb) recovery to baseline values [23]. The physiological significance of these NIRS parameters was previously described by [21–23]. We also included the maximum ΔtHb amplitude during the relaxation period (ΔtHb_max) and the ΔtHb analysis at specific time points: at the start of contraction (ΔtHb_start), at the end of contraction (ΔtHb_end), at 6s and 15s after the end of contraction (ΔtHb_end and ΔtHb_end).  

**Determination of muscle volume by MRI**

MRI was performed using a 1.5 Tesla MRI apparatus (Magnetom Area Avento, Siemens Medical Erlangen Germany) with "lower limbs" surface antennas without injection of contrast dye and the participants in supine dorsal position, lower limbs at rest. T1-weighted 3 dimensional (3D) flash sequences were obtained in three orthogonal planes centered on the thighs. All images were treated with the Myrian® 64 1.14.2 software (Intrasense, Montpellier, France). MRI images were analyzed by drawing a two-dimensional (2D) “mask” for each ten transversal leg images. Then, the software recognized all transversal masks and determined a 3D mask for the muscle group volume in both legs. To build the 3D mask, first we verified in six patients how many 2D masks (one 2D mask/each 5, 10, 15, 20 or 30 images, respectively) were required to sustain the 3D mask quality. The same procedure was used for assessing the fat infiltration and muscular tissues.

The muscle morphological analysis was carried out by using the thresholding technique and by contour recognition allowing the quantification of morphologically healthy muscle volume (i.e. without evidence of fatty degeneration). To detect and quantify the volume of muscle not affected by the dystrophic processes, we determined an intensity threshold (55–130 cm³) for the identification and quantification of the non-affected muscle volume. Volumes were expressed in cm³. More than 300 images per participant were treated.

**Determination of muscle regions**

To standardize and minimize the probability of erroneous interpretations of the MRI images, all measurements were determined from a standardized bone position between the little trochanter and the proximal extremity of the patella. The determination of the regions of interest (ROI) was performed in each muscle group, outside the fascia and vascular structures. The first ROI, named QD, included all quadriceps muscles (rectus femoris, vastus lateralis, vastus intermedius and vastus medialis). The second ROI, named POST, included the medial muscles (pectineus, gracilis and the three adductors: long, short and wide), hamstrings (semi-membranosus, semi-tendon and biceps femoris) and sartorius. The lipid ratio (RL) was determined by the formula:

\[
RL = \frac{\bar{O}[\text{Muscle Signal}] - (\text{Noise Signal})^2}{\bar{O}[\text{Bone Marrow Signal})^2 - (\text{Noise Signal})^2] \times 100
\]

**Skeletal muscle biopsy**

A muscle biopsy of the lateral part of the vastus lateralis was performed under local anesthesia (xylocaine) by using the percutaneous Bergstrom technique currently employed in our laboratory. Samples were then dissected free of visible connective tissue and fat. Each muscle biopsy was divided in two parts: one part to evaluate the tissue organization by hematoxylin-eosin staining of transversal sections and to investigate the mitochondria morphology (30mg) by transmission electron microscopy (TEM) and one part to assess the fiber typology.

**Muscle fiber typology**

To determine the muscle fiber typology, muscle tissues were frozen in precooled isopentane and then liquid nitrogen. Tissues were cut in fine slices of 10 microns thickness, mounted and stored at -20℃. For immunofluorescence analysis, all incubations were
carried out at room temperature on a shaker and in the dark, in the case of fluorescent immunoreagents. The following primary antibodies were used: anti-myosin heavy chain slow (MHCs) (1:1000 in PBS; Sigma ref M8421) and anti-myosin heavy chain fast (MHCf) (1:800 in PBS; Sigma ref M4276). After washes, samples were incubated with the secondary goat anti-mouse Alexa 488 antibody (1:1000 in PBS; Invitrogen ref A11017). Images were acquired with an Axio Imager M1 fluorescence microscope (Zeiss) and treated with Image J 1.47K and Adobe Photoshop CS3.

Mitochondrial morphology

The mitochondrial morphology was determined by TEM. Fresh muscle samples (5 mg) were immediately fixed in 2% paraformaldehyde, 2.5% glutaraldehyde and 0.1M cacodylate buffer, at 4°C overnight, and then rinsed with 0.1M cacodylate buffer. Samples were then prepared as previously described [24]. TEM analyses were carried out at the Centre Régional d’Imagerie Cellulaire (CRIC) of Montpellier (France).

Statistical Analysis

In this exploratory ancillary study, a total of 17 patients with FSHD completed the study (evaluation of muscle oxygenation by NIRS, muscle function parameters and oxidative stress markers). These patients were individually matched with healthy controls on age and BMI. No sample size was calculated. Data were summarized as mean ± standard deviation (SD). Groups were compared by the unpaired Student t-test or by the non-parametric Kruskal-Wallis (KW) test in case of skewed distributions. The association between any two variables was assessed by the Spearman correlation coefficient. Results were considered significant at the 5% critical level (p<0.05). All statistical calculations were performed with SAS version 9.4 (SAS Institute, Cary, North Carolina). In order to verify the effect size in the comparisons of averages between CONT and FSHD groups, Cohen’s d was observed, and the values of d were considered small if 0.20 ≤ d < 0.50; 0.50 ≤ d < 0.80 and large if d ≥ 0.80 [25]. Analysis of covariance (ANCOVA using SigmaPlot 13 software) was performed, with “NIRS parameters” as the dependent variable and MVCA and group (patient with FSHD/control) as independent covariates.

Results

Subjects

Among 53 adult patients with FSHD enrolled within the main study (Passerieux et al. FRBM, 2015) (Figure 2), 24 patients were assessed for NIRS parameters. Seven patients with FSHD and one healthy control (CONT) were excluded due to the impossibility of interpretation of the NIRS data (excess of background noise), possibly due to the lack of muscle tissue and/or skin-subcutaneous thick fat layer tissue at the probe site and the absence of arterial occlusion during the MVCA indicated by similar values of ΔtHb0 and ΔtHben. A total of 17 patients with FSHD and 14 healthy controls completed the study. Both groups were comparable with respect to age (FSHD 40.8 ±10.8 vs. CONT 42.1 ± 9.8 years), height (176.8 ± 8.5 vs. 172.7 ± 10.6 cm), body weight (BW: 74.1 ± 13.0 vs. 70.1 ± 11.5 kg) and body mass index (BMI): 23.6 ± 3.2 vs. 23.4 ± 2.6 kg/m² (Table 1).

Figure 2 Enrollment, randomization, and study population (modified from Passerieux et al., 2015).
Table 1 Functional muscle parameters in the FSHD (n = 17) and CONT (n = 14) groups.

<table>
<thead>
<tr>
<th></th>
<th>Patients with FSHD</th>
<th>Control subjects</th>
<th>Cohen’s d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=14</td>
<td>N=17</td>
<td></td>
</tr>
<tr>
<td>Age (Yrs)</td>
<td>40.8 ±10.8</td>
<td>42.1 ± 9.8</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>176.8 ± 8.5</td>
<td>172.7 ± 10.6</td>
<td></td>
</tr>
<tr>
<td>Body weight (Kg)</td>
<td>74.1 ± 13.0</td>
<td>70.1 ± 11.5</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.6 ± 3.2</td>
<td>23.4 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>MVC₅₀(N)</td>
<td>160 ± 111*</td>
<td>308 ± 103</td>
<td>1.39</td>
</tr>
<tr>
<td>MVC₅₀/BW (N.kg⁻¹)</td>
<td>2.1 ± 1.5*</td>
<td>4.3 ± 1.1</td>
<td>1.69</td>
</tr>
<tr>
<td>ROFD (N.s⁻¹)</td>
<td>80.5 ± 75.4*</td>
<td>362 ± 264</td>
<td>1.45</td>
</tr>
<tr>
<td>ROFD/BW (N.s⁻¹.kg⁻¹)</td>
<td>1.1 ± 1.1*</td>
<td>5.0 ± 3.0</td>
<td>1.73</td>
</tr>
<tr>
<td>ROFDt200 (N.s⁻¹)</td>
<td>402 ± 396*</td>
<td>1642 ± 941</td>
<td>1.71</td>
</tr>
<tr>
<td>ROFDt200/BW (N.s⁻¹.kg⁻¹)</td>
<td>5.5 ± 5.7*</td>
<td>23.0 ± 12.5</td>
<td>1.79</td>
</tr>
<tr>
<td>Δtime (s)</td>
<td>2.2 ± 1.0*</td>
<td>1.1 ± 0.3</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± SD. * p<0.001 (vs CONT). MVC₅₀: maximum voluntary contraction, ROFD: rate of force development, BW: body weight, Δtime: time to reach the MVC₅₀.

Antioxidant status and blood stress markers in patients with FSHD and control subjects

Whole blood CuZn-SOD and GSH-Px activities were significantly higher in the FSHD group (n=17) than in the CONT group (n=7) (GSH-Px: 47.1 ± 10.0 vs 38.1 ± 5.0 UI/g Hb, p<0.01, d=1.13; CuZn-SOD: 2166 ± 575 vs 1179 ± 206 UI/g Hb; p<0.001, d=2.29). Further, GSH levels (reference interval: 715-1090) were significantly lower and GSSG levels (reference interval: 0.96-10) were significantly higher in the FSHD group (GSH 761 ± 143 vs 823 ± 67.4 μmol/l, p<0.05, d=0.55; GSSG 8.6 ± 9.7 vs 2.6 ± 1.2 μmol/l, p<0.001, d=0.85). No group difference was observed for total GSH pool (reference interval: 717-1110) and GSH/GSSG ratio (reference interval: 111-747). Finally, oxidized DNA (reference interval: 0-20) (FSHD 25.5 ± 13.5 vs. CONT 8.1 ± 2.4 μg/l, d=1.8) and lipid peroxides (reference interval <432) (FSHD 407 ± 258 vs. CONT 148 ± 31.3 μmol/l, d=1.41) were significantly higher (p<0.001) in FSHD than in control subjects. All parameters presented a large effect size (Cohen’s d) except for GSH levels where it was medium.

Quadriceps mechanical performance

Results obtained during quadriceps isometric contraction at the maximum voluntary intensity (see representative curves in Figure 3) indicated that MVC₅₀ (160 ±111 vs 308 ± 103 N), ROFD (80.5 ± 75.4 vs. 362 ± 264 N.s⁻¹) and ROFDt200 (402 ± 396 vs. 1642 ± 941 N.s⁻¹) were significantly lower in the FSHD than in the CONT group (Table 1). These results did not change after normalization to body weight (Table 1). The Δtime also was significantly higher in patients with FSHD than in CONT subjects. All mechanical parameters presented a large effect size (Cohen’s d).

Figure 3 Maximum voluntary isometric contraction of the quadriceps. Representative curves showing the changes in force development during the maximum voluntary isometric contraction of the quadriceps (MVC₅₀) in a patient with FSHD and a healthy control (CONT).
Muscle oxygenation during contraction

The rates of $\Delta$TOI decrease ($\Delta$TOI_{slope} $\pm$ 1.9 $\pm$ 1.4 vs. -3.1 $\pm$ 1.5 %s$^{-1}$; $p<0.05$, $d=0.83$) to reach the minimum value ($\Delta$TOI_{min}) after the end of each contraction were significantly different in the FSHD and CONT groups (see representative curves in Figure 4A) while $\Delta$TOI_{min} amplitudes were comparable (-14.0 $\pm$ 10.7 vs. -14.0 $\pm$ 6.1%). After $\Delta$TOI_{min} normalization to the level of force, the $\Delta$TOI_{min}/MVC ratio was also significantly different between groups (-0.13 $\pm$ 0.14 vs. -0.05 $\pm$ 0.01 %/N; $p<0.001$, $d=0.88$). During the relaxation phase, $\Delta$TOI increased at a significantly higher rate ($\Delta$TOI_{slope}) towards baseline values in the FSHD than in the CONT group (4.3 $\pm$ 4.2 vs. 1.3 $\pm$ 1.4 %s$^{-1}$; $p<0.001$, $d=0.96$), to reach a significantly higher $\Delta$TOI_{max} in the FSHD than CONT group (-1.2 $\pm$ 3.0 vs. -5.9 $\pm$ 4.3 %; $p<0.01$, $d=1.27$).

The $t_{50}$ value was significantly lower in the FSHD than in the CONT group (9.91 $\pm$ 5.97 vs. 15.85 $\pm$ 3.96 s; $p<0.01$, $d=1.15$). All muscle oxygenation parameters also presented a large effect size (Cohen’s $d$).

The $\Delta$Hb decreased rapidly at the onset of contraction in both groups (Figure 4B). $\Delta$Hb was nearly constant during the contraction phase ($\Delta$Hb_{mean}) in both groups, suggesting blood flow occlusion due to muscle contraction. Then, $\Delta$Hb quickly increased after the end of the contraction (reperfusion), and $\Delta$Hb_{15} and $\Delta$Hb_{max} were significantly higher in the FSHD than in the CONT group ($\Delta$Hb_{15}: 58.2 $\pm$ 59.6 vs. 20.5 $\pm$ 33.0 µmol.l$^{-1}$.cm, $p<0.05$; $\Delta$Hb_{max}: 112 $\pm$ 84.0 vs. 40.1 $\pm$ 43.1 µmol.l$^{-1}$.cm, $p<0.01$) with a large effect size. All oxygenation/deoxygenation results during the MVC contraction and relaxation phases are summarized in Table 2.

Figure 4 Muscle oxygenation kinetics during Maximum voluntary isometric contraction of the quadriceps. Representative curves of the changes in oxygenated ($\Delta$HbO$_2$) and deoxygenated ($\Delta$Hb) hemoglobin values (A), tissue oxygenation index ($\Delta$TOI) (B) and total hemoglobin (tHb) values (C) of one patient with FSHD and one healthy control (CONT) obtained after analysis of the near-infrared diffuse optical spectroscopy (NIRS) data collected during maximum voluntary isometric contraction (MVC) of the quadriceps. $\Delta$TOI_{slope}: $\Delta$TOI slope, $\Delta$HbO$_2$: maximum $\Delta$Hb amplitude, $\Delta$HBO$_2$: $\Delta$HbO$_2$ recovery slope, $\Delta$HBO$_2$: maximum $\Delta$Hb amplitude, $\Delta$HbO$_2$: mean $\Delta$Hb amplitude, $\Delta$HbO$_2$: maximum $\Delta$Hb amplitude, $t_{50}$: the half time of $\Delta$HbO$_2$ recovery to baseline values.
**Table 2** Total hemoglobin volume variations during maximum isometric quadriceps contraction.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta \text{Hb}_\text{rest}$ (µmol l$^{-1}$ cm$^{-3}$)</th>
<th>$\Delta \text{Hb}_0$ (µmol l$^{-1}$ cm$^{-3}$)</th>
<th>$\Delta \text{Hb}_{QD}$ (µmol l$^{-1}$ cm$^{-3}$)</th>
<th>$\Delta \text{Hb}_{QD}$ (µmol l$^{-1}$ cm$^{-3}$)</th>
<th>$\Delta \text{Hb}_{POST}$ (µmol l$^{-1}$ cm$^{-3}$)</th>
<th>$\Delta \text{Hb}_{POST}$ (µmol l$^{-1}$ cm$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSHD</td>
<td>6.0 ± 25.0</td>
<td>10.1 ± 27.0</td>
<td>-6.6 ± 32.3</td>
<td>27.2 ± 42.7</td>
<td>58.2 ± 59.6*</td>
<td>112 ± 84.0**</td>
</tr>
<tr>
<td>CONT</td>
<td>7.9 ± 15.4</td>
<td>2.0 ± 4.8</td>
<td>-16.2 ± 23.8</td>
<td>10.7 ± 25.6</td>
<td>20.5 ± 33.0</td>
<td>40.1 ± 43.1</td>
</tr>
<tr>
<td>Cohen’s $d$</td>
<td>0.09</td>
<td>0.41</td>
<td>0.33</td>
<td>0.49</td>
<td>0.78</td>
<td>1.07</td>
</tr>
</tbody>
</table>

Data are the mean ± SD of 17 patients with FSHD and 14 controls (CONT). * $p<0.05$, ** $p<0.01$; $\Delta \text{Hb}_\text{rest}$: total hemoglobin volume at rest; $\Delta \text{Hb}_0$, $\Delta \text{Hb}_{QD}$, and $\Delta \text{Hb}_{POST}$: total hemoglobin difference before the contraction start, at the end of contraction, at 6s and 15s after the end of the contraction and maximum amplitude, respectively.

**Muscle structure determined by MRI**

The total volumes of the QD and POST muscle regions (TV$_{QD}$ and the TV$_{POST}$) were not significantly different between groups. However, the absolute volume and the percentage of healthy QD (MV$_{QD}$ and %MV$_{QD}$) and POST muscles (MV$_{POST}$ and %MV$_{POST}$) were significantly lower in the FSHD than in the CONT group (Table 3). Conversely, the volume of fat infiltration in QD and POST muscle regions (FV$_{QD}$ and FV$_{POST}$) was significantly higher in the FSHD than in the CONT group (FV$_{QD}$: 292 ± 193 vs. 18.5 ± 7.5 cm$^3$; FV$_{POST}$: 746 ± 425 vs. 155 ± 59.9 cm$^3$) as observed also in the MRI images (Figure 5). Excepted MV$_{QD}$ that presented a medium size effect, all others significantly different parameters presented a large effect size.

**Table 3** Muscle volume analysis by magnetic resonance imaging.

<table>
<thead>
<tr>
<th></th>
<th>MV$_{QD}$ (cm$^3$)</th>
<th>FV$_{QD}$ (cm$^3$)</th>
<th>TV$_{QD}$ (cm$^3$)</th>
<th>%MV$_{QD}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSHD</td>
<td>995 ± 706*</td>
<td>292 ± 193***</td>
<td>1288 ± 576</td>
<td>67.2 ± 30.7***</td>
</tr>
<tr>
<td>CONT</td>
<td>1433 ± 427</td>
<td>18.5 ± 7.5</td>
<td>1451 ± 424</td>
<td>98.6 ± 0.9</td>
</tr>
<tr>
<td>Cohen’s $d$</td>
<td>0.75</td>
<td>2.01</td>
<td>0.32</td>
<td>1.44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>MV$_{POST}$ (cm$^3$)</th>
<th>FV$_{POST}$ (cm$^3$)</th>
<th>TV$_{POST}$ (cm$^3$)</th>
<th>%MV$_{POST}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSHD</td>
<td>822 ± 587**</td>
<td>746 ± 425**</td>
<td>1569 ± 381</td>
<td>49.2 ± 30.9**</td>
</tr>
<tr>
<td>CONT</td>
<td>1542 ± 457</td>
<td>155 ± 59.9</td>
<td>1696 ± 427</td>
<td>90.1 ± 5.1</td>
</tr>
<tr>
<td>Cohen’s $d$</td>
<td>1.37</td>
<td>1.95</td>
<td>0.31</td>
<td>1.85</td>
</tr>
</tbody>
</table>

Data are the mean ± SD of 17 patients with FSHD and 7 controls (CONT). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ (vs CONT). MV: volume of healthy muscle; FV: volume of fat infiltrations and connective tissue; TV: MV + FV; % MV: percentage of healthy muscle volume. QD: quadriceps muscles (rectus femoris, vastus lateralis, vastus intermedius and vastus medialis); POST: medial muscles (pectineus, gracilis and the three adductors: long, short and wide), hamstrings (semi-membranous, semi-tendon and biceps femoris) and sartorius.

**Vastus lateralis fiber typology**

The vastus lateralis fiber typology parameters (number, diameter, surface and percentage of MHCs and MHCF fibers) were not significantly different between FSHD and CONT groups (Table 4).

**Mitochondrial morphology by TEM**

TEM analysis showed morphological changes in muscle mitochondria, particularly aggregation of subsarcolemmal mitochondria near blood capillary (Figure 6).

**Correlations between MVC$_Q$ and muscle oxygenation parameters in FSHD AND CONT**

Analysis of the correlations between mechanical and muscle oxygenation parameters in all study healthy controls (n=14) and FSHD (n=17) showed significant correlations between MVC$_Q$ and both $\Delta$TOI$_{slope}$ and $\Delta$TOI$_{min}$ in FSHD group ($r = -0.78$, $p<0.005$) (Table 5, Fig7a). Similarly, these correlations were also observed in CONT group ($r = -0.609$, $p<0.05$). For $\Delta$TOI$_{slope}$ and $\Delta$TOI$_{min}$, analysis of variance with an interaction term showed that there was no significant interaction between the groups (patients with FSHD and healthy controls) and MVC$_Q$ ($P=0.816$ and $P = 0.097$ respectively). However, for $\Delta$TOI$_{min}$ this interaction tended to be significant suggesting that the relation between MVC$_Q$ and MVC$_D$ differed between groups. In the model without interaction $\Delta$TOI$_{min}$ adjusted on MVC$_Q$ remained significantly different ($p < 0.001$) between control and patients with FSHD and the coefficient of covariate MVC$_Q$ was significantly different from zero ($p <0.001$) suggesting that the covariate MVC$_Q$ significantly affects the $\Delta$TOI$_{min}$. 
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**Figure 5** Muscle volume of the quadriceps and posterior muscles. In selected muscle group, MRI images showing fat infiltration (in yellow) and muscle (in red) in the quadriceps and posterior muscles (rectus femoris, vastus lateralis, vastus intermedius and vastus medialis) of both legs in a patient with FSHD and a healthy control. Dashed lines delimit the two muscle regions.

**Table 4** Histological analysis of vastus lateralis fiber typology

<table>
<thead>
<tr>
<th></th>
<th>MHC slow</th>
<th></th>
<th>MHC fast</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Numbers</td>
<td>Diameters</td>
<td>Surface</td>
<td>% of fibers</td>
</tr>
<tr>
<td>FSHD</td>
<td>31.8±19.3</td>
<td>139±36.7</td>
<td>1.72±0.53</td>
<td>49.0±11.9</td>
</tr>
<tr>
<td>CONT</td>
<td>29.2±9.9</td>
<td>131±21.2</td>
<td>1.85±0.42</td>
<td>50.7±9.7</td>
</tr>
<tr>
<td>Cohen’s d</td>
<td>0.17</td>
<td>0.25</td>
<td>0.26</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Values are mean ± SD of histological parameters analysed in fibers of vastus lateralis biopsies stained with anti-Myosin Heavy Chain Slow (MHCslow) and Myosin Heavy Chain Fast (MHCfast) antibodies. Data are the means ± SD of 17 patients with FSHD and 7 controls (CONT).

**Figure 6** Mitochondrial morphology by transmission electron microscopy (TEM). TEM analysis showed morphological changes in muscle mitochondria, particularly aggregation of subsarcolemmal mitochondria (Mss) near blood capillary (Ca).
Table 5 Correlations between studied parameters in patients with FSHD and control subjects.

<table>
<thead>
<tr>
<th>Variables</th>
<th>FSHD patients</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVC/G (%)</td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;slope&lt;/sub&gt;</td>
<td>-0.78</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;min&lt;/sub&gt;</td>
<td>-0.81</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;reox&lt;/sub&gt;</td>
<td>-0.69</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;deo&lt;/sub&gt;</td>
<td>-0.77</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;deo&lt;/sub&gt;</td>
<td>-0.64</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;deo&lt;/sub&gt;</td>
<td>-0.46</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;deo&lt;/sub&gt;</td>
<td>0.69</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;deo&lt;/sub&gt;</td>
<td>0.63</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;deo&lt;/sub&gt;</td>
<td>-0.68</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;deo&lt;/sub&gt;</td>
<td>0.82</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MVCQ/ΔTOI&lt;sub&gt;deo&lt;/sub&gt;</td>
<td>0.73</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

MVCQ, volume of healthy quadriceps muscle; % MVCQ, percentage of healthy quadriceps muscle volume; ΔTOI<sub>slope</sub>, ΔTOI<sub>deo</sub> oxygenation slope; ΔTOI<sub>min</sub>, minimum ΔTOI amplitude; ΔTOI<sub>reox</sub>, ΔTOI reoxygenation slope. CuZn-SOD, copper–zinc-dependent superoxide dismutase; GSH-Px, glutathione peroxidase; GSSG, total glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione. It was used the Spearman correlation coefficient.

Correlations between muscle parameters, oxidative stress and antioxidant markers in FSHD and CONT

No correlation between muscle parameters and antioxidant status or oxidative stress markers could be found in healthy controls (n=7). In patients with FSHD, the blood level of lipid peroxides was negatively correlated with the MVCQ (r = -0.69, p<0.01) and with MVCQ/BW (r = -0.64, p<0.01). By contrast, blood concentrations of the antioxidant marker GSH-Px increased with MVCQ/BW values (r = 0.46, p<0.05) (Table 5). Similarly, a negative correlation was observed between ΔTOI<sub>slope</sub> and GSSG values (r = -0.64, p<0.01). The ΔTOI<sub>slope</sub> was also positively correlated with the GSH/GSSG ratio and the blood levels of lipid peroxides (oxidative stress markers) (r = 0.57, p<0.05 and r = 0.43, p<0.05, respectively). A significant positive correlation was also observed between ΔTOI<sub>deo</sub>/MVCQ and the concentration of the antioxidant GSH (r = 0.45, p<0.05) (Table 5). MVCQ and MVCQ/BW were significantly related with MVCQ (r = 0.92, p<0.005 and r = 0.83, p<0.001, respectively) and % MVCQ (r = 0.82, p<0.001 and r = 0.73, p<0.05, respectively) (Table 5 and Figure 7b). Moreover, ROFD and ROFD/BW were correlated with MVCQ (r = 0.69, p<0.05 and r = 0.63, p<0.05, respectively). Finally, the reduction in muscle volume in patients with FSHD resulted in a reduced O<sub>2</sub> consumption during muscle contraction. Hence, a negative and significant correlation between the MVCQ and the deoxygenation rate (r = -0.68, p<0.01) was observed in this group (Figure 7c).
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Discussion

The results of this study suggest that, compared to healthy controls, patients with FSHD evidence a number of salient findings: 1) oxidative stress markers and antioxidant values are altered; 2) torque production is significantly decreased and is associated with reduced local $O_2$ consumption per unit of time; 3) muscle oxygenation and muscle performance parameters ($\Delta TOI_{\text{Slope}}$, MVCQ) are significantly correlated with oxidative stress markers; 4) skeletal muscle volume is significantly reduced and fat tissue increased for the same volume, although muscle typology is comparable between groups; 5) the reduction in muscle volume is related to reduced force production. The present data provide novel insights into the muscle oxidative metabolism, muscle volume and the correlations of these parameters with oxidative stress in patients with FSHD and contribute to explaining the reduction in force production observed in these patients.

The levels of oxidative stress markers and antioxidant molecules (GSH, GSSG, GSH-Px, CuZn-SOD, lipid peroxides, and oxidized DNA) were significantly altered in the FSHD group compared with the CONT group. These findings confirm the higher oxidative stress levels previously observed in patients with FSHD [3,4]. The higher oxidative stress levels are correlated with lower force development and ROFD in the FSHD group compared with the CONT group. A significant between-group difference in the oxidative metabolic demands (i.e., $O_2$ consumption relative to $O_2$ supply) in the quadriceps was observed during MVCQ, in accordance with a previous study [14].

The difference in $O_2$ demand between groups could be explained by the negative correlation between MVCQ and $\Delta TOI_{\text{Slope}}$ (Fig. 7a), observed also in control group. This suggests that the different muscle $O_2$ demands represent a different activation of the quadriceps muscle volume [21,25] and/or that in FSHD, less muscle mass is involved during contraction (due to the presence of muscle fatty infiltration). However, $\Delta TOI_{\text{Slope}}$ was not significantly different between groups after normalization by MVCQ and there was no significant interaction between groups (patients with FSHD and healthy controls) and the covariate MVCQ. But we cannot exclude lack of statistical power to detect between group differences. The finding that $\Delta TOI_{\text{min}}$ was significantly different between groups only after normalization to force production (Table 3) confirms that dystrophic quadriceps muscles in FSHD produce less force for a lower $O_2$ consumption. Therefore, the reduced $\Delta TOI_{\text{Slope}}$, in conjunction with arterial occlusion during MVCQ [28], as indicated by the similar values of $\Delta HBD$ and $\Delta HB_{\text{endr}}$, shows that the volume of $O_2$ consumption by dystrophic quadriceps is reduced in the FSHD group compared with controls. This suggests a reduction in the ability of mitochondria to provide energy during MVCQ, which is consistent with previous results from our laboratory [3]. Indeed, in FSHD muscle biopsies, we observed mitochondrial dysfunction (decreased cytochrome c oxidase and ATP synthesis activities) that was correlated with lower quadriceps force production [3].

A novel finding of the present study is that the significant difference in deoxygenation kinetic parameters during MVCQ between the FSHD and CONT groups is linked to the oxidative stress status in patients with FSHD. Mitochondrial dysfunction and oxidative stress might play a crucial role in FSHD pathology [4,29–32]. Interestingly, the $\Delta TOI_{\text{Slope}}$ was negatively correlated with the blood level of lipid peroxides and GSSG and positively correlated with the GSH/GSSG ratio in the FSHD group (Table 5). The link between lower $O_2$ demand and higher lipid peroxide levels could be the result of morphological alterations in muscle mitochondria induced by oxidative stress that might lead to lower ATP synthesis in patients with FSHD [3]. Indeed, it was previously shown that NIRS data are correlated with the mitochondrial capacity in healthy subjects [5]. Moreover, the imbalance between lower blood GSH and higher blood GSSG values, observed in the FSHD group, could be explained by modifications in the intracellular redox environment and is consistent with the observation that GSH reduction reflects an increase in oxidative stress and mitochondrial dysfunction [33].

The significant reduction in force production and ROFD in patients with FSHD compared with controls is consistent with the muscle loss and weakness that characterize this disease [3,34–36]. This is associated with alterations in the energy metabolite levels [37] and lower quadriceps force development during MVCQ in patients with FSHD compared with healthy subjects [3]. An additional novel aspect of the current study was to investigate muscle volume and fat infiltration in the thigh by MRI and not only the cross-section surface area [37,38]. To our knowledge, this is the first study assessing muscle volume in patients with FSHD. We did not find any difference in the total volume of quadriceps and posterior muscles (TVQD and TVPOST) between the FSHD and CONT groups. However, we report a significant reduction in the percentage of healthy muscle volume (MVQD and MVPOST) in patients with FSHD compared with CONT. This could be explained by the significant increase in fat infiltration and fibrous tissue (FVQD and FVPOST) in FSHD muscle (Table 3) and is consistent with previous observations obtained on cross-sectional area surface [35].

Moreover, muscle volume and fat infiltration as well as the deoxygenation kinetics were linked to force production (Fig. 7c) and were correlated with oxidative stress markers and the antioxidant status in patients with FSHD (Table 5). Therefore, our results suggest that the relationship between increased fat infiltration and lower
muscle strength is coherent with the loss of muscle mass [37]. Additional studies are needed to confirm that muscle atrophy occurs in response to oxidative stress and the effects of antioxidant supplementation to counteract dystrophy progression.

In addition to these structural reorganizations inside FSHD muscles, we observed morphological changes characterized by abnormal aggregation of subsarcolemmal mitochondria near blood capillaries. This reorganization occurred without changes in muscle fiber typology. Indeed, we found similar muscle fiber typology in \textit{vastus lateralis} biopsies from both groups, in accordance with the typology observed in healthy muscles [39]. Furthermore, Statland et al. (2015) observed a decrease in the capillary density in the vastus lateralis of patients with FSHD that could result in lower intracellular O$_2$ tension. Thus, mitochondria aggregation near blood capillaries could be the result of a migratory movement inside skeletal muscle regions with a better O$_2$ pressure gradient. The exact mechanism of this internal mitochondria reorganization is an important question for future studies.

Conclusions

The findings of this study provide clear evidence that patients with FSHD present a lower O$_2$ demand during maximal isometric contraction related to higher oxidative stress and reduced muscle volume, leading to reduced muscle functional performance compared with healthy sedentary controls. While the present work provides clear evidence that NIRS can be a useful tool to assess the kinetics of deoxygenation and reoxygenation in FSHD during maximal isometric exercise, other studies are necessary to determine the mechanisms by which muscle function is altered in FSHD. Nevertheless, this study supports the idea that oxidative stress could play an important role in FSHD pathophysiological mechanisms [29–32].

Acknowledgments

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Conflict of interest statement

The authors declare that they have no conflict of interest

References


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