



Structural and Functional Alterations of Skeletal Muscle Microvasculature in Dystrophin-Deficient mdx Mice.

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30 The authors declare no conflict of interest.

31

32 **ABSTRACT**

33 Duchenne Muscular Dystrophy (DMD) is a progressive neuromuscular disease, caused by an
34 absence of dystrophin, inevitably leading to death. Although muscle lesions are well
35 characterised, blood vessel alterations that would have major impact on muscle regeneration,
36 remain poorly understood. Our aim was to elucidate alterations of the vascular network
37 organisation, taking advantage of Flk1^{GFP/+} crossed with mdx mice (model for human DMD
38 where all blood vessels express GFP) and functional repercussions using *in vivo* nuclear
39 magnetic resonance (NMR), combining arterial spin labeling imaging of perfusion, and ³¹P-
40 spectroscopy of phosphocreatine kinetics. For the first time, our study focused on old (12
41 month-old) mdx mice, displaying marked chronic muscle lesions, very similar to the lesions
42 observed in human DMD, in comparison to young-adult (3 month-old) mdx mice displaying
43 only mild muscle lesions with no fibrosis. Using an original approach combining specific
44 animal model, state of the art histology/morphometry techniques, and functional NMR, we
45 demonstrated (i) that the microvascular system is almost normal in young-adult in contrast to
46 old mdx mice, displaying marked microvessel alterations, and (ii) functional repercussions on
47 muscle perfusion and bioenergetics after a hypoxic stress, that vary depending on stage of
48 pathology. This original approach clarifies disease evolution and paves the way for setting up
49 new diagnostic markers or therapeutic strategies.

50

51 INTRODUCTION

52 Duchenne muscular dystrophy (DMD) is the most frequent genetic neuromuscular disorder
53 affecting 1:3500 school-age boys worldwide. This X-linked muscle disease is characterised
54 by progressive skeletal muscle weakness and cardiomyopathy, leading to premature death
55 generally because of respiratory and/or cardiac failure. The cause of DMD is the absence of
56 dystrophin, a key component of the dystrophin-associated protein complex involved in the
57 linkage between myofiber cytoskeleton and extracellular matrix. When linkage is disrupted,
58 muscle fibers develop normally but are more susceptible to damage due to mechanical
59 stretch. Despite presence of satellite cells (muscle stem cells) and successive regeneration
60 attempts, myofibers undergo necrosis and are eventually replaced by connective and adipose
61 tissue¹.

62 Muscle lesions in DMD have been widely investigated, with studies focusing principally on
63 myofibers and/or satellite cells (SC). Although (i) skeletal muscle is one of the most
64 vascularised tissues, (ii) endothelial cells are essential in muscle regeneration process, and
65 (iii) dystrophin is expressed in endothelial/smooth muscle cells, disease impact on blood
66 vessels and effect of blood vessel alteration in disease expression remain poorly understood.

67 In recent years, interest in DMD vascular network has increased with primary focus on
68 vasculature-related therapeutic strategies² such as methods to increase vasculature by
69 modulating VEGF/VEGFR pathways³. These strategies were initially based on: (i)
70 observation of “grouped necrosis” in muscles of DMD patients, *i.e.* simultaneous necrosis of
71 contiguous myofibers, suggesting local failure in capillary blood supply and muscle ischemic
72 necrosis⁴, and (ii) membrane-associated nitric oxide synthase (NOS) deficiency in
73 dystrophin-deficient muscle⁵. The hypothesis of an ischemic process has been strongly
74 discussed, as other studies could not detect any vascular bed abnormality in DMD either

75 morphologically using electron microscopy⁶ or physiologically by studying muscle blood
76 flow⁷⁻⁹. More recent studies carried out in DMD patients confronted blood vessel alteration
77 with tissue fibrosis. They suggested that endomysial fibrosis plays an essential role, causing
78 an increase in capillary-to-myofiber distances, which impairs both muscle fiber mechanical
79 function and gas exchanges¹⁰. Moreover, increased distances between capillaries and
80 myofibers could potentially impede their reciprocal stimulation by soluble factors secreted
81 during muscle repair¹¹.

82 Rare studies addressed the relevance of muscle vascular network in dystrophinopathy
83 pathophysiology in animal models, focusing on muscle vascular density and characterisation
84 of a possible hypoxic condition in dystrophic muscle. Part of these studies pointed to a
85 decreased vascular density and an impaired angiogenesis in 6 week- to 6 month-old mdx
86 mice¹²⁻¹⁴ the dystrophin-deficient murine model of human DMD. However, contradictory
87 results were also published in mdx mice, showing a higher hindlimb perfusion one week after
88 femoral artery dissection and significant increase in arteriole length density in 2 month-old
89 animals¹⁵. These discrepancies could be related in part to the effect of aging in disease
90 progression; age appears to be an important parameter to consider when studying vascular
91 changes³. Thus, involvement of blood vessels in the pathogenesis of dystrophy is still not
92 completely understood.

93 In the present study, we investigated both structural organisation and *in vivo* function of
94 vascular system in young-adult (3 month-old) mdx mice, displaying only moderate subacute
95 muscle lesions with no fibrosis, and old (12 month-old) mdx mice, displaying marked muscle
96 lesions with persistent inflammation and fibrosis¹⁶, more relevant for the study of DMD
97 pathophysiology in human. We used complementary morphological approaches based on
98 genetically-modified mice that allowed for the first time to reconstruct the 3-dimensional

99 microvascular network in mdx mouse. These were confronted to innovative histological
100 techniques and dynamic and non-invasive multiparametric and functional nuclear magnetic
101 resonance (NMR).

102

103 **MATERIAL AND METHODS**

104 **Mice**

105 C57Bl/6J control mice were obtained from Charles River Laboratory (l'Arbresle, France),
106 mdx-4Cv with C57Bl/6 background mice, model for human DMD, were kindly provided by
107 Pr. Gherardi (Hôpital Henri Mondor, France), Flk-1^{GFP/+} mice, in which green fluorescent
108 protein (GFP) is targeted in vascular endothelial growth factor (VEGF) receptor-2 gene locus,
109 exhibiting a bright GFP signal in all endothelial cells, were kindly provided by A. Medvinsky
110 (Institute for Stem Cell Research, University of Edinburgh, UK), and Flk-1^{GFP/+}::mdx-4Cv
111 mice were obtained by crossing Flk-1^{GFP/+} with mdx-4Cv mice. Only male animals were used
112 *i.e.* young-adults (3 month-old) or old (12 month-old).

113 Animals were housed in animal facilities of the Institut Pasteur licensed by the French
114 Ministry of Agriculture and complying with European Union regulations. Protocols were
115 approved by the Institut Pasteur Animal Experimentation Ethics Committee (01332.01).

116

117 **Microvascular network organisation in three dimensions**

118 Young-adult and old Flk-1^{GFP/+} and Flk-1^{GFP/+}::mdx-4Cv mice were anaesthetised with
119 isoflurane inhalation (Forene, Abbott, Rungis, France) and killed by cervical dislocation.
120 *Gastrocnemius* muscles were removed and imaging of vascular network was carried out in
121 two conditions: thick cryo-sections or whole muscle. *Gastrocnemius* muscle was snap frozen

122 in liquid nitrogen-cooled isopentane before cryosectionning (100 μm -thick sections).
123 Confocal acquisitions were performed using a spinning disk microscope (Leica, Wetzlar,
124 Germany), laser femto-second was used: Chameleon Ultra, 20x/0.7 and 40x/0.75 objectives
125 and a CoolSnap HQ2 camera. Optical slices were taken every 0.5 or 0.3 μm interval along
126 the z-axis (80 μm).

127 For whole muscle conditions, images of *Gastrocnemius* blood vessels were obtained from the
128 entire muscle using multi-photon scanner resonant confocal Leica TCS-SP5 with 20x/0.95
129 objective. Optical slices were taken every 0.5 μm along the z-axis.

130

131 **Histological/Immunohistochemical analysis**

132 *Gastrocnemius* muscles were collected from mice after NMR experiments, snap frozen in
133 liquid nitrogen-cooled isopentane and kept at -80°C. Six different levels of 7 μm -thick
134 sections were cut and stained with hematoxylin-eosin (HE) to describe histopathological
135 modifications of muscle tissue, and Sirius red for visualisation of collagen. For
136 immunohistochemistry analyses, muscle cryosections were incubated with antibodies
137 directed against endothelial cells (anti-CD31; Pharmingen), satellite cells (anti-Pax7; DSHB,
138 Iowa city, IA, USA), pericytes (anti-NG2; Millipore), smooth muscle cells (α SMA; Sigma)
139 and basal lamina (anti-laminin; Sigma). Primary antibodies were incubated overnight at 4°C
140 and revealed by cy3- or TRITC-labeled secondary antibodies (Jackson ImmunoResearch
141 Laboratories).

142

143 **Morphometric analysis**

144 Two-dimension analysis was performed to evaluate distribution of muscle fiber diameter,
145 percentage of centro- or peri-nucleated fibers, microvessel count and distribution around each
146 myofiber using ImageJ (NIH, Bethesda, MD, USA) and NIS-Element (Nikon) softwares. At
147 least 200 fibers were considered for each muscle.

148 Three-dimensional analysis was performed to evaluate organisation of vascular network. For
149 each muscle, 10 z-stack image reconstructions were achieved on 80 to 150 μm -thick frozen
150 sections. Analysis was carried out using IMARIS (ImarisBitplane, Zurich, Switzerland)
151 software (quantification of vessel density, tortuosity, volume, anastomose count, diameter
152 and distance between microvessels).

153

154 **Quantitative RT-PCR**

155 We used real-time PCR to determine the level of angiogenesis-related mRNA expression in
156 young-adult and old mdx mice. Total *Gastrocnemius* muscle RNA was extracted using
157 RNeasy Mini Kit (Qiagen). One μg of total RNA was reverse transcribed into first-strand
158 cDNA using Superscript II Reverse Transcriptase (Life technologies). Quantitative PCR was
159 carried out on StepOne Plus RealTime PCR system (Applied Biosystems, Carlsbad, CA,
160 USA). Reaction mixtures had a final volume of 20 μl , consisting of 1 μl of cDNA, 10 μl of
161 Sybr Green Master (Roche) and 10 μM of primers, listed in Table 1. After initial
162 denaturation, amplification was performed at 95°C (10 s), 60°C (5 s), 72°C (10 s) for 45
163 cycles. Calculation of relative expression was determined by the StepOnePlus software
164 (Applied Biosystems) and fold change was normalized to 18S rRNA housekeeping gene.

165

166 **Nuclear Magnetic Resonance analysis**

167 NMR experiments were performed on: 3 month-old mdx-4Cv (n=6) and control C57Bl/6J
168 (n=9) and on 12 month-old mdx-4Cv (n=5) and control C57Bl/6J (n=7).

169 *Hyperaemic response paradigm:* To highlight differences between normal and altered
170 muscles we classically applied a stress to increase the global need for perfusion. Ischemia-
171 reperfusion stress was applied to the mouse left hindlimb which provokes maximal
172 vasodilatation and limited resistance of arteries/arterioles ¹⁷ just after tourniquet release.

173 In practice, anaesthesia was induced with 4% isoflurane delivered in 1.5 L/min air and
174 maintained with 1.75% isoflurane. During experiments, a water heating pad ensured a
175 constant temperature of 37°C and breathing was monitored. After a 24 min NMR acquisition
176 at rest (baseline), ischemia of the leg was induced by occlusion of femoral artery by two
177 surgical threads placed around the thigh and pulled tight by application of a weight ¹⁷. After
178 30 min of ischemia, the weight was instantly removed, inducing a hyperaemic response
179 which was monitored over the next 30 min. During whole protocol, dynamic acquisitions of
180 NMR scans of interleaved perfusion imaging and ³¹P-spectroscopy (³¹P-NMRS) were
181 collected.

182 *Multiparametric functional NMR (mpf-NMR) acquisitions.* *In vivo* NMR experiments were
183 conducted in a 4 Tesla Biospec system equipped with a 20 cm diameter 200 mT.m⁻¹ gradient
184 insert (BrukerBioSpin MRI GmbH, Ettlingen, Germany). Mice were placed supine in a 6 cm
185 diameter, 12 cm length volume transmitter ¹H coil for whole-body signal excitation. An
186 actively decoupled 2 cm diameter surface ¹H coil, positioned below the left calf, was used for
187 image signal reception. Muscle metabolites were probed by a 10 mm ³¹P saddle-shaped coil
188 placed around the left leg.

189 As described in detail elsewhere^{17,18}, Arterial Spin Labeling (ASL)-NMR imaging and ³¹P-
190 NMRS acquisitions were interleaved using the dedicated Bruker MultiScanControl software
191 (BrukerBioSpin GmbH) in order to follow simultaneously and non-invasively: (i) muscle
192 perfusion signal by SATuration-Inversion Recovery (SATIR) (time resolution: 10 sec), and
193 (ii) mitochondrial activity by dynamic ³¹P-NMRS (time resolution: 2.5 sec). In brief, ASL
194 imaging is based on non-invasive alternate magnetic tagging of blood water spins to provide
195 endogenous markers of muscle perfusion, measured in regions of interest (ROI) drawn in
196 posterior compartment of the leg. Muscle bioenergetics and pH were assessed from ratios of
197 energetic phosphates measurable by ³¹P-NMRS at rest, *in vivo* mitochondrial oxidative
198 capacity was directly assessed from the rate of creatine rephosphorylation at the end of
199 ischemia, and intramuscular pH was calculated from chemical shift between phosphocreatine
200 (PCr) and inorganic phosphate (Pi). A minimum of 50% PCr depletion at the end of ischemia
201 was necessary to reliably measure dynamics for PCr recovery, and examinations which did
202 not reach this threshold were rejected.

203 *NMR perfusion analysis.* Images were acquired after positive or negative labeling alternately.
204 To avoid large vessels, ROI were drawn in the posterior compartment of the leg. Muscle
205 perfusion f was calculated from the normalized difference between consecutive images
206 according to the equation¹⁹:

$$f = -\lambda/T_{ev} \times \ln \left[\frac{(M^+ - M^-)}{(M^+ + M^-)} \times (1 - \exp(r_1 T_{ev})) + 1 \right]$$

207 where r_1 is the longitudinal relaxation rate for muscle (measured by saturation-recovery
208 acquisition for each mouse at the end of 30 minutes hyperaemic period), M^+ and M^- are the
209 signals of positive and negative labelled perfusion images and λ is the blood-tissue partition
210 coefficient ($\lambda = 0.9$).

211 *³¹P-NMR Spectroscopy analysis.* Successive ³¹P Free Induction Decays were acquired
212 throughout rest, ischemia and hyperaemia. ³¹P-spectroscopy gives access to principal
213 metabolites implicated in energetic metabolism such as phosphocreatine (PCr), the three α, β,
214 γ ATP and inorganic phosphate (Pi). Signal intensity of these resonances is directly
215 proportional to their concentrations, which allows the quantitative following of these
216 metabolite variations.

217 At ischemia and recovery, PCr recovery was fitted by a mono-exponential function with a
218 least mean squares algorithm and pH was calculated from the chemical shift δ_{Pi} between PCr
219 and Pi according to the formula ²⁰:

$$pH = 6.75 + \log \left[\frac{(3.27 - \delta_{Pi})}{(\delta_{Pi} - 5.69)} \right]$$

220 **Statistics**

221 Perfusion data were analysed by repeated measurements ANOVA. Analyses were performed
222 with NCSS-2007 software (Kaysville, UT, USA). Group comparisons for perfusion
223 parameters and phosphorus spectroscopy analysis were performed using Mann-Whitney test.

224 Statistical analysis of histological data was performed with GraphPad-Prism software (La
225 Jolla, CA, USA). Fiber diameter repartition was evaluated by a chi-square test followed by a
226 multi-t-test corrected for multiple comparisons using Holm-Sidak method. Same multi-t-test
227 was used to evaluate capillary count/fiber repartition.

228 Statistical significance was taken at p<0.05 and p-values indicated on figures are *p<0.05,
229 **p <0.01, and ***p<0.001. Numerical NMR and histological data are reported as mean±SD.

230

231 **RESULTS**

232 Microvessels were defined as the small blood vessels located at the periphery of myofibers,
233 in the endomysium, displaying a diameter of less than 20 μm and a wall sometimes
234 containing one layer of αSMA -expressing cells, thus including capillaries, terminal arterioles
235 and terminal venules²¹.

236 **Young-adult Flk1^{GFP/+}::mdx mice display a normal microvascular network organisation
237 but a mild decrease in terminal arteriole density.**

238 Polyphasic subacute lesions, characterised by small inflammatory infiltrates and centrally
239 nucleated fibers, were observed in *Gastrocnemius* muscle (Figure 1A-B). Surprisingly, these
240 lesions had no impact on blood microvascular network organisation. In both Flk1^{GFP/+} and
241 Flk1^{GFP/+}::mdx mice, vascular network was indeed well organised with straight microvessels
242 located along myofibers, parallel to each other with few anastomoses oriented
243 perpendicularly to myofibers (Figure 1C-F). Microvessel diameter, measured using diameter
244 of endothelial cell fluorescence, was similar in both groups (13-14 μm), as well as
245 anastomose count (1,200-1,650 anastomoses/mm³) (Figure 1G-H).

246 Immunofluorescence analyses did not detect any significant difference between young-adult
247 wild-type and mdx mice (Figure 2). Both muscles displayed the same myofiber cross-section
248 diameter, fiber size distribution, and microvessel density, quantified by microvessel count per
249 fiber. No macrovascular modification was detected either (data not shown). To characterise
250 further the microvascular network and identify terminal arterioles, we carried out an
251 immunohistochemistry against αSMA , highlighting perivascular smooth muscle cells. We
252 quantified a 26% loss of αSMA expression in mdx mice, suggesting a decrease in terminal
253 arteriole density (Figure 3I, J, L). Collectively, these results highlighted a normal

254 microvascular network organisation in muscles of both groups, but a mild decrease in
255 terminal arteriole density, in mdx mice.

256 Capillary-to-fiber perimeter exchange index (CFPE) has been used to calculate the contact
257 surface area between capillaries and myofibers. It provides an indirect quantitative criterion
258 to evaluate movement of oxygen from capillaries to muscle fibers ²². CFPE index was not
259 affected in young-adult mdx mice (Figure 2F).

260 As almost no alteration of the microvascular network was detected at the morphological
261 level, we investigated the expression of angiogenesis-related mRNA (VEGF and its receptors
262 Flk1 and Flt1, CD31, Ang1, Ang2 and Tie1, Tie2 receptors and nNOS) (Figure 4A). We did
263 not observe any significant modification of these mRNA expression in young-adult mdx
264 mice, suggesting no stimulation of the angiogenesis process. In contrast, nNOS expression
265 was significantly decreased in young-adult mdx mice.

266

267 **Similar pericyte density but increase in satellite cell count in young-adult mdx mice.**

268 Using immunohistochemistry analysis, we focused on important partners of endothelial cells:
269 pericytes and satellite cells. Concerning pericytes (NG2+ cells located at the periphery of
270 blood vessels in muscle sections ²³), no difference in density per mm² was detected between
271 wt (151.6 ± 14.3 pericytes/mm², n=5) and mdx (154.3 ± 21.2 pericytes/mm², n=5) mice (Figure
272 3I-K). Satellite cells (SC; Pax7+ cells) are in a close relationship with endothelial cells and
273 coupling between myogenesis and angiogenesis takes place concomitantly during muscle
274 regeneration ²⁴. In young-adult mdx mice, we demonstrated using immunofluorescence an
275 increase in SC count per mm² (wt: 14.5 ± 0.3 SC/mm², n=4; mdx: 29.7 ± 3.5 SC/mm², n=6;
276 p<0.01) and per fibre (wt: 0.04 ± 0.01 SC/fibre, n=4; mdx: 0.07 ± 0.01 SC/fibre, n=5; p<0.05),
277 in comparison to wt (Figure 3A-D).

278

279 **Muscle blood perfusion is modified in young-adult mdx mice.**

280 In accordance with our previous observations, profiles of reactive hyperaemia were
281 significantly different in mdx (n=6) and wt (n=9) mice (Figure 5A, $p<10^{-6}$ with ANOVA).
282 The release of ischemia provoked an instantaneous increase of perfusion which was lower in
283 wt mice (mdx: 78.7 ± 27.1 ml/min/100 g; wt: 41.3 ± 32.3 ml/min/100 g, 20 s post-release). In
284 wt mice, this first perfusion peak was followed by a drop to reach a plateau around a value of
285 26.6 ± 9.2 ml/min/100 g peaking at 270 s post-ischemia. In contrast, mdx muscle perfusion
286 slightly increased to a mean perfusion value of 84.8 ± 24.8 ml/min/100 g at 300 s post-
287 ischemia and reduced to 26.3 ± 25.9 ml/min/100 g only 850 s after stress release (Figure 5A).
288 Moreover, the global volume repaid after ischemia was significantly higher in mdx mice (wt:
289 474.3 ± 216.3 ml/100 g; mdx: 1017.0 ± 369.2 ml/100 g, $p<0.05$). The response to ischemic
290 stress was therefore different and enhanced in young-adult mdx mice while almost no
291 morphological modification of microvessels was detected.

292

293 **Muscle bioenergetics in young-adult mice** (Table 2).

294 At rest, mdx mice displayed a slightly higher Pi/PCr ratio compared to wt which reflects an
295 increase in ADP concentration in dystrophic mice. In addition, a lower PCr/ γ ATP ratio was
296 observed in mdx mice, reflecting a decrease in metabolically functional muscle tissue.
297 The 30 min ischemic stress induced a higher Pi/PCr ratio in mdx mice while PCr depletion
298 tended to be accelerated compared to wt (mdx: Δ PCr = $65\pm9\%$; wt: Δ PCr = $58\pm6\%$; $p=0.09$).
299 At reactive hyperaemia, release revealed a significant acceleration of PCr resynthesis rate in
300 mdx mice compared to wt, reflecting higher mitochondrial ATP production in the mdx.

301 However, contrarily to wt, combined ^{31}P -NMRS and perfusion results showed a very tight
302 correlation between time of rephosphorylation τPCr and various parameters reflecting
303 perfusion: maximum perfusion ($r^2=0.66$, $p<0.05$), time-perfusion integrals ($r^2=0.99$, $p<0.001$
304 until 30 sec; $r^2>0.93$, $p<0.01$ until 150s) (Figure 6). In wt, none of the correlations between
305 τPCr and perfusion were significant.

306 In summary, phosphate metabolism was accelerated during ischemia in 3 month-old mdx
307 mice. At recovery, mitochondrial oxidative rephosphorylation was unexpectedly faster and
308 perfusion was increased in comparison to age-matched control mice. Moreover, perfusion in
309 mdx was directly related to mitochondrial ATP production. This is unlike normal healthy
310 case where a luxury perfusion is observed and is neither limiting nor correlated to τPCr .

311

312 **Alteration of microvascular network organisation in old Flk1^{GFP/+}::mdx mouse.**

313 Old mdx mice displayed marked histological lesions; some already observed in young-adult
314 as anisocytosis or centrally nucleated myofibers, others included persistence of chronic
315 inflammation, and presence of endomysial/perimysial fibrosis (Figure 7A-D). The
316 microvascular network was as well organised in old Flk1^{GFP/+} as in young-adult mice (Figure
317 7E). In contrast, Flk1^{GFP/+}::mdx mice displayed significant alterations, characterised by a
318 marked increase in tortuosity and irregular scattering of microvessels (Figure 7F).

319 Microvessel diameter was similar in both groups ($12 \mu\text{m}$), but we identified a higher
320 anastomose count, from more than 50,000 anastomoses/ mm^3 for Flk1^{GFP/+}::mdx mice to less
321 than 1,000 anastomoses/ mm^3 for control Flk1^{GFP/+} ($p<0.01$) (Figure 7J). Collectively, these
322 results pointed to an anarchic blood vessel organisation in this context of dystrophinopathy.

323 Immunofluorescence analyses showed that (i) myofiber cross-section mean diameter was
324 smaller in mdx mice (mdx: $47.4\pm4.2 \mu\text{m}$; wt: $61.2\pm3.9 \mu\text{m}$; $p<0.001$) (Figure 8C), (ii) the

325 smaller myofibers ($<50\mu\text{m}$) were clearly under-vascularised (Figure 8E), (iii) perinucleated
326 (Figure 8G), and (iv) represented almost 60% of total muscle fibers in mdx mice in contrast
327 to 35% in controls (Figure 8D). Alterations were also detected at the terminal arteriole level,
328 as a loss of 70% of α SMA expression was detected in old mdx mice (wt: $88.6\pm2.4\%$, n=7;
329 mdx: $18.0\pm2.1\%$, n=8; p<0.001), suggesting a marked decrease in terminal arteriole density
330 (Figure 3M, N, P). These data suggested either a progressive degradation of tissue with no
331 maintenance of microvascular network with time or a defect of neo-angiogenesis. The CFPE
332 was not affected in old mice (Figure 8F).

333 Angiogenesis-related mRNA expression analysis revealed a collapse of VEGF expression
334 and its decoy receptor Flt1 (Figure 4B). As observed in young-adult mice, nNOS was also
335 significantly decreased in old mdx mice.

336 Collectively, these results pointed out severe alterations of microvessel organisation,
337 especially around small/atrophic myofibers, associated with alteration of angiogenesis,
338 suggesting chronic alteration of endothelial-myogenic cell interface.

339

340 **Pericytes and satellite cells in old mdx mice.**

341 The density of pericytes was similar between wt and mdx mice but, in contrast to young-adult
342 mdx mice, a decrease in satellite cell count was observed for old mdx mice (wt: 12.4 ± 0.6
343 SC/mm², n=8; mdx: 10.0 ± 0.8 SC/mm², n=9; p<0.05) (Figure 3E-H).

344

345 **Alteration of muscle perfusion in old mdx mice.**

346 Despite severe alterations in mdx muscle microvascular network organisation, at rest, no
347 difference in muscle perfusion was observed between mdx and wt mice (mdx: 12.09 ± 5.90

348 ml/min/100 g; wt: 8.19 ± 2.19 ml/min/100 g). After tourniquet release, rapid increase of
349 perfusion was detected in muscles of the posterior hindlimb compartment; this increase was
350 significantly lower in old mdx mice (mdx perfusion maximal value at 380 s post-ischemia:
351 60.5 ± 39.3 ml/min/100 g; wt perfusion maximal value at 400 s post-ischemia: 106.1 ± 38.1
352 ml/min/100 g, $p < 0.05$), in contrast to what was seen in young-adult mice.

353 Analysis of variance of perfusion time-courses demonstrated differing profiles between wt
354 and mdx mice ($p < 10^{-4}$), with specific differences in the early phase of reperfusion. A similar
355 initial peak of perfusion, as the one observed in young-adult wt mice, was detected in the old
356 wt group, 20 s post-ischemia, but was absent in mdx mice (Figure 5B).

357 Thus at 12 months, both mdx and wt showed different profiles from young-adult animals
358 (ANOVA, $p < 10^{-6}$), and in contrast to wt and young-adult mdx, old mdx mice displayed a
359 decrease in muscle perfusion and a modified perfusion profile after an ischemic stress.

360

361 **Muscle bioenergetics in 12 month-old mice (Table 3).**

362 At rest, no difference in pH was observed between wt ($n=7$) and mdx ($n=5$) mice but hypoxic
363 stress induced a significant acidosis in both groups ($p < 0.005$), more pronounced in mdx (wt:
364 $\Delta pH = 0.22 \pm 0.06$; mdx: $\Delta pH = 0.30 \pm 0.04$; $p < 0.05$). Ischemia was associated with a
365 significant increase in PCr depletion in old dystrophic mice compared to wt, though the
366 difference in Pi/PCr ratio between the two groups was not significant. Unlike in young-adult
367 mice, the rephosphorylation rate was comparable in both groups. Indeed τ PCr was shorter in
368 the old compared to the young-adult wt mice ($p < 0.01$), but was unchanged with age in the
369 mdx mice. Thus no alteration of oxidative capacities was observed in old mdx mice in
370 response to hypoxic stress compared to age-matched control mice, despite reduced perfusion.
371 In older mice (wt and mdx), no correlation was found between τ PCr and perfusion variables.

372

373 **DISCUSSION**

374 Our study deciphers lesions of muscle microvascular network, in a model of
375 dystrophinopathy, using combination of state of the art histology/morphometry techniques
376 and totally non-invasive functional approach. This experimental paradigm, combining
377 histopathology and mpf-NMR, clearly relevant for clinical diagnosis and research, allowed us
378 to associate for the first time the fine 3D-alterations of muscle microvascular network with
379 functional repercussions on muscle.

380 Concerning the animal model, previous studies used 6 week to 6 month-old mdx mice^{12, 14, 25},
381 which display very few chronic lesions with no fibrosis, in contrast to what happens in
382 human¹⁶. We therefore worked on 12 month-old mdx mice, displaying persistence of
383 endomysial inflammation and fibrosis, more representative of human DMD and thus more
384 relevant for chronic myopathy and DMD pathophysiology study, in contrast to young-adult
385 mdx mice displaying no chronic lesions. We demonstrated (i) strong alterations of
386 microvascular network structure associated with reduced muscle perfusion in old mdx mice,
387 (ii) functional increase in muscle perfusion and mitochondrial oxidative phosphorylation with
388 normal microvascular network organisation in young-adult mdx mice, and (iii) a different
389 impact of age on wild-type and mdx mouse muscles.

390 In young-adult and old wild-type mice, no alteration could be detected in muscle histology or
391 microvascular network organisation. Perfusion is primarily regulated by smooth muscles that
392 control blood flow distribution and capillary recruitment²⁶. Capillary resistance, at rest, plays
393 only a minor role in perfusion regulation. Using ischemia-reperfusion, we provoke maximal
394 arteriolar dilatation in order to limit arteriolar resistance, and thus microvessel network
395 becomes predominant in control of muscle perfusion²⁷. Analysis of perfusion profiles

396 revealed the existence of a “peak” of perfusion in the first seconds after ischemia release, for
397 young-adult and old wild-type mice. This initial “peak” suggests specific regulation of
398 perfusion in early phase after ischemia release, probably coordinated by perivascular smooth
399 muscles and/or pericytes²⁸.

400 Surprisingly and in contrast to previous studies describing decreased vascular density^{12,14}, we
401 did not detect any alteration in vascular network 3D-organisation in young-adult mdx mice.
402 However, muscle post-ischemic perfusion was higher than in aged-match control mice, and
403 time resolution of mpf-NMR allowed to demonstrate the absence of the initial “peak” of
404 perfusion. In the same time, we also demonstrated a 26% loss of αSMA expression in young-
405 adult mdx muscle (and more than 70% loss in old mdx), suggesting a drop in perivascular
406 smooth muscle cells, responsible for part of these deleterious effects. It has indeed been
407 demonstrated *in vivo* that the re-expression of dystrophin only in smooth muscle cells
408 significantly ameliorates vasoregulation in mdx mice²⁹ confirming the importance of
409 perivascular cells (smooth muscle for example) in blood flow regulation. One of the possible
410 key factor is NO production alteration³⁰ or impairment of neuronal NO synthase (nNOS)³⁰⁻³²,
411 very probably explaining the significant decrease in nNOS expression both in young-adult
412 and old mdx mice, in our study. Concerning pericytes, Yemisci *et al.* demonstrated in the
413 brain, after a 2h ischemic stress, that pericytes remain contracted despite successful re-
414 opening of blood flow, impairing microcirculatory reflow²⁸. These experiments were carried
415 out *ex vivo*, and no functional *in vivo* validation was done. Our data seem therefore to
416 highlight functional alterations of smooth muscles and/or pericytes after ischemic stress. This
417 alteration is severe enough to significantly impact perfusion profiles between control and
418 mdx mice, and we are currently carrying out new experiments to better understand the effect
419 of an absence of dystrophin in perivascular cells and their involvement in dystrophinopathy
420 pathophysiology.

421 Old Flk1^{GFP/+}::mdx model allowed us to highlight disorganisation of microvascular network.

422 A marked increase in microvessel tortuosity, an irregular scattering, and an increase in

423 anastomose count were observed. Existence of these highly abundant anastomoses, suggests

424 that “radial” as well as “longitudinal” (parallel to myofibers) blood flow is important, and in

425 turn, that “longitudinal” flow is abnormally heterogeneous, microvessel longitudinal

426 resistance being likely to vary a lot from microvessel to microvessel which would be the

427 driven force for collateral flow.

428 Considering the close association between microvessels and myofibers, we demonstrated in

429 old mdx mice that more than 60% of myofibres were atrophic with peripheral nuclei and

430 displayed less microvessels at their periphery, resulting in a global undercapillarisation and

431 loss of terminal arterioles. In parallel, NMR analysis revealed a two-fold decrease in

432 perfusion after ischemia release. The significant decrease of microvessel (capillary and

433 terminal arteriole) density around small myofibers is likely responsible for these functional

434 alterations. Our data are thus in agreement with other studies demonstrating the effect of age

435 on dystrophinopathy pathophysiology ^{15,25}. Our hypothesis is that interaction between

436 angiogenesis and myogenesis could be affected in old mdx mice; the increasingly scarce

437 microvessels would provoke an alteration of myofiber regeneration that in turn could lead to

438 impairment of remaining microvascular network support, maintaining a vicious circle. With

439 this idea, we focused on the dynamic of satellite cell density in the muscle tissue. While SC

440 density in young-adult mdx mice was increased, very probably because of the stimulation of

441 muscle regeneration, it collapsed in old mdx mice, with pathology evolution, suggesting a

442 worsening of the situation and an increase in the severity of chronic muscle lesions. Decline

443 in SC number and activity has already been observed with age in mdx mice, in association

444 with attenuated Notch signalling transduction ³³. Christov *et al.* already introduced the idea

445 that angiogenesis and myogenesis are coupled during muscle regeneration, these processes

446 involving several growth factors, such as VEGF²⁴. In our study, we also observed
447 modifications of VEGF and its receptor Flt1 expression profiles. Flt1 is the decoy receptor of
448 VEGF³⁴, acting as a negative regulator of endothelial cell growth and differentiation. A
449 previous study demonstrated that mdx mice knock out for the Flt1 receptor (mdx:Flt1^{+/-})
450 presented an improved muscle histology associated with a better muscle perfusion and force
451 production compared to mdx mice³⁵. These data underlines the link between vascular
452 remodelling and muscle regeneration, even in severe chronic diseases. Concerning VEGF, the
453 2-fold decreased expression, detected in old mdx mice, was in accordance with previous
454 studies demonstrating that treatment with VEGF strongly ameliorates mdx phenotype, with
455 improvement of functional parameters, increase in capillary density, improved muscle
456 regeneration, and decrease in interstitial fibrosis^{36,37}. Fibrosis is incidentally a key parameter
457 influencing perfusion, and is increased in DMD¹⁰. However, fibrosis might not be the most
458 limiting factor to perfusion, as it only represents 10% of old mdx mice muscle tissue in our
459 study. Moreover, distance between capillaries and myofibers, generally modified with
460 endomysial fibrosis²², is similar between young-adult and old wild-type and mdx mice.
461 The *in vivo* increase of post-ischemic muscle perfusion with old age in wt animals was found
462 to be reproducible in different mouse strains, but no explanation is currently put forward,
463 while effect of aging on perfusion is still debated even in humans^{38,39}.
464 In parallel to perfusion analysis, acquisition of ³¹P-spectroscopy revealed moderate energetic
465 metabolism alterations, in agreement with previous literature, and contrarily to what might be
466 expected from alterations of enzymatic activities or defects of mitochondrial localization *in*
467 *vitro*. Compensatory mechanisms must thus exist in dystrophic muscle⁴⁰⁻⁴².
468 As for perfusion, anomalies in phosphorus metabolites in wild-type animals depended on age,
469 as already evoked in early studies of mdx metabolism^{41,43}. Anomalies at rest were more

470 marked in younger mdx: Pi/PCr reflecting resting ADP production was increased while
471 PCr/ATP proportional to functional muscle was reduced (-11%), coherently with other
472 reports ⁴⁰⁻⁴² (-50% or more in DMD children ⁴⁴).

473 Unlike a recent study in 3 month-old mdx mice ⁴⁵, where 10min ischemia was used as stress
474 protocol, we found greater depletion after 30min ischemia in both young-adult and old mdx.
475 During prolonged ischemia, two energetic pathways are activated to supply ATP demand:
476 ATP-PCr system and glycolytic pathway. Production of ATP directly from PCr consumption
477 is very small; it is therefore unlikely that PCr would be consumed to compensate for
478 defective glycolytic pathway. The higher depletion observed in both young-adult and old
479 mdx could more likely reflect a higher ATP demand to maintain ionic homeostasis.

480 Confrontation between perfusion and metabolic data obtained simultaneously by NMR
481 revealed that despite strongly reduced perfusion in old mdx mice, oxidative metabolism was
482 preserved, suggesting existence of a “luxury perfusion”, *i.e.* reserve of perfusion that can be
483 eliminated without impact on muscle physiology, as previously evidenced in a model of
484 peripheral artery disease ⁴⁶. It generally explains the absence, or very loose correlation,
485 between perfusion and PCr recovery rates in wt animals and in old mdx. This contrasts with
486 the tight correlation between initial perfusion and τ PCr of young-adult mdx mice, which
487 display faster τ PCr recovery and stronger perfusion than controls. Using optical spectroscopy
488 to analyse myoglobin and haemoglobin oxygen desaturation in parallel to ³¹P-NMRS,
489 Percival observed strong uncoupling between ATP synthesis and O₂ consumption in 4 month-
490 old mdx, dystrophic muscle producing 39% ATP less per O₂ consumed than controls ⁴⁵. We
491 might thus hypothesize that at this young age, increased perfusion might be a means to
492 compensate for mitochondrial inefficiency.

493

494 In conclusion, we demonstrated strong structural and functional alterations in muscle
495 microvascular network of dystrophin-deficient mdx mice, with an increasing severity in
496 parallel to aging. Our approach combining 3D-morphological analyses with non-invasive
497 functional evaluation, allowed to better characterise the impact of histological lesions on
498 tissue function. Collectively, our data pointed out that vascular network has a key role in
499 dystrophinopathy pathophysiology and would be very important target for the set-up of new
500 innovative therapeutic strategies.

501

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510

511 **Statement of author contributions:**

512 GJ, FC, CW, CL and PGC designed experiments. CL, BM, AMB and DB carried out
513 experiments and GJ, FC, PGC, CW, BM and CL analysed data. GJ, CL, BC, BM and CW
514 were involved in writing the paper and all authors gave final approval of the submitted and
515 published versions.

516 **List of abbreviations:**

517 ADP: Adenosine diphosphate, ASL: Arterial spin labeling, ATP: Adenosine triphosphate,
518 CFPE: capillary to fiber perimeter exchange, DMD: Duchenne muscular dystrophy, GFP:
519 green fluorescent protein, HE: haematoxylin and eosin, NMR: nuclear magnetic resonance,
520 NOS: nitric oxide synthase, PCr: Phosphocreatine, Pi: phosphate inorganic, SATIR:
521 SATuration-Inversion Recovery, VEGF/R: vascular endothelial growth factor/Receptor

522

523 **REFERENCES**

- 524 1. De Paepe B, De Bleecker JL: Cytokines and chemokines as regulators of skeletal
525 muscle inflammation: presenting the case of Duchenne muscular dystrophy,
526 *Mediators Inflamm* 2013, 2013:540370
- 527 2. Ennen JP, Verma M, Asakura A: Vascular-targeted therapies for Duchenne muscular
528 dystrophy, *Skelet Muscle* 2013, 3:9
- 529 3. Shimizu-Motohashi Y, Asakura A: Angiogenesis as a novel therapeutic strategy for
530 Duchenne muscular dystrophy through decreased ischemia and increased satellite
531 cells, *Front Physiol* 2014, 5:50
- 532 4. Engel WK, Hawley RJ: Focal lesions of muscle in peripheral vascular disease, *J
533 Neurol* 1977, 215:161-168
- 534 5. Rando TA: Role of nitric oxide in the pathogenesis of muscular dystrophies: a "two
535 hit" hypothesis of the cause of muscle necrosis, *Microsc Res Tech* 2001, 55:223-235
- 536 6. Koehler J: Blood vessel structure in Duchenne muscular dystrophy. I. Light and
537 electron microscopic observations in resting muscle, *Neurology* 1977, 27:861-868
- 538 7. Bradley WG, O'Brien MD, Walder DN, Murchison D, Johnson M, Newell DJ: Failure
539 to confirm a vascular cause of muscular dystrophy, *Arch Neurol* 1975, 32:466-473
- 540 8. Gudrun B, Andrew GE, Boysen G, Engel AG: Effects of microembolization on the
541 skeletal muscle blood flow. A critique of the microvascular occlusion model of
542 Duchenne dystrophy, *Acta Neurol Scand* 1975, 52:71-80
- 543 9. Leinonen H, Juntunen J, Somer H, Rapola J: Capillary circulation and morphology in
544 Duchenne muscular dystrophy, *Eur Neurol* 1979, 18:249-255
- 545 10. Desguerre I, Mayer M, Leturcq F, Barbet J-P, Gherardi RK, Christov C: Endomysial
546 fibrosis in Duchenne muscular dystrophy: a marker of poor outcome associated with

- 547 macrophage alternative activation, Journal of Neuropathology & Experimental
548 Neurology 2009, 68:762-773
- 549 11. Christov C, Chretien F, Abou-Khalil R, Bassez G, Vallet G, Authier FJ, Bassaglia Y,
550 Shinin V, Tajbakhsh S, Chazaud B, Gherardi RK: Muscle satellite cells and
551 endothelial cells: close neighbors and privileged partners, Mol Biol Cell 2007,
552 18:1397-1409
- 553 12. Loufrani L: Absence of Dystrophin in Mice Reduces NO-Dependent Vascular
554 Function and Vascular Density: Total Recovery After a Treatment with the
555 Aminoglycoside Gentamicin, Arterioscler Thromb Vasc Biol 2004, 24:671-676
- 556 13. Landisch RM, Kosir AM, Nelson SA, Baltgalvis KA, Lowe DA: Adaptive and
557 nonadaptive responses to voluntary wheel running by mdx mice, Muscle Nerve 2008,
558 38:1290-1303
- 559 14. Matsakas A, Yadav V, Lorca S, Narkar V: Muscle ERRgamma mitigates Duchenne
560 muscular dystrophy via metabolic and angiogenic reprogramming, Faseb J 2013,
561 27:4004-4016
- 562 15. Straino S: Enhanced Arteriogenesis and Wound Repair in Dystrophin-Deficient mdx
563 Mice, Circulation 2004, 110:3341-3348
- 564 16. Grounds MD, Radley HG, Lynch GS, Nagaraju K, De Luca A: Towards developing
565 standard operating procedures for pre-clinical testing in the mdx mouse model of
566 Duchenne muscular dystrophy, Neurobiol Dis 2008, 31:1-19
- 567 17. Bertoldi D, Loureiro de Sousa P, Fromes Y, Wary C, Carlier PG: Quantitative,
568 dynamic and noninvasive determination of skeletal muscle perfusion in mouse leg by
569 NMR arterial spin-labeled imaging, Magn Reson Imaging 2008, 26:1259-1265

- 570 18. Baligand C, Gilson H, Menard JC, Schakman O, Wary C, Thissen JP, Carlier PG:
571 Functional assessment of skeletal muscle in intact mice lacking myostatin by
572 concurrent NMR imaging and spectroscopy, Gene Ther 2010, 17:328-337
- 573 19. Raynaud JS, Duteil S, Vaughan JT, Hennel F, Wary C, Leroy-Willig A, Carlier PG:
574 Determination of skeletal muscle perfusion using arterial spin labeling NMRI:
575 validation by comparison with venous occlusion plethysmography, Magn Reson Med
576 2001, 46:305-311
- 577 20. Taylor DJ, Bore PJ, Styles P, Gadian DG, Radda GK: Bioenergetics of intact human
578 muscle. A ^{31}P nuclear magnetic resonance study, Mol Biol Med 1983, 1:77-94
- 579 21. Granger DN, Senchenkova E: Edited by San Rafael (CA), 2010, p.
- 580 22. Hepple RT: A new measurement of tissue capillarity: the capillary-to-fibre perimeter
581 exchange index, Can J Appl Physiol 1997, 22:11-22
- 582 23. Wanjare M, Kusuma S, Gerecht S: Perivascular cells in blood vessel regeneration,
583 Biotechnol J 2013, 8:434-447
- 584 24. Christov C, Chretien F, Abou-Khalil R, Bassez G, Vallet Gg, Authier Fo-Jrm,
585 Bassaglia Y, Shinin V, Tajbakhsh S, Chazaud Bnd, others: Muscle satellite cells and
586 endothelial cells: close neighbors and privileged partners, Mol Biol Cell 2007,
587 18:1397-1409
- 588 25. Palladino M, Gatto I, Neri V, Straino S, Smith RC, Silver M, Gaetani E, Marcantoni
589 M, Giarretta I, Stigliano E, Capogrossi M, Hlatky L, Landolfi R, Pola R: Angiogenic
590 impairment of the vascular endothelium: a novel mechanism and potential therapeutic
591 target in muscular dystrophy, Arterioscler Thromb Vasc Biol 2013, 33:2867-2876
- 592 26. Clifford PS: Vasodilatory mechanisms in contracting skeletal muscle, J Appl Physiol
593 (1985) 2004, 97:393-403

- 594 27. Baligand C, Jouvion G, Schakman O, Gilson H, Wary C, Thissen JP, Carlier PG:
595 Multiparametric functional nuclear magnetic resonance imaging shows alterations
596 associated with plasmid electrotransfer in mouse skeletal muscle, J Gene Med 2012,
597 14:598-608
- 598 28. Yemisci M, Gursoy-Ozdemir Y, Vural A, Can A, Topalkara K, Dalkara T: Pericyte
599 contraction induced by oxidative-nitrative stress impairs capillary reflow despite
600 successful opening of an occluded cerebral artery, Nat Med 2009, 15:1031-1037
- 601 29. Ito K: Smooth muscle-specific dystrophin expression improves aberrant
602 vasoregulation in mdx mice, Hum Mol Genet 2006, 15:2266-2275
- 603 30. Loufrani L, Matrougui K, Gorny D, Duriez M, Blanc I, Levy BI, Henrion D: Flow
604 (shear stress)-induced endothelium-dependent dilation is altered in mice lacking the
605 gene encoding for dystrophin, Circulation 2001, 103:864-870
- 606 31. Brenman JE, Chao DS, Xia H, Aldape K, Bredt DS: Nitric oxide synthase complexed
607 with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular
608 dystrophy, Cell 1995, 82:743-752
- 609 32. Sander M, Chavoshan B, Harris SA, Iannaccone ST, Stull JT, Thomas GD, Victor
610 RG: Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal
611 muscle of children with Duchenne muscular dystrophy, Proc Natl Acad Sci U S A
612 2000, 97:13818-13823
- 613 33. Jiang C, Wen Y, Kuroda K, Hannon K, Rudnicki MA, Kuang S: Notch signaling
614 deficiency underlies age-dependent depletion of satellite cells in muscular dystrophy,
615 Disease Models & Mechanisms 2014, 7:997-1004
- 616 34. Fong GH, Rossant J, Gertsenstein M, Breitman ML: Role of the Flt-1 receptor
617 tyrosine kinase in regulating the assembly of vascular endothelium, Nature 1995,
618 376:66-70

- 619 35. Verma M, Asakura Y, Hirai H, Watanabe S, Tastad C, Fong GH, Ema M, Call JA,
620 Lowe DA, Asakura A: Flt-1 haploinsufficiency ameliorates muscular dystrophy
621 phenotype by developmentally increased vasculature in mdx mice, *Hum Mol Genet*
622 2010, 19:4145-4159
- 623 36. Deasy BM, Feduska JM, Payne TR, Li Y, Ambrosio F, Huard J: Effect of VEGF on
624 the regenerative capacity of muscle stem cells in dystrophic skeletal muscle, *Mol Ther*
625 2009, 17:1788-1798
- 626 37. Messina S, Mazzeo A, Bitto A, Aguennouz M, Migliorato A, De Pasquale MG,
627 Minutoli L, Altavilla D, Zentilin L, Giacca M, Squadrito F, Vita G: VEGF
628 overexpression via adeno-associated virus gene transfer promotes skeletal muscle
629 regeneration and enhances muscle function in mdx mice, *Faseb J* 2007, 21:3737-3746
- 630 38. Trinity JD, Layec G, Lee JF: Heterogeneity of blood flow: impact of age on muscle
631 specific tissue perfusion during exercise, *J Physiol* 2014, 592:1729-1730
- 632 39. Rudroff T, Weissman JA, Bucci M, Seppanen M, Kaskinoro K, Heinonen I,
633 Kallikoski KK: Positron emission tomography detects greater blood flow and less
634 blood flow heterogeneity in the exercising skeletal muscles of old compared with
635 young men during fatiguing contractions, *J Physiol* 2014, 592:337-349
- 636 40. Cole MA, Rafael JA, Taylor DJ, Lodi R, Davies KE, Styles P: A quantitative study of
637 bioenergetics in skeletal muscle lacking utrophin and dystrophin, *Neuromuscul*
638 *Disord* 2002, 12:247-257
- 639 41. Dunn JF, Frostick S, Brown G, Radda GK: Energy status of cells lacking dystrophin:
640 an in vivo/in vitro study of mdx mouse skeletal muscle, *Biochim Biophys Acta* 1991,
641 1096:115-120

- 642 42. Heerschap A, Bergman AH, van Vaals JJ, Wirtz P, Loermans HM, Veerkamp JH:
643 Alterations in relative phosphocreatine concentrations in preclinical mouse muscular
644 dystrophy revealed by in vivo NMR, NMR Biomed 1988, 1:27-31
- 645 43. Dunn JF, Tracey I, Radda GK: Exercise metabolism in Duchenne muscular
646 dystrophy: a biochemical and [31P]-nuclear magnetic resonance study of mdx mice,
647 Proc Biol Sci 1993, 251:201-206
- 648 44. Kemp GJ, Taylor DJ, Dunn JF, Frostick SP, Radda GK: Cellular energetics of
649 dystrophic muscle, J Neurol Sci 1993, 116:201-206
- 650 45. Percival JM, Siegel MP, Knowels G, Marcinek DJ: Defects in mitochondrial
651 localization and ATP synthesis in the mdx mouse model of Duchenne muscular
652 dystrophy are not alleviated by PDE5 inhibition, Hum Mol Genet 2013, 22:153-167
- 653 46. Vidal G WC, Giacomini E, Emmanuel F, Carlier PG: A truly non-invasive set-up for
654 the study of perfusion and energy metabolism in the rat calf in vivo: application to a
655 model of peripheral arterial disease (Abstract), Magma 2002, 25:15

656

657

658 **FIGURE LEGENDS**

659 **FIGURE 1.** Normal microvascular network organisation in 3 month-old mdx mice.

660 In contrast to wild-type mice (A), mdx mice (B) display subacute lesions in *Gastrocnemius*
661 muscle, characterised by small inflammatory infiltrates (star) associated with regenerated
662 myofibers displaying central nuclei (arrows) (HE staining).

663 Microvessel 3D organisation of Flk1^{GFP/+} (C,E) and Flk1^{GFP/+}::mdx (D,F) mice: normal blood
664 microvessel organisation, with microvessels regularly scattered along myofibers (C-F) (Scale
665 bars: 50 μm). Morphometric analyses revealed similar diameter (G) and anastomose
666 count/mm³ (H) between microvessels from wild-type and mdx mice.

667

668 **FIGURE 2.** Microvessel morphometry in 3 month-old mice.

669 Young-adult wild-type (n=5) and mdx (n=3) mice display similar: microvessel distribution in
670 *Gastrocnemius* muscle (A,B) (laminin-FITC and CD31-TRITC immunohistochemistry to
671 label basal lamina (green) and blood vessels (red); Scale bar: 50 μm), fiber size repartition
672 (C), microvessel count per fiber (D), microvessel diameter (E), and capillary to fiber
673 perimeter exchange index (CFPE) (F).

674

675 **FIGURE 3.** Satellite cells, terminal arterioles and pericytes are affected in mdx mice.

676 Pool of SC was analysed by immunohistochemistry (A, B, E, F, white arrows) (Pax7-FITC
677 and Laminin-Cy3). SC density is increased in young-adult mdx mice (C, D) while we
678 observed a significant depletion of the SC pool in old mdx mice (G, H). Pericyte density,
679 assessed using NG2 immunolabeling (NG2-FITC), was similar for all groups, at all ages (I-

680 K, M-O). We also evaluated the expression of α SMA (Smooth Muscle Actin) by perivascular
681 smooth muscle cells, surrounding terminal arterioles (α SMA-Cy3). We observed a decrease
682 of 26% α SMA expression for young-adult mdx mice in comparison to wt animals (I, J, L),
683 reaching 70% loss for old mdx (M, N, P), suggesting a marked drop in terminal arterioles,
684 increasing with the disease progression (scale bars: 25 μ m) (*p<0.05, **p<0.01,
685 ***p<0.001).

686

687 **FIGURE 4.** Analysis of angiogenesis-related gene expression by RT-qPCR.

688 Total RNA was extracted and complementary DNA was analysed by qPCR. Gene expression
689 was measured in the *Gastrocnemius* of young-adult (A) and old (B) mdx mice. Results were
690 normalized relative to the expression of the 18s rRNA housekeeping gene. Data are presented
691 as fold change mean \pm SEM. Symbol ** indicates statistical difference (p<0.01) observed
692 between mdx and wild-type mice from the same age.

693

694 **FIGURE 5.** Muscle blood perfusion during ischemia-reperfusion.

695 After release of ischemia, a rapid and important increase in perfusion is detected.

696 (A) Different profiles of perfusion are obtained in young-adult mice: total perfusion is higher
697 in mdx mice and a first “peak” of perfusion followed by a rapid decrease in muscle perfusion
698 is only detected in wild-type mice.

699 (B) A first “peak”, similar to what is observed in young-adult wild-type mice, is also
700 observed in 12 month-old control animals. This first “peak” does not exist in mdx mice that

701 also displayed a reduced perfusion, with a maximum perfusion equivalent to half the value
702 observed in wild-type mice during the hyperaemia phase.

703 As the release of ischemia induced movements of the leg, images affected by these
704 movement artefacts are removed from analysis of muscle perfusion.

705

706 **FIGURE 6.** Correlation between perfusion and time of creatine rephosphorylation (τ PCr) in
707 3 month-old mice.

708 Correlation between τ PCr and maximal perfusion (A) or time-perfusion integrals until 30 sec
709 (B) is significant in mdx mice (* $p<0.05$ and *** $p<0.001$, respectively) with a coefficient of
710 determination r^2 of 0.66 and 0.99, respectively. In wt, none of the correlations were
711 significant.

712

713 **FIGURE 7.** Alteration of microvascular network in 12 month-old mdx mice.

714 Twelve month-old wild-type mice display histologically normal muscles (A), with no fibrosis
715 (C), and regularly scattered microvessels along myofibers, with few anastomoses (E,G,J). In
716 contrast, 12 month-old mdx mice display chronic histological lesions (B), characterised by
717 multifocal inflammatory infiltrates (mostly macrophages), included in endomysial collagen
718 tissue (fibrosis; stars), associated with a marked variation in myofiber size (anisocytosis) and
719 the presence of atrophic and regenerating myofibers displaying centrally-located nuclei
720 (arrows). Sirius red staining and fluorescence microscopy reveal a moderate to marked
721 endomysial fibrosis (D) and microvascular network alterations (F,H), characterised by
722 irregularly scattered tortuous microvessels (Scale bars: 50 μ m). Even if microvessel diameter
723 is similar between mdx and wild-type mice (I), a clear increase in anastomose count/mm³ is

724 detected for old mdx mice (J). A, B: HE staining. C, D: Sirius red staining (specific for
725 collagen staining). **p<0.01.

726

727 **FIGURE 8.** Twelve month-old mdx mice display atrophic myofibers with low capillarisation
728 and terminal arteriole density.

729 Anisocytosis is more pronounced in old mdx mice, with the presence of small atrophic
730 myofibers (A-C) (Laminin-FITC and CD31-TRITC immunohistochemistry; Scale bars: 50
731 μm). Atrophic myofibers (with a diameter up to 60 μm) represent more than 60% of the total
732 myofibers in mdx mice (D). These atrophic myofibers display less microvessels at their
733 periphery (E). The distance between microvessels and myofibers, calculated using the
734 capillary to fiber perimeter exchange index (CFPE) is similar in both groups (F). Small
735 myofibers with low capillarisation are mostly perinucleated myofibers (G) (**p<0.01,
736 ***p<0.001).

737

738

739 **TABLES**

740 Table 1. Oligonucleotide Primers Used for qPCR

Primer	Sequence
18S rRNA F	5'-CGGACAGGATTGACAGATTG-3'
18S rRNA R	5'-CAAATCGCTCCACCAACTAA-3'
Flk1 F	5'-CAGTGGTACTGGCAGCTAGAAG-3'
Flk1 R	5'-ACAAGCATACGGGCTTGT-3'
Flt1 F	5'-GGCCCGGGATATTATAAGAAC-3'
Flt1 R	5'-CCATCCATTAGGGGAAGTC-3'
VEGF F	5'-GGCGTGGTGGTGACATGGTT-3'
VEGF R	5'-ACCTCACCAAGCCAGCACA-3'
CD31 F	5'-CGGTGTTCAGCGAGATCC-3'
CD31 R	5'-ACTCGACAGGATGGAAATCAC-3'
Ang1 F	5'-GACAGTAATACAACACCGGGAAGA-3'
Ang1 R	5'-CAAAACCCATTATACTCCTCCA-3'
Ang2 F	5'-ACTACGACGACTCAGTGCAAAG-3'
Ang2 R	5'-TCTGGTTCTGCACCACATTG-3'
Tie1 F	5'-AGGGCAGCTTCCAGAGTATG-3'

Tie1 R 5'-GGTTGCCAGCAATGTTAAG-3'

Tie2 F 5'-GGCTATAAGGATACGGACCATGAA-3'

Tie2 R 5'-TCCCCTGTCCACGGTCATA-3'

nNOS F 5'-GGCGTTCGTGATTACTGTGA-3'

nNOS R 5'-TCTTCCTCATGTCCAATCCA-3'

741

742

743 Table 2. Energetic metabolism analysis from ^{31}P -spectroscopy in young-adult mice.

	wt (n=9)	mdx (n=6)
$\Delta\text{PCr} (\%)$	58 ± 6	65 ± 9
$\tau\text{PCr} (\text{s})$	118 ± 34	$76 \pm 34^*$
pH at rest (pH_{rest})	7.20 ± 0.04	7.17 ± 0.03
pH end ischemia (pH_{end})	7.00 ± 0.08	6.98 ± 0.03
$\text{Pi/PCr at rest } (\text{Pi/PCr}_{\text{rest}})$	0.08 ± 0.03	$0.10 \pm 0.01^*$
$\text{Pi/PCr end ischemia } (\text{Pi/PCr}_{\text{end}})$	0.93 ± 0.25	$1.71 \pm 0.52^*$
$\text{PCr/ATP}\gamma \text{ at rest } (\text{PCr/ATP}\gamma_{\text{rest}})$	3.39 ± 0.25	$3.01 \pm 0.27^*$
$\text{PCr/ATP}\gamma \text{ end ischemia } (\text{PCr/ATP}\gamma_{\text{end}})$	1.80 ± 0.62	1.39 ± 0.51

744

745 Ischemia stress was sufficient as the mean depletion of phosphocreatine (PCr) for wt and
 746 mdx mice was above 50%. Pi/PCr at rest and after ischemia were higher in mdx compared to
 747 wt mice while PCr/ATP γ at rest was lower (*p<0.05).

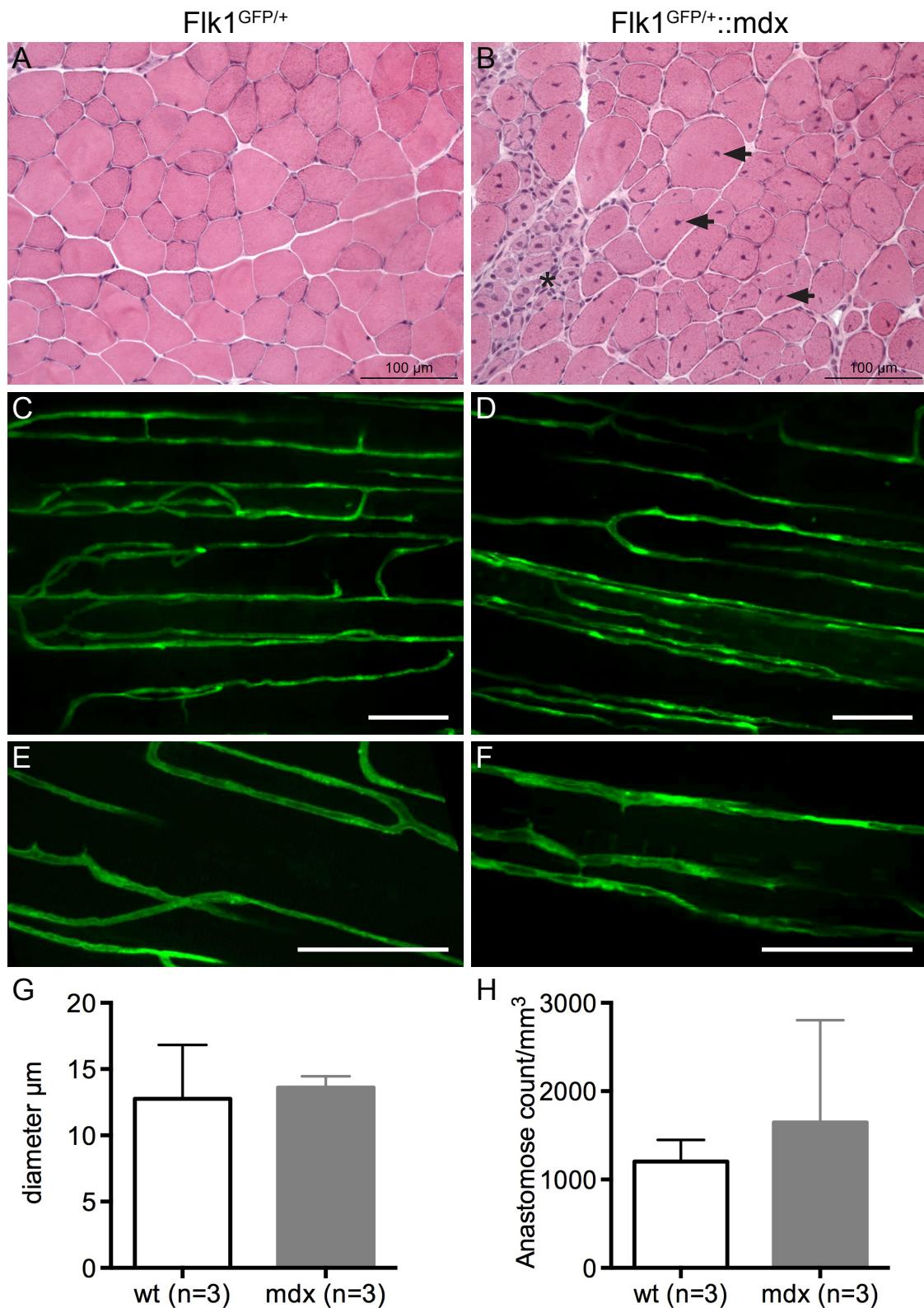
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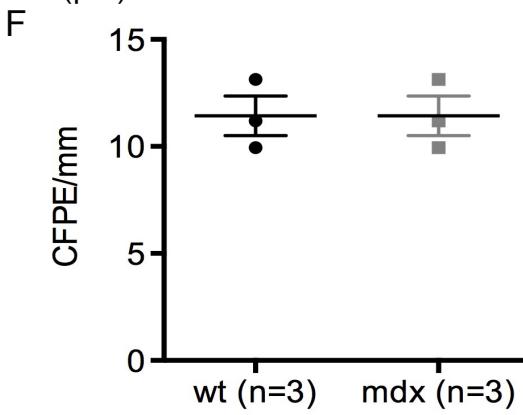
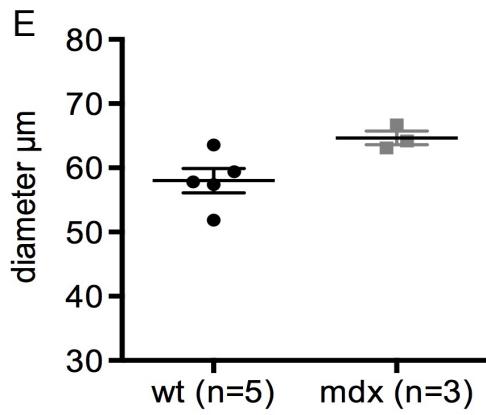
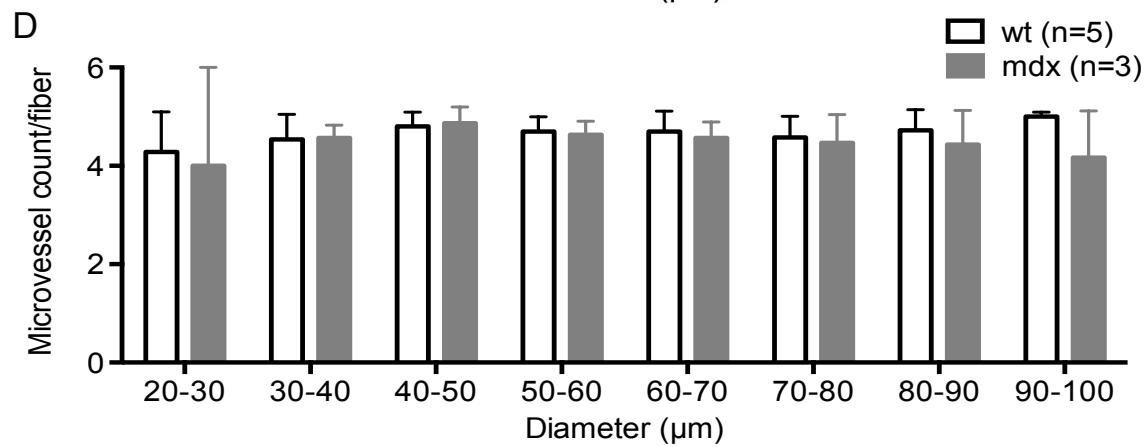
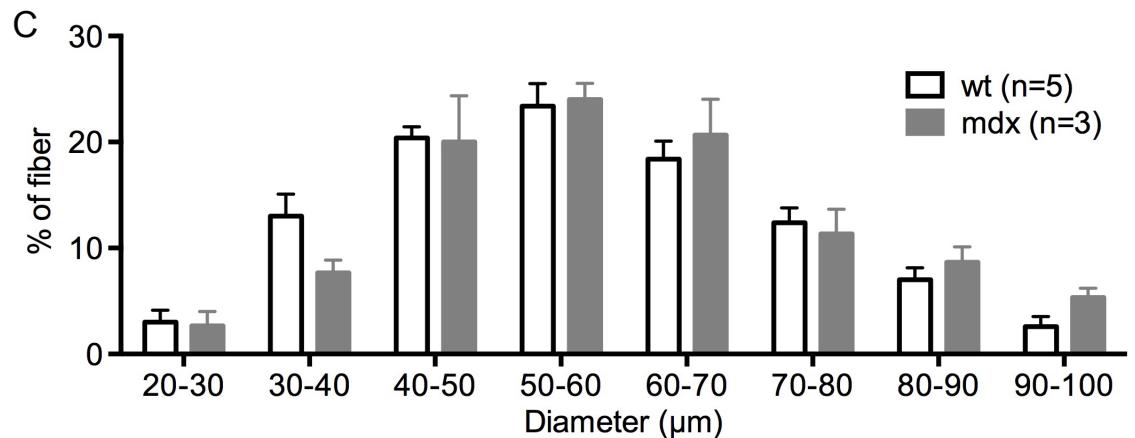
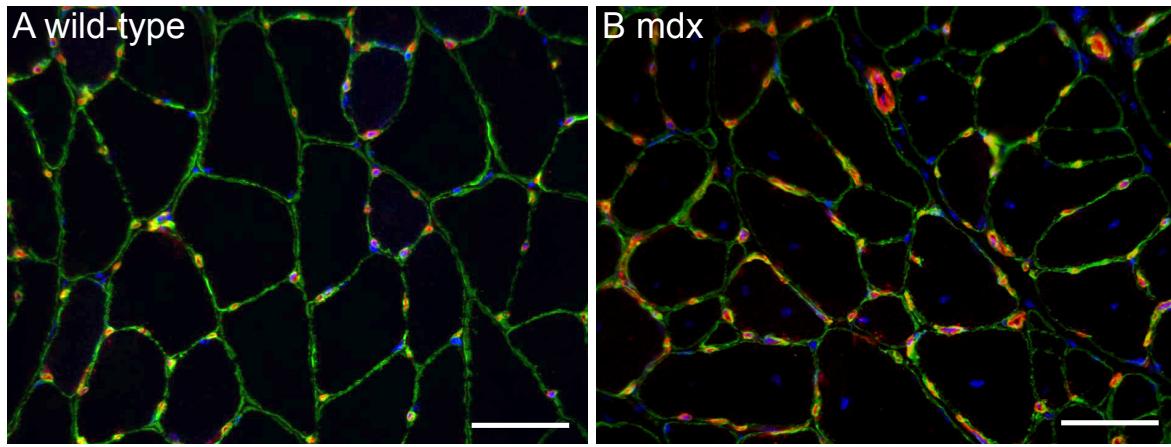
749 Table 3. Energetic metabolism analysis from ^{31}P -spectroscopy in old mice.

	wt (n=7)	mdx (n=5)
$\Delta\text{PCr} (\%)$	54 ± 4	$63 \pm 2^{**}$
$\tau \text{ PCr (s)}$	66 ± 25	80 ± 20
pH at rest (pH_{rest})	7.16 ± 0.07	7.18 ± 0.04
pH end ischemia (pH_{end})	6.94 ± 0.04	$6.87 \pm 0.04^*$
$\text{Pi/PCr at rest } (\text{Pi/PCr}_{\text{rest}})$	0.08 ± 0.04	0.10 ± 0.01
$\text{Pi/PCr end ischemia } (\text{Pi/PCr}_{\text{end}})$	1.24 ± 0.40	1.34 ± 0.20
$\text{PCr/ATP}\gamma \text{ at rest } (\text{PCr/ATP}\gamma_{\text{rest}})$	2.98 ± 0.35	3.05 ± 0.16
$\text{PCr/ATP}\gamma \text{ end ischemia } (\text{PCr/ATP}\gamma_{\text{end}})$	1.20 ± 0.35	1.36 ± 0.30

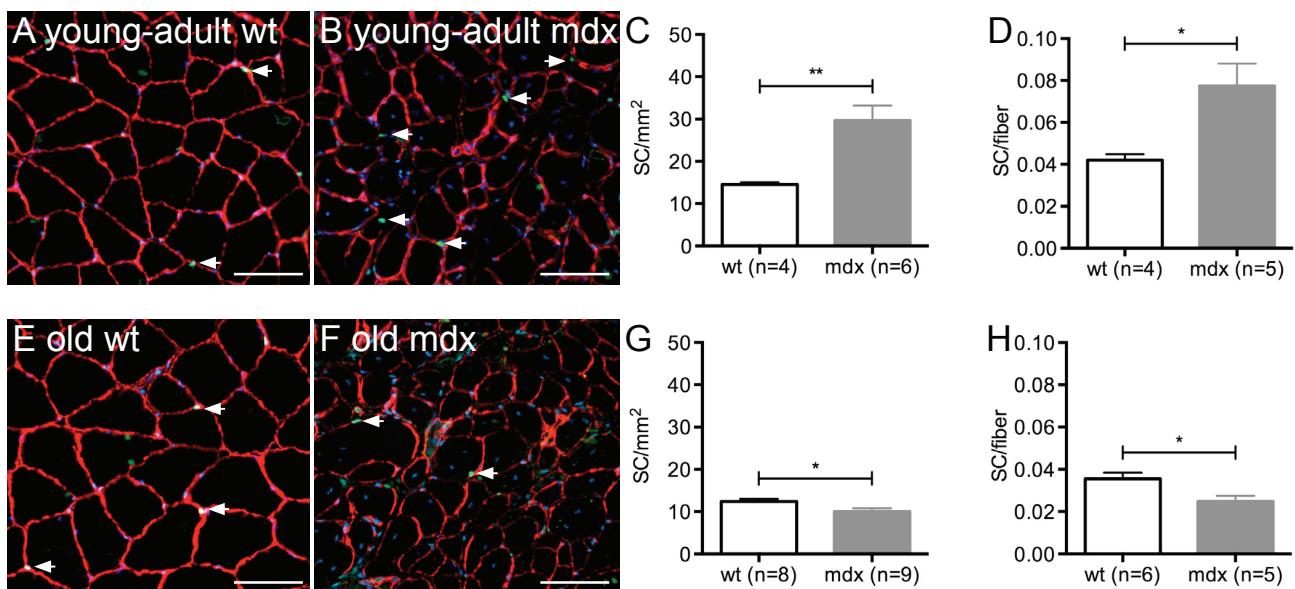
750

751 Ischemia stress was sufficient as the mean depletion of phosphocreatine (PCr) for wt and
 752 mdx mice was above 50%. ΔPCr is higher in mdx mice; pH decreased for both wt and mdx
 753 mice after ischemia, but mdx mice suffered a more severe acidosis. Others energetic
 754 parameters did not change in our experimental conditions (* $p<0.05$, ** $p<0.01$).

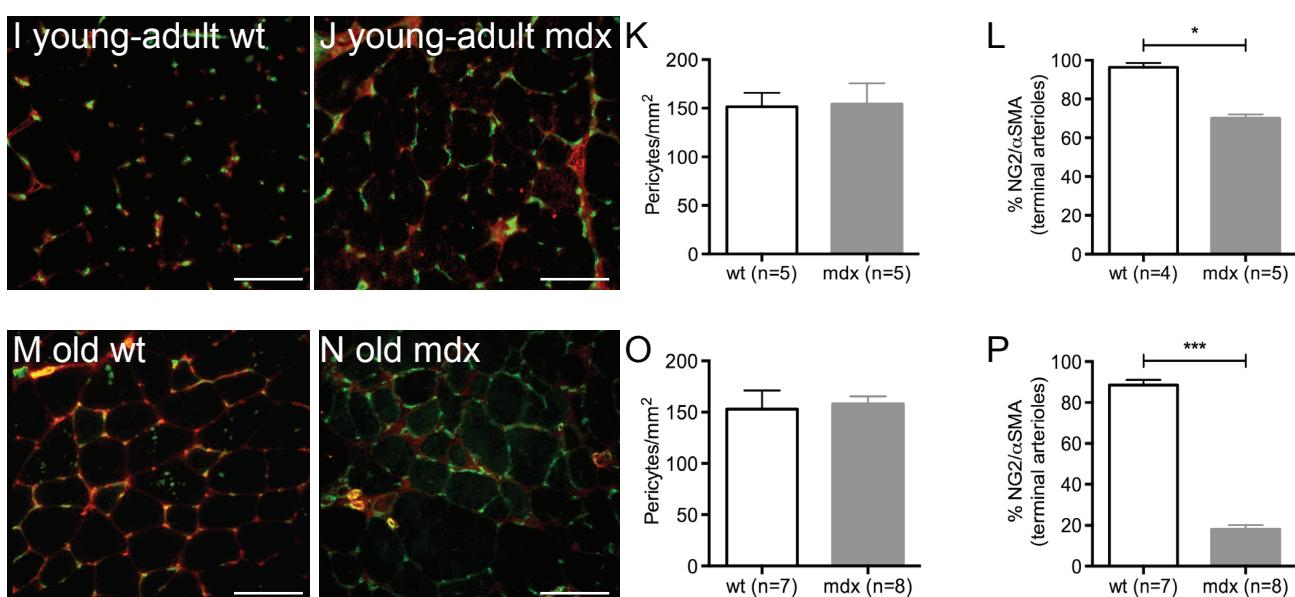




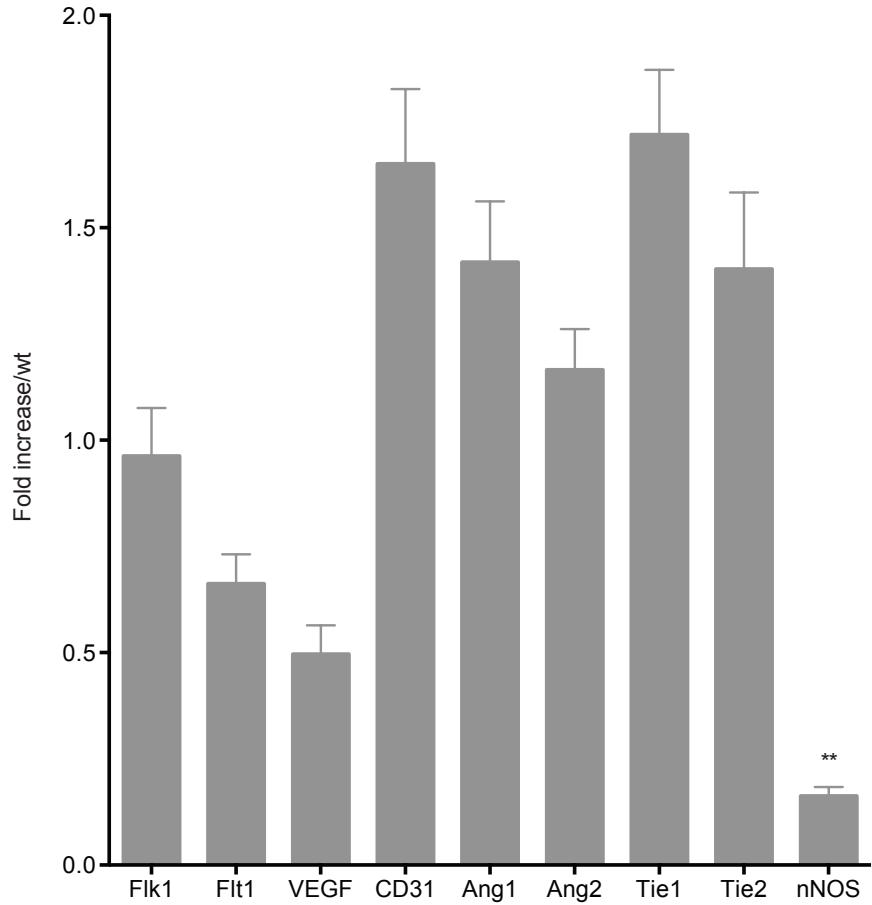
Satellite cells



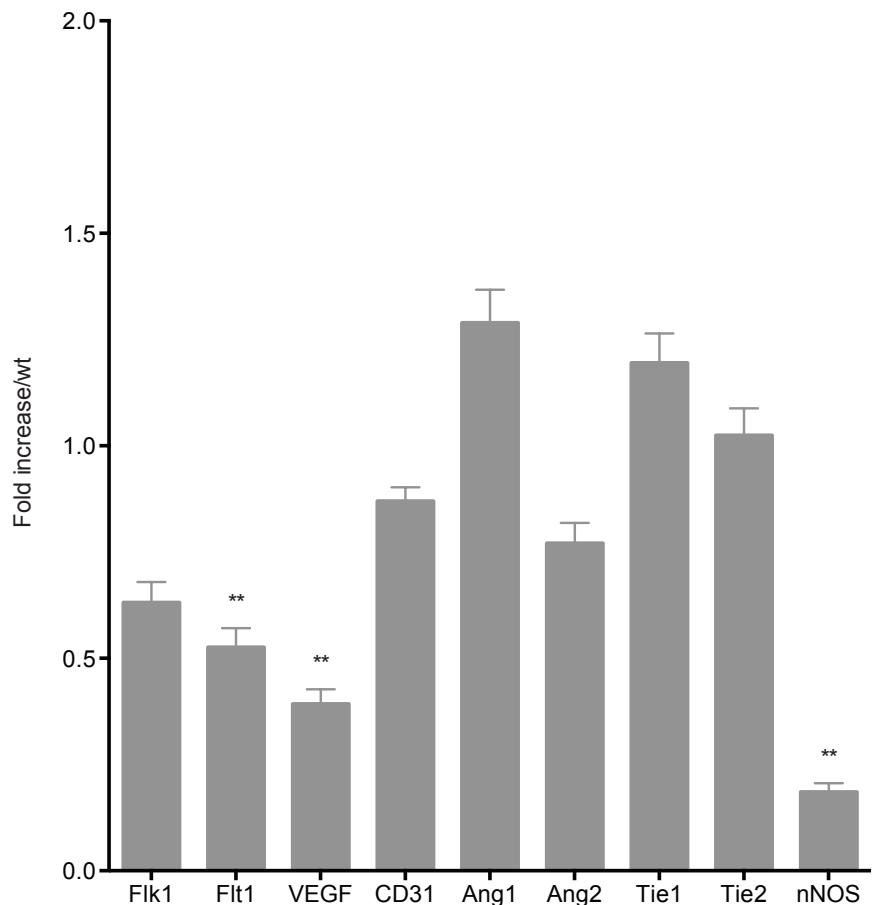
Pericytes and terminal arterioles



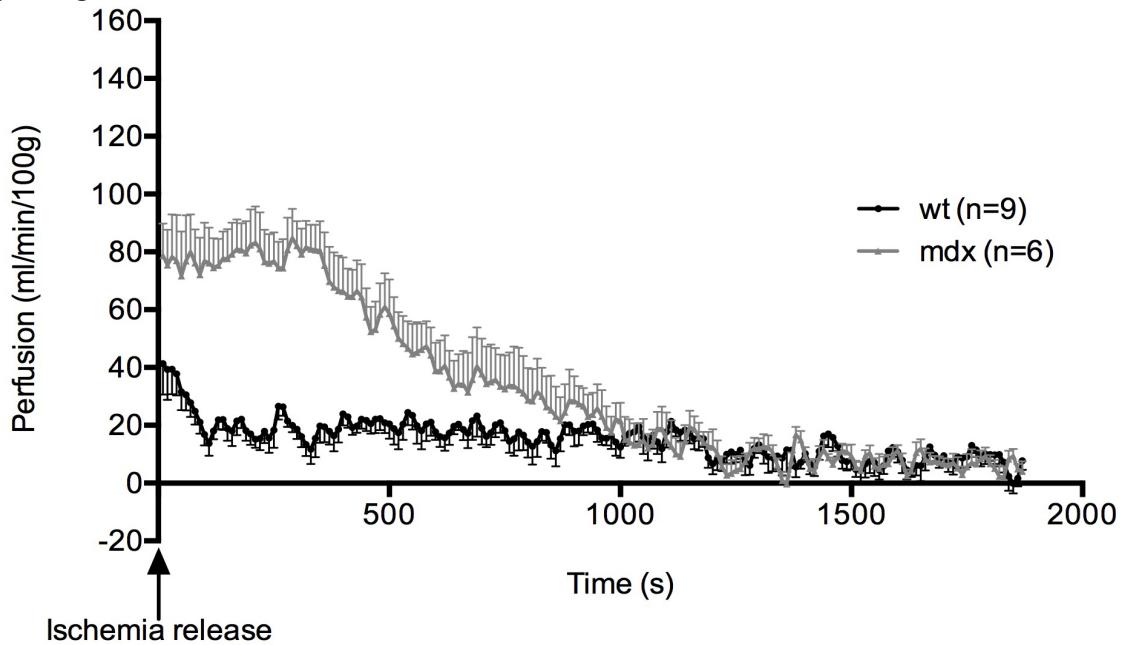
A young adult mdx



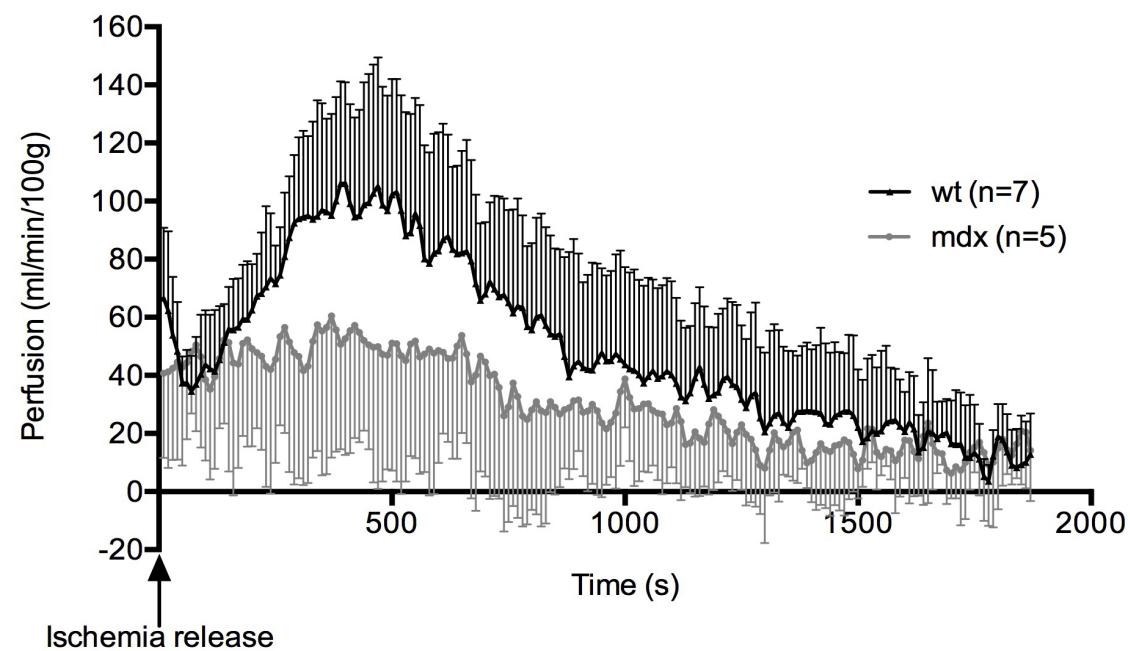
B old mdx



A young adult mice

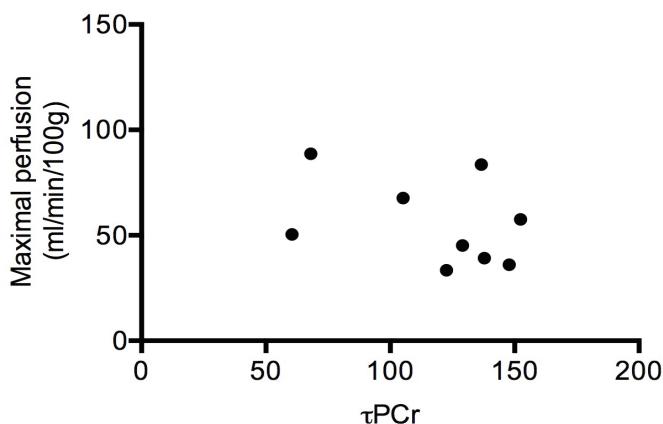


B old mice

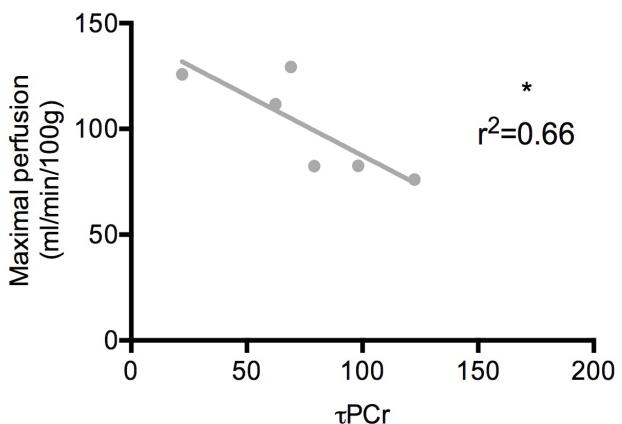


A Correlation maximal perfusion/ τ PCr

Young adult wt mice

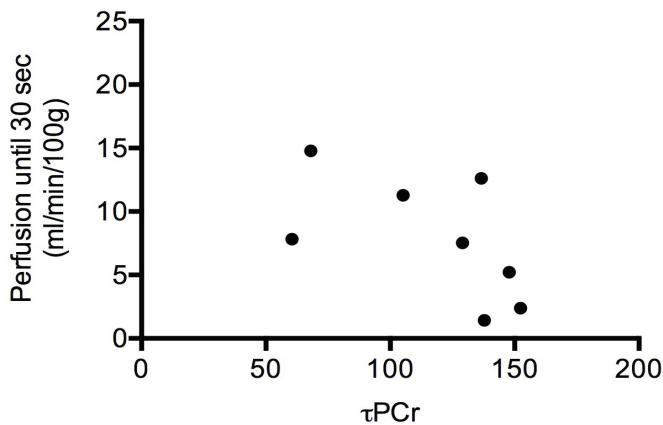


Young adult mdx mice



B Correlation perfusion integral until 30 sec/ τ PCr

Young adult wt mice



Young adult mdx mice

