

1 **The effect of high-fat diet on the morphological properties of the forelimb**  
2 **musculature in hypertrophic myostatin null mice.**

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27

28 **Abstract**

29 Obesity is a worldwide nutritional disorder affecting body performance including  
30 skeletal muscle. Inhibition of myostatin not only increases the muscle mass but also it  
31 reduces body fat accumulation. We examined the effect of high-fat diet on the  
32 phenotypic properties of forelimb muscles from myostatin null mice. **Male wild-type and**  
33 **myostatin null mice were fed on either normal diet or high-fat diet (45 % fat) for ten**  
34 **weeks. *M. triceps brachii Caput longum*; *M. triceps brachii Caput laterale*; *M. triceps***  
35 ***brachii Caput mediale*; *M. extensor carpi ulnaris* and *M. flexor carpi ulnaris* were**  
36 processed for fiber type composition using immunohistochemistry and morphometric  
37 analysis. Although the muscle mass revealed no change under high-fat diet, there were  
38 morphometric alterations in the absence of myostatin. **We show that high-fat diet**  
39 **reduces the cross-sectional area of the fast (IIB and IIX) fibers in *M. triceps brachii***  
40 ***Caput longum* and *M. triceps brachii Caput laterale* of both genotypes. In contrast,**  
41 **increases of fast fibers area were observed in both *M. extensor carpi ulnaris* of wild-**  
42 **type and *M. flexor carpi ulnaris* of myostatin null. Meanwhile, a high-fat diet increases**  
43 **the area of the fast IIA fibers in wild-type, myostatin null displays a muscle-dependent**  
44 **alteration in the area of the same fiber type. The combined high-fat diet and myostatin**  
45 **deletion shows no effect on the area of slow type I fibers. Despite, a high-fat diet causes**  
46 **a reduction in the area of the peripheral IIB fibers in both genotypes, only myostatin**  
47 **null shows an increase in the area of the central IIB fibers. We provide evidence that a**  
48 **high-fat diet induces a muscle-dependent fast to slow myofiber shift in the absence of**  
49 **myostatin. Taken together, the data suggest that the morphological alterations of**  
50 **muscle fibers under combined high-fat diet and myostatin deletion reflect a functional**  
51 **adaptation of the muscle to utilize the high energy intake.**

52 **Keywords:** skeletal muscle, high-fat diet, myostatin, myosin heavy chain, muscle fiber

53

54 **Running Headline/ Short title**

55 High-fat diet in myostatin null mice

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## 58 **1. Introduction**

59 Obesity has become a challenging chronic nutritional threat affecting human health,  
60 performance and quality of life worldwide (Apovian, 2016). Obesity originates in an  
61 imbalance between energy consumption and energy expenditure (Lebrasseur, 2012).  
62 As a metabolic disorder, obesity increases the risk of cardiovascular disease, type 2  
63 diabetes, and cancer (Muio & Newgard, 2006). However, the impact of obesity on  
64 skeletal muscle remains controversial, although obesity coincides with impaired  
65 locomotor ability of skeletal muscle (reviewed by Tallis et al. 2018). Beyond the  
66 essential contractile function, skeletal muscle is one of the main regulator for glucose  
67 disposal and glycogen storage in human body and is crucial for minimizing the risk of  
68 insulin resistance (Stump et al. 2006).

69 Skeletal muscle is an adapting tissue altering its physiological components under the  
70 effect of either external or internal stimuli to overcome functional demands (Egan &  
71 Zierath, 2013). Phenotypic evaluation of skeletal muscle is often based on profiling the  
72 heterogeneous population of muscle fibers categorized on their speed of contraction  
73 (underpinned by the expression of specific myosin heavy chain isoforms- MHC) or their  
74 mode of metabolism (oxidative versus glycolytic). Generally, glycolytic muscles tend to  
75 be fast twitching, fatigue susceptible and rely on anaerobic metabolism to produce  
76 energy whereas oxidative muscles are slow contracting, fatigue resistant and generate  
77 energy via aerobic metabolism (Zierath & Hawley, 2004; Matsakas & Patel, 2009). It  
78 has been reported that obesity impairs muscle mass and function as in case of  
79 sarcopenic obesity (Prado et al. 2012). The accumulation of fatty tissue can induce  
80 skeletal muscle inflammation. Studies have shown an increase of macrophage and  
81 inflammatory cytokines involving tumor necrosis factor- alpha (TNF $\alpha$ ) and monocyte  
82 chemoattractant protein-1 (MCP-1) were associated with obesity (Valerio et al. 2006;  
83 Varma et al. 2009; Lumeng & Saltiel, 2011). At the molecular level, it has been reported  
84 that obesity interferes with calcium signaling and 5-adenosine monophosphate-  
85 activated protein kinase (AMPK) causing muscle fiber shift and alteration in the  
86 contractile properties (Tallis et al. 2018). High-fat diet has been shown to elicit changes  
87 in mouse skeletal muscle gene expression and increase MHC type I in the quadriceps  
88 muscle (Lange et al. 2007; Wilde et al. 2008). Conversely, skeletal muscle composition  
89 from obese humans and animals has shown an increased proportion of fast twitch at  
90 the expense of slow twitch type I fibers (Matsakas & Patel, 2009). In addition, cytokines

91 and other molecules produced by adipocytes have been shown to impair the contractile  
92 function of the muscle leading to muscle fiber atrophy, inflammation and insulin  
93 resistance (Srikanthan et al. 2010; Pellegrinelli et al. 2015). Additionally, development  
94 of obesity is associated with a reduction of mitochondrial contents in skeletal muscle  
95 which results in impairment of fatty acid oxidation (Holloway et al. 2009).

96 Myostatin is a member of transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, acts as a  
97 powerful negative regulator for skeletal muscle development (Sharma et al. 2001).  
98 Myostatin interferes with the cell-cycle progression and inhibits myoblasts proliferation  
99 (Thomas et al. 2000). Genetic deletion of myostatin results in significant increase of  
100 muscle mass due to a combination of hyperplasia (an increase of muscle fiber number)  
101 and hypertrophy (an increase of muscle fiber size) (McPherron et al. 1997).  
102 Interestingly, as myostatin deletion reduced the body fat accumulation concomitantly,  
103 it has become a suitable target for therapeutic investigations against obesity  
104 (McPherron & Lee, 2002) as well as type 2 diabetes mellitus (Allen et al. 2011).  
105 Moreover, myostatin inhibition alters the phenotype of white adipose tissue into brown  
106 adipose tissue indicating a higher energy utilization and enhanced fatty acid oxidation  
107 (Zhang et al. 2012).

108 The present study examined for the first time, the effect of high-fat diet (HFD) on the  
109 myofiber morphology of forelimb musculature **in the myostatin null (*Mstn*<sup>-/-</sup>) mice in  
110 comparison to C57/BL6 wild-type (*Mstn*<sup>+/+</sup>) counterpart**. After 10 weeks of HFD intake,  
111 **five anatomically and phenotypically different forelimb muscles nominated as follow;**  
112 ***M. triceps brachii* Caput longum (T.long); *M. triceps brachii* Caput laterale (T.lateral),**  
113 ***M. triceps brachii* Caput mediale (T.medial); *M. extensor carpi ulnaris* (ECU) and *M.***  
114 ***flexor carpi ulnaris* (FCU) were processed for immunohistochemistry using antibodies**  
115 **against myosin heavy chain type IIB, IIA and type I isoforms. Evaluation of body mass,**  
116 **wet muscle specific-weight, cross-sectional area (CSA) and fiber type composition**  
117 **were analyzed. Our data demonstrates that although the muscle mass revealed no**  
118 **change under HFD, there were a remarkable alteration in the absence of myostatin in**  
119 **terms of CSA and phenotype of all muscle fibers types. Our detailed analysis revealed**  
120 **that HFD reduced the CSA of the fast glycolytic (IIB and IIX) fibers in T.lateral and**  
121 **T.long of both genotypes. In contrast, the CSA of the same fiber type was increased in**  
122 **T.medial and ECU of *Mstn*<sup>+/+</sup> as well as, T.medial and FCU muscles of *Mstn*<sup>-/-</sup>. HFD**  
123 **increases CSA of the fast oxidative IIA fibers in a muscle and genotype-dependent**

124 manner. We show that slow type I fibers of *Mstn*<sup>-/-</sup> were less liable to the effect of HFD  
125 intake. We found evidence of fiber size alteration based on their anatomical distribution  
126 following combined myostatin deletion and HFD intake. We provide evidence that  
127 myostatin deletion together with HFD induced a muscle-dependent fast to slow  
128 myofiber shift. The data suggest that HFD modulates the phenotypic properties of the  
129 myostatin null muscles to maximize energy utilization.

130

## 131 **2. Materials and Methods**

### 132 2. 1. Animals

133 **Males 4-5 months *Mstn*<sup>+/+</sup> and *Mstn*<sup>-/-</sup> mice** were raised in the biological unit, University  
134 of Reading, United Kingdom. *Mstn*<sup>-/-</sup> mice were donated by Dr. Lee (McPherron et al.  
135 1997). All the standard procedures were approved by the Animal Care and Ethical  
136 Review Committee (AWERB) and performed under a project license (number 7516)  
137 from the United Kingdom Home Office under Animals (Scientific Procedures) Act 1986.  
138 *Mstn*<sup>+/+</sup> and *Mstn*<sup>-/-</sup> mice were administered *ad libitum* either a standard chow (normal  
139 diet, ND) or a high-fat diet (HFD), composed of 45 % fat, 20 % protein and 35%  
140 carbohydrates (Special diet services (SDS) code: 824053). The animals were kept  
141 under standard environmental conditions at 21 C and cyclic 12 hours light/dark groups  
142 up to 10 weeks. After the completion of the experiment, the animals were humanely  
143 euthanized using Schedule 1 procedure.

144

### 145 2. 2. Tissue collection

146 In order to evaluate whether HFD affects the phenotype of the muscles in the absence  
147 of myostatin, five representative muscles from the upper and lower forelimb were  
148 surgically removed as follow; M. triceps brachii Caput longum (T.long); M. triceps  
149 brachii Caput laterale (T.lateral), M. triceps brachii Caput mediale (T.medial); M.  
150 extensor carpi ulnaris (ECU) and M. flexor carpi ulnaris (FCU). The dissected muscles  
151 were frozen using isopentane pre-cooled with liquid nitrogen and were embedded in  
152 tissue tech OCT (Sakura, VWR) using ethanol precooled in dry ice. 10 µm thickness  
153 transverse mid-belly cryosections from each muscle were placed on poly-L-lysine  
154 coated slides (VWR, Germany) and left to dry at room temperature at least for one  
155 hour before stored at -80C.

## 156 2. 3. Immunohistochemistry

157 Muscle sections were washed three times in PBS for 15 minutes, then permeabilized  
158 in buffer solution composed of 20 mM Hepes (Biochrom, Germany), 300 mM sucrose  
159 (Merck, Germany), 50 mM NaCl (Roth, Germany), 3 mM MgCl<sub>2</sub> (Roth, Germany) and  
160 0.5% Triton-X100 (pH7, Roth, Germany) for 15 minutes at room temperature. The  
161 muscle sections were preblocked in wash buffer containing 5% fetal calf serum (v/v)  
162 (Gibco, Thermo Fisher Scientific) and 0.05 % Triton X-100 (v/v) in phosphate buffered  
163 saline (PBS) for 30 minutes at room temperature. The myofiber type was investigated  
164 based on the expression of certain myosin heavy chain protein. The myofiber type IIB,  
165 IIA and type I were identified using BFF3 mouse IgM (1:1), A.474 mouse IgG (1:4) and  
166 A4.840 mouse IgM (1:1) monoclonal primary antibodies (DSHB) respectively as  
167 previously reported (Matsakas et al. 2009). Type IIB and IIA myofibers were  
168 immunostained in the same muscle section. However, type I myofibers were identified  
169 on serial sections. Muscle sections with no primary antibody added were used as a  
170 negative control (Fig. 1a and b). All the primary antibodies were incubated with muscle  
171 sections overnight at 4C. After five times washing steps for 10 minutes, the primary  
172 antibodies were detected using Alexa Fluor 633 goat anti-mouse IgM (Molecular  
173 Probes, A21046 diluted in wash buffer 1:200) for MHC I and MHC IIB and Alexa Fluor  
174 488 Goat-anti-mouse IgG (Molecular probes, A11029 diluted in wash buffer 1:200)  
175 secondary antibody. The muscle sections were incubated with the secondary  
176 antibodies for 45-60 minutes at room temperature in the dark. The nuclei were  
177 counterstain with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Thermo  
178 Fisher scientific) and the slides were mounted using a fluorescent mounting medium  
179 (Mowiol 4-88, Calbiochem).

## 180 2. 4. Imaging and myofiber quantifications

181 Immunostained muscle sections were morphologically evaluated and photographed  
182 using a Zeiss Axioscop2 fluorescence microscope connected to a digital camera and  
183 a computer operated with the Axiovision software (Zeiss). Briefly, uniform, transverse  
184 and undamaged muscle sections were selected for photography. To evaluate the total  
185 myofiber number and the percentage of each fiber type (IIB, IIA and, type I), the whole  
186 muscle cross section was photographed in separate images and was reconstructed  
187 using the photo merge tool in Photoshop CS6 (64 Bit). The total fiber number, number  
188 of IIB, IIA and type I fibers were quantified via manual tracking of the immunostained

189 fibers. The quantification of type IIX fibers was performed via subtracting the number  
190 of all immune positive fibers from the total fiber number per muscle section.  
191 Measurement of the cross sectional area (CSA) for each fiber type was performed for  
192 all muscles by using the measuring tool in the Axiovision software (Zeiss). Minimum of  
193 250 fibers was measured for each fiber type. For some muscles, the available fibers  
194 from certain type were insufficient. Therefore, all the available fibers from that particular  
195 type were measured.

## 196 2. 5. Statistical analysis

197 In order to analyze the effect of genotype ( $Mstn^{+/+}$  vs.  $Mstn^{-/-}$ ) and high caloric  
198 supplement (ND vs. HFD) on total muscle fiber number, measurement of CSA and the  
199 percentage of type IIB, IIX, IIA and I fibers of T.lateral, T.long, T.medial, ECU and FCU  
200 muscles, a two-way ANOVA was performed. Multiple comparisons and the variables  
201 interactions were evaluated using Tukey's and Sidak's Post hoc test. Statistical  
202 analysis was conducted by using Graph Pad Prism 6 software. All values are presented  
203 as mean  $\pm$  SEM and  $p < 0.05$  was considered to be significant.

204

## 205 3. Results

### 206 3. 1. Effect of high-fat diet on the total body mass and individual muscle weight

207 The present study examined the effect of high-fat diet on the phenotypic properties of  
208 forelimb muscles in the myostatin null and wild type mice. As expected, body mass  
209 was significantly higher ( $p < 0.05$ ) in  $Mstn^{-/-}$  ND compared to  $Mstn^{+/+}$  ND mice. We found  
210 that a 10-week high-fat diet regime resulted in significantly greater ( $p < 0.01$ ) body mass  
211 (g) independent of genotype (i.e. 20% and 14% increase in  $Mstn^{+/+}$  and  $Mstn^{-/-}$   
212 respectively) (Table 1). Next, we evaluated the effect of HFD on specific muscle weight  
213 (mg). No significant differences were detected in  $Mstn^{+/+}$  and  $Mstn^{-/-}$  muscles after HFD  
214 compared to ND (Table 1).

### 215 3. 2. Evaluation of the muscle fiber number following a high-fat diet

216 We next determined the effect of HFD on muscle fiber number studied (Fig. 1a-f). As  
217 expected, myostatin deletion induced muscle-dependent hyperplasia of  $232 \pm 12$  %,   
218  $142 \pm 2$  %,  $140 \pm 2$  %,  $130 \pm 8$  % and  $98 \pm 1$  % for T.lateral, FCU, T.long, ECU and T.medial

219 muscles respectively in comparison to *Mstn*<sup>+/+</sup> kept on a ND. T.lateral, FCU and T.long  
220 muscles of *Mstn*<sup>-/-</sup> demonstrated the highest increases in myofiber number ( $p < 0.0001$ )  
221 followed by ECU and T.medial muscles ( $p < 0.01$ ) compared to matched muscles of  
222 *Mstn*<sup>+/+</sup> ND. However, HFD did not induce any significant changes in total muscle fiber  
223 number in either genotype (Fig. 1g). These data indicate that neither myofiber  
224 hyperplasia nor fiber splitting nor fiber loss occurs under HFD. These data also suggest  
225 that muscle-specific differences in the amount of myofiber hyperplasia may be due to  
226 differential levels of myostatin expression within individual muscle.

### 227 **3.3. The effect of high-fat diet on the CSA of muscle fiber type**

228 Evaluation of the muscle fiber CSA would indicate whether high-fat diet alters  
229 myofiber type lead to either atrophy (a decrease of the muscle fiber size) or  
230 hypertrophy (an increase of the muscle fiber size). Therefore, measurement of the CSA  
231 of different fiber types IIA, IIX and IIB for each muscle of both genotypes was  
232 performed. The analysis showed that *Mstn*<sup>+/+</sup> and *Mstn*<sup>-/-</sup> muscles responded differently  
233 regarding the CSA of type IIB fiber following HFD. *Mstn*<sup>+/+</sup>HFD displayed significant  
234 reduction of IIB fiber CSA in T.long and FCU muscles ( $p < 0.0001$  and  $p < 0.001$ ).  
235 Conversely, IIB fiber CSA was increased in T.medial and ECU muscles ( $p < 0.0001$  and  
236  $p < 0.01$ ) compared to the matched *Mstn*<sup>+/+</sup> fed on a ND (Fig. 2c, f, i, and l). Similarly,  
237 *Mstn*<sup>-/-</sup>HFD showed reduction in CSA of IIB fiber in T.lateral and T.long muscles  
238 ( $p < 0.001$  and  $p < 0.0001$ ), in contrast, there were increased CSA of IIB in T.medial and  
239 FCU muscles ( $p < 0.001$ ) compared to matched muscles of *Mstn*<sup>-/-</sup>ND (Fig. 2a, c, f and  
240 l). The data analysis for T.long and FCU muscles demonstrated significant interactions  
241 ( $p < 0.05$  and  $p < 0.0001$ ) between the effects of genotype and the diet on the CSA of IIB  
242 fibers. Thus, it might suggest that both genotypes responded differently under HFD  
243 (Fig. 2c and l). The same trend was observed when we examined the CSA of type IIX  
244 fibers. HFD resulted in significantly smaller CSA of type IIX fibers ( $p < 0.001$  and  
245  $p < 0.0001$ ) for T.lateral muscles of *Mstn*<sup>+/+</sup> and T.long of *Mstn*<sup>-/-</sup> compared to genotype-  
246 matched mice on a ND. Interestingly, however both *Mstn*<sup>+/+</sup> and *Mstn*<sup>-/-</sup> showed  
247 decreased CSA of type IIX of FCU muscle under HFD ( $p < 0.001$  and  $0.01$ ) compared  
248 to genotype-matched mice kept on ND (Fig. 2b, d, and m). Although ECU muscle of  
249 *Mstn*<sup>+/+</sup>HFD exhibited an elevation of the CSA of type IIX fibers ( $p < 0.001$ ), such  
250 increase was stronger in *Mstn*<sup>-/-</sup>HFD involving both T.medial and ECU muscles ( $p < 0.05$   
251 and  $p < 0.0001$ ) compared to the corresponding muscles from mice kept on a ND (Fig.

252 2g, and j). The data analysis revealed that four out of five muscles showed significant  
253 interactions ( $p < 0.001$ ,  $p < 0.0001$ ,  $p < 0.05$  and  $p < 0.001$ ) between the effects of HFD and  
254 the genotype on the CSA of type IIX fibers. The results suggest that myostatin deletion  
255 affect the response of fast fibers (type IIB and IIX) under HFD.

256 Next, the CSA of MHC type IIA fibers referred to the fast oxidative fibers for all muscles  
257 under ND and HFD was analyzed. Under HFD, an increased CSA of type IIA fibers  
258 was detected in T.long and T.medial muscles of  $Mstn^{+/+}$  ( $p < 0.05$  and  $p < 0.0001$ )  
259 compared to  $Mstn^{+/+}$ ND (Fig. 2e and h). In contrast, the response of the  $Mstn^{-/-}$ HFD  
260 was slightly variable, meanwhile, CSA of type IIA fibers was decreased in T.long and  
261 T.medial muscles ( $p < 0.0001$  and  $0.01$ ), there was an increase in type IIA CSA in ECU  
262 and FCU muscles ( $p < 0.001$ ) compared to  $Mstn^{-/-}$  ND muscles (Fig. 2e, h, k, and n).  
263 The analysis demonstrated significant interactions between the effect of HFD and  
264 genotype ( $p < 0.0001$ ,  $p < 0.001$ ,  $p < 0.001$  and  $p < 0.0001$ ) for T.long, T.medial, ECU and  
265 FCU muscles. Due to the absence of the sufficient number of MHC type I referred to  
266 the slow oxidative fibers in T.lateral, T.long, ECU and FCU muscles for CSA  
267 measurement, only T.medial muscle could be analyzed. The data showed a significant  
268 reduction in the CSA of type I fibers in the  $Mstn^{+/+}$  under HFD ( $p < 0.05$ ) compared to  
269  $Mstn^{+/+}$  kept on a ND. However, the  $Mstn^{-/-}$  demonstrated no difference in the CSA of  
270 type I fiber of T.medial muscle following HFD period (Fig. 2o). These data were  
271 accompanied with significant interactions ( $p < 0.01$ ) between the effect of HFD and the  
272 effect of genotype suggesting the influence of genotype on the slow fiber type under  
273 HFD (Fig. 2o).

#### 274 **3.4. Effect of high-fat diet on the CSA of myofibers based on their regional** 275 **distribution**

276 Given our data, we next examined whether high-fat consumption alters the myofiber  
277 CSA based on their anatomical distribution. Therefore, we analyzed the CSA of MHC  
278 type IIB, IIX and type IIA fibers from the peripheral (PF) and central (CT) regions within  
279 the mid-belly of  $Mstn^{+/+}$  and  $Mstn^{-/-}$  muscles. Type I fibers were excluded from the  
280 analysis due to insufficient numbers in some of the muscles under investigation. The  
281 CSA of PFIIB fibers of  $Mstn^{+/+}$ HFD were reduced in T.lateral, T.long and FCU muscles  
282 ( $p < 0.0001$ ,  $p < 0.0001$  and  $p < 0.01$ ) compared to PFIIB fibers of  $Mstn^{+/+}$ ND (Fig. 3a, c  
283 and k). Similarly,  $Mstn^{-/-}$ HFD showed a reduction in the CSA of PF type IIB fibers of  
284 T.lateral and T.long muscles ( $p < 0.0001$  and  $0.0001$ ) compared to the matched region

285 of  $Mstn^{-/-}$ ND (Fig. 3a and c). In contrast, PFIIB fibers of T.medial and FCU muscles of  
286  $Mstn^{-/-}$ HFD demonstrated an increase of the CSA of the same fiber type ( $p<0.05$  and  
287  $p<0.0001$ ) compared to  $Mstn^{-/-}$ ND (Fig. 3e and k). Furthermore, we quantified the CSA  
288 of CTIIB fibers in each genotype following HFD in comparison to ND. Under HFD,  
289  $Mstn^{+/+}$  showed an increase of CSA in the CTIIB fibers of T.lateral and T.medial  
290 muscles ( $p<0.0001$ ). However, T.long and FCU muscles of the same genotype  
291 demonstrated a decrease in the CSA of CTIIB fibers ( $p<0.0001$  and  $p<0.01$ ) compared  
292 to the matched muscle region of  $Mstn^{+/+}$ ND (Fig. 3a, c, e, h and k). Similarly, significant  
293 increases in the CSA of CTIIB fibers of T.lateral, ECU and FCU muscles of  $Mstn^{-/-}$ HFD  
294 ( $p<0.0001$ ) compared to the corresponding muscle region of  $Mstn^{-/-}$ ND (Fig. 3a, c, e,  
295 h, and k). The analysis revealed, significant interactions in almost all muscles  
296 ( $p<0.0001$ ) between the effect of HFD, the effect of genotype and most importantly the  
297 anatomical localization of the muscle fiber. Furthermore, under high-fat diet, a common  
298 trend of loss in the CSA of PFIIB compared to the same muscle region on a ND could  
299 be detected. Although, there were either increased or decreased CSA of CTIIB in  
300  $Mstn^{+/+}$ , at least three muscles of  $Mstn^{-/-}$  showed an increase in the CSA of CTIIB fibers  
301 following HFD.

302 Subsequently, we evaluated whether HFD alters the CSA of MHC type IIX fibers in  
303 terms of the anatomical distribution (PF vs. CT) of the myofiber. Although, the majority  
304 of  $Mstn^{+/+}$ HFD displayed no change in the CSA of PFIIX fibers compared to the  
305 matched region of  $Mstn^{+/+}$ ND, only  $Mstn^{-/-}$  HFD had larger CSA in PFIIX fibers of  
306 T.medial and ECU muscles ( $p<0.0001$  and  $p<0.001$ ) compared to the matched region  
307 of  $Mstn^{-/-}$ ND (Fig. 3b, d, f, i and l). Regarding the CSA of CTIIX fibers measurement,  
308  $Mstn^{+/+}$ HFD showed an elevation in the CSA of CTIIX fibers of T.medial and ECU  
309 muscles ( $p<0.0001$  and  $0.01$ ) in comparison to the matched region of  $Mstn^{+/+}$ ND. In  
310 contrast, both T.lateral and FCU muscles of  $Mstn^{+/+}$  HFD showed a reduction in the  
311 CSA of the CTIIX fibers ( $p<0.0001$ ) compared  $Mstn^{+/+}$ ND. Similarly,  $Mstn^{-/-}$ HFD  
312 displayed increase in the CTIIX fibers of T.long, T.medial and ECU muscles ( $p<0.0001$ )  
313 compared to matched-muscle region of  $Mstn^{-/-}$ ND (Fig. 3b, d, f, i and l). The data was  
314 accompanied with significant interaction ( $p<0.0001$ ) indicating the effect of genotype  
315 under HFD on all muscles.

316 Furthermore, we examined the impact of HFD on the CSA of MHC type IIA fibers from  
317 PF and CT regions of T.medial, ECU and FCU muscles of both genotypes following

318 the HFD course. The analysis revealed a significant increase in the CSA of PF and CT  
319 IIA fibers of ECU ( $p < 0.01$  and  $0.01$ ) and FCU muscles ( $p < 0.001$  and  $0.001$ ) of  $Mstn^{-/-}$   
320 HFD compared to the matched region of  $Mstn^{-/-}$ ND. However, T.medial muscle of  $Mstn^{-/-}$   
321 HFD showed a decrease in the CSA of only PFIIA fibers ( $p < 0.0001$ ) compared to the  
322 corresponding PFIIA region in  $Mstn^{-/-}$ ND. Moreover, CTIIA fibers of  $Mstn^{+/+}$ HFD muscle  
323 showed either increase in T.medial or a decrease in ECU muscles compared to  
324 matched-muscle region kept on ND (Fig. 3g, j, and m).

### 325 **3. 5. Morphological evaluation of the muscle phenotype following a high-fat diet**

326 Having shown that the muscles of both genotypes responded differently in terms of the  
327 CSA of the muscle fibers. We next examined whether HFD affects the muscle  
328 phenotypic profile. The morphological evaluation revealed marked increase of slow  
329 MHC fibers compared to fast MHC in  $Mstn^{+/+}$  and  $Mstn^{-/-}$  under HFD compared to  
330 matched genotype muscle kept on a ND (Fig. 4f). Thus, quantifications of the  
331 percentage of MHC type I, IIA, IIX and IIB fibers in the mid-belly of each muscle were  
332 analyzed. All the muscles except the T.lateral muscle displayed a common observation  
333 of myofiber shift from fast to slow MHC isoform due to the HFD. T.long and FCU  
334 muscles showed a reduction in the percentage of type IIB ( $p < 0.001$  and  $p < 0.01$ ) and  
335 ( $p < 0.05$  and  $0.01$ ) in  $Mstn^{+/+}$ HFD and  $Mstn^{-/-}$ HFD compared to matched genotype  
336 muscle kept on a ND. The reduction of MHC type IIB fibers was accompanied by a  
337 shift towards an increase of the percentage of MHC type IIX fiber as shown in T.long  
338 ( $p < 0.001$ ) and FCU muscles ( $p < 0.01$  and  $0.05$ ) of the  $Mstn^{+/+}$ HFD and  $Mstn^{-/-}$ HFD  
339 respectively when compared to the corresponding genotype kept on a ND (Fig. 4a and  
340 b). Furthermore, the fast to slow myofiber shift was also observed in T.medial muscle  
341 of  $Mstn^{-/-}$ HFD via a reduction of the percentage of MHC type IIX ( $p < 0.001$ ) at the  
342 expense of an increase of MHC type IIA ( $p < 0.05$ ) compared to  $Mstn^{-/-}$ ND (Fig. 4c). This  
343 finding was supported with significant interaction ( $p < 0.0001$ ) indicative for the effect of  
344 myostatin deletion under HFD. Alternatively, ECU m of  $Mstn^{+/+}$ HFD displayed another  
345 form of fast to slow MHC shift, as a reduction in the percentage of MHC type IIX fibers  
346 ( $p < 0.0001$ ) towards an increase in the percentage of MHC type IIA fibers ( $p < 0.01$ ) was  
347 detected compared to  $Mstn^{+/+}$ ND. Interestingly, however, in ECU muscle of  $Mstn^{-/-}$ HFD  
348 only MHC type IIB into IIX fiber shift ( $p < 0.01$ ) was detected in comparison to  $Mstn^{-/-}$   
349 ND. In addition, a significant interaction ( $p < 0.0001$ ) between the effects of genotype  
350 and HFD could be detected (Fig. 4d). In contrast, no significant change was observed

351 in the myofiber composition of T.lateral muscle for both  $Mstn^{+/+}$  and  $Mstn^{-/-}$  following  
352 HFD course (Fig. 4e). These data not only provides strong evidence that a muscle-  
353 dependent attempt of fast to slow MHC shift but also point out the effect of myostatin  
354 deletion on the muscle response in the course of HFD intake.

#### 355 **4. Discussion**

356 The aim of this study was to determine the effect of high-fat diet intake on the  
357 morphological properties and fiber composition of five phenotypically different forelimb  
358 muscle of myostatin null mice.

359 Our data revealed that after 10 weeks of HFD intake, both  $Mstn^{+/+}$ HFD and  $Mstn^{-/-}$ HFD  
360 showed increased body mass with no increase in the muscle-specific weight compared  
361 to genotype-matched littermates under ND. The higher body mass may be attributed  
362 to accumulation of abdominal body fat as shown previously (Matsakas et al. 2015).  
363 Similarly, a study on rats showed no change in muscle weight following 16 weeks of  
364 high-fat diet intake (Campbell et al. 2015). However, another report found that an  
365 increase in body mass and a reduction in fat deposition were present following  
366 inhibition of myostatin and HFD in skeletal muscle but not in adipose tissue (Guo et al.  
367 2009).

368 Although the deletion of myostatin results in an increase in muscle fiber number as  
369 previously shown (McPherron & Lee, 1997; Elashry et al. 2009; Elashry et al. 2017),  
370 HFD for 10 weeks showed no alteration in fiber number which excludes not only the  
371 possibility of myofiber hyperplasia but also myofiber apoptosis/loss due to lipotoxicity.  
372 In agreement with our results, a study demonstrated that HFD intake for up to 16 weeks  
373 failed to induce autophagy or even apoptosis in soleus and plantaris muscles of rat  
374 (Campbell et al. 2015).

375 Despite the absence of muscle mass changes under HFD, there were remarkable  
376 alterations in myofiber CSA of  $Mstn^{-/-}$  mice. We have observed a reduction of CSA of  
377 fast type IIB fibers in (T.long, FCU) and (T.lateral, T.long) muscles for  $Mstn^{+/+}$  and  $Mstn^{-/-}$   
378  $^{-/-}$  respectively. However, there was an increase in the CSA of the same fiber isoform  
379 in T.medial and ECU muscles of  $Mstn^{+/+}$  and T.medial and FCU muscles of  $Mstn^{-/-}$   
380 under HFD. There was a significant interaction between genotype and diet on the CSA  
381 of IIB and IIX fibers. These data suggest that the phenotype of muscles affects their  
382 response to HFD. In this respect, a study using mixed high-fat and sucrose diet in rats

383 to evaluate muscle performance under obesity, showed that despite the quadriceps  
384 muscle was functionally attenuated, the slow twitch soleus muscle was resistant to  
385 diet-based muscle alteration (Collins et al. 2017). Taken together, the alteration of CSA  
386 of type IIB and IIX fibers under HFD seems to be relevant to the muscle phenotype as  
387 well as, to its metabolic activity. Furthermore, simultaneous myostatin deletion and  
388 HFD induce muscle- dependent adaptation in order to utilize the excess of energy  
389 intake. The present results also revealed that both genotypes responded differently  
390 under HFD regarding the CSA of MHC type IIA fibers.

391 Our data revealed a reduction in CSA of IIB fibers from PF region under HFD as shown  
392 in T.lateral and T.long muscles of both genotypes. The data point out that PF fibers  
393 are more prone to HFD-induced fiber size reduction compared to the corresponding  
394 muscle region under ND. Consequently, there was an increase in the CSA of CTIIB  
395 fibers in T.lateral, ECU and FCU muscles of *Mstn*<sup>-/-</sup> under HFD as well as, in CTIIX  
396 fibers as shown in T.long, T.medial and ECU muscles compared to matching muscle  
397 region for ND. These data were accompanied by a significant interaction indicating the  
398 different response of *Mstn*<sup>-/-</sup> under HFD. Moreover, such regional alteration might be a  
399 compensatory adaptation to restore the PF fiber loss. Indeed, the PFIIB and PFIIX  
400 fibers are the major store of muscle protein plus their glycolytic nature which it could  
401 point out towards an alteration of the muscle metabolism. In the same line, we have  
402 shown previously that fast myofibers are more liable to age-related muscle loss  
403 (Elashry et al. 2009) as well as, being more prone to atrophy after five weeks of dietary  
404 restriction (Elashry et al. 2017). The results suggest a compensatory mechanism in  
405 order to maximize energy utilization in the course of the HFD regime by either reducing  
406 the size of the fast MHC fibers or increasing the size of the slow fiber isoform. In the  
407 same line, it has been reported that following three weeks of HFD, the whole body  
408 metabolism was directed toward lipid oxidation with fast to slow myofiber shift in order  
409 to maintain muscle-dependent insulin sensitivity (Trajcevski et al. 2013). MHC type IIA  
410 fibers increased in CSA for both PF and CTIIA fibers in ECU and FCU muscles of *Mstn*<sup>-/-</sup>  
411 <sup>-</sup>HFD. The data revealed enhanced oxidative response in the absence of myostatin.  
412 In agreement with our results, a study in cats reported increased CSA of the oxidative  
413 fibers in the oxidative region of the muscle compared to the same fiber type in glycolytic  
414 region suggesting that the fiber size adaptation is more likely to compensate functional  
415 demands (Gonyea, 1979).

416 Despite four out of five muscles displaying fast to slow myofiber shift, the mechanism  
417 of fiber type shift varied between the muscles in the absence of myostatin. Along the  
418 same lines, it has been shown in rats that HFD intake promoted the oxidative  
419 metabolism in the fast twitch Extensor digitorum longus (EDL) muscle via upregulation  
420 of mitochondrial uncoupling protein 3 and pyruvate dehydrogenase kinase 4 and porin  
421 expression in conjunction with, fast to slow shift of MHC expression (Mizunoya et al.  
422 2013). Similarly, it has been reported that the muscle with high levels of oxidative  
423 muscle fibers showed enhanced oxidation and reduction of body fat accumulation  
424 (Abou Mrad et al. 1992). On the other hand, the muscle oxidative capacity is affected  
425 by the quality and the number of mitochondria (Chanséaume & Morio, 2009). Taken  
426 together, we postulate that HFD may enhance the oxidative metabolism of the muscle  
427 fiber perhaps by increasing the mitochondrial biogenesis leading to slow fiber type shift.  
428 This is in line with our data regarding the reduction of the MHC IIB and IIX isoforms  
429 CSA in combination with the increased size of MHC IIA isoform. In agreement with this  
430 concept, similar report revealed changes in the oxidative metabolism in case of insulin  
431 resistance due to suppression of mitochondrial biogenesis (Johannsen & Ravussin,  
432 2009; Abdul-Ghani & DeFronzo, 2010). Similarly, a study in rats revealed that HFD  
433 intake was associated with intramyocellular lipid (IMCL) that require a comparable  
434 increase of mitochondrial contents to maintain proper oxidative capacity (Van den  
435 Broek et al. 2010).

436 Our data revealed no change in the myofiber composition of the T.lateral muscle of  
437 both genotypes following HFD treatment. This muscle is composed primarily of IIB  
438 fibers and responds only by losing the size of the fast fibers rather than enhancing the  
439 slow phenotype shift under HFD. In the same line, a study in mouse extensor digitorum  
440 longus m (EDL) showed that although HFD induced a fast to slow fiber type shift in  
441  $Mstn^{+/+}$ ,  $Mstn^{-/-}$  under HFD displayed no changes in terms of MHC quantification and  
442 succinate dehydrogenase (SDH) analysis (Matsakas et al. 2015). It has been  
443 documented that, following five weeks of HFD in mice, the fast twitch EDL  
444 demonstrated neither increase in fatty acid content nor increase in mitochondrial  
445 oxidation, in contrast, slow twitch soleus muscle displayed a reduced force production  
446 and fast to slow shift in troponin C type (Ciapaite et al. 2015). A similar study revealed  
447 that, although no change in the muscle mass following 12 weeks of HFD in mice, there  
448 was reduction in the contractile properties, increase of mitochondrial oxidation and

449 increased percentage of type IIX MHC in the EDL muscle indicating a phenotypic  
450 alteration (Eshima et al. 2017).

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## 452 **5. Conclusion**

453 The present study elucidated the effect of HFD on the morphological properties of the  
454 forelimb skeletal musculature in the myostatin null mice. Although the significant  
455 increase in the body weight, no change in the muscle mass was detected after 10  
456 weeks of HFD intake. We provide evidence that HFD induces a reduction in the CSA  
457 of the fast MHCIIB and MHCIIX myofibers of myostatin null in muscle and genotype-  
458 dependent manner. HFD increases CSA of oxidative type IIA fibers of T.long and  
459 T.medial of *Mstn*<sup>+/+</sup> and ECU and FCU muscles of the *Mstn*<sup>-/-</sup>. Moreover, type I fibers  
460 of *Mstn*<sup>-/-</sup> was less liable to HFD intake. We show that the anatomically located  
461 peripheral fibers are more prone to HFD-induced fiber atrophy compared to the central  
462 fibers. We provide evidence that combined HFD together with myostatin absence  
463 induced a muscle-dependent fast to slow myofiber shift. Thus, it can be assume that  
464 HFD reverses the hypermuscular phenotype of the myostatin null mice to utilize the  
465 high energy intake. Our work provides a platform for further investigations interms of  
466 using myostatin inhibition as a therapeutic application for metabolic diseases including  
467 obesity.

## 468 **Competing interest**

469 All the authors have declared no competing interests regarding the publication of this  
470 article.

## 471 **Author contributions**

472 MIE, AE, KG and AM have contributed to data collection and analysis, designed the  
473 experiments, conceived and prepared the manuscript draft. SA and SW have  
474 contributed to the manuscript preparation and data interpretation. AM and KP has  
475 revised, formulated and finalized the submitted manuscript.

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608 **Table legends**

609 **Table (1)** Effect of high-fat diet on body mass and individual muscle weight

610 Average body mass (g) and muscle wet weight (mg) of normal diet (ND) and high food  
611 diet (HFD) Mstn<sup>+/+</sup> and Mstn<sup>-/-</sup> mice (N= 8 for each experimental group). Significant  
612 increase in body mass of Mstn<sup>+/+</sup> and Mstn<sup>-/-</sup> following 10 weeks of HFD. No significant  
613 differences were detected in Mstn<sup>+/+</sup> and Mstn<sup>-/-</sup> muscles after HFD compared to ND.  
614 All values presented as mean± SEM. \*\*=p<0.01 for the comparison within the same  
615 genotype and Φ =p<0.05 for the comparison between different genotype kept on a ND.

616 **Figures legends**

617 **Fig. 1** Evaluation of the muscle fiber number following high-fat diet

618 (a-f) Representative double labelled immunofluorescent images of ECU muscle show  
619 MHCII B (red), MHCII A (green) and MHCII X (black) positive fibers of Mstn<sup>+/+</sup> and Mstn<sup>-/-</sup>  
620 mice kept on ND and HFD. (g) Average total muscle fiber number of T.lateral, T.long,  
621 T.medial, ECU and FCU muscles of ND and HFD Mstn<sup>+/+</sup> and Mstn<sup>-/-</sup> mice (N=8 for  
622 each experimental group). The muscles of Mstn<sup>-/-</sup> show various degrees of myofiber  
623 number increase compared to Mstn<sup>+/+</sup>. T.medial muscle displays no changes following  
624 Mstn<sup>-/-</sup>. The muscles of both Mstn<sup>+/+</sup> and Mstn<sup>-/-</sup> on HFD demonstrate no detectable  
625 difference in the muscle fiber number compared to genotype-matched muscles on ND.  
626 All values presented as mean± SEM. \*\*=p<0.01 and \*\*\*\*=p<0.001. Scale bar in a, b=  
627 20 μm and in c, d, e, f= 200 μm. DAPI used as a nuclear counter stain in a, b.

628 **Fig. 2** Effect of high-fat diet on CSA of muscle fiber type

629 (a-o) Average CSA measurements (μm<sup>2</sup>) of MHC type IIB, IIX, IIA and type I myofibers  
630 of ND and HFD Mstn<sup>+/+</sup> and Mstn<sup>-/-</sup> mice (250 fibers per each type and group were  
631 measured using the axiovision software). Average CSA of types IIB (a) and IIX (b)  
632 myofibers of T.lateral muscle; type IIB (c), IIX (d) and IIA (e) myofibers of T.long  
633 muscle; type IIB (f), IIX (g), IIA (h) and type I (o) myofibers of T.medial muscle; type IIB  
634 (i), IIX (j), IIA (k) of ECU muscle and type IIB (l), IIX (m), IIA (n) myofibers of FCU  
635 muscle. All values presented as mean± SEM. \*=p<0.05, \*\*=p<0.01, \*\*\*=p<0.001 and  
636 \*\*\*\*=p<0.001.

637 **Fig. 3** Effect of high-fat diet on the CSA of myofibers based on their regional distribution  
638 (a-m) Average CSA measurement of peripheral (PF) and central (CT) myofibers of ND  
639 and HFD Mstn<sup>+/+</sup> and Mstn<sup>-/-</sup> mice (250 fibers per each type and group were measured  
640 using the axiovision software). Average CSA measurements of type IIB (a) and IIX (b)

641 myofibers of T.lateral muscle; type IIB (c) and IIX (d) myofibers of T.long muscle; type  
642 IIB (e), IIX, (f) and IIA (g) myofibers of T.medial muscle; type IIB (h), IIX (i) and IIA (j)  
643 myofibers of ECU muscle and types IIB (k), IIX (l) and IIA (m) myofibers of FCU muscle.  
644 All values presented as mean± SEM. \*=p<0.05, \*\*=p<0.01, \*\*\*=p<0.001 and  
645 \*\*\*\*=p<0.001.

646 **Fig. 4** Morphological evaluation of the muscle phenotype following high-fat diet  
647 (a-e) Average percentage of type IIB, IIX, IIA and I myofibers of ND and HFD Mstn<sup>+/+</sup>  
648 and Mstn<sup>-/-</sup> mice (N= 8 for each experimental group). (a) T.long, (b) FCU, (c) T.medial,  
649 (d) ECU and (e) T.lateral muscles. (f) Reconstructive double immunofluorescent  
650 images for ECU muscle of Mstn<sup>+/+</sup> and Mstn<sup>-/-</sup> following ten weeks on a ND and HFD.  
651 Immunofluorescence of the muscle mid-belly cryo-sections show muscle phenotypic  
652 composition based on MHCIIIB (red), MHCIIIX (unstained) and MHCIIA (green)  
653 isoforms. All values presented as mean± SEM. (IIB) \*=p<0.05, \*\*=p<0.01 and  
654 \*\*\*=p<0.001; (IIX) σ=p<0.05, σ σ =p<0.01, σ σ σ =p<0.001 and σ σ σ σ =p<0.0001;  
655 (IIA) θ=p<0.05; θ θ=p<0.01. Scale bar in f = 200 μm.

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Weight	Mstn <sup>+/+</sup>		Mstn <sup>-/-</sup>	
	ND	HFD	ND	HFD
<b>Total body weight</b>	30.8±0.4	36.9±0.6**	36±0.9 <sup>Φ</sup>	40.7±0.3**
<b>M. triceps brachii Caput laterale</b>	3.5±0.2	3.3±0.28	7±0.2	7.52±0.6
<b>M. triceps brachii Caput longum</b>	127±0.7	138±6.5	223±7.6	242±3.8
<b>M. triceps brachii Caput mediale</b>	10±1	9.2±0.4	13.4±0.5	15.2±1.2
<b>M. extensor carpi ulnaris</b>	9.7±0.1	9.5±0.2	14±0.8	15.6±0.3
<b>M. flexor carpi ulnaris</b>	3.5±0.2	3.3±0.3	7±0.3	7.5±0.6

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678 **Table (1)** Effect of high-fat diet on body mass and individual muscle weight

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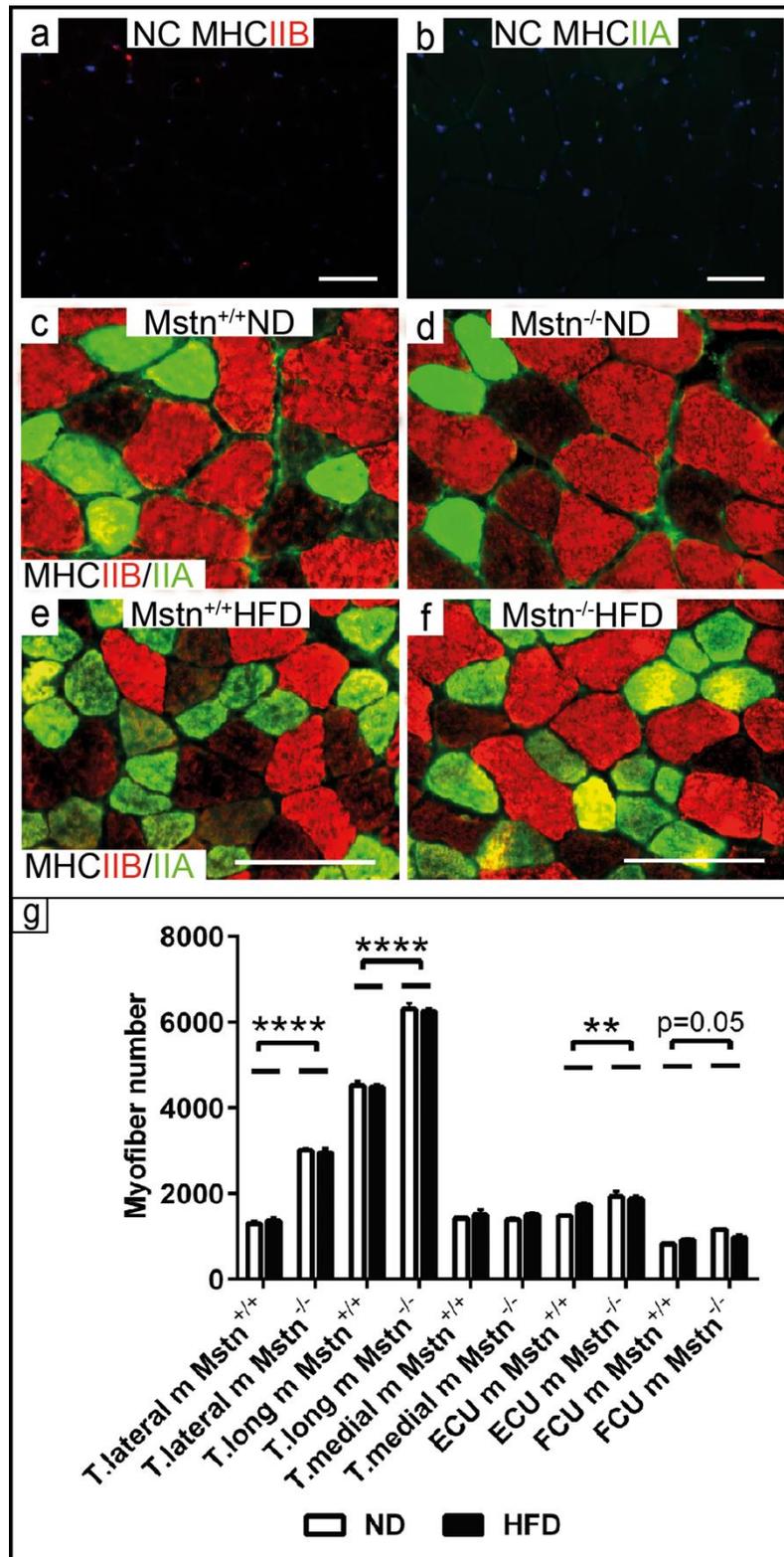
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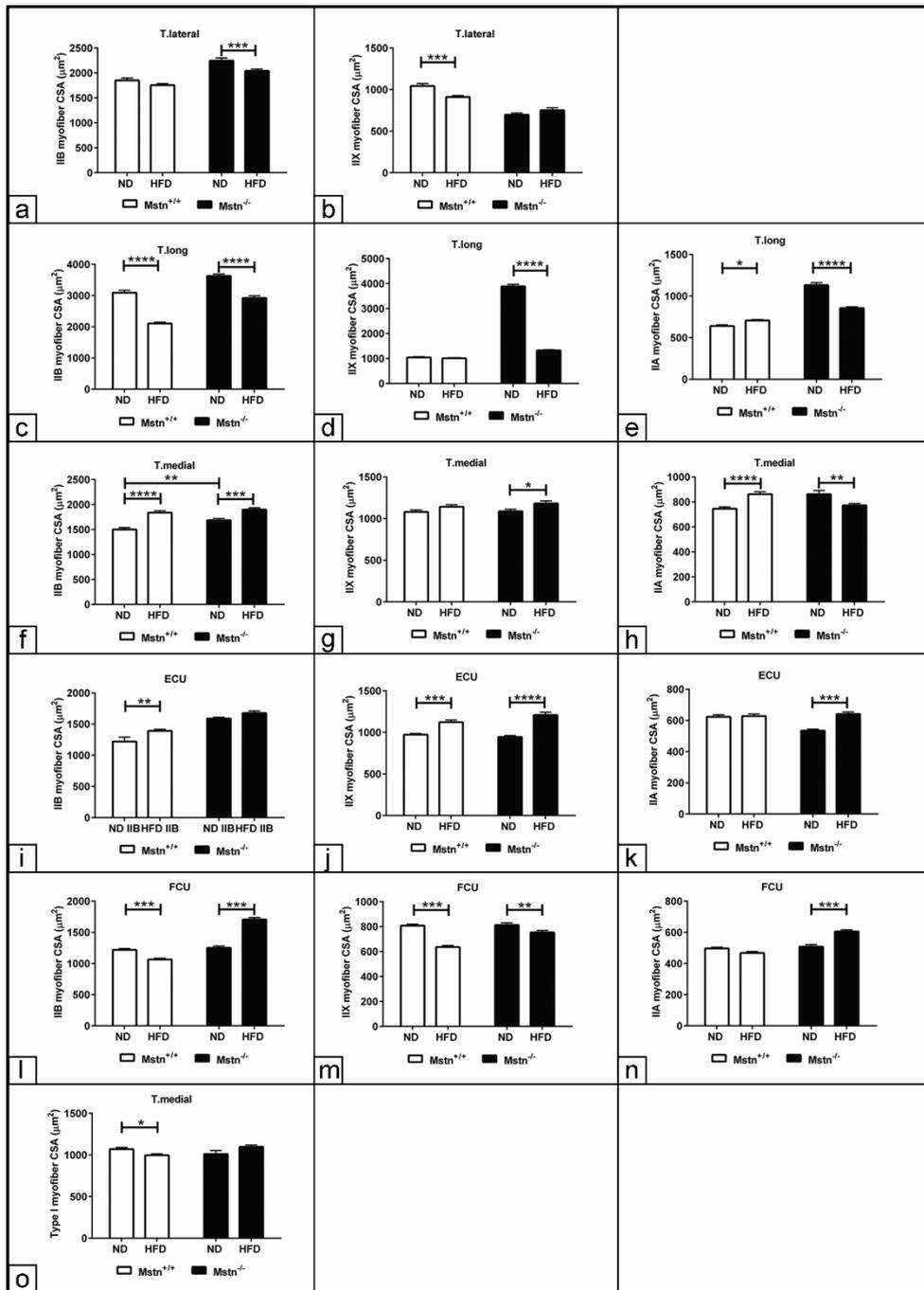
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**Fig. 1** Evaluation of the muscle fiber number following high-fat diet



**Fig. 2** Effect of high-fat diet on CSA of muscle fiber type

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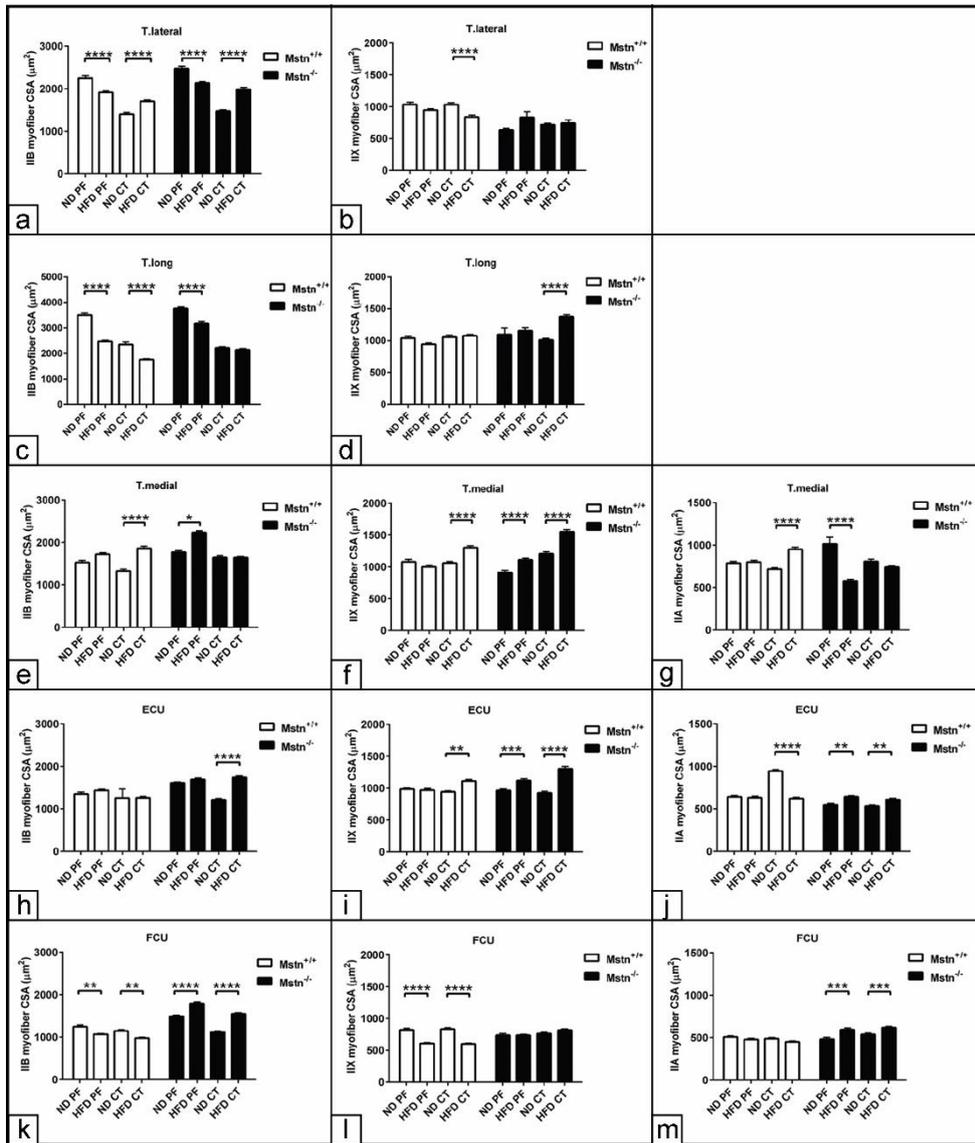
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729 **Fig. 3** Effect of high-fat diet on the CSA of myofibers based on their regional distribution

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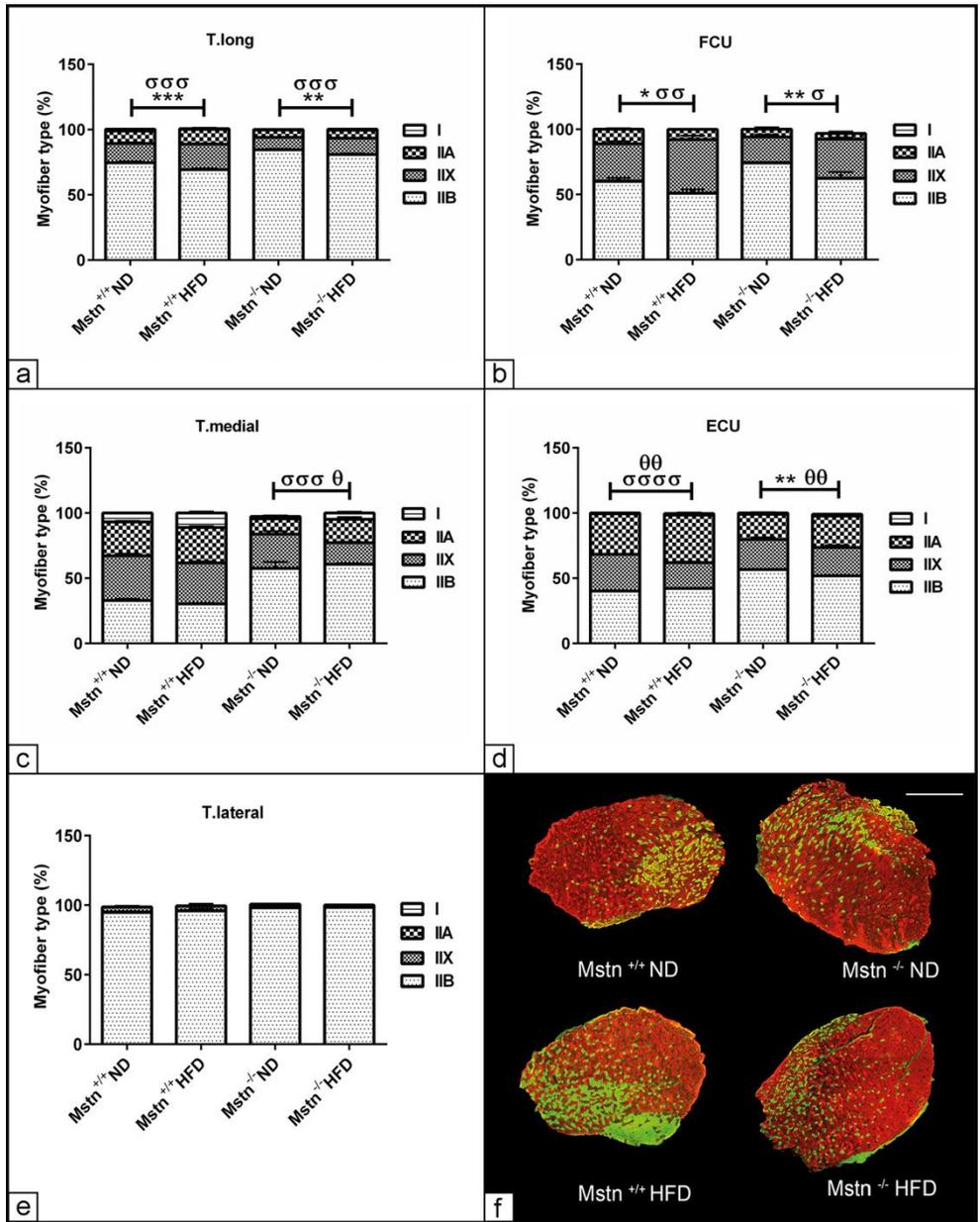
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741 **Fig. 4** Morphological evaluation of the muscle phenotype following high-fat diet