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

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

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Abstract

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

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

Running Headline/ Short title

High-fat diet in myostatin null mice

1. Introduction

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

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

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

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

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

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

2. Materials and Methods

2. 1. Animals

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

2. 2. Tissue collection

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

2. 3. Immunohistochemistry

Muscle sections were washed three times in PBS for 15 minutes, then permeabilized in buffer solution composed of 20 mM Hepes (Biochrom, Germany), 300 mM sucrose (Merck, Germany), 50 mM NaCl (Roth, Germany), 3 mM $MgCl_2$ (Roth, Germany) and 0.5% Triton-X100 (pH7, Roth, Germany) for 15 minutes at room temperature. The muscle sections were preblocked in wash buffer containing 5% fetal calf serum (v/v) (Gibco, Thermo Fisher Scientific) and 0.05 % Triton X-100 (v/v) in phosphate buffered saline (PBS) for 30 minutes at room temperature. The myofiber type was investigated based on the expression of certain myosin heavy chain protein. The myofiber type IIB, IIA and type I were identified using BFF3 mouse IgM (1:1), A.474 mouse IgG (1:4) and A4.840 mouse IgM (1:1) monoclonal primary antibodies (DSHB) respectively as previously reported (Matsakas et al. 2009). Type IIB and IIA myofibers were immunostained in the same muscle section. However, type I myofibers were identified on serial sections. Muscle sections with no primary antibody added were used as a negative control (Fig. 1a and b). All the primary antibodies were incubated with muscle sections overnight at 4C. After five times washing steps for 10 minutes, the primary antibodies were detected using Alexa Fluor 633 goat anti-mouse IgM (Molecular Probes, A21046 diluted in wash buffer 1:200) for MHC I and MHC IIB and Alexa Fluor 488 Goat-anti-mouse IgG (Molecular probes, A11029 diluted in wash buffer 1:200) secondary antibody. The muscle sections were incubated with the secondary antibodies for 45-60 minutes at room temperature in the dark. The nuclei were counterstain with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Thermo Fisher scientific) and the slides were mounted using a fluorescent mounting medium (Mowiol 4-88, Calbiochem).

2. 4. Imaging and myofiber quantifications

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

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

2. 5. Statistical analysis

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

3. Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

5. Conclusion

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

Competing interest

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

Author contributions

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

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Table legends

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

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

Figures legends

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

(a-f) Representative double labelled immunofluorescent images of ECU muscle show MHCIIB (red), MHCIIA (green) and MHCIIX (black) positive fibers of $Mstn^{+/+}$ and $Mstn^{-/-}$ mice kept on ND and HFD. (g) Average total muscle fiber number of T.lateral, T.long, T.medial, ECU and FCU muscles of ND and HFD $Mstn^{+/+}$ and $Mstn^{-/-}$ mice (N=8 for each experimental group). The muscles of $Mstn^{-/-}$ show various degrees of myofiber number increase compared to $Mstn^{+/+}$. T.medial muscle displays no changes following $Mstn^{-/-}$. The muscles of both $Mstn^{+/+}$ and $Mstn^{-/-}$ on HFD demonstrate no detectable difference in the muscle fiber number compared to genotype-matched muscles on ND. All values presented as mean± SEM. **=p<0.01 and ****=p<0.001. Scale bar in a, b= 20 μm and in c, d, e, f= 200 μm. DAPI used as a nuclear counter stain in a, b.

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

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

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

(a-m) Average CSA measurement of peripheral (PF) and central (CT) myofibers of ND and HFD $Mstn^{+/+}$ and $Mstn^{-/-}$ mice (250 fibers per each type and group were measured using the axiovision software). Average CSA measurements of type IIB (a) and IIX (b)

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

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

(a-e) Average percentage of type IIB, IIX, IIA and I myofibers of ND and HFD $Mstn^{+/+}$ and $Mstn^{-/-}$ mice (N= 8 for each experimental group). (a) T.long, (b) FCU, (c) T.medial, (d) ECU and (e) T.lateral muscles. (f) Reconstructive double immunofluorescent images for ECU muscle of $Mstn^{+/+}$ and $Mstn^{-/-}$ following ten weeks on a ND and HFD. Immunofluorescence of the muscle mid-belly cryo-sections show muscle phenotypic composition based on MHCIIB (red), MHCIIX (unstained) and MHCIIA (green) isoforms. All values presented as mean± SEM. (IIB) *=p<0.05, **=p<0.01 and ***=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; (IIA) θ=p<0.05; θ θ=p<0.01. Scale bar in f = 200 μm.

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

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

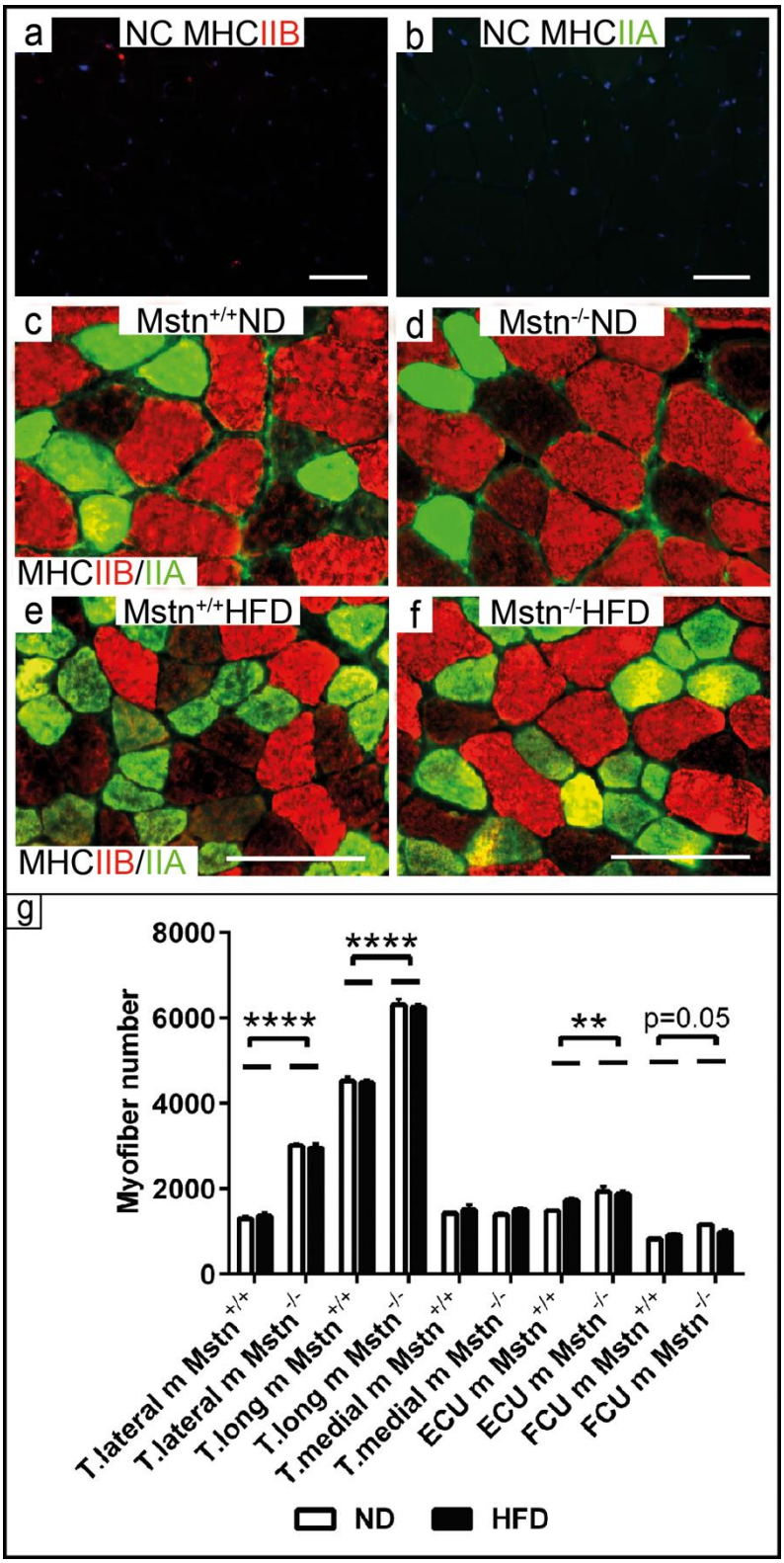

Fig. 1 Evaluation of the muscle fiber number following high-fat diet

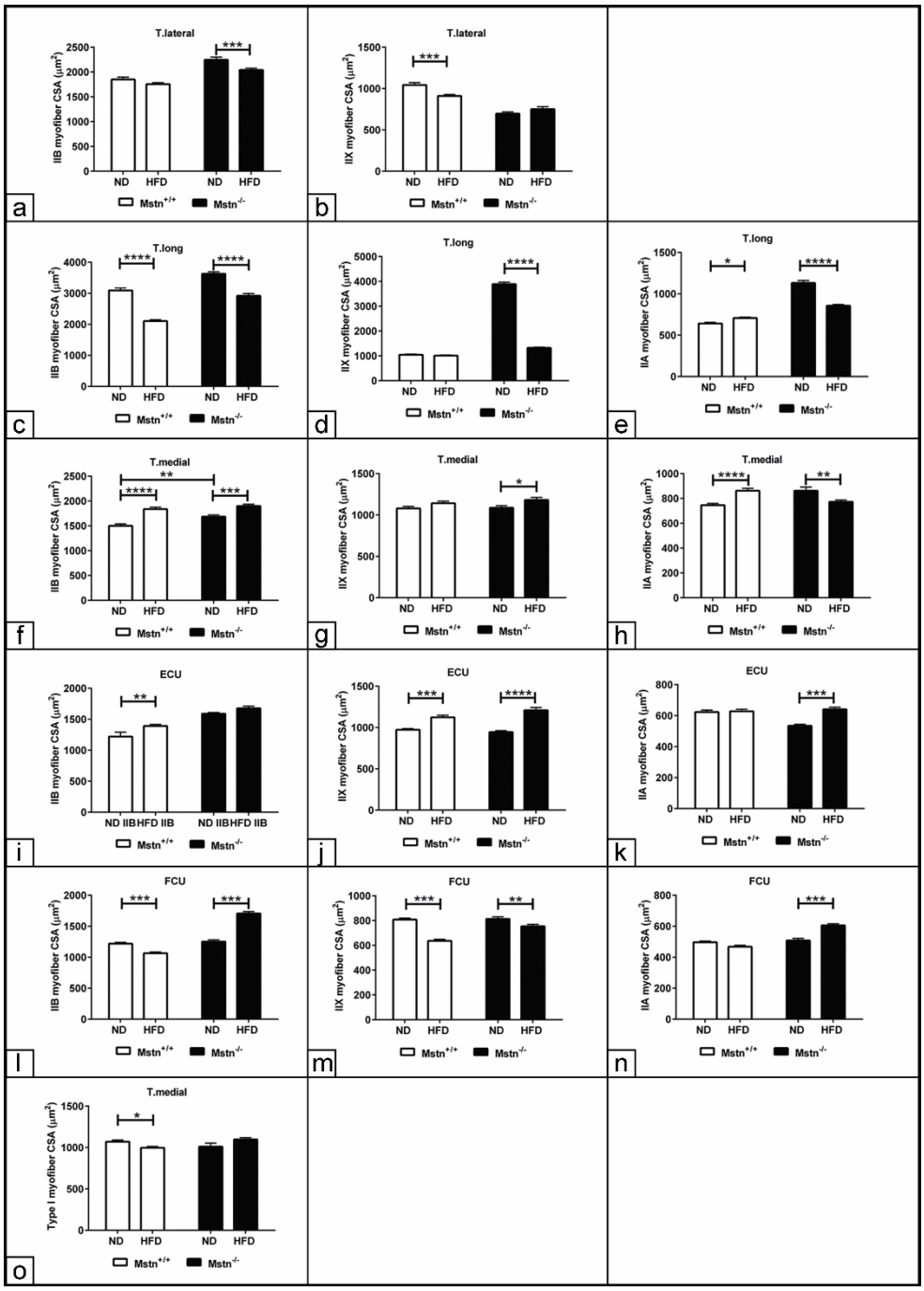

Fig. 2 Effect of high-fat diet on CSA of muscle fiber type

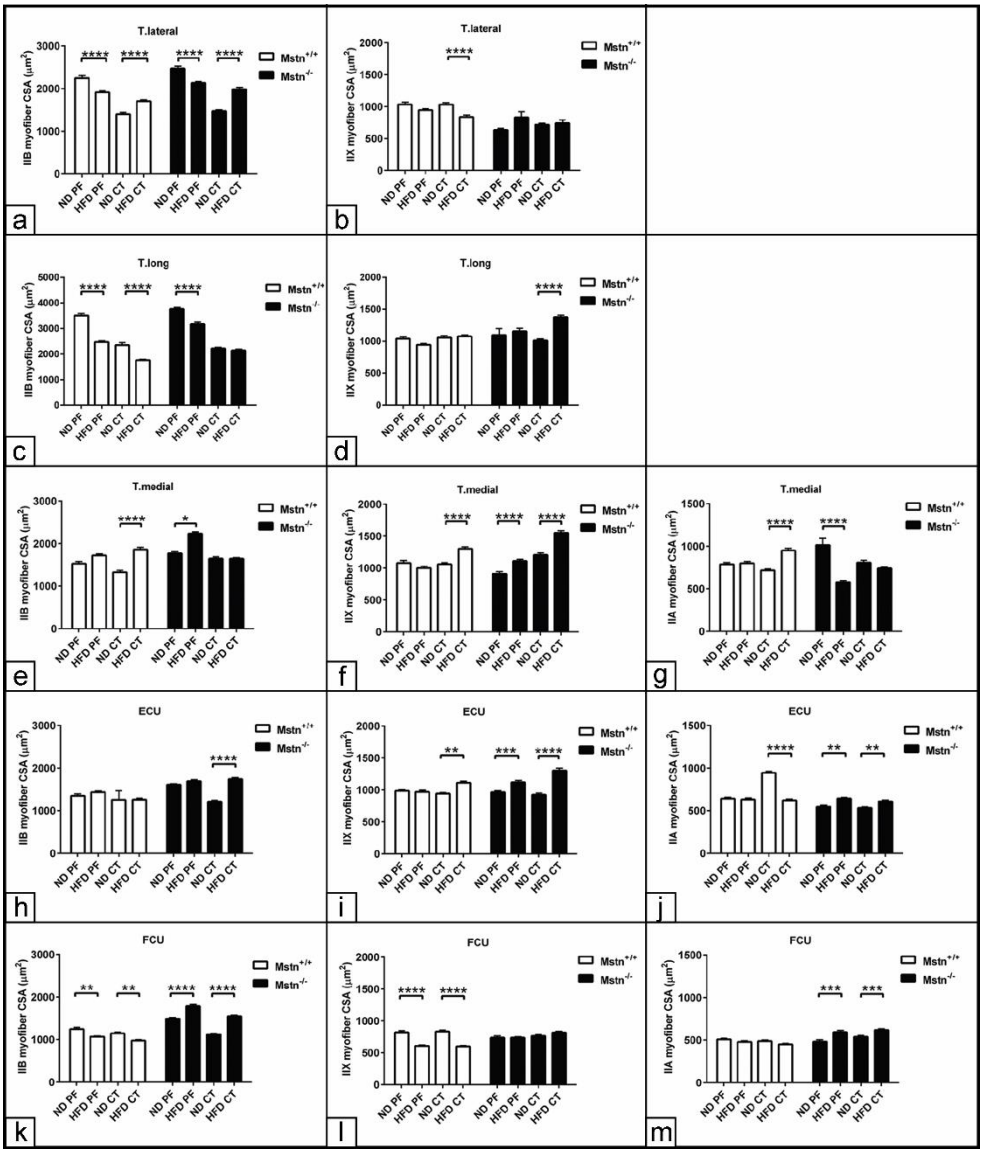

Fig. 3 Effect of high-fat diet on the CSA of myofibers based on their regional distribution

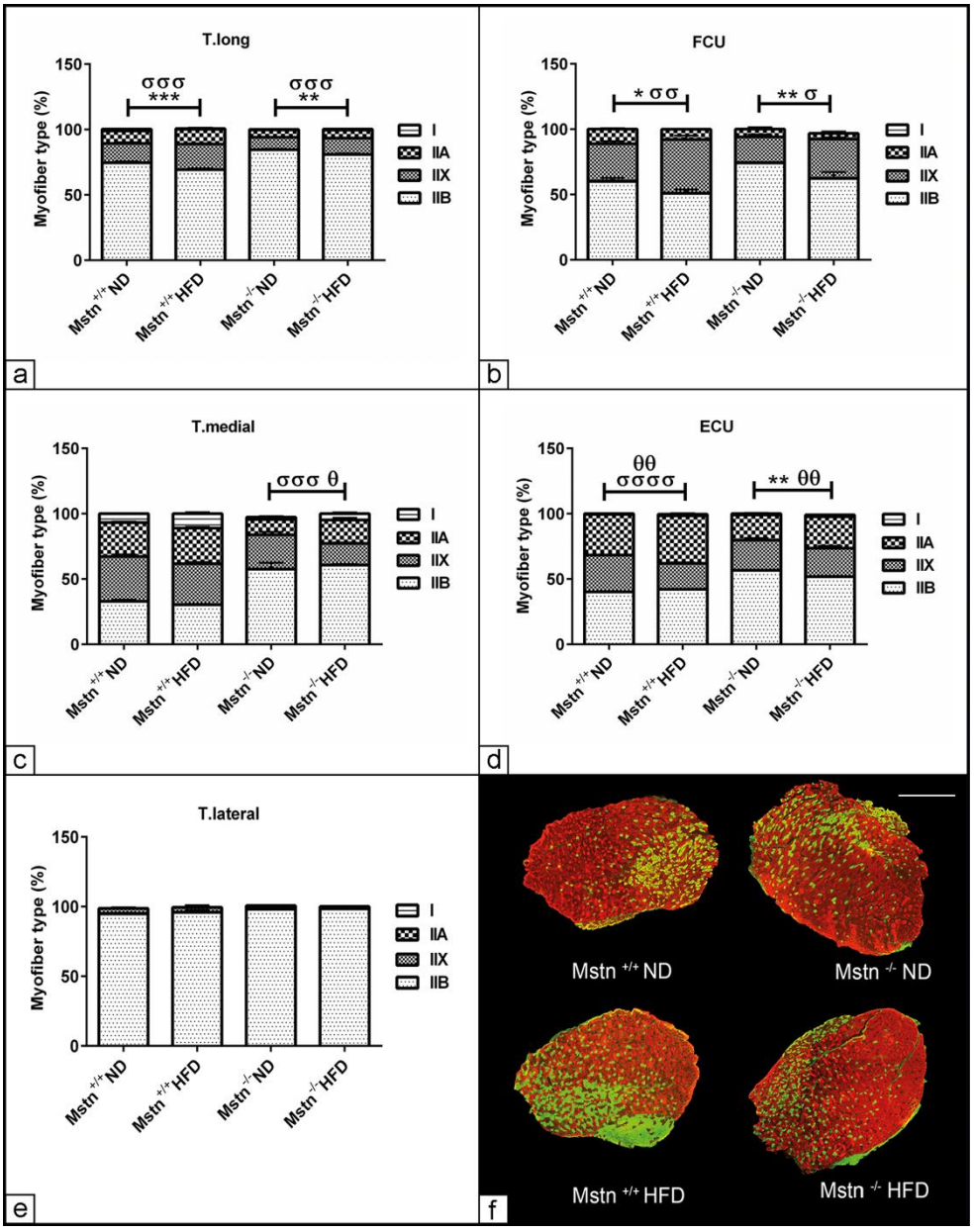

Fig. 4 Morphological evaluation of the muscle phenotype following high-fat diet