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## Accepted Manuscript

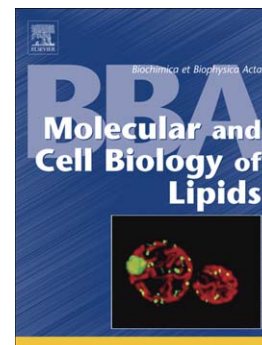
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**Myostatin deficiency is associated with  
lipidomic abnormalities in skeletal muscles**

Narjes Baati<sup>1</sup>, Christine Feillet-Coudray<sup>1</sup>, Gilles Fouret<sup>1</sup>, Barbara Vernus<sup>1</sup>, Bénédicte Goustard<sup>1</sup>, Charles Coudray<sup>1</sup>, Jérôme Lecomte<sup>2</sup>, Véronique Blanquet<sup>3</sup>, Laetitia Magnol<sup>3</sup>, Anne Bonnieu<sup>1a</sup> & Christelle Koechlin-Ramonatxo<sup>1a\*</sup>

<sup>1</sup> INRA, UMR866 Dynamique Musculaire Et Métabolisme, Université Montpellier, 34000 Montpellier, France

<sup>2</sup> CIRAD, UMR IATE, Montpellier F-34398, France

<sup>3</sup> INRA, UMR1061 Unité de Génétique Moléculaire Animale, 87060 Limoges, France

**\*Correspondance:** Christelle Koechlin-Ramonatxo

INRA, UMR866 Dynamique Musculaire et Métabolisme, Université Montpellier,  
F-34060, Montpellier, France

Email: [christelle.ramonatxo@umontpellier.fr](mailto:christelle.ramonatxo@umontpellier.fr)

Tel: +33 4 99 61 23 38

Fax: +33 4 67 54 56 94

<sup>a</sup> These authors are co-senior authors

**Running Headline:** Myostatin deficiency and lipidome metabolism in skeletal muscles

**Highlights**

Lipidomic abnormalities in hypertrophied muscle of mstn deficient mice

Unbalanced fatty acid composition in mstn deficient muscle: towards saturated profile

Impaired cardiolipin pathway in skeletal muscle with oxidative mitochondrial disorders

**Abbreviations**

ACC	Acetyl-CoA carboxylase	PE	Phosphatidyletanolamine
Alcat1	ER-associated monolysosomal acyltransferase	PG	Phosphatidylglycerol
ATP	Adenosin triphosphate	PGC1- $\alpha$	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
AU	Arbitrary Unit	PGPS	Phosphatidylglycerolphosphate synthase
$\beta$ -HAD	beta-hydroxyacyl CoA dehydrogenase	PI	Phosphatidylinositol
CHL	Cholesterol	PPAR- $\delta$	Peroxisome proliferator-activated receptor delta
CL	Cardiolipin	PS	Phosphatidylserine
CLS	Cardiolipin synthase	PTPMT1	Mitochondrial Phosphatase
COX	Cytochrome c oxidase	PUFA	Polyunsaturated fatty acids
CPT1	Carnitine palmitoyltransferase I	Rf	Retention factor
CS	Citrate synthase	RQ	Relative Quantity
DAG	1,2-diacylglycerol	SAM	Sorting and assembly machinery of the outer membrane
DMD	Duchenne muscular dystrophy	SCD1	Stearoyl-CoA desaturase
EDL	Extensor digitorum longus muscle	SFA	Saturated fatty acids
FA	Fatty acid	SM	Sphingomyelin
FABP3	Fatty acid binding protein 3	TAM41	The translocator maintenance protein
FAS	Fatty acid synthase	TAZ	Tafazzin
FAT/CD36	Fatty acid translocase	TCA	Tricarboxylic acid cycle
FATP1	Fatty acid transport proteins 1	TG	Triglycerides
FATP4	Fatty acid transport proteins 4	TGF- $\beta$	Transforming Growth Factor Beta
FFA	Free fatty acid	TIM22	Carrier translocase of the inner membrane
IMF	Intermyofibrillar	TIM23	Presequence translocase of the inner membrane
KO	Knock-out	TLC	Thin layer chromatography
GLUT4	Glucose transporter-4	TOM	Translocase of the outer membrane
LPC	Lysophosphatidylcholine	Tub	Tubulin
MAG	Monoacylglycerol	WT	Wild Type
MIM	Mitochondrial import complex		
Mstn	Myostatin		
MUFA	Monounsaturated fatty acids		
OXA	The oxidase assembly translocase		
PA	Phosphatidic acid		
PC	Phosphatidylcholine		
PCR	Polymerase chain reaction		

## Abstract

Myostatin (Mstn) deficiency leads to skeletal muscle overgrowth and Mstn inhibition is considered as a promising treatment for muscle-wasting disorders. Mstn gene deletion in mice also causes metabolic changes with decreased mitochondria content, disturbance in mitochondrial respiratory function and increased muscle fatigability. However the impact of MSTN deficiency on these metabolic changes is not fully elucidated. Here, we hypothesized that lack of MSTN will alter skeletal muscle membrane lipid composition in relation with pronounced alterations in muscle function and metabolism. Indeed, phospholipids and in particular cardiolipin mostly present in the inner mitochondrial membrane, play a crucial role in mitochondria function and oxidative phosphorylation process. We observed that Mstn KO muscle had reduced fat membrane transporter levels (FAT/CD36, FABP3, FATP1 and FATP4) associated with decreased lipid oxidative pathway (citrate synthase and  $\beta$ -HAD activities) and impaired lipogenesis (decreased triglyceride and free fatty acid content), indicating a role of mstn in muscle lipid metabolism. We further analysed phospholipid classes and fatty acid composition by chromatographic methods in muscle and mitochondrial membranes. Mstn KO mice showed increased levels of saturated and polyunsaturated fatty acids at the expense of monounsaturated fatty acids. We also demonstrated, in this phenotype, a reduction in cardiolipin proportion in mitochondrial membrane versus the proportion of others phospholipids, in relation with a decrease in the expression of phosphatidylglycerolphosphate synthase and cardiolipin synthase, enzymes involved in cardiolipin synthesis. These data illustrate the importance of lipids as a link by which MSTN deficiency can impact mitochondrial bioenergetics in skeletal muscle.

Key words: GDF-8, thin layer chromatography, delta-9 desaturase, neutral lipids, phospholipids

## Introduction

Myostatin (*Mstn*), a secreted growth factor and member of the TGF- $\beta$  superfamily, regulates levels of lean muscle mass and also body fat content in mice [1]. Inactivation of the *Mstn* gene in mice, or mutations in the bovine [2,3] ovine [4] and human *Mstn* [5] genes result in a similar phenotype of increased muscle growth. Thus, targeted inhibition of *Mstn* gene has been considered as a promising treatment for various muscle-wasting disorders [6,7,8,9]. In this case, strategies have been developed to treat muscle dystrophies, muscle wasting and myopathies by blocking the Myostatin/ActRIIB pathway. Many clinical trials are in process but results are associated with undesirable adverse effects [10]. Therefore, a better understanding of the mechanisms underlying the muscle phenotype in *Mstn* knock-out (KO) model is warranted that may ultimately improve treatments.

Indeed, beyond muscle hypertrophy, *Mstn* KO mice show a disturbed muscle function with loss of muscle strength and endurance *in vivo* or *ex vivo* accompanied with a decrease in mechanical performance, and ATP production during exercise [11,12,13,14]. In parallel, many other metabolic changes have been documented in *Mstn* KO muscle such as a decrease in mitochondrial content, disturbance in mitochondrial respiratory function with a decay in the respiratory control ratio in intermyofibrillar mitochondria, and a decline in porine activity [11,13,15]. In addition, a decrease of fatty acid translocase (FAT/CD36) level, a lipid transporter, and the citrate synthase (CS) activity, one of the main mitochondrial oxidative enzyme, associated with a lower peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1- $\alpha$ ) expression has been observed [15,16]. Although it is clear that lack of MSTN impairs muscle oxidative metabolism and mitochondrial function, the underlying mechanisms by which this alteration occurs have not been fully elucidated.

It is known that muscle mitochondrial function and oxidative metabolism are both highly reliant on the lipid pathway. Indeed, fatty acids (FA) are the main substrate for ATP production in muscle via the  $\beta$ -oxidation pathway [17]. Entering muscle fibers from blood via different transporters, lipids are then oxidized to Acyl-CoA in the cytosol then to acylcarnitine before translocating through mitochondria membranes by carnitine palmitoyltransferase I and II. These substrates in turn are subjected to oxidation by beta-hydroxyacyl CoA dehydrogenase ( $\beta$ -HAD) and CS to produce ATP. Lipogenesis encompasses the processes of fatty acid and triglyceride synthesis from glucose [18]. After glycolysis, pyruvate is produced from glucose which then enters mitochondria. Through the tricarboxylic acid cycle (TCA), pyruvate is converted to acetyl-CoA and exported to cytosol to be used as a precursor for lipid synthesis. Indeed, acetyl-CoA carboxylase (ACC) converts acetyl-CoA into malonyl-CoA, which finally activates synthesis of triglycerides, using the fatty acid synthase enzyme (FAS) [18]. In the case of high-fat diet, fatty acids are also stored as triglycerides.

In addition to their energetic role, lipids are crucial components of cell membranes, including mitochondrial membranes. Moreover, mitochondrial characteristics and function are largely dependent

of FA and phospholipid composition [19]. For example, being the most abundant phospholipid in both the outer mitochondrial membrane and the inner mitochondrial membrane, phosphatidylcholine (PC) modulates skeletal muscle mitochondrial function [20]. Genetic studies in mice or humans with defects in choline kinase  $\beta$ , enzyme catalysing the synthesis of PC, demonstrate that reduction of mitochondrial membrane PC content induced enlarged mitochondria, reduced inner membrane potential and caused muscle weakness [20–23]. The proper lipid composition of the membranes is crucial for membrane-bound protein machineries to maintain optimal mitochondrial biogenesis and function. The recent review of Martensson et al., 2016 illustrated that phospholipids affect outer (TOM complex, MIM, SAM) and inner (TIM23, TIM22 and OXA) membrane protein translocases and thus play a crucial role in mitochondrial protein import [24]. The role of phospholipids on mitochondria protein is also highlighted by their impact on respiratory chain supercomplexes [24]. In these studies, one phospholipid, cardiolipin (CL) attracted scientific attention. Mostly present in the inner mitochondrial membrane, CL undergoes two main phases for its activity [24,25]. The first is its biosynthesis that takes place on the matrix face of the inner mitochondrial membranes, pathway catalysed by numerous and successive enzymes as Tam41, Pgs1, PTPMT1 and finally by CL synthase (also called Crd1), to form premature (nascent) CL. The second phase is the remodelling of its fatty acids, with recruitment of a new set of tissue-specific fatty acids, and its assembly into the inner membrane. Nascent CL is deacylated by CL deacylases/phospholipases to monolysocardiolipin (MLCL) which is reacylated principally by tafazzin to mature CL [19,25]. CL interacts with a number of inner mitochondrial membrane proteins, enzymes and metabolite carriers, such as the electron transport chain complexes involved in oxidative phosphorylation and ADP/ATP carriers, which optimize efficient electron/proton flux and hence, ATP synthesis [26]. CL is also involved in the  $\beta$ -oxidation of fatty acids, as a reduction of mature CL impairs skeletal muscle FA oxidation [27,28] and a positive correlation has been demonstrated between mitochondrial COX activity and CL content [29]. Patients with Barth Syndrome (a X-linked disorder resulting from mutations in the gene encoding for tafazzin) or mice models which lack mature CL, have shown exercise intolerance, cardiac and skeletal muscle dysfunction, mitochondrial dysfunction, ATP deficiency and premature mortality [30,31,32]. *In vitro*, mitochondrial respiration has been shown to be impaired in inducible pluripotent stem cell (iPSC)-derived human cardiomyocytes from Barth Syndrome patients [33]. Possible mechanisms for this disease-associated mitochondrial dysfunction include mitochondrial supercomplex destabilization [34], higher reduced levels of mitochondrial cardiolipin [35] and abnormal mitochondrial morphology [30]. Recently, Bashir et al., 2016 [36] found higher resting skeletal muscle [PCr]/[Pi] in children/adolescents and adults with Barth syndrome, suggesting that individuals with Barth syndrome rely on glycolytic metabolism to a greater extent than unaffected, age-matched controls, likely due to an impairment in fat oxidation. These findings highlight the crucial role that mitochondrial CL plays in skeletal muscle oxidative metabolism and mitochondria bioenergetics processes [26].

Due to energetic and structure function of fatty acids and phospholipids in muscle, we hypothesized that in MSTN deficient hypertrophied skeletal muscle, hence a change in the muscle and mitochondrial lipid composition could exist, in relation with metabolic and mitochondrial abnormalities observed in this phenotype. In this study, we evaluated the lipid profile of muscle and mitochondrial membranes from Mstn KO mice, and explored muscle lipid metabolism pathway. We demonstrated in MSTN deficient muscle a decrease of both lipogenesis and lipid oxidation associated with important modifications in the fatty acid composition of total muscle lipid profile. Interestingly, we also found that muscle mitochondrial proportion of cardiolipin is significantly decreased in Mstn KO mice correlating with the reduced gene expression of two enzymes involved in cardiolipin synthesis. These results reveal alterations of the phospholipid profile in skeletal muscle of Mstn KO mice. We suggest that this could be mechanism through which lack of MSTN impacts mitochondrial function.

ACCEPTED MANUSCRIPT

## Material and Methods

### *Animals*

Male Mstn KO mice (10 wk old) used in this study have been described previously and were generously provided by L. Grobet (Faculty of Veterinary Medicine, University of Liège, Belgium) [37]. These mice harbor a constitutive deletion of the third Mstn exon leading to the deletion of the entire mature COOH-terminal region of MSTN and were therefore null for MSTN function. The mice were generated on a FVB/N-C57BL genetic background. WT (n=20) and Mstn KO mice (n=20) were produced from homozygous matings. Parental genotyping were determined by polymerase chain reaction analysis of tail DNA. Mice were fed ad libitum and kept under a 12:12-h light-dark cycle.

The experimental protocols of this study were handled in strict accordance with European directives (86/609/CEE) and approved by the Ethical Committee of Region Languedoc Roussillon (APAFIS#2551-2015110311365663v2).

### *Muscle preparation and sample*

Mice were weighed and killed by cervical elongation. Tibialis anterior, extensor digitorum longus (EDL), and quadriceps muscles were quickly excised and immediately placed in ice-cold buffer (100 mM KCl, 5 mM MgSO<sub>4</sub>, 5 mM EDTA, and 50 mM Tris·HCl, pH 7.4) for total mitochondria extraction. Gastrocnemius was removed and then frozen in liquid nitrogen. Gastrocnemius was powdered to perform analysis of protein, mRNA expression and analysis of fatty acid and enzymatic activity measures.

### *Mitochondria isolation*

Total mitochondria were fractionated by differential centrifugation as described previously [38]. Briefly, muscles were freed of connective tissues, minced, and homogenized with a Potter-Elvehjem homogenizer. Then, mitochondria were obtained from the initial pellet following a treatment with Subtilisin A (0.25 mg/g wet muscle) and subsequent centrifugations at 800 and 9,000 g. Total mitochondria were resuspended in 100 mM KCl and 10 mM MOPS, pH 7.4. Mitochondrial protein content was determined using the Bradford assay, and the yield was expressed as milligram of mitochondrial proteins per gram of muscle wet weight.

### *Lipids extraction and thin layer chromatography (TLC)*

#### *Triglyceride (TG) and free fatty acids (FFA) content in muscle lipid extracts by TLC-densitometry*

Muscle lipids were extracted using a mixture of chloroform–methanol (2:1 by volume) in the presence of butylated hydroxytoluene (50mg/l) according to [39]. The application of lipid extracts of

muscle homogenate on silica gel 60 HPTLC plates (250  $\mu\text{m}$ , 20 $\times$ 10 cm; Merck, Germany), pre-treated with 1.5% w/v boric acid in ethanol, was automatically performed on a 4-mm band width using a CAMAG ATS4 apparatus (Muttenez, Switzerland). Two developments were performed, first with pentane–chloroform–methanol (52:45:3, by volume) on 60-mm migration distance and second with pentane–diethyl ether (97:3 by volume) on 67-mm migration distance, which allowed the separation of neutral lipids. The scanning of the plates was carried out using a CAMAG TLC scanner 3 (Muttenez, Switzerland) operating in the reflectance mode. The plates were scanned at 550 nm after dipping in a solution of copper sulphate 640 mM in H<sub>3</sub>PO<sub>4</sub> 1.18 M and heating for 20 min at 180°C. The 1-monoacylglycerol (MAG), 1,2- diacylglycerol (DAG) and 1,3-DAG, TG, cholesterol (CHL) and FFA contents were finally identified by comparing their retention factor (R<sub>f</sub>) with authentic standards and were quantified using calibration curves of the same standards [40].

#### ***FFA composition of muscle by gas chromatography***

Fatty acid methyl esters were prepared by incubation with acidified methanol, according to the method of Lepage and Roy [41], after the addition of an internal standard (500  $\mu\text{g}$  C17:0/mL) to lipids in the Folch extract. Briefly, the methylation reagent was generated by mixing sulphuric acid (5% v.v) with methanol (95% v.v) and butylated hydroxytoluen as antioxidant (50 mg/L), and the sample was heated at 90°C for 45 min to get the fatty acid methyl esters. After addition of sodium bicarbonate, distilled water and hexane, the sample was vortexed, centrifuged, and the upper hexane layer was transferred to a glass vial, evaporated under a nitrogen stream at 37°C, and dissolved in isooctane for gas chromatography analysis. Individual fatty acid methyl esters were identified according to the retention times of standards by gas liquid chromatography (Trace GC Ultra, Thermo Fisher Scientific, Courtaboeuf, France), with a Supelco Vax capillary column [30mx0.32mm idx0.25 $\mu\text{m}$  df], an AS-3000 autosampler and flame-ionization detector (Thermo Electron Corporation, Thermo Fisher Scientific). The fatty acid methyl esters were quantified using the chromatographic peak area according to the internal standard method. The FA composition of total lipids and mitochondrial lipids were compared in terms of the percentage content of various fatty acids. The sum of the dimethylactals DMA16:0 and DMA18:0, the main plasmalogens were also quantified, as controls and showed limited proportion in skeletal muscles.

#### ***Tissue desaturase indices and unsaturation index***

Since it is not possible to directly measure the activity of the enzymes that catalyze the desaturation and elongation reactions in the fatty acid metabolic pathways, results in animals have supported the use of surrogate measures of desaturase activity such as the use of desaturase indices [42]. These indices are based on the ratio of product to precursor of individual fatty acids. The desaturase indices were calculated as follows:  $\Delta 9$ , [16:1(n-7)/16:0];  $\Delta 6$ , [18:3(n-6)/18:2(n-6)]; and  $\Delta 5$ , [20:4(n-6)/20:3(n-

6)]. The unsaturation index (UI) was calculated from the relative percentage of each type of mono- and polyunsaturated fatty acid multiplied for the number of double bonds present in the molecule [43].

#### ***Phospholipid composition of muscle mitochondrial membrane by TLC-densitometry***

Muscle mitochondrial suspensions were extracted by a mixture of chloroform/methanol (2:1 by volume) according to [39] in the presence of 50 mg/L of butylated hydroxytoluene. Phosphorus was quantified on Folch extracts of mitochondrial suspensions in order to determine total phospholipid quantity as previously described [44]. The application of Folch extracts of muscle mitochondrial suspensions on silica gel 60 HPTLC plates (250  $\mu\text{m}$ , 20x10 cm, Merck, Germany) pretreated with 2.3% w/v boric acid in ethanol, was automatically performed on a 4 mm band width using a CAMAG ATS4 apparatus (Muttentz, Switzerland). The development was performed with methanol/acetic acid/pentane/chloroform (15/10/30/45, by volume), which allowed the separation of phospholipids on a 60 mm total migration distance. The scanning of the plates was carried out using a CAMAG TLC scanner 3 (Muttentz, Switzerland), operating in the reflectance mode. The plates were scanned at 715 nm after dipping in a solution of Blue Spray (Sigma, France) (Blue Spray/H<sub>2</sub>SO<sub>4</sub> 4.2 M/acetone, 1/2/3 by volume,) and heating for 3 mn at 55°C. The different classes of phospholipids (SM, LPC, PC, PI, PS, PE, PG, PA, CL) were identified by comparing their retention factor (R<sub>f</sub>) to authentic standards and quantified using calibration curves of the same standards [45]. Results are expressed as percentage of nmol of phosphorus in total phospholipids, and indicate for each phospholipid its proportion (%) in the composition of mitochondrial membrane. We also estimate the mitochondria CL content expressing in  $\mu\text{mol}$  pf phosphorus in CL per mg of mitochondrial protein.

#### ***Enzyme activity assays***

Lipids oxidation was evaluated by CS and  $\beta$ -HAD activities. CS activity was measured according to Srere et al., 1969 [46] : the activity of the enzyme was measured spectrophotometrically at 412 nm by following the yellow color of 5-thio-2-nitrobenzoic acid, which is generated from the reaction of 5,5'-dithiobis-2-nitrobenzoic acid with the free -CoA liberated from the reaction of acetyl-CoA and oxaloacetate under citrate synthase action to synthesize citrate.  $\beta$ -HAD activity was measured according to [47]. The activity of the enzyme was measured spectrophotometrically at 340 nm by following the disappearance of NAD. The  $\beta$ -HAD converted the acetoacetyl-CoA in alcohol inversely  $\beta$ -oxidation reaction.

#### ***Protein extraction and Western blot analysis***

50 mg gastrocnemius muscle powder was homogenized using an Potter-Elvehjem homogenizer Craig recrystallization tube homogenizer (KONTES GLASS CO, Sigma, VINELAND NJ) in an ice cold extraction buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM  $\beta$ -glycerophosphate, 1 mM dithiothreitol, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10% glycerol, 1% Triton X-100, 10  $\mu\text{M}$  leupeptin, 5  $\mu\text{M}$  pepstatin A, 3 mM benzamidine, 10  $\mu\text{g}/\text{mL}$  aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Proteins were

denatured by mixing with an equal volume of Leamli Buffer and the linearization was achieved by heating the samples for 5 min at 95°C. Proteins (50 µg) were separated on SDS-PAGE and then transferred (Tris-glycine 1x, ethanol 100%, H<sub>2</sub>O) to a nitrocellulose membrane (nitrocellulose, 0.2 µm, Bio-RAD, CAS#9004-70-0) (90 minutes, 400mA). Membranes were blocked in (Tris-buffered saline) TBS1X/0.1% Tween-20 containing either 5% fat-free milk or 5% BSA for 1 hour at room temperature.

Then, membranes were incubated overnight with primary antibody against, **FAT/CD36**, **FATP1**, **FATP4**, **SCD1** (1/500; Santa Cruz Biotechnology, CA), **FABP3**, **CLS** (1/500; Abcam, Cambridge, UK), **FAS** (1/1000, Cell Signaling Technology 3180S, Danvers, MA), **CS** (1/1000; Genetex) and **α-tubulin** (1/5000; Sigma-Aldrich, St Louis, MO) in blocking buffer. After washes in TBS1X/0.1% Tween-20 under gentle agitation, membrane was incubated for 1 hour with horseradish peroxidase-labelled antibody (Anti mouse NA931V, GE, UK, Anti Rabbit NA934V, GE, UK, Anti-Goat, SC-2768, Santa Cruz Biotechnology, CA).

After further washes, antibody bound proteins were revealed using enhanced chemiluminescence detection reagents (ECL, ThermoScientific, Courtaboeuf, France). Signals detected with ChemiDoc™ Touch Imaging System instrument (Bio-RAD, 1.1.0.4, 732BR1121). α-tubulin was used as loading reference. Gel band intensities in the membranes were measured using the Image Lab software (Version 5.2.1 for Windows 7).

#### ***Quantification of mRNA expression by real time reverse transcription (RT-qPCR)***

Total RNAs were isolated from gastrocnemius muscle powder using the RNeasy Fibrous Tissue Mini Kit following the manufacturer's instructions (Qiagen, Venlo, The Netherlands). RNA concentration was determined by spectrophotometric analysis (Eppendorf AG, Hamburg, Germany), and purity was checked by the OD<sub>260nm</sub>/OD<sub>280nm</sub> absorption ratio (>1.7).

Reverse transcription reaction was performed with 1µg of total RNA using the TAKARA kit (TAKRR037A, PrimeScript RT reagent Kit, Perfect Real Time) according to the manufacturer's instructions. One-tenth of the cDNA was used in each polymerase chain reaction (PCR).

Quantitative PCR (qPCR) analysis was performed in a MiniOpticon detection system (Bio-Rad, Hercules, CA) with 18µL of Mastermix (SYBR Premix Ex Taq II, Takara), 300nM of both forward and reverse primers, 2µL of diluted cDNA template and water to a final volume of 20µL. The forward and reverse primers used to amplify genes are listed in **Table 1**. We used Cpt-1 and Ppar-δ genes for lipid oxidation; Pgps for cardiolipin synthesis, Taz and Lcat1 for cardiolipin remodelling.

All PCRs were performed in duplicate using the following cycle parameters: 30s at 98°C, 40 cycles of 1s at 95°C and 15s at 60°C. Relative mRNA levels were normalized to ribosomal protein ARP and α-tubulin housekeeping gene levels, which were unaffected by treatments. Results are expressed using the comparative cycle threshold (CT) method to generate ΔΔCT values. PCR amplification was

performed with template dilutions ranging from  $10^1$  to  $10^6$  copies. Overall efficiencies (E) of PCR were calculated from the slopes of the standard curves according to the equation  $E = [10^{(-1/\text{slope})}] - 1$ . All PCR efficiencies were above 95%. The relative abundance of each sample was then normalized according to the equation: Relative Quantity =  $2^{-\Delta\Delta Ct}$  [48].

Role	Gene	Reverse Primer (5'-3')	Forward Primer (5'-3')
<b>Housekeeping Gene</b>	Arp	5'-ACTGGTCTAGGACCCGAGAAG-3'	5'-TCCCACCTGTCTCCAGTCT-3'
	$\alpha$ -tubulin	5'CTGGAACCCACGGTCATC-3'	5'GTGGCCACGAGCATAGTTATT-3'
<b>Lipid oxidation</b>	Cpt1	5'CATCCCAGGCAAAGAGACA-3'	5'AAGCGACCTTTGTGGTAGACA-3'
	Ppar- $\delta$	5'GTATGCGCATGGGACTCAC-3'	5'GTCTGAGCGCAGATGGACT-3'
<b>Cardiolipin synthesis enzyme</b>	Pgps	5'CTCACGCTGATTGGCTCTC-3'	5'CAGGGGTTGGACCTTTCAT-3'
<b>Cardiolipin Remodeling enzyme</b>	Taz	5'CCAGCCCATGTTTAACTGCT-3'	5'GTCTACCAGAAAGGGATGGAC-3'
	Alcat1	5'ACACCTGCCCTCAACACAG-3'	5'CCAGCCCATGTTTAACTGCT-3'

**Table 1. Primers used for real-time quantitative PCR**

### *Statistical analysis*

All data are presented as means  $\pm$  SEM. Data were compared between the two groups of mice WT and Mstn KO using an unpaired t-test or a Mann-Whitney rank sum test when normality was not obtained. The significance level was set at 0.05. The data were analyzed using the statistical package Graphpad Prism version 6.02 for windows (GraphPad Software, La Jolla, California).

## Results

### *Animals and muscle characteristics*

Consistent with the hypermuscular phenotype, at 10 weeks of age, Mstn KO mice displayed greater body and muscle (gastrocnemius, EDL, soleus) weights compared to their WT littermates (**Table 2**).

Weight	WT	Mstn KO
<b>Body (g)</b>	25.7 ± 0.5	34.1 ± 0.3*
<b>EDL (mg)</b>	9.8 ± 0.2	18.6 ± 0.6***
<b>Gastrocnemius (mg)</b>	140.2 ± 0.2	259.9 ± 0.4***
<b>Soleus (mg)</b>	7.5 ± 0.2	14.9 ± 0.4*

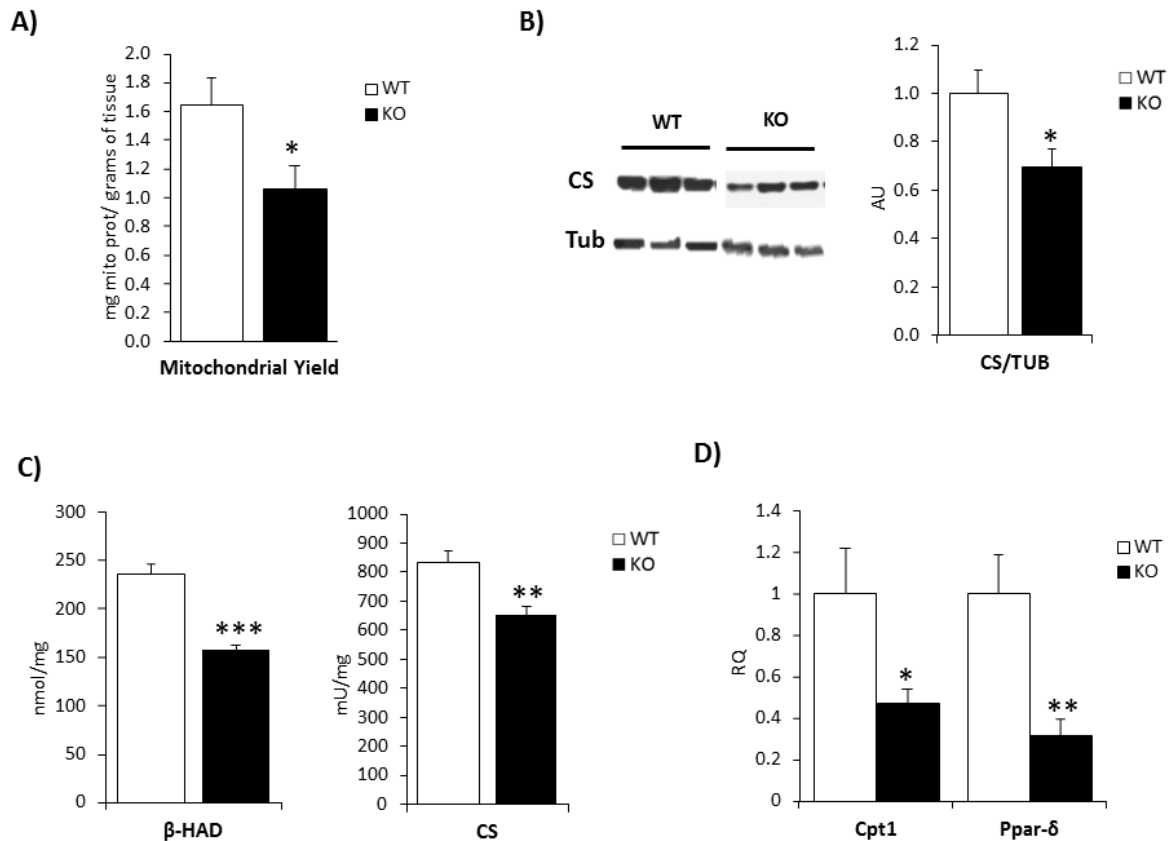
**Table 2. Body and muscle weights in WT and Mstn KO Mice .**

Data are presented as means ± SEM; n = 20 mice in each group. WT; wild type; KO, knockout; EDL. Extensor digitorum longus, \* P < 0.05 and \*\*\* P < 0.001 vs. WT.

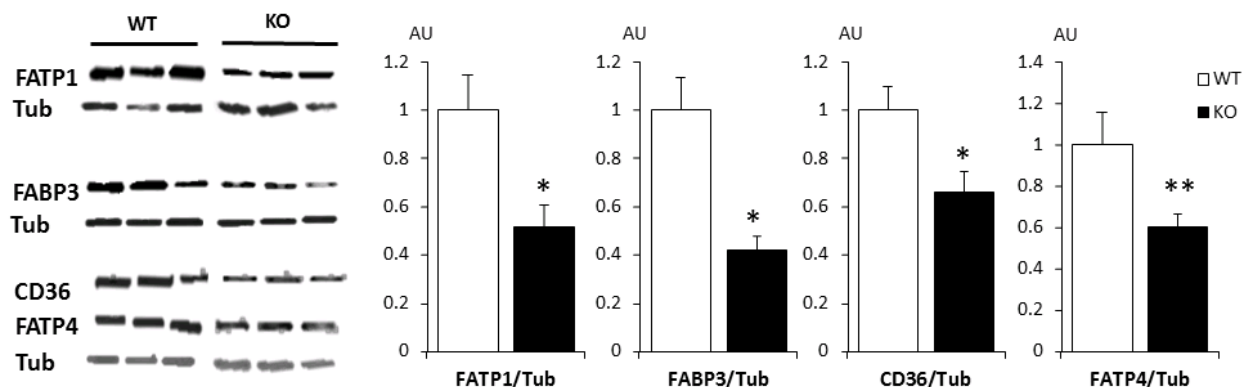
### *Decreased lipid oxidation in Mstn KO muscle*

We and others have recently shown that inhibition of Mstn represses muscle endurance and metabolic mitochondrial function [13,15]. As this metabolic phenotype is greatly dependent on lipids, we first investigated whether myostatin deletion would impact mitochondrial lipid oxidation in muscle.

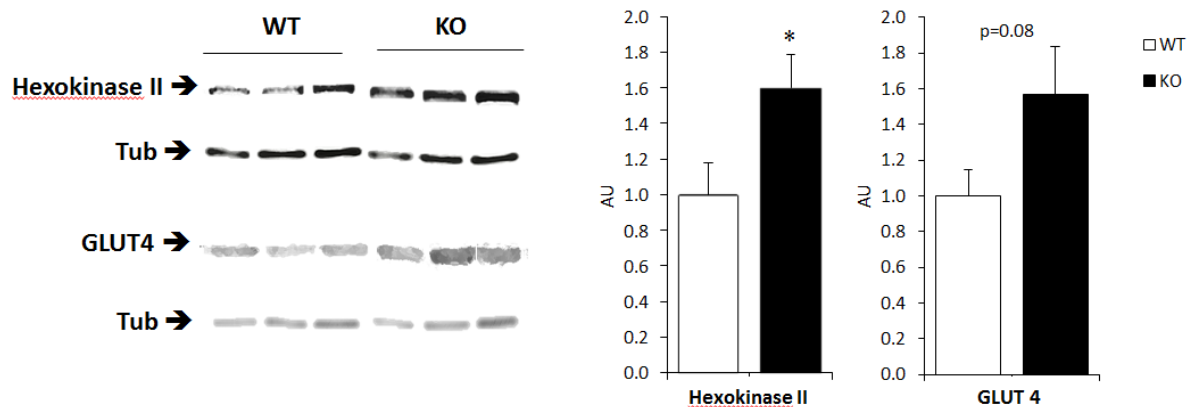
Mitochondrial yield and CS quantity were significantly reduced in mstn gastrocnemius muscles (Figure 1A and B) confirming the reduced mitochondrial pool in mstn KO mice vs WT. The activity of isolated key mitochondrial enzymes, CS and  $\beta$ -HAD, was decreased in gastrocnemius muscle homogenates from KO compared to WT littermates (**Figure 1C**). Supporting the decrease of lipid oxidation, we found reduced mRNA level of Cpt1, a membrane mitochondrial lipid transporter (**Figure 1D**). Furthermore, mRNA levels of Ppar- $\delta$ , a key transcription factor promoting oxidative metabolism is also markedly reduced in mstn deficient muscle (**Figure 1D**). Altogether, these data suggest that oxidative metabolism and lipid  $\beta$ -oxidation is down-regulated in the absence of myostatin. In agreement with these results, we observed decreased protein content of CD36, FABP3 (fatty acid binding protein 3), FATP1 and FATP4 (fatty acid transport proteins 1 and 4), all of which are involved in lipid membrane and cytosolic transport, in gastrocnemius muscle of KO mice compared to WT mice (**Figures 2A, 2B**). Inversely, we showed that protein involved in muscle glucose uptake, as hexokinase II (HKII) and glucose transporter type 4 (GLUT4) protein content were respectively significantly increased or tended to be increased in mstn KO muscle in comparison with control group (**Figure 3**).



**Figure 1: Markers of mitochondrial content and  $\beta$ -oxidation in WT and *Mstn* KO mice** **A)** Mitochondrial yield in mixed muscle homogenates from WT (open bars) and *mstn* KO mice (filled bars). **B)** Citrate synthase quantity evaluated by Western blot in *mstn* KO muscle compared to WT muscle. A representative Western blot is presented here (each lane depicts signal obtained from 50 $\mu$ g of muscle protein from one animal after western-blot analysis) and quantification of CS protein levels expressed relative to  $\alpha$ -tubulin levels in gastrocnemius muscle from *Mstn* KO mice compared to WT mice. The ratio is expressed relative to control WT (arbitrary taken as 1). **C)** Enzymatic activities evaluated by spectrophotometry of  $\beta$ -HAD and citrate synthase in gastrocnemius muscle homogenates from WT (open bars) and *mstn* KO mice (filled bars). **D/** Cpt1 and Ppar- $\delta$  gene expression evaluated by RT-PCR in WT and *Mstn* KO muscle. Values are shown as means  $\pm$  SEM. P-Values were calculated using the parametric test T-student, except for  $\beta$ -HAD and Ppar- $\delta$  (\* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001 vs WT) mU/mg: units of activity per mg of total muscular protein. AU: Arbitrary Units. RQ: Relative Quantity. (n=10 per group).



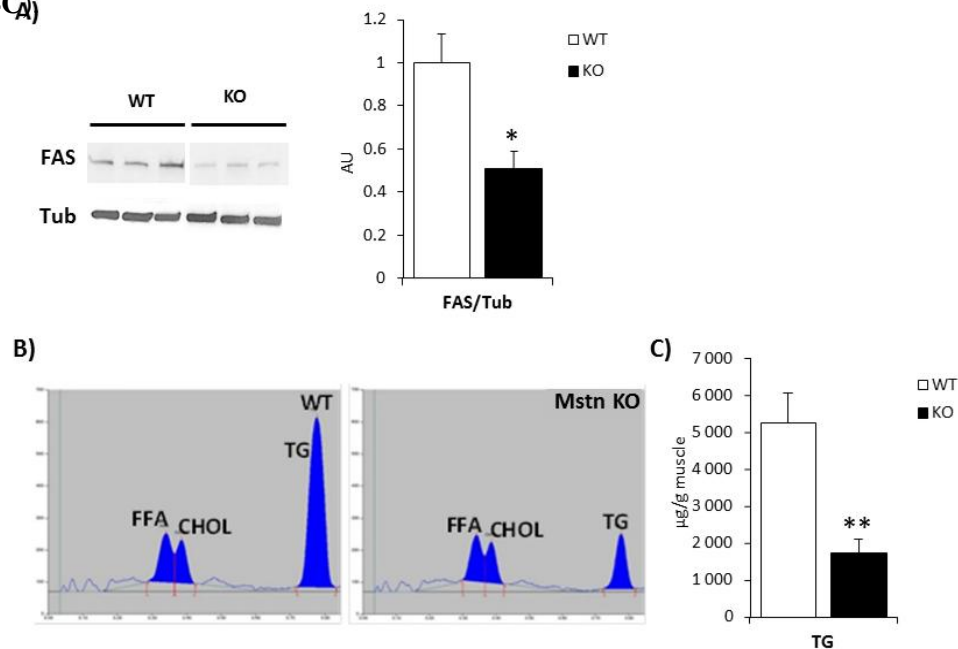
**Figure 2: Lipid transporters in WT and *Mstn* KO mice** **A)** Representative immunoblots showing protein expression levels of the lipid membrane and cytosolic transporters FATP1, FABP3, CD36 and FATP4 within the gastrocnemius of WT and *Mstn* KO mice. Each lane depicts signal obtained from 50 $\mu$ g of muscle protein from one animal after western-blot analysis **B)** Quantifications of FATP1, FABP3, CD36 and FATP4 are normalized to  $\alpha$ -tubulin. The ratio is expressed relative to control WT (arbitrary taken as 1). Values are shown as means  $\pm$  SEM. P-Values were calculated using the parametric test T-student (\* $p$ <0.05. \*\* $p$ <0.01). AU: Arbitrary Units. (n=10 per group).



**Figure 3: Glycolytic markers in WT and Mstn KO mice** A) Representative immunoblots showing protein expression levels of the Hexokinase II and glucose transporter GLUT4 within the gastrocnemius of WT and Mstn KO mice. Each lane depicts signal obtained from 50 $\mu$ g of muscle protein from one animal after western-blot analysis B) Quantifications of hexokinase II and GLUT4 are normalized to  $\alpha$ -tubulin. The ratio is expressed relative to control WT (arbitrary taken as 1). Values are shown as means  $\pm$  SEM. P-Values were calculated using the parametric test T-student (\* $p$ <0.05). AU: Arbitrary Units. (n=10 *per* group).

### Decreased lipogenesis in Mstn KO muscles

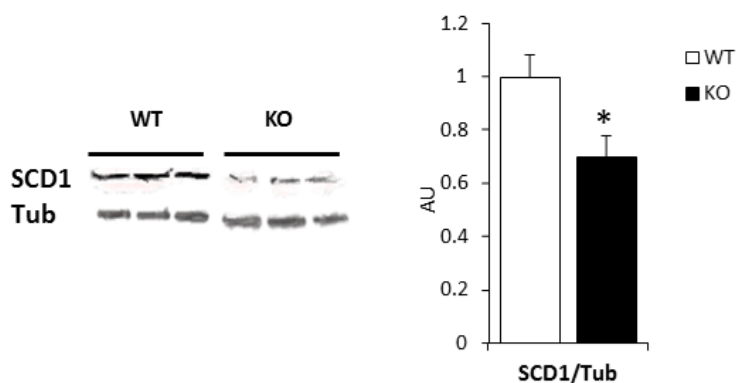
Since lipogenesis involves synthesis of fatty acids from excess carbohydrates leading to TG storage, we next tested whether MSTN deficiency altered this metabolic pathway. Western blot analysis showed that FAS levels were decreased in Mstn KO gastrocnemius compared to WT (Figure 4A). In agreement with this, we found that TG levels were 4-fold lower in Mstn KO mice compared with WT (Figures 4B, 4C)



**Figure 4: Lipogenesis markers in WT and Mstn KO mice** A) Representative immunoblots showing protein expression levels and quantification of FAS evaluated by western blot (normalized to  $\alpha$ -tubulin) in Mstn KO gastrocnemius compared with WT (n=10 *per* group) B) Representative thin layer chromatography analysis: the profile indicates the amounts of TG, CHOL and FFA in gastrocnemius C) Triglyceride quantity decreased in Mstn KO muscle compared with WT (n=7 *per* group). Values are shown as means  $\pm$  SEM. FAS, fatty acid synthase; Tub:  $\alpha$ -tubulin; FFA: free fatty acids; CHOL: cholesterol, TG: triglycerides. P-Values were calculated using the parametric test T-student (\* $p$ <0.05. \*\* $p$ <0.01) AU: Arbitrary Units.

### Modification of fatty acid composition in *Mstn* KO muscle

The structural unit of lipids is the FA, which is divided in three groups: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). **Table 3** shows distribution of 11 FAs of gastrocnemius muscle in WT and KO mice. The ratio 22:6n-3/20:4n-6 is 2 to 3, both in WT and KO mouse muscle, as previously reported in skeletal muscle tissue [49,50]. *Mstn* KO mice contained higher percent of SFAs ( $46.2 \pm 3.7\%$  vs  $40.3 \pm 2.8\%$ ,  $p < 0.01$ ), as well as PUFAs ( $38.6 \pm 1.5\%$  vs  $36.3\% \pm 1.4\%$ ,  $p < 0.01$ ) with increased proportion of 20:3n-6, 20:4n-6, 20:5n-3 and 22:5n-3. Interestingly, among PUFAs, an exception with the 18:2n-6, linoleic acid is noticed with a trend of reduced expression in *mstn* KO gastrocnemius muscle compared to controls ( $p = 0.092$ ). *Mstn* KO mice contained lower percent of MUFA ( $15.2 \pm 3.4\%$  vs  $23.4 \pm 3.7\%$ ,  $p < 0.001$ ) than WT. We further focused our attention on the fatty acid desaturases ( $\Delta$ -9-desaturase and  $\Delta$ -5-desaturase) that are involved in the synthesis of unsaturated fatty acids. Interestingly, we found a strong decrease of the index of desaturase 9 activity (16:1n-7/16:0 and 18:1n-9/18:0) (**Table 3**), which was paralleled by a strong decrease in  $\Delta$ -9-desaturase protein levels (also known as stearoyl-CoA desaturase SCD) in *Mstn* KO muscle (**Figure 5**).



**Figure 5 Stearoyl-CoA desaturase is reduced in gastrocnemius muscles of *Mstn* KO mice**

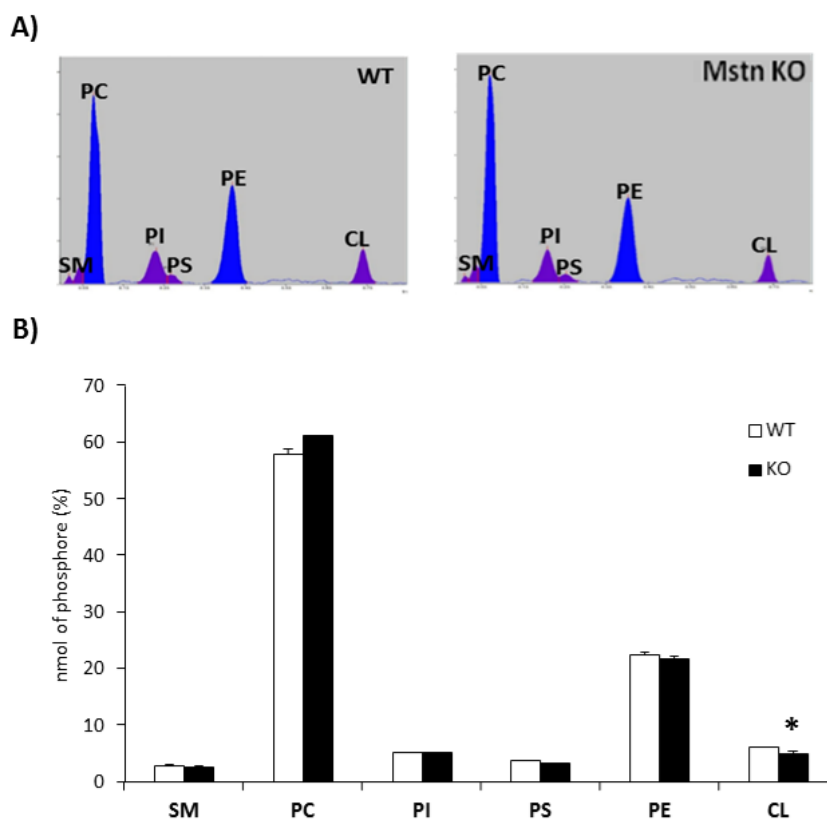
Quantification of SCD1 protein levels expressed relative to  $\alpha$ -tubulin levels in gastrocnemius muscles of WT and KO mice revealed by Western blot. Each lane depicts signal obtained from 50 $\mu$ g of muscle protein from one animal after western-blot analysis. Values are means  $\pm$  SEM. P-Values were calculated using the parametric test T-student ( $*p < 0.05$ );  $n = 8$  per group.

	Fatty acid	WT (n=8)	KO (n=7)	p values
Total SFA		40.3±2.8	46.2±3.7**	0.0043
16:0	Palmitic acid	26.8±1.3	29.5±1.7**	0.0056
18:0	Stearic acid	10.8±1.5	13.9±1.4**	0.0016
Total MUFA		23.4±3.7	15.2±3.4***	0.0008
16:1n-7	Palmitoleic acid	5.4±1.2	2.4±0.9***	0.0001
18:1n-9	Oleic acid	14.0±2.6	8.8±2.6**	0.0022
18:1n-7	Vaccenic acid	2.4±0.1	2.4±0.1	0.61
Total PUFA		36.3±1.4	38.6±1.5**	0.0078
18:2n-6	Linoleic acid	16.5±1.4	14.7±2.3	0.092
20:3n-6	Dihomo- $\gamma$ -linolenic acid	0.5±0.7	0.7±0.1***	0.0001
20:4n-6	Arachidonic acid	4.5±0.6	6.6±0.5***	<0.0001
20:5n-3	Eicosapentaenoic acid	0.1±0.0	0.3±0.1**	0.0058
22:5n-3	Clupanodonic acid	1.4±0.2	2.1±0.3***	0.0002
22:6n-3	Docosahexaenoic acid	11.1±1.7	11.8±1.6	0.4
Total (n-6)		22.6±0.7	23.5±1.8	0.23
Total (n-3)		13.7±1.8	15.1±1.8	0.17
(n-6)/(n-3)		1.7±0.2	1.6±0.2	0.53
$\Delta$ -9-désaturase				
18 :1n-9/18 :0		1.3±0.4	0.6±0.2**	0.005
c16:1n-7/16:0		0.2±0.1	0.1±0.0***	0.0002
$\Delta$ -5-desaturase		9.4±0.7	9.5±0.6	0.66
$\Sigma$ Dimethylacetals		0.6±0.1	0.8±0.1	0.07

**Table 3: Lipid fatty acids composition in gastrocnemius muscle** (mg FA/g muscle) Fatty acids distribution in WT (n=8) and Mstn KO (n=7) muscle evaluated by gas chromatography. SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids. Relative plasmalogens amount were evaluated by quantification of the relative dimethylacetals 16 and 18 amount (DMA16:0 and DMA18:0). Values are shown as means  $\pm$  SEM. P-Values were calculated using the parametric test T-student or Mann-Whitney test when the normality was not obtained (\*\*p<0.01;\*\*\*p < 0.001).

### Modification in phospholipids composition in *Mstn* KO muscle

Then, we identified and compared the lipid patterns of gastrocnemius muscle from wild type and *Mstn* KO mice between the different phospholipids quantity by thin layer chromatography. Among different phospholipids analyzed, we observed a significant decrease in CL levels in *Mstn* KO suggesting modifications in mitochondria membrane phospholipid composition (**Figure 6**).



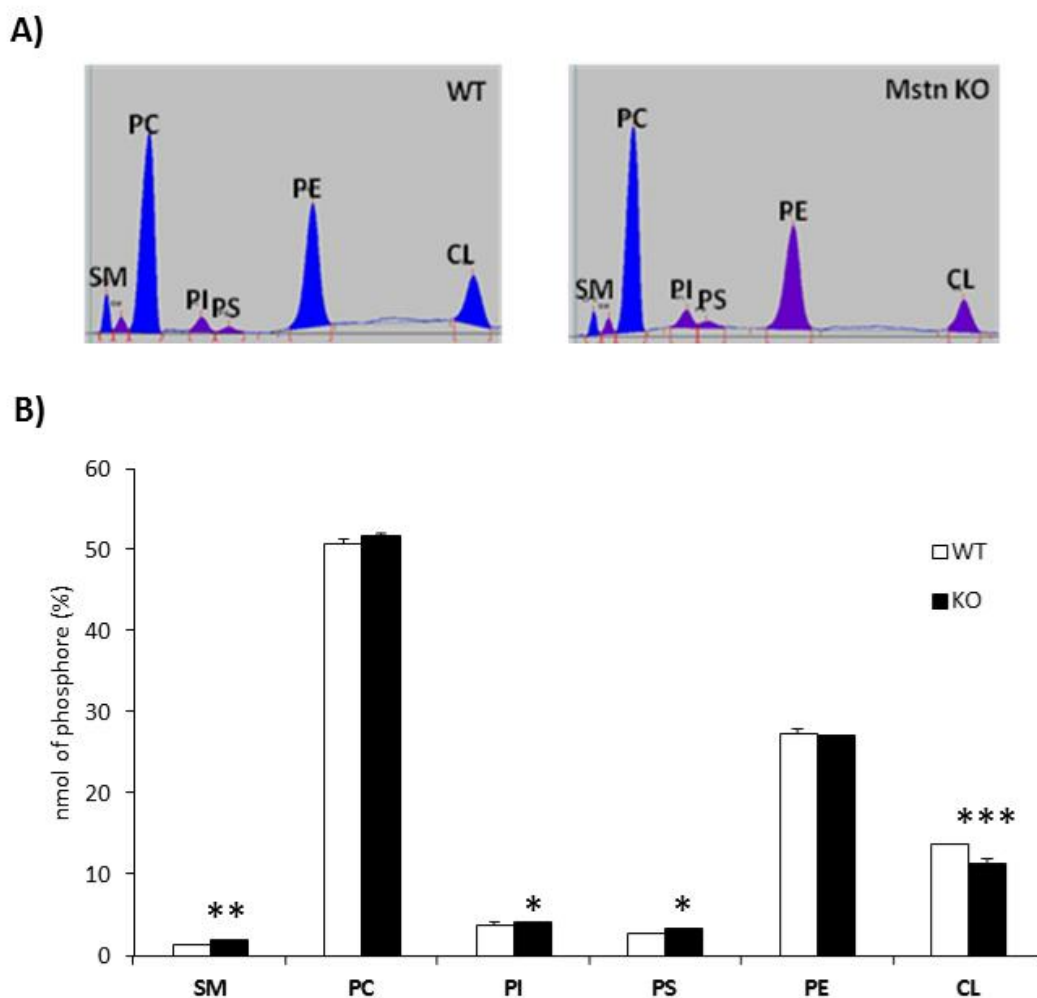
**Figure 6: Phospholipid composition of gastrocnemius muscle in WT and *Mstn* KO mice.**

**A)** Representative thin-layer chromatographic profile of the extracted lipids. Sphingomyelin (SM), Phosphatidylcholine (PC), Phosphatidylinositol (PI), Phosphatidylserine (PS), Phosphatidylethanolamine (PE) and Cardiolipin (CL) were detected with phospholipid separation. **B)** Quantification of phospholipids in gastrocnemius muscle of WT and *Mstn* KO mice. Results are expressed as percentage of nmol of phosphorus in total phospholipids. Values are shown as means  $\pm$  SEM, p-values were calculated using the parametric test T-student, except for CL; \* p < 0.05 vs WT; n = 8 per group).

### Impaired phospholipid mitochondrial membrane composition in *Mstn* KO muscle.

As *Mstn* deficiency led to a congenital shift towards fast glycolytic muscle fibers and a reduction in mitochondrial content, afterwards we identified and compared the lipid patterns of mitochondria membranes from wild type and *Mstn* KO mice using the same technique using mixed muscle samples.

**Figure 7A** shows the thin-layer chromatographic profile of the extracted lipids. The amount of SM, PI and PS was higher in *Mstn* KO mitochondria compared with WT (**Figure 7B**) whereas no change in major membrane phospholipids PC and PE was observed in the two groups of mice.

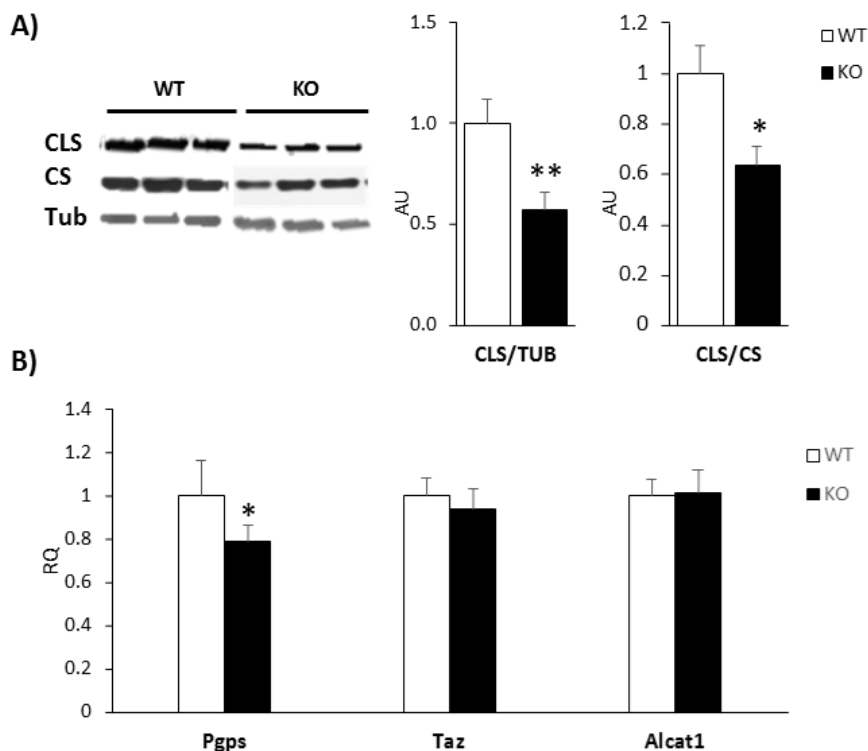


**Figure 7: Phospholipids composition of mitochondrial membrane in mixed limb muscles in WT and Mstn KO mice.** **A)** Representative thin-layer chromatographic profiles of the extracted lipids. Sphingomyelin (SM), Phosphatidylcholine (PC), Phosphatidylinositol (PI), Phosphatidylserine (PS), Phosphatidyletanolamine (PE) and cardiolipin (CL) were detected with phospholipid separation. **B)** Quantification of phospholipids in mitochondrial membranes of mixed limb muscles of WT and Mstn KO mice. Results are expressed as percentage of nmol of phosphorus in total phospholipids. Values are shown as means  $\pm$  SEM, p-values were calculated using the parametric test T-student (\* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs WT). (n=8 per group).

Interestingly, there was a reduction in CL proportion (mean 12%) in mitochondria membrane versus the proportion of others phospholipids in mstn KO mice compared to WT (Figure 7B), revealing a qualitative impact of mstn deficiency on mitochondrial membrane composition. We estimated the mitochondria CL content expressing in  $\mu\text{mol}$  of phosphorus in CL per mg of mitochondrial protein, and confirmed the reduced content (reduction of 23%) in mstn KO muscle compared with WT ( $0.07\mu\text{mol}$  vs  $0.09\mu\text{mol}$  of Phosphorus in CL/mg mitochondria protein, respectively,  $p < 0.05$ ) suggesting a quantitative impact of mstn deficiency on CL. To determine if these impacts on CL metabolism were specific to skeletal muscles, complementary control analysis on heart mitochondria was made. Contrary to skeletal muscle, results revealed similar cardiolipin proportion and content in heart mitochondria membranes in mstn KO mice compared to WT ( $12.8 \pm 1.3$  vs  $12.4 \pm 1.1$  respectively, expressed as percentage of nmol of phosphorus in total phospholipids and  $0.13 \pm 0.02$  vs  $0.09 \pm 0.01$  respectively, expressed in  $\mu\text{mol}$  of phosphorus in CL per mg of mitochondrial protein).

**Cardiolipin synthesis pathway in *Mstn* KO muscle mitochondria**

Thereafter, we examined the enzymes involved in CL synthesis (CLS: Cardiolipin synthase, Pgps: phosphatidylglycerolphosphate synthase) and CL remodeling (Alcat1: ER-associated monolyso-CL acyltransferase, Taz: tafazzin). At the protein level, the amount of CLS is lower in the *mstn* deficient muscle normalized by  $\alpha$ -tubulin or by CS abundance (**Figure 8A**). Furthermore mRNA level of Pgps was also significantly decreased in *Mstn* KO muscle (**Figure 8B**) whereas the expression of the genes involved in the remodeling of CL acyl chains (Alcat1, Taz) did not change.



**Figure 8: Expression of enzymes of cardiolipin synthetic route.** **A)** Endogenous CLS, CS and  $\alpha$ -tubulin (as a loading control) were detected by western blot analysis using specific antibodies. CLS quantity was evaluated by Western Blot in KO gastrocnemius muscle and WT standardised either with  $\alpha$ -tubulin or CS in mitochondria. Each lane depicts signal obtained from 50 $\mu$ g of muscle protein from one animal after western-blot analysis **B)** Gene expression of Pgps, Taz and Alcat1 enzymes evaluated by RT-PCR in KO and WT muscle. Values are shown as means  $\pm$  SEM. p-values were calculated using the parametric test T-student (\*p<0.05; \*\*p<0.01). AU: Arbitrary Units, RQ: Relative Quantity. CLS: cardiolipin synthase, CS: citrate synthase, Pgps: phosphatidylglycerolphosphate synthase, Taz: tafazzin, Alcat1: ER-associated monolyso-CL acyltransferase (n=8 per group).

## Discussion

MSTN is a potent inhibitor of skeletal muscle mass. Mice lacking MSTN exhibit a strong hypermuscular phenotype and a faster and more glycolytic muscle phenotype. Previous work in our laboratory revealed in *Mstn* KO mice significant functional abnormalities of inter-myofibrillar mitochondria, that are primarily involved in the process of muscle contraction [13]. These abnormalities are characterized by a decrease in the efficiency of mitochondrial respiratory coupling, resulting in excess oxygen consumption for the same production of ATP. Such mitochondrial oxidative and metabolic alterations have also been reported in mice with partial inhibition of MSTN using anti-MSTN antibody [15,51,52]. However it is still unknown how *Mstn* inhibition would influence the mitochondrial function. We explored the hypothesis that MSTN can impact the regulation of lipid synthesis and mitochondrial lipid content, thus affecting mitochondrial function.

In this study, we demonstrated for the first time *Mstn* KO mice showed alterations in lipid composition of mitochondrial membranes. In fact, we demonstrated in *Mstn* KO muscle a full decline in lipid pathways such as fatty acid uptake, fatty acid oxidation and fatty acid synthesis (lipogenesis) coupled to a shift in fatty acids composition illustrated by an increase in SFA and PUFA levels at the expense of MUFA levels. Interestingly, we observed a significant decrease in the proportion of CL in *Mstn* KO mitochondrial membranes. These findings provide evidence that at least part of the mechanism by which MSTN regulates mitochondria function is by modulating the lipid metabolism and mitochondrial membrane phospholipid composition.

### Myostatin and muscle lipid metabolism

Here, we showed that *Mstn* gene deletion results in reduced lipolytic machinery in *Mstn* deficient hypertrophic muscles (impaired mitochondrial yield, CS and  $\beta$ -HAD activities and *Cpt1* and *Ppar- $\delta$*  gene expressions), in line with previous studies showing that oxidative metabolism is diminished in several models of *Mstn* deficiency [12,13, 15,16,51]. In addition, we demonstrated that the levels of cytosolic (FABP3) and sarcolemmal lipid transporters (FATP1, FATP4) are reduced, in agreement with our previous observation showing a significant reduction of FAT/CD36 levels [16]. It should be noted that CD36, FABP3, FATP1 and FATP4 are co-expressed in skeletal muscle, and all of these proteins contributed to transport, with recent evidence suggesting that CD36 and FATP4 are qualitatively the most important [53]. Our results indicated that the process regulating fatty acids uptake into muscle by protein-mediated transport is negatively and strongly affected by MSTN deficiency. Interestingly, by evaluating lipogenesis in *Mstn* KO muscle, we found a marked decrease in triglycerides levels (70%) compared to WT muscles, correlating with the decline of FAS activity recorded. Collectively, our data pointed a role for MSTN in the regulation of muscle lipid metabolism, with a lower capacity to store, mobilize and oxidize lipids. This deficit in lipid pathway observed in our present study is counterbalanced by the increased glucose oxidation previously shown in MSTN deficient muscle by the group of McPherron. Indeed, *Mstn* KO mice have increased glucose utilization

and insulin sensitivity thanks to an increase in glucose uptake in muscle and adipose tissues [54]. In the same vein, Relizani et al. reported that the blockade of *mstn* decreased mRNA levels of Pdk4, a regulatory protein switch of substrate utilization from glucose towards fatty acids [15]. In a previous study, we observed in aged *Mstn* KO EDL muscle a significant increase in GLUT4 protein levels, and a decrease in FAT/CD36 protein levels compared to aged WT mice [16]. In the present study, we confirm the increased content of muscle glucose uptake markers in *mstn* KO muscle. Given that FA and glucose are the main energy sources in skeletal muscle, these results suggest that MSTN could contribute to the fine regulation for the use of these two substrates. Our results demonstrate that *mstn* deficient muscle malfunctions for fat handling that could explain metabolic shift towards glycolytic pathway.

### Myostatin and membrane phospholipids composition

Phospholipids affect function of protein machineries involved in respiratory metabolism, protein import, membrane architecture and mitochondrial dynamics [24]. At this point, we hypothesized that muscle lipid metabolism alterations related to MSTN deficiency can impact muscle lipid composition. We observed changes in FA composition, and components of phospholipids, in deficient *Mstn* muscles, concomitant with an increase in SFAs at the expense of MUFAs. This was accompanied by a down-regulation of SCD1. Of note, the promoter of *Scd1* gene contains a Ppar response element sequence [55]; therefore the down-regulation of Ppar $\delta$  that we have seen in absence of *mstn* could result in decreased gene transcription of *Scd1*. As *Scd1* converts SFA to MUFA, the down-regulation of this enzyme supports a shift away MUFA towards SFA. Biosynthesis PUFAs of the n-6 and n-3 series occurs via sequential desaturation, elongation and partial degradation step [56]. In our work, the impact of *mstn* deficiency on increased  $\Delta$ -5 and  $\Delta$ -6 desaturase activity is unlikely, as we noticed no significant change in  $\Delta$ -5 desaturase activity between mice groups. Moreover, 22:6n-3 proportion (docosahexaenoic acid), reflecting indirectly  $\Delta$ -6 desaturase activity, was not modified. The hypothesis of increased elongase and/or  $\Delta$ -8 desaturase activities in *mstn* KO muscle cannot be excluded. Finally, as *mstn* KO muscle exhibited higher significant proportion of 22:5n-3, but not of 22:6n-3, the hypothesis of an inhibited peroxysomal  $\beta$ -oxidation can be also advanced [56].

The lipid tails of the phospholipids composing the plasma membranes can affect mechanical properties. Membrane fluidity is promoted by lipids with short, unsaturated fatty acids [57]. In DMD differentiated muscle cells, Le Borgne et al., identified an increase in PUFA and a decrease in MUFA as well as cholesterol contents. That changes affected mechanical properties, as reduced membrane fluidity was observed by fluorescence anisotropy [58]. In line with our results and these previous studies, we hypothesize a trend towards stiff and rigid membranes in *Mstn* KO skeletal muscles. The functional and clinical relevance of these modifications on muscle membrane fluidity has yet to be investigated in *mstn* KO model.

We next focused on mitochondrial membranes, where localized proteins and complexes of the respiratory chain work together to produce ATP. In *mstn* KO mitochondrial membranes, we found abnormalities in the proportion of specific lipids, including a decrease (about 12%) in the proportion of CL while SM, PI and PS are slightly but significantly increased. This result revealed a qualitative impact of *mstn* deficiency on mitochondria membrane composition, with reduced proportion of mitochondrial CL levels, an important regulator of mitochondrial metabolism. Furthermore, mitochondrial CL content reduced of 23% in *mstn* KO mice suggesting also quantitative impact of *mstn* deficiency on CL metabolism. This reduction approximates values published in previous studies related to cardiolipin and diseases, in particular those from group of Paradies et al indicating that low levels of cardiolipin in heart mitochondria (approximating 30-40% reduction) from aged or hypothyroid rats were accompanied by lower activities of the pyruvate carrier and cytochrome oxidase and dysfunction [59–63]. Our results suggest both qualitative (reduction of 12% in the proportion) and quantitative (reduction of 23% in quantity) impacts of *mstn* deficiency on mitochondrial cardiolipin pathway.

If it is difficult to distinguish between a causal role of cardiolipin and a correlative effect, in one study, replenishment with cardiolipin of hypothyroid mitochondria restored the activity of cytochrome oxidase to control levels [60]. Synthesized in the inner mitochondrial membrane, nascent CL undergoes a remodelling process to form the mature CL [24]. Our results on enzymes involved in CL formation indicated that *MSTN* deficiency likely negatively affected synthesis, rather than remodeling process, that induced the lower abundance of CL. It should be added that we showed in *mstn* KO gastrocnemius a tendency towards the reduction of linoleic acid (18:2n-6), the most abundant fatty acid in CL [64]. As a close interplay between CL biosynthesis and oxidative phosphorylation is recognized [24], our results raise the question of the link with the compromised bioenergetic capacity observed in this hypertrophic muscle. We recognize that direct evidence is still missing, and should be discussed with caution. However, our results suggest a specific effect on skeletal muscle. Indeed, no impact of *mstn* deficiency has been observed on mitochondrial CL proportion /content in heart, an organ without functional impairment in this mice model. These results strengthen and highlight correlation between cardiolipin pathway and mitochondrial impairment, as only skeletal muscle from *mstn* KO mice presents both qualitative and quantitative impacts on mitochondrial membrane cardiolipin pathway with oxidative mitochondrial and functional disorders. CL plays an essential role in maintaining an adequate membrane potential [65], but arguably, the most important function of CL in mitochondria is the effect it has on the association of protein complexes and supercomplexes [25]. Actually, this specific phospholipid of the mitochondria plays an important role in respiratory chain (biogenesis, stability and activity of the respiratory complexes), in the  $\beta$ -oxidation of fatty acids and is involved in the last step of energy conversion, namely the production of ATP [24–26]. Crystallographic and nuclear magnetic resonance studies showed the presence of CL, in

particular the species rich in 18:2n-6, associated with complex I, III and IV, with the ATPsynthase and the ANT [66,25] and its presence is essential for the functionality structure of these proteins, and thus for their optimal activity ([25] For Review). For instance, the full transport activity of the ADP/ATP carrier depends on the presence of CL [67]. Inversely, the oligomerization state of ADP/ATP carrier in mitochondria, and its association with other protein complexes are altered in the absence of CL [68]. Interestingly, our previous data highlighted metabolic perturbations exclusively in the respiration of intermyofibrillar (IMF) mitochondria from *Mstn*-deficient muscle [13]. In diabetes mellitus type 1, cardiolipin content is decreased in the IMF subpopulation, but not in the in the sub-sarcolemmal subpopulation [69,70]. The decrease in CL mitochondria content, demonstrated in our study, could thus lead to the uncoupling in the mitochondria respiration (decay in the respiratory ratio control in the IMF mitochondria) and the decrease in ATP production previously reported in *mstn* KO muscle [13,71]. Consequently, we propose that the significant decrease in CL in *Mstn* KO mice, pointing to a defect of IMF, as one causative mechanism for the metabolic phenotype.

Our results reveal alterations in mitochondrial membrane lipids related to MSTN deficiency. How can we link membrane lipid changes to MSTN? We showed that MSTN deficiency down-regulates expression of PPAR $\delta$  transcription factor, which regulates fatty acid transport and oxidation in skeletal muscle [72–75]. Indeed, genes involved in energy metabolism including *Fatp*, *Pdk4* are induced by a PPAR $\delta$  specific agonist. In addition, Wang et al. showed that the PPAR $\delta$ -mediated reprogramming of muscle fiber involves the increased expression of genes related to fatty acid oxidation, mitochondrial respiration, oxidative metabolism, and slow-twitch contractile apparatus [73]. This suggests that MSTN might regulate membrane lipid changes via PPAR activity. Since *Mstn* KO mice have fiber hypertrophy in muscles composed predominantly of fast glycolytic fibers [12,[76], it is possible that the decrease in oxidative metabolism and  $\beta$ -oxidation is an indirect effect and related to the fast-twitch phenotype. In fact, PPAR $\delta$  is preferentially found in oxidative rather than glycolytic myofibers [73]. Nevertheless, reduction of oxidative metabolism has been demonstrated using post-natal *Mstn* inhibition, where fiber type composition was not affected [15,69,77]. Furthermore, no difference has been noted in the percentage of CL in mitochondria from muscles with varying oxidative potentials, as well as in the percent of phospholipid head groups or major fatty acid subclasses [70]. However, rates of palmitate oxidation were positively correlated with both the unsaturation index and relative abundance of cardiolipin within mitochondria [69]. Overall, this suggests that the glycolytic shift cannot be the only mechanism to explain oxidative metabolism decline and membrane lipid changes in *Mstn* KO mice.

## Conclusion and perspectives

To conclude, the results of the present study show that MSTN absence significantly alters the mitochondrial lipidome. This abnormality may be related to the impairment in mitochondrial function and the higher susceptibility to fatigue in this hypertrophic model, as lipids and in particular cardiolipin play central roles in optimal mitochondrial function. Future studies that modulate the membrane phospholipid composition in mitochondria and muscle in *Mstn* deficient models will be required for rigorous examination of how the abundance of each class of phospholipids becomes optimized, and in turn affects mitochondrial respiratory function and oxidative metabolism. In this context, nutritional regimes or endurance training are two potential strategies to induce a remodelling of mitochondrial membrane phospholipid composition [78,79], and thus should be studied in the future to elucidate the link between membrane lipid changes and mstn signalling pathway.

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## Ethical statements

The manuscript does not contain clinical studies or patient data. The experimental protocols of this study were handled in strict accordance with European directives on animal right (86/609/CEE) and approved by the Ethical Committee of Region Languedoc Roussillon (APAFIS#2551-2015110311365663v2).

Furthermore, the authors declare that they have no conflict of interest.

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