Combined MRI and $^{31}$P-MRS Investigations of the ACTA1(H40Y) Mouse Model of Nemaline Myopathy Show Impaired Muscle Function and Altered Energy Metabolism

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Abstract

Nemaline myopathy (NM) is the most common disease entity among non-dystrophic skeletal muscle congenital diseases. Mutations in the skeletal muscle $\alpha$-actin gene (ACTA1) account for $\sim$25% of all NM cases and are the most frequent cause of severe forms of NM. So far, the mechanisms underlying muscle weakness in NM patients remain unclear. Additionally, recent Magnetic Resonance Imaging (MRI) studies reported a progressive fatty infiltration of skeletal muscle with a specific muscle involvement in patients with ACTA1 mutations. We investigated strictly noninvasively the gastrocnemius muscle function of a mouse model carrying a mutation in the ACTA1 gene (H40Y). Skeletal muscle anatomy (hindlimb muscles and fat volumes) and energy metabolism were studied using MRI and $^{31}$Phosphorus magnetic resonance spectroscopy. Skeletal muscle contractile performance was investigated while applying a force-frequency protocol (from 1–150 Hz) and a fatigue protocol (80 stimuli at 40 Hz). H40Y mice showed a reduction of both absolute ($\sim$40%) and specific ($\sim$25%) maximal force production as compared to controls. Interestingly, muscle weakness was associated with an improved resistance to fatigue (+40%) and an increased energy cost. On the contrary, the force frequency relationship was not modified in H40Y mice and the extent of fatty infiltration was minor and not different from the WT group. We concluded that the H40Y mouse model does not reproduce human MRI findings but shows a severe muscle weakness which might be related to an alteration of intrinsic muscular properties. The increased energy cost in H40Y mice might be related to either an impaired mitochondrial function or an alteration at the cross-bridges level. Overall, we provided a unique set of anatomic, metabolic and functional biomarkers that might be relevant for monitoring the progression of NM disease but also for assessing the efficacy of potential therapeutic interventions at a preclinical level.

Introduction

Although considered a rare disease, nemaline myopathy (NM) is the most common of the non-dystrophic congenital myopathies and is characterized by muscle weakness and the presence of rod shaped structures in the muscle fibers [1]. NM has been divided into six different subtypes based, amongst several features, on the severity of the disease and the age of onset [2,3]. Mutations in seven genes have been identified so far as causing NM: actin alpha 1 (ACTA1), alpha-tropomyosin-3 and beta-tropomyosin (TPM3 and TPM2), nebulin (NEB), troponin T type 1 (TNNT1), cofilin-2 (CFL2), and kelch repeat and BTB (POZ) domain containing 13 (KBTBD13) genes. The majority of these genes encode proteins associated with the thin filament of the sarcomere so that NM is considered as a thin filament myopathy [4,5].

NM related to ACTA1 mutations represents 15% to 25% of NM cases and up to 50% of the most severely affected patients [6,7]. The transmission of ACTA1 mutations may be either dominant or recessive and most of the patients with actin mutations have a sporadic disease [8,9]. The clinical phenotype in NM associated with ACTA1 mutation is often severe resulting in early death from respiratory failure within the first year of life [10]. However, mild and even adult-onset disease has been observed in a few patients [11,12].

So far, the mechanisms underlying muscle weakness in NM are poorly understood mainly because of the limitations linked to the analyses of human biopsy samples [13]. Typically, they are of limited size, only provide a snapshot of the muscle status at a particular time and location so that the findings are limited to a specific region and cannot be easily extrapolated to the whole muscle inasmuch as different muscles have distinct contractile and enzymatic characteristics. Additionally, iterative muscle biopsies are painful and not ethically acceptable especially in young...
atrophy in patients with
Resonance Imaging (MRI) study showing a marked muscle
H40Y mice, a finding consistent with a recent Magnetic
both muscle weight and myofiber diameter has been reported in
soleus and EDL muscles and the marked decrease in forearm grip
specific force production
reduction in H40Y mice was larger than could be expected from
Asp286Gly mutation showed a significant rightward shift of both
sensitivity in NM patients [21,22]. Similarly, mice carrying the
muscular properties [20]. Recent investigations reported an
the loss of muscle mass is secondary to alteration of intrinsic
in vivo
phosphorylated compounds
expected to provide relevant information about potential meta-
bolic alterations in H40Y muscles.
In the present study, we aimed at characterizing strictly noninvasively the functional, anatomical and metabolic conse-
quences of the H40Y mutation in a mouse model. Both muscles
and fat volumes were quantified using MRI in order to investigate
whether this mouse model displays similar MRI findings than NM
patients. Additionally, skeletal muscle contractile performance was
investigated throughout a ramp frequency protocol in order to
determine the underlying mechanisms responsible for the muscle
weakness in H40Y muscles. Finally, 31P-MRS investigations were
performed throughout a standardized fatigue protocol in order to
detect potential energy defects in NM mouse muscles.

Materials and Methods

Animals
Fourteen-week old ACTA1 knock-in females (H40Y) and wild-
type female littermates (WT) were used for the experiments (n = 9
for each group) conducted in agreement with the French
guidelines for animal care and in conformity with the European
convention for the protection of vertebrate animals used for
experimental purposes and institutional guidelines n˚ 86/609/ CEE
November 24, 1986. All animal experiments were approved
by the Institutional Animal Care Committee of Aix-Marseille
University (permit number: #15-1402012). Experiments were
only performed on females given that the majority of males
typically die within the first 6–8 weeks after birth [16]. Mice were
housed in an environment-controlled facility (12–12 hour light-
dark cycle, 22°C), received water and standard food ad libitum. An
ophthalmic ointment (Rifamycine Chibret, Clermont-Ferrand,
France) was applied on the eyes of the H40Y mice in order to
avoid infections caused by ptosis. Mice were identified through
PCR genotyping from mouse tail DNA as previously described
[16,30].

In vivo Experiments

Animal preparation. Mice were initially anesthetized in an
induction chamber using 4% isoflurane in 33% O2 (0.5 l/min) and
66% NO2 (1 l/min). The left hindlimb was shaved before an
electrode cream was applied at the knee and heel regions to
optimize electrical stimulation. Each anaesthetized mouse was
placed supine in a home-built cradle which has been specially
designed for the strictly non-invasive functional investigation of the
left hindlimb muscles [31]. Throughout a typical experiment,
anesthesia was maintained by gas inhalation through a facemask
continuously supplied with 1.75% isoflurane in 33% O2 (0.2 l/
min) and 66% N2O (0.4 l/min). Exhaled and excess gases were
removed through a canister filled with activated charcoal (Smiths
Industries Medical System, Sheffield, UK) mounted on an
electrical pump extractor (Equipement Vétérinaire Minerve,
Esterney, France). Physiological temperature was adjusted with an
electrical heating blanket. The foot was positioned on the pedal of
the ergometer with a 90° flexion ankle joint. The hindlimb was
centered inside a 20 mm-diameter 1H Helmholz imaging coil and
the belly of the gastrocnemius muscle was located above an
elliptical (8x12 mm) 31P-MRS surface coil. Muscle contractions
were achieved by transcutaneous electrical stimulation using two
rod-shaped 1.5 mm-diameter surface electrodes integrated in the
cradle and connected to an electrical stimulator (type 215/T;
Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-
Hugstetten, Germany). One electrode was placed at the heel level
and the other one was located just above the knee joint. The
gastrocnemius muscle was chosen because it is easily accessible for

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31P-MRS measurements and preferentially activated by our in vivo experimental set-up [31].

**Study design.** Mice were tested twice over a one-week period in order to assess mechanical performance, muscles and fat volumes using MRI and metabolic changes during a fatigue protocol.

During the first testing session, transcutaneous stimulation was first elicited with square-wave pulses (0.5 ms duration) on the gastrocnemius muscle. The individual maximal stimulation intensity was determined by progressively increasing the stimulus intensity until there was no further peak twitch force increase. This intensity was then maintained to elicit tetanic stimulations (duration = 0.75 sec; rest interval = 30 sec) at various incremental frequencies (from 1 to 150 Hz).

During the second testing session, MRI measurements were performed at rest to get information about anatomy (i.e., muscles and fat volumes). Additionally, metabolic changes were investigated using 31P-MRS during a fatigue protocol consisting of 80 contractions (frequency = 40 Hz; pulse train duration = 1.73 sec; rest interval = 6.92 sec).

**Force output measurements.** The analog electrical signal from the force transducer was amplified with a home-built amplifier (Operational amplifier AD620; Analog Devices, Norwood, MA, USA; gain = 70 dB; bandwidth = 0–5 kHz) and converted to a digital signal (PCI-6220; National Instruments, Austin, TX, USA) monitored and recorded on a personal computer using the WinATS software (Sysma, Aix-en-Provence, France).

**MR experiments.** Investigations were performed in a 4.7-Tesla horizontal superconducting magnet (47/30 Biospec Avance, Bruker, Ettlingen, Germany) equipped with a Bruker 120-mm BGA12SL (200 mT/m) gradient insert.

**MR imaging.** Ten contiguous axial slices (thickness = 1.1 mm; spaced = 0.1 mm), covering the region from the knee to the ankle, were acquired at rest using a spin echo sequence (TE = 18.2 ms; TR = 1000 ms; two accumulations; field of view = 50 x 30 mm; matrix size = 256 x 256; acquisition time = 8 - min 32 sec).

**31P-MRS measurements.** Spectra (8-kHz sweep width; 2048 data points) from the gastrocnemius region were continuously acquired at rest and throughout the fatigue protocol. A fully relaxed spectrum (8 accumulations, TR = 30 sec) was acquired at rest followed by a total of 384 free induction decays (FID) (TR = 1.73 sec). The first 64 FIDs were acquired at rest and summed together. The next 320 FIDs were acquired during the stimulation period and were summed by blocks of 64, providing a temporal resolution of ~110 sec.

**Data Processing**

Mechanical performance. For each stimulation train, isotropic peak force was calculated and the corresponding data were fitted to the Hill equation providing $F_0$ (frequency giving 50% of the maximal force). Regarding the fatigue protocol, the peak force of each contraction was measured and the corresponding tetanic force was averaged every 5 contractions. Additionally, the force time integral (FTI; mN.sec/mm²) of each contraction was calculated and then summed together. A fatigue index corresponding to the ratio between the last five and the first five contractions was determined.

For all stimulation protocols, force was divided by the corresponding hindlimb muscles volume (see below) in order to obtain specific force (in mN/mm²).

MRI data. The hindlimb muscles, intermuscular and subcutaneous fat volumes (in mm³) were calculated as the sum of the five cross-sectional areas of the six consecutive largest slices using an automatic method adapted from Positano et al. [32]. Briefly, different groups of pixels were separated according to their respective signal intensities and on that basis the volumes of muscle tissue, intermuscular adipose tissue (IMAT), subcutaneous adipose tissue (SAT) and bone/vessels/connective tissues were quantified (Fig. 1). Fatty infiltration was quantified from the ratio between IMAT and muscles volumes.

**31P-MRS data.** Data were processed using a proprietary software developed using IDL (Interactive Data Language, Research System, Inc., Boulder, CO, USA) [33]. Relative concentrations of phosphocreatine (PCr), inorganic phosphate (Pi) and ATP were obtained with a 110 sec time-resolution by a time-domain fitting routine using the AMARES-MRUI Fortran code and appropriate prior knowledge of the ATP multiplets. PCr to Pi-ATP ratios were calculated from the peak areas of the spectrum acquired at rest. Intracellular pH (pHi) was calculated from the chemical shift of the Pi signal relative to PCr [34].

**Statistical Analyses**

Statistical analyses were performed with the Statistica software version 9 (StatSoft, Tulsa, OK, USA). Normality was checked using a Kolmogorov-Smirnov test. Two-factor (group x contraction number or stimulation frequency) analysis of variance (ANOVAs) with repeated measures on contraction number or stimulation frequency were used to compare force production. Two-factor (group x time) ANOVAs with repeated measures on time were used to compare PCr consumption, Pi production and pH. When a main effect or a significant interaction was found, Newman-Keuls post-hoc analysis was used. Unpaired t-tests were used for other comparisons. Data are presented as mean ± standard error of mean (SEM). Significance was accepted when $P<0.05$.

**Results**

**Body Weight, Muscles and Fat Volumes**

H40Y mice had a significantly reduced body weight ($-16\%$, $P<0.05$) and a reduced volume of hindlimb muscles ($-20\%$, $P<0.05$) as compared to WT mice. Subcutaneous fat volume was significantly lower in H40Y mice as compared to controls whereas intermuscular fat volume was not significantly different between the two groups. Interestingly, the extent of fatty infiltration, assessed by the IMAT-to-muscle ratio, was similar between the two groups (Table 1 & Fig. 1).

**Mechanical Performance**

As illustrated in figure 2A, a 40% reduction ($P<0.05$) in absolute maximal tetanic force was quantified in H40Y mice as compared to WT mice. Interestingly, the specific tetanic force was significantly lower ($P<0.05$) in H40Y mice as compared to WT mice at 30, 50, 70, 100 and 150 Hz (Fig. 2B). A relative force-frequency curve was constructed using force values expressed as a percentage of the maximally generated force at 150 Hz. As illustrated in figure 2G, these curves were similar between the two groups and the corresponding $F_0$ values were identical (Fig. 2G).

During the fatigue protocol, force production was significantly lower ($P<0.05$) in H40Y mice as compared to WT mice from the first to the 15th contraction (Fig. 3A). FTI during the whole protocol was significantly lower ($P<0.05$) for H40Y (1.25 ± 0.01 mN.sec/mm²) as compared to controls (1.40 ± 0.03 mN.sec/mm²). On the contrary, the fatigue index was significantly higher ($P<0.05$) in H40Y mice compared to WT mice.
mice (Fig. 3B), thereby suggesting an improved resistance to fatigue in the H40Y.

Metabolic Changes

\([\text{PCr}] / [\text{ATP}]\) resting ratios were similar between H40Y (4.2 ± 0.5; Fig. 4A) and WT groups (4.0 ± 0.3; Fig. 4B). For both groups, [PCr] fell rapidly throughout the fatigue protocol and reached a steady state at the end of the stimulation bout. No significant difference was observed between the two groups throughout the stimulation period (Fig. 5A). As expected, the \([\text{P}_i]\) time-course evolved as a mirror of the [PCr] time-dependent changes. For both groups, \([\text{P}_i]\) increased during the fatigue protocol and reached a plateau after 3 min of exercise (Fig. 5B). At rest, \(p\text{Hi}\) was not significantly different for WT (7.13 ± 0.04) and H40Y groups (7.15 ± 0.04). \(p\text{Hi}\) decreased throughout the stimulation session so that the acidosis extent was similar for the two groups at the end of the fatigue protocol (Fig. 5C). [ATP] slightly decreased during the stimulation protocol but reached similar values for the two groups at the end of exercise (90.9 ± 15.7% vs. 85.4 ± 9.1% of resting value for H40Y and WT groups, respectively). Taken together, the fatigue protocol-induced metabolic changes were comparable between the two groups despite lower FTI values in H40Y mice as compared to WT mice, thereby suggesting an increased energy cost in H40Y.

Discussion

Considering the rarity of NM and the well-known limitations linked to the analysis of biopsy samples, we originally aimed at investigating the functional, anatomical and metabolic conse-
quences of the H40Y mutation in a recently generated NM mouse model on the basis of a strictly noninvasive approach. We observed a large decrease in maximal force production in H40Y mice which may be partially related to muscle atrophy. Interestingly, muscle weakness was associated with an improved resistance to fatigue and an increased energy cost. On the contrary, the force frequency relation was not modified in H40Y mice and the extent of fatty infiltration was minor and not different from the WT group.

In the present study, we performed MRI investigations in H40Y mice in order to assess whether this mouse model replicates the MRI findings in NM patients. Using a methodological approach adapted from Positano et al. [32], fatty infiltration was originally quantified from MR images of hindlimb muscles of both H40Y and control mice. In contrast to NM patients carrying ACTA1 mutations in whom a progressive replacement of skeletal muscle by fatty tissue has been recently reported [17,35], intramuscular fat content was negligible in H40Y muscles and did not differ from

Table 1. Anatomical measurements in H40Y and WT groups.

|                | Body weight (g) | Muscle volume (mm³) | SAT volume (mm³) | IMAT volume (mm³) | IMAT/muscle ratio (%) |
|----------------|-----------------|---------------------|------------------|-------------------|-----------------------|
| WT             | 23.8±0.9        | 106.1±8.1           | 34.8±1.5         | 6.8±0.7           | 6.8±0.9               |
| H40Y           | 20.1±0.5        | 85.3±5.3            | 29.7±1.2         | 5.9±0.7           | 7.4±1.3               |

SAT: subcutaneous adipose tissue, IMAT: intermuscular adipose tissue. Ratios have been expressed in percentage of muscle volume. Values are presented as mean ± SEM. Significantly different from WT group *P<0.05.
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Figure 2. Absolute maximal force production (A), specific (B) and relative (C) force production during the force-frequency protocol in H40Y (○) and WT (●) groups. Force was normalized to hindlimb muscles volume (B) and to maximal force obtained at 150 Hz (C). f₅₀ (inset fig. C) represents the frequency providing 50% of maximal force. Maximal force was lower in H40Y group as compared to WT group whereas f₅₀ was similar between the two groups. Values are presented as mean ± SEM. Significantly different between groups *P<0.05.
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Figure 3. Specific force production during the stimulation protocol (A) and fatigue index (B) in H40Y (○) and WT (■) groups. Force was normalized to hindlimb muscle volume. H40Y group showed a lower force production and an improved resistance to fatigue as compared to the WT group. Values are presented as mean ± SEM. Significantly different between groups *P<0.05.
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Figure 4. Example of resting $^{31}$P-MR spectra in H40Y group (A) and WT group (B). PCR to ATP ratios were calculated from the peak area of the PCR and β-ATP.
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controls. Our results totally agree with the small amount of fatty tissue infiltration in the hindlimb muscles (i.e., 5%) of mdx mice [18,36] whereas large fat infiltration is a typical feature in dystrophic patients [29]. Alternatively, considering that the current experiments were performed in fourteen-week old mice, one could also assume that fatty infiltration might occur at a more advanced stage of the disease. Overall, the present MRI data indicated that the H40Y mouse model did not reproduce the large fatty infiltration typically observed in NM patients.

We reported the first in vivo characterization of hindlimb muscle function in H40Y mice. Absolute maximal tetanic force of the gastrocnemius muscle was largely reduced, i.e., 40% in H40Y mice as compared to controls, which is in agreement with the severe muscle weakness previously reported in vitro in isolated EDL muscle [16]. Interestingly, our MRI data also showed a 20% reduction in hindlimb muscles volume which is consistent with the 15–20% reduction of gastrocnemius muscle weight recently reported in vitro [16], illustrating that the H40Y mutation lead to muscle atrophy. Considering that serum response factor signaling, which is known to control muscle growth and differentiation [37], is altered in patients with actin-based myopathy [38], one could speculate that similar defects might occur in H40Y and thereby explain the atrophic phenotype in H40Y mice. It is noteworthy that the specific force was also ~25% lower in H40Y as compared to controls, thereby indicating that muscle atrophy alone cannot solely explain the severe muscle weakness found in H40Y mice.

One could thereby suggest that intrinsic muscular properties are likely altered as a result of the mutation. Considering that an alteration of calcium sensitivity has been recently reported in both NM patients with NEB or TPM mutations [22,39] and in a mouse model carrying the Asp286Gly mutation in the ACTA1 gene [15], we indirectly assessed this parameter on the basis of in vivo measurements of force production resulting from incremental stimulation frequencies. The corresponding force-frequency curve was not shifted in the H40Y group as compared to control mice leading to similar f50 values between the two groups. Accordingly, one could assume that calcium sensitivity was not altered in H40Y mice even though further studies are needed in order to directly assess the effects of H40Y mutation on calcium homeostasis. While it has been recently suggested that Ca2+ sensitizers agents might counterbalance muscle weakness [40] in NM patients carrying mutation in the NEB gene [22], our data indicated that these pharmaceutical agents would be ineffective for counteracting the deleterious effects of H40Y mutation on muscle function.

31P-MRS investigations were performed on both H40Y and control gastrocnemius muscles in order to record the metabolic changes during a fatigue protocol. We observed similar PCr consumption, Pi production and pH changes for the two groups during our fatigue protocol, indicating that the metabolic changes induced by the stimulation protocol were similar for the two groups. However, the force production was largely reduced in H40Y as compared to WT mice. As a consequence, for a given

Figure 5. Changes in gastrocnemius PCr (% resting value; A), Pi (% resting value; B), and pH (C) during the stimulation protocol were similar in H40Y (○) and WT (□) groups. Values are presented as mean ± SEM. doi:10.1371/journal.pone.0061517.g005
energy consumption, the mechanical output was lower in the transgenic mice, indicating that the energy cost of the contraction was higher for the H40Y mice as compared to controls. Interestingly, our findings are similar to those recently reported in adipose triglyceride lipase knockout (ATGL-KO) mice in which the force production was lower as compared to controls while a similar PCr depletion was observed for the two groups. The authors also concluded that ATGL-KO mice had a higher energy cost per contraction (or a lower contractile efficiency) as compared to controls [41].

Considering that subsarcolemmal mitochondria were abnormally large in H40Y muscles [13], the higher energy cost might be related to an impaired mitochondrial function. Over the last decade, analysis of PCr recovery kinetics has been extensively used in order to investigate in vivo skeletal muscle mitochondrial oxidative capacity [41,42]. Previous animal studies reported PCr recovery times typically ranging from ~50 sec to ~160 sec according to the end-of-exercise pH values [31,41,43]. From a methodological point of view, it should be pointed out that PCr recovery is usually fitted with a monoexponential function so that the temporal resolution of the raw dataset is of utmost importance for an accurate measurement of the corresponding variables. The temporal resolution is directly related to the signal to noise ratio (SNR), which is related to several variables such as the sampled muscle volume, the magnetic field strength, the repetition time and the total acquisition time. In our previous 31P-MRS study [31], PCr recovery time was obtained with a temporal resolution of ~60 sec in C57BL/6 mice in which the hindlimb muscles volume was roughly 200 mm3 [44]. In the present experiment, hindlimb muscles volume of both WT (R1–129 genetic background) and H40Y mice was ~106 and ~85 mm3, respectively. These values were thereby ~1.9–2.3 fold lower than those previously obtained in C57BL/6 control mice [31], thereby leading to a lower SNR and to a temporal resolution higher than 100 sec (i.e., ~110 sec). As a consequence, the corresponding temporal resolution of our experiments was too high to accurately measure the PCr recovery time. Consequently, further 31P-MRS investigations at higher magnetic field [45] could improve the corresponding temporal resolution and provide important insights to the effects of NM-causing mutations on mitochondrial oxidative capacity.

Alternatively, the higher energy cost could also be related to mechanisms occurring at the cross-bridges level. First of all, it should be emphasized that the H40Y mutation is located in the actin domain which interacts with the myosin and may thereby disturb the attachment of the myosin to the actin filament [16,47]. Moreover, alterations in cross-bridges cycling kinetics have been recently reported in both NM patients [19,22,39] and mouse models of NM [48,49]. For example, the combination of both the slack/release approach and the simultaneous force-ATPase measurements illustrated a large reduction of the rate of force redevelopment and an increased tension cost in muscle fibers from patients with NEB mutations [19,39]. These findings indicated that the rate of cross-bridges attachment was reduced while the rate of cross-bridges detachment was increased in NM muscles. Additional investigations are therefore warranted in order to determine the effects of H40Y mutation on the cross-bridges cycling kinetics.

Analysis of the mechanical performance during the fatigue protocol showed a higher fatigue index in H40Y as compared to controls, thereby indicating an improved resistance to fatigue in H40Y mice. This result might be related to the increased number of slow oxidative fibers recently reported in this mouse model [16]. However, our 31P-MRS results showed an increased energy cost in H40Y mice that was inconsistent with a potential shift toward a slower phenotype in H40Y. Indeed, it has been well established that muscles composed of slow twitch fibers typically showed lower acidosis [50,51] and reduced energy cost [52,53] during isometric contractions as compared with fast-glycolytic muscles. However, it should be pointed out that previous determination of fiber-type composition was performed on both slow soleus and fast flexor digitorum brevis muscles. Considering that NM is associated with specific muscle involvement, one could speculate that the gastrocnemius muscle might be less or not affected by this fast-to-slow phenotypic transition even though further studies are needed to assess potential changes in fiber type composition in gastrocnemius muscle. The discrepancies between our 31P-MRS results and the higher resistance to fatigue in H40Y might also be related to the criterion used to assess muscle fatigue. Indeed, our fatigue index was determined as the ratio between the last five and the first five contractions and did not take into account the lower baseline force level in H40Y. For instance, human studies showed that endurance time for sustained submaximal isometric contraction was inversely related to the absolute target force exerted during the voluntary task [54,55]. Consequently, additional analyses would be required in order to determine the potential cause-effect relationship between the lower absolute force level and the improved resistance to fatigue in H40Y.

In conclusion, our strictly noninvasive methodological approach provides compelling evidence of an altered muscle function in H40Y mice. We clearly demonstrated that the H40Y mutation led to a reduced specific force production which might be related to an alteration of intrinsic muscular properties. Although the underlying mechanisms remain to be determined, 31P-MRS investigations indicated an increased energy cost in H40Y mice despite an improved resistance to fatigue. We also clearly demonstrated that intramuscular fat content was negligible in H40Y muscles, indicating that this NM mouse model does not replicate MRI findings of NM patients. Overall, we have provided a unique set of information about the anatomic, metabolic and functional consequences of the H40Y mutation that might be considered as relevant biomarkers for monitoring the severity and/or the progression of NM disease but also for assessing the efficacy of potential therapeutic interventions at a preclinical level including for example dietary L-tyrosine supplementation [16].

Author Contributions
Conceived and designed the experiments: CG JG. Performed the experiments: CG JG. Analyzed the data: CG JG. Contributed reagents/materials/analysis tools: YL EP CV EH AL PJC DB. Wrote the paper: CG JG DB.

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