Myostatin: a Circulating Biomarker Correlating with Disease in Myotubular Myopathy Mice and Patients

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Myotubular myopathy, also called X-linked centronuclear myopathy (XL-CNM), is a severe congenital disease targeted for therapeutic trials. To date, biomarkers to monitor disease progression and therapy efficacy are lacking. The Mtm1+/−y mouse is a faithful model for XL-CNM, due to myotubulin 1 (MTM1) loss-of-function mutations. Using both an unbiased approach (RNA sequencing [RNA-seq]) and a directed approach (qRT-PCR and protein level), we identified decreased Mstn levels in Mtm1+/−y muscle, leading to low levels of myostatin in muscle and plasma. Myostatin (Mstn or growth differentiation factor 8 [Gdf8]) is a protein released by myocytes and inhibiting muscle growth and differentiation. Decreasing Dnm2 by genetic cross with Dnm2−/− mice or by antisense oligonucleotides blocked or postponed disease progression and resulted in an increase in circulating myostatin. In addition, plasma myostatin levels inversely correlated with disease severity and with Dnm2 mRNA levels in muscles. Altered Mstn levels were associated with a generalized disruption of the myostatin pathway. Importantly, in two different forms of CNMs we identified reduced circulating myostatin levels in plasma from patients. This provides evidence of a circulating biomarker that may be used to monitor disease state in XL-CNM mice and patients and supports monitoring circulating myostatin during clinical trials for myotubular myopathy.

INTRODUCTION

Many potential therapies for neuromuscular diseases have moved from proof-of-concept toward clinical trials in the past decade.1 However, in many cases, reaching predefined endpoints to show efficacy in clinical trials can be challenging. Simple and practical biomarkers that are highly sensitive and respond rapidly to treatment are urgently required. Blood-based biomarkers can be time and cost efficient, and importantly, provide less-invasive and more global means of sampling from the patient than, for example, a muscle biopsy. In a research setting, mouse models of disease provide an excellent tool for biomarker discovery. Mouse lines that recapitulate the disease phenotype can be used to identify potential blood-based biomarkers and map in a temporal and dose-dependent manner response to treatment. This is especially true in the context of rare diseases, where large collection of samples is challenging and the absence of approved medication make sensitivity to positive change impossible to validate.

Myostatin (Mstn or growth differentiation factor 8 [Gdf8]) is a protein produced and released by myocytes. Myostatin acts in an autocrine function to inhibit muscle growth and differentiation.2 Low levels of myostatin were identified in muscle biopsies and in serum from patients with different myopathies.3 Myostatin was also recently shown to be reduced in muscle biopsies from Mtm1+/− mice, a faithful mouse model for X-linked centronuclear myopathy due to MTM1 mutations.4 Centronuclear myopathies (CNMs) are non-dystrophic, debilitating rare congenital diseases, associated with muscle weakness and abnormally located nuclei in skeletal muscle.4 Several forms of CNMs have been characterized. The main forms are: X-linked CNM (XL-CNM, OMIM: 310400), also called myotubular myopathy, due to mutations in the phosphoinositides phosphatase myotubularin (MTM1),5 autosomal recessive and dominant CNM (OMIM: 255200) caused by mutations in the membrane remodeling protein amphiphysin 2 (BIN1),5,6 autosomal dominant CNM (AD-CNM, OMIM: 160150) due to mutations in dynamin 2 (DNM2),7 and autosomal recessive CNM–like disease due to mutations in ryanodine receptor (RYR1).8 There are approximately 4,000 living patients with CNM, in the USA, EU, Japan, and Australia.10

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Circulating Myostatin Levels Respond to DNM2 Therapy in a Time-Dependent Manner, Correlating with Reduced Disease Severity

We next wanted to determine whether myostatin levels correlated with disease severity and reduction of Dnm2, in a temporal manner. To do this, we first established a protocol to correlate Dnm2 levels and disease phenotype in mice over time. We performed a single ASO injection in WT or Mtm1<sup>-/-</sup> mice at 3 weeks of age, near the onset of the disease phenotype (study 3). Indeed, a single injection of DYN101-m significantly delayed the progression of the disease phenotype in mice by approximately 1 month (Figure 2A). A significant reduction in Dnm2 mRNA was observed in tibialis anterior (TA) muscles of Mtm1<sup>-/-</sup> DYN101-m mice both 1 and 2 weeks post injection relative to age-matched ASO-Ctrl injected Mtm1<sup>-/-</sup> mice, which returned to WT levels 4 weeks post injection (Figure 2B; Figure S2A), coinciding with the timing of decline in the clinical phenotype in these mice. Plasma myostatin levels in untreated Mtm1<sup>-/-</sup> mice were decreased at all time points measured compared to WT mice (1–4 weeks post injection; Figure 2C), corresponding with increased severity of disease. However, Mtm1<sup>-/-</sup> mice injected with DYN101-m displayed a significant relative myostatin increase compared to Mtm1<sup>-/-</sup> control mice 2 and 4 weeks post injection (Figure 2C). The increase was apparent 1 week after ASO administration resulting in Dnm2 mRNA reduction, and at a time where mice displayed a significant improvement in disease phenotype (Figures 2A and 2B). Furthermore, myostatin levels did not purely reflect body weight or muscle mass (Figures S2B and S2C) suggesting myostatin levels are not altered solely due to a change in muscle mass.

We analyzed one cohort in detail, at 4 weeks post single injection, a time point where Mtm1<sup>-/-</sup> mice were still alive, and Mtm1<sup>-/-</sup> mice treated with DYN101-m displayed a significant improvement in phenotypes. Mtm1<sup>-/-</sup> muscles from this cohort were clearly affected by disease, with smaller fibers containing centralized nuclei, and an abnormal intracellular SDH staining pattern with accumulation observed in the center and/or the periphery of affected fibers (Figures 2D–2F). Muscles from Mtm1<sup>-/-</sup> mice treated with DYN101-m were clearly improved, with the majority of nuclei at the periphery of the fiber, and SDH staining greatly improved (Figures 2D and 2F). However, no improvement in fiber size was observed in response to a single injection of DYN101-m (Figures 2D and 2E), in contrast to the rescue observed upon repeated injections, and further suggesting the increase in myostatin may be reflective of improved muscle strength rather than muscle mass.

To further understand the relationship between circulating myostatin levels and disease rescue, we performed a dose-response study. Three doses were tested in Mtm1<sup>-/-</sup> mice, and phenotype and myostatin analysis was performed (study 5). All doses resulted in improved survival, with the middle and high doses resulting in similar levels of disease improvement at 12 weeks of age (Figures S3A and S3B). Correspondingly, myostatin levels were increased in all 3 doses compared to untreated 7-week-old mice (Figure 1E; Figure S3C). However, a clear dose response was not observed, suggesting a more complex
Mstn mRNA was reduced in TA skeletal muscles from (Table S3). This is as expected given that myostatin is a myokine pro-

ure S4), supported also by transcriptomics data from (A) Plasma myostatin protein levels (ng/mL) from WT and

studies 1, 3, 5), myostatin was identi-

tin levels relative to hanging test ability was completed across studies (studies 1, 3, 5), myostatin was identified as a predictor for hanging test ability, with a myostatin value of 37 ng/mL corresponding to a true positive rate of over 0.8 for hanging test ability of 10 s or more (Figure 2G; Table S4). Combined, these results show circulating myostatin levels respond to treatment, in alignment with Dnm2 reduction and improvement in disease phenotype from baseline, suggesting that myostatin may be a useful biomarker for disease severity or for monitoring treatment efficacy.

Underlying Mechanisms Leading to Myostatin Alteration in Mtm1−/y Mice

We next wanted to investigate the molecular mechanism supporting reduced circulating myostatin levels in Mtm1−/y mice and increase in response to Dnm2 reduction. To do this, we analyzed the effects of Dnm2 reduction mediated by ASO administration or genetic cross, in Mtm1−/y mice at different ages from 4 independent studies including multiple cohorts performed in parallel from different mouse colonies (studies 1–4, Table 1, see Methods section for details). The level of Mstn mRNA was reduced in TA skeletal muscles from Mtm1−/y mice from all 4 studies including independent cohorts (Table 2; Figure S4), supported also by transcriptomics data from Mtm1−/y mice (Table S3). This is as expected given that myostatin is a myokine produced from muscles. This defect was limited to the TA muscles as Mstn mRNA in cardiac muscle was unaltered in Mtm1−/y mice. Surprisingly, despite the clear and rapid increase in circulating myostatin levels correlating with improved disease phenotype upon reduction of Dnm2, Mstn mRNA did not increase in TA muscles in response to Dnm2 reduction either through ASO treatment or genetic cross with Dnm2−/− mice in any cohort (Table 2; Figure S4). The TA muscle is one of the most severely affected muscles in Mtm1−/y mice, and variable myostatin levels have been observed in different muscles from 2 XL-CNM mouse models. Therefore we next hypothesized that the increase in circulating myostatin may be produced from other skeletal muscle tissues. Mstn production from gastrocnemius, diaphragm, and extensor digitorum longus (EDL) muscles confirmed a clear reduction in Mstn mRNA in Mtm1−/y mice, akin to TA muscles (Table 2; Figure S4). No significant increase (p < 0.059) in Mstn mRNA levels was detected in gastrocnemius muscles from Mtm1−/y mice upon reduction of Dnm2, however a significant increase was observed in the diaphragm and EDL skeletal muscles. Of note, a significant improvement in the diaphragm muscle histology in response to Dnm2 reduction through a genetic cross was previously observed. These results suggest the increased circulating myostatin may come from increased transcription of Mstn in certain skeletal muscles (Figure S4). We next investigated myostatin protein levels. Myostatin is produced in skeletal muscles with an N-terminal prodomain.
of enhancer binding proteins C/EBP regulate Mtm1 mRNA was reduced in pre- and post-symptomatic stages in Cebpg ASO-mediated reduction of muscles that was partially normalized in response to genetic and ure S5; Table S3). However mature miR-27 levels were not altered post-transcriptional gene expression. miR-27a binds to the 3'UTR of Myostatin regulators of myostatin transcription. MicroRNAs (miRNAs) are small non-coding RNAs involved in regulating RNA silencing and post-transcriptional gene expression. miR-27a binds to the 3'UTR of Myostatin. Micro-RNAs or "MiomiRs" play a role in physiological and pathological muscle functions. The MyomiR miR-206 is found predominantly in skeletal muscle, and a signifi cant amelioration upon treatment was observed (Figures 4A and 4B). Therefore, the increase in circulating myostatin levels in response to Dnm2 reduction (Figure 4A), supporting downregulation of the myostatin pathway in Mtm1−/− mice. Myostatin binds to the activin IIb receptor (AcvRIIb) on the sarcolemma of skeletal muscles to activate signaling pathways. AcvRIIb levels were not altered in Mtm1−/− mice (Figure 4B; Figure S5), consistent with previous results. Follistatin is a protein that acts in an antagonistic manner to myostatin through direct binding. Fstn mRNA levels were increased in Mtm1−/− mice (Table S3; Figure 4C; Figure S5) in the inverse direction to Mstn (Figure S4); however, levels did not return to WT upon reduction of Dnm2 by genetic cross or ASO injections.

We next looked downstream of the myostatin pathway. Myostatin activates SMAD2/3 phosphorylation and atrophic signaling pathways (reviewed in Walker et al. 25). While transcriptomics data identified changes in transcription of Smad family members, at the functional level we did not observe a change in total or phosphorylated levels of SMAD2/3 in any cohorts and groups (Figure S5; Table S3), suggesting myostatin-mediated effects may be modulated independent of SMAD2/3 signaling pathway in Mtm1−/− mice. Skeletal muscle miRNAs or “MomiRs” play a role in physiological and pathological muscle functions. The MyomiR miR-206 is found predominantly in skeletal muscle, and its expression may be negatively regulated by the myostatin pathway.26 According in Mtm1−/− mice where myostatin levels are reduced, miR-206 was increased both in disease (Figure S5) and late (Figures 4D, E) stages of disease. This level was reduced in response to reduction of Dnm2 via genetic cross or ASO-mediated reduction of Dnm2 (Figures 4D and 4E; Figure S5). These changes were confirmed by analyzing expression of the mature miR-206 (Figure 4F). Furthermore, downstream dysregulation of the myostatin pathway was observed, in particular myogenin and Murf1 levels were altered, which were partially or fully rescued by reducing Dnm2 (Table S3). These results suggest that the myostatin pathway signaling is globally modified both in disease state (Figure 5) and in response to treatment.

Taken together, these results suggest that myostatin is altered early during development of disease pathogenesis, and these changes result in global downstream dysregulation of the myostatin pathway in mice.

Table 1. Summary of Individual Studies Analyzed in This Manuscript

| Study | Laboratory | Study Design | Results Presented |
|-------|------------|--------------|--------------------|
| 1 A   | weekly ASO injections, from 2/3–7 weeks of age (WT ASO-Ctrl, Mtm1−/− ASO-Ctrl, Mtm1−/− DYN101-m), 25 mg/kg | Figures 1, 2, 3, 4; Figures S1, S4, S5 |
| 2 B   | as for (1), 2nd independent cohort, 25 mg/kg | Figure 4; Figures S4 and S5 |
| 3 A   | single ASO injection at 3 weeks, analysis 1, 2, 4, 8 weeks post injection (WT ASO-Ctrl, Mtm1−/− ASO-Ctrl, Mtm1−/− DYN101-m), 25 mg/kg | Figure 2; Figures S2, S4, S5 |
| 4 B   | genetic cross cohort, analyzed at 2 weeks (WT, Mtm1−/−, Mtm1−/− Dnm2−/−) | Figure 4; Figures S4 and S5 |
| 5 A   | weekly ASO injections, from 5 to 12 weeks of age (WT ASO-Ctrl, Mtm1−/− ASO-Ctrl, Mtm1−/− DYN101-m), 6.25, 12.5, and 25 mg/kg | Figure 2; Figure S3 |
| 6 A   | weekly ASO injections, from 8 to 12 weeks of age (WT ASO-Ctrl, Dnm2−/− ASO-Ctrl, Dnm2−/− DYN101-m), 25 mg/kg | Figure S6 |

myostatin expression potentially acting through miR-27, was altered in Mtm1−/− muscle (Table S3). Overall, decreased expression of the myostatin gene, potentially due to alteration in its transcriptional regulators, may result in decreased circulating myostatin.

General Alterations of the Myostatin Pathway in Mtm1−/− Mice
To gain a better insight into the alterations of the myostatin pathway, we investigated growth differentiation factor 11 (GDF11), due to the high degree of similarity to myostatin (GDF8). Gdf11 levels were significantly increased in Mtm1−/− mice and variable following Dnm2 reduction (Figure 4A), supporting downregulation of the myostatin pathway in Mtm1−/− mice. Myostatin binds to the activin IIb receptor (AcvRIIb) on the sarcolemma of skeletal muscles to activate signaling pathways. AcvRIIb levels were not altered in Mtm1−/− mice (Figure 4B; Figure S5), consistent with previous results. Follistatin is a protein that acts in an antagonistic manner to myostatin through direct binding. Fstn mRNA levels were increased in Mtm1−/− mice (Table S3; Figure 4C; Figure S5) in the inverse direction to Mstn (Figure S4); however, levels did not return to WT upon reduction of Dnm2 by genetic cross or ASO injections.

To explore further potential alterations in the myostatin pathway leading to decreased circulating myostatin in disease, we investigated regulators of myostatin transcription. MicroRNAs (miRNAs) are small non-coding RNAs involved in regulating RNA silencing and post-transcriptional gene expression. miR-27a binds to the 3'UTR of Mstn and regulates post-transcriptional expression of myostatin. We observed an increase in pre-miR-27a in Mtm1−/− TA skeletal muscles that was partially normalized in response to genetic and ASO-mediated reduction of Dnm2, both by qRT-PCR and RNA sequencing (RNA-seq) analyses in several cohorts (Figure 3E; Figure S5; Table S3). However mature miR-27 levels were not altered (Figure 3F), suggesting other cellular mechanisms may be involved in the reduction in myostatin in Mtm1−/− mice. The CCAAT enhancer binding proteins C/EBP regulate MSTN expression. Transcriptomics data suggests Cebpb and Cebpd are unchanged, whereas Cebpg mRNA was reduced in pre- and post-symptomatic stages in the Mtm1−/− muscle and partially normalized through Dnm2 reduction by genetic cross (Table S3). Similarly, c-Myc, another regulator of...
be dramatically reduced (3-fold) compared to age-matched control samples (Figure 6, control 3,588±328 pg/mL versus 1,122 ± 445 pg/mL in XL-CNM), suggesting the results generated in mice from this study may be translatable to patients.

We next wanted to see whether myostatin levels were also altered in an autosomal form of CNM. A faithful murine model for AD-CNM due to DNM2 mutations has been created (Dnm2Rw/+ mice), which recapitulates the adult-onset form of the disease linked to the most common R465W mutation.30,31 No significant change in circulating myostatin levels were detected at the symptomatic age of 12 weeks (study 6, Table 1; Figure S6), which may be due to the mild phenotype presentation in this model,17,31 with minimal reduction in fiber size (14%, Figure S6). Importantly, circulating myostatin levels were
significantly reduced (>4-fold) in patients with AD-CNM compared to age-matched controls (Figure 6, control 3.588±328 pg/mL versus 823 ± 367 pg/mL in AD-CNM). Overall these results suggest myostatin may be a relevant biomarker for disease in patients with several forms of CNMs.

**DISCUSSION**

The myokine myostatin is known to inhibit muscle growth, and based on this concept, several anti-myostatin therapeutic clinical trials have been launched for neuromuscular disease patients, with the goal of inhibiting the myostatin pathway and therefore improving muscle mass and function. The publicly released data from these trials are so far disappointing (https://clinicaltrials.gov/; NCT02310763, NCT02515669). Recent data, including data presented here, suggest a common mechanism whereby myostatin levels in neuromuscular disease patients are naturally reduced,\(^3,32\), which may help explain in part the lack of positive data generated in clinical trials to date, and the limited therapeutic efficacy observed following AcvRIIb inhibition in two XL-CNM mouse models.\(^{18,19}\) Results generated here from several cohorts in independent laboratories now support that circulating myostatin is a biomarker for disease progression and severity in a mouse model of myotubular myopathy and is responsive to upstream treatment. The reduction in circulating myostatin is consistent with observations in XL-CNM and AD-CNM patients.

**Defects in the Myostatin Pathway in XL-CNM**

Myostatin was previously shown to be reduced in serum from patients with different neuromuscular disorders;\(^2\) however, this had not been investigated in plasma or serum from patients or animal models of centronuclear myopathies. Mstn mRNA levels were previously shown to be reduced in skeletal muscles from XL-CNM mice and dogs.\(^{3,33}\) In this study, we confirmed a reduction of myostatin at the mRNA level in muscle biopsies from XL-CNM mice and identified low levels of circulating myostatin in plasma. The shutdown of the myostatin pathway may be linked to a parallel increase in follistatin, an inhibitor of myostatin, and/or altered transcriptional regulation. While Fstn levels were inversely increased in our model, AcvRIIb levels were unaltered, in contrast to Duchenne Muscular Dystrophy patient muscles where AcvRIIb but not Fstn levels were altered,\(^3\) suggesting differential regulation of the myostatin pathway between neuromuscular disorders. Our data support a hypothesis where altered myostatin regulation occurs in the myopathic tissue, where myostatin levels are reduced due at least in part to direct signaling mechanisms altering transcription of Mstn and activation of the receptor and associated pathway. Furthermore, our data support a specific downregulation of myostatin (Gdf8), as transcription of the highly homologous family member Gdf11 is inversely increased in Mtm1\(^{-/+}\) mice. Increased Gdf11 has previously been associated with aging muscles;\(^{34}\) however, the significance of this in Mtm1\(^{-/+}\) mice is unclear. Interestingly this was not observed in other neuromuscular disorders,\(^3\) suggesting a specific regulation in XL-CNM.

A resulting increase in the MyomiR-206 in response to reduced myostatin in Mtm1\(^{-/+}\) mice was observed, supporting previous data that showed an increase in the MyomiR-206 in response to loss of myostatin\(^2\) and in newly formed muscle fibers of mdx mice.\(^{35}\) MyomiRs have been previously shown to be altered from XL-CNM patient muscle biopsies.\(^{36}\) While a decrease in myostatin would in principle promote muscle hypertrophy, the muscles of the Mtm1\(^{-/+}\) mice are very hypotrophic. Our results suggest the net loss in muscle mass alone is not responsible for the reduction in circulating myostatin levels observed in animal models of neuromuscular disease and that an overall dysregulation of the myostatin pathway occurs in Mtm1\(^{-/+}\) mice (Figure 5).

**Circulating Myostatin as a Biomarker for Disease Severity and Therapy Efficacy**

Because centronuclear myopathies are non-dystrophic structural muscle diseases, blood-based markers such as creatine kinase levels are not informative. Here we identify that circulating myostatin levels are decreased in both the XL-CNM mouse model (Mtm1\(^{-/+}\) mice) and in patients with XL or AD-CNM. In addition, in Mtm1\(^{-/+}\) mice myostatin levels correlated with the progression of the disease severity across several studies. Therefore, myostatin is a potential biomarker to follow disease progression. Sampling patients at different disease severity in a mouse model of myotubular myopathy and is responsive to upstream treatment. The reduction in circulating myostatin is consistent with observations in XL-CNM and AD-CNM patients.

**Table 2. mRNA Analysis of Mstn Levels across Cohorts**

| Study Number | Tissue | Group | Mtm1\(^{-/+}\) C0 | Mtm1\(^{-/+}\) Reduced |
|--------------|--------|-------|-------------------|-----------------------|
| 1            | TA     | WT    | 1.04 ± 0.09 (10)  | 0.19 ± 0.03 (5)*      |
|              | GAS    | WT    | 1.03 ± 0.13 (5)   | 0.27 ± 0.01 (2)*      |
|              | heart  | WT    | 1.43 ± 0.50 (5)   | 0.92 ± 0.25 (2)       |
| 2            | TA     | WT    | 1.00 ± 0.17 (6)   | 0.32 ± 0.09 (4)*      |
|              | GAS    | WT    | 1.01 ± 0.14 (2)   | 0.20 ± 0.03 (5)       |
|              | heart  | WT    | 1.14 ± 0.55 (2)   | 1.24 ± 0.35 (5)       |
|              | soleus | WT    | 1.03 ± 0.25 (2)   | 0.79 ± 0.37 (5)       |
|              | diaphragm | WT | 1.03 ± 0.23 (2) | 0.22 ± 0.06 (5)*     |
| 4, analyzed at 2 weeks | TA | WT | 1.00 ± 0.07 (2) | 0.15 ± 0.03 (5)* |
| 4, analyzed at 7 weeks | TA | WT | 1.00 ± 0.10 (5) | 0.27 ± 0.06 (5)* |

\(^*\)Significant difference from WT.
\(^\text{a}\)Significant difference from Mtm1\(^{-/+}\).
stages and with different disease severity will be needed to confirm the relevance in humans. Patient age/gender will need to be taken into account when interpreting myostatin levels in clinic. Moreover, the differential regulation of this pathway in XL-CNM may suggest novel therapeutic targets.\textsuperscript{18} Given the rarity of the disease, a formal validation of the biomarker on large cohort of patients is not possible, but it is noteworthy that the different clinical programs in CNMs (https://clinicaltrials.gov; NCT04033159, NCT03199469) will help to verify whether the increase of myostatin level observed in treated animals translates into humans.

Therapeutic Dnm2 reduction resulted in a rapid increase in circulating myostatin, again supporting myostatin as a potential blood-based biomarker for XL-CNM. An increase in Mstn mRNA was observed in several skeletal muscles, akin to previously published data testing another therapeutic approach in mice and dogs.\textsuperscript{3,33} suggesting a possible molecular mechanism for increased plasma myostatin levels. These results suggest that circulating myostatin may be a relevant biomarker that may be used to monitor therapeutic efficacy in animal models of disease with different treatments. Of note, a correlation was observed between myostatin and increased whole-body strength even when muscle size was unaltered (Figure 2). Given the natural low level, myostatin is probably not a good treatment target but could be considered as a possible target in treated patients. These results confirm improvement in disease phenotype in animal models of CNMs by reduction of DNM2.\textsuperscript{14,17,37} Here we show that myostatin levels respond rapidly to treatment coinciding with improved muscle function and reduced Dnm2 mRNA from muscle biopsies in a temporal manner and that myostatin may act as a surrogate marker for treatment efficacy.

In conclusion, we show that reducing dynamin 2 increased survival and improved the myopathy phenotype in a time-dependent manner in Mtm1\textsuperscript{1−/y} mice, a mouse model for XL-CNM. In addition, we show here that repeated treatments may be required for patients with CNMs. Reduction of Dnm2 to treat CNMs is now being developed toward the clinic; however, supportive blood-based biomarkers are not yet available for monitoring of non-dystrophic muscle diseases. While skeletal muscles are the primary tissue affected by the disease, repeated muscle biopsies to monitor disease progression and efficacy of treatment are highly invasive for patients and not only result in permanent damage to muscles but also place a heavy burden on patients and the health care system. Here we show that centronuclear myopathies display a significant reduction in circulating myostatin. Our data therefore suggest myostatin as a novel blood-based biomarker for CNM that responds to treatment and may therefore be used to monitor disease state and rescue in X-linked centronuclear myopathy.

**Figure 3. Underlying Mechanisms Leading to Myostatin Alteration in Mtm1\textsuperscript{1−/y} Mice**

(A) Immunoblot for protein expression of Myostatin prodomain and GAPDH (protein loading control) in TA muscles. (B) Myostatin prodomain expression quantified relative to GAPDH loading control from (A). (C) Immunoblot for protein expression of Myostatin prodomain and ponceau (protein loading control) in plasma. (D) Densitometry analysis of myostatin prodomain expression from (C). (E) mRNA analysis of Pre-miR-27a relative to Rp27 expression from TA skeletal muscles of WT and Mtm1\textsuperscript{1−/y} mice, following weekly injections of DYN101-m targeting Dnm2 or ASC-Ctrl. (F) Mature miR-27 analysis relative to U6+Snord61. Results presented as violin plots, individual mouse data shown. *p < 0.0125, **p < 0.01, ***p < 0.001, ****p < 0.0001. All results from study 1.

**MATERIALS AND METHODS**

**Generation of Mtm1\textsuperscript{1−/y} Mice**

Mtm1\textsuperscript{1−/y} or WT 129Sv/PAS mice were previously generated and characterized by crossing Mtm1 heterozygous females obtained by homologous recombination with WT males.\textsuperscript{14,38} Animals were housed in a temperature-controlled room (19°C–22°C) with a 12:12 h light/dark cycle, with free access to food. Animal
experimentation was approved by the institutional ethical committee Com’Eth IGBMC-ICS; APAFIS#5453-2016052510176016 and APAFIS#3026-201512041851445. Mice were humanely sacrificed when required according to national and European legislations on animal experimentation. Male mice were analyzed in this study.

**ASOs**

ASOs were chemically modified with phosphorothioate in the backbone and constrained ethyl (cEt) modifications on the wings with a deoxy gap (3-10-3 design). ASOs were synthesized by IONIS Pharmaceuticals using an Applied Biosystems 380B automated DNA synthesizer (PerkinElmer Life and Analytical Sciences-Applied Biosystems, Waltham, MA, USA) and purified.39 The 16 nucleotide ASO candidate (DYN101-m) was previously tested and shown to efficiently reduce *Dnm2* expression levels in vivo, while the random control sequence has no homology to the mouse genome and does not reduce *Dnm2* RNA nor protein.14,17 The sequence of each ASO is listed in Table S1. ASOs were dissolved in filtered and autoclaved sterile D-PBS (Life Technologies, #14190-144). Intraperitoneal injections of up to 25 mg/kg of ASOs were performed in WT and *Mtm1<sup>−/−</sup>* mice. Generation of Mouse Cohorts

Six independent mouse experiments were performed. Study 1: weekly ASO intraperitoneal injections of DYN101-m targeting *Dnm2* mRNA or ASO control (ASO-Ctrl) were administered to WT and *Mtm1<sup>−/−</sup>* mice, from 2/3–7 weeks of age (25 mg/kg, laboratory A, Dynacure). Study 2: experiment performed as for study 1 (ASO injections weeks 3–7), this second experiment was performed from an independent colony of mice (laboratory B, IGBMC), thus further validating the protocol for study 1. Study 3: single injection of ASO targeting *Dnm2* (DYN101-m) or ASO control (ASO-Ctrl) was performed at 3 weeks of age in WT and *Mtm1<sup>−/−</sup>* mice. Individual cohorts (6) were sacrificed 1, 2, 4, 8, 12, or 16 weeks post injection (laboratory A). Study 4: WT, *Dnm2<sup>−/−</sup>*, *Mtm1<sup>−/−</sup>*, and *Mtm1<sup>−/−</sup>*-*Dnm2<sup>−/−</sup>* mice were generated and sacrificed at 2 or 7 weeks of age (laboratory B). Study 5: weekly ASO intraperitoneal injections of DYN101-m (6.25, 12.5, 25 mg/kg)
targeting Dnm2 mRNA or ASO control (ASO-Ctrl, 25 mg/kg) were administered to WT and Mtm1<sup>R465W/+</sup> mice, from 5 to 12 weeks of age (25 mg/kg, laboratory A). Study 6: weekly ASO injections (25 mg/kg) were performed from 8 to 12 weeks of age in WT or Dnm2<sup>R465W/+</sup> mice (WT ASO-Ctrl, Dnm2<sup>R465W/+</sup> ASO-Ctrl, Dnm2<sup>R465W/+</sup>DYN101<sup>y</sup>, laboratory A). A summary of all studies can be found in Table 1.

**Disease Severity Score (DSS)**

DSS was performed to monitor the clinical appearance of mice. The DSS was designed to evaluate the clinical evolution of six centronuclear myopathy features; body weight, hanging test ability, kyphosis, hindlimb position while walking, breathing ability, and ptosis, as described previously.14

**Hanging Test**

Mice were placed on a grid (cage lid) and then turned upside down; the suspending animal should hold on to the grid to avoid falling when the grid was then inverted. The latency to fall was measured three times for each mouse. The three trials were performed with a minimum of 5 min interval to allow a recovery period. The latency time measurements began from the point when the mouse was hanging free on the wire and ended with the animal falling to the cage underneath the wire or grid. The maximum time measured was 60 s. The data were expressed as an average of three trials.

**Blood Collection**

Blood samples were collected on EDTA-coated tubes (Microvette 500 K3E, Sarstedt) or Heparin-coated tubes (Microvette 500 LH, Sarstedt), by mandibular puncture or by cardiac puncture on anaesthetized mice. Samples were centrifuged at +4°C during 10 min at 2,000 × g to collect plasma, and then plasma was stored at −20°C until analysis.

**Myostatin ELISA**

Myostatin plasma levels were quantified using a Quantikine ELISA GDF-8/Myostatin Immunoassay kit and according to the manufacturer’s instructions (catalog #DGDF80, RnDSys-tems). The latent form of myostatin was dissociated with an acid activation with 1 N HCl followed by a neutralization step (12 N NaOH/0.5 M HEPES). Total pool of plasma myostatin was then quantified with the quantitative sandwich enzyme immunoassay technique. The optical density was measured at 450 and 540 nm with a Multiskan Go (Thermo Fisher Scientific).

**Quantitative RT-PCR**

Studies 1 and 3: RNA was isolated from organs using NucleoSpin RNA kit (Macherey Nagel). For muscle tissue, the manufacturer “isolation of RNA from fibrous tissue” supplemental protocol was used. Reverse transcription was carried out on 500 ng aliquot using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific). qPCR was done with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) in a Quant Studio 3 Real-Time PCR System (Thermo Fisher Scientific). Studies 2 and 4: Trizol reagent (Invitrogen, UK) was used to extract total RNA from mouse TA muscles, then 1 μg of total RNA was used for reverse transcription using Super- script IV reverse transcriptase (Thermo Fisher Scientific). qPCR was performed in Lightcycler 480 (Roche) using primers mixed in Sybr-Green (QIAGEN). The list of primers used is available in Table S2. The relative expression of mRNAs was normalized to Rpl27. Rpl27 was selected as the normalizing gene after testing of four different candidate genes (Hprt, Rpl27, Rpl41, and P0) in 2 different tissues (liver and TA skeletal muscle) of untreated and treated WT and Mtm1<sup>R465W/+</sup> mice. The Thermo Fisher cloud application was used to select the gene with the best score. This process follows most MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) standards.

**Total RNA Extraction and miRNA Quantification**

Total RNA including miRNA was isolated from muscle tissues with the NucleoSpin miRNA kit (Macherey Nagel) using the manufacturer’s “isolation of RNA from fibrous tissue” supplemental protocol. Reverse transcription of mature miRNA was carried out on 500 ng using the miScript II RT kit (QIAGEN). qPCR was performed using miScript SYBR Green PCR Kit (QIAGEN), miRNA specific forward primers (miR-27 5′-TTCCAGTGGCTAAGTTCGCG-3′; miR-206 5′-TGGGAATGGAATGTTGG-3′) and miScript universal reverse primer (QIAGEN). The expression of mature miRNAs was normalized by the average of Rnu6-2 and Snord61 small non-coding
were loaded, separated in 4% fi
Thermo Fisher Scienti
coupled to horseradish peroxidase (1:5,000, catalog #115-036-068, Jackson ImmunoResearch) or goat anti-rabbit coupled to horseradish peroxidase (1:2,000, catalog #111-036-045, Jackson ImmunoResearch) was incubated for 1 h at room temperature. Immobilon ECL (catalog #WBKLS0100, Millipore) or Pierce ECL (catalog #11517271, Thermo Fisher Scientific) were used and membranes were visualized with a Fusion FX imager (Vilber). Images were quantified using the FIJI software.

Histological Analysis of Skeletal Muscle
Air-dried transverse cryosections (8 μm) were fixed and stained with hematoxylin and eosin (H&E) or succinate dehydrogenase (SDH), and image acquisition performed with a slide scanner NanoZoomer 2 HT equipped with the fluorescence module L11600-21 (Hamamatsu Photonics, Japan). Cross-sectional area (CSA) was analyzed in WGA sections from TA mouse skeletal muscle, using a plugin developed in ImageJ. CSA (μm²) was calculated in >500 fibers per mouse. The percentage of TA muscle fibers with centralized or internalized nuclei was counted in >500 fibers using the cell counter plugin in ImageJ (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD, USA, http://rmb.info.nih.gov/jij/, 1997–2009) or FIJI analysis software. Qualitative SDH staining analysis was performed. Fiber to fiber variation in intensity in SDH staining is normal and indicative of the oxidative state of the fiber. SDH staining is normally relatively homogeneous within each individual fiber. Accumulation within the center or the periphery of a fiber of SDH staining indicates an abnormal distribution.

Human Sample Collection and Analysis
30 healthy control K2 EDTA plasma samples were purchased from Zenbio (catalog #5ER-PLE2ML), including 15 men and 15 women, aged from 19 to 61 years old. Plasma were obtained from consenting adult donors undergoing an elective procedure in the United States who had signed an IRB or FDA validated donor consent form that technically lists both the intended uses for non-clinical research and confirms the procedures for processing the samples are Standard Operating Procedure (SOP) managed GLP protocols in compliance with ethical regulations. Plasma samples from 4 MTM1 and 13 DNM2 mutated patients between 17–72 years old were obtained from the NatHis-CNM Natural History study. The clinical study has been approved by the ERB Paris V1, local ERB in Belgium and Germany, and by the French Regulatory Agency ANSM. It was recorded on clinicaltrials.gov (NCT02057705 / NCT03351270, IDRCB 2013-A00974-41 (France). Blood was collected on K2 EDTA tubes and centrifuged at 2,000 × g for 10 min. Plasma was collected and stored at −20°C or lower.

Statistics
Statistical analyses were performed using ANOVA unless otherwise stated in the figure legend (one-way, or two-way if matched genotype/treatment groups), with p values of <0.05 considered significant. Multiple comparisons were done using t test with the significance threshold corrected by the number of comparisons (0.05/nb comparisons). t tests were also performed when comparing only two groups,
unless otherwise stated in the figure legend. Correlations were done using Pearson’s method.

**ROC Method**

For different thresholds of hanging time (10 s intervals from 10 s to 60 s), a ROC curve was constructed based on the level of myostatin. This descriptive analysis allows us to represent the capability of the predictor (myostatin) to correctly classify the phenotypic reaction (hanging time). The ROC curves translate the true positive rate (well-classified mice with good phenotypic reaction) and the false positive rate (rate of mice classified as having good phenotypic reaction when not the case).

**CONSORTIA**

NatHis-CNM study group contributors: Mélanie Annoussamy, Andreea Seferian, Jonathan Baets, Nicole Voermans, Antony Behin, U Schara, Adele D’Amico, Arturo Hernandez, Capucine de Lattre, Jean-Michel Arnal, Michèle Mayer, Jean-Marie Cuisset, Carole Vullerot, Stéphanie Fontaine, Rémy Bellance. Please see supplemental information for NatHis-CNM study group author affiliations.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.04.022.

**AUTHOR CONTRIBUTIONS**

B.S.C. and J.L. designed the study. C. Koch, S.B., A.M., S.D., and A.R. performed experiments, A.R. performed statistical analysis, C. Kretz, R.G.O., and M.D. provided technical support, L.T. provided scientific advice, L.S. and NatHis-CNM study group provided samples from XL-CNM and AD-CNM patients, J.L. and B.S.C. directed the research, and B.S.C. wrote the manuscript with input from coauthors.

**CONFLICTS OF INTEREST**

B.S.C. and J.L. are coinventors of a patent on targeting DNM2 for the treatment of centronuclear myopathies, and cofounders of Dynacure. B.S.C., C. Koch, S.B., A.M., A.R., R.G.O., M.D., and L.T. are currently employed by Dynacure, and L.S. is on the medical advisory board.

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