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Molecular Therapy
Methods & Clinical Development
Original Article

# miR-223-3p and miR-24-3p as novel serum-based biomarkers for myotonic dystrophy type 1

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**Myotonic dystrophy type 1 (DM1) is the most common adult-onset muscular dystrophy, primarily characterized by muscle wasting and weakness. Many biomarkers already exist in the rapidly developing biomarker research field that aim to improve patients' care. Limited work, however, has been performed on rare diseases, including DM1. We have previously shown that specific microRNAs (miRNAs) can be used as potential biomarkers for DM1 progression. In this report, we aimed to identify novel serum-based biomarkers for DM1 through high-throughput next-generation sequencing. A number of miRNAs were identified that are able to distinguish DM1 patients from healthy individuals. Two miRNAs were selected, and their association with the disease was validated in a larger panel of patients. Further investigation of miR-223-3p, miR-24-3p, and the four previously identified miRNAs, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, showed elevated levels in a DM1 mouse model for all six miRNAs circulating in the serum compared to healthy controls. Importantly, the levels of miR-223-3p, but not the other five miRNAs, were found to be significantly downregulated in five skeletal muscles and heart tissues of DM1 mice compared to controls. This result provides significant evidence for its involvement in disease manifestation.**

## INTRODUCTION

Muscular dystrophies are a group of heterogeneous genetic disorders characterized by progressive loss of skeletal muscle mass. The most common form of muscular dystrophy in adults is myotonic dystrophy type 1 (DM1), affecting ~1 in 8,000 individuals globally. DM1 is an inherited autosomal dominant neuromuscular disorder with increasing disease severity through the generations.[1] The disease initially affects the skeletal muscles, causing progressive skeletal muscle weakness, wasting, and myotonia. Furthermore, DM1 is a multisystemic disorder with many clinical symptoms including heart and central nervous system (CNS) abnormalities.[2] DM1 is caused by a trinucleotide CTG repeat expansion located in the 3′ UTR of the *dystrophia myotonica protein kinase* (*DMPK*) gene.[3,4] Healthy individuals have 5–35 CTG triplets, whereas the number of repeats in DM1 patients varies from 50 to over 1,000 triplets. Individuals with

Received 28 January 2021; accepted 7 September 2021;
https://doi.org/10.1016/j.omtm.2021.09.007.

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36–49 repeats are asymptomatic with a high risk to have offspring with DM1 syndrome.[5]

Genetic tests combined with electromyography, skeletal muscle histopathology, and magnetic resonance are currently the gold standard for the diagnosis of DM1.[6] Creatine kinase activity is the only biochemical marker measured in DM1 patients, but it is not a disease-specific marker and it is not increased in all the patients.[7–9] Moreover, alternative splicing alterations in skeletal muscle tissue have been proposed as disease biomarkers; however, this requires an invasive procedure that causes discomfort and pain to the patients.[10] To date, limited studies have been performed regarding the development of non-invasive biomarkers for DM1, showing promising results on the potential use of microRNAs (miRNAs) as candidate biomarkers.[11–15] miRNAs are small non-coding RNA molecules that regulate numerous biological processes, through mRNA degradation or repression of translation, leading to the regulation of protein production. miRNAs were reported to circulate stably in blood and suggested as candidate biomarkers for the diagnosis, prognosis, or monitoring of various diseases.[16–19] The stability of miRNAs in blood has been ascribed either to the formation of complexes with proteins or to their encapsulation within membrane-bound vesicles, such as exosomes.[20–22]

In DM1, several miRNAs, including miR-1, miR-27b, miR-133a, miR-133b, miR-140-3p, miR-206, miR-454, and miR-574, were reported to be deregulated in DM1 patients compared to healthy individuals. These results suggest that they can be used as diagnostic biomarkers for DM1.[13,14,23,24] Moreover, four miRNAs, miR-1, miR-133a, miR-133b, and miR-206, that are highly expressed in muscle tissues and are also known as myomiRs, were previously reported by our group to be increased in the serum of DM1 patients with progressive muscle wasting compared to patients with non-progressive muscle wasting, suggesting their use as monitoring biomarkers for the progression of the disease.[11] Importantly, we reported that the four myomiRs are encapsulated within small extracellular vesicles (EVs), which were found to be enriched in exosomes, in the blood of DM1 patients and their levels are correlated to the progression of the disease.[12]

The aim of this study was to identify novel serum-based biomarkers for DM1 by analyzing for the first time the entire small RNA population in the serum of DM1 patients and healthy individuals. Using high-throughput small RNA next-generation sequencing (NGS) analysis, we identified a unique signature of miRNAs that are altered in the serum of DM1 patients in comparison to healthy participants. After bioinformatics analysis, we identified two miRNAs, miR-24-3p and miR-223-3p, with high potential to be used as biomarkers in DM1. We further validated the levels of these two miRNAs in a larger cohort of patients and showed that the levels of miR-223-3p and miR-24-3p are elevated in the serum of DM1 patients compared to healthy individuals. We next examined the levels of miR-223-3p, miR-24-3p, and the previously identified four myomiRs, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, in the DM1 animal model DMSXL mice. We showed that the circulating levels of all six miRNAs are elevated in the serum of the DMSXL mice compared to healthy mice. Notably, increased circulating levels of the same miRNAs were detected in the bloodstream of DMSXL mice relative to controls and encapsulated in small EVs, in agreement with the findings in DM1 patients and normal individuals.[12] By examining the levels of these six miRNAs in the skeletal muscles and heart of DMSXL mice, we showed that only the levels of miR-223-3p are downregulated in these tissues in DMSXL mice, thus providing significant evidence for its involvement in the pathogenesis of DM1.

## RESULTS

### miR-223-3p and miR-24-3p levels are elevated in the serum of DM1 patients compared to healthy individuals

Small RNA NGS was initially performed to profile the entire spectrum of small RNA molecules, including miRNAs, present in serum samples of nine DM1 patients and eight gender- and age-matched healthy participants (Table S1). All the samples underwent NGS analysis to identify their small RNA content, revealing an average of total 7,034,228 reads per run. Bioinformatics analysis of the small RNA NGS data revealed a number of circulating miRNAs whose levels are either higher or lower in DM1 patients compared to healthy individuals (Figure 1). Since the RNA NGS run was performed on a low number of samples, miRNAs were selected for further investigation based on the uncorrected p values with significant difference ($p < 0.05$) between DM1 patients and healthy individuals. The miRNAs were further analyzed in order to select the subset of the best biomarker candidates for validation in a larger number of samples. The first criterion used to narrow down the list of the identified miRNAs was their abundance in serum, thus ensuring accurate detection by real-time PCR. Their abundance in serum was evaluated based on their counts per million reads revealed by the NGS analysis. The second criterion was the expression of the identified miRNAs in skeletal muscle tissue. Since DM1 is a disorder primarily affecting skeletal muscles, we excluded the identified miRNAs that were poorly expressed in muscle tissues.

Based on these criteria, two miRNAs, miR-223-3p and miR-24-3p, were chosen for experimental validation in a larger number of patients by real-time PCR. All patients were previously diagnosed with DM1, characterized with variable disease severity, progression, and age of onset, and assigned a Muscular Impairment Rating Scale (MIRS) grade (Table S1). Both miR-223-3p and miR-24-3p showed a statistically significant difference between the DM1 patients and healthy individuals in small RNA NGS analysis ($p < 0.05$) and are highly expressed in skeletal muscle tissue, known to promote muscle cell differentiation (Figure 1).[25–29] Notably, miR-223-3p was also reported to be dysregulated in skeletal muscle tissues affected by another muscular dystrophy, Duchenne muscular dystrophy (DMD), in both the DMD animal model mdx mice and DMD patients.[30]

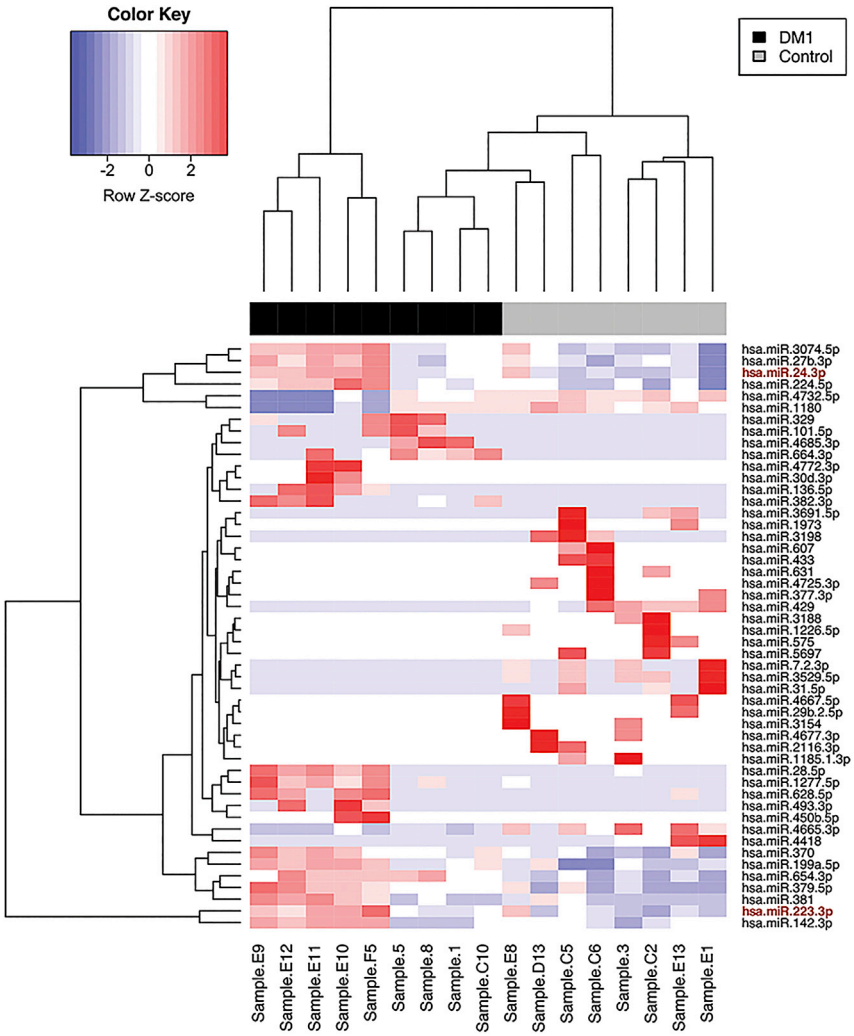



**Figure 1. Heatmap of the most differentially expressed miRNAs and hierarchical clustering**

Nine DM1 and eight gender- and age-matched healthy participants were analyzed by NGS. The controls group (healthy individuals) is shown in gray color, and the DM1 patients group is shown in black color. The color key panel shows the *Z* score values calculated for each miRNA by subtracting the row mean and then dividing by the standard deviation. *Z* scores describe the expression of each miRNA in relation to the mean. Overexpressed miRNAs are shown in red and underexpressed miRNAs in blue. White color indicates expression change close to 0. miRNAs in red font were selected for further analysis.

Serum samples were isolated from sixty DM1 patients and sixty healthy participants, followed by miRNA extraction. Real-time PCR analysis showed that the levels of miR-223-3p and miR-24-3p are significantly elevated in the serum samples of DM1 patients compared to healthy participants ($p < 0.001$) (Figures 2A and 2B; Figure S2). The levels of these two miRNAs were normalized to the levels of the ubiquitously expressed miR-16-5p, which was used as an internal control.[31–33] In order to confirm that miR-16-5p is a suitable normalization control for the two miRNAs, statistical analysis was performed showing no correlation of miR-16-5p levels with age ($p \approx 0.68$), DM1 pathology, and gender (Figure S1). Receiver operating characteristic (ROC) analysis was obtained by plotting the true positive (sensitivity) versus false positive (1 − specificity). The area under the curve (AUC > 0.98 for miR-223-3p and > 0.86 for miR-24-3p) suggests that the serum levels of miR-223-3p and miR-24-3p can discriminate DM1 patients from healthy individuals with high specificity (Figures 2C and 2D). Additional statistical analysis revealed that both miR-223-3p and miR-24-3p levels are independent of gender, age of onset, severity, progression, and MIRS grade of the patients (Figures S3 and S4).

### The circulating levels of miR-24-3p, miR-223-3p, and the four myomiRs are elevated in the serum of DMSXL mice

miRNAs were reported to circulate in the blood in a cell-free manner either encapsulated within membranous particles, such as exosomes, or in complexes with proteins.[22,34–36] We have previously reported that the four myomiRs, miR-1, miR-133a, miR-133b, and miR-206, are promising monitoring biomarkers for DM1 since they were detected to be increased in the blood circulation of DM1 patients.[11] Although there are some promising reports regarding the presence of circulating biomarkers in DM1 patients, there is no information regarding any disease biomarkers in DM1 mouse models. The establishment of blood biomarkers in disease mouse models would allow proper monitoring of their symptoms during the development of therapeutic strategies, thus enabling the efficient assessment of candidate drugs and therapies before clinical trials. Several DM1 mouse models have been developed, but few recapitulate the multisystemic character of the disease, with DMSXL being one of them.[37,38] DMSXL mice are transgenic mice carrying the human DM1 locus with over 1,000 CTG repeats. Mice that carry both transgenes (homozygous; DMSXL) exhibit the majority of DM1 symptoms, whereas mice that have one (heterozygous) or no (wild type; WT) copy of the transgene do not show any of the DM1 symptomatology.[39,40] In this study, gender- and age-matched DMSXL and WT mice were employed to further examine miR-223-3p, miR-24-3p, and the four myomiRs as possible biomarkers in the DMSXL mouse model. Total RNA, enriched in miRNAs, was extracted from the serum of 6-week-old DMSXL and WT mice and investigated for the presence of the six miRNAs. At this age, DMSXL mice of both genders weigh 8–12 g, whereas WT mice of the same litter weigh 16–18 g. For the normalization of the six miRNA levels, spike-in control cel-miR-39 was employed. Statistical analysis revealed that exogenous cel-miR-39 is independent of

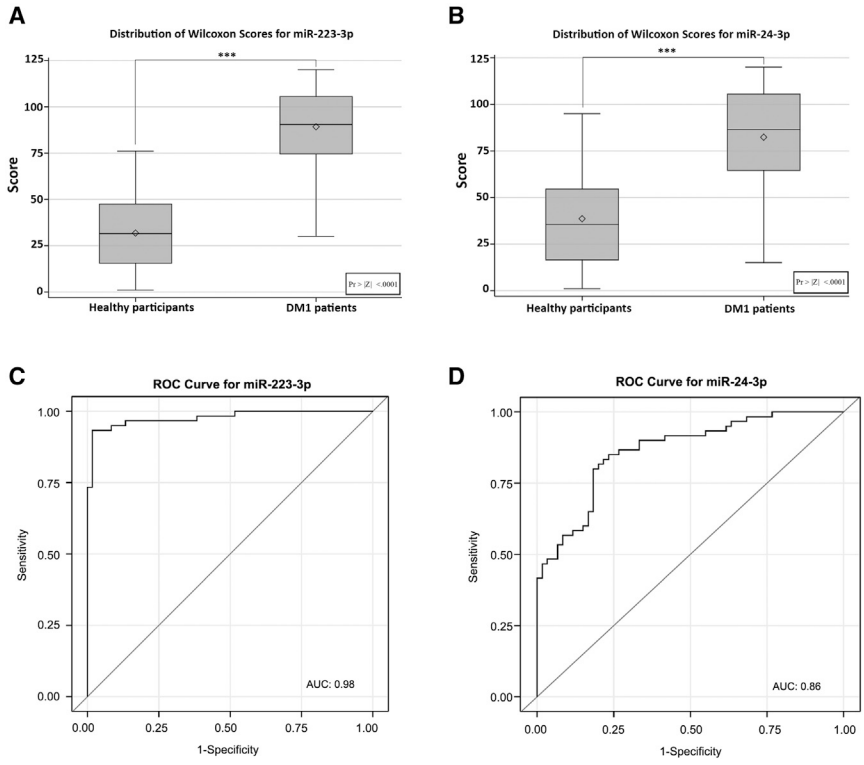



**Figure 2. miR-223-3p and miR-24-3p levels are elevated in the serum of DM1 patients**

Serum samples from sixty DM1 patients and sixty healthy participants were analyzed with real-time PCR for the presence of miR-223-3p and miR-24-3p. (A and B) Distribution charts of miR-223-3p (A) and miR-24-3p (B) levels in DM1 patients and healthy participants show elevated levels of the two miRNAs in DM1 patients' serum compared to healthy participants. Horizontal lines inside the boxes mark the medians. Mean expression values are marked with rhombus. Two-sided Pr > |Z| p values of Wilcoxon test are indicated in the boxes. ***$p < 0.0001$. (C and D) ROC curve analyses using serum miR-223-3p (C) and miR-24-3p (D) for discriminating healthy participants from DM1 patients. AUC values suggest that serum levels of miR-223-3p and miR-24-3p can discriminate DM1 patients from healthy individuals extremely well.

the DM1-related pathology in mice (Figure S5A). Real-time PCR analysis revealed that the six miRNAs under investigation are elevated in the serum of DMSXL mice in comparison to the unaffected controls (Figure 3).

### The miR-223-3p, miR-24-3p, and four myomiR encapsulated levels within small EVs are increased in the serum of DMSXL mice

We have previously reported that the whole population of the four myomiRs is encapsulated within circulating small EVs, enriched in exosomes, in the serum of DM1 patients.[12] We therefore next investigated whether the levels of the six miRNAs, miR-24-3p, miR-223-3p, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, that are elevated in the blood of DM1 patients and DMSXL mice are also encapsulated within small EVs circulating in the blood of mice. EVs were initially isolated from the serum of 6-week-old mice by employing a commercial polymer-based precipitation method. The purity of this method is not optimal, since it isolates vesicular and non-vesicular components of the serum, including exosomes. Scanning electron microscopy (SEM) and protein analysis of the exosomal marker proteins indicated that the isolated serum small EVs contain exosomes (Figure 4). SEM micrographs of the small EVs revealed round exosome-like structures (Figure 4A). The diameter of the isolated small EVs was determined by tunable resistive pulse sensing (TRPS) analysis to range from 70 to 140 nm, with the majority of them in the range of 90–110 nm, similar to previously described exosomes (Figure 4B).[41] The isolation of small EVs was further confirmed by western blot for the exosomal marker proteins. The exosomal marker molecules CD63, CD81, and TSG101 were detected in the small EV samples at higher amounts compared to whole cell lysate extracts, further supporting the presence of exosomes in the lysates (Figure 4C). After the analysis of the small EV properties, we investigated whether the six miRNAs, miR-24-3p, miR-223-3p, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, are encapsulated within the small EVs in the serum of 6-week-old DMSXL and WT littermates. Real-time PCR analysis confirmed the presence of all six miRNAs within the small EVs isolated from the serum of both DMSXL and WT mice (Figures 4D–4I). Importantly, the encapsulated levels of the six miRNAs were found to be significantly elevated in the serum of DMSXL mice compared to control mice ($p < 0.05$) (Figures 4D–4I). The spike-in control cel-miR-39 was used for the normalization of the six miRNA levels within the small EVs. Statistical analysis confirmed that cel-miR-39 levels are stable in all EV samples (Figure S5B). We next investigated whether the differences in the six miRNA levels encapsulated within small EVs observed between the DMSXL and control samples are associated with the different number of small EVs circulating in the serum of the affected and unaffected mice. Quantification analysis with the TRPS method therefore followed, showing that the small EVs isolated from the serum of either DMSXL or WT mice do not have significant differences (Figure S6A). Subsequent normalization of the six miRNA levels over the number of small EVs confirmed that small membranous vesicles isolated from the serum of DMSXL mice are highly abundant in miRNAs relative to the WT mice (Figures S6B–S6G). Consequently, the elevated miRNA levels encapsulated in serum small EVs of the affected mice are possibly associated with the increased packaging of the specific miRNAs in the small EVs in the tissues of origin of the circulating EVs, which may be related to the DM1 multisystemic pathogenesis.

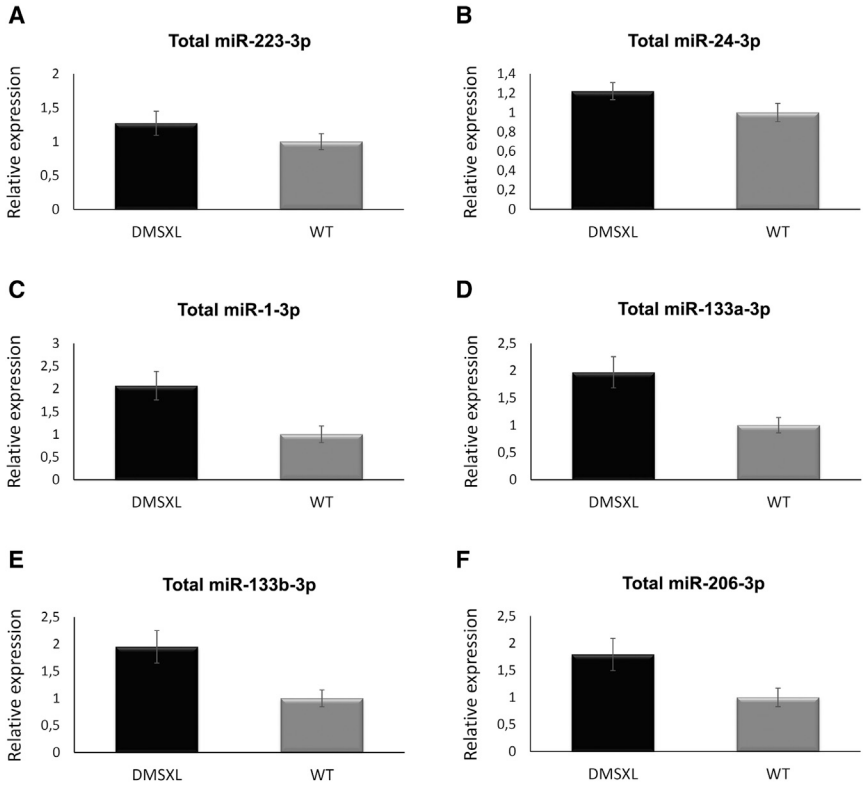



**Figure 3. Cell-free miR-223-3p, miR-24-3p, and the four myomiRs are increased in the bloodstream of DMSXL mice**

Total RNA, enriched in miRNAs, was isolated from serum samples of 6-week-old DMSXL and WT mice (n = 5) and analyzed for the abundances of miR-223-3p (A), miR-24-3p (B), miR-1-3p (C), miR-133a-3p (D), miR-133b-3p (E), and miR-206-3p (F). All miRNA levels were found to be elevated in DMSXL serum samples compared to WT mice. Graphs represent the relative expression of miRNA levels in DMSXL mice compared to controls.

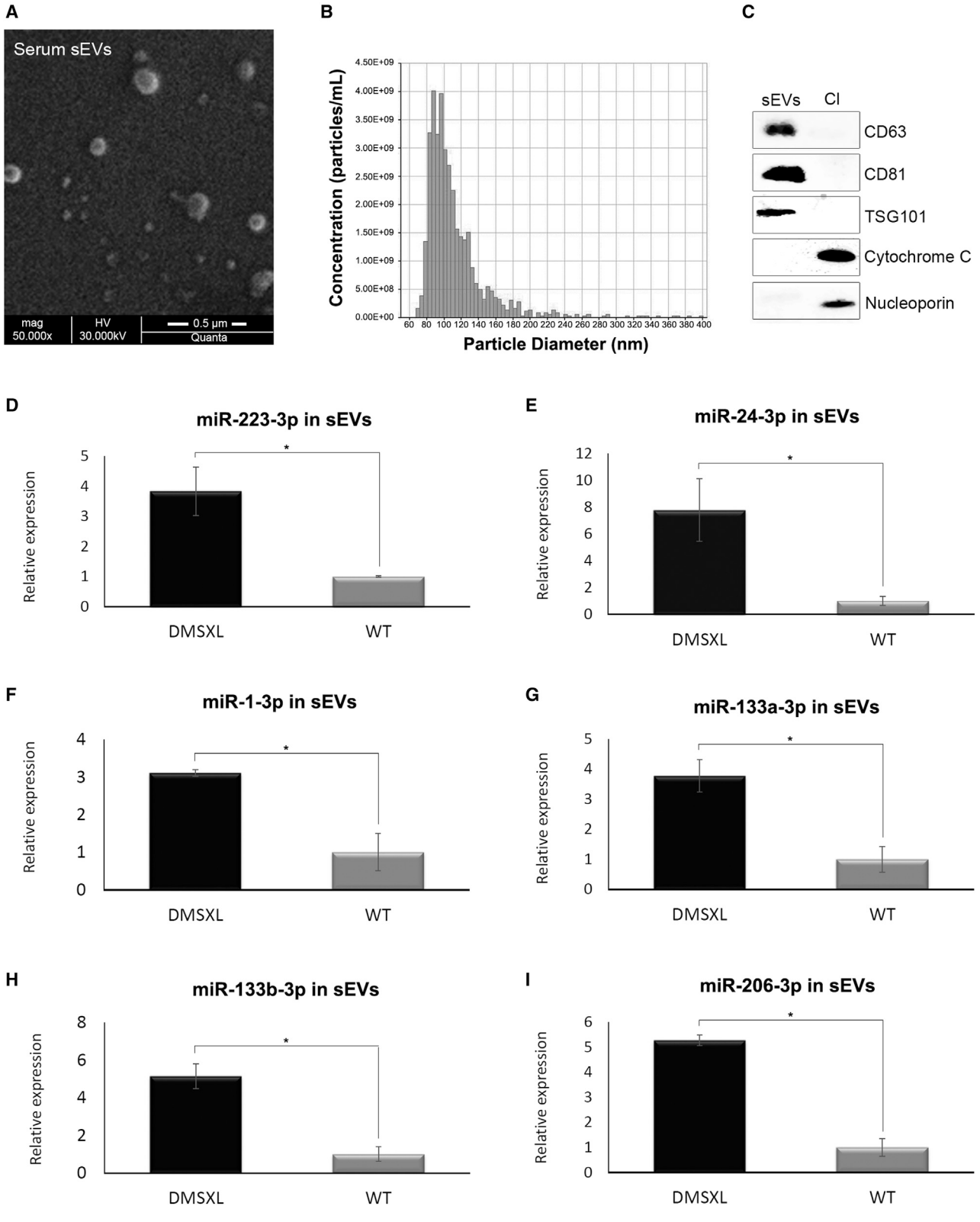
### miR-223-3p is downregulated in skeletal muscle and heart tissues of DMSXL mice

After the identification of the six elevated miRNA levels in small EVs circulating in the blood of DMSXL mice compared to controls, their endogenous levels in five skeletal muscles of mouse hindlimbs were next investigated, to identify any disease-related alterations of the miRNA expression in DM1-affected tissues. More specifically, tibialis anterior (TA), soleus, extensor digitorum longus (EDL), gastrocnemius, and quadriceps were collected from 6-week-old DMSXL and WT mice and analyzed for the endogenous levels of miR-223-3p, miR-24-3p, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p. The endogenous levels of the six miRNAs were normalized over the endogenous control, snoR-135. Statistical analysis showed that the levels of snoR-135 are stable between skeletal muscle tissues of DMSXL and WT mice (Figures S7A–S7E). Analysis of the real-time PCR data revealed that miR-223-3p levels expressed in all five skeletal muscle tissues are significantly decreased in DMSXL mice as opposed to littermate controls ($p < 0.05$) (Figure 5A). In contrast, the endogenous expression levels of miR-24-3p and the four myomiRs, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, showed no difference between DMSXL mice and controls (Figures 5B–5F).

The heart is a smooth muscle tissue affected in DM1 patients.[42,43] The two miRNAs identified in the present study, miR-223-3p and miR-24-3p, were previously reported to be expressed in heart tissue and to regulate cardiac fibrosis after myocardial infarction.[25,44,45] miR-24-3p was also reported to act as a pro-survival molecule that inhibits the apoptosis of cardiomyocytes.[46] Furthermore, the four myomiRs, miR-1, miR-133a, miR-133b, and miR-206, were found to be involved in the development of heart diseases.[47,48] The endogenous expression levels of the six miRNAs in heart tissues of DMSXL and WT mice were therefore examined next. Total RNA, enriched in miRNAs, was isolated from the heart tissues of 6-week-old DMSXL mice and controls and analyzed for the endogenous levels of the six miRNAs. The endogenous levels of miR-223-3p in the heart tissue of DMSXL mice were significantly decreased compared to asymptomatic mice ($p < 0.05$) (Figure 6A). In contrast, the endogenous levels of miR-24-3p, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p showed no significant difference between heart tissues of DMSXL mice and littermate controls, similar to the results obtained from the investigation of the five skeletal muscles (Figures 6B–6F).

### The six miRNAs are predicted to target a diverse set of genes and biological processes

The human miR-223-3p and miR-24-3p identified as biomarkers in DM1 patients in our study were screened computationally against a number of available miRNA databases to identify known and predicted gene targets and to provide information regarding their involvement in biological pathways. Through this analysis five gene targets were predicted to be common in both miR-24-3p and miR-223-3p (*ZXDA*, *EMC2*, *ERCC6L2*, *MARCHF10*, and *PLEKHH2*) (Figure 7A). The same analysis was also performed for the mouse miRNAs, miR-24-3p, miR-223-3p, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, revealing a number of unique and shared predicted gene targets (Figure 7B). miR-133a-3p and miR-133b-3p were predicted to share almost the same gene targets, as they differ by a single nucleotide at the 3′ end, and therefore these two miRNAs were grouped as miR-133. Most of the targets of miR-206 are common with miR-1-3p, due to the fact that these miRNAs share the same seed region and differ by only four nucleotides. Considering the four myomiRs analyzed, miR-1-3p and miR-133-3p share a common gene target, whereas miR-1-3p, miR-133-3p, and miR-206-3p

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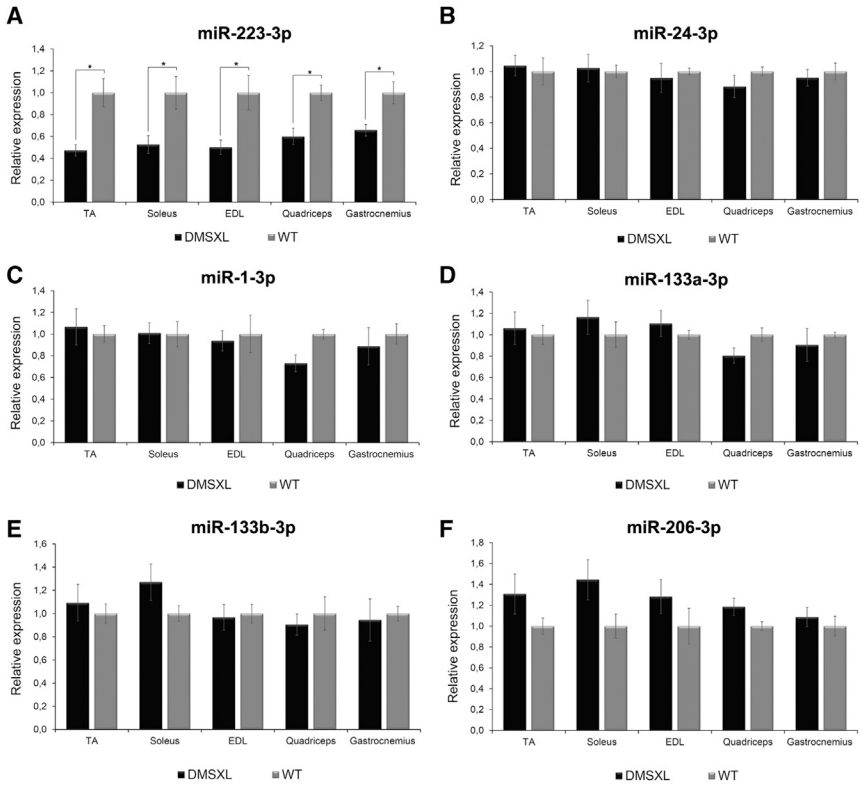



**Figure 5. The endogenous levels of miR-223-3p are decreased in five skeletal muscle tissues in DMSXL mice**

Five skeletal muscle tissues, TA, soleus, EDL, gastrocnemius, and quadriceps, located on the hindlimbs of 6-week-old DMSXL and control mice (n = 5) were harvested and analyzed for the endogenous levels of miR-223-3p (A), miR-24-3p (B), miR-1-3p (C), miR-133a-3p (D), miR-133b-3p (E), and miR-206-3p (F). miR-223-3p expression levels were decreased in all five skeletal muscle tissues of DMSXL mice compared to WT mice. The endogenous levels of miR-24-3p and the four myomiRs showed no significant changes in DMSXL mice as opposed to controls. Graphs represent the relative expression of miRNA levels in DMSXL mice relative to WT mice. *p < 0.05.

share two common gene targets in mice. Furthermore, one gene target is common in miR-1-3p, miR-206-3p, and miR-24-3p (*Matr3*), two gene targets are common in miR-1-3p, miR-206-3p, and miR-223-3p (*Eva1a* and *Hsp90b1*), and three targets are common in miR-133-3p and miR-223-3p (*Glra2*, *Septin6*, and *Tm45f1*) (Figure 7B).

To investigate the potential functional and biological impact when these gene targets are dysregulated, a functional analysis using the PANTHER Classification System through the Gene Ontology (GO) web server was next performed.[49,50] The functional analysis aimed to identify the most relevant GO biological processes in which the predicted target genes of the dysregulated miRNAs are involved. The most enriched GO biological processes (levels 4 up to 16) were found to relate to muscle and neuron developmental and functional processes (Figures 7C–7F). In all miRNA groups tested (hsa-miR-24-3p and hsa-miR-223-3p; mmu-miR-24-3p and mmu-miR-223-3p; mmu-miR-1-3p, mmu-miR-133a-3p, mmu-miR-133b-3p, and mmu-miR-206-3p), we found several such related processes, although mmu-miR-24-3p was predicted to be involved in most biological processes of interest (Figure 7D). Although the two mouse miRNAs, miR-24-3p and miR-223-3p, were predicted to regulate common biological processes such as development of the nervous system, several non-overlapping processes were also predicted (Figures 7D and 7E). An example of these biological processes is the involvement of miR-223-3p in response to stress in cardiac muscle adaptation and cardiac muscle hypertrophy. The full set of the predicted gene targets and the corresponding functional analysis are given in the Supplemental information (Tables S2, S3, S4, and S5).

## DISCUSSION

DM1 is the most common type of muscular dystrophy in adults and the second most common type of muscular dystrophy, after DMD. In recent years, emphasis has been placed on examining the potential use of circulating miRNAs as a promising tool for the diagnosis and monitoring of various diseases, including muscular dystrophies.[15,51] Although there are several studies focusing on the development of biomarkers in muscular dystrophies, few reports exist regarding the use of such circulating miRNAs as biomarkers for DM1.[11–14,23,24] Initially, nine miRNAs were found to be deregulated in plasma of DM1 patients compared to control subjects and suggested as diagnostic biomarkers for DM1.[13] In a following report, we showed that the levels of four myomiRs, miR-1-3p, miR-133a-3p, miR-133b-3p,

**Figure 4. The levels of miR-223-3p, miR-24-3p, and the four myomiRs are elevated in serum small EVs of DMSXL mice**

(A) Small EVs isolated from serum samples of 6-week-old DMSXL and control mice were imaged by SEM. SEM images at 60,000× magnification revealed rounded exosome-like structures. Scale bar, 500 nm. (B) Quantification and size analysis of serum small membranous particles using TRPS showed that small EVs isolated from murine serum were within the size range of exosomes. (C) Western blot analysis of exosomal markers CD63, CD81, and TSG101 confirmed that the isolated EVs from murine serum samples include exosomes. Cytochrome C and nucleoporin were also analyzed as negative controls. Lysates from C2C12 cells served as controls. (D–I) Serum small EVs of DMSXL and WT mice (n = 5) were further analyzed for the presence of miR-223-3p (D), miR-24-3p (E), miR-1-3p (F), miR-133a-3p (G), miR-133b-3p (H), and miR-206-3p (I). All miRNA levels under investigation were found to be increased in circulating small EVs in DMSXL mice compared to WT. Graphs represent the relative expression of miRNA levels in DMSXL mice relative to controls. *p < 0.05; sEVs, small extracellular vesicles; Cl, cell lysates.

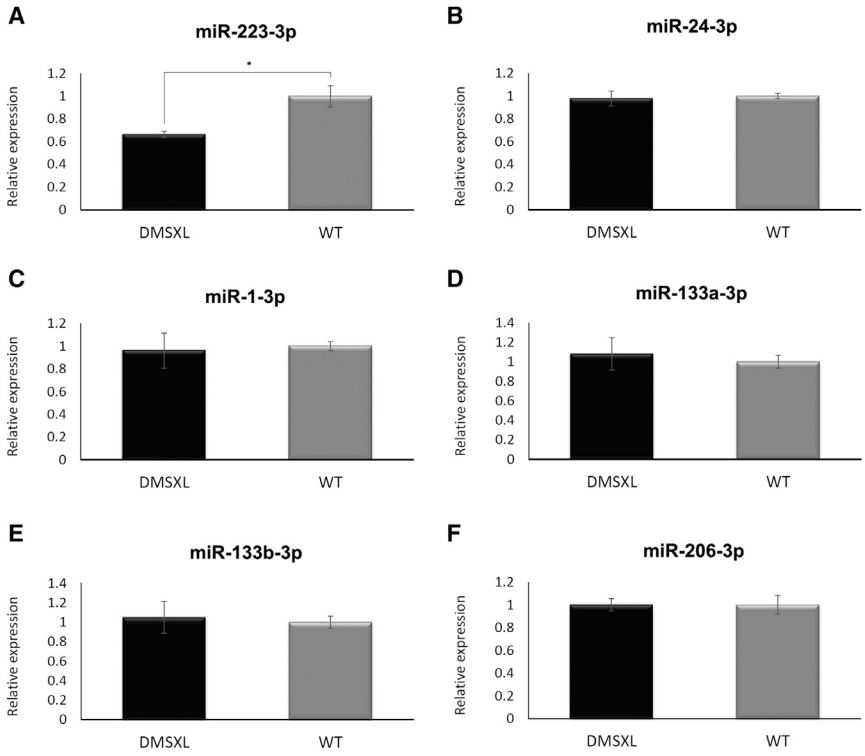


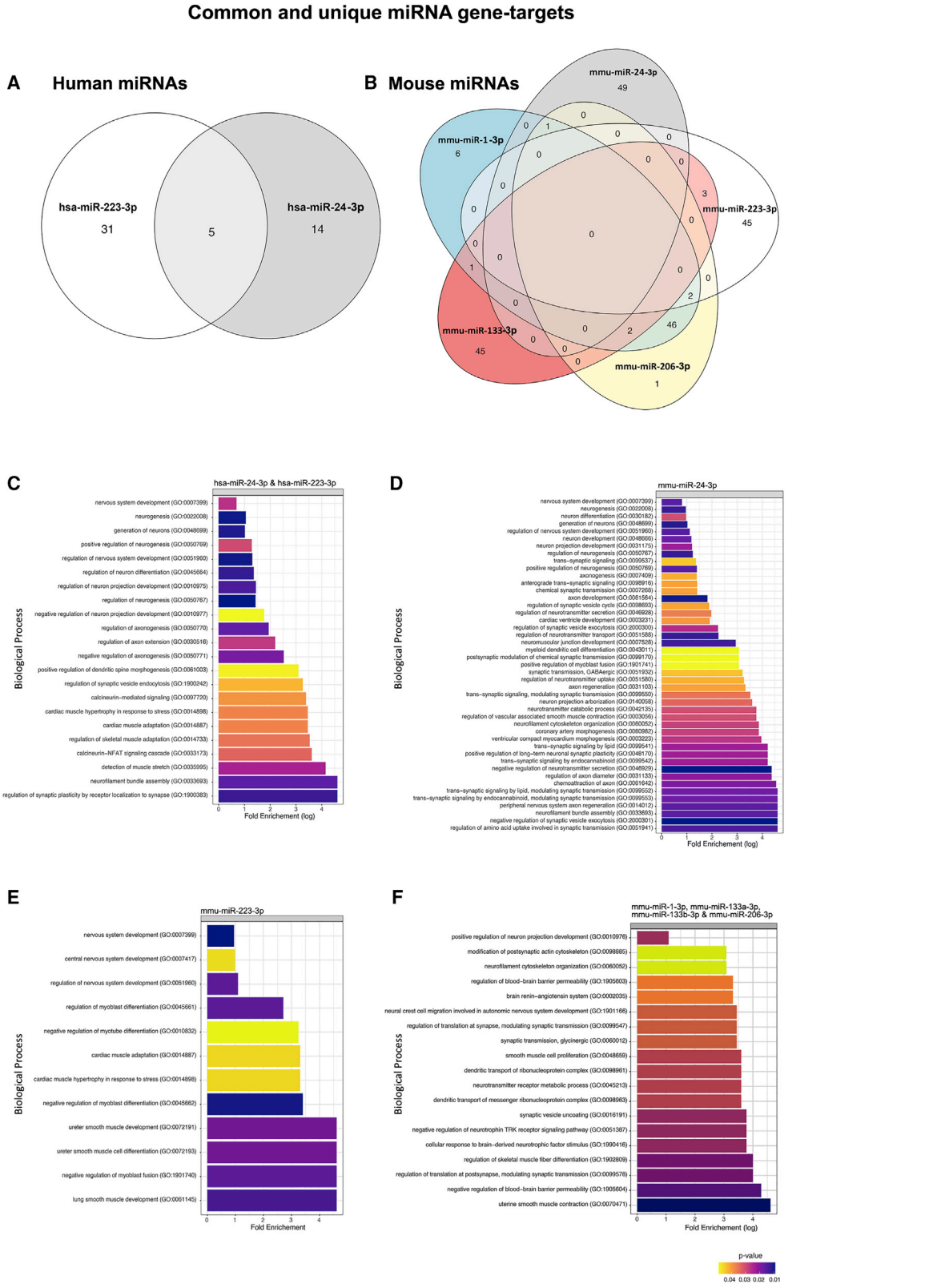

**Figure 6. The endogenous levels of miR-223-3p are decreased in the heart of DMSXL mice**

Heart tissues were harvested from 6-week-old DMSXL and control mice (n = 5), and the endogenous levels of miR-223-3p (A), miR-24-3p (B), miR-1-3p (C), miR-133a-3p (D), miR-133b-3p (E), and miR-206-3p (F) were measured. The expression levels of miR-223-3p were decreased in the cardiac tissue of DMSXL mice compared to WT mice. miR-24-3p and the four myomiR levels revealed no significant changes in DMSXL mice compared to controls. Graphs represent the relative expression of miRNA levels in DMSXL mice relative to WT mice. *p < 0.05.

and miR-206-3p, are elevated in the serum of DM1 patients compared to control subjects and, more importantly, their levels were determined to be associated with the disease progression.[11] Specifically, the myomiR levels were reported to be significantly elevated in DM1 patients with progressive muscle wasting compared to patients with non-progressive muscle wasting (stable patients), suggesting their potential use as monitoring biomarkers for the disease progression.[11] In a larger group of DM1 patients, eight miRNAs, miR-1, miR-27b, miR-133a, miR-133b, miR-140, miR-206, miR-454, and miR-574, were also validated as plasma-based biomarkers for DM1.[14] Interestingly, we reported that the whole population of the four myomiRs is encapsulated within small EVs, which were also found to be enriched in exosomes, in the serum of DM1 patients. Their encapsulated levels were correlated to the progression of the disease.[12]

In this work we aimed to identify novel miRNA biomarkers for DM1 by analyzing, for the first time, the entire population of small RNA molecules in the serum samples from DM1 patients and healthy individuals. By performing high-throughput small RNA NGS analysis, we identified miRNAs that are altered in the serum of DM1 patients. Based on bioinformatics analysis and expression studies in literature, two novel miRNAs, miR-223-3p and miR-24-3p, were chosen for further investigation for their potential use as miRNA biomarkers. Notably, the four myomiRs, miR-1, miR-133a, miR-133b, and miR-206, that have been previously validated as blood biomarkers for DM1 were not listed as differentially expressed miRNAs with high statistical significance.[12,16,23,24] The low circulating myomiR levels in the blood of patients and the low sensitivity of the current NGS technology led to their detection with low statistical significance. miR-223-3p and miR-24-3p were further validated in a larger cohort of patients showing promising results. Specifically, both miRNAs were found to be significantly increased in the serum of DM1 patients compared to healthy individuals. ROC analysis showed that miR-223-3p is characterized by higher sensitivity and specificity than miR-24-3p. The sensitivity and specificity of the miR-223-3p are almost the same as the four myomiRs previously reported as serum biomarkers of DM1, thus suggesting that these five miRNAs, miR-223-3p, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, can discriminate DM1 patients from healthy individuals extremely well (AUC = 0.96–0.98).[11,12] Conversely, miR-24-3p can discriminate DM1 patients from healthy individuals with slightly lower sensitivity and specificity (AUC = 0.88). Taking into consideration the interpatient variability observed in the DM1 patients of this study, which arises from the differences in the severity and progression of the symptoms of each patient, both miRNAs miR-223-3p and miR-24-3p can be suggested as reliable DM1 biomarkers.

We have previously reported that the four myomiRs, miR-1, miR-133a, miR-133b, and miR-206, circulating in the blood of DM1 patients are encapsulated within small EVs.[12] EVs are a heterogeneous group of membranous vesicles released from cells and tissues.[52,53] They can be found in most of the biological fluids like serum, saliva, and urine, and their molecular cargo was suggested to be a promising source for the development of biomarkers.[54,55] In order to further investigate the possible reasons underlying the existence of the six miRNAs in the blood of DM1 patients, the transgenic animal model for DM1, DMSXL, was used. DMSXL mice show molecular DM1 features such as muscle wasting, nuclear foci, and splicing defects as well as muscle dysfunction, reduced muscle strength, and low motor performance.[39] These mice also recapitulate some molecular features of DM1 leading to physiological abnormalities.[39] Although DMSXL mice share similar features with DM1 patients, there are no reports

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regarding the development of biomarkers in this animal model. Serum miRNA analysis showed that all six miRNAs, miR-24-3p, miR-223-3p, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, are increased in the blood circulation of the diseased DMSXL mice relative to the healthy littermate controls. More importantly, we demonstrated that small EVs circulating in the serum of the DMSXL mice encapsulate elevated levels of these miRNAs compared to WT mice. This implies that all six miRNAs are abnormally packaged and released in the blood circulation in the DMSXL mouse model. A limitation of the present study is that the method employed for the isolation of the small EVs results in the co-isolation of non-vesicular impurities together with the membranous vesicles. However, small EVs, such as exosomes, were also isolated, as demonstrated by the analysis of the vesicles' properties. It is noteworthy that the encapsulation of the four myomiRs within small EVs in the blood of DMSXL mice is consistent with the reported results in DM1 patients, implying that DM1 patients and DMSXL mice share similar abnormal pathways for the miRNA-sorting mechanisms within the secreted small EVs.[12] These results provide novel evidence for the potential use of these miRNAs as biomarkers in the DMSXL animal model, facilitating their use not only for the study of DM1 pathogenesis but also for the development of efficient DM1 therapies. Whether the two new biomarkers reported in this study, miR-223-3p and miR-24-3p, are specific to the DM1 pathology remains to be elucidated in future studies.

An in-depth investigation of the levels of the six miRNAs in skeletal muscle and cardiac tissues in DMSXL mice revealed notable differences in the endogenous expression levels of these miRNAs. In particular, the endogenous levels of miR-24-3p as well as the four myomiRs showed no significant changes in the five skeletal muscle tissues of the hindlimbs of DMSXL mice compared to controls. Similarly, none of the four myomiRs or miR-24-3p showed any difference in their endogenous expression levels in heart tissues of DMSXL mice relative to unaffected mice. These results show that the endogenous expression of the five miRNAs, miR-24-3p, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, is not affected by the disease, either in skeletal muscle or in the cardiac tissues in DMSXL mice. Thus, it is suggested that DM1-associated pathways possibly underlying the abnormal release of the five miRNAs in the blood of the affected DMSXL mice are yet to be discovered. Further investigation of the involvement of these miRNAs in the pathogenesis of secondary symptoms will shed light on the multisystemic character of DM1 in human patients.

Interestingly, the endogenous expression levels of miR-223-3p were found to be significantly decreased in five skeletal muscles and the heart of DMSXL mice compared to asymptomatic WT mice. miR-223-3p is an inflammatory miRNA and has been reported to be involved in the promotion of skeletal muscle regeneration after injury in mice, by regulating pro- and anti-inflammatory signals.[76] Furthermore, miR-223-3p has been associated with dysregulated glucose uptake in murine cardiomyocytes and with heart failure in diabetic patients.[77] Taking into consideration our findings and published reports, it is suggested that miR-223-3p is involved in pathways underlying the pathogenesis of DM1-related symptoms in the DMSXL muscles and, therefore, probably associated with similar phenotypes in DM1 patients. For instance, the decreased miR-223-3p levels in skeletal muscles of DMSXL mice may be related to dysregulated muscle regeneration activity, leading to the observed muscle wasting and atrophy. Moreover, the low endogenous levels of miR-223-3p and its elevated circulating levels in blood may be ascribed to the inability of tissues to compensate the loss of this specific miRNA following its secretion into the extracellular environment. Furthermore, the lower levels of miR-223-3p in skeletal muscle tissues and heart possibly affect the expression of its targets, thus causing downstream pathogenic DM1 events in these tissues, such as insulin resistance.[77]

Bioinformatics analysis of the human miR-24-3p and miR-223-3p showed that both miRNAs are implicated in the regulation of the nervous system development and mediate CNS homeostasis. It has been previously reported that miR-24-3p is implicated in pathways regulating neuronal differentiation, and, more importantly it was proposed as a plasma-based biomarker for CNS diseases such as Parkinson disease.[56,57] In addition, miR-223-3p has been described to protect neural cells from degeneration by regulating the expression of neuroprotective genes.[58,59] The CNS is affected in DM1 patients, who show patterns of psychological dysfunction, intellectual disability, excessive daytime sleepiness, and neuropathological abnormalities. Taking into consideration the reported brain-specific functions of the two miRNAs, the CNS-related symptoms in DM1 may be associated with the observed upregulated detection of these miRNAs in the blood of DM1 patients.[60]

In-depth bioinformatics analysis of mouse miR-24-3p, miR-223-3p, and the four myomiRs showed that only miR-24-3p shares a common biological process with the four myomiRs, neurofilament cytoskeleton organization, whereas miR-223-3p was found to be involved in independent biological processes with the other investigated miRNAs, such as CNS development and cardiac muscle function. These results provide evidence that the two novel miRNAs reported in this study are involved in different pathways in comparison with the four myomiRs. Overall, the decreased miR-223-3p endogenous levels and the different biological processes compared to the other

**Figure 7. Target prediction and pathways analysis of miR-223-3p, miR-24-3p, and the four myomiRs**

(A) Gene target prediction analysis of the investigated human miR-223-3p and miR-24-3p. Human miR-223-3p and miR-24-3p were predicted to share five common gene targets. (B) Gene target prediction analysis of the mouse miRNAs showed that miR-223-3p and miR-24-3p have not any common predicted targets; myomiRs share five common gene targets with miR-233-3p and only one with miR-24-3p. (C–F) Pathway analysis of the predicted target genes of the miRNAs showed different biological processes that are predicted to be involved: pathway analysis of human miR-223-3p and miR-24-3p (C), mouse miR-24-3p (D), mouse miR-223-3p (E), and mouse myomiRs (F). hsa, *Homo sapiens*; mmu, *Mus musculus*.

miRNAs that involve miR-223-3p provide information for possibly novel pathways that may be altered in DM1.

In conclusion, we investigated for the first time the entire small RNA population in the serum of DM1 patients, and we suggested two novel miRNAs, miR-24-3p and miR-223-3p, that can be used as potential biomarkers for the disease. We further examined the levels of miR-223-3p, miR-24-3p, and the four myomiRs, miR-1-3p, miR-133a-3p, miR-133b-3p, and miR-206-3p, which were previously suggested as monitoring biomarkers for the disease progression, in the DMSXL mouse model. Both miRNAs as well as the four myomiRs were found to be increased circulating in the serum of DMSXL mice compared to controls and were proposed for the first time as potential biomarkers for this DM1 animal model. Importantly, we showed that the endogenous levels of miR-223-3p are reduced in skeletal muscles and heart tissues of DMSXL mice, providing significant evidence for its role in the manifestation of the disease.

## MATERIALS AND METHODS

### Participant inclusion, blood collection, and isolation of serum

The study was permitted by the National Bioethics Committee of Cyprus, and experiments were carried out according to the Declaration of Helsinki principles. All human individuals provided a written informed consent to participate and deliver blood specimens for the project. All DM1 patients were formerly diagnosed by (1) the Diagnostic Department, Cyprus Institute of Neurology and Genetics using Southern blot technique, (2) the Diagnostic Department, Eginitio Hospital in Greece, and (3) the Medical University of Warsaw in Poland. For the purposes of the project, all patients were physically examined prior to study enrollment. Healthy participants did not have a family history of muscle disease. Information regarding the gender, age, progression, age of onset, MIRS scale, and severity of the DM1 patients is included in Table S1. The age and gender of the healthy participants are also summarized in Table S1. After clinical examination, a total of 4 mL of whole blood was drawn from all participants and placed in serum collection tubes (BD Vacutainer, Franklin Lakes, NJ, USA). For DM1 patients, blood collection for miRNA analysis was performed after their last clinical examination. Blood samples were allowed to form a clot for an hour at room temperature before being centrifuged at 3,000 rpm for 5 min at room temperature. Serum was subsequently isolated from the samples and stored at −80°C in DNase- and RNase-free tubes.

### Small RNA-next-generation sequencing

After serum collection, total RNA, including miRNAs, was extracted from serum samples with the miRNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Small RNA-NGS libraries were constructed from 5 μL of RNA with the QIAseq miRNA Library Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Library concentrations were measured with Qubit dsDNA HS Assay Kit (Thermo Fisher, Waltham, MA, USA). Quality and concentration of libraries were determined by real-time PCR. Libraries were sequenced on a NextSeq 500 System (Illumina, San Diego, CA, USA) in 1 × 75 bp single-end sequencing mode.

### Raw RNA-NGS data analysis

The raw data were processed for QIASeq adaptor filtering and unique molecular identifier (UMI)-demultiplexing with Trimmomatic and UMI-tool software.[61] The UMI sequences were initially extracted, the raw reads were then mapped to Genome Reference Consortium GRCh37 with Bowtie1 version 1.2.2, and then deduplication was performed with UMI-tools in order to collapse the duplicated reads (i.e., the reads with the same UMI).[62] Quantification of mapped reads was performed with the HTSEQ-count tool.[63] All mature miRNAs were quantified according to the General Feature Format (GFF) file that was retrieved from the miRBase database in July 2019.[64] Differential expression analysis was performed using the EdgeR package version 3.8 of R Bioconductor for the identification of differentially expressed miRNAs (DEMs) between patient and control samples.[65] The miRNA count matrices were normalized for RNA composition between libraries with a trimmed mean of M-values (TMM) normalization.[66] We kept the miRNAs with a minimum requirement of 1 count per million (CPM) across two or more libraries. The Quasi-Likelihood F-test (QLF) was used as a statistical method to calculate the DEMs provided by the EdgeR package.[67] The EdgeR analysis returned the following: log2FC, logCPM, p value, false discovery rate (FDR) and F-value for each miRNA. Sequencing data are available through the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/browser/home) supported by the European Bioinformatics Institute (EBI) under the following accession ENA: PRJEB46413.

### Animal experiments

Housing and handling of all mice were performed according to European Directive 2010/63/EE and Cyprus Legislation for the protection and welfare of animals, Laws 1994–2013. Heterozygous transgenic DMSXL breeders were provided by G. Gourdon (Institute of Myology, Paris, France). The transgenic mouse model DMSXL (>90% C57BL/6 background) carries 45 kb genomic DNA cloned from a patient with DM1.[40] Mice carrying two copies of the transgene (homozygous; DMSXL) are distinguishable from the 2$^{nd}$ to 3$^{rd}$ week of their life, as they show very slow growth. Moreover, DMSXL mice are characterized by high mortality rate, muscle weakness, and mental abnormalities compared to healthy mice of the same litter.[39] Six-week-old gender-matched DMSXL mice and control mice carrying no copy of the transgene (WT) of both sexes were used for this study. Five skeletal muscle tissues located on the hindlimbs of mice (EDL, soleus, TA, gastrocnemius, quadriceps) and hearts were harvested from the mice and homogenized with Precellys 24 (Bertin Technologies, Montbonnot-Saint-Martin, France). Total endogenous RNA, enriched in miRNAs, was extracted from tissue lysates with the mirVana miRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. Blood samples were also collected by orbital blood sampling technique in EDTA-free tubes and left to clot for 20 min at room temperature before centrifugation at 2,000 rcf at room temperature. Serum was subsequently isolated from the samples and stored at −80°C until further use. Total RNA, enriched in

**Table 1. Source edge list databases for miRNA–gene target predictions**

| Source DB | Database file | Edge list | Kappa score threshold |
|---|---|---|---|
| Human miRNA edge lists | | | |
| CluePedia | CluePedia_microRNA.org-human_predictions_S_C_aug2010.txt.gz | mirsvr_score*(−1) human_predictions_S_C_aug2010 | 0.6 |
| miRDB[72] | miRDB_v6.0_prediction_hsa_based_on_miRBase_22_17.04.2019.txt.gz | miRanda-hsa-Score_miRDB_v6.0_prediction_based_on_miRBase_22 | 0.8 |
| mirTarBase[73] | mirTarBase.validated.miRNAs_15.06.2016.txt | validated miRTarBase | 0.6 |
| miRecords[74] | mirecords.umn.edu.validated.miRNAs.2010-11-25.txt.gz | validated miRNA | 0.6 |
| Mouse miRNA edge lists | | | |
| CluePedia | CluePedia_microRNA.org-mouse_predictions_S_C_aug2010.txt.gz | mirsvr_score*(−1) mouse_predictions_S_C_aug2010 | 0.6 |
| miRDB[72] | miRDB_v6.0_prediction_mmu_based_on_miRBase_22_17.04.2019.txt.gz | miRanda-hsa-Score_miRDB_v6.0_prediction_based_on_miRBase_22 | 0.8 |
| mirTarBase[73] | mirTarBase.validated.miRNAs_17.06.2016.txt | validated miRTarBase | 0.6 |
| miRecords[74] | mirecords.umn.edu.validated.miRNAs.2010-11-25.txt.gz | validated miRNA | 0.6 |

miRNAs, was extracted from 100 μL serum samples with the mirVana PARIS Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### Isolation of small EVs and encapsulated miRNAs

Small EVs were isolated from 100 μL serum samples of mice with ExoQuick Exosome Precipitation Solution (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. Small EVs were allowed to precipitate at 4°C overnight, followed by centrifugation at 1,500 rcf for 30 min. Total RNA, enriched in miRNAs, was extracted from the small EV pellets with the Total Exosome RNA and Protein Isolation Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### Scanning electron microscopy

Small EVs isolated from serum of mice were subjected to fixation with 4% paraformaldehyde (PFA) for 10 min followed by phosphate-buffered saline (PBS) washes. Samples were mounted on aluminum specimen stubs and sputtered with gold/palladium (Au/Pd) for 15 s. High-resolution scanning electron microscopic analysis was performed at 20.00 kV (magnification of 60,000) in a FEI Quanta 200 microscope, and images were processed with the MountainsMap SEM Topo version 7.3 software.

### Tunable resistive pulse sensing analysis

Quantification and size analysis of small EVs was performed with the qNano Gold platform (Izon Science, Oxford, UK) combining tunable nanopores with proprietary data capture. Small EVs were diluted in filtered electrolyte and compared to calibration particles CPC200, all provided by Izon Science. Samples were measured with the nanopore NP200 (A44687, Izon Science, Oxford, UK) at 45-mm stretch (voltage: 0.78 V and pressure: 10 mbar). Particles were detected as short pulses of the current, and data analysis was carried out with Izon Control Suite software v3.3 (Izon Science, Oxford, UK).

### Western blot analysis of small EVs

Protein samples were prepared by lysing the serum small EVs in buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH = 7.6), 10% glycerol, 0.5% Tween, and 10 mM mercaptoethanol, supplemented with 2× EDTA-free Protease Inhibitor Cocktail. The lysate was sonicated and then centrifuged at 4°C. Protein extracts of 40–60 μg were separated on a 12% SDS-PAGE, transferred on a membrane, and then incubated overnight at 4°C with primary antibodies specific for anti-CD63 (Abcam, Cambridge, UK), anti-CD81 (Abcam, Cambridge, UK), anti-TSG101 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-Nucleoporin p62 (BD Biosciences, Franklin Lakes, NJ, USA), and anti-Cytochrome C (BD Biosciences, Franklin Lakes, NJ, USA). Subsequently, the membranes were incubated for 2 h at room temperature with anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). Proteins were visualized with the UVP BioSpectrum 810 Imaging System.

### Quantitative real-time PCR of miRNAs

A total of 10 ng of the extracted RNA was subjected to reverse transcriptase PCR using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Real-time PCR amplification was performed with TaqMan MicroRNA Assays specific for miR-223-3p (Assay ID: 002295; Cat. Num.: 4427975), miR-24-3p (Assay ID: 000402; Cat. Num.: 4427975), miR-1-3p (Assay ID: 002222; Cat. Num.: 4427975), miR-133a-3p (Assay ID: 002246; Cat. Num.: 4427975), miR-133b-3p (Assay ID: 002247; Cat. Num.: 4427975), and miR-206-3p (Assay ID: 000510; Cat. Num.: 4427975) (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. miRNA levels

were normalized to miR-16-5p (Assay ID: 000391; Cat. Num.: 4427975) for patients' serum samples, to snoR-135 (Assay ID: 001230; Cat. Num.: 4427975) for mouse tissues, and to the spike-in control cel-miR-39 (Assay ID: 000200; Cat. Num.: 4427975) for the encapsulated miRNA levels in the small EVs (Applied Biosystems, Foster City, CA, USA). Data analysis was performed with SDS 2.4 Real-Time PCR data analysis software (Applied Biosystems, Foster City, CA, USA).

### Statistical analysis

Statistical analysis was performed as described previously.[68] ΔThreshold cycle (Ct) values were calculated as Ct (miR-16-5p for human serum, snoRNA-135 for mouse tissue, and cel-miR-39 for mice serum small EVs) minus Ct (miRNA). Normality of the distribution of each of the miRNA variables was assessed with the Shapiro-Wilk test; nonparametric methods (exact Wilcoxon and Kruskal-Wallis tests) were used in the analyses. A two-tailed p value of 0.05 was used to determine statistical significance. A Bonferroni adjustment was made to the alpha level (from 0.05 to 0.00069) to account for multiple comparisons (number of comparisons = 72). Spearman's correlation analyses were carried out to assess correlations between miRNA levels and study participant demographic, clinical, and molecular characteristics. Differences between DM1 patients and healthy participants were assessed with chi-square (categorical variables) and Wilcoxon (continuous variables) tests. In addition, ROC curves were used to determine the sensitivity and specificity of the assays in discriminating between DM1 patients and healthy participants. The AUC for the ROC curves was calculated. All analyses were performed with SAS v.9.4 (SAS Institute) software. Data illustrated in figures are mean values ± SE after normalization. Data in illustrations are relative expressions of DMSXL mice compared to controls. Regarding the statistical analysis of the serum small EVs in DMSXL mice, p values were calculated assuming a one-sided test based on the hypothesis that the miRNA values would be elevated in diseased mice compared to WT, following the patients' results.

### Bioinformatics analysis

The human and mouse miRNAs of interest were imported in Cytoscape v3.7.2. using the ClueGO v2.5.5[69] and CluePedia v1.5.5.[70,71] plugins and analyzed based on the following grouping:

(a) Human: hsa-miR-24-3p and hsa-miR-223-3p
(b) Mouse: mmu-miR-24-3p
(c) Mouse: mmu-miR-223-3p
(d) Mouse: mmu-miR-1-3p, mmu-miR-206-3p, mmu-miR-133a-3p, and mmu-miR-133b-3p

The first group was created to identify any common prediction target genes in human between miR-223-3p and miR-24-3p. The other three groups were formed for the identification of the predicted targets in mouse of miR-223-3p and miR-24-3p as well as the four myomiRs. Each set was screened against a number of reference miRNA target edge lists that are available through CluePedia sourced from the databases shown in Table 1. Using CluePedia's enrichment function, we constructed bipartite networks connecting the miRNAs in each group with their respective predicted gene targets with the default kappa score for each edge list in Table 1 and an arbitrary limit of 50 genes per miRNA. The predicted gene targets for each disease were used as an input in the GO enrichment analysis tool run by the PANTHER Classification System server.[72–74] Functional analysis was performed against the *Homo sapiens* and *Mus musculus* GO Biological Process database, returning the most significantly enriched GO terms. The GO term set was further processed in R in order to isolate the terms that are related to neuronal and muscle-related processes. GO term level mapping was performed through ClueGO's *preselected functions* mode and plotted with R's ggplot2 package.[75]

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.09.007.

## ACKNOWLEDGMENTS

This Project (POST-DOC/0916/0235) was co-financed by the European Regional Development Fund and the Republic of Cyprus through the Research and Innovation Foundation. This work was also supported by A. G. Leventis Foundation. The authors would like to thank Anna Katsioloudi for her support on TRPS analysis. H.L. receives support from the Canadian Institutes of Health Research (Foundation Grant FDN-167281), the Canadian Institutes of Health Research and Muscular Dystrophy Canada (Network Catalyst Grant for NMD4C), the Canada Foundation for Innovation (CFI-JELF 38412), and the Canada Research Chairs program (Canada Research Chair in Neuromuscular Genomics and Health, 950-232279). M.T., A.O., A.C.K., and G.M.S. were funded by European Commission Research Executive Agency Grant BIORISE [number 669026], under the Spreading Excellence, Widening Participation, Science with and for Society Framework.

## AUTHOR CONTRIBUTIONS

L.A.P. conceived the study. L.A.P., A.K., and D.K. designed the study. D.K. conducted most of the experiments and developed the data. A.K. and C.M. performed the mouse handling and the tissue isolation. C.M. performed the WB analysis. D.K. and C.M. performed real-time PCR analysis. A.C.K., A.O., M.T., and G.M.S. performed the bioinformatics analysis. A.A., M.P., and K.K. performed SEM and TRPS experiments. A.L., K.J., N.N., G. Gorman, C.T., H.L., G.K.P., C.P., E.K., and E.Z.P. provided the serum samples, clinical data, and clinical expertise. G. Gourdon provided the mouse animal model. T.C.K. carried out statistical analysis. L.A.P., D.K., and A.K. analyzed and interpreted the data. D.K. and A.K. wrote the manuscript. L.A.P. coordinated the study. All authors read and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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