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Original Article

ORIGINAL ARTICLE

Identification of exosomal muscle-specific miRNAs in serum of myotonic dystrophy patients relating to muscle disease progress

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Abstract

Myotonic dystrophy type 1 (DM1) is the most common form of adult-onset muscular dystrophy, which is characterised by progressive muscle wasting and the discovery of reliable blood-based biomarkers could be useful for the disease progress monitoring. There have been some reports showing that the presence of specific miRNAs in blood correlates with DM1. In one of these, our group identified four muscle-specific miRNAs, miR-1, miR-133a, miR-133b and miR-206, which correlated with the progression of muscle wasting observed in DM1 patients. The levels of the four muscle-specific miRNAs were elevated in the serum of DM1 patients compared to healthy participants and were also elevated in the serum of progressive muscle wasting DM1 patients compared to disease-stable DM1 patients. The aim of this work was to characterise the ontology of these four muscle-specific miRNAs in the blood circulation of DM1 patients. Here we show that the four muscle-specific miRNAs are encapsulated within exosomes isolated from DM1 patients. Our results show for the first time, the presence of miRNAs encapsulated within exosomes in blood circulation of DM1 patients. More interestingly, the levels of the four exosomal muscle-specific miRNAs are associated with the progression of muscle wasting in DM1 patients. We propose that exosomal muscle-specific miRNAs may be useful molecular biomarkers for monitoring the progress of muscle wasting in

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DM1 patients. There has been a growing interest regarding the clinical applications of exosomes and their role in prognosis and therapy of various diseases and the above results contribute towards this way.

Introduction

Myotonic dystrophy type 1 (DM1) which is also called Steinert disease, is the most common form of muscular dystrophy in adult population caused by an expansion of a highly unstable CTG triplet repeat in the 3′ untranslated region (3′UTR) of *dystrophia myotonica protein kinase* (*DMPK*) gene located on chromosome 19ql3.3 (1,2). In healthy individuals, the number of CTG repeats is in the range of 5-35, while individuals with 36-49 CTG repeats are considered to have the pre-mutation of the disease and are at high risk of having children with further expanded repeats. In DM1 patients, the number of CTG repeats increases to 50 up to thousands. CTG repeat length is unstable in DM1 patients in both somatic and germ lines (3). DM1 is an autosomal dominant disease in which symptoms become apparent at an earlier age and disease severity increases with each generation. DM1 is a highly variable multisystemic disorder primarily characterized by progressive muscle weakness and wasting (4). DM1 patients very commonly face weakness of distal muscles of the extremities which later spreads to the proximal muscles (5). The symptoms and severity of DM1, ranges from mild to severe, and frequently result in death due to respiratory deficiency (6). DM1 multisystemic features include cataract, diabetes and cardiac conduction abnormalities (4).

The diagnosis of DM1 includes genetic testing, electromyography, skeletal muscle biopsy histopathology, magnetic resonance and serum creatine kinase measurements (7). Creatine kinase activity is the only blood biomarker that is currently being performed during the diagnosis of DM1 although it is not a disease-specific biomarker. There is a strong pressing need to develop biomarkers for monitoring the disease progression and response to therapeutic interventions. Alternative splicing changes in skeletal muscle have been suggested as biomarkers for the disease however these biomarkers require the collection of biopsies which is an invasive procedure (8).

The severity and the rate of muscle wasting are highly variable among DM1 patients (7,9). There are DM1 patients that will be very affected by the disease while others will show very mild signs of it. Other DM1 patients can also be carriers of the genetic expansion but show no sign at all of the disease. DM1 heterogeneity is also seen within different families as well as within the same family. In DM1, certain muscles are more affected than others with a severe effect on the daily life of patients. The facial muscles are often the first to show weakness, resulting in a lack of facial expression, slurred speech (dysarthria) and droopy eyelids (ptosis) (5,7,9). The muscles of the lower leg, ankle, foot, forearm and hand are usually the next group of muscles to show wasting and weakness thus leading to difficulty with walking, and with finger and hand movements (5,10). The muscles involved with breathing and swallowing may become weak over time. The extent to which muscle wasting and weakness will affect a person's ability to function however, is variable and unpredictable. The progression of muscle wasting observed in DM1 varies greatly from patient to patient. For instance a DM1 patient can face progressive muscle wasting and after a period of time the patient could become stable without any further muscle wasting. Muscle wasting, has not been correlated with CTG repeat expansions or any other clinical biomarker, and is being currently monitored through comparisons between regular physical examinations and electromyography (7,11,12). The development of molecular biomarkers would provide an additional diagnostic tool which could complement the existing monitoring methods and help the clinicians to have a greater understanding of their patients' progress independently of their prior clinical examination.

MicroRNAs (miRNAs) are small non-coding RNA molecules that are implicated in the regulation of a wide variety of cellular processes and disease conditions. It is well established that miRNAs are present at significant levels in extracellular bodily fluids, including blood serum and plasma (13–18). Extracellular circulating miRNAs show an unexpected and remarkable nuclease resistance thus providing them the ability to be considered as potential clinical non-invasive biomarkers for various diseases and situations (19–22). In DM1, a signature of nine deregulated miRNAs in plasma samples of DM1 patients was initially identified (23). Our previously published results showed that the levels of muscle-specific miRNAs, miR-1, miR-133a, miR-133b and miR-206, are elevated in DM1 patients compared to controls and interestingly they are correlated with the progression of muscle wasting and weakness observed in DM1 patients (24). Recently, Perfetti and colleagues in a larger cohort of DM1 patients validated the use of eight miRNAs as potential biomarkers for DM1 including the four muscle-specific miRNAs identified by our research group (24,25).

Although extracellular miRNAs have been identified in DM1 patients, their nature in the blood circulation has not been clarified yet. Up to date, two mechanisms have been reported to be responsible for the unexpected nuclease resistance of miRNAs in the RNase-rich environment of blood (18,26–30). Specifically, extracellular circulating miRNAs have been identified either to be bound to proteins or to be encapsulated within membrane-vesicles. A significant amount of the extracellular circulating miRNAs were determined to be associated with Argonaute1 (Ago1) and/or Argonaute2 (Ago2) proteins - parts of the RNA-induced silencing complex (RISC) - both in blood plasma, serum and cell culture media (18,26,27). Some miRNAs were also identified to be associated with the high-density lipoprotein (HDL) and stably exist in the blood circulation (28,29). In other published reports miRNAs were determined to be encapsulated within membrane-vesicles such as exosomes (30).

The identification of the ontology of the miRNAs in blood circulation of DM1 patients is very important in order to provide information regarding their release mechanism and the pathogenic mechanism of the disease. Additionally, an understanding of the ontology of serum miRNAs would be important for a precise clinical interpretation which could in turn render them more reliable biomarkers in clinical practice. The aim of this study was to provide an in-depth investigation of the circulating muscle-specific miRNAs, miR-1, miR-133a, miR-133b and miR-206, in blood circulation of DM1 patients by identifying their nature. In the present work, we show that the four circulating muscle-specific miRNAs are not bound to proteins such as Ago1, Ago2 or HDL, in serum of DM1 patients. Notably, the four muscle-specific miRNAs that are elevated in serum of DM1 patients were found to be encapsulated within exosomes. More interestingly the exosomal levels of the four muscle-specific miRNAs correlate with the progression of muscle wasting

observed in DM1 patients which is the most significant symptom representing DM1 disease progression. Our results indicate that the exosomal levels of muscle-specific miRNAs can potentially be used for monitoring the progress of DM1 muscle wasting. Furthermore, our results suggest a mechanism by which the four muscle-specific miRNAs are packed and/or released within exosomes in blood circulation of DM1 patients. Additionally, our results can imply that the circulating exosomal muscle-specific miRNAs may be involved in secondary complications observed in DM1.

Results

MiRNAs are deregulated in serum samples of DM1 patients

Circulating miRNAs were identified as good non-invasive biomarker candidates for diagnosis or even prognosis of various diseases (31–33). A signature of twelve miRNAs was identified to be deregulated in plasma and serum samples of DM1 patients in previous studies (23,24). Specifically, eight miRNAs were identified to be elevated (miR-133a, miR-193b, miR-191, miR-140-3p, miR-454, miR-574, miR-885-5p, miR-886-3p) and one miRNA to be decreased (miR-27b) in the plasma samples of DM1 patients compared to controls (25). In our previous published report, we showed that the four muscle-specific miRNAs (miR-1, miR-133a, miR-133b and miR-206) were elevated in the serum samples of DM1 patients compared to healthy participants (24). Notably, the serum levels of miR-1, miR-133a, miR-133b and miR-206 were correlated with the progression of muscle wasting in DM1 patients (24). In another recent report, the eight out of twelve miRNAs that are deregulated in plasma samples of a large cohort of DM1 patients compared to controls were validated (25). In particular, miR-1, miR-133a, miR-133b, miR-206, miR-140-3p, miR-454 and miR-574 were found to be elevated, while miR-27b was found to be decreased (25).

As a first step in the present work, we wanted to validate the association of the four miRNAs, miR-1, miR-133a, miR-133b and miR-206 in a larger patient cohort. The levels of the four muscle-specific miRNAs, miR-1, miR-133a, miR-133b and miR-206 in DM1 patients were compared to the levels in healthy individuals. Serum samples from sixty three DM1 patients and sixty three healthy participants were isolated from blood samples. Total RNA, including miRNA extraction from serum samples was followed and Real-Time PCR analysis was performed for the four muscle-specific miRNAs. In agreement with our previous published report the levels of miR-1, miR-133a, miR-133b and miR-206 were significantly higher in the serum of the DM1 patients compared to healthy participants who showed minimal levels of the muscle-specific miRNAs ($P < 0.0001$) (24) (Fig. 1A; Table 1). The levels of the four miRNAs were normalized to the levels of the ubiquitously expressed miR-16 which was used as an internal control (34,35). Means and standard deviation for miRNA levels are provided in Table 1. Using receiver-operator characteristics (ROC) analytical methods plotting, the true positive (sensitivity) versus false positive (1-specificity) was plotted. The area under the curve (AUC > 0.96) suggests that serum levels of miR-1, miR-133a, miR-133b and miR-206 can discriminate DM1 patients from healthy individuals with high specificity (Fig. 1B). Among DM1 patients, a high correlation was observed between some of the four muscle-specific miRNAs (correlation coefficients, ρ: 0.48–0.91; $P < 0.001$). The highest correlation was observed between miR-133a and miR-206 ($\rho = 0.79$; $P < 0.0001$). Further statistical analysis was performed taking into consideration the average relative quantitation (RQ) values of the four muscle-specific miRNAs (Fig. 1C). ROC analysis shows that a variable constructed by taking the average expression of the four miRNAs has almost the same specificity with the individual specificities of the four miRNAs (AUC = 0.98) (Fig. 1B and C). These results show that the levels of the four muscle-specific miRNAs in the serum of DM1 patients are significantly higher than that of the healthy individuals (controls).

In our previous published report, we showed that the serum levels of miR-1, miR-133a, miR-133b and miR-206 correlate to the progress of muscle wasting which is the primary characteristic in DM1 patients (24). All the patients participated in this study were monitored and followed for more than two years by their neurologists. The neurological examination of the patients included detailed muscle power evaluation on all muscle groups (based on the Medical Research Council (MRC) scale) as described previously (24). For the purposes of this study, patients without any change in the MRC scoring for the last two years were considered as disease stable (non-progressive patients). On the other hand, patients who during the last two years had scored worse in the MRC scale were considered as disease progressive patients with muscle wasting (24). The correlation of the levels of the four muscle-specific miRNAs and the progression of muscle wasting was investigated in a larger number of DM1 patients. Statistical analysis showed that, compared to non-progressive DM1 patients, progressive DM1 patients had significantly higher levels of miR-1, miR-133a, miR-133b and miR-206 levels ($P < 0.0001$) (Fig. 2A). Means and standard deviation of miRNA levels are provided in Table 2. ROC analyses (using miR-1, miR-133a, miR-133b and miR-206 serum levels) showed high specificity in discriminating between progressive and non-progressive DM1 patients (AUC > 0.79) (Fig. 2B). Among progressive DM1 patients, the correlation between the four muscle-specific miRNAs was observed ($\rho = 0.35$–0.75; $P < 0.05$). The highest correlation was observed between miR-206 and both miR-1 and miR-133a ($\rho = 0.75$; $P < 0.0001$). Further statistical analysis was performed taking into consideration the average RQ values of the four muscle-specific miRNAs (Fig. 2C). ROC analysis shows that the variable constructed as the average of the four miRNAs values has almost the same specificity with the individual specificities of miR-133a, miR-133b and miR-206 (AUC = 0.90).

The eight additional miRNAs (miR-193b, miR-191, miR-140-3p, miR-454, miR-574, miR-885-5p, miR-886-3p) initially identified by another group in plasma, were investigated, this time in serum of DM1 patients and healthy participants. Some miRNAs were found to be different between DM1 patients and healthy participants whereas some other miRNAs showed no significant difference (Supplementary Material, Fig. S1). Specifically, we identified significantly increased levels of miR-140-3p ($P = 0.047$), miR-191 ($P = 0.0256$), miR-193b ($P < 0.0001$), miR-574-3p ($P = 0.0003$), miR-885-5p ($P < 0.0001$) and miR-886-3p ($P = 0.015$). The levels of miR-454 were not found to be significantly different in DM1 patients compared to healthy participants ($P = 0.39$). There is also a different association of miR-27b. In plasma samples of DM1 patients miR-27b levels were found to be decreased compared to controls, whereas in the present study, it was found that miR-27b levels are significantly elevated in serum samples of DM1 patients compared to healthy participants ($P < 0.0001$) (Supplementary Materials, Fig. S1; Table S1) (23,25). In order to exclude that the discrepancies determined for the two miRNAs, miR-27b and miR-454, are due to the extraction method and/or the normalization to the particular internal control, their levels were determined using an additional extraction method. Additionally, the levels of the two miRNAs were also normalized to the internal control,

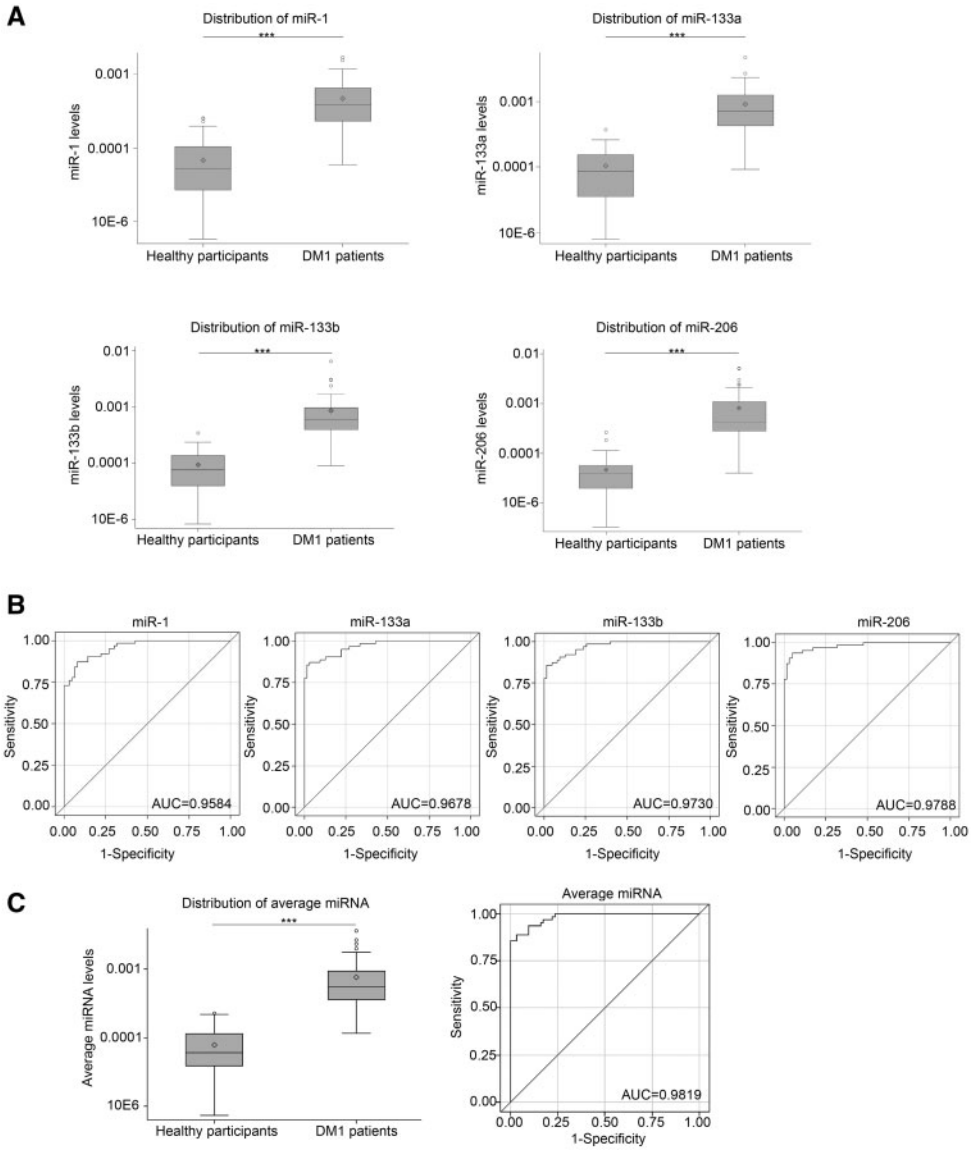

Figure 1. Muscle-specific miRNAs are elevated in serum of DM1 patients. Serum samples from sixty three DM1 patients and sixty three healthy participants were analysed for the presence of muscle-specific miRNAs. (**A**) Distribution charts show that miR-1, miR-133a, miR-133b and miR-206 are significantly elevated in the serum of DM1 patients compared to the serum of healthy participants. $^{***}P < 0.0001$. Horizontal lines inside the boxes mark the medians. Mean expression values are marked with rhombus. (**B**) Receiver-operator characteristics (ROC) curve analyses using serum miR-1, miR-133a, miR-133b and miR-206 discriminate DM1 patients from healthy participants. Area under the curve (AUC) values are presented. (**C**) The average relative quantitation (RQ) values of the four muscle-specific miRNAs were calculated and analysed. Distribution chart shows that the average of miRNA expression levels is elevated in DM1 patients compared to healthy participants. ROC curve analysis shows that the average expression of the four miRNAs has almost the same specificity with the individual specificities of the four miRNAs. AUC value is presented. $^{***}P < 0.0001$.

Table 1. Muscle-specific serum miRNA levels in healthy participants and DM1 patients

miRNA	Healthy participants (Mean ± SD)	DM1 patients (Mean ± SD)	*P*-value
miR-1	$6.9 \times 10^{-5} \pm 5.8 \times 10^{-5}$	$46.9 \times 10^{-5} \pm 33.6 \times 10^{-5}$	$P < 0.0001$
miR-133a	$10.4 \times 10^{-5} \pm 7.9 \times 10^{-5}$	$91.9 \times 10^{-5} \pm 75.7 \times 10^{-5}$	$P < 0.0001$
miR-133b	$9.4 \times 10^{-5} \pm 6.8 \times 10^{-5}$	$86.7 \times 10^{-5} \pm 95.7 \times 10^{-5}$	$P < 0.0001$
miR-206	$4.6 \times 10^{-5} \pm 4.2 \times 10^{-5}$	$81.2 \times 10^{-5} \pm 99.8 \times 10^{-5}$	$P < 0.0001$

miR-106a (Supplementary Materials, Fig. S1C and D; Tables S2 and S3). Similar to the other extraction method, the levels of miR-27b were significantly elevated in DM1 patients compared to healthy participants using the new extraction method and both internal controls ($P < 0.005$) (Supplementary Material, Fig. S1C and D; Tables S2 and S3). The levels of miR-454 showed variation between the extraction methods and internal controls used for normalization. Specifically, miR-454 levels were found increased in

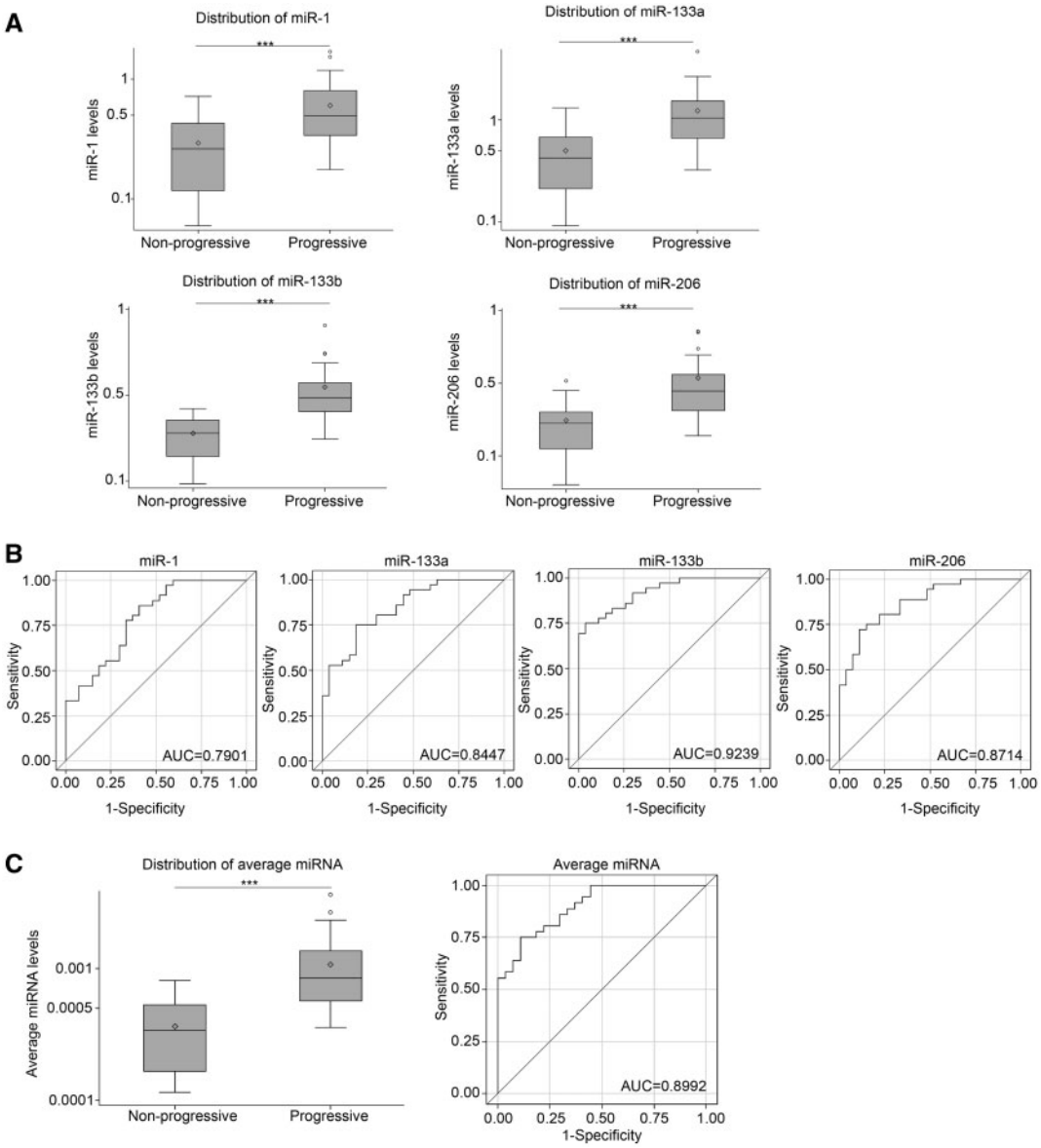

Figure 2. Elevated muscle-specific miRNAs are correlated to muscle disease progress. The DM1 patients were classified as progressive and non-progressive based on the progression of the muscle wasting that the patients faced at the time of blood sample collection. Serum samples from twenty-seven non-progressive DM1 patients and thirty-six progressive DM1 patients were analysed for the presence of muscle-specific miRNAs. (**A**) Distribution charts show that miR-1, miR-133a, miR-133b and miR-206 serum levels are significantly higher in the progressive DM1 patients compared to the non-progressive DM1 patients. ***$P < 0.0001$. Horizontal lines inside the boxes mark the medians. Mean expression values are marked with rhombus. (**B**) Receiver-operator characteristics (ROC) curve analyses using serum miR-1, miR-133a, miR-133b and miR-206 discriminate DM1 patients from healthy participants. Area under the curve (AUC) values are presented. (**C**) The average RQ values of the four muscle-specific miRNAs were calculated and analysed. Distribution chart shows that the average of miRNA expression levels is elevated in progressive DM1 patients compared to non-progressive DM1 patients. ROC curve analysis shows that the average expression of the four miRNAs has almost the same specificity with the individual specificities of miR-133a, miR-133b and miR-206. AUC value is presented. ***$P < 0.0001$.

Table 2. Muscle-specific serum miRNA levels of DM1 patients in non-progressive and progressive DM1 patients

miRNA	Non-progressive DM1 patients (Mean ± SD)	Progressive DM1 patients (Mean ± SD)	P-value
miR-1	$2.93 \times 10^{-4} \pm 1.98 \times 10^{-4}$	$6.01 \times 10^{-4} \pm 3.6 \times 10^{-4}$	$P < 0.0001$
miR-133a	$4.99 \times 10^{-4} \pm 3.27 \times 10^{-4}$	$12.34 \times 10^{-4} \pm 8.35 \times 10^{-4}$	$P < 0.0001$
miR-133b	$3.57 \times 10^{-4} \pm 1.8 \times 10^{-4}$	$12.49 \times 10^{-4} \pm 11.17 \times 10^{-4}$	$P < 0.0001$
miR-206	$3.09 \times 10^{-4} \pm 2.36 \times 10^{-4}$	$11.99 \times 10^{-4} \pm 11.76 \times 10^{-4}$	$P < 0.0001$

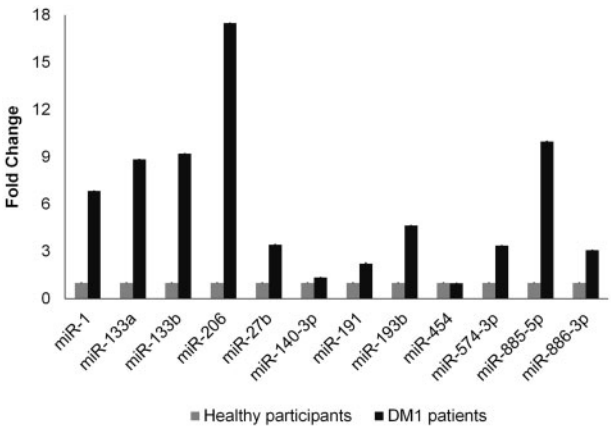

Figure 3. Muscle-specific miRNAs show higher fold change compared to the other miRNAs. The differences in serum levels of miR-27b, miR-140-3p, miR-454 and miR-574-3p previously validated in the plasma of DM1 patients, miR-191, miR-193b, miR-885-5p and miR-886-3p previously identified in the plasma of DM1 patients and the original four muscle-specific miRNAs (miR-1, miR-133a, miR-133b and miR-206) among healthy participants and DM1 patients were determined. The four muscle-specific miRNAs, miR-1, miR-133a, miR-133b and miR-206 were found to show higher fold change in serum samples of healthy participants and DM1 patients. Each bar represents the fold change of the mean value of all the patients investigated for each miRNA. The standard deviation values are calculated using the mean values.

serum samples of DM1 patients compared to healthy participants using the second extraction method and miR-16 internal control ($P < 0.05$) (Supplementary Materials, Fig. S1C; Table S2). On the other hand, miR-454 levels were not found to be significantly different in DM1 patients compared to healthy participants using the second extraction method and miR-106a internal control ($P = 0.1199$) (Supplementary Materials, Fig. S1D; Table S3).

Having examined all the candidate miRNAs it can be concluded that the differences in the levels of the four muscle-specific miRNAs (miR-1, miR-133a, miR-133b and miR-206) between DM1 patients and healthy individuals are higher compared to the other miRNAs previously studied (Fig. 3).

Muscle-specific miRNAs are not associated with proteins in serum of DM1 patients

The four muscle-specific miRNAs were further processed for an in-depth investigation of their ontology in the blood circulation of DM1 patients. The specific miRNAs were chosen for further investigation since they showed the highest change between healthy participants and DM1 patients when they were compared with the four previously validated miRNAs (miR-27b, miR-140-3p, miR-454 and miR-574-3p) (25) (Fig. 3). Furthermore, the four muscle-specific miRNAs were found to correlate with the progression of muscle wasting observed in DM1 patients suggesting that their presence in blood circulation represents the situation of muscle tissue (24) (Fig. 2). The purpose of this study was to identify serum-based biomarkers specific for muscle wasting and weakness which is considered to be a very important and serious characteristic of DM1 patients therefore, miR-1, miR-133a, miR-133b and miR-206 were chosen to be further investigated. A possible mechanism ascribed to the RNase resistance of the miRNAs in blood circulation is their binding to proteins such as Ago1 or Ago2 proteins or HDL (18,26–29). To investigate if the four muscle-specific miRNAs are bound to proteins in the blood circulation, immunoprecipitation assays were performed initially against Ago1 and Ago2 proteins. Protein analysis using the immunoprecipitates showed that Ago1 and Ago2 proteins were successfully immunoprecipitated from human serum samples. The specific precipitation of Ago1 and Ago2 was verified with the absence of Ago1 and Ago2 in negative control IgG immunoprecipitates (Fig. 4A). To further confirm the successful immunoprecipitation experiments the levels of miR-16 which was previously identified to be bound on Ago1 and Ago2 proteins were examined in Ago1 and Ago2 immunoprecipitates (18,26,27). miR-16 was successfully enriched in both Ago1 and Ago2 immunoprecipitates relative to IgG immunoprecipitates used as negative controls (Fig. 4B). Immunoprecipitation assays and Real-Time PCR analysis demonstrated that none of the miRNAs under investigation is bound to Ago1 or Ago2 proteins in blood circulation of DM1 patients (Fig. 4C). Human ApoA-I protein is the main protein component of HDL and was found to be bound with miRNAs in blood circulation thus protecting them from nuclease degradation (28,29). Immunoprecipitation assays using ApoA-I were also performed to investigate whether miR-1, miR-133a, miR-133b and miR-206 are bound to ApoA-I. The successful immunoprecipitation of ApoA-I from human serum samples was confirmed using protein analysis. The absence of ApoA-I in negative control IgG immunoprecipitates showed that ApoA-I was specifically precipitated from human serum (Fig. 4D). miR-877, previously identified to be bound on ApoA-I in plasma, was enriched in ApoA-I immunoprecipitates relative to IgG negative control immunoprecipitates thus confirming the successful completion of the immunoprecipitation assays (29) (Fig. 4E). Immunoprecipitation assays and Real-Time PCR analysis showed that none of the four muscle-specific miRNAs is associated with ApoA-I protein in blood circulation of DM1 patients (Fig. 4F). The Ago1, Ago2 and ApoA-I immunoprecipitation assays were also performed in serum samples isolated from healthy participants showing no binding of the four muscle-specific miRNAs with the proteins under investigation (Supplementary Material, Fig. S2).

Muscle-specific miRNAs are encapsulated within exosomes in DM1 patients

An additional reported mechanism identified to protect extracellular miRNAs from degradation in blood circulation is their encapsulation in lipid membrane-bound vesicles such as exosomes (30,36,37). These vesicles stabilize miRNAs to the otherwise hostile, RNases-rich environment of the blood (30,38). Since the four muscle-specific miRNAs were not identified to be bound to either Ago1/2 or ApoA-I proteins in serum of DM1 patients, the possibility that they are found encapsulated within exosomes was next investigated. Exosomes are small membranous vesicles that originate from internal multivesicular bodies (39). Exosomes have been found in body fluids such as plasma, urine, saliva, breast milk and synovial fluid and contain cell-specific protein, mRNA and miRNA. Several studies showed that exosomal miRNA is stable in blood because exosomes have a protective role against degradation from enzymes such as RNases (40,41). For the isolation of exosomes, the ExoQuick™ Exosome precipitation solution was used which is a widely used polymer that enables high-throughput isolation of exosomes (42–44). ExoQuick reagent was chosen for this study because it requires lower sample volumes, which is very important when human serum samples are used, and has fewer steps that minimizes human error compared to other isolation methods like ultracentrifugation (45,46). Ultracentrifugation which has been broadly used for exosomes isolation requires a large initial volume of the sample and also is a relatively crude methodology

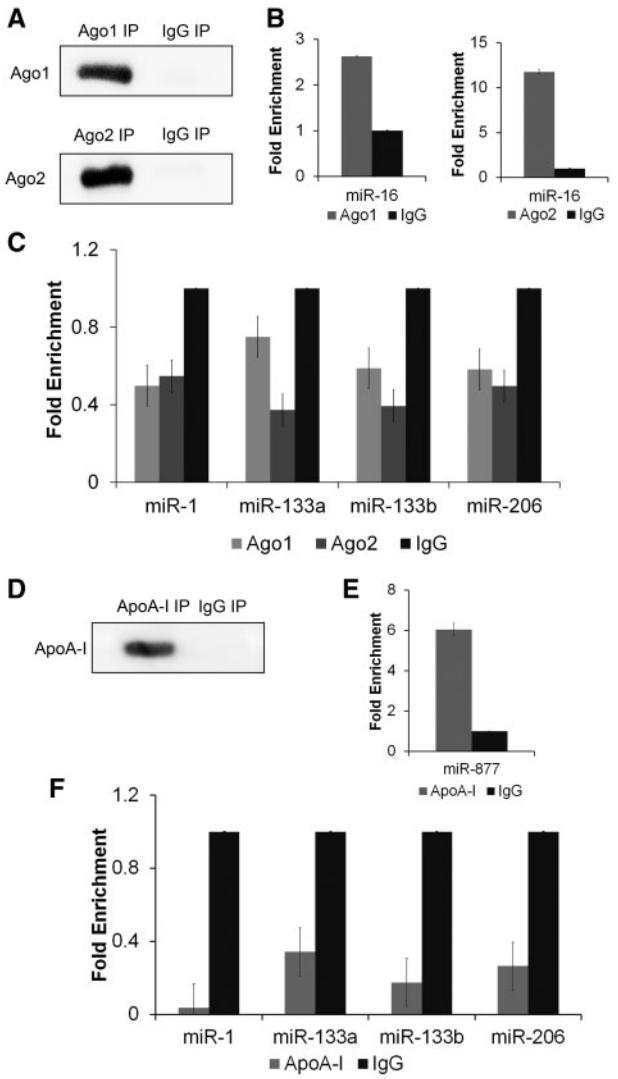

Figure 4. Muscle-specific miRNAs are not associated with proteins in serum of DM1 patients. (**A**) Circulating Ago1, Ago2 were immunoprecipitated from serum samples of DM1 patient and confirmed by western blotting. Immunoprecipitation with IgG served as a negative control. (**B**) miR-16 levels were enriched in Ago1 and Ago2 immunoprecipitates relative to IgG negative control immunoprecipitates. (**C**) Serum samples from five DM1 patients were immunoprecipitated using anti-Ago1, anti-Ago2, and anti-IgG antibodies. The levels of the four muscle-specific miRNAs were lower in the Ago1 and Ago2 immunoprecipitates compared to IgG immunoprecipitates implying that circulating muscle-specific miRNAs are not associated with Ago1 or Ago2 proteins in serum samples of DM1 patients. (**D**) ApoA-I was immunoprecipitated from serum samples of DM1 patient and detected by western blotting. Immunoprecipitation with IgG served as a negative control. (**E**) Real-time PCR analysis showed that the levels of miR-877 were enriched in ApoA-I immunoprecipitates relative to IgG immunoprecipitates which were served as negative control. (**F**) Serum samples from five DM1 patients were immunoprecipitated using anti-ApoA-I and anti-IgG antibodies. The levels of the four muscle-specific miRNAs were lower in the ApoA-I immunoprecipitates compared to IgG immunoprecipitates implying that circulating muscle-specific miRNAs are not associated with ApoA-I protein in serum samples of DM1 patients. The levels of the four muscle-specific miRNAs were normalized to the spike-in control cel-miR-39.

identified to causing aggregation, fusion and/or rupture of extracellular vesicles due to the high speed centrifugation (47).

Initially, the successful isolation of exosomes was confirmed (Fig. 5). The verification of the exosomes in isolated vesicles was examined by scanning electron microscopy (SEM), Tunable resistive pulse sensing (TRPS) analysis and protein analysis of the proteins that are displayed on exosome membranes. Exosome purity was confirmed by SEM (Fig. 5A). The scanning electron micrographs of the exosomes revealed rounded structures (roundness of 0.701) with a mean size of 100 nm, which is consistent with imaging by this method due to changes caused by adherence, fixation and desiccation (Fig. 5B and C). The diameter of exosomes was verified by the qNano Gold platform ranging from 60-180nm with the majority of them to fit in the range of 95-135nm similar to previously described exosomes (48,49) (Fig. 5D). To further visualize the exosomal membrane proteins in association with isolated exosomes, immunogold labeling techniques were utilized in combination with antibodies against CD63 and CD81 exosomal protein markers. The detection of colloidal gold on the outer layer of the exosomes confirmed the successful isolation of exosomes (Fig. 5E). The identity of the studied vesicles was further confirmed as exosomes by western blot analysis and detection of commonly used exosomal marker proteins. The exosomal marker molecules CD63, CD81 and TSG101 were detected in the exosomes sample at higher amounts compared to whole cell lysate extracts (50) (Fig. 5F).

Following the confirmation of successful isolation of exosomes, the possibility that the four muscle-specific miRNAs are encapsulated within exosomes was investigated. Exosomes were isolated from serum samples of DM1 patients followed by total RNA, including small RNA, extractions from both the supernatant and exosome fractions, and the abundance of the four muscle-specific miRNAs was quantified by Real-Time PCR. The supernatant fraction which corresponds to the non-exosomal miRNAs contains little muscle-specific miRNAs relative to the exosomes fractions showing that indeed miR-1, miR-133a, miR-133b and miR-206 are encapsulated within exosomes (Fig. 6). The levels of the four muscle-specific miRNAs were normalized to cel-miR-39 spike-in control.

To further confirm that the four muscle-specific miRNAs are encapsulated within exosomes, the exosomes were permeabilized using saponin detergent. Specifically, the exosomes were treated with 10% saponin followed by total RNA including small RNA extractions. Real-Time PCR analysis revealed that permeabilization of exosomes results in a decrease in miR-1, miR-133a, miR-133b and miR-206 implying that when the muscle-specific miRNAs are exposed to the RNases present in serum they are degraded (Fig. 7A). When the serum samples were treated with 10% saponin, Proteinase K and RNase the levels of miR-1, miR-133a, miR-133b and miR-206 were further reduced. Serum samples were treated with RNase only and Proteinase K, followed by RNase and served as negative controls, showing no difference in the levels of the four muscle-specific miRNAs compared to untreated samples (Fig. 7A).

The levels of the four muscle-specific miRNAs present in total serum samples were next compared to those identified in exosomes in order to confirm that the whole population of miR-1, miR-133a, miR-133b and miR-206 exists within exosomes (Fig. 7B). Statistical analysis shows that there are no significant differences between the total muscle-specific miRNA levels compared to the exosomal muscle-specific miRNAs ($P > 0.14$) (Fig. 7B).

Exosomal muscle-specific miRNAs correlate with muscle wasting observed in DM1 patients

Following the identification of miR-1, miR-133a, miR-133b and miR-206 within exosomes, the previously correlation reported by our group of these muscle-specific miRNAs with DM1 disease

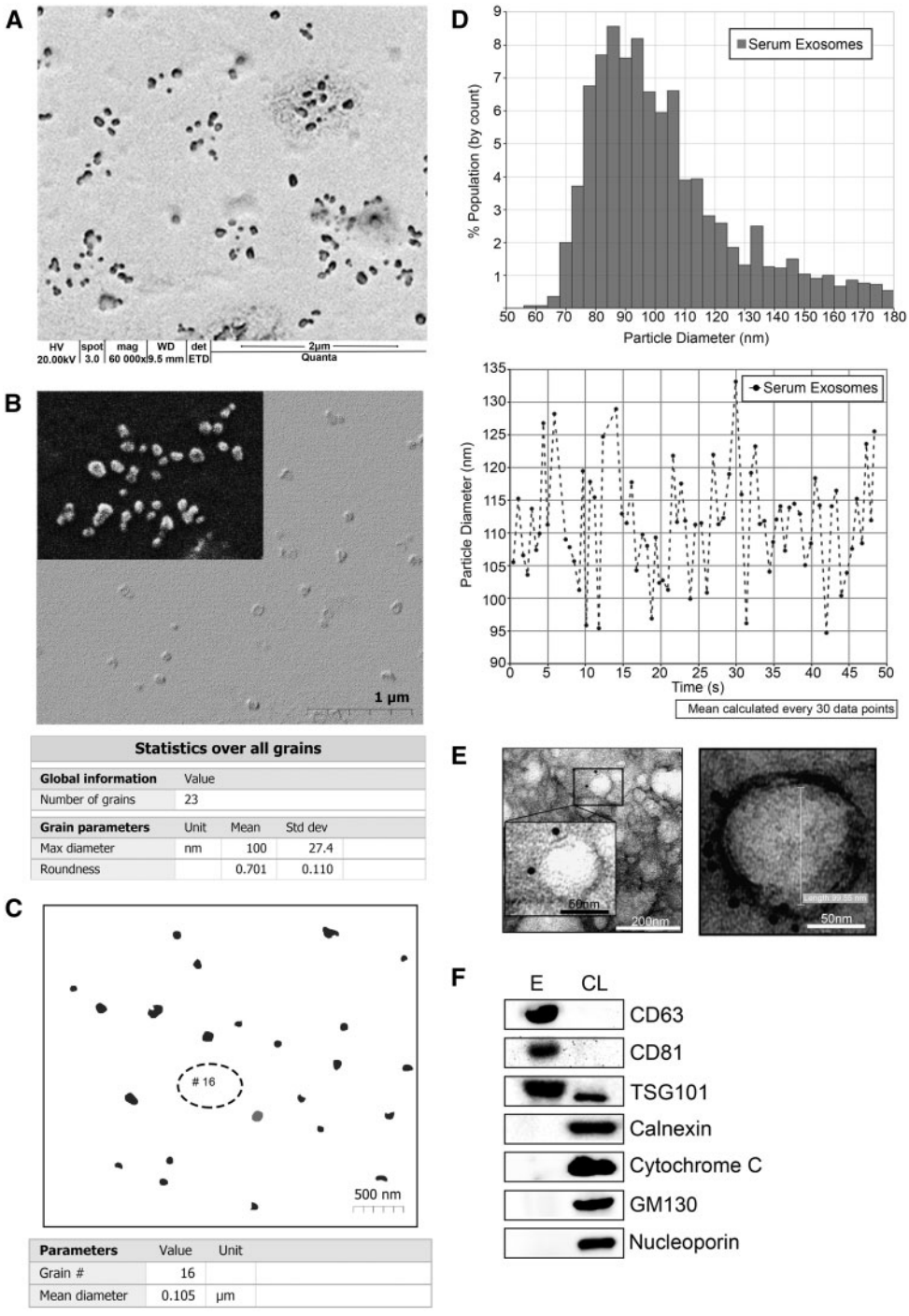

Figure 5. Exosomes are present in serum of DM1 patients. Exosomes were isolated from serum samples of DM1 patients and healthy participants. The isolated exosomes were initially characterized by SEM, TRPS and western blot analysis. (**A**) SEM micrographs of round exosomes at 60,000x magnification, (**B**) SEM micrograph and anaglyph analysis shown statistical information of the maximum mean diameter (100 nm) of the exosomes and their roundness level (0.701), (**C**) Advance surface analysis of specific exosomes e.g. #16 of mean diameter 0.105µm, (**D**) Quantification and size analysis of exosomes using TRPS. Replicates of patient exosomes compared to known size and concentration of polysterine beads. Once the exosomes sample is calibrated to the reference beads, the recorded blockades in nA, can be calculated to absolute sizes in nm. The particles rates are used to calculate the concentration of exosomes. (**E**) Exosomes were immunogold labeled with anti-CD63 and anti-CD81. (**F**) Western blot analysis of the exosomal markers CD63, CD81 and TSG101 of the exosomal lysates isolated from serum samples of DM1 patients confirmed the successful isolation of the exosomes. Calnexin, Cytochrome C, GM130 and Nucleoporin were also analysed as negative controls. Lysates from human muscle cell lines were served as controls. E, exosomes; CL, cell lysates.

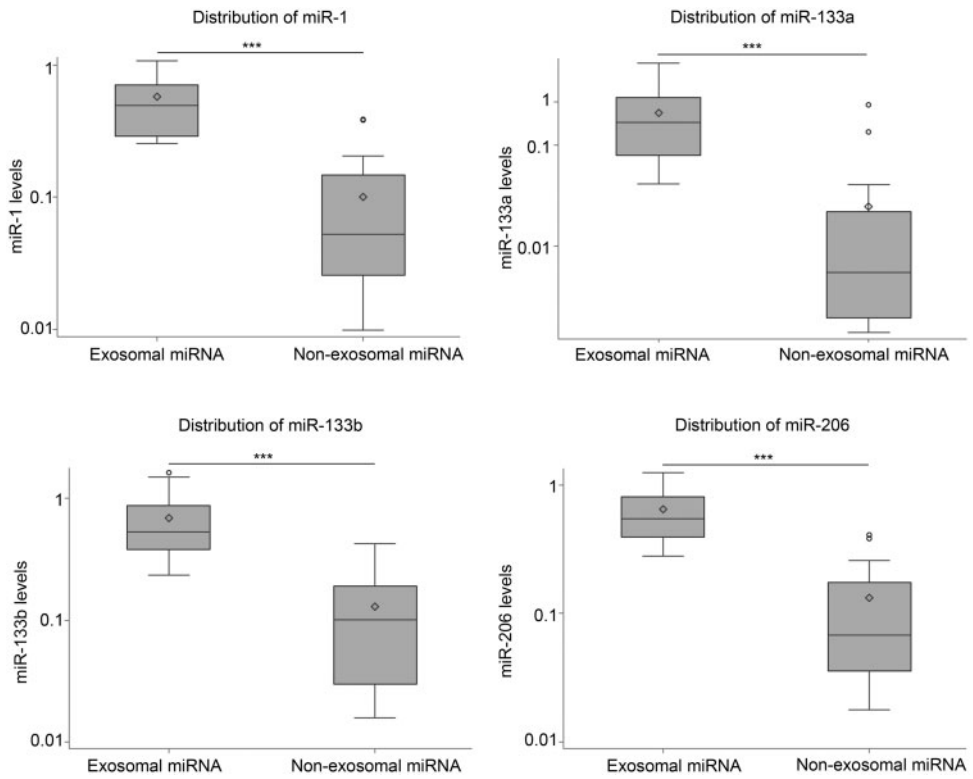

Figure 6. Muscle-specific miRNAs are encapsulated within exosomes in serum of DM1 patients. Serum samples isolated from fifteen DM1 patients (eight progressive and seven non-progressive DM1 patients) were treated with ExoQuick reagent and total RNA, including small RNA, was extracted from both the exosomal (pellet) and non-exosomal (supernatant) fractions. Real-time PCR analysis shows that the levels of miR-1, miR-133a, miR-133b and miR-206 are significantly elevated in exosomal fractions compared to non-exosomal fractions which contain very low amounts of muscle-specific miRNAs. ***$P < 0.0001$. Spike in cel-miR-39 was used as a control for isolation efficiency in all samples.

and also with muscle wasting observed in DM1 patients was investigated taking into consideration their levels within exosomes (24).

Initially, muscle-specific miRNA levels encapsulated within exosomes isolated from DM1 patients were compared to the levels encapsulated within exosomes isolated from healthy participants. Exosomes were isolated from serum samples of fifteen DM1 patients and ten healthy participants followed by extraction of total RNA, including miRNA. Real-Time PCR analysis was performed specific for the four muscle-specific miRNAs. The levels of miR-1, miR-133a, miR-133b and miR-206 were significantly higher in the exosomes isolated from serum samples of the DM1 patients compared to healthy participants ($P \leq 0.001$) (Fig. 8A; Table 3). The levels of the four miRNAs were normalized to the cel-miR-39 spike-in control. Means and standard deviation for miRNA levels are provided in Table 3. ROC analysis generated plots of true positivity (sensitivity) versus false positivity (1-specificity). The area under the curve (AUC > 0.88) suggests that the exosomal levels of miR-1, miR-133a, miR-133b and miR-206 in serum can discriminate DM1 patients from healthy individuals extremely well (Fig. 8B). Further statistical analysis was performed taking into consideration the average RQ values of the four muscle-specific miRNAs (Fig. 8C). ROC analysis shows that a variable constructed by taking the average levels of the four miRNAs has almost the same specificity with the individual specificities of miR-1 and miR-206 (AUC = 0.98) (Fig. 8C). These results demonstrate that the exosomal levels of muscle-specific miRNAs in the serum of DM1 patients are higher compared to the control healthy individuals.

Exosomes present in serum of DM1 patients and healthy participants were next quantified using qNano Gold. Exosomes were isolated from serum samples of six DM1 patients and six healthy participants showing an increase in the number of exosomes present in DM1 patients compared to healthy participants (Supplementary Material, Fig. S3).

The correlation of the levels of exosomal muscle-specific miRNAs with the progression of muscle wasting observed in DM1 patients was next investigated. Real-Time PCR analysis showed that the exosomal levels of miR-1, miR-133a, miR-133b and miR-206 are significantly higher in progressive DM1 patients compared to non-progressive DM1 patients ($P < 0.05$) (Fig. 9A, Table 4). Means and standard deviation of miRNA data are provided in Table 4. ROC analyses using miR-1, miR-133a, miR-133b and miR-206 exosomal levels showed high specificity in discriminating between progressive and non-progressive DM1 patients (AUC > 0.88) (Fig. 9B). Further statistical analysis was performed taking into consideration the average RQ values of the four exosomal muscle-specific miRNAs (Fig. 9C). ROC curve analysis shows that a variable constructed by taking the average expression of the four miRNAs has similar specificity with the individual specificities of the four miRNAs (AUC = 0.88) (Fig. 9C).

Discussion

Currently, there is much evidence that miRNAs are released from tissues into the blood circulation (13–18). The biological and clinical significance of circulating extracellular miRNAs

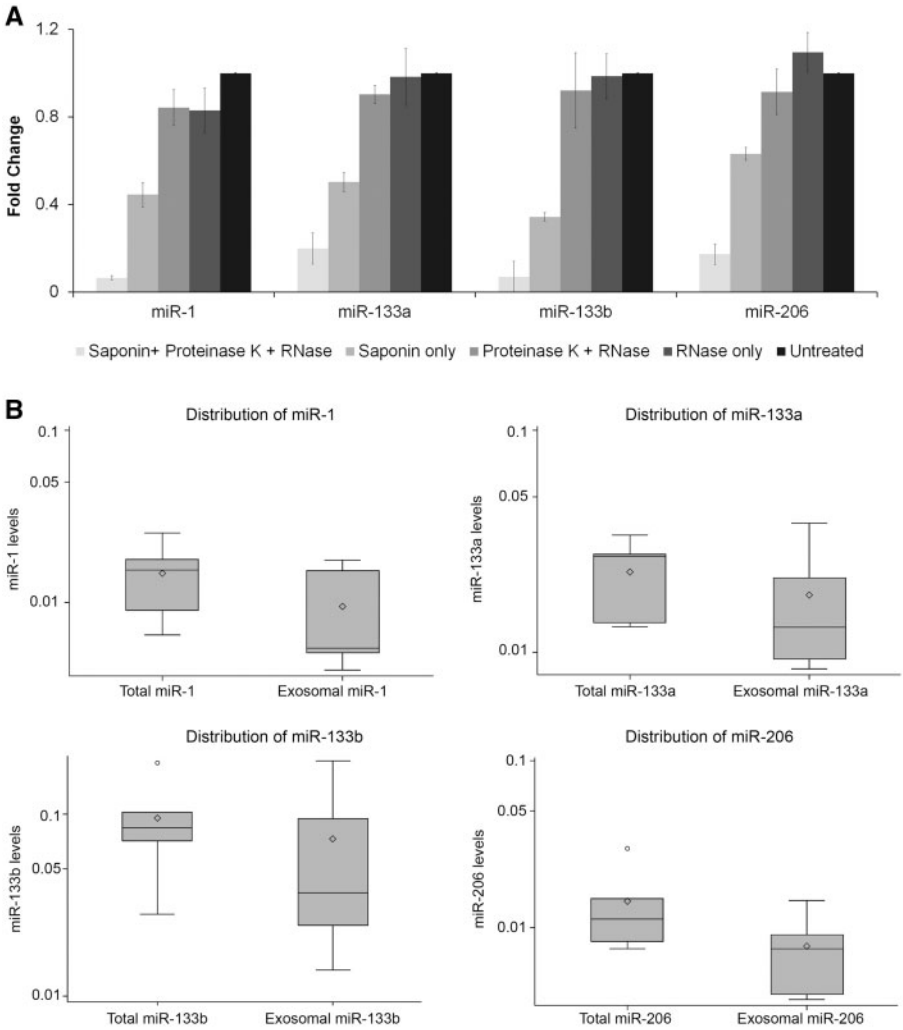

Figure 7. Validation of muscle-specific miRNAs encapsulation within exosomes. (**A**) Permeabilization of exosomes reduced the levels of the muscle-specific miRNAs. Serum samples from DM1 patients were treated with 10% saponin for permeabilization of their membranes. Real-time PCR and statistical analyses reveal that following the permeabilization of the exosomes the levels of miR-1, miR-133a, miR-133b and miR-206 were significantly decreased. When the serum samples were treated with 10% saponin, Proteinase K and RNase the levels of miR-1, miR-133a, miR-133b and miR-206 were further reduced. $^{*}P < 0.05$. Serum samples from DM1 patients were treated with RNase only and Proteinase K followed by RNase and served as negative controls. The levels of the four muscle-specific miRNAs were normalized to cel-miR-39 spike-in control. Each bar represents mean values ± standard deviation of five independent experiments. (**B**) The levels of the four muscle-specific miRNAs present in total serum samples from five DM1 patients were compared to those present within exosomes. No significant difference observed in the total and exosomal levels of miR-1, miR-133a, miR-133b and miR-206. $P > 0.14$. Spike-in cel-miR-39 was used as a control for isolation efficiency in all samples.

however remains largely unknown. The aim of this study was to investigate in depth the nature of muscle-specific miRNAs, miR-1, miR-133a, miR-133b and miR-206, in the blood circulation of DM1 patients and evaluate their use as clinical biomarkers for muscle wasting progression observed in DM1 patients.

In our previous published report, the levels of the four muscle-specific miRNAs, miR-1, miR-133a, miR-133b and miR-206, were found to be elevated in the serum samples of twenty three DM1 patients compared to serum samples of healthy participants (24). More interestingly, the levels of the four muscle-specific miRNAs were determined to correlate with muscle wasting progression in DM1 patients (24). In a parallel study, a signature of nine deregulated miRNAs in plasma samples of DM1 patients was identified and suggested that these miRNAs can be used as diagnostic biomarkers for DM1 (23). Very recently, in a larger cohort of DM1 patients the same group validated eight miRNAs in a large number of plasma samples and suggested that miRNAs might be useful as DM1 humoral biomarkers (25). Specifically, miR-1, miR-133a, miR-133b, miR-206, miR-140-3p, miR-454 and miR-574 were identified to be elevated in plasma samples of DM1 patients compared to plasma samples of healthy participants, whereas miR-27b was reduced (25). In the present study, the levels of the four muscle-specific miRNAs were evaluated in serum samples of sixty three DM1 patients and compared to sixty three healthy participants. miRNA analysis followed by statistical analysis shows that muscle-specific miRNA levels are significantly increased in serum of DM1 patients, as compared to levels in normal participants which were found to be minimal, in agreement with our previous report and elsewhere (24,25). Similar reports were published showing this association in the serum and/or plasma with other diseases such as DMD and congenital muscular dystrophy type 1A (MDC1A) suggesting that muscle-specific

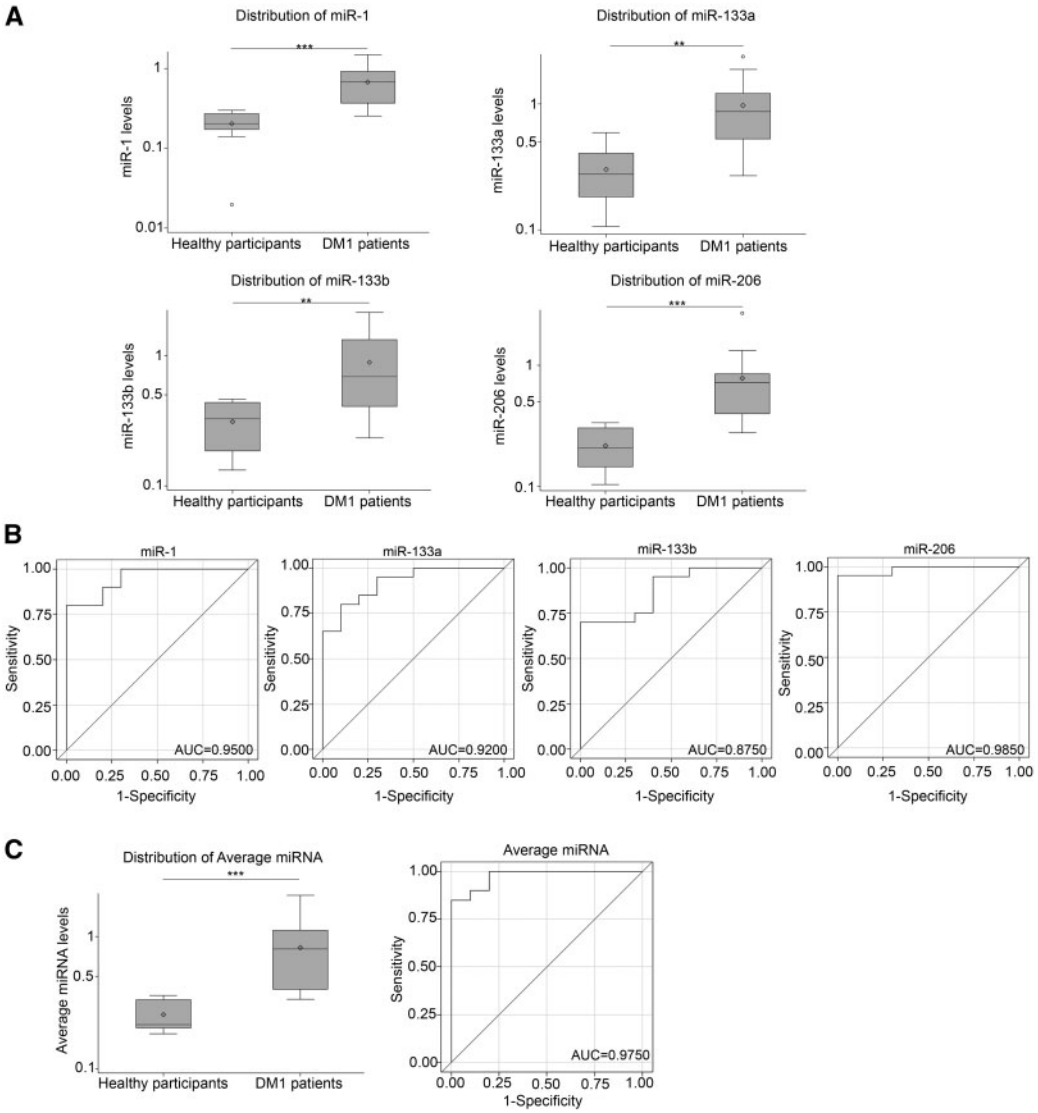

Figure 8. Exosomal muscle-specific miRNAs are elevated in the serum of DM1 patients. Exosomes were isolated from serum samples of fifteen DM1 patients and ten healthy participants and analysed for the presence of the four muscle-specific miRNAs. (**A**) Distribution charts show that the exosomal levels of miR-1, miR-133a, miR-133b and miR-206 are significantly higher in DM1 patients compared to healthy participants which are minimal. $^{**}P > 0.001$. The levels of the four muscle-specific miRNAs were normalized to cel-miR-39 spike-in control. Horizontal lines inside the boxes mark the medians. Mean expression values are marked with rhombus. (**B**) Receiver-operator characteristics (ROC) curve analyses using serum exosomal miR-1, miR-133a, miR-133b and miR-206 discriminate DM1 patients from healthy participants. Area under the curve (AUC) values are presented. (**C**) The average RQ values of the four muscle-specific miRNAs were calculated and analysed. Distribution chart shows that the average of exosomal miRNA levels is elevated in DM1 patients compared to healthy participants. ROC curve analysis shows that the average expression of the four miRNAs has almost the same specificity with the individual specificities of miR-1 and miR-206. AUC value is presented. $^{***}P < 0.0001$.

Table 3. Exosomal muscle-specific serum miRNA levels in healthy participants and DM1 patients

miRNA	Healthy participants (Mean ± SD)	DM1 patients (Mean ± SD)	*P*-value
miR-1	$2.03 \times 10^{-1} \pm 0.8 \times 10^{-1}$	$6.73 \times 10^{-1} \pm 3.48 \times 10^{-1}$	$P < 0.0001$
miR-133a	$3.02 \times 10^{-1} \pm 1.59 \times 10^{-1}$	$9.69 \times 10^{-1} \pm 5.76 \times 10^{-1}$	$P = 0.0002$
miR-133b	$3.09 \times 10^{-1} \pm 1.27 \times 10^{-1}$	$8.84 \times 10^{-1} \pm 5.46 \times 10^{-1}$	$P = 0.001$
miR-206	$2.17 \times 10^{-1} \pm 0.84 \times 10^{-1}$	$7.86 \times 10^{-1} \pm 5.51 \times 10^{-1}$	$P < 0.0001$

miRNAs may be considered valid biomarkers for muscular dystrophies (51,52). In the present work, we also studied the possible association of the other four miRNAs (miR-140-3p, miR-454, miR-574 and miR-27b) that were previously identified and validated to be deregulated in plasma samples of DM1 patients (23,25). Two of these miRNAs (miR-140-3p and miR-574-3p) agree with the previously validated miRNAs using our initial extraction method (25). miR-454 identified to be elevated in DM1 patients compared to healthy participants when a different extraction method was used in agreement with the previously published results (25). The levels of miR-27b were found to be significantly elevated in DM1 patients compared to healthy

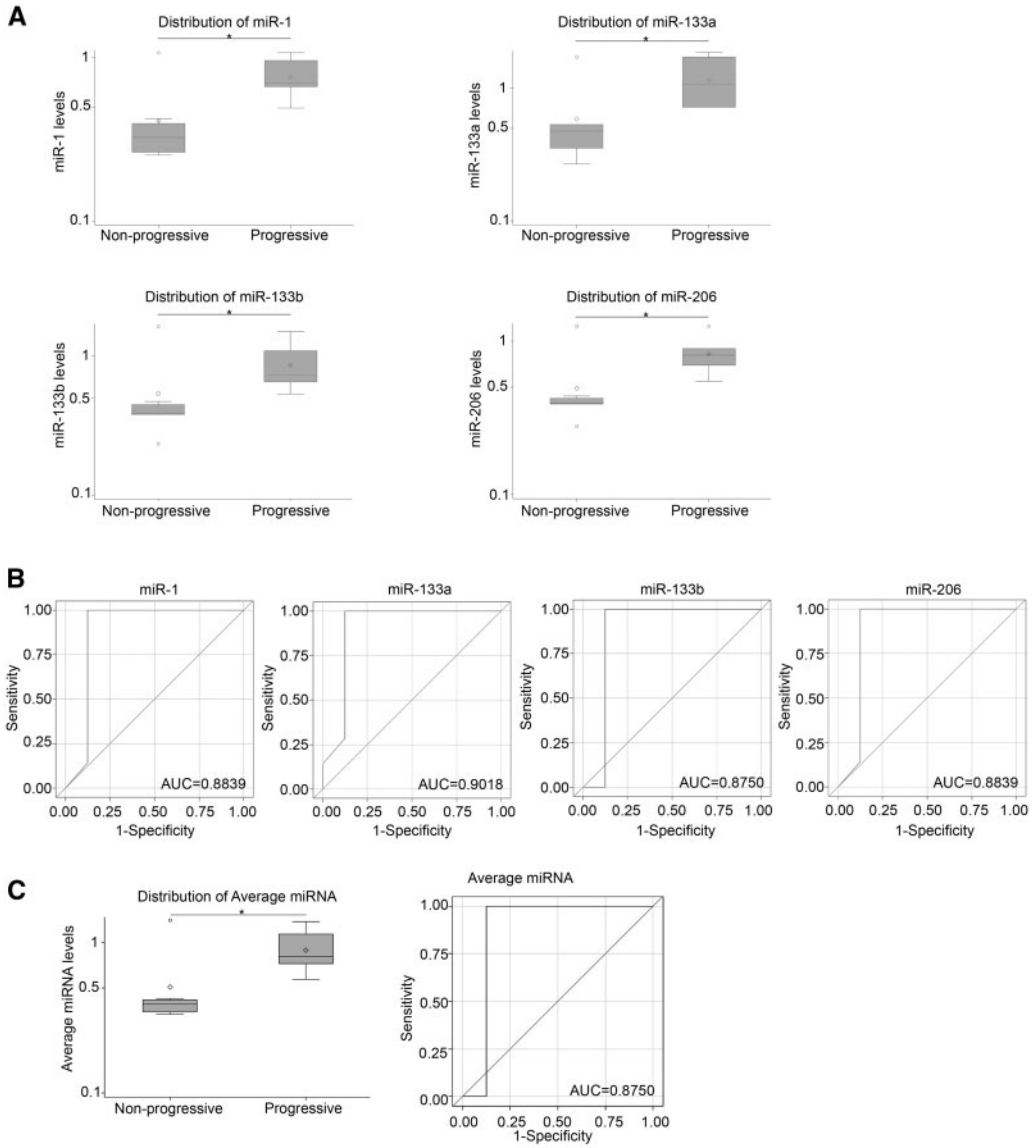

Figure 9. Exosomal muscle-specific miRNAs are correlated with the progression of muscle wasting observed in DM1 patients. The DM1 patients were classified as progressive and non-progressive DM1 patients. Exosomes were isolated from serum samples of seven progressive and eight non-progressive DM1 patients. (**A**) Distribution charts show that the exosomal levels of miR-1, miR-133a, miR-133b and miR-206 are significantly higher in progressive DM1 patients compared to non-progressive DM1 patients. $^{*}P < 0.05$. The levels of the four muscle-specific miRNAs were normalized to cel-miR-39 spike-in control. Horizontal lines inside the boxes mark the medians. Mean expression values are marked with rhombus. (**B**) Receiver-operator characteristics (ROC) curve analyses using serum exosomal muscle-specific miRNAs discriminate DM1 patients from healthy participants. Area under the curve (AUC) values are presented. (**C**) The average RQ values of the four muscle-specific miRNAs were calculated and analysed. Distribution chart shows that the average of exosomal miRNA levels is elevated in progressive DM1 patients compared to non-progressive DM1 patients. ROC curve analysis shows that the average expression of the four miRNAs has almost the same specificity with their individual specificities. AUC value is presented. $^{*}P < 0.05$.

Table 4. Exosomal muscle-specific serum miRNA levels in non-progressive and progressive DM1 patients

miRNA	Non-progressive DM1 patients (Mean ± SD)	Progressive DM1 patients (Mean ± SD)	*P*-value
miR-1	$4.13 \times 10^{-1} \pm 2.74 \times 10^{-1}$	$7.56 \times 10^{-1} \pm 1.96 \times 10^{-1}$	$P = 0.0148$
miR-133a	$5.89 \times 10^{-1} \pm 4.67 \times 10^{-1}$	$11.37 \times 10^{-1} \pm 4.73 \times 10^{-1}$	$P = 0.0107$
miR-133b	$5.35 \times 10^{-1} \pm 4.44 \times 10^{-1}$	$8.59 \times 10^{-1} \pm 3.33 \times 10^{-1}$	$P = 0.0175$
miR-206	$4.92 \times 10^{-1} \pm 3.09 \times 10^{-1}$	$8.24 \times 10^{-1} \pm 2.16 \times 10^{-1}$	$P = 0.0148$

participants using both extraction methods and two different internal controls in contrast to the previously published results (25). There are several reasons that can explain the discrepancies in the results. Investigations in the previous reports were carried out in plasma whereas in our studies were carried out in serum (23,25). Although, serum and plasma samples are both widely used for the identification and development of biomarkers, variations in their molecular constituents have been identified and reported (53). Another reason for the difference in the results for some instances could be the different method of extraction of the material. In the present study, we tried two different extraction methods and determined that for one miRNA the extraction method affects the results whereas for the second miRNA there is no change.

Muscle weakness and wasting is a primary characteristic of DM1 patients and the main cause of their disability. Muscle wasting generally worsens over time, but the rate of deterioration varies between patients. In our previous published report, we showed that the levels of the four muscle-specific miRNAs correlate with the progression of muscle weakness and wasting in DM1 patients (24). Specifically, miR-1, miR-133a, miR-133b and miR-206 levels were found to be significantly higher in progressive DM1 patients, compared to non-progressive DM1 patients (24). In this study, we validated the relation of the four muscle-specific miRNAs present in serum samples of DM1 patients with the progression of muscle wasting in a larger cohort of DM1 patients. The results of this study further support and strengthen our previous published results regarding the use of the four muscle-specific miRNAs as clinical non-invasive biomarkers for muscle wasting in DM1.

The presence of miRNAs in the RNase-rich blood environment has been widely accepted however the mechanisms that are responsible for the release of miRNAs are not clearly understood although their significance (54,55). By identifying the ontology of the miRNAs in blood circulation evidence will be provided regarding their release mechanism and more widely the pathogenic mechanism of the disorder. Possible mechanisms have been identified to be responsible for the release and stability of the miRNAs in blood circulation. Published reports showed that the encapsulation of the miRNAs into membrane-vesicles provide a general protection for the extracellular circulating miRNAs (30). Specifically, it has been found that extracellular miRNAs are enclosed in exosomes isolated from peripheral blood and culture media of several cell lines thus protecting them from the RNases activity (30,56). In other published reports it has been demonstrated that a significant amount of the extracellular circulating miRNAs is associated with Ago1 and/or Ago2 proteins both in blood plasma, serum and cell culture media (18,26,27). Ago1 and Ago2 are the proteins that miRNAs are naturally associated within the cells and are part of the RNA-induced silencing complex (RISC). The stability of Ago proteins in protease rich environment explained the resistance of Ago-bound miRNAs in nucleases that exist in biological fluids (18,55). Some miRNAs were also identified to be associated with HDL and stably exist in the blood circulation (28,29). This study aimed to investigate the nature of the four muscle-specific miRNAs in blood circulation of DM1 patients. In particular, the possibilities that miR-1, miR-133a, miR-133b and miR-206 are bound on Ago1/2 or ApoA-I proteins or encapsulated within exosomes were investigated.

This study shows that the four muscle-specific miRNAs that are present in blood circulation of DM1 patients are encapsulated within exosomes and are not bound to either Ago1/2 or ApoA-I. Exosomes are nanovesicles that originate from internal multivesicular bodies and are secreted into the extracellular environment. Exosomes are found in most of the biological fluids like plasma, urine, saliva, breast milk and synovial fluid. These extracellular vesicles seem to have specialized functions and play an important role in processes such as intercellular signalling, waste management and coagulation (57). Exosomes are shed by cells under both normal and pathological conditions. They carry nucleic acids and proteins from their host cells that are indicative of pathophysiological conditions, and they are widely considered to be crucial for biomarker discovery for clinical diagnostics. Exosomes are stable structures within blood circulation. Studies showed that exosomes are stable in plasma over 90 days under various storage conditions (58). In *in vitro* experiments, it has been reported that exosomes are stable in cell culture medium for at least 7 days (59). The precise molecular mechanisms for their secretion and uptake, along with their composition, cargo, and resulting functions, are only beginning to unravel (60–62).

This is the first report regarding the investigation of the ontology of miRNAs in blood circulation of DM1 patients however some reports have been published regarding the nature of the four muscle-specific miRNAs in DMD. Roberts and colleagues reported that in DMD only a minority of serum muscle-specific miRNAs are found in extracellular vesicles, such as exosomes, whereas the majority is protected from serum nucleases by association with protein/lipoprotein complexes (63). On the other hand Matsuzaka and colleagues reported that muscle-specific miRNAs (miR-1, miR-133a and miR-206) are both encapsulated within exosomes and also are bound to proteins in serum of DMD patients (64). Our results show that in DM1 miR-1, miR-133a, miR-133b and miR-206 are encapsulated within exosomes. These results were further confirmed by the permeabilization of the exosomes using a detergent. Following permeabilization of the exosomes, the RNases that naturally exist in serum samples were free to enter the vesicles thus degrading the miRNAs. Addition of Proteinase K and RNase, in order to eliminate the effect of Proteinase K to the natural existing RNases, further decreased the levels of the four muscle-specific miRNAs thus suggesting that possibly miR-1, miR-133a, miR-133b and miR-206 are bound to proteins within exosomes in DM1 blood circulation. Quantification of the exosomes present in serum of DM1 patients and healthy participants showed that DM1 patients have an increased number of exosomes compared to healthy participants. This observation suggests that an increased amount of exosomes is possibly produced and/or released in DM1 patients compared to healthy participants. Although the number of exosomes present in serum samples of DM1 patients is increased, the elevated levels of the four muscle-specific miRNAs are not related to the increase observed in the number of exosomes. More specifically, the increase observed in the four muscle-specific miRNAs is much higher compared to the increase observed in the number of exosomes. These data however, have been collected from a limited number of patients and further investigation with a larger sample size is necessary. Further investigation and comparison of the total levels of the four muscle-specific miRNAs and the exosomal levels in blood circulation of DM1 patients showed that miR-1, miR-133a, miR-133b and miR-206 are exclusively encapsulated within exosomes. These observations are in contrary to what was observed in DMD. This difference could be explained due to the different pathogenic mechanisms that are responsible or involved in the two types of muscular dystrophy. These observations imply a novel mechanism by which skeletal muscle cells abnormally pack and release muscle constituents within blood circulation. It has been reported that the miRNAs identified in

exosomes can be taken up by neighbouring or distant cells and subsequently modulate recipient cells (49,65,66). The existence of elevated levels of the four muscle-specific miRNAs in exosomes isolated from DM1 patients compared to healthy participants possibly imply that are implicated in DM1 pathogenesis. Additionally, taking into consideration that the exosomes can be taken up by other cells/tissues evidence could be provided for any secondary complications that are observed in DM1 patients.

Muscle weakness and wasting is the primary characteristic of DM1 patients and its progress cannot be predicted or diagnosed using any relevant biomarker. A convenient and non-invasive method to monitor muscle wasting progression would therefore be of value for DM1 patients and their neurologists. Additionally, the development of such biomarkers will be beneficial for monitoring the outcomes of therapeutic interventions in patients. Comparison between the exosomal levels of the four muscle-specific miRNAs and the situation of muscle wasting of DM1 patients revealed that the exosomal levels of muscle-specific miRNAs correlate with muscle wasting progression observed in DM1 patients. Specifically, the levels of miR-1, miR-133a, miR-133b and miR-206 encapsulated within exosomes in DM1 patients are significantly elevated in progressive DM1 patients compared to non-progressive DM1 patients.

Exosomal miRNAs have a potential role as a diagnostic biomarker in patients with lung and ovarian cancer (67,68). This is the first report however investigating the exosomal miRNAs in DM1 patients. The identification of clinical molecular biomarkers within exosomes in blood circulation of DM1 patients and their association with muscle wasting progression in DM1 will provide added value to the field of the development of non-invasive clinical biomarkers. A significant body of articles published lately validated that miRNAs encapsulated within exosomes hold great potential as innovative biomarkers for clinical diagnosis. The enrichment of diagnostic markers at the exosomal source facilitates the discovery of biomarkers that normally would go undetected. Published data suggest that exosome purification may represent a more informative diagnostic tool than whole body fluid (69). The power of exosomes as biomarkers relies on the enrichment of highly selected markers during exosomal sorting, which otherwise constitute only a very small proportion of the total of body fluids (69).

The understanding of the ontology of serum miRNAs would be important for a precise clinical interpretation which could in turn render them more reliable biomarkers in clinical practice. Additionally, the observation that muscle-specific miRNAs are encapsulated within exosomes in DM1 patients imply a situation of DM1 disorder during of which muscle constituents are abnormally encapsulated within exosomes and released to the blood circulation of the patients. The development of a reliable biomarker for monitoring and characterizing muscle wasting in DM1 patients will give the opportunity to the clinicians to have a regular and better monitoring of patient progress. Moreover, the detection of these miRNAs can help towards a better understanding of the efficacy of current drugs and the evaluation of any therapeutic intervention.

Materials and Methods

Participant inclusion, blood collection and isolation of serum

The study was approved by the National Bioethics Committee of Cyprus and participants provided a written informed consent to participate and provide blood specimens to the study. All DM1 patients were previously diagnosed by (a) the Diagnostic Department, Cyprus Institute of Neurology and Genetics using Southern blot technique, (b) the Diagnostic Department, Eginitio Hospital using Long-PCR and Fragment Analysis and TP-PCR methods and (c) the Department of Neurology, Medical University of Warsaw using PCR and modified RP-PCR. For the purposes of the project all DM1 patients were physically examined prior to study enrolment. None of the participants were taking steroids or diabetes treatment (such medicines may affect the levels of circulating miRNAs). All the patients participated in this study were monitored and followed for more than two years by their neurologists. The neurological examination of the patients included detailed muscle power evaluation on all muscle groups (based on the MRC scale) as described previously (24). General hematological and biochemical examinations were performed twice yearly and cardiological assessments took place yearly. For the purposes of this study, patients without any change in the MRC scoring for the last two years were considered as disease stable (non-progressive patients). On the other hand, patients who during the last two years had scored worse in the MRC scale were considered as disease progressive patients with muscle wasting (24). Healthy participants completed a health-status questionnaire in order to verify they were free of any serious medical history or recent illness (more than a year) and were not being treated for a chronic medical condition. The healthy participants did not have a family history of muscle disease. Following clinical examination, a total of 4 ml of blood was drawn from all study participants and placed in plain serum collection tubes (BD Vacutainer, New Jersey, U.S.A.). For DM1 patients, blood collection for miRNA analysis was performed following their last clinical examination. Serum was subsequently isolated from the samples.

miRNA immunoprecipitation

miRNA immunoprecipitation was performed using Immunoprecipitation Kit Dynabeads® Protein G (Novex, Waltham, MA, U.S.A.), according to the manufacturer's instructions. 300 μl of serum samples were immunoprecipitated with 10 μg of one of the following antibodies: anti-AGO1 (Sigma-Aldrich, St. Louis, Missouri, U.S.A.), anti-AGO2 (Sigma-Aldrich St. Louis, Missouri, U.S.A.), anti-ApoA-I (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.) or anti-IgG (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.). Immune complexes were pulled down using 50 μl of Protein G magnetic Dynabeads. The beads were collected using a magnet and proceeded to extraction of either protein or total RNA, including miRNAs. For protein extraction, 20 μl of Elution Buffer and 5 μl of SDS was added to the beads and incubated at 70 °C for 10 min. For the extraction of total RNA, including miRNAs, 700 μl of Denaturing Buffer was added to the beads and the mirVana PARIS Kit (Applied Biosystems, Foster City, CA, U.S.A.) was used according to the manufacturer's instructions.

Isolation of exosomes from serum

Exosomes were isolated from serum samples using ExoQuick™ Exosome Precipitation Solution (System Biosciences, Palo Alto, CA, U.S.A.). 63 μl of ExoQuick™ Exosome Precipitation Solution was added to 250 μl of serum samples. Exosomes were precipitated by refrigeration at 4 °C for 12 h. Exosome pellets were collected by centrifugation at 1500 × g for 30 min.

Tunable resistive pulse sensing (TRPS) analysis

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

Scanning electron microscopy (SEM)

Serum isolated exosomes were subject to fixation using 4% paraformaldehyde (PFA) for 10 min followed by Phosphate Buffered Saline (PBS) washes. Samples were mounted on aluminum specimen stubs and sputtered using gold/palladium (Au/Pd) for 15 s. High resolution scanning electron microscopic analysis was performed at 20.00 kV (magnification range of 60,000-80,000x) in a FEI Quanta 200 microscope and images were processed using the MountainsMap SEM Topo version 7.3 software.

Transmission electron microscopy (TEM)

Isolated exosomes were fixed to formvar-carbon coated copper grids using 2% glutaraldehyde/2% paraformaldehyde and incubated with anti-CD63 (Abcam, Cambridge, U.K.) or anti-CD81 (Abcam, Cambridge, U.K.) primary antibody and anti-rabbit or anti-mouse secondary antibody, which contains the gold particles. 2% uranyl acetate was used to negatively stain the exosomes. The grids were viewed TEM using a JEM 1010 transmission electron microscope (JEOL) equipped with a Mega View III digital camera (Olympus).

Western blot analysis

30–40 μg of protein extracts were incubated with anti-AGO1 (Sigma-Aldrich, St. Louis, Missouri, U.S.A.), anti-AGO2 (Sigma-Aldrich, St. Louis, Missouri, U.S.A.), anti-ApoA-I (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.), anti-CD63 (Abcam, Cambridge, U.K.), anti-CD81 (Abcam, Cambridge, U.K.), anti-TSG101 (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.), anti-Calnexin (Abcam, Cambridge, U.K.), anti-GM130 (BD Biosciences, New Jersey, U.S.A.), anti-Nucleoporin p62 (BD Biosciences, New Jersey, U.S.A.) and anti-Cytochrome C (BD Biosciences, New Jersey, U.S.A.) followed by incubation with either goat anti-mouse IgG or donkey anti-rabbit IgG or goat anti-rat IgG secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.).

miRNA isolation and analysis

Total RNA, including miRNAs, was extracted from serum samples and immunoprecipitation assays using the mirVana PARIS Kit (Invitrogen, Carlsbad, CA, U.S.A.) and from exosomes using the Total Exosome RNA & Protein Isolation Kit (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions. Cell-free total RNA, including miRNAs, was also extracted from serum samples using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. 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, U.S.A.), according to the manufacturer's instructions. Real-Time PCR amplification was performed using TaqMan MicroRNA Assays to measure miRNA levels. miRNA detection assays specific for miR-1, miR-133a, miR-133b, miR-206, miR-193b, miR-191, miR-140-3p, miR-454, miR-574, miR-885-5p, miR-886-3p,miR-27b and miR-877 (Applied Biosystems, Foster City, CA, U.S.A.) were carried out according to the manufacturer's instructions. miRNA expression was normalized to miR-16, miR-106a or spike-in control cel-miR-39 (Applied Biosystems, Foster City, CA, U.S.A.). Data analysis was performed using the SDS 2.4 Real-Time PCR data analysis software (Applied Biosystems, Foster City, CA, U.S.A.). Where appropriate, serum samples were treated with either 20 mg/ml Proteinase K (Invitrogen, Carlsbad, CA, U.S.A.) at 53 °C and 10% Saponin (Fluka Analytical, Mexico City, Mexico), and aliquots were sampled at specific time points followed by total RNA, including miRNAs extractions using the mirVana PARIS Kit (Invitrogen, Carlsbad, CA, U.S.A.).

Statistical analysis

Statistical analysis was performed as described before (24,70). ΔCt values were calculated from Ct (miR-16/miR-106a/cel-miR-39) minus Ct (miRNA). Normality of the distribution of each of the miRNA variables was assessed using the Shapiro-Wilk test; non-parametric methods (exact Wilcoxon 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 to account for multiple comparisons. 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 using chi-square (categorical variables) and Wilcoxon tests (continuous variables). In addition, receiver-operating characteristic (ROC) curves were used to determine the sensitivity and specificity of the assays in discriminating between (a) DM1 patients and healthy participants and (b) progressive vs non-progressive DM1 patients. The area under the curve (AUC) for the ROC curves was calculated. All analyses were performed using SAS, v.9.3 (SAS Institute Inc., Cary, NC, USA) software. In this project, the primary comparisons were those comparing the four exosomal muscle-specific miRNAs in progressive DM1 patients versus non-progressive DM1 patients. All comparisons were significant at the alpha = 0.05 level. For secondary comparisons ($n = 13$), a corrected alpha level of 0.004 (0.05/13) was used to establish significance. The remaining comparisons were of exploratory nature.

Supplementary Material

Supplementary Material is available at *HMG* online.

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