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Plasma microRNAs as biomarkers for myotonic dystrophy type 1

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Abstract

Myotonic dystrophy type 1 (DM1) lacks non-invasive and easy to measure biomarkers, still largely relying on semi-quantitative tests for diagnostic and prognostic purposes. Muscle biopsies provide valuable data, but their use is limited by their invasiveness. microRNA (miRNAs) are small non-coding RNAs regulating gene expression that are also present in biological fluids and may serve as diseases biomarkers. Thus, we tested plasma miRNAs in the blood of 36 DM1 patients and 36 controls. First, a wide miRNA panel was profiled in a patient subset, followed by validation using all recruited subjects. We identified a signature of nine deregulated miRNAs in DM1 patients: eight miRNAs were increased (miR-133a, miR-193b, miR-191, miR-140-3p, miR-454, miR-574, miR-885-5p, miR-886-3p) and one (miR-27b) was decreased. Next, the levels of these miRNAs were used to calculate a "DM1-miRNAs score". We found that both miR-133a levels and DM1-miRNAs score discriminated DM1 from controls significantly and Receiver–Operator Characteristic curves displayed an area under the curve of 0.94 and 0.97, respectively. Interestingly, both miR-133a levels and DM1-miRNAs score displayed an inverse correlation with skeletal muscle strength and displayed higher values in more compromised patients.

In conclusion, we identified a characteristic plasma miRNA signature of DM1. Although preliminary, this study indicates miRNAs as potential DM1 humoral biomarkers.

Keywords: microRNA; Myotonic dystrophy; Patients; Biomarker; Plasma

1. Introduction

Myotonic dystrophy (DM) is the most common type of muscular dystrophy in adults characterized by muscle weakness, myotonia and multisystemic features, like cardiac and central nervous system involvement [1]. Indeed, after respiratory failure, cardiac arrhythmias are the second most frequent death cause for DM patients [1]. The most common and severe form of DM is named type 1 or Steinert's disease (DM1, OMIM 160900) and is caused by $(CTG)_n$ expansions in the 3′untranslated region of the DMPK gene [2].

The main analytical methods for DM1 diagnosis and prognosis are based on muscle biopsy immunohistochemistry, electromyography, and DNA mutation analysis [1]. Albeit very informative, the invasive nature of muscle biopsy analysis precludes its repeated use to monitor disease progression and therapy efficacy. Another important limitation is the variability between different skeletal muscles, that allows the

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comparison only between biopsies originating from the same muscle. Manual (MMT) and quantitative (QMT) muscle test are useful instrument in clinical practice, but require trained personnel and are prone to high variability between operators. Indeed, standard protocols have not yet defined [3]. Also magnetic resonance imaging is very useful for DM patient care, but it incurs in high costs and requires specialized personnel and protocols to provide quantitative data [4,5]. Among analytical measurements of humoral markers, CK activity is seldom used in clinical settings since it lacks disease specificity and it is not always increased in DM1 patients [6]. Thus, the identification of a minimally invasive analytical biomarker correlating with disease conditions is a clearly unmet need for DM1 care.

microRNAs (miRNAs) are small non-coding RNA modulating gene expression at post-transcriptional level [7] and their expression and intracellular distribution is deregulated in many human diseases, including muscular dystrophies [8–11]. Interestingly, Chen et al. demonstrated that miRNAs derived from various tissues and organs are easily detectable in both plasma and serum, being stable and resistant to nuclease digestion [12]. In addition, serum/plasma levels of miRNAs have been found to be reproducible and indicative of the disease state [12] also in mouse models and in patients affected by Duchenne muscle dystrophy and limb-girdle muscular dystrophy type 2C and 2D [13–15]. Given the lack of humoral biomarkers for DM1 and the established potential of circulating miRNAs as prognostic and diagnostic biomarkers, we profiled miRNAs in plasma of DM1 patients.

2. Materials and methods

2.1. Patients selection and plasma collection

This study was authorized by the Institutional Ethics Committee (Azienda Sanitaria Locale Milano 2) and was conducted according to the principles expressed in the Declaration of Helsinki, the institutional regulation and Italian laws and guidelines. All blood samplings were taken after specific written informed consent. Clinical diagnosis of DM1 patients was based upon International Consortium for myotonic dystrophies guidelines [16] and genetic analysis was carried out to confirm DM1 diagnosis, as previously described [17–19]. Stage of disease was determined using Muscular Impairment Rating Scale (MIRS) [20]: 1 = no muscular impairment (controls), 2 = minimal signs, 3 = distal weakness, 4 = mild–moderate proximal weakness, 5 = severe proximal weakness. Five-point MRC scale (Medical Research Council) in the upper and lower limbs for a total maximum score of 150 (MRC megascore) was used to evaluated muscle strength [21]. Specifically, the score was obtained measuring on both sides 7 muscle groups of the upper limbs (shoulder abductors and adductors, elbow flexors and extensors, wrist flexors and extensors, finger flexors) and 7 muscle groups of the lower limbs (hip flexors and extensors, knee flexors and extensors, ankle dorsiflexors and plantar flexors, extensor hallucis) as well as neck flexors and extensors. Myotonia was evaluated qualitatively during neurological examination and confirmed by needle electromyography. To test insulin resistance, HOMA-IR score was calculated from fasting glucose and insulin level. A score >2.6 is considered as insulin resistance.

Table 1
DM1 patient clinical characteristics ($n = 36$).

Age (years ± se) (Range)	42.2 ± 2.4 (17–72)
Sex (male/female)	18/18
Skeletal muscle strength (MRC megascore, max 150, mean ± se)	128.0 ± 2.9
Myotonia (%)	100
Glucose (mg/dL, normal values 70–110 mg/dL, mean ± se)	77.6 ± 1.9
Insulin resistance (%)	38.5
CPK (mg/dL, normal range, $m < 190$, $f < 167$ mg/dl, mean ± se)	Males: 307.8 ± 59.7 Females: 187.9 ± 34.6
Calcium (mg/dL, normal values 8.60–10.20 mg/dL, mean ± se)	9.3 ± 0.1
Stage of disease (MIRS) (range 1–5%)	Stage 2: 15.6 Stage 3: 28.1 Stage 4: 56.3
Arrhythmia (%)	46.9
Cataract (%)	36.8
ECG–QRS duration (ms, normal range 60–100 ms, mean ± se)	104.6 ± 5.4

Blood samples were obtained by venous punctures of 36 DM1 (Table 1) patients and of 36 sex and age matched subjects displaying no sign of neuromuscular disorders (47.6 ± 2.3 years old, range 25–76 years, 20 females and 16 males). EDTA-tubes were used for plasma preparation. Cell- and platelet-free plasma was prepared following a 2 step centrifugation protocol: samples were centrifuged at 1.500 g for 15′ at 4 °C. Next, the supernatant was centrifuged again at 14.000 g for 15′ at 4 °C and stored at −80 °C [22].

2.2. RNA isolation

Total RNA was extracted from plasma using NucleoSpin miRNA Plasma (Macherey-Nagel) according to the manufacturer's instructions. Briefly, 0.3 ml of plasma were used for total RNA extraction and 25 fmoles of cel-miR-39 synthetic spike-in (mirVana miRNA Mimic, Life technologies) were added before protein precipitation step. Pellets were resuspended in 50 µl of RNAse-free water. NanoDrop (Thermo Scientific) spectrophotometer quantification was not reliable, likely because of the very low amount of RNA yielded. Thus, cel-miR-39 was measured by quantitative PCR (qPCR) to assess the efficiency of extraction.

2.3. miRNAs profiling and validation

TaqMan Human MicroRNA A Array version 3.0 (Applied Biosystems) was used for miRNA expression screening of 381 miRNAs. Reverse transcription (RT) was performed using 3 µl of total RNA and card A Megaplex RT primers (10×), according to the manufacturer instructions. Then, 2.5 µl of cDNA were pre-amplified. Finally, each sample was uploaded in each card and 50 cycles qPCR were performed using a 7900 HT Fast-Real Time PCR apparatus (Applied Biosystem). After median Ct values normalization, relative miRNA expression was calculated with the ΔΔCt method as previously described [9]. In order to validate profiling data, we filtered raw data (Table S1) for those miRNAs with a $Ct \leqslant 30$ and a significant $DDCt \geqslant |1|$. Single TaqMan microRNA assays were performed according to the manufacturer instruction (Applied Biosystems) and cel-miR-39, miR-17-5p and miR-106a were used as normalizers. Intra-assay variability for detection of individual miRNAs is indicated in Table S2. For the "DM1-miRNAs score" calculation, the module of the fold changes obtained for all validated miRNAs was averaged.

2.4. Statistical analysis

Continuous variables are expressed as mean ± standard error (SE) unless indicated differently. For group-wise comparisons, Mann–Whitney test or *t*-test were used as appropriate. The ability to discriminate between the DM1 and control groups was characterized by the receiver operating characteristic curve, and the area under the curve (AUC) was calculated. A ROC curve allows to visualize the reciprocal relationship between sensitivity (true positive fraction) and specificity (false positive fraction) and is an effective method to evaluate the performance of diagnostic tests. The calculation of sensitivity and specificity depends on the threshold value used to separate the two classes. As the thresholds gets higher, specificity increases and sensitivity decreases; conversely, lower threshold levels correspond to decreased specificity and increased sensitivity [23,24]. The Area Under Curve (AUC) is a value of diagnostic accuracy that quantifies the overall ability of the test to discriminate between control and diseased individuals. It ranges from totally non-informative ($AUC = 0.5$) to perfect test ($AUC = 1$) [25,26]. Spearman rank correlation was used to compare the DM1 miRNAs-score with muscle strength measured by MRC megascore [21]. Kruskal Wallis non parametric assay was used to compare the miR-133a and DM1 miRNAs-score levels with MIRS score. All tests were performed 2-sided and a $p \leqslant 0.05$ was considered as statistically significant. For statistical analysis and heat map data visualization, GraphPad Prism v.4.03 (GraphPad Software Inc.) and Genesis 1.7.2 (Graz University of Technology, Institute for Genomics and Bioinformatics) softwares were used, respectively.

3. Results

3.1. Identification of differentially expressed miRNAs in DM1 plasma

Plasma was derived from the peripheral blood of 36 consecutive DM1 patients and from 36 age and sex matched controls (CTR), displaying no neuromuscular disorders. As expected, most DM1 displayed myotonia, cataract and loss of muscle strength (Table 1). Total RNA was extracted from plasma samples and microRNAs profiling was performed by multiplex qPCR in small patient groups (DM1, $n = 24$ and CTR, $n = 26$). Out of 381 miRNAs measured (Table S1), 113 were detectable (average $Ct \leqslant 30$, highlighted in yellow in Table S1) in at least one of the groups and 14 were significantly modulated at least 2-fold, either positively or negatively (Fig. S1). Validation was performed by miRNA single qPCR assays (no multiplexing) and was extended to all recruited patients and CTR. Given the existing controversy on the most precise normalization method for circulating miRNAs [27], we decided to use, alone or in combination, both an exogenous spike-in normalizer (cel-miR-39) and the average of 2 stable endogenous miRNA normalizers (hsa-miR-106a and hsa-miR-17) [22]. Thus, one miRNA was considered as validated only if displayed significant differences after normalization with all of the following references: (1) the average of the 3 normalizers (Fig. 1); (2) cel-miR-39 values (Fig. S2A); (3) the average of miR-106a and miR-17 (Fig. S2B). Using these stringent normalization criteria, we found 9 significantly deregulated miRNAs in DM1 patients: miR-133a, miR-193b, miR-191, miR-454, miR-574, miR-885-5p and miR-886-3p were increased, while the levels of

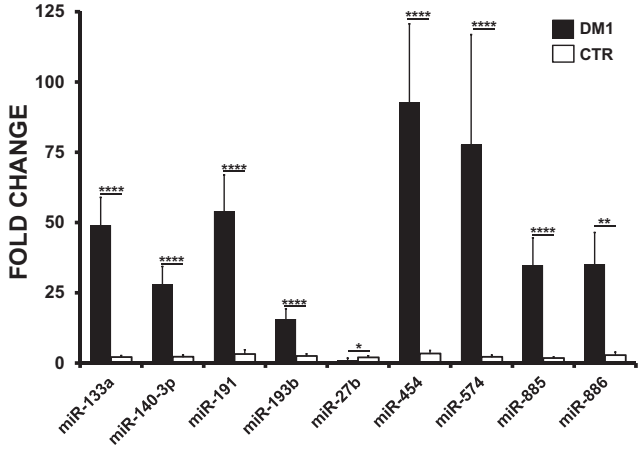

Fig. 1. Validation of miRNAs modulation in plasmas of DM1 patients. The bar graph represents mean values of the indicated miRNAs in plasmas of DM1 patients compared to CTR (DM1 $n = 36$, CTR $n = 36$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$).

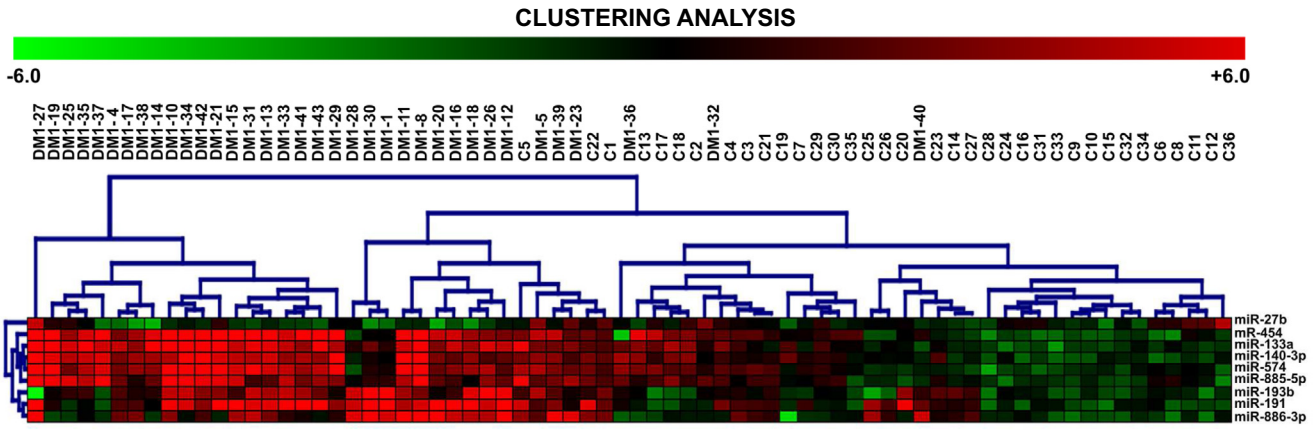

Fig. 2. Complete linkage clustering of miRNAs expression segregates DM1 from CTR samples. Each row represents a validated miRNA and each column represents an individual. Data are normalized versus the average of three normalizers (cel-miR-39, hsa-miR-17 and hsa-miR-106a) and are expressed in $\log_2$ scale. The color coding represents miRNAs expression, where red indicates an up-regulation and green a down-modulation.

miR-27b were decreased. It is worth noting that, for these 9 miRNAs, values obtained with the 3 normalization methods were very similar.

As expected, cluster analysis shows that the combination of these values, with only few exceptions, discriminated DM1 patients from CTR (Fig. 2).

3.2. miR-133a and DM1-miRNAs score accurately discriminate the two populations and correlate with muscle strength and MIRS class

To test the differentiating value of the identified miRNAs, we determined miRNA specific cutoff levels

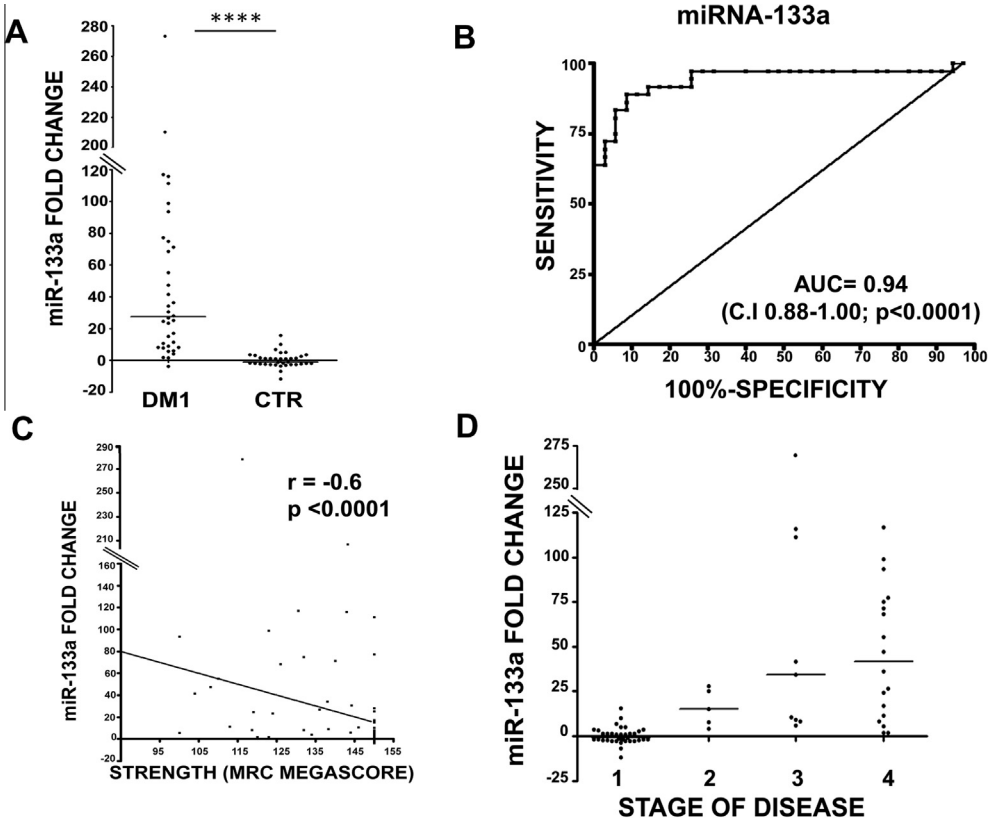

Fig. 3. miRNA-133a levels identify DM1 patients and correlate with muscle strength. (A) Dot plot of the miR-133a levels in DM1 and CTR. The segments among dots indicate median values for each group ($^{****}p < 0.0001$). (B) ROC curve illustrating sensitivity/specificity of miRNA-133a in discriminating between DM1 and CTR groups (AUC = 0.94, C.I. 0.88–1.00; $p < 0.0001$; DM1 $n = 36$, CTR $n = 36$). (C) Spearman's inverse correlation between miRNA-133a fold change compared to CTR and muscle strength measured by MRC megascore ($r = -0.6$; $p < 0.0001$; DM1 $n = 36$, CTR $n = 36$). (D) miR-133a data were divided according to MIRS score. Kruskal Wallis analysis identified a significant ($p < 0.0001$) increase of median miR-133a values in subjects belonging to higher MIRS classes.

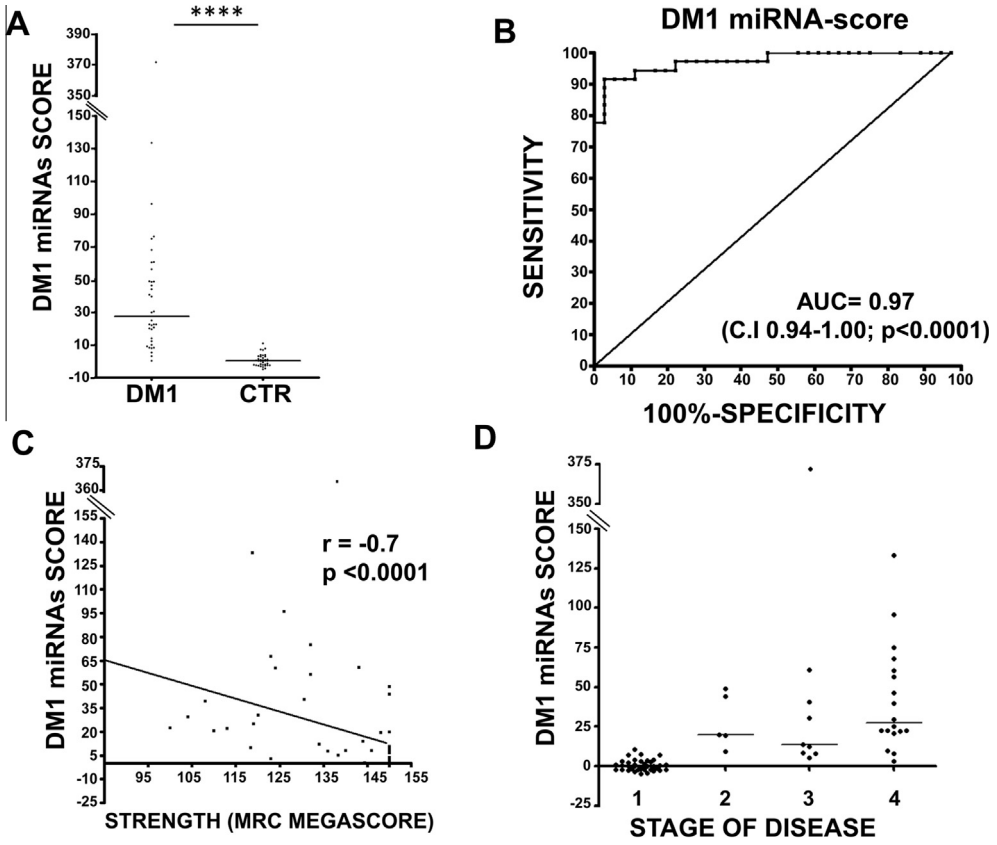

Fig. 4. DM1-miRNAs score identifies DM1 patients and correlates with muscle strength. A "DM1-miRNAs score" averaging the fold changes obtained for all miRNAs was calculated (DM1 $n = 36$, CTR $n = 36$). (A) Dot plot of the DM1-miRNAs score in DM1 and CTR. The segments among dots indicate median values for each group ($^{****}p < 0.0001$). (B) ROC curve illustrating sensitivity/specificity of DM1-miRNAs score in discriminating between DM1 and CTR groups (AUC = 0.97, C.I. 0.94–1.00; $p < 0.0001$). (C) Spearman's inverse correlation ($r = -0.7$; $p < 0.0001$) between DM1-miRNAs score and muscle strength measured by MRC megascore. (D) DM1-miRNAs score data were divided according to MIRS score. Kruskal Wallis analysis identified a significant ($p < 0.0001$) increase of median DM1 miRNA-score values in subjects belonging to higher MIRS classes.

deduced from Receiver–Operator Characteristic (ROC) curves (Fig. S3), and we found that miR-133a (Fig. 3A) had the highest area under the curve (AUC = 0.94) (Fig. 3B).

Moreover, we also tested whether combining the values of all differentially expressed miRNA allowed even better performances. To this aim, we calculated a "DM1-miRNAs score" averaging the fold changes obtained for all miRNAs. As expected, DM1-miRNAs score values were significantly different for DM1 and CTR groups ($p < 0.0001$, Fig. 4A). By analyzing the ROC curve, the obtained AUC of 0.97 was further increased compared to the one of miR-133a alone (Fig. 4B). Indeed, by using a threshold score of 6.95, both sensitivity and specificity of 91.7% for the identification of DM1 patients were achieved.

The low number of analyzed patients did not allow stratification and limited correlation analysis with clinically relevant parameters. However, considering all tested subjects, we identified a negative correlation of both miR-133a values (Spearman $r = -0.6$, Fig. 3B) and DM1-miRNAs score values (Spearman $r = -0.7$, Fig. 4C) with global muscle strength. Moreover, there was a significant increase of median miR-133a (Fig. 3D) and DM1 miRNA-score values (Fig. 4D) in subjects belonging to higher MIRS classes.

4. Discussion

Circulating miRNAs profile has been found to dynamically change in correlation with the pathological state in many diseases such as cancer, myocardial infarction, heart failure and Duchenne Muscular Dystrophy [28–30].

In this study, we identified 9 deregulated miRNAs in the plasma of DM1 patients. Particular attention was devoted to the still controversial issue of data normalization, adopting criteria that are at the same time stringent and easily transferable to a possible diagnostic routine.

Interestingly, when cluster analysis was performed, DM1 and control subjects segregated in different classes, albeit few exceptions were present. When considered as potential biomarkers, each miRNAs displayed different sensitivity and specificity levels, with AUC values ranging

from a minimum of 0.67 for miR-27b, to a maximum of 0.94 for miRNA-133a. Indeed, miRNA-133a, expressed in skeletal and cardiac muscles, was increased in DM1 plasmas compared to controls, and, considered alone, it allowed to distinguish DM1 patients almost as efficiently as the global signature of 9 miRNAs. MiR-133a plasma levels increase is unlikely due to passive release from damaged myofibers. Indeed, other equally or more abundant myomiRs such as miR-1, and miR-133b [11], did not display significant alterations of plasma levels in DM1 patients, as reported for Duchenne Muscle Dystrophy instead [13–15]. Interestingly, while myomiR levels are not modulated in DM1 skeletal muscle biopsies, their intracellular distribution is aberrant [11], possibly leading also to increased extracellular release of miR-133a, with unknown selection mechanisms. Another evidence is that DM1-miRNA levels in the plasma do not simply reflect passive release due to tissue damage is the fact miR-27b is down-modulated in DM1 patients; thus, either miR-27b release in the extracellular space is decreased or cell re-uptake of miR-27b is increased in DM1 patients.

Another muscle-related DM1-miRNA is miR-885, that has been associated to bovine muscle type-specific tissue formation and maintenance [31]. The other identified plasma DM1-miRNAs are not muscle specific/enriched. Indeed, miR-140-3p is highly expressed in the cartilage [32] and miR-27b controls fat deposition in dystrophic skeletal muscle by regulating fibro-adipogenic precursor differentiation [33]. Intriguingly, we previously found that miR-193b is down-regulated in skeletal muscle biopsies of DM2 patients [9]. It remains to be determined if this also observed in DM1. Finally, miR-886 is not even properly a miRNA, since it has been found to be identical to a fragment of Vault RNA 2 (VTRNA2) [34].

Given the fact that DM1 is a multisystemic disorder, it is possible that the tissue of origin of many DM1-miRNAs might not be skeletal muscles. In this respect, circulating miRNAs hold the potential to reflect the overall clinical state of the patient, rather than that of a specific tissue or organ. This seems particularly important given that DM1 is a multisystemic disease, with involvement of the heart, the eye, the central nervous system and of the insulin pathway [1].

The identification of new disease biomarkers is particularly important to monitor DM1 progression and effectiveness of new drug treatments. In this respect, a minimally invasive, relatively simple analytical assay is particularly desirable and currently not available for DM1 patient care. We identified one specific miRNA, miR-133a, and devised a DM1-miRNAs score that both correlate with muscle strength measurement and increased in patients with higher MIRS score, potentially reflecting disease severity. One interesting question that remains to be elucidated is whether the identified miRNAs are deregulated also in DM type 2 and other muscular dystrophies. Indeed, increased levels of miR-133a and miR-193b have been observed also in Duchenne Muscle Dystrophy [13–15]. Moreover, in a longitudinal study, it will be necessary to test miR-133a and DM1-miRNAs score changes over time during disease evolution.

In conclusion, despite the preliminary character of our observations, the results provide sufficient rationale for a larger confirmation study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nmd.2014.02.005.

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