EMBO
Molecular Medicine

Manuscript EMM-2010-00552

miRNAs as serum biomarkers for Duchenne Muscular Dystrophy

Davide Cacchiarelli, Ivano Legnini, Julie Martone, Valentina Cazzella, Adele D'Amico, Enrico Bertini and Irene Bozzoni

Corresponding author: Irene Bozzoni, SAPIENZA, University of Rome

---

Review timeline:

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   20 December 2010

Thank you for the submission of your manuscript "miRNAs as serum biomarkers for Duchenne Muscular Dystrophy" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, reviewer #1 highlights that a direct comparison between CK and miRNA levels is crucial to convincingly demonstrate the prognostic advantage of miRNA detection. Importantly, both reviewers #2 and #3 feel that additional data regarding the correlation between the serum levels of the miRNAs and severity of disease are required to strengthen the study. On a more editorial note, it would also be very important to provide more clinical information about the patients (see Reviewer #2) and to include a statement about informed consent to obtain patient material in the Material and Methods section of the manuscript, where it can be easily found. Regarding the previously published Fig 3a, the figure legend should specify that the figure was reproduced from a previous publication and please make sure you have obtained authorization from the publisher to reproduce this figure.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript, Cacchiarelli et al. propose the measurement of miRNA in the blood of DMD patients as a biomarker of disease progression and to monitor effectiveness of therapeutic interventions. The authors show that muscle-specific miRNAs can be detected in the bloodstream of DMD patients, and that increased expression levels correlates with the severity of the disease.
The search for novel, reliable DMD biomarkers is currently an imperative task to select patients for clinical trials and to monitor the effectiveness of therapeutic interventions. As such, the topic of this work is highly relevant. However, I found that the finding reported in this work is still preliminary and needs to be strengthened by additional studies, as outlined below.
A key point of this study is to demonstrate that measurement of miRNAs in the blood of DMD patients provides a prognostic advantage over currently used parameters, such as CK levels. As such, it is important that the authors show a direct comparison between miRNA and CK in the serum of patients or mdx mice. This comparison is the only meaningful parameter for this manuscript to effectively advance the current status of knowledge and the consequent applications in the field of DMD.
Indeed, one conceptual hurdle to be overcome here relates to the common origin of elevated blood levels of CK and miRNAs. The authors propose that the presence of miRNAs in the serum of DMD patients is due to passive release of "muscle content" from degenerating muscles. As such, serum miRNAs would reflect the "leakage" of dystrophic muscles, which is typically responsible also for the elevated levels of CK in the serum of DMD patients and mdx mice. Thus, it is not clear the conceptual advantage of measuring serum miRNAs, instead of CK, unless there is clear evidence in support of this claim. Therefore, the authors should monitor in parallel the CK and miRNA levels in the blood of DMD patients and mdx mice (treated or not), possibly at different stages of the disease, and see if there is any statistically meaningful difference in the association of these parameters with the severity of the disease (for DMD patients) or functional amelioration of muscle performance (in mdx mice treated with exon skipping).
In other words, the authors should show that miRNA actually provide a better parameter of disease progression, as compared to CK levels in patient samples and in mice. Are miRNA levels still elevated in conditions in which CK levels drop, in coincidence of muscle consumption? And if so, why degenerating muscles would still released their miRNA content, but not CK? Thus, in case miRNAs would show a more reliable biomarker of disease progression as compared to CK levels, it should be important to define why miRNA levels would provide more sensitive, long lasting and stable biomarker.

Other points
A direct comparison of muscle content release should be performed using the same technique (PCR). CK measurement is typically performed by detecting the enzymatic activity, while miRNAs are detected by PCR. This could be an intrinsic experimental caveat - i.e. PCR (and not miRNAs by themselves) might just provide a more stable and sensitive technology for detection of disease markers in the blood of patients. I'm wondering if PCR can be used for direct comparison of miRNAs versus the RNA of muscle genes, such as CK.
Not sure the size of the patient samples is adequate to support solid conclusion from a statistic standpoint. How many mdx mice have been analyzed in figure 3?

Referee #2 (Novelty/Model system Comments for Author):

This is an interesting manuscript, in which specific muscle-miRNAs, apparently released in the bloodstream of DMD patients from dystrophic muscles, as a consequence of muscle degeneration, correlate the severity of the disease (i.e.more elevated in DMD with more advanced disease

compared to DMD earlier in the course of the diseases, and to BMD)

The manuscript reports for the first time 3 miRNAs elevated in DMD; and propose that their determination could represent a novel biomarker for monitoring disease progression and response to novel curative treatment such as exon skipping.

The task is certainly ambitious and very interesting. Several observations are also novel and interesting; there are however several issues that the authors should consider to address as at the moment these weaknesses affect the impact that these finding have and also, potentially, the significance of the biological observation.

From a pure biological perspective, an issue which is not discussed is that the difference between "severe" older DMD and "milder" younger DMD is the amount of residual muscle which is left partially functional to perform its activity. The authors suggest that the "severe DMD" have higher levels of miRNA compared to the milder- younger DMD. However - as the miRNA are produced by muscle fibres and released in the extracellular space, there will be much less muscle in the later stage of the condition compared to the ealier pauci-symptomatic phases. As degeneration and regeneration are present from birth in DMD, it is not clear why the same disease process would at a later stage determine such a dramatic increase in miRNA- if corrected for muscle bulk the increase is phenomenally higher in older DMD- much higher than the authors claim.

Another issue- again from a general perspective- which should have been addressed is to have children who did follow a more severe disease course (and not who were simply at a more advanced stage of their condition and older) and compare the miRNA at the same age. This would have provided more compelling evidence of a correlation between severity and miRNA.

More specific methodological issues are ;

1) There is not detailed information about the patients - their age, how many were ambulant/non-ambulant, how many on steroids/not on steroids. Only limited information is provided in the legends of Figure 1 but this is crucial information which should be provided as a table. The same applies for the controls - how many, their age, gender. A significant difference between the age of the patients and the controls might lead to false positive or negative results.
2) The correlation between miRNAs and NSAA scores reached statistical significance but as NSAA can be used only for ambulant patients it is not clear whether the levels of miRNAs can be used as biomarker in severe cases. Also, it is not clear in how many patients the NSAA was not applicable, nor this reviewer knows which is the lower age limit at which this assessment tool can be used..
MiR-206 was able to discriminate DMD from WT and BMD but it was not clear in the paper how well the level of serum miRNAs (miR-1, miR-133, miR-206) correlate with the clinical severity of the disease.
3) It will be important to confirm where miRNA are expressed in muscle (see my previous comment on the decrease muscle bulk in aged DMD), and if levels in this tissue correlate with the serum miR-1, miR-133 and miR-206 in patients.
4) Figure 3 a is already publish in an article by the same group (Cell Metab. 2010 Nov 3;12(5):425-6.)
5) Figure 1 - the ROC curves for distinguishing DMD vs. WT, DMD vs. WT+BMD, DMD vs. BMD are presented using different colours. When the paper is printed in black and white it is difficult to distinguish between the three groups. Graphs with different pattern should be used.
6) Page 3 - "These results show that miR-206 is able to discriminate DMD from WT and BMD cases with almost absolute specificity (AUC always >0,94, p<0,001) but also miR-1 and miR-133 display a very good statistical score (AUC always >0,84 and >0,76 respectively)". The p-values for miR-1 and miR-133 should be presented as well.
7) Results. Serum samples from healthy, Becker and Duchenne children were collected under informative consensus? .

8. Discussion. The reference to Cozzi et al is not clear. Are the authors suggesting that there is otherwise no clear evidence from previously published literature on intense degeneration / regeneration in young dystrophic boys?

Referee #2 (Remarks to the Author):

1) No clear information about the patients - how many patients were included, their age, gender, ambulant/non-ambulant, on steroids/not on steroids. Some data about the patients is given in the text of Figure 1 but I think that is should be given clearly in a table in the paper. The same applies for the controls - how many, their age, gender. A significant difference between the age of the patients and the controls might lead to false positive or negative results.
2) The results show that the level of the studied miRNAs is enriched in DMD patients with a magnitude of 100-fold compared to healthy controls. MiR-206 was able to discriminate DMD from WT and BMD but there is no data in the paper showing how well the level of serum miRNAs (miR-1, miR-133, miR-206) correlates with the clinical severity of the disease. The correlation between miRNAs and NSAA scores reached statistical significance but as NSAA can be used only for ambulant patients it is not clear whether the levels of miRNAs can be used as biomarker in severe cases.
3) It will be interesting to see how the levels of miR-1, miR-133 and miR-206 expressed in muscles correlate with the serum miR-1, miR-133 and miR-206 in patients.
4) "miRNAs as biomarkers for therapeutic outcome measurements" - the authors treated mdx mice with a recombinant adeno-associated viral vector carrying a U1-chimeric antisense construct which induced skipping of exon 23. The exon skipping was very effective and in these mice the levels of studied miRNAs was reduced to almost wild type level. Based on that finding the authors concluded that the miRNAs (miR-1, miR-133, miR-206) can be utilized for measuring the outcome of the therapeutic intervention in humans. There is no direct experimental data in the paper supporting that statement as the exon skipping in human might be not as effective as in mice and the levels of miR-1, miR-133, miR-206 might be different in the treated human muscles.
5) Figure 3 a is already publish in an article by the same group (Cell Metab. 2010 Nov 3;12(5):425-6.)
6) Figure 1 - the ROC curves for distinguishing DMD vs. WT, DMD vs. WT+BMD, DMD vs. BMD are presented using different colours. When the paper is printed in black and white it is difficult to distinguish between the three groups. Graphs with different pattern should be used.
7) Page 3 - "These results show that miR-206 is able to discriminate DMD from WT and BMD cases with almost absolute specificity (AUC always >0,94, p<0,001) but also miR-1 and miR-133 display a very good statistical score (AUC always >0,84 and >0,76 respectively)". The p-values for miR-1 and miR-133 should be presented as well.

Referee #3 (Novelty/Model system Comments for Author):

I am convinced that the author's have demonstrated new biomarkers for dystrophinopathy, that correlate closely with the degree of overt pathology in patients. This is important because, as the authors discuss in their introduction, none of the present biomarkers reflect consistently the severity of the disease. I am also fairly convinced that these biomarkers can be used to check the efficacy of treatment in the Mdx murine model, subject to the caveat that I request data to clarify whether the miRNA levels correlate sensitively with the rescue of dystrophin expression in myofibres, as I discuss in the comments to the authors. I have requested two minor modifications that would clarify this point. With these modifications I would have no hesitation to recommend the article for publication, as it is of significant clinical importance and offers a useful and timely research tool.

Referee #3 (Remarks to the Author):

The authors wanted to identify bloodstream biomarkers that would reflect the severity and the progression of Duchenne muscular dystrophy. For this purpose, they measured in patient sera the level of microRNAs-miR-1, miR-133 and miR-206- by qRT-PCR using artificial microRNAs as internal controls. Quantifying miR206 in the blood- but also miR1, and miR133- the authors are able to distinguish DMD patients from BMD and Healthy subjects. The authors show convincing inverse correlations between the amount of miRNAs and the severity of the disease, suggesting that miRNAs, especially miR206, can be good serum biomarkers for dystrophinopathies.
miR206 and miR1 are also detected in mdx mice, a murine model for DMD. The authors wanted to show the level of those biomarkers in mdx mice, after treating the mice with AAV-U1#23. They observed a dramatic decrease in miRNAs in treated mdx mice. In parallel, they show that dystrophin

expression is rescued to 10% of wild type values, as shown by western blot analysis. However, miRNAs levels are reduced by >80% in treated mice (to 13-19% of the levels in untreated mdx mice). The disparity between the dystrophin amount and the miRNA levels, suggest that more than 10% of the fibers are rescued: it seems likely that most fibres are expressing dystrophin at a low level, as opposed to 10% of myofibres expressing dystrophin at normal levels. This interpretation would fit with the author's previous paper in which immunostaining shows that most of the fibers in TA, EDL Gastrocnemius and Heart were dystrophin positive (Denti et al. 2008). In this context, it would be good to know the proportion of fibers that are positive for dystrophin in mdx treated mice of the present study, to clarify whether the dramatic decrease in miRNA levels fits with the number of fibers that are rescued, and confirm that the reduction in miRNA levels measured in the blood of treated mdx mice does not overestimate the rescue by AAV treatment. The authors present HE staining showing a dramatic decrease in the inflammatory response in the muscle, supporting the idea that more than 10% of the fibers are rescued: most of the fibers shown are not necrotic. Despite this lack of certainty concerning the sensitivity of miRNA levels as an indicator of treatment efficacy, the strong inverse correlation in human samples between miRNA levels and disease severity supports the author's assertion that these miRNAs represent useful indicators of dystrophinopathic pathology.

Minor modifications:
I suggest two minor modifications:
1. The data are convincing in terms of the usefulness of these miRNAs as indicators of disease severity in patients but, as discussed above, there is a questionmark over the sensitivity of miRNA level as an indicator of the efficacy of treatment. It is not clear whether the 10% expression level of dystrophin represents complete rescue of a small proportion of myofibres or partial rescue of a large proportion of myofibres. The HE staining and near-complete rescue of serum miRNA level suggests the latter, but the point is important and requires clarification because the authors assert that the levels of these miRNAs represent an indicator of treatment efficacy. The authors need to demonstrate that miRNA levels correlate with treatment efficacy. I suggest that to do this they show western blots and dystrophin immunostains from several muscles, measuring levels of dystrophin protein and proportions of dystrophin-expressing myofibres in the treated mice.
2. Figure 3C shows HE staining. The right panel image is of poor quality, appearing blurred. A better quality image should be provided to show the degree to which necrosis is rescued.

1st Revision – Authors' Response   18 January 2011

Please find enclosed the revised version of the manuscript "miRNAs as serum biomarkers for Duchenne Muscular Dystrophy" by Cacchiarelli et al. that we have revised according to the requests of the referees.

Before going into the details of our revision, let me summarize the new data and changes introduced in the new version of the paper:

1) CK values for DMD patients have been introduced in the new Fig.2a and discussed in the text.

2) CK levels were measured in *mdx* versus wild type and exon-skipping treated animals. The results have been included in the new Fig.3b.

3) Symbols instead of colours have been used in figure 2a.
4) A Table with information about the patients is provided as supplementary material.
5) A new H/E staining in Fig.3c has been utilized.
6) The indication about informative consensus has been included
7) The p-values for miR-1 and miR-133 have been included in the figures

Moreover, the comparison of miRNA values in patients belonging to different classes of ages has been added (supplementary figure 1).

Here below the description of the new results and the point-by-point response to the referees.

Referee #1:

A key point of this study is to demonstrate that measurement of miRNAs in the blood of DMD patients provides a prognostic advantage over currently used parameters, such as CK levels. As such, it is important that the authors show a direct comparison between miRNA and CK in the serum of patients or mdx mice. This comparison is the only meaningful parameter for this manuscript to effectively advance the current status of knowledge and the consequent applications in the field of DMD.

.....In other words, the authors should show that miRNA actually provide a better parameter of disease progression, as compared to CK levels in patient samples and in mice. Are miRNA levels still elevated in conditions in which CK levels drop, in coincidence of muscle consumption? And if so, why degenerating muscles would still released their miRNA content, but not CK? Thus, in case miRNAs would show a more reliable biomarker of disease progression as compared to CK levels, it should be important to define why miRNA levels would provide more sensitive, long lasting and stable biomarker.

As the reviewer indicated, this is an important point.

We obtained from our clinician collaborators the CK values for all the patients who were analyzed for ambulatory activity test (NSAA), which is a gold standard functional test to score the motor ability of DMD patients. Values are reported in the new panel inserted in Figure 2a (bottom panel). It appears that, at difference with miRNAs showing a very clear inverse correlation with NSAA values, the CK values have fluctuations unrelated to the severity of the disease.

CK levels were also measured in mice. The new panel in Fig.3d indicates that, at difference with the statistically significant increase of miRNA levels in mdx versus the wt (p<0.01), the increase in CK levels is very low and not statistically significant (p=0.396).

Support to our findings derives also from previous published work on clinical assessments through MR imaging which indicated that there was a significant positive correlation between the T2 of the gluteus maximus muscle, the patient's age, CFS, timed Gower score and time to run 30 feet, while there was no statistically significant correlation with serum CK levels (Kim et al., Radiology. 2010 255:899-908)

Therefore, our data provide evidence that miRNA quantification in the serum is more effective for distinguishing patients with different degrees of DMD severity.

We would like to point to the fact that in order to correlate miRNA abundance with disease severity we purposely selected young ambulating patients since very well established and reliable clinical assessments (NSAA) were available. The situation in older, not ambulant, patients is more complicated due to heterogeneous clinical conditions and to the progressive loss of muscle mass. This can have strong effects on the amount of muscle cellular material released into the blood, as previously described for CK (Zatz et al., 1991). In agreement with this, in older patients we indeed observed decrease of serum miRNA levels (see new supplementary Figure1).

Finally, we agree with the referee comment that miRNA quantification "*might just provide a more stable and sensitive technology for detection of disease markers in the blood of patients*" due to the use of PCR technology, better than CK enzymatic assay. In fact, direct comparison of miRNA and CK quantifications indicate that:

- in mice, miR-1 and miR-206 quantification display 20- and 40-folds increase respectively, in mdx versus WT, while the CK increment is only 6 folds with no clear statistical significance (p=0.396).

- CK activity is sensitive to stress conditions and has to be measured in fresh serum preparations; in fact, we have verified that freezing and thawing strongly reduced its activity. On the contrary,

quantification of miRNAs is not altered upon freezing of the serum samples providing an easy way for maintaining and analyzing specimen even after long periods of time.

A direct comparison of muscle content release should be performed using the same technique (PCR). CK measurement is typically performed by detecting the enzymatic activity, while miRNAs are detected by PCR. This could be an intrinsic experimental caveat - i.e. PCR (and not miRNAs by themselves) might just provide a more stable and sensitive technology for detection of disease markers in the blood of patients. I'm wondering if PCR can be used for direct comparison of miRNAs versus the RNA of muscle genes, such as CK.

mRNAs are in general very unstable in the blood/serum; therefore, the quantification by PCR of CK mRNA can be very unreliable. At variance with mRNAs, miRNAs are instead particularly stable in the blood/serum because they are associated in vesicles called esosomes which protect them from serum RNases. RNase treatment of DMD sera have proven that these RNA species are indeed very resistant to this type of degradation (not shown).

Not sure the size of the patient samples is adequate to support solid conclusion from a statistic standpoint. How many mdx mice have been analyzed in figure 3?

26 DMD patients and 10 mdx have been analysed. Even the lowest values are at least more than 10 times higher that the highest wt controls. This is also confirmed by the low p-values now indicated in figure 1a (DMD) and figure 3d (mdx).

Referee #2

From a pure biological perspective, an issue which is not discussed is that the difference between "severe" older DMD and "milder" younger DMD is the amount of residual muscle which is left partially functional to perform its activity. The authors suggest that the "severe DMD" have higher levels of miRNA compared to the milder- younger DMD (if comparing kids below 6-years). However - as the miRNA are produced by muscle fibres and released in the extracellular space, there will be much less muscle in the later stage of the condition compared to the earlier pauci-symptomatic phases. As degeneration and regeneration are present from birth in DMD, it is not clear why the same disease process would at a later stage determine such a dramatic increase in miRNA- if corrected for muscle bulk the increase is phenomenally higher in older DMD- much higher than the authors claim.

As replied to ref.#1, we purposely selected young ambulant patients (kids below 6-years) since very well established clinical assessments (NSAA) were available for them. Moreover, in these patients, even if degeneration is quite intense, the total muscle mass is not expected to decrease due to conspicuous regeneration and physiological growth.

As also requested in the following point, the comparison between patient DMD 10 (5.5 years-old) and DMD 9 (5.7 years-old) provides an additional answer to this point: while the first has a NSAA value of 16 and more than 400×10^4/ml copies of miR-1, the second has NSAA of 25 and 15 $\times 10^4$/ml copies of miR-1.

The situation is instead very different in older, not ambulant, patients who have more heterogeneous clinical conditions and progressive loss of muscle mass due to reduced regenerating capacity. This can affect the amount of muscle cellular material released into the blood; in agreement with previous observations on CK (Zatzet al., 1991), older patients show an overall decrease of miRNA serum levels (see new supplementary Figure1).

When we refer to the diagnostic use of miRNAs to monitor the outcome of therapeutic interventions we refer to the "young" class of patients, namely those that still have enough muscle mass to be "cured" by the therapeutic treatment.

We have tried to better discuss this issue.

Another issue- again from a general perspective- which should have been addressed is to have children who did follow a more severe disease course (and not who were simply at a more advanced stage of their condition and older) and compare the miRNA at the same age. This would have provided more compelling evidence of a correlation between severity and miRNA.

See previous point the discussion on patients DMD9 and DMD10.

1) There is not detailed information about the patients - their age, how many were ambulant/non-ambulant, how many on steroids/not on steroids. Only limited information is provided in the legends of Figure 1 but this is crucial information which should be provided as a table. The same applies for the controls - how many, their age, gender. A significant difference between the age of the patients and the controls might lead to false positive or negative results.

That was an important omission. We have added a table (in supplementary material) where the following data are reported: type of mutation, age, NSAA values, walking ability and steroid treatment.

2) The correlation between miRNAs and NSAA scores reached statistical significance but as NSAA can be used only for ambulant patients it is not clear whether the levels of miRNAs can be used as biomarker in severe cases. Also, it is not clear in how many patients the NSAA was not applicable, nor this reviewer knows which is the lower age limit at which this assessment tool can be used. MiR-206 was able to discriminate DMD from WT and BMD but it was not clear in the paper how well the level of serum miRNAs (miR-1, miR-133, miR-206) correlate with the clinical severity of the disease.

The NSAA test was not applicable only in 3 very young patients (<3y/o). The reason was that the NSAA is reliable in patient older then 4 years (see reference Mazzone et al., 2010); however all of them had normal muscle strength at neurological assessment and they are indeed indicated with maximum NSAA score according to normal ambulation. NSAA score is a test that can be performed in about 10 minutes where several motor actions have to be performed (http://www.muscular-dystrophy.org/assets/0000/6388/NorthStar.pdf). NSAA is applicable to any ambulant patient and is the gold standard to monitor disease severity because motor activity is the primary measurable defect of DMD.

For non-ambulating patients, where NSAA reaches the score=0, there are other indicators like muscle measurements, adipose and fibrose tissue deposition measured by NMR imaging. Unfortunately, no uniform functional, non-invasive, tests to evaluate disease progression are available. In future work, we plan to extend our analysis also to a large number of such "old" cases in order to define:

   1) whether the levels of miR-206 (specifically expressed in precursor muscle cells) is an appropriate marker for establishing the regenerating potentiality of the patient
   2) whether miR-1 and miR-133 (present in mature fibers) levels can represent indicators of the residual muscle mass.

When we refer to the diagnostic use of miRNAs to monitor the outcome of therapeutic interventions we refer to the "young" class of patients, namely those that still have enough muscle mass to be

"cured" by the therapeutic treatment.

3) It will be important to confirm where miRNA are expressed in muscle (see my previous comment on the decrease muscle bulk in aged DMD), and if levels in this tissue correlate with the serum miR-1, miR-133 and miR-206 in patients.

We previously showed that dystrophic muscles contain such miRNAs even if their levels are altered with respect to wild type conditions. In particular: miR-1 and miR-133 undergo 2-fold reduction while miR-206 levels undergo 2-fold increase (Cacchiarelli et al., 2010). Interestingly, we described that miR-206 is specifically expressed in activated satellite cells which are known to increase in the dystrophic muscle. In spite of these 2-fold variations between DMD and control muscle cells, the differences we observed in the serum are much higher (100 fold) indicating that the miRNA serum contribution derives from the intensive degeneration occurring in DMD muscles rather than constitutive muscle leakage.

4) There is no direct experimental data in the paper supporting that statement as the exon skipping in human might be not as effective as in mice and the levels of miR-1, miR-133, miR-206 might be different in the treated human muscles.

It is our intention to test our hypothesis whenever such human samples will be available. We have rephrased this statement

5) Figure 3a is already publish in an article by the same group (Cell Metab. 2010 Nov 3;12(5):425-6.)

Indeed one of the mdx analyzed in the present study was one included in the analysis of the Cell Metabolism paper. We have selected a different photograph.

6) Figure 1 - the ROC curves for distinguishing DMD vs. WT, DMD vs. WT+BMD, DMD vs. BMD are presented using different colours. When the paper is printed in black and white it is difficult to distinguish between the three groups. Graphs with different pattern should be used.

We have modified the figure following this suggestion.

7) Page 3 - "These results show that miR-206 is able to discriminate DMD from WT and BMD cases with almost absolute specificity (AUC always >0,94, p<0,001) but also miR-1 and miR-133 display a very good statistical score (AUC always >0,84 and >0,76 respectively)". The p-values for miR-1 and miR-133 should be presented as well.

We have introduced such values.

8) Results. Serum samples from healthy, Becker and Duchenne children were collected under informative consensus?

Yes, this has been specified.

9) Discussion. The reference to Cozzi et al is not clear. Are the authors suggesting that there is otherwise no clear evidence from previously published literature on intense degeneration/regeneration in young dystrophic boys?

The sentence was unclear, we meant that in dogs there was the formal proof that also immature/regenerating fibers undergo degeneration in Duchenne muscles. We have clarified the sentence.

Referee #3

1. It is not clear whether the 10% expression level of dystrophin represents complete rescue of a small proportion of myofibres or partial rescue of a large proportion of myofibres. The HE staining and near-complete rescue of serum miRNA level suggests the latter, but the point is important and requires clarification because the authors assert that the levels of these miRNAs represent an indicator of treatment efficacy. The authors need to demonstrate that miRNA levels correlate with treatment efficacy. I suggest that to do this they show western blots and dystrophin immunostains from several muscles, measuring levels of dystrophin protein and proportions of dystrophin-expressing myofibres in the treated mice.

In the last 5 years we have analyzed quite many mdx mice (> 100) injected with AAV recombinant viruses able to induce exon skipping. We have standardized the procedures such that we can be confident that similar transduction efficiencies and dystrophin rescue is obtained in different experiments. Our data have previously shown (Denti et al., 2006a and b; Denti et al., 2008) that a large numbers of fibers are transduced (GFP analysis), each one with little rescue of dystrophin (immunostaining). Morpho-functional analysis further proved that the benefit involved a large number of cells. Moreover, our recent work identified the molecular basis by which little dystrophin rescue is able to revert the dystrophic phenotype to almost wild type conditions (Cacchiarelli et al., 2010).

2. Figure 3C shows HE staining. The right panel image is of poor quality, appearing blurred. A better quality image should be provided to show the degree to which necrosis is rescued.

This has been done.

I hope that the additional data and the responses to the referees' criticisms will make now the paper suitable for publication.

2nd Editorial Decision   15 February 2011

Thank you for the submission of your revised manuscript "miRNAs as serum biomarkers for Duchenne Muscular Dystrophy" to EMBO Molecular Medicine. We have now received the enclosed reports from the reviewers who were asked to re-assess it.

As you will see, the reviewers acknowledge that the manuscript was significantly improved during revision. However, while reviewer #3 indicates that that the manuscript is suitable for publication, reviewer #1 and #2 raise concerns that should be convincingly addressed. Since we do acknowledge the potential interest of your findings, we would therefore be open to allow a second revision of the manuscript that would address the outstanding issues.

Importantly, both reviewers #1 and #2 highlight that it is essential to tone down the conclusions

regarding the definite prognostic potential of the reported set of miRNA biomarkers and discuss explicitly that replication in a larger cohort will be needed to validate your findings. This should be reflected in the Abstract and Discussion section.

Revised manuscripts should be submitted within one month of a request for revision; they will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS

Referee #1 (Remarks to the Author):

While the authors have addressed most of the concerns raised by the reviewers, it is still unclear why miRNA release from dystrophic muscles should differ from CK release. This should be at least explained in the discussion. Furthermore, the authors should tone down their statements on the actual prognostic value of this assay for DMD patients, as it should be validated in a larger cohort of patients. So far, the most parsimonious conclusion to deliver to the scientific community is that measuring circulating miRNAs can provide a more accurate non-invasive test (for technical reasons, in the absence of biological insight) to evaluate the disease progression in DMD patients. And future studies should be performed to confirm this interesting idea, and determine if specific miRNA profiles can discriminate DMD patients from patients affected by other muscular dystrophies or neuromuscular disorders or myotrauma.

Referee #2 (Novelty/Model system Comments for Author):

The finding is of inteerst and I would be enclined to publish it. However the authors do not appear to be aware of limitations of this observation. It will have to be replicated in a independent and much larger population (they have studied only 10 DMD and 4 or 5 BMD). The discussion and abstract do need to be substantially modified to take into account this limitation. While ut is appropriate for this pilot to be published, it certainly is not a definitive paper , although the authors make it sounds like it is.

Referee #2 (Remarks to the Author):

The authors have made several changes including a table with information about the patients (mutation, age, NSAA, ambulant, steroids).

The main finding is the correlation between microRNA and NSAA - the authors showed inverse correlation between the serum levels of mir-1, miR-133 and miR-206 and NSAA (the progressive decrease in NSAA is associated with increase in miRNAs serological levels). They used data from 10 young, ambulant DMD patients (Figure 2). The result indicated "independently from age, the increase in miRNA levels correlate with the severity of muscle damage" which raises the question what is the correlation between the tree microRNAs and the NSAA in older but still ambulant patients (DMD patients 13, 14, 15, 23, 24, 25; Supplementary table 1).

I think the findings of the authors are certainly interesting, but they are still preliminary. My main problem is with the strength of the claiming, based on a very small patient population.
I have no doubt that these interesting result needs to be replicated in larger numbers of boys with DMD, as the total sample size tested by the authors is very small by genetic association standards (10 DMD and 4 BMD patients): the joint p value of the whole study was 0.001, whereas convincing association evidence requires a p value less than 10_7. Furthermore, the true effect on disease

progression cannot be measured accurately from the same data used to discover variant association as the discovery of genetic associations often overstates the effect size. Thus, the magnitude of effect of miRNAs on disease severity is yet to be established and the discussion needs to reflect this limitation. The final conclusion:
"In conclusion, our results indicate that quantification in the serum of the described miRNAs (dystromiR), or even of only one of them, can be utilized as a biomarker tool to evaluate the severity and progression of the disease in human patients. In consideration of the results with mdx mice "cured" through exon skipping, we propose to test whether this method can be applied as an objective parameter for measuring the outcomes and effectiveness of different therapeutic interventions." Is therefore not fully substantiated by the study findings.

Similarly, the statement in the discussion "Interestingly, patient DMD10, coetaneous of DMD9 but with worst motor ability (NSAA 16 and 25 respectively), displays a 26 fold increase of miRNA levels in comparison to DMD9 (right panel of Figure 2a). This indicates that, independently from age, the increase in miRNA levels correlate with the severity of muscle damage".
Is a very strong statement based on the trend observed in just a few boys.

Other minor comments:
1. "These results show that miR-206 is able to discriminate DMD from WT and BMD cases with almost absolute specificity (AUC always >0,94, p<0,001) but also miR-1 and miR-133 display a very good statistical score (AUC always >0,84 and >0,76 respectively)".

The p-values for miR-1 and miR-133 should be presented as well.

Figure 2 legend: "Patients DMD 1, 2 and 3, due to their age (3 years), have a non-collaborative behaviour to perform the entire NSAA;".

However In the supplementary table 1 DMD2 and DMD3 are 4.1 and 4.8 years respectively.

Serum CK vs miRNA determination. Were these 2 values obtained at the same time?

Referee #3 (Novelty/Model system Comments for Author):

The authors have addressed my minor modifications requests satisfactorily. I now have no hesitation to recommend the article for publication, as it is of significant clinical importance and offers a useful and timely research tool.

Referee #3 (Remarks to the Author):

The authors have addressed my minor modifications requests satisfactorily. I now have no hesitation to recommend the article for publication, as it is of significant clinical importance and offers a useful and timely research tool.

2nd Revision - Authors' Response	18 February 2011

Thank you for the consideration and interest given to our paper. We have now produced a revised version including the last requirements of the referees.

In particular, we have toned down our conclusions on the abstract and discussion introducing these two sentences:

Abstract

Even though the analysis on a larger number of patients should allow to obtain more refined correlations with the different stages of disease progression, we propose that miR-1, miR-133 and miR-206, are new and valuable biomarkers for the diagnosis of DMD and, possibly, also monitoring the outcomes of therapeutic interventions on humans

Discussion (p.6)

Even though a larger collection of data from DMD patients will be required in order to establish a precise correlation between miRNA levels, extent of muscle degeneration and stage of the disease (age, ambulating conditions and time since wheel chair use), our studies indicate that the serum quantification of specific miRNAs (dystromiR), or even of only one of them, can be utilized as a biomarker tool to reveal DMD conditions in humans.

Here below the other specific points:

Referee #1

While the authors have addressed most of the concerns raised by the reviewers, it is still unclear why miRNA release from dystrophic muscles should differ from CK release. This should be at least explained in the discussion.

We have better explained this point in the discussion, (p. 6)

Further advantage of miRNA also relates to the fact that CK activity is very sensitive to stress conditions and has to be measured in fresh serum preparations, while the serum stability of miRNA is very long lasting (Mitchell et al, 2008).

Furthermore, as previously explained by ref#1 "CK measurement is typically performed by detecting the enzymatic activity, while miRNAs are detected by PCR. PCR might just provide a more stable and sensitive technology for detection of disease markers in the blood of patients."

Here below there is also the demonstration of different stability between CK and miR-1 in fresh serum samples versus 2 cycles of freeze-thawing. This indicates that CK activity is altered by sample treatments (in this case freeze-thaw cycles) while miR titration is unaffected.
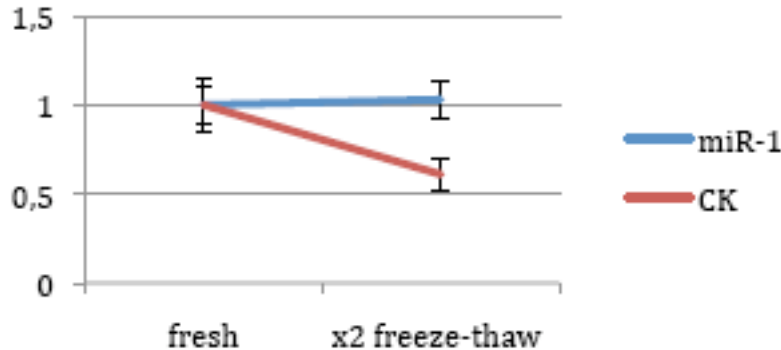
.....And future studies should be performed to confirm this interesting idea, and determine if specific miRNA profiles can discriminate DMD patients from patients affected by other muscular dystrophies or neuromuscular disorders or myotrauma.

See new abstract and discussion

Referee #2

The finding is of interest and I would be enclined to publish it. However the authors do not appear to be aware of limitations of this observation. It will have to be replicated in an independent and much larger population (they have studied only 10 DMD and 4 or 5 BMD). The discussion and abstract do need to be substantially modified to take into account this limitation. While it is appropriate for this pilot to be published, it certainly is not a definitive paper, although the authors make it sounds like it is.

See new abstract and discussion

Similarly, the statement in the discussion "Interestingly, patient DMD10, coetaneous of DMD9 but with worst motor ability (NSAA 16 and 25 respectively), displays a 26 fold increase of miRNA levels in comparison to DMD9 (right panel of Figure 2a).

This indicates that, independently from age, the increase in miRNA levels correlate with the severity of muscle damage".

Is a very strong statement based on the trend observed in just a few boys.

We have attenuated the statement

Other minor comments:

The p-values for miR-1 and miR-133 should be presented as well.

Done

Figure 2 legend: "Patients DMD 1, 2 and 3, due to their age (3 years), have a noncollaborative behaviour to perform the entire NSAA". However, in the supplementary table 1 DMD2 and DMD3 are 4.1 and 4.8 years respectively.

Sorry for DMD2 and DMD3 there was a mistake: in the table their current age was introduced and not that at the time of the NSAA test. The table has been corrected.

Serum CK vs miRNA determination. Were these 2 values obtained at the same time?

Yes.

Referee #3

The authors have addressed my minor modifications requests satisfactorily. I now have no hesitation to recommend the article for publication, as it is of significant clinical importance and offers a useful and timely research tool.

I hope that the changes introduce will now satisfy the referees' requests.