Downregulation of microRNA-29, -23, and -21 in urine of Duchenne muscular dystrophy patients

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Abstract

Aim

To study the signature of 87 urinary miRNAs in Duchenne muscular dystrophy (DMD) patients, select the most dysregulated and determine statistically significant differences in their expression between controls, ambulant and non-ambulant DMD patients, and patients on different corticosteroid regimens.

Patients/materials & methods

Urine was collected from control (*n* =20), ambulant (*n* =31) and non-ambulant (*n* =23) DMD patients. MiRNA expression was measured by RT-qPCR.

Results

MiR-29c-3p was significantly downregulated in ambulant DMD patients while miR-23 -3p and miR-21-5p were significantly downregulated in non-ambulant DMD patients compared to age matched controls.

Conclusions

MiR-29c-3p, miR-23 -3p and miR-21-5p are promising novel non-invasive biomarkers for DMD, and miR-29c-3p levels are differentially affected by different steroid regimens, supporting the antifibrotic effect of steroid therapy.

Keywords: Duchenne Muscular Dystrophy, microRNA, exosome, biomarker, deflazacort, prednisolone

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked and progressive neuromuscular disorder affecting 1 in 5000 newborn males [1]. It is more commonly caused by out-of-frame deletions or, more rarely, duplications, nonsense or other small mutations affecting the dystrophin gene (*DMD*) [2]. *DMD* is the largest gene in the human genome. Its sequence, spanning ~2.3 megabases, [3] encodes dystrophin, a 427 kDa protein principally expressed in skeletal and cardiac muscle, connecting the sarcolemma to the actin cytoskeleton. Dystrophin plays a significant structural role by giving plasticity and flexibility to the muscle fibers, ensuring stability over the contraction-relaxation phase [4]. Affected children are typically diagnosed between 3 and 5 years of age and the progressive skeletal muscle weakness and wasting used to lead to loss of ambulation at a mean age of 9.5 years. Due to the implementation of current standards of care and corticosteroid administration, the mean age at which ambulation is lost has shifted to 12-14 years [5].

Prednisolone and deflazacort are the most commonly used corticosteroids, administered on either a daily or intermittent regimens [6]. The mechanism underlying the pharmacological action of these corticosteroids is not completely understood, but is likely to involve both an anti-inflammatory action and anabolic effects in dystrophic muscle via the activation of a metabolic transcription factor [7-9]. Progressive weakness nevertheless continues leading to premature death between the second and fourth decade of life [10, 11].

DMD is currently an incurable disease, although encouraging results are emerging from different clinical trials, and two personalised medicine drugs, eteplirsen and ataluren, have received conditional approval in the US and Europe, respectively [10, 12-14]. A number of other therapeutic approaches are being trialled [15].

The diagnosis of DMD is usually made by combining genetic, clinical and biochemical tests and, in selected cases, by muscle biopsy. Levels of creatine kinase (CK), an enzyme involved in energy production and utilisation [16], are generally elevated in serum from patients. Serum CK is not however considered to be a reliable circulating biomarker because it is subject to fluctuations, mainly related to age and its modulation by physical activity [17, 18]. In addition, the progressive loss of muscle mass in DMD leads to a secondary reduction of CK levels, which therefore does not adequately capture the progressive nature of the condition. Serum matrix metalloproteinase-9 (MMP-9) is being studied as potential serum biomarker for dystrophinopathies as it increases significantly with age in patients' serum. Nevertheless, its efficacy in monitoring disease progression and therapeutic response remains to be confirmed [19, 20]. Elevated levels of myomesin 3 (MYOM3) protein fragments have been found in serum from DMD patients and are promising candidate for monitoring experimental therapies [21]. Also, urinary levels of the amino terminal fragments of the sarcomeric protein titin (N-ter titin) represent a potential non-invasive biomarker useful for the diagnosis and to monitor the response to therapies [22]. Despite these encouraging results obtained from proteomic studies, there is still a pressing need for novel, non-invasive and reliable biomarkers in DMD sensitive to disease progression and to assess their efficacy in response to therapeutic intervention.

MicroRNAs are small (~22 nucleotides) endogenous non-coding RNAs implicated in post-transcriptional regulation by binding the 3' untranslated region (UTR) of their messenger RNA (mRNA) targets [23]. Through this inhibitory mechanism, microRNAs modulate the expression of genes involved in pathways regulating skeletal muscle formation [24], differentiation [25] and homeostasis [26]. Moreover, miRNA dysregulation in serum has

been associated with a few paediatric neuromuscular conditions including DMD and spinal muscular atrophy (SMA) and in their respective murine animal models [27-29]. MiRNAs are also present in urine where they are included in small microvesicles called exosomes (40-100 nm) that protect them from degradation by RNases [30].

Exosomes are secreted by different cell types, including myoblasts, allowing cell-to-cell signalling by transferring their cargo molecules (mainly composed of miRNA, mRNA, lipids and proteins) [31]. Urinary exosomes have been investigated in urinary tract related diseases [32, 33], breast cancer [34] and neurodegenerative conditions [35]. However, there is no information on the contribution (if any) of skeletal muscle cells to the exosome population in urine, nor has there been any study on the expression of urinary miRNA in patients with muscular dystrophies.

In order to investigate the potential of urinary miRNAs as a novel non-invasive biomarker in DMD, we profiled their pool isolated from urinary exosomes of ambulant and non-ambulant DMD patients and age matched controls and found >50 miRNAs downregulated in DMD patients. From these, 5 candidate miRNAs were selected for further validation, based on previous reports indicating their involvement in skeletal muscle related diseases [36-41]. Finally, to test if there was any association between miRNA levels in urine and corticosteroid treatment, we studied the expression of the most dysregulated miRNAs in patients treated with prednisolone or deflazacort following a daily or intermittent regimens.

2. Materials & Methods

2.1. Subject selection and urine collection

The patients included in this cross-sectional study are part of a cohort of DMD boys taking part in a multicenter natural history study registered in clinicaltrials.gov (NCT02780492). Patients are assessed every six months according to a standardized protocol. Samples from 54 patients recruited in London, Paris, Newcastle and Leiden were analysed. This study was approved by the London-Bromley Research Ethics Committee (REC 12/LO/0442) and all Ethical Committees in the countries involved. All patients and their families signed the informed consent and assent for the Biobank for Neuromuscular Disorders (approved by The Hammersmith and Queen Charlotte's and Chelsea Research Ethics Committee - 06/Q0406/33).

Twenty healthy age-matched volunteers were recruited from patients' families and friends at Great Ormond Street Hospital. Urine samples (~ 20 ml) were collected on the day of the study visit (morning, not fasting) and immediately frozen at -80^{o}C until analyses were performed. The demographic, clinical data and corticosteroid therapy regimen administered are shown in **Supplementary Table 1**.

2.2. Exosome isolation

Exosomes were extracted from urine using the miRCURY™ Exosome Isolation Kit – Cells, Urine & CSF (Exiqon) according to the manufacturer's instructions. Briefly, 1.6 mL of urine was centrifuged for 5 min at 10,000 x g to remove cell debris, and 1.5 mL of the resulting supernatant was incubated overnight at 4°C after the addition of 600 µl of Precipitation Buffer B. In the last step, the supernatant was completely removed by centrifugation (30 minutes at 10,000 x g at 20°C), and the pellet was used for RNA isolation.

153

154 **2.3. RNA isolation**

155 To isolate microRNAs contained in exosomes, the miRCURY™ RNA Isolation Kit – Cell &
156 Plant (Exiqon) was used according to the manufacturer's instructions. Briefly, the pellet
157 obtained from the exosome isolation was re-suspended in 350 µl lysis solution containing
158 1.25 µl MS2 RNA carriers (Roche) and 1 µl of synthetic UniSp2, UniSp4, UniSp5 RNA
159 spike-in mix (Exiqon) required to monitor the isolation efficiency. After vortexing, 200 µl of
160 96 – 100% ethanol were added to the solution, then it was transferred onto a Mini Spin
161 Column and centrifuged for 1 minute at > 3500 x g. Subsequently, the column was subjected
162 to three washing cycles by adding 400 µl of Wash Solution and centrifuged for 1 minute at
163 14000 x g.
164 Total RNA, including small RNAs from exosomes, was obtained by adding 100 µl of Elution
165 Buffer followed by two centrifugation steps (2 minutes at 200 x g and 1 minute at 14000 x g).
166

167 **2.4. microRNA profiling**

168 Profiling analysis were performed on urinary exosomes from 15 healthy controls, 15
169 ambulant DMD and 17 non-ambulant DMD patients.
170 *RT:* cDNA was generated by reverse transcription using the Universal cDNA synthesis kit II
171 (Exiqon) according to the manufacturer's instructions. A fixed volume of 4 µl of total RNA (5
172 ng/µl)/sample was used as the starting material. The quality of the samples was verified by
173 adding to the mix 1 µl of synthetic UniSp6/cel-miR-39 spike-in mix (Exiqon).
174 *qPCR:* The reactions were performed using a miRCURY LNA™ Pick-&-Mix microRNA
175 PCR SYBR green- ased panels (containing primers for 87 urinary miRNAs, **Table S2)** and a
176 StepOne Plus 96 well Real-time PCR System (ThermoFisher). A total volume of 10 µl of

177 cDNA/sample was added in the mix for the profiling analysis, according to the
178 manufacturer's instructions (Exiqon).
179 *Normalization:* Expression analysis was performed using the GeneX software (Exiqon).
180 Global mean value normalisation using the global mean of all miRNAs that had CT values
181 <34 for the microRNA profiling.
182 *Heatmaps:* Heatmaps and average linkage hierarchical clusters showing the miRNA signature
183 pattern within the samples were designed on http://www1.heatmapper.ca/expression/ [42].
184

185 **2.5. microRNA validation**

186 In the validation step, we studied the expression of five selected candidates (miR-21-5p, miR-
187 22-3p, miR-23 -3p, miR-29c-3p, and miR-103a-3p) in urinary exosomes from 20 controls
188 (average age=9 years), 31 ambulant (average age=8 years) and 23 non-ambulant DMD
189 patients (average age=14 years), including the samples used for miRNA profiling.
190 *RT:* cDNA was generated by reverse transcription using the TaqMan™ Advanced miRNA
191 cDNA Synthesis Kit (ThermoFisher) according to the manufacturer's instructions. 2 µl of
192 total RNA (5 ng/µl)/sample was used as the starting material. The quality of the samples was
193 verified by adding to the mix 1 µl of synthetic UniSp6/cel-miR-39 spike-in mix (Exiqon).
194 *qPCR:* The reactions were performed using a qPCR TaqMan small RNA Assay (Life
195 Technology) and a StepOne Plus 96 well Real-time PCR System (ThermoFisher).
196 *Normalization:* Normalisation using the ΔΔCt method to a stable reference gene (miR-16c-
197 5p) detected by NormFinder algorithm were performed at the validation stages [43, 44].
198

199 **2.6. Negative controls**

200 Before the exosomal miRNA isolation, we added three synthetic non-human spike in
201 controls, UniSp2, UniSp4 and UniSp5, in the lysis buffer in order to monitor the efficiency of

the process. As suggested in the manufacturer's protocol, UniSp2 should amplify at the level of highly expressed microRNAs, whereas UniSp4 should amplify approximately 6.6 cycles later and UniSp5 might not always be detectable.

Our data confirm that the isolation process in all samples was successful. The detected average cycle threshold (Ct) for UniSp2 was 21.46 and UniSp4 was amplified for an average of 6.56 cycles later than UniSp2 (average Ct=28.02). Little or no expression of UniSp5 was detected across the samples.

Two additional synthetic controls, *cel-miR-39* and UniSp6, were added to the mix immediately before the retro-transcription. These two controls were expressed in all samples, indicating that the cDNA used for the profiling was of high quality (**Fig S1**).

2.7. Statistical analysis

The Mann-Whitney test was used for statistical analysis of two groups of data, whereas One-way analysis of variance was used to determine statistical significance between three and four groups of subjects. The Bonferroni test for the correction of the p-value was performed for multiple comparisons (profiling). Data are presented as mean ± standard error of the mean (Mean ± SEM). GraphPad Prism 7.0 software was used for statistical analysis and graph design.

3. Results

3.1. Exosomal microRNA profiling in urine of DMD patients and healthy controls

34 microRNAs were either undetected or weakly expressed and were excluded from the study. 53 miRNAs were detected in at least 60% of the samples and included in subsequent

statistical comparisons. There was an overall trend of miRNA downregulation in DMD patients (ambulant and non-ambulant) compared to healthy controls (**Fig S2**). No microRNAs were upregulated in DMD compared to controls.

3.1.1. miRNA profiling in urinary exosomes from all DMD patients compared to healthy controls

After Bonferroni correction of the p-value, three miRNAs were significantly different between DMD patients and controls. There was significant downregulation of miR-21-5p (P<0.001, **Fig 1A**), miR-22-3p (P<0.001, **Fig 1B**) and miR-29c-3p (P<0.001, **Fig 1C**) when the DMD patients group (ambulant and non-ambulant) was compared to controls.

3.1.2. miRNA profiling in urinary exosomes from ambulant DMD patients compared to healthy controls

To investigate the possibility that the levels of urinary miRNA might be influenced by the ambulatory status of the DMD boys, further statistical analyses were carried out by stratifying the DMD samples into 15 ambulant (A) and 17 non-ambulant (NA). Of three miRNAs that were significantly downregulated in DMD compared to controls, only miR-29c-3p was significantly downregulated (P<0.01, **Fig 2A**) in ambulant DMD patients compared to the healthy controls. There was significant downregulation of two additional identified in the first part of the study, miRNAs - miR-92a-3p (P<0.01, **Fig 2B**) and miR-103a-3p (P<0.01, **Fig 2C**) when ambulant patients were compared to healthy controls.

3.1.3. Expression of miR-29c-3p, miR-92a-3p and miR-103a-3p correlates with preserved ambulation in DMD

Receiving operating characteristic (ROC) curves were generated to test sensitivity and specificity of miR-29c-3p, miR-92a-3p and miR-103a-3p. Regarding miR-29c-3p, when comparison was made between the healthy controls and the group including all patients (A+NA), the area under the curve (AUC) was 0.8086 (95%CI=0.6678-0.9494, **Fig 3A**). When comparison was made between healthy controls and ambulant patients (A), the AUC was 0.8267 (95%CI=0.6786-0.9748, **Fig 3B**). These results indicate that miR-29c-3p levels better correlate with preserved ambulation in DMD patients. Similarly, for miR-103a-3p, the areas under the curves (AUC) were 0.8244 (95%CI=0.6914-0.9575, **Fig 3C**) in patients (A+NA) and 0.8533 (95%CI=0.6966-1.01, **Fig 3D**) in ambulant patients. In addition, AUC values for miR-92a-3p, were 0.7126 (95%CI=0.5465-0.8788, **Fig 3E**) in patients (A+NA) and 0.8178 (95%CI=0.6586-0.977, **Fig 3F**) in ambulant patients. All the AUC results indicate that miR-29c-3p, miR-103a-3p and miR-92a-3p levels correlate best with preserved ambulation in DMD patients.

3.1.4. miRNA profiling in urinary exosomes from non-ambulant DMD patients compared to healthy controls

There were no significant differences in the levels of urinary miRNAs between non-ambulant DMD patients and healthy controls.

3.1.5. miRNA profiling in urinary exosomes from DMD ambulant compared to DMD non-ambulant patients

There were no significant differences in miRNA expression between ambulant and non-ambulant DMD patients.

3.2. Selection of microRNA candidates for further validation studies

From the most dysregulated miRNA identified through profiling analysis, we selected 5 miRNAs for further validation studies (miR-21-5p, miR-22-3p, miR-23 -3p, miR-29c-3p and miR-103a-3p). Among these, miR-23 -3p was downregulated in all the statistical comparisons, although the significance was eventually lost when applying the Bonferroni corrections. Moreover, the dysregulation of miR-21, miR-22 and miR-29 in muscular dystrophy has already been reported in literature (**Table 1**).

Table 1

Selected candidate involvement in skeletal muscle

microRNA	Previous findings	Model
miR-29	downregulated in *mdx* mouse model of DMD [36]	*mdx* muscles
	loss of miR-29 in myoblasts contributes to dystrophic muscle pathogenesis[36]	*mdx* primary myoblasts
	myogenic factor[39]	C2C12 cells
	reduced in DMD patients[37]	DMD patient muscle and myoblasts
	downregulated in quiescent satellite cells during myogenesis *in vitro* [38]	Human satellite cells
miR-22	upregulated in Facio scapulo humeral muscular dystrophy (FSHD)[40]	FSHD patient myoblasts
	upregulated in Limb-girdle muscular dystrophy type 2D (LGMD2D)[41]	*Sgca*-null mouse serum
miR-21	increased in DMD[37]	DMD patient muscle and myoblasts

3.2.1. Bioinformatic prediction of the targets

In order to predict the target genes of the 5 selected candidates, Pathway Analysis with the online tool DianaMirpath [45] was performed. The algorithm allows the identification not only of the potential target genes of a specific microRNA, but also to locate them to the related Kyobo Encyclopedia of Genes and Genomes (KEGG) pathway [46].

As showed in **Table S3**, our miRNA affected pathways included the following: extracellular matrix (ECM)-receptor interaction, focal adhesion, ErbB signaling pathway, TGF- eta signaling pathway, mTOR signaling pathway, apoptosis and MAPK signaling pathway.

294

295 **Table S3**

296 **Predicted target genes of the candidate microRNAs**

microRNA	Predicted target genes from DIANA miRPath	p-value	KEGG pathway
hsa-miR-21-5p	*ITGB8, THBS1, COL5A2, CD47*	1.05E-11	ECM-receptor interaction
	ERBB2, ITGB8, THBS1, BCL2, EGFR, PTK2, PIK3R1, PDGFD, VEGFA, PTEN, COL5A2	2.12E-06	Focal adhesion
	ERBB2, EGFR, PTK2, MYC, PIK3R1	2.31E-06	ErbB signaling pathway
	TGFBR1, ZFYVE16, MYC, TGFB2, TGFBR2, BMPR2	8.30355E-05	TGF-beta signaling pathway
	TSC1, PIK3R1, RPS6KA3, VEGFA, PTEN, RRAGC	0.000527381	mTOR signaling pathway
	BID, BCL2, APAFI, PIK3R1, FAS	0.007398499	Apoptosis
	TGFBR1, EGFR, MAP3K1, RASA1, RASGRP1, MYC, DUSP8, FAS, RPS6KA3, TGFB2, MAP3K2, RASGRP3, MKNK2, TGFBR2	0.0173412	MAPK signaling pathway
hsa-miR-22-3p	*SP1, BMP7*	8.30355E-05	TGF-beta signaling pathway
	PRKACA	0.007398499	Apoptosis
	PRKACA	0.0173412	MAPK signaling pathway
hsa-miR-23b-3p	*STAT5B*	2.31E-06	ErbB signaling pathway
hsa-miR-29c-3p	*COL3A1, COL4A2, COL1A1, COL1A2, LAMC1, Col6a2, COL4A1*	1.05E-11	ECM-receptor interaction
	BCL2, COL3A1, JUN, COL4A2, COL1A1, COL1A2, LAMC1, AKT3, Col6a2, VEGFA, COL4A1	2.12E-06	Focal adhesion
	AKT2, JUN,AKT3	2.31E-06	ErbB signaling pathway
	AK2, AKT3, VEGFA	0.000527381	mTOR signaling pathway
	BCL2, AK2, AK3	0.007398499	Apoptosis
	AKT2, JUN, AKT3	0.0173412	MAPK signaling pathway
hsa-miR-103a-3p	*ITGA2*	1.05E-11	ECM-receptor interaction
	BCL2, ZYX, ITGA2	2.12E-06	Focal adhesion
	ABL2, RPS6KB1	2.31E-06	ErbB signaling pathway
	ACVR2B, SMAD7, RPS6KB1	8.30355E-05	TGF-beta signaling pathway
	RPS6KB1	0.000527381	mTOR signaling pathway
	FGF2, MAP3K7	0.007398499	Apoptosis

	FGF2, MAP3K7	0.0173412	MAPK signaling pathway

297

298 *3.2.2. Validation of the candidates*

299 The Normfinder [44] algorithm was used to discover the most suitable reference gene (among

300 the 87 microRNAs analysed at the profiling stage) which was miR-16 -5p.

301

302 *3.2.3. miR-29 downregulation in ambulant DMD patients*

303 The significant dysregulation of miR-29c-3p detected in the original profiling step was

304 confirmed further, with 54 DMD patients (including both ambulant and non-ambulant)

305 having lower miR-29c-3p levels compared to the 20 healthy controls ($P<0.05$, **Fig 4A**). This

306 miRNA remained significantly downregulated when only ambulant DMD patients were

307 compared to the healthy controls ($P<0.05$, **Fig 4B**).

308

309 We also compared the non-ambulant DMD patients to controls, and although we observed a

310 trend towards downregulation, this was not statistically significant (**Fig 4C**). These results

311 indicate that the extent of miR-29c-3p downregulation is more marked in ambulant than in

312 non-ambulant DMD patients, but not sufficiently different between the 2 groups to be of

313 significance.

314

315 Moreover, to determine if miR-29c-3p levels correlated with the age of DMD patients, we

316 performed linear regression analyses. Although the levels of miR-29c-3p in urine of DMD

317 patients decrease with age, there was no significant correlation between their expression and

318 the age of the patients (**Fig S3A**).

319

320 *3.2.4. miR-23b-3p and miR-21-5p downregulation in non-ambulant DMD patients*

There was a significant downregulation of miR-23 -3p (P<0.01, **Fig 4F**) and miR-21-5p (P<0.05, **Fig 4I**) in non-ambulant DMD patients compared with the controls but not in controls vs all DMD patients (**Fig 4D, 4G**), nor in controls vs ambulant DMD patients (**Fig 4E, 4H**) . There was no significant difference in the relative expression of the other selected candidates (miR-22-3p and miR-103a-3p) in patients compared to the controls; hence, they were excluded from further analysis. Finally, as for miR-29c-3p, linear regression analyses did not show a significant correlation between miR-23 -3p and miR-21-5p expression and age of patients (**Fig S3B, S3C**).

In summary, validation analysis confirmed the significant downregulation of 3 urinary exosomal microRNAs: - miR-29c-3p in DMD ambulant, miR-23 -3p and miR-21-5p in DMD non-ambulant patients respectively.

3.3. miRNA response to the corticosteroid therapy

In order to determine if there were differences in the selected candidate miRNA expression profiles between patients on different corticosteroid regimens, statistical comparisons were performed on patients receiving prednisolone vs deflazacort, and on daily versus intermittent (10/10) steroid regimens.

3.3.1. Deflazacort compared to Prednisolone

When comparisons were made between the two different steroid regimens (deflazacort vs prednisolone), none of the selected candidates showed significant differences (**S4 Fig**).

3.3.2. Daily compared to intermittent regimen

To test if there was any association between expression of selected candidates in urine and corticosteroid regimen, statistical comparisons were made among three groups: healthy controls, patients undergoing a daily treatment and those receiving the drugs intermittently (regardless of the corticosteroid administered). Interestingly, miR-29c-3p was significantly downregulated in patients undergoing intermittent corticosteroid treatment compared to controls, but not in those receiving daily treatment (**Fig 5D**). There was no correlation between the expression levels of the remaining miRNAs: miR-21-5p (**Fig 5A**), miR-22-3p (**Fig 5B**), miR-23b-3p (**Fig 5C**) and miR-103a-3p (**Fig 5E**) and the corticosteroid regimen.

4. Discussion

Our study is the first to investigate the expression of exosomal urinary miRNAs in DMD patients or in any form of muscular dystrophy. In the last decade, several studies have focused on differential miRNA expression in DMD, providing new insights into their role in the modulation of pathological signalling pathways [47, 48], and also indicating their potential role as non-invasive biomarkers to monitor disease progression [29, 49].

We show that miR-29 was significantly downregulated in ambulant DMD patients and that miR-23 and miR-21 were significantly downregulated in non-ambulant DMD patients compared to age matched controls. Contrary to several studies focused on noncoding RNA dysregulation in serum from DMD patients and *mdx* mice, in which a large number of miRNAs were significantly upregulated compared to controls [27, 50-52], we found no upregulated miRNAs in urine from DMD patients.

The downregulation of mir-29c-3p, a member of the miR-29 family, which is composed of five miRNAs having identical seed regions (thus sharing the same target genes) [53] has been reported in muscles from DMD patients [37] and *mdx* mice [36]. This miRNA is a key promoter of skeletal muscle regeneration in *mdx* mice, and myogenic differentiation of primary *mdx* myoblasts *in vitro* [36]. Moreover, miR-29 agonists have potential therapeutic application in a broad spectrum of fibrotic diseases [54], as shown by the demonstration that systemic delivery of miR-29 significantly reduced diaphragm fibrosis in *mdx* mice [36].

Fibrosis is a particular hallmark of DMD, and contributes to the skeletal and cardiac muscle pathology by altering the functionality [55, 56]. In DMD, TGFβ is considered to be one of the

strongest profibrogenic factors. It is stored in the extracellular matrix and when activated, as a consequence of tissue damage, exerts its effects through binding to the TGFβ Type I and TGFβ Type II receptors [57, 58]. In the *mdx* diaphragm, TGF-β1 upregulation occurs at early stages of fibrogenesis [36, 58, 59]. In DMD patients, TGFβ-1 triggers the fibrotic process, and reaches peak levels in muscles during the early stages of the disease (6 years) promoting a massive connective tissue proliferation. After this phase, TGF-β1 levels decline while the proliferation process continues [60]. TGFβ promotes fibrosis in *mdx* skeletal muscle by inhibiting mir-29 expression [36] which is a key player in controlling ECM modifications [61]. Other evidence implicating TGF-β1 in promoting fibrosis by inhibiting miR-29 expression comes from studies on pulmonary fibrosis, which showed that miR-29 modulates the fibrotic process by binding a large number of genes involved in ECM synthesis and remodelling including *COL1A1*, *MMP2* and *MMP14* [62]. Moreover, previous studies focused on renal fibrosis showed that this process was correlated with the loss of miR-29 mediated by a TGFβ/SMAD3 dependent mechanism [63].

We speculate that reduced levels of miR-29 observed in the ambulant DMD boys are the result of the progressive fibrosis, promoted by TGF-β1 signalling, which characterises the early stages of the disease. Interestingly, we found that miR-29 levels are affected by the regimen of corticosteroid therapy, drugs which are prescribed as part of the standards of care in DMD patients [64, 65]. In particular, we found significant downregulation of miR-29 in patients receiving the intermittent steroid regimen but not in those receiving a daily treatment, indicating that a constant administration of corticosteroids might be more efficient in maintaining miR-29 levels closer to those in healthy controls, and hence in slowing down the fibrotic process. This hypothesis is supported by the recent report of the inhibitory action of prednisolone on TGF-β1, a repressor of miR-29 [36], in *mdx* diaphragm [66]. Since miR-29

downregulation in muscle [37] was mirrored in urine from DMD patients and its levels in urine were normalised by corticosteroid therapy, this microRNA represents to date, the most promising urinary non-invasive biomarker for DMD.

In our validation studies, we found a significant downregulation of miR-23 -3p in non-ambulant patients. This is the first time miR-23 has been reported to be implicated in DMD, and consequently, its role in the disease is still unclear. However, a link between miR-23 and TGFβ have been reported in liver, where it downregulates *Smad* genes in mouse fetal liver cells and consequently the TGFβ signalling [67] and also in murine airway smooth muscle, where it controls the proliferation of the cells through inactivating TGFβ signalling [68]. Moreover, it has been shown that fibrosis in human fibroblasts is induced by TGFβ via the PAK2 pathway which in turn, stimulates matrix synthesis through the activation of the Smad1 protein [69]. As miR-23 targets a large number of genes in human, including *PAK2* [70], we speculate that it might counteract the fibrotic process in DMD, through the TGFβ signaling inhibition. However further studies aimed at investigating the association between miR-23 and fibrosis are needed to confirm this hypothesis and to address its involvement in DMD pathogenesis.

We also found a significant downregulation of miR-21 in urine of non-ambulant DMD patients. Our results differ those from Zanotti et al, where miR-21 was upregulated in muscle biopsies (quadriceps) and fibroblasts from DMD patients aged 1-8 years [37]. MiR-21 promotes TGFβ-1 related fibrosis by inducing the transdifferentiation of fibroblasts to myofibroblasts [37] in which collagen synthesis is augmented leading to fibrosis [71]. Further investigations aimed at clarifying the mechanisms underlying differential miR-21 expression observed in muscle [37] and urine will be beneficial to understand its contribution

in DMD. In particular studies aimed at determining the source of exosomes would clarify whether they are synthesised by the renal epithelial cells as proposed by Pisiktun et al. [72], or produced by other cells elsewhere in the body and merely transit the renal epithelium before being released into urine. Whether urinary exosomes actually originate from cells within skeletal muscle, or other organs [73-75] is not known.

4.1. Conclusions

Our findings indicate that exosomal urinary miR-29c-3p, miR-23 -3p and miR-21-5p are promising novel non-invasive biomarkers for DMD, and that miR-29c-3p levels are differentially affected by different steroid regimens, supporting the antifibrotic effect that steroid therapy have, and indicating for the first time that the determination of urinary miRNA levels allow to capture differences between different steroids regimens, which likely reflect the differences in clinical benefit between daily vs intermittent steroids therapies [76, 77].

4.2. Executive Summary

- Duchenne muscular dystrophy (DMD) is an X-linked and progressive neuromuscular disorder affecting 1 in 5000 newborn males leading to progressive skeletal muscle wasting and death.

- Levels of creatine kinase (CK), are generally elevated in serum from patients, however this enzyme is not considered to be a reliable circulating biomarker because

it is subject to fluctuations, mainly related to age and its modulation by physical activity.

- There is still a pressing need for novel, non-invasive and reliable biomarkers in DMD that are sensitive to disease progression and able to reliably monitor the efficacy of any therapeutic intervention.

- MicroRNAs are small (~22 nucleotides) endogenous non-coding RNAs implicated in post- transcriptional regulation of their messenger RNA (mRNA) targets, that modulate the expression of genes involved in pathways regulating skeletal muscle formation, differentiation and homeostasis.

- MiRNAs are present in urine where they are included in small microvesicles called exosomes (40-100 nm) that protect them from degradation by RNases.

- We studied the signature of 87 urinary miRNAs from controls (n =20), ambulant (n = 31) and non-ambulant (n = 23) DMD patients.

- MiR-29c-3p is significantly downregulated in ambulant DMD patients compared to age matched controls and its levels are affected by different steroid regimens.

- MiR-23b-3p and miR-21-5p are significantly downregulated in non-ambulant DMD patients compared to age matched controls.

4.3. Future perspective

Changes in urinary miRNA levels are a potential non-invasive means of determining disease progression and the efficacy of any therapeutic intervention in neuromuscular conditions such as DMD.

Acknowledgements

All research at Great Ormond Street Hospital NHS Foundation Trust and UCL Great Ormond Street Institute of Child Health is made possible by the NIHR Great Ormond Street Hospital Biomedical Research Centre. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

This research was performed at the Dubowitz Neuromuscular Centre (London) with the collaboration of the Institute of Myology (Paris), the Muscular Dystrophy Research Centre (Newcastle), the Leiden University Medical Center (Leiden) and the UMC St Radboud (Nijmegen), and supported by a grant from the Association Francaise Contre Les Myopathies (AFM).

Funding

This work was supported by the Association Francaise Contre Les Myopathies

Disclosures

VR is currently an employee of Solid Biosciences

FM has received consulting fees from Biogen, Italfarmaco, Pfizer, PTC Therapeutics, Roche, Sarepta Therapeutics, and Wave Therapeutics; and is supported by the National Institute of Health Research Biomedical Research Centre at Great Ormond Street Hospital for Children NHS Foundation Trust, and University College London. The support of the MRC Neuromuscular Centre Biobank and of the Muscular Dystrophy UK is also gratefully acknowledged.

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Supplementary Fig. 2. Expression profiles of exosomal microRNAs in the urine of DMD patients and healthy controls. Heat map and average linkage hierarchical clusters showing the miRNA signature pattern within the samples (n=15 controls, n=15 ambulant DMD, n=17 non-ambulant DMD). Upregulated miRNAs are depicted in green and downregulated in red. A prevalence of downregulated miRNAs is evident in the DMD population when compared to controls.

Fig. 1. Expression of miR-21-5p, miR-22-3p and miR-29c-3p in urine samples from DMD patients.

Expression of the three microRNA in urinary exosomes isolated from healthy controls (n=15) ambulant DMD patients (n=15) and non-ambulant DMD patients (n=17). There was significant downregulation of miR-21-5p (**A**), miR-22-3p (**B**) and miR-29c-3p (**C**) in the DMD patient group (ambulant and non-ambulant patients) compared to controls. Data are presented as Mean ± SEM. ***p< 0.001. GMV= global mean value.

Fig. 2. Expression of miR-29c-3p, miR-92a-3p and miR-103a-3p in urine samples from ambulant DMD patients.

Expression of the three microRNAs in urinary exosomes isolated from healthy controls (n=15) and ambulant DMD patients (n=15). There was significant downregulation of miR-29c-3p (**3A**), miR-92a-3p (**3B**) and miR-103a-3p (**3C**) in the DMD patients (including both ambulant and non-ambulant patients) compared to controls. Data are presented as Mean ± SEM. **p< 0.01. GMV= global mean value.

Fig. 3. ROC curve analysis of urinary miR-29c-3p, miR-92a-3p and miR-103a-3p.

ROC curves based on miR-29c-3p urinary levels, for differentiating between the group including all the DMD (n=32) (**A**) and ambulant patients (n=15) (**B) .** The same analysis was performed for miR-103a-3p (**C**, **D**) and miR-92a-3p (**E**, **F**).

Fig. 4. Validated expression of miR-29c-3p, miR-23b-3p and miR-21-5p in urine samples from DMD patients.

Expression of miR-29c-3p, miR-23 -3p and miR-21-5p in urinary exosomes isolated from healthy controls (n=20) ambulant (n=31) and non-ambulant (n=23) DMD patients. There was significant downregulation of miR-29c-3p in patients compared to controls (**A**) and in ambulant patients compared to controls (**B**). MiR-23 -3p was significantly downregulated in non-ambulant patients compared to controls (**F**). MiR-21-5p was significantly downregulated in non-ambulant patients compared to controls (**I**) Data are presented as Mean ± SEM. *p< 0.05; **p< 0.01.

Supplementary Fig. 3. Lack of correlation between miR-29c-3p, miR-23b-3p and miR-21-5p expression with age in DMD patients.

Linear regression analyses between the levels of and miR-29c-3p (**A**), miR-23 -3p (**B**) and miR-21-5p (**C**), in urine and the Age of DMD patients (N=54). The regression line is presented.

Fig. 5. Effects of different corticosteroid regimens on candidate miRNA expression.

Expression of miR-21-5p (**A**), miR-22-3p (**B**), miR-23 -3p (**C**), miR-29c-3p (**D**) and miR-103a-3p (**E**) in urinary exosomes isolated from healthy controls (n=20), DMD patients treated

771 with any daily corticosteroids (n=25) and DMD patients treated with any intermittent
772 corticosteroids (n=18). Data are presented as Mean ± SEM. *p< 0.05.
773
774 **Supplementary Fig. 1 Expression of the synthetic controls.** UniSp6 (orange) and *cel-miR-*
775 *39* (light blue) expression in the samples (n=47) used for microRNA profiling. The x-axis
776 represents the samples (both patients and controls) while the y-axis represents Ct values.
777
778 **Supplementary Fig. 4. Validated microRNA expression in DMD patients receiving**
779 **different corticosteroids (prednisolone compared to deflazacort).**
780 Expression of miR-21-5p (**A**), miR-22-3p (**B**), miR-23b-3p (**C**), miR-29c-3p (**D**) and miR-
781 103a-3p (**E**) in urinary exosomes isolated from healthy controls (n=20), Prednisolone treated
782 DMD patients (n=36) and Deflazacort treated DMD patients (n=7). Data are presented as
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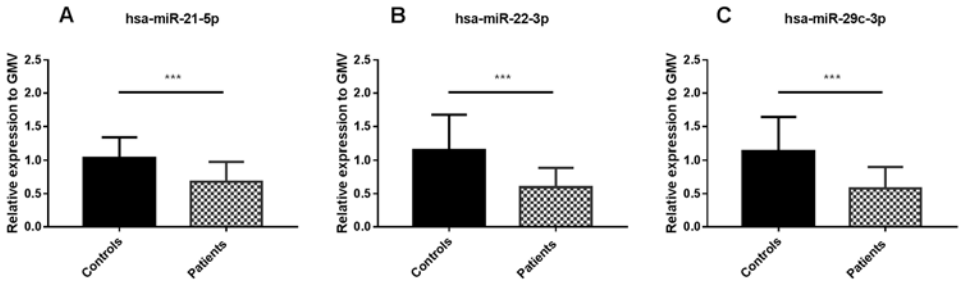

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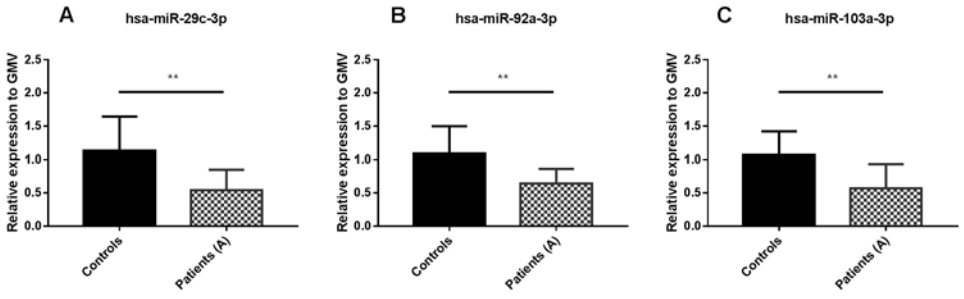

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Expression of the three microRNAs in urinary exosomes isolated from healthy controls (n=15) and ambulant DMD patients (n=15). There was significant downregulation of miR-29c-3p (3A), miR-92a-3p (3B) and miR-103a-3p (3C) in the DMD patients (including both ambulant and non-ambulant patients) compared to controls. Data are presented as Mean ± SEM. **p< 0.01. GMV= global mean value.

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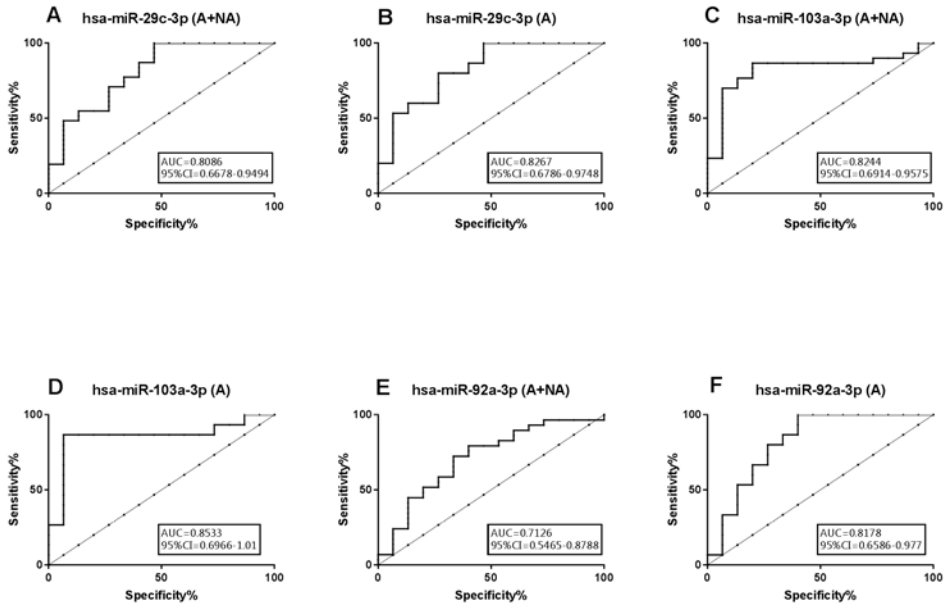

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ROC curves based on miR-29c-3p urinary levels, for differentiating between the group including all the DMD (n=32) (A) and ambulant patients (n=15) (B). The same analysis was performed for miR-103a-3p (C, D) and miR-92a-3p (E, F).

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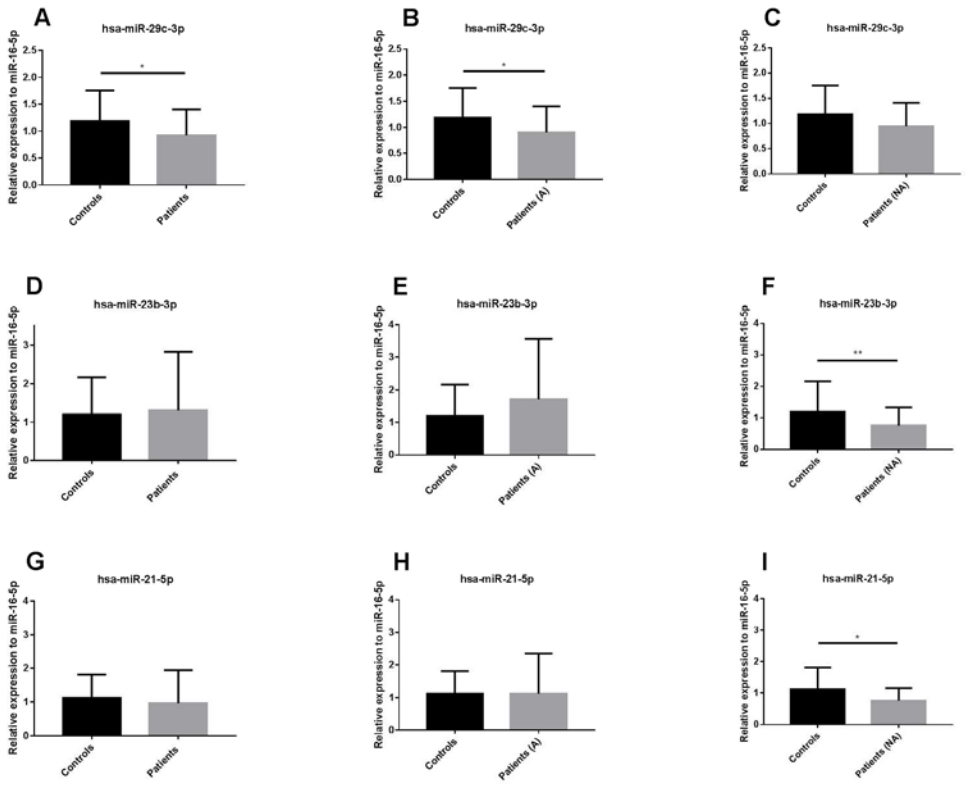

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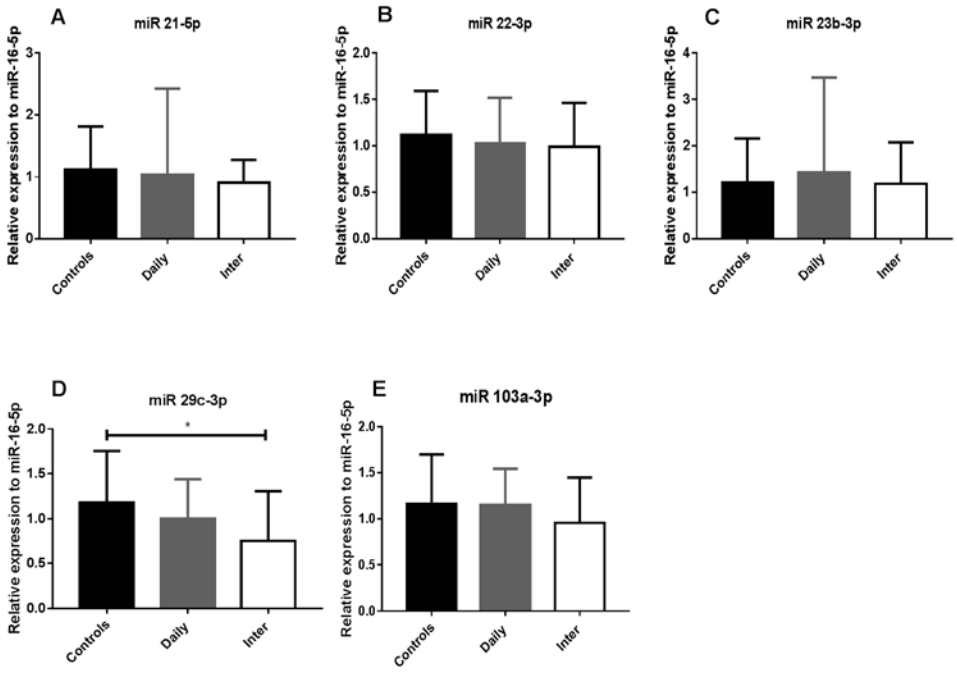

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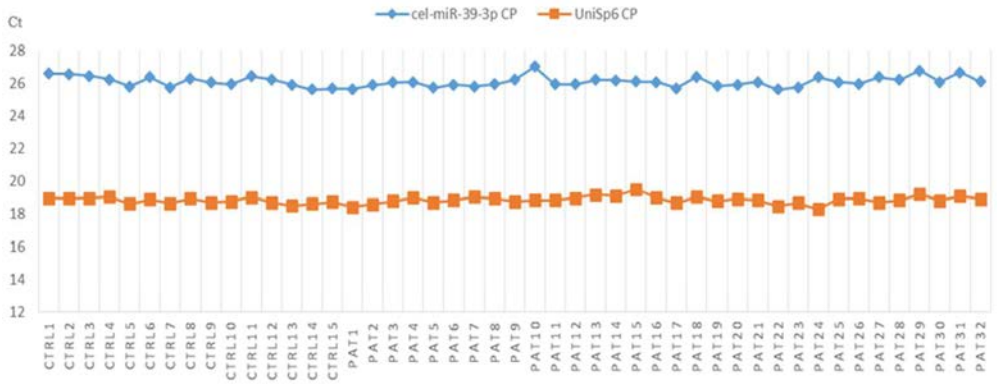

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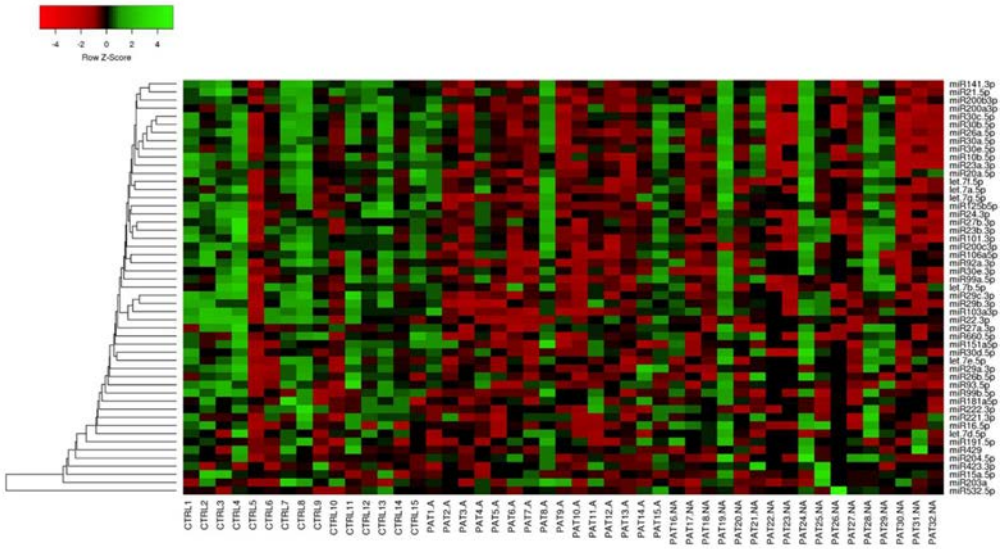
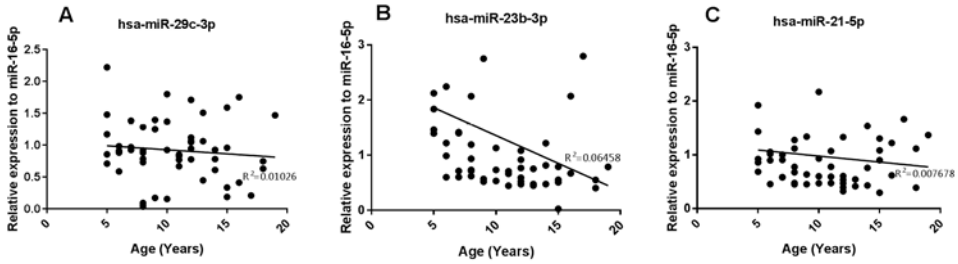


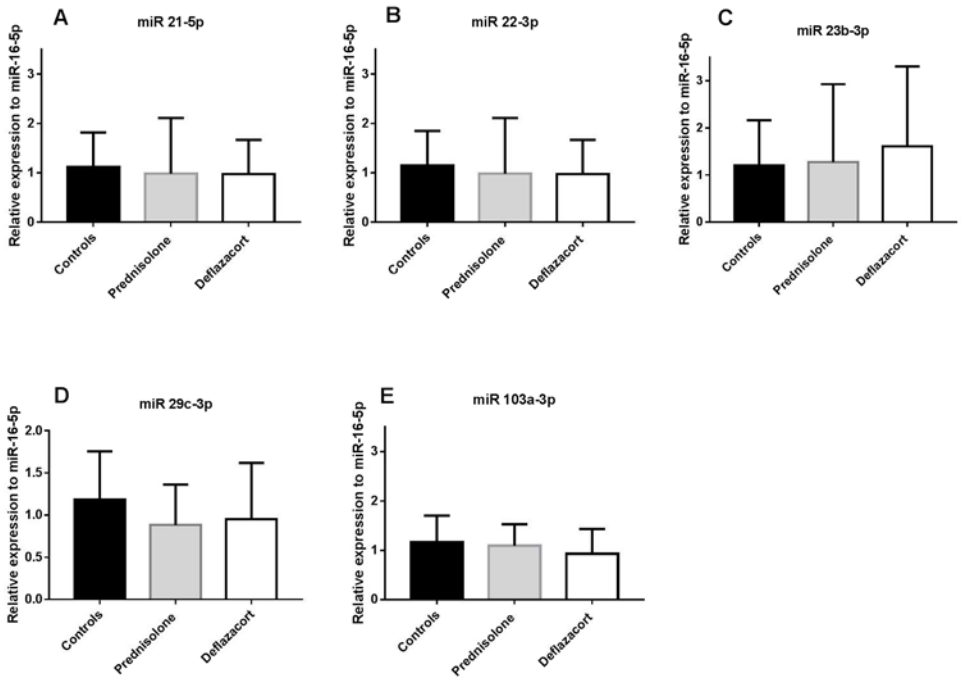
Supplementary Fig. 2. Expression profiles of exosomal microRNAs in the urine of DMD patients and healthy controls. Heat map and average linkage hierarchical clusters showing the miRNA signature pattern within the samples (n=15 controls, n=15 ambulant DMD, n=17 non-ambulant DMD). Upregulated miRNAs are depicted in green and downregulated in red. A prevalence of downregulated miRNAs is evident in the DMD population when compared to controls.

189x107mm (150 x 150 DPI)



Supplementary Fig. 3. Lack of correlation between miR-29c-3p, miR-23b-3p and miR-21-5p expression with age in DMD patients.
Linear regression analyses between the levels of and miR-29c-3p (A), miR-23b-3p (B) and miR-21-5p (C), in urine and the Age of DMD patients (N=54). The regression line is presented.

555x179mm (96 x 96 DPI)



Supplementary Fig. 4. Validated microRNA expression in DMD patients receiving different corticosteroids (prednisolone compared to deflazacort).
Expression of miR-21-5p (A), miR-22-3p (B), miR-23b-3p (C), miR-29c-3p (D) and miR-103a-3p (E) in urinary exosomes isolated from healthy controls (n=20), Prednisolone treated DMD patients (n=36) and Deflazacort treated DMD patients (n=7). Data are presented as Mean ± SEM.

555x419mm (96 x 96 DPI)

Table 1. Selected candidate involvement in skeletal muscle.

microRNA	Previous findings	Model
miR-29	downregulated in *mdx* mouse model of DMD [36]	*mdx* muscles
	loss of miR-29 in myoblasts contributes to dystrophic muscle pathogenesis[36]	*mdx* primary myoblasts
	myogenic factor[39]	C2C12 cells
	reduced in DMD patients[37]	DMD patient muscle and myoblasts
	downregulated in quiescent satellite cells during myogenesis *in vitro* [38]	Human satellite cells
miR-22	upregulated in Facio scapulo humeral muscular dystrophy (FSHD)[40]	FSHD patient myoblasts
	upregulated in Limb-girdle muscular dystrophy type 2D (LGMD2D)[41]	*Sgca*-null mouse serum
miR-21	increased in DMD[37]	DMD patient muscle and myoblasts

S1 Table. Demographic and clinical characteristics of the samples used for profiling and validations.

Sample	Amb	Age at sampling	Steroids	Steroids regime	Genetic mutation
CONTROL 1	A	4	n/a	n/a	no
CONTROL 2	A	4	n/a	n/a	no
CONTROL 3	A	5	n/a	n/a	no
CONTROL 4	A	5	n/a	n/a	no
CONTROL 5	A	6	n/a	n/a	no
CONTROL 6	A	7	n/a	n/a	no
CONTROL 7	A	8	n/a	n/a	no
CONTROL 8	A	8	n/a	n/a	no
CONTROL 9	A	8	n/a	n/a	no
CONTROL 10	A	8	n/a	n/a	no
CONTROL 11	A	8	n/a	n/a	no
CONTROL 12	A	9	n/a	n/a	no
CONTROL 13	A	9	n/a	n/a	no
CONTROL 14	A	9	n/a	n/a	no
CONTROL 15	A	9	n/a	n/a	no
CONTROL 16	A	11	n/a	n/a	no
CONTROL 17	A	12	n/a	n/a	no
CONTROL 18	A	13	n/a	n/a	no
CONTROL 19	A	15	n/a	n/a	no
CONTROL 20	A	18	n/a	n/a	no
DMD1	A	5	Prednisolone	Intermittent	Deletion exons 49-52
DMD2	A	5	Prednisolone	Daily	Deletion exon 51
DMD3	A	5	No	n/a	Deletion exons 46-48
DMD4	A	5	Prednisolone	Intermittent	Deletion exons 48-50
DMD5	A	5	Prednisolone	Every other day	Deletion exons 48-52
DMD6	A	6	Prednisolone	Every other day	Deletion exons 45-52
DMD7	A	6	Prednisolone	Intermittent	Deletion exons 49-50
DMD8	A	6	Prednisolone	Daily	Deletion exons 52-54
DMD9	A	6	Prednisolone	Intermittent	Deletion exons 14-43
DMD10	A	7	Prednisolone	Intermittent	Deletion exon 45
DMD11	A	7	Prednisolone	Daily	Deletion exons 48-50
DMD12	A	7	Deflazacort	Daily	Deletion exons 45-52
DMD13	A	7	Prednisolone	Daily	Deletion exons 46,47,48,49
DMD14	A	8	Prednisolone	Intermittent	Deletion exons 45-54
DMD15	A	8	Prednisolone	Intermittent	Deletion exons 49-50
DMD16	A	8	Prednisolone	Intermittent	Deletion exons 42-43
DMD17	A	8	Prednisolone	Daily	Deletion exon 51

DMD18	A	8	Prednisolone	Daily	Deletion exon 45
DMD19	A	8	Prednisolone	Daily	Deletion exons 53-54
DMD20	A	8	Prednisolone	Daily	Deletion exons 45-52
DMD21	A	8	Prednisolone	Daily	Deletion exon 43
DMD22	A	9	Prednisolone	Intermittent	Deletion exons 54-52
DMD23*	A	9	Prednisolone	Daily	Deletion exons 51-54
DMD24	A	9	Prednisolone	Daily	Deletion exon 51
DMD25	A	10	Deflazacort	Daily	Deletion exons 45-52
DMD26	A	10	Deflazacort	Daily	Deletion exons 45-52
DMD27	A	10	Prednisolone	Intermittent	Deletion exons 42-43
DMD28	A	11	Prednisolone	Daily	Deletion exons 49-50
DMD29	A	11	Prednisolone	Intermittent	Stop mutation exon 19
DMD30	A	12	Prednisolone	Intermittent	Deletion exon 44
DMD31	A	14	Prednisolone	Intermittent	Duplication exons 49-50
DMD32	A	14	Prednisolone	Daily	Deletion exons 48-54
DMD33	NA	10	Prednisone	Daily	Deletion exons 45-50
DMD34	NA	11	Prednisone	Daily	Deletion exons 45-50
DMD35	NA	11	Prednisolone	Every other day	Deletion exon 50
DMD36	NA	12	Prednisone	Daily	Deletion of exons 30-50
DMD37	NA	12	No	n/a	Deletion exons 46-48
DMD38	NA	12	No	n/a	Deletion exon 46
DMD39	NA	12	No	n/a	Deletion exons 46-52
DMD40	NA	12	Deflazacort	Intermittent	Deletion exons 46-49
DMD41	NA	13	Prednisolone	Intermittent	Deletion exons 48-50
DMD42	NA	13	Deflazacort	Intermittent	Deletion exon 53
DMD43	NA	13	Deflazacort	Intermittent	Point mutation exon 44
DMD44*	NA	13	Deflazacort	Daily	Deletion exons 48-50
DMD45	NA	14	Prednisolone	Intermittent	Deletion exon 45
DMD46	NA	15	Prednisolone	Intermittent	Deletion exon 44
DMD47	NA	15	Prednisolone	Intermittent	Deletion exons 46-51
DMD48	NA	15	No	n/a	Deletion exons 46-55
DMD49	NA	15	Prednisone	Daily	Deletion exons 45
DMD50	NA	15	Prednisolone	Intermittent	Deletion exons 48-52
DMD51	NA	16	Prednisolone	Intermittent	Deletion exon 51
DMD52	NA	16	No	n/a	Deletion exon 52
DMD53	NA	17	Deflazacort	Daily	Deletion exons 49-52
DMD54	NA	18	Prednisone	Daily	Deletion exons 45-52
DMD55	NA	18	No	n/a	Deletion exons 48-52
DMD56	NA	19	Prednisone	Daily	Deletion exon 53

A=ambulant patients, NA= non-ambulant patients, Intermittent =administered every 10 days, n/a= not applicable, * samples used only for profiling

S2 Table. Exosomal microRNAs tested.

miRNA	Target sequence
hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
hsa-let-7d-3p	CUAUACGACCUGCUGCCUUUCU
hsa-let-7d-5p	AGAGGUAGUAGGUUGCAUAGUU
hsa-let-7e-5p	UGAGGUAGGAGGUUGUAUAGUU
hsa-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU
hsa-let-7g-5p	UGAGGUAGUAGUUUGUACAGUU
hsa-let-7i-5p	UGAGGUAGUAGUUUGUGCUGUU
hsa-miR-10a-5p	UACCCUGUAGAUCCGAAUUUGUG
hsa-miR-10b-5p	UACCCUGUAGAACCGAAUUUGUC
hsa-miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG
hsa-miR-15b-5p	UAGCAGCACAUCAUGGUUUACA
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG
hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG
hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG
hsa-miR-21-5p*	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-22-3p*	AAGCUGCCAGUUGAAGAACUGU
hsa-miR-22-5p	AGUUCUUCAGUGGCAAGCUUUA
hsa-miR-23a-3p	AUCACAUUGCCAGGGAUUUCC
hsa-miR-23b-3p*	AUCACAUUGCCAGGGAUUACC
hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG
hsa-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA
hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-26b-5p	UUCAAGUAAUUCAGGAUAGGU
hsa-miR-27a-3p	UUCACAGUGGCUAAGUUCCGC
hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC
hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA
hsa-miR-29b-3p	UAGCACCAUUUGAAAUCAGUGUU
hsa-miR-29c-3p*	UAGCACCAUUUGAAAUCGGUUA
hsa-miR-30a-5p	UGUAAACAUCCUCGACUGGAAG
hsa-miR-30b-5p	UGUAAACAUCCUACACUCAGCU
hsa-miR-30c-5p	UGUAAACAUCCUACACUCUCAGC
hsa-miR-30d-5p	UGUAAACAUCCCCGACUGGAAG
hsa-miR-30e-3p	CUUUCAGUCGGAUGUUUACAGC
hsa-miR-30e-5p	UGUAAACAUCCUUGACUGGAAG
hsa-miR-31-3p	UGCUAUGCCAACAUAUUGCCAU
hsa-miR-31-5p	AGGCAAGAUGCUGGCAUAGCU
hsa-miR-34a-5p	UGGCAGUGUCUUAGCUGGUUGU
hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU

miRNA	Target sequence
hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG
hsa-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU
hsa-miR-107	AGCAGCAUUGUACAGGGCUAUCA
hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA
hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG
hsa-miR-128-3p	UCACAGUGAACCGGUCUCUUU
hsa-miR-133a-3p	UUUGGUCCCCUUCAACCAGCUG
hsa-miR-135b-5p	UAUGGCUUUUCAUUCCUAUGUGA
hsa-miR-141-3p	UAACACUGUCUGGUAAAGAUGG
hsa-miR-145-5p	GUCCAGUUUUCCCAGGAAUCCCU
hsa-miR-148a-3p	UCAGUGCACUACAGAACUUUGU
hsa-miR-148b-3p	UCAGUGCAUCACAGAACUUUGU
hsa-miR-149-5p	UCUGGCUCCGUGUCUUCACUCCC
hsa-miR-151a-5p	UCGAGGAGCUCACAGUCUAGU
hsa-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-187-3p	UCGUGUCUUGUGUUGCAGCCGG
hsa-miR-191-5p	CAACGGAAUCCCAAAAGCAGCUG
hsa-miR-193b-3p	AACUGGCCCUCAAAGUCCCGCU
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC
hsa-miR-197-3p	UUCACCACCUUCUCCACCCAGC
hsa-miR-200a-3p	UAACACUGUCUGGUAACGAUGU
hsa-miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA
hsa-miR-200c-3p	UAAUACUGCCGGGUAAUGAUGGA
hsa-miR-203a	GUGAAAUGUUUAGGACCACUAG
hsa-miR-204-5p	UUCCCUUUGUCAUCCUAUGCCU
hsa-miR-210-3p	CUGUGCGUGUGACAGCGGCUGA
hsa-miR-221-3p	AGCUACAUUGUCUGCUGGGUUUC
hsa-miR-222-3p	AGCUACAUCUGGCUACUGGGU
hsa-miR-301a-3p	CAGUGCAAUAGUAUUGUCAAAGC
hsa-miR-342-3p	UCUCACACAGAAAUCGCACCCGU
hsa-miR-365a-3p	UAAUGCCCCUAAAAAUCCUUAU
hsa-miR-375	UUUGUUCGUUCGGCUCGCGUGA
hsa-miR-378a-3p	ACUGGACUUGGAGUCAGAAGGC
hsa-miR-423-3p	AGCUCGGUCUGAGGCCCCUCAGU
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
hsa-miR-425-5p	AAUGACACGAUCACUCCCGUUGA
hsa-miR-429	UAAUACUGUCUGGUAAAACCGU
hsa-miR-500a-5p	UAAUCCUUGCUACCUGGGUGAGA
hsa-miR-532-5p	CAUGCCUUGAGUGUAGGACCGU

hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG
hsa-miR-99a-5p	AACCCGUAGAUCCGAUCUUGUG
hsa-miR-99b-5p	CACCCGUAGAACCGACCUUGCG
hsa-miR-101-3p	UACAGUACUGUGAUAACUGAA
hsa-miR-103a-3p*	AGCAGCAUUGUACAGGGCUAUGA

hsa-miR-574-3p	CACGCUCAUGCACACACCCACA
hsa-miR-582-5p	UUACAGUUGUUCAACCAGUUACU
hsa-miR-598-3p	UACGUCAUCGUUGUCAUCGUCA
hsa-miR-660-5p	UACCCAUUGCAUAUCGGAGUUG

* Validated miRNAs

S3 Table. Predicted target genes of the candidate microRNAs.

microRNA	Predicted target genes from DIANA miRPath	p-value	KEGG pathway
hsa-miR-21-5p	*ITGB8, THBS1, COL5A2, CD47*	1.05E-11	ECM-receptor interaction
	ERBB2, ITGB8, THBS1, BCL2, EGFR, PTK2, PIK3R1, PDGFD, VEGFA, PTEN, COL5A2	2.12E-06	Focal adhesion
	ERBB2, EGFR, PTK2, MYC, PIK3R1	2.31E-06	ErbB signaling pathway
	TGFBR1, ZFYVE16, MYC, TGFB2, TGFBR2, BMPR2	8.30355E-05	TGF-beta signaling pathway
	TSC1, PIK3R1, RPS6KA3, VEGFA, PTEN, RRAGC	0.000527381	mTOR signaling pathway
	BID, BCL2, APAFI, PIK3R1, FAS	0.007398499	Apoptosis
	TGFBR1, EGFR, MAP3K1, RASA1, RASGRP1, MYC, DUSP8, FAS, RPS6KA3, TGFB2, MAP3K2, RASGRP3, MKNK2, TGFBR2	0.0173412	MAPK signaling pathway
hsa-miR-22-3p	*SP1, BMP7*	8.30355E-05	TGF-beta signaling pathway
	PRKACA	0.007398499	Apoptosis
	PRKACA	0.0173412	MAPK signaling pathway
hsa-miR-23b-3p	*STAT5B*	2.31E-06	ErbB signaling pathway
hsa-miR-29c-3p	*COL3A1, COL4A2, COL1A1, COL1A2, LAMC1, Col6a2, COL4A1*	1.05E-11	ECM-receptor interaction
	BCL2, COL3A1, JUN, COL4A2, COL1A1, COL1A2, LAMC1, AKT3, Col6a2, VEGFA, COL4A1	2.12E-06	Focal adhesion
	AKT2, JUN,AKT3	2.31E-06	ErbB signaling pathway
	AK2, AKT3, VEGFA	0.000527381	mTOR signaling pathway
	BCL2, AK2, AK3	0.007398499	Apoptosis
	AKT2, JUN, AKT3	0.0173412	MAPK signaling pathway
hsa-miR-103a-3p	*ITGA2*	1.05E-11	ECM-receptor interaction
	BCL2, ZYX, ITGA2	2.12E-06	Focal adhesion
	ABL2, RPS6KB1	2.31E-06	ErbB signaling pathway
	ACVR2B, SMAD7, RPS6KB1	8.30355E-05	TGF-beta signaling pathway
	RPS6KB1	0.000527381	mTOR signaling pathway
	FGF2, MAP3K7	0.007398499	Apoptosis
	FGF2, MAP3K7	0.0173412	MAPK signaling pathway