

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

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28 **Abstract**

29

30 **Aim**

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

35

36 **Patients/materials & methods**

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

39

40 **Results**

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

44

45 **Conclusions**

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

49

50 **Keywords:** Duchenne Muscular Dystrophy, microRNA, exosome, biomarker, deflazacort,
51 prednisolone

52

53 1. Introduction

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

66

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

73

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

78

79 The diagnosis of DMD is usually made by combining genetic, clinical and biochemical tests
80 and, in selected cases, by muscle biopsy. Levels of creatine kinase (CK), an enzyme involved
81 in energy production and utilisation [16], are generally elevated in serum from patients.

82 Serum CK is not however considered to be a reliable circulating biomarker because it is
83 subject to fluctuations, mainly related to age and its modulation by physical activity [17, 18].

84 In addition, the progressive loss of muscle mass in DMD leads to a secondary reduction of
85 CK levels, which therefore does not adequately capture the progressive nature of the
86 condition. Serum matrix metalloproteinase-9 (MMP-9) is being studied as potential serum
87 biomarker for dystrophinopathies as it increases significantly with age in patients' serum.
88 Nevertheless, its efficacy in monitoring disease progression and therapeutic response remains
89 to be confirmed [19, 20]. Elevated levels of myomesin 3 (MYOM3) protein fragments have
90 been found in serum from DMD patients and are promising candidate for monitoring
91 experimental therapies [21]. Also, urinary levels of the amino terminal fragments of the
92 sarcomeric protein titin (N-ter titin) represent a potential non-invasive biomarker useful for
93 the diagnosis and to monitor the response to therapies [22]. Despite these encouraging results
94 obtained from proteomic studies, there is still a pressing need for novel, non-invasive and
95 reliable biomarkers in DMD sensitive to disease progression and to assess their efficacy in
96 response to therapeutic intervention.

97

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

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

107

108 Exosomes are secreted by different cell types, including myoblasts, allowing cell-to-cell
109 signalling by transferring their cargo molecules (mainly composed of miRNA, mRNA, lipids
110 and proteins) [31]. Urinary exosomes have been investigated in urinary tract related diseases
111 [32, 33], breast cancer [34] and neurodegenerative conditions [35]. However, there is no
112 information on the contribution (if any) of skeletal muscle cells to the exosome population in
113 urine, nor has there been any study on the expression of urinary miRNA in patients with
114 muscular dystrophies.

115

116 In order to investigate the potential of urinary miRNAs as a novel non-invasive biomarker in
117 DMD, we profiled their pool isolated from urinary exosomes of ambulant and non-ambulant
118 DMD patients and age matched controls and found >50 miRNAs downregulated in DMD
119 patients. From these, 5 candidate miRNAs were selected for further validation, based on
120 previous reports indicating their involvement in skeletal muscle related diseases [36-41].

121 Finally, to test if there was any association between miRNA levels in urine and corticosteroid
122 treatment, we studied the expression of the most dysregulated miRNAs in patients treated
123 with prednisolone or deflazacort following a daily or intermittent regimens.

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128 **2. Materials & Methods**

129 **2.1. Subject selection and urine collection**

130 The patients included in this cross-sectional study are part of a cohort of DMD boys taking
131 part in a multicenter natural history study registered in clinicaltrials.gov (NCT02780492).
132 Patients are assessed every six months according to a standardized protocol. Samples from 54
133 patients recruited in London, Paris, Newcastle and Leiden were analysed. This study was
134 approved by the London-Bromley Research Ethics Committee (REC 12/LO/0442) and all
135 Ethical Committees in the countries involved. All patients and their families signed the
136 informed consent and assent for the Biobank for Neuromuscular Disorders (approved by The
137 Hammersmith and Queen Charlotte's and Chelsea Research Ethics Committee -
138 06/Q0406/33).

139

140 Twenty healthy age-matched volunteers were recruited from patients' families and friends at
141 Great Ormond Street Hospital. Urine samples (~ 20 ml) were collected on the day of the study
142 visit (morning, not fasting) and immediately frozen at -80°C until analyses were performed.

143 The demographic, clinical data and corticosteroid therapy regimen administered are shown in

144 **Supplementary Table 1.**

145

146 **2.2. Exosome isolation**

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

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

205

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

210

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

214

215 **2.7. Statistical analysis**

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

222

223 **3. Results**

224 **3.1. Exosomal microRNA profiling in urine of DMD patients and healthy controls**

225 34 microRNAs were either undetected or weakly expressed and were excluded from the
226 study. 53 miRNAs were detected in at least 60% of the samples and included in subsequent

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

230

231 *3.1.1. miRNA profiling in urinary exosomes from all DMD patients compared to healthy* 232 *controls*

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

237

238 *3.1.2. miRNA profiling in urinary exosomes from ambulant DMD patients compared to* 239 *healthy controls*

240 To investigate the possibility that the levels of urinary miRNA might be influenced by the
241 ambulatory status of the DMD boys, further statistical analyses were carried out by stratifying
242 the DMD samples into 15 ambulant (A) and 17 non-ambulant (NA). Of three miRNAs that
243 were significantly downregulated in DMD compared to controls, only miR-29c-3p was
244 significantly downregulated (P<0.01, **Fig 2A**) in ambulant DMD patients compared to the
245 healthy controls. There was significant downregulation of two additional identified in the first
246 part of the study, miRNAs - miR-92a-3p (P<0.01, **Fig 2B**) and miR-103a-3p (P<0.01, **Fig**
247 **2C**) when ambulant patients were compared to healthy controls.

248

249 *3.1.3. Expression of miR-29c-3p, miR-92a-3p and miR-103a-3p correlates with preserved* 250 *ambulation in DMD*

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

264

265 *3.1.4. miRNA profiling in urinary exosomes from non-ambulant DMD patients compared to*
266 *healthy controls*

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

269

270 *3.1.5. miRNA profiling in urinary exosomes from DMD ambulant compared to DMD non-*
271 *ambulant patients*

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

274

275 **3.2. Selection of microRNA candidates for further validation studies**

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

282

283 Table 1

284 Selected candidate involvement in skeletal muscle

microRNA	Previous findings	Model
miR-29	downregulated in <i>mdx</i> mouse model of DMD [36]	<i>mdx</i> muscles
	loss of miR-29 in myoblasts contributes to dystrophic muscle pathogenesis[36]	<i>mdx</i> primary myoblasts
	myogenic factor[39]	C2C12 cells
	reduced in DMD patients[37]	DMD patient muscle and myoblasts
	downregulated in quiescent satellite cells during myogenesis <i>in vitro</i> [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]	<i>Sgca</i> -null mouse serum
miR-21	increased in DMD[37]	DMD patient muscle and myoblasts

285

286 *3.2.1. Bioinformatic prediction of the targets*

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

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

microRNA	Predicted target genes from DIANA miRPath	p-value	KEGG pathway
hsa-miR-21-5p	<i>ITGB8, THBS1, COL5A2, CD47</i>	1.05E-11	ECM-receptor interaction
	<i>ERBB2, ITGB8, THBS1, BCL2, EGFR, PTK2, PIK3R1, PDGFD, VEGFA, PTEN, COL5A2</i>	2.12E-06	Focal adhesion
	<i>ERBB2, EGFR, PTK2, MYC, PIK3R1</i>	2.31E-06	ErbB signaling pathway
	<i>TGFBR1, ZFYVE16, MYC, TGFB2, TGFBR2, BMPR2</i>	8.30355E-05	TGF-beta signaling pathway
	<i>TSC1, PIK3R1, RPS6KA3, VEGFA, PTEN, RRAGC</i>	0.000527381	mTOR signaling pathway
	<i>BID, BCL2, APAFI, PIK3R1, FAS</i>	0.007398499	Apoptosis
	<i>TGFBR1, EGFR, MAP3K1, RASA1, RASGRP1, MYC, DUSP8, FAS, RPS6KA3, TGFB2, MAP3K2, RASGRP3, MKNK2, TGFB2</i>	0.0173412	MAPK signaling pathway
hsa-miR-22-3p	<i>SPI1, BMP7</i>	8.30355E-05	TGF-beta signaling pathway
	<i>PRKACA</i>	0.007398499	Apoptosis
	<i>PRKACA</i>	0.0173412	MAPK signaling pathway
hsa-miR-23b-3p	<i>STAT5B</i>	2.31E-06	ErbB signaling pathway
hsa-miR-29c-3p	<i>COL3A1, COL4A2, COL1A1, COL1A2, LAMC1, Col6a2, COL4A1</i>	1.05E-11	ECM-receptor interaction
	<i>BCL2, COL3A1, JUN, COL4A2, COL1A1, COL1A2, LAMC1, AKT3, Col6a2, VEGFA, COL4A1</i>	2.12E-06	Focal adhesion
	<i>AKT2, JUN, AKT3</i>	2.31E-06	ErbB signaling pathway
	<i>AK2, AKT3, VEGFA</i>	0.000527381	mTOR signaling pathway
	<i>BCL2, AK2, AK3</i>	0.007398499	Apoptosis
	<i>AKT2, JUN, AKT3</i>	0.0173412	MAPK signaling pathway
hsa-miR-103a-3p	<i>ITGA2</i>	1.05E-11	ECM-receptor interaction
	<i>BCL2, ZYX, ITGA2</i>	2.12E-06	Focal adhesion
	<i>ABL2, RPS6KB1</i>	2.31E-06	ErbB signaling pathway
	<i>ACVR2B, SMAD7, RPS6KB1</i>	8.30355E-05	TGF-beta signaling pathway
	<i>RPS6KB1</i>	0.000527381	mTOR signaling pathway
	<i>FGF2, MAP3K7</i>	0.007398499	Apoptosis

	<i>FGF2, MAP3K7</i>	0.0173412	MAPK signaling pathway
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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*

321 There was a significant downregulation of miR-23 -3p ($P < 0.01$, **Fig 4F**) and miR-21-5p
322 ($P < 0.05$, **Fig 4I**) in non-ambulant DMD patients compared with the controls but not in
323 controls vs all DMD patients (**Fig 4D, 4G**), nor in controls vs ambulant DMD patients (**Fig**
324 **4E, 4H**). There was no significant difference in the relative expression of the other selected
325 candidates (miR-22-3p and miR-103a-3p) in patients compared to the controls; hence, they
326 were excluded from further analysis. Finally, as for miR-29c-3p, linear regression analyses
327 did not show a significant correlation between miR-23 -3p and miR-21-5p expression and
328 age of patients (**Fig S3B, S3C**).

329

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

333

334 **3.3. miRNA response to the corticosteroid therapy**

335 In order to determine if there were differences in the selected candidate miRNA expression
336 profiles between patients on different corticosteroid regimens, statistical comparisons were
337 performed on patients receiving prednisolone vs deflazacort, and on daily versus intermittent
338 (10/10) steroid regimens.

339

340 *3.3.1. Deflazacort compared to Prednisolone*

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

343

344 *3.3.2. Daily compared to intermittent regimen*

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

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356

357 4. Discussion

358

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

364

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

371

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

379

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

382 strongest profibrogenic factors. It is stored in the extracellular matrix and when activated, as a
383 consequence of tissue damage, exerts its effects through binding to the TGF β Type I and
384 TGF β Type II receptors [57, 58]. In the *mdx* diaphragm, TGF- β 1 upregulation occurs at early
385 stages of fibrogenesis [36, 58, 59]. In DMD patients, TGF- β 1 triggers the fibrotic process,
386 and reaches peak levels in muscles during the early stages of the disease (6 years) promoting
387 a massive connective tissue proliferation. After this phase, TGF- β 1 levels decline while the
388 proliferation process continues [60]. TGF β promotes fibrosis in *mdx* skeletal muscle by
389 inhibiting mir-29 expression [36] which is a key player in controlling ECM modifications
390 [61]. Other evidence implicating TGF- β 1 in promoting fibrosis by inhibiting miR-29
391 expression comes from studies on pulmonary fibrosis, which showed that miR-29 modulates
392 the fibrotic process by binding a large number of genes involved in ECM synthesis and
393 remodelling including *COL1A1*, *MMP2* and *MMP14* [62]. Moreover, previous studies
394 focused on renal fibrosis showed that this process was correlated with the loss of miR-29
395 mediated by a TGF β /SMAD3 dependent mechanism [63].

396

397 We speculate that reduced levels of miR-29 observed in the ambulant DMD boys are the
398 result of the progressive fibrosis, promoted by TGF- β 1 signalling, which characterises the
399 early stages of the disease. Interestingly, we found that miR-29 levels are affected by the
400 regimen of corticosteroid therapy, drugs which are prescribed as part of the standards of care
401 in DMD patients [64, 65]. In particular, we found significant downregulation of miR-29 in
402 patients receiving the intermittent steroid regimen but not in those receiving a daily treatment,
403 indicating that a constant administration of corticosteroids might be more efficient in
404 maintaining miR-29 levels closer to those in healthy controls, and hence in slowing down the
405 fibrotic process. This hypothesis is supported by the recent report of the inhibitory action of
406 prednisolone on TGF- β 1, a repressor of miR-29 [36], in *mdx* diaphragm [66]. Since miR-29

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

410

411 In our validation studies, we found a significant downregulation of miR-23 -3p in non-
412 ambulant patients. This is the first time miR-23 has been reported to be implicated in DMD,
413 and consequently, its role in the disease is still unclear. However, a link between miR-23
414 and TGF β have been reported in liver, where it downregulates *Smad* genes in mouse fetal
415 liver cells and consequently the TGF β signalling [67] and also in murine airway smooth
416 muscle, where it controls the proliferation of the cells through inactivating TGF β signalling
417 [68]. Moreover, it has been shown that fibrosis in human fibroblasts is induced by TGF β via
418 the PAK2 pathway which in turn, stimulates matrix synthesis through the activation of the
419 Smad1 protein [69]. As miR-23 targets a large number of genes in human, including *PAK2*
420 [70], we speculate that it might counteract the fibrotic process in DMD, through the TGF β
421 signaling inhibition. However further studies aimed at investigating the association between
422 miR-23 and fibrosis are needed to confirm this hypothesis and to address its involvement in
423 DMD pathogenesis.

424

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

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

437

438

439 **4.1. Conclusions**

440 Our findings indicate that exosomal urinary miR-29c-3p, miR-23 -3p and miR-21-5p are
441 promising novel non-invasive biomarkers for DMD, and that miR-29c-3p levels are
442 differentially affected by different steroid regimens, supporting the antifibrotic effect that
443 steroid therapy have, and indicating for the first time that the determination of urinary
444 miRNA levels allow to capture differences between different steroids regimens, which likely
445 reflect the differences in clinical benefit between daily vs intermittent steroids therapies [76,
446 77].

447

448 **4.2. Executive Summary**

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

452

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

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

457

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

461

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

466

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

469

470

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

473

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

476

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

479

480 **4.3. Future perspective**

481

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

485

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723

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

730

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

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

738

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

741 Expression of the three microRNAs in urinary exosomes isolated from healthy controls
742 (n=15) and ambulant DMD patients (n=15). There was significant downregulation of miR-
743 29c-3p (**3A**), miR-92a-3p (**3B**) and miR-103a-3p (**3C**) in the DMD patients (including both
744 ambulant and non-ambulant patients) compared to controls. Data are presented as Mean \pm
745 SEM. **p< 0.01. **GMV= global mean value.**

746

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

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

751

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

754 Expression of miR-29c-3p, miR-23b-3p and miR-21-5p in urinary exosomes isolated from
755 healthy controls (n=20) ambulant (n=31) and non-ambulant (n=23) DMD patients. There was
756 significant downregulation of miR-29c-3p in patients compared to controls (A) and in
757 ambulant patients compared to controls (B). MiR-23b-3p was significantly downregulated in
758 non-ambulant patients compared to controls (F). MiR-21-5p was significantly downregulated
759 in non-ambulant patients compared to controls (I) Data are presented as Mean \pm SEM. *p<
760 0.05; **p< 0.01.

761

762 **Supplementary Fig. 3. Lack of correlation between miR-29c-3p, miR-23b-3p and miR-**
763 **21-5p expression with age in DMD patients.**

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

767

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

769 Expression of miR-21-5p (A), miR-22-3p (B), miR-23b-3p (C), miR-29c-3p (D) and miR-
770 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 \pm 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
783 Mean \pm SEM.

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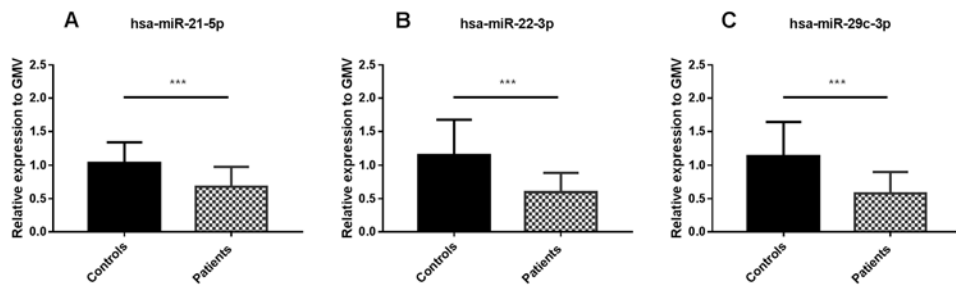


Fig. 1. Expression of miR-21-5p, miR-22-3p and miR-29c-3p in urine samples from DMD patients. Expression of the three microRNA in urinary exosomes isolated from healthy controls (n=15) ambulant DMD patients (n=15) and non-ambulant DMD patients (n=17). There was significant downregulation of miR-21-5p (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 \pm SEM. ***p < 0.001. GMV= global mean value.

555x177mm (96 x 96 DPI)

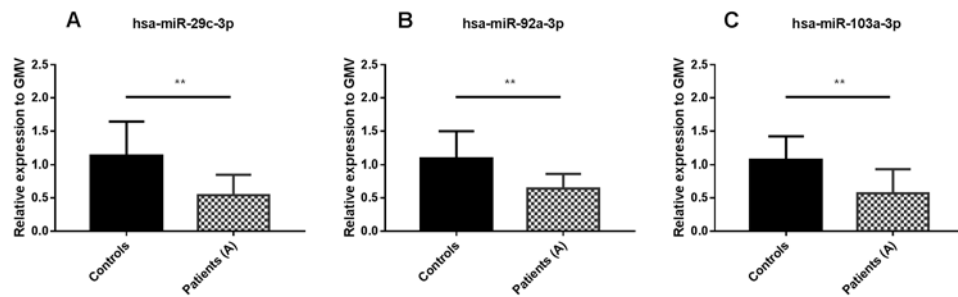


Fig. 2. Expression of miR-29c-3p, miR-92a-3p and miR-103a-3p in urine samples from ambulant DMD patients.

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 \pm SEM. **p< 0.01. GMV= global mean value.

555x184mm (96 x 96 DPI)

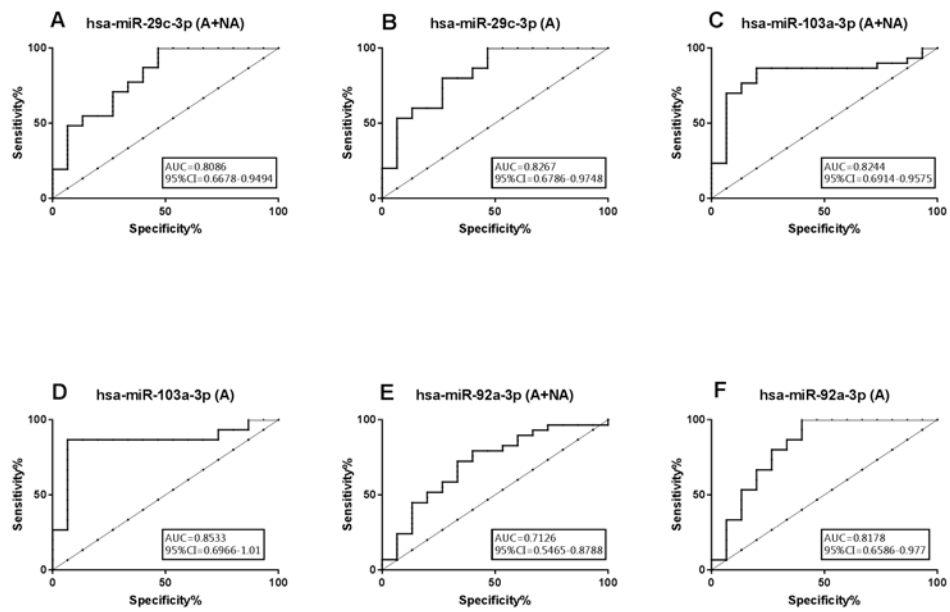


Fig. 3. ROC curve analysis of urinary miR-29c-3p, miR-92a-3p and miR-103a-3p. ROC curves based on miR-29c-3p urinary levels, for differentiating between the group including all the DMD (n=32) (A) and ambulant patients (n=15) (B). The same analysis was performed for miR-103a-3p (C, D) and miR-92a-3p (E,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

555x356mm (96 x 96 DPI)

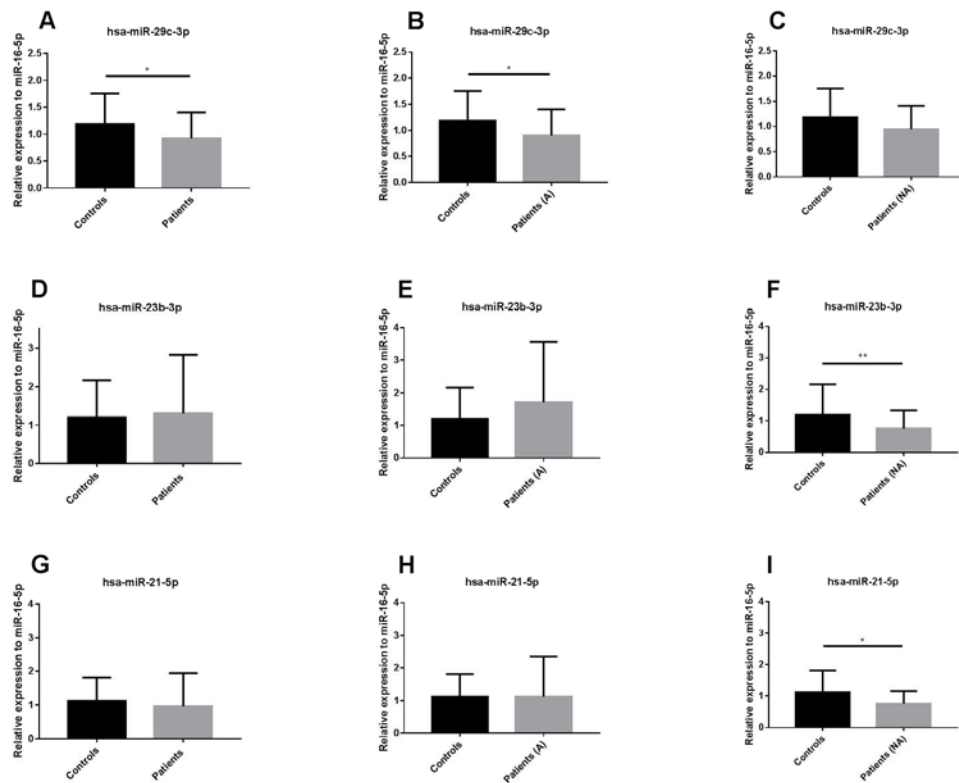


Fig. 4. Validated expression of miR-29c-3p, miR-23b-3p and miR-21-5p in urine samples from DMD patients. Expression of miR-29c-3p, miR-23b-3p and miR-21-5p in urinary exosomes isolated from healthy controls (n=20) ambulant (n=31) and non-ambulant (n=23) DMD patients. There was significant downregulation of miR-29c-3p in patients compared to controls (A) and in ambulant patients compared to controls (B). MiR-23b-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 \pm SEM. *p < 0.05; **p < 0.01.

555x471mm (96 x 96 DPI)

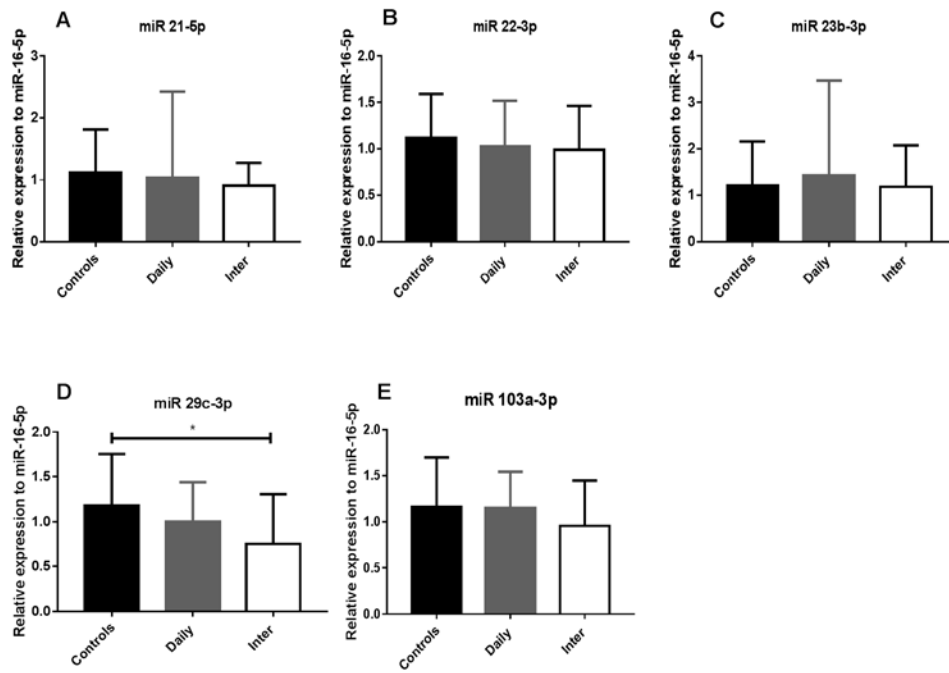
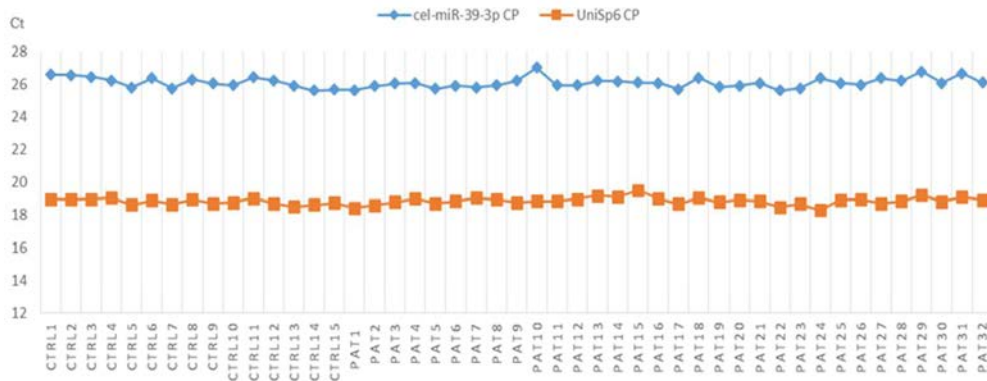


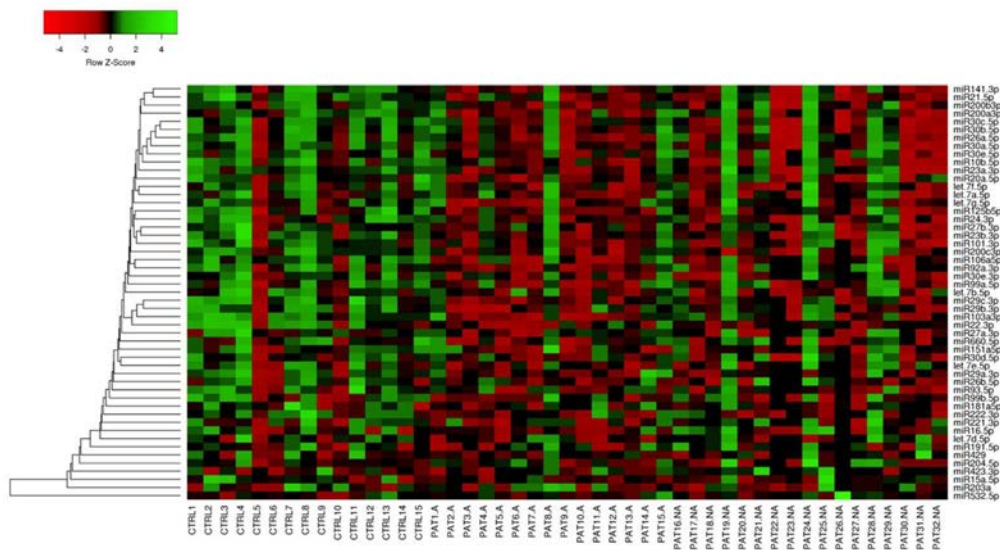
Fig. 5. Effects of different corticosteroid regimens on candidate miRNA expression. Expression of miR-21-5p (A), miR-22-3p (B), miR-23b-3p (C), miR-29c-3p (D) and miR-103a-3p (E) in urinary exosomes isolated from healthy controls (n=20), DMD patients treated with any daily corticosteroids (n=25) and DMD patients treated with any intermittent corticosteroids (n=18). Data are presented as Mean \pm SEM. *p < 0.05.

555x419mm (96 x 96 DPI)



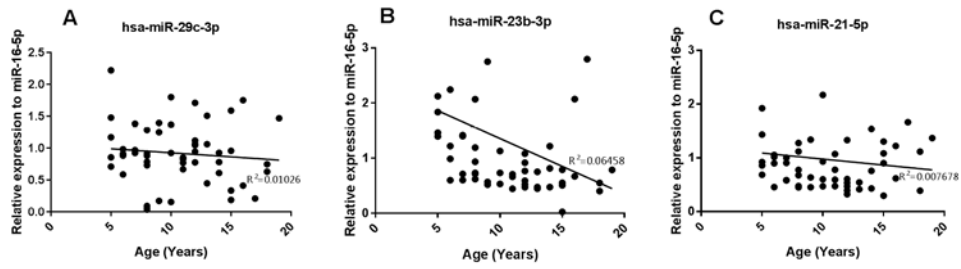
Supplementary Fig. 1 Expression of the synthetic controls. UniSp6 (orange) and cel-miR-39 (light blue) expression in the samples (n=47) used for microRNA profiling. The x-axis represents the samples (both patients and controls) while the y-axis represents Ct values.

180x70mm (150 x 150 DPI)



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.

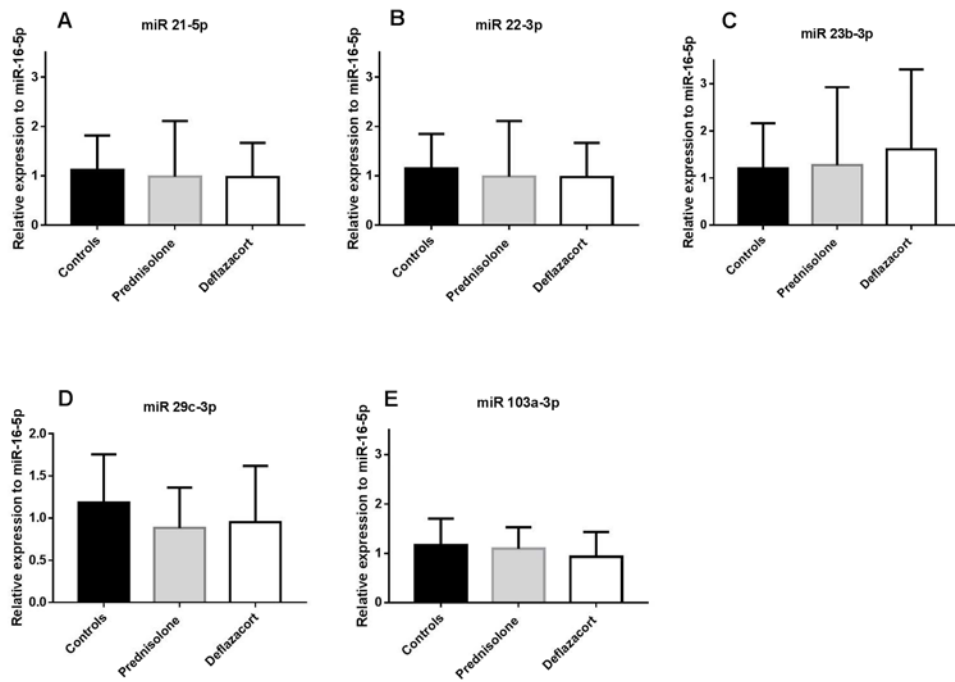
189x107mm (150 x 150 DPI)



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 \pm SEM.

555x419mm (96 x 96 DPI)

Table 1. Selected candidate involvement in skeletal muscle.

microRNA	Previous findings	Model
miR-29	downregulated in <i>mdx</i> mouse model of DMD [36]	<i>mdx</i> muscles
	loss of miR-29 in myoblasts contributes to dystrophic muscle pathogenesis[36]	<i>mdx</i> primary myoblasts
	myogenic factor[39]	C2C12 cells
	reduced in DMD patients[37]	DMD patient muscle and myoblasts
	downregulated in quiescent satellite cells during myogenesis <i>in vitro</i> [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]	<i>Sgca</i> -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	miRNA	Target sequence
hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU	hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU	hsa-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU
hsa-let-7d-3p	CUAUACGACCUGCUGCCUUUCU	hsa-miR-107	AGCAGCAUUGUACAGGGCUAUCA
hsa-let-7d-5p	AGAGGUAGUAGGUUGCAUAGUU	hsa-miR-125b-5p	UCCUGAGACCCUAACUUGUGA
hsa-let-7e-5p	UGAGGUAGGAGGUUGUAUAGUU	hsa-miR-126-3p	UCGUACCGUGAGUAAUAAUGCG
hsa-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU	hsa-miR-128-3p	UCACAGUGAACCGGUCUCUUU
hsa-let-7g-5p	UGAGGUAGUAGUUUGUACAGUU	hsa-miR-133a-3p	UUUGGUCCCCUUAACCAGCUG
hsa-let-7i-5p	UGAGGUAGUAGUUUGUGCUGUU	hsa-miR-135b-5p	UAUGGCUUUUUUCALUCCUAUGUGA
hsa-miR-10a-5p	UACCCUGUAGAUCCGAAUUUGUG	hsa-miR-141-3p	UAACAGUCUCUGGUAAGAUCC
hsa-miR-10b-5p	UAGCCUGUAGACCCGAAUUUGUG	hsa-miR-145-5p	GUCCAGUUUUUCCGAGCAUUGCCU
hsa-miR-15a-5p	UAGCAGCACAUAAUGGUUUUGUG	hsa-miR-148a-3p	UCAGUGCACUACAGAACUUUGU
hsa-miR-15b-5p	UAGCAGCACAUCAUGGUUUACA	hsa-miR-148b-3p	UCAGUGCAUCACAGAACUUUGU
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	hsa-miR-149-5p	UCUGGCUCCGUGUCUUCACUCCC
hsa-miR-17-5p	CAAAGUGCUCUACAGUGCAGGUAG	hsa-miR-151a-5p	UCGAGGAGCUCACAGUCUAGU
hsa-miR-20a-5p	UAAAGUGCUCUUAAGUGCAGGUAG	hsa-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-21-5p*	UAGCUUAUCAGACUGAUGUUGA	hsa-miR-187-3p	UCGUGUCUUGUGUUGCAGCCGG
hsa-miR-22-3p*	AAGCUGCCAGUUGAAGAACUGU	hsa-miR-191-5p	CAACGGAAUCCAAAAGCAGCUG
hsa-miR-22-5p	AGUUCUUCAGUGGCAAGCUUUA	hsa-miR-193b-3p	AACUGGCCCUCAAAGUCCCGCU
hsa-miR-23a-3p	AUCACAUUGCCAGGGAUUUC	hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC
hsa-miR-23b-3p*	AUCACAUUGCCAGGGAUUACC	hsa-miR-197-3p	UUCACCACCUUCCACCCAGC
hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG	hsa-miR-200a-3p	UAAACUGUCUGGUAACGAUGU
hsa-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA	hsa-miR-200b-3p	UAAUACUGCCUGGUAUUGAUGA
hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU	hsa-miR-200c-3p	UAAUACUGCCGGGUAUUGAUGGA
hsa-miR-26b-5p	UUCAAGUAAUUCAGGAUAGGU	hsa-miR-203a	GUGAAAUGUUUAGGACCACUAG
hsa-miR-27a-3p	UUCACAGUGGCUAAGUCCGC	hsa-miR-204-5p	UUCCUUUGUCAUCCUAUGCCU
hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC	hsa-miR-210-3p	CUGUGCGUGUGACAGCGGCUGA
hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA	hsa-miR-221-3p	AGCUACAUUGUCUGCGGUUUC
hsa-miR-29b-3p	UAGCACCAUUUGAAAUCAGUGUU	hsa-miR-222-3p	AGCUACAUCUGGCUACUGGGU
hsa-miR-29c-3p*	UAGCACCAUUUGAAAUCGGUUA	hsa-miR-301a-3p	CAGUGCAAUAGUAUUGUCAAAAGC
hsa-miR-30a-5p	UGUAAACAUCUCCGACUGGAAG	hsa-miR-342-3p	UCUCACACAGAAAUCGCACCCGU
hsa-miR-30b-5p	UGUAAACAUCUACACUCAGCU	hsa-miR-365a-3p	UAAUGCCCUAAAAUCCUUUAU
hsa-miR-30c-5p	UGUAAACAUCUACACUCUCAGC	hsa-miR-375	UUUGUUCGUUCGGCUCGCGUGA
hsa-miR-30d-5p	UGUAAACAUCUCCGACUGGAAG	hsa-miR-378a-3p	ACUGGACUUGGAGUCAGAAGGC
hsa-miR-30e-3p	CUUUCAGUCGGAUGUUUACAGC	hsa-miR-423-3p	AGCUCGGUCUGAGGCCUUCAGU
hsa-miR-30e-5p	UGUAAACAUCUUGACUGGAAG	hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
hsa-miR-31-3p	UGCUAUGCCAACAUAUUGCCA	hsa-miR-425-5p	AAUGACACGAUCACUCCCGUUGA
hsa-miR-31-5p	AGGCAAGAUGCUGGCAUAGCU	hsa-miR-429	UAAUACUGUCUGGUAACCCGU
hsa-miR-34a-5p	UGGCAGUGUCUUAGCUGGUUGU	hsa-miR-500a-5p	UAAUCCUUGCUACCUGGGUGAGA
hsa-miR-92a-3p	UAUUGCACUUGUCCCGCCUGU	hsa-miR-532-5p	CAUGCCUUGAGUGUAGGACCGU

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

hsa-miR-574-3p	CACGCUCAUGCACACCCACA
hsa-miR-582-5p	UUACAGUUGUUAACCAGUUACU
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	<i>ITGB8, THBS1, COL5A2, CD47</i>	1.05E-11	ECM-receptor interaction
	<i>ERBB2, ITGB8, THBS1, BCL2, EGFR, PTK2, PIK3R1, PDGFD, VEGFA, PTEN, COL5A2</i>	2.12E-06	Focal adhesion
	<i>ERBB2, EGFR, PTK2, MYC, PIK3R1</i>	2.31E-06	ErbB signaling pathway
	<i>TGFBRI, ZFYVE16, MYC, TGFB2, TGFB2, BMPR2</i>	8.30355E-05	TGF-beta signaling pathway
	<i>TSC1, PIK3R1, RPS6KA3, VEGFA, PTEN, RRAGC</i>	0.000527381	mTOR signaling pathway
	<i>BID, BCL2, APAFI, PIK3R1, FAS</i>	0.007398499	Apoptosis
	<i>TGFBRI, EGFR, MAP3K1, RASA1, RASGRP1, MYC, DUSP8, FAS, RPS6KA3, TGFB2, MAP3K2, RASGRP3, MKNK2, TGFB2</i>	0.0173412	MAPK signaling pathway
hsa-miR-22-3p	<i>SP1, BMP7</i>	8.30355E-05	TGF-beta signaling pathway
	<i>PRKACA</i>	0.007398499	Apoptosis
	<i>PRKACA</i>	0.0173412	MAPK signaling pathway
hsa-miR-23b-3p	<i>STAT5B</i>	2.31E-06	ErbB signaling pathway
hsa-miR-29c-3p	<i>COL3A1, COL4A2, COL1A1, COL1A2, LAMC1, Col6a2, COL4A1</i>	1.05E-11	ECM-receptor interaction
	<i>BCL2, COL3A1, JUN, COL4A2, COL1A1, COL1A2, LAMC1, AKT3, Col6a2, VEGFA, COL4A1</i>	2.12E-06	Focal adhesion
	<i>AKT2, JUN, AKT3</i>	2.31E-06	ErbB signaling pathway
	<i>AK2, AKT3, VEGFA</i>	0.000527381	mTOR signaling pathway
	<i>BCL2, AK2, AK3</i>	0.007398499	Apoptosis
	<i>AKT2, JUN, AKT3</i>	0.0173412	MAPK signaling pathway
	hsa-miR-103a-3p	<i>ITGA2</i>	1.05E-11
<i>BCL2, ZYX, ITGA2</i>		2.12E-06	Focal adhesion
<i>ABL2, RPS6KB1</i>		2.31E-06	ErbB signaling pathway
<i>ACVR2B, SMAD7, RPS6KB1</i>		8.30355E-05	TGF-beta signaling pathway
<i>RPS6KB1</i>		0.000527381	mTOR signaling pathway
<i>FGF2, MAP3K7</i>		0.007398499	Apoptosis
<i>FGF2, MAP3K7</i>		0.0173412	MAPK signaling pathway