DIFFERENCES IN MICRORNA EXPRESSION PROFILE BETWEEN VASTUS LATERALIS SAMPLES AND MYOTUBES IN COPD CACHEXIA

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ABSTRACT

Quadriceps muscle weakness and wasting are common comorbidities in COPD. MicroRNA expression upregulation may favor muscle mass growth and differentiation. We hypothesized whether the profile of muscle-enriched microRNAs in cultured myotubes differs between COPD patients of a wide range of body composition and healthy controls and whether expression levels of those microRNAs from COPD and controls differ between in vivo and in vitro conditions. Twenty-nine COPD patients (n=15 with muscle wasting, FFMI 15 kg/m^2 and n=14 with normal body composition, FFMI 18 kg/m^2) and 10 healthy controls (FFMI, 19 kg/m^2) were consecutively recruited. Biopsies from the vastus lateralis were obtained in all study subjects. A fragment of each biopsy was used to obtain primary cultures, in which muscle cells were first proliferated to be then differentiated into actual myotubes. In both sets of experiments (in vivo biopsies and in vitro myotubes) the following muscle-enriched microRNAs were analyzed using qRT-PCR: miR-1, miR-133, miR-206, miR-486, miR-29a, miR-27a, and miR-181a from all the study subjects. While the expression of miR-1, miR-206, miR-486, and miR-29a was upregulated in the muscle biopsies of COPD patients compared to those of healthy controls, levels of all the study microRNAs in the myotubes (primary cultured cells) did not significantly differ between COPD patients and the controls. We conclude from these findings that environmental factors (blood flow, muscle metabolism, inflammation) taking place in vivo (biopsies) in muscles may account for the differences observed in microRNA expression between COPD patients and controls. In the myotubes, however, the expression of the same microRNAs did not differ between the study subjects as such environmental factors were not present. These findings suggest that therapeutic strategies should rather target environmental factors in COPD muscle wasting as the profile of microRNA expression in myotubes was similar in patients to that observed in the healthy controls. **Word count:** 300

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51 **KEY WORDS:** lower limb muscle atrophy; severe COPD; muscle-enriched microRNAs;
52 myotubes; muscle specimens

53 **NEW & NOTEWORTHY**

54 Environmental factors taking place in vivo (biopsies) in the muscles may explain differences
55 observed in microRNA expression between COPD patients and controls. In the myotubes,
56 however, the expression of the same microRNAs did not differ between the study subjects as
57 such environmental factors were not present. These findings suggest that therapeutic
58 strategies should rather target environmental factors in COPD muscle wasting and cachexia as
59 microRNA expression profile in myotubes was similar between patients and controls. **Word**
60 **count: 75**

61 **Abbreviations:** COPD, Chronic obstructive pulmonary disease; DNA, deoxyribonucleic acid;
62 RNA, ribonucleic acid; mRNA, messenger RNA; VL, vastus lateralis; FFMI, fat-free mass
63 index; BMI, body mass index; QMVC, maximum voluntary contraction; DMEM, Dulbecco's
64 modified Eagle's medium; PM, proliferation medium; FBS, fetal bovine serum; PSF,
65 penicillin/streptomicin/fungizone-solution; bFGF, basic fibroblast growth factor; EGF,
66 epidermal growth factor; PBS, phosphate-buffered saline; HS, horse serum; DMSO, dimethyl
67 sulfoxide; qRT-PCR, quantitative real time-PCR amplification; MGB, taqMan-minor groove
68 binder; FAM, fluorescein amidite; qPCR, real-time polymerase chain reaction; snU6, small
69 nuclear RNA U6; ANOVA: analysis of variance; CRP, C-reactive protein; GSV, globular
70 sedimentation velocity.

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72 **INTRODUCTION**

73 Skeletal muscle dysfunction and mass loss are commonly present in patients with chronic
74 respiratory and cardiac conditions and cancer. In patients with chronic obstructive pulmonary
75 disease (COPD), the prevalence of muscle dysfunction and atrophy is approximately 30%
76 even in those with a mild-to-moderate disease (2; 3; 21-24; 37; 43). Muscle mass loss and
77 dysfunction were also shown to predict disease progression and mortality irrespective of the
78 airflow limitation in COPD patients (18; 36; 40). Quality of life and physical activity are also
79 impaired in these patients as a result of skeletal muscle loss and dysfunction (5; 18; 21; 38;
80 40).

81 Dysfunction of the respiratory and lower limb muscles has been described in patients
82 with severe COPD (3; 6; 18; 21; 38). However, the latter are usually more severely affected,
83 since factors such as reduced physical activity, malnutrition, and prolonged bed-rest due to
84 acute exacerbations lead to muscle atrophy of the lower limbs. Other factors, namely hypoxia,
85 hypercapnia, cigarette smoking, and comorbidities were also demonstrated to contribute
86 further to muscle dysfunction especially of the lower limbs (3; 4; 7; 8; 18; 21; 38). Moreover,
87 several biological mediators have also been shown to be part of the etiology of skeletal
88 muscle dysfunction and mass loss in COPD. Our group and others have demonstrated widely
89 the contribution of redox potential, metabolic derangements, systemic inflammation,
90 proteolysis signaling and catabolism, structural alterations, muscle remodeling, and epigenetic
91 changes to muscle dysfunction and mass loss in patients with COPD, particularly in those
92 with a severe disease (3; 4; 7; 8; 18; 21; 38).

93 Epigenetics regulate gene expression without inducing any modification in the primary
94 DNA sequence. Gene expression is regulated through direct remodeling of chromatin
95 structure (epigenetics) in cooperation with transcription factors and the translational
96 machinery. In skeletal muscles as in other cell types, epigenetics regulate myogenesis, muscle

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97 development, and adaptation to the environment, thus controlling mechanisms of atrophy and
98 hypertrophy (4-6; 8; 13; 20; 28-30). Among several epigenetic mechanisms, the role of
99 microRNAs in muscle development and myogenesis has been most widely studied in the last
100 decade. MicroRNAs are small, regulatory, non-coding RNA molecules that are mostly located
101 within the cells, although they may also be identified in biological fluids and cell culture
102 media (25). MicroRNAs regulate gene transcription by either inducing RNA silencing or by
103 inhibiting the translation of the target mRNA within the cells. Muscle-specific microRNAs
104 have been shown to regulate muscle adaptation to disuse and overloading. MicroRNAs have
105 also been shown to play a significant role in the satellite cell niche, which is essential for
106 these cells to proliferate and differentiate during myogenesis and muscle development
107 following injury (4-6; 8; 13; 20; 28-30).

108 As such miR-133 was shown to favor myoblast proliferation as a result of the inhibition
109 of myotube formation (10), while cell differentiation and innervation is favored by the action
110 of miR-1 and miR-206 (27). Other microRNAs that are ubiquitously expressed in other cell
111 types are also abundantly expressed in skeletal muscle and may also be involved in muscle
112 phenotype and myogenesis. For instance, miR-27, a target of Paired box protein (Pax)3, was
113 also expressed in embryonic myotomes, satellite cells, and adult muscle fibers (12), myoblast
114 differentiation through the downregulation of Pax7 was also induced by the action of miR-
115 486 (1), MyoD expression may be regulated by miR-181 actions (26), and miR-29 may also
116 favor muscle development through inhibition of transcription factors such as Yin Yang (44).

117 Importantly, a differential regulation of muscle-specific microRNAs has been
118 demonstrated in patients with COPD and normal body composition (20) and in those with
119 severe muscle weakness and atrophy (30). Muscle differentiation rather than proliferation was
120 promoted in the vastus lateralis (VL) of the sarcopenic COPD patients in that study (30).
121 Whether similar findings may be expected in cultured myogenic progenitor cells, a well-

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122 validated model to study biological mechanisms of muscle dysfunction (7; 25), obtained from
123 muscle specimens of patients with severe COPD and cachexia remain to be elucidated.
124 Therefore, we hypothesized whether the profile of muscle-enriched microRNAs in
125 cultured myotubes would differ between COPD patients of a wide range of body composition
126 and healthy controls and whether expression levels of the same microRNAs from both COPD
127 and the control subjects would also differ between in vivo (muscle specimens) and in vitro
128 (myotubes) conditions. Accordingly, our primary objectives were: 1) to explore expression
129 levels of muscle-enriched miRNAs involved in muscle proliferation and differentiation in
130 muscle specimens and in vitro primary cell cultures obtained from the same muscle samples
131 in severe COPD patients with and without cachexia and 2) to analyze potential differences in
132 the expression levels of the muscle-specific microRNAs between study patients in both states
133 (primary cultures and in vivo specimens). For the purpose of the investigation, specimens
134 from VL were additionally obtained in sedentary healthy controls, and all groups of patients
135 and control individuals were clinically and functionally evaluated.
136

137 **METHODS**

138 **Study subjects**

139 Twenty-nine patients with severe COPD according to currently available guidelines (24; 25;
140 43) and 10 age-matched sedentary controls with normal respiratory function were
141 prospectively and consecutively enrolled in a cross-sectional study from the COPD Clinic at
142 Hospital del Mar (Barcelona). The patients and healthy controls were recruited and properly
143 evaluated by some of the investigators involved in the current study. Patients were further
144 subdivided into two different groups according to their body composition: 1) non-cachectic (n
145 = 14) and 2) cachectic (n = 15), following the international consensus on cachexia and muscle
146 wasting (14; 15) and previously published criteria (7; 16; 30; 31). In all patients, muscle

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147 wasting was defined as a body mass index (BMI) ≤ 21 kg/m^2 and a fat-free mass index
148 (FFMI) ≤ 18 kg/m^2, cut-off value established for a Mediterranean population in accordance
149 with both previously published criteria (7; 16; 30; 31) and the international consensus on the
150 definition of cachexia (14; 15). Control subjects were non-smoker (50% ex-smokers and 50%
151 never-smokers) male and female sedentary control subjects recruited from the general
152 population (patients' relatives or friends), while patients in both groups were active smokers
153 (43% and 40%), or ex-smokers (57% and 60%), respectively. All patients were on inhaled
154 bronchodilators. They were clinically stable at the time of the study, without episodes of
155 exacerbation or oral steroid treatment in the previous four months. None of them presented
156 significant comorbidities. All groups of individuals were Caucasian.
157 *Exclusion criteria.* Exclusion criteria for COPD patients and control subjects included other
158 chronic respiratory or cardiovascular disorders, acute exacerbations in the last 3 months,
159 limiting osteoarticular condition, chronic metabolic diseases, suspected para-neoplastic or
160 myopathic syndromes, and/or treatment with drugs known to alter muscle structure and/or
161 function including oral corticosteroids. COPD patients and healthy controls were qualified as
162 sedentary after being specifically inquired about whether they were conducting any regular
163 outdoor physical activity, going regularly to the gymnasium, or participating in any specific
164 training program. Specifically, sedentarism was defined on the basis of the following criteria:
165 1) if subjects were not engaged in one or more of these activities: walking, running, bike
166 riding, swimming, dancing, gardening, or weight lifting more than five times per week, 2) not
167 performing at least 3 hours/week of endurance-type physical activity, and/or 3) inactive
168 general state in which leisure time physical activity was minimal (32). Moreover, the time
169 spent in sedentary postures (lying and sitting) was also considered in the assessment of
170 sedentarism in the study groups (17; 41).

171 The current cross-sectional, prospective investigation was designed in accordance

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172 with both the ethical standards on human experimentation in our institutions and the World
173 Medical Association guidelines (Helsinki Declaration of 2008) for research on human beings.
174 Approval was obtained from the institutional Ethics Committee on Human Investigation
175 (*Hospital del Mar,* Barcelona). Informed written consent was obtained from all individuals.

176 **Anthropometrical and Functional Assessment**

177 Anthropometrical evaluation included BMI and determination of the FFMI by bioelectrical
178 impedance (7; 16; 30; 31). Nutritional parameters were also evaluated through conventional
179 blood tests. Diagnostic criteria for muscle wasting were BMI < 21 kg/m^2 and FFMI < 18
180 kg/m^2 in all patients (7; 16; 30; 31).

181 Lung function was evaluated through determination of spirometric values, static lung
182 volumes, diffusion capacity, and blood gases using standard procedures and established
183 reference values (33-35). Exercise capacity was assessed through the six-minute walking
184 distance following current guidelines (7; 16; 30; 31). The test consisted of two attempts (with
185 at least a 30-minute rest between them) in a 30-meter corridor. Encouragement was given
186 every minute and the test was interrupted if symptoms of exhaustion appeared. Quadriceps
187 muscle strength was evaluated in both patients and controls through the determination of the
188 isometric maximum voluntary contraction (QMVC) of the dominant lower limb as formerly
189 described (7; 16; 30; 31). Briefly, patients were seated with both trunk and thigh fixed on a
190 rigid support of an exercise platform (Domyos HGH 050, Decathlon, Lille, France). The
191 highest value from three brief reproducible maneuvers (<5% variability among them) was
192 accepted as the QMVC.

193 **Muscle biopsy and blood samples**

194 With the aim to prevent potentially exhausting physical exercise that could interfere with the
195 results obtained in muscles, all study subjects were recommended to maintain their regular
196 daily physical activity as usually 10-14 days before undergoing the surgical biopsy procedures

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197 on outpatient basis. Additionally, patients and healthy subjects rested for one hour on a chair
198 with legs half-flexed, time at which blood samples were obtained, right before initiation of the
199 surgical procedures (16; 30; 31). Specimens from the *vastus lateralis* (VL) portion of the
200 quadriceps muscle were obtained from all subjects using the open biopsy technique as
201 previously described (2; 29-31). Primary myogenic cultures were conducted on the muscle
202 biopsy specimens obtained from both patients and healthy controls.
203 Blood samples were drawn at 8:00h am after an overnight fasting period in both patients and
204 healthy controls.

205 **Myogenic precursor cell isolation and culture**

206 Muscle samples were placed on sterile petri dishes in Dulbecco's modified Eagle's medium
207 (DMEM) and trimmed of connective and fat tissues. Muscle specimens were minced finely
208 and digested in 0.2 % collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for
209 one hour with occasional agitation. The resulting suspension of particles was further digested
210 with 0.05 % trypsin at 37 °C for 30 minutes. Samples were then filtered through a 100-
211 micrometer cell strainer. Cell suspensions were cultured on 1.5% gelatin-coated petri dishes
212 in proliferation medium (PM) consisting of 3:1 mixture of DMEM/Medium-199 and
213 supplemented with the following compounds: 20% fetal bovine serum (FBS), 2 mM L-
214 glutamine, 1% penicillin/streptomicin/fungizone-solution (PSF), recombinant human basic
215 fibroblast growth factor (bFGF, 2.5 ng/ml), recombinant human insulin (10 microgram/mL)
216 and recombinant human epidermal growth factor (EGF,10 ng/mL). All the study experiments
217 described below were performed on muscle cells between passages 4-6 of the primary
218 cultures, time at which the number of cells needed to perform all the molecular biology
219 analyses was reached and the myotubes still preserved their differentiating features (as seen
220 under light microscopy, fusion of myoblasts to form multinucleated myotubes, Figure 1).
221 Myogenic purity was determined under light microscopy (x 200) in 20 randomly selected

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222 fields for each muscle specimen after identification of the number of desmin positive cells. In
223 all samples from both patients and controls, more than 94 % desmin positive cells were
224 identified.

225 *Myogenic purity assessment.* As desmin is a muscle-specific type III intermediate filament
226 of the cytoskeleton of muscle cells and is one of the earliest muscle specific proteins
227 expressed by undifferentiated muscle progenitor cells, even prior to myotube formation, this
228 protein was used to assess myogenic purity in the cultures as also previously described (7; 11;
229 19; 42).To evaluate the myogenic purity, cell monolayers grown on chamber culture slides
230 were fixed with 1:1 methanol-acetone at -20ºC for 10 minutes before immunocytochemical
231 characterization. Myoblasts were identified using anti-desmin antibody (Thermo Scientific,
232 Waltham, MA, USA). After a 30-minute incubation with the antibody, slides were washed
233 and incubated for 30 minutes with biotinylated universal secondary antibody followed by
234 another 30-minute incubation with horseradish-conjugated streptavidin and diaminobenzidine
235 for 5 minutes (kit LSAB+HRP Dako Cytomation Inc., Carpinteria, CA, USA) as a substrate.
236 Slides were counterstained with hematoxylin for two more minutes, dehydrated and mounted
237 for conventional microscopy. Images were captured under light microscopy (x200, Olympus
238 BX 61, Olympus Corporation, Tokyo, Japan) in 20 randomly selected fields of each muscle
239 specimen. In all samples from both patients and controls, more than 94 % desmin positive
240 cells were identified (11). An efficient and reproducible isolation and culture of enriched
241 muscle precursor cells/myoblasts from the human muscles was possible using this protocol as
242 also previously described (7; 11). We monitored the myogenic purity of cells during the initial
243 passages to verify that non-myogenic cells had not overgrown the myoblasts. At passages 4-6,
244 most of the cells were desmin+ (94%), and cells were not used in the study thereafter (7; 11).

245 **Experimental procedures and groups**

246 Cell cultures of differentiated myotubes were conducted at the same time from each muscle

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247 specimen obtained from both groups of patients and healthy control subjects.

248 Myogenic differentiation from the muscle precursor cells was induced by plating cells at a
249 high density (10^4 cells/cm^2) and allowing them to adhere in proliferation medium (PM)
250 overnight. After a 24-hour period of cell attachment in PM, cells were washed twice in
251 phosphate-buffered saline (PBS) and induced to differentiate over a five-day period in
252 differentiation medium (DMEM/M-199 (3:1 mixture) supplemented with 2% horse serum
253 (HS): 2mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in the presence
254 of 0.1% dimethyl sulfoxide (DMSO).

255 **Molecular biology analyses**

256 *RNA isolation***.** Total RNA was isolated from cultured cells (in vitro) as well as from VL
257 specimens (in vivo) using the Trizol reagent and following the manufacturer's instructions
258 (Life technologies, Carlsbad, CA), and previous studies (7; 28-31). Total RNA concentrations
259 were determined spectrophotometrically using the NanoDrop 1000 (Thermo Scientific,
260 Waltham, MA, USA).

261 *MicroRNA reverse transcription (RT)***.** MicroRNA RT was performed using TaqMan
262 microRNA assays (Life Technologies) following the manufacturer's instructions and previous
263 studies (28-31). RNA was preserved frozen at -80ºC until further use.

264 *Quantitative real time-PCR amplification (qRT-PCR)*. TaqMan-minor groove binder (MGB)
265 moiety (TaqMan® primers, fluorescein amidite (FAM) dye labelled, Life Technologies)
266 based real-time polymerase chain reaction (qPCR) reactions were performed using the ABI
267 PRISM® 7900HT Sequence Detector System (Applied BioSystems, Foster City, CA, USA),
268 together with a commercially available predesigned microRNA assay as shown in Table 1.
269 Taqman microRNA assay for small nuclear RNA U6 (snU6) was used to normalize the
270 miRNAs amplifications. MicroRNA data were collected and subsequently analyzed using the
271 Sequence Detection System Relative Quantification Software version 2.1 (Applied

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272 BioSystems), in which the comparative C_T method ($2^{-\Delta\Delta CT}$) for relative quantification was
273 employed (7; 30), as also reported in previous studies (7; 16; 30; 31). All the study samples
274 were run in triplicates for all the analyses of each marker.

275 **Statistical Analysis**

276 The sample size chosen was based on previous studies (7; 16; 30; 31). Statistical power was
277 calculated using specific software (StudySize 2.0, CreoStat HB, Frolunda, Sweden). FFMI
278 was selected as the target variable on the basis of the ANOVA test to estimate the statistical
279 power in the study. On the basis of a standard power statistics established at a minimum of
280 80% and assuming an alpha error of 0.05 ($P \leq 0.05$), the statistical power was sufficiently high
281 to detect a minimum difference of four points between groups in the sample size and standard
282 deviation. Statistical power was calculated using specific software (StudySize 2.0, CreoStat
283 HB, Frolunda, Sweden). Shapiro-Wilk test was used to explore the normality of the
284 distributions of the variables. Data are expressed either as mean (standard deviation) or as
285 deltas (Δ), defined as the average change in each sample specimen between differentiating
286 cells and the specimens obtained from the VL (myotubes versus in vivo conditions). The
287 following sets of comparisons and tests were used in the investigation: 1) comparisons of
288 results obtained either in the muscle specimens or the myotubes among the study groups were
289 assessed using one-way and *Tukey's post-hoc* analysis of variance (ANOVA); 2) for each
290 study group separately, comparisons of results between myotubes and in vivo conditions were
291 explored using paired T-test; 3) comparisons of the results obtained from the microRNA
292 expression analyses in either myotubes or in vivo conditions within each study group of
293 subjects (healthy controls, non-cachectic and cachectic COPD patients) were determined
294 using the general linear model of repeated measures and Bonferroni's multiple comparison
295 correction. Additionally, Pearson's Chi-Square test was employed to assess potential
296 differences in smoking history variables between patients and healthy controls.

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297 **RESULTS**

298 **Clinical characteristics**

299 Table 2 shows all clinical and functional variables of controls and COPD patients recruited in
300 the study. The number of male COPD patients and healthy controls were greater than that of
301 females (Table 2). Age did not significantly differ among the study subjects. Body
302 composition as measured by body mass index (BMI) and fat-free mass index (FFMI) was
303 significantly reduced in cachectic COPD patients compared to non-cachectic and the controls
304 (Table 2). The number of packs-year was similar in the three study groups (Table 2). Both
305 groups of patients exhibited severe airflow limitation, functional signs of emphysema,
306 moderate hypoxemia, and reduced both exercise capacity and quadriceps strength compared
307 to healthy controls (Table 2). Moreover, in cachectic patients, QMVC was lower than in non-
308 cachectic patients (Table 2). Levels of C-reactive protein (CRP), fibrinogen, and globular
309 sedimentation velocity (GSV) were higher in the two groups of COPD patients than in
310 healthy controls (Table 2). Furthermore, CRP and fibrinogen levels were greater in cachectic
311 than in non-cachectic patients (Table 2).

312 **MicroRNA expression in muscle specimens and myotubes of patients and controls**

313 *In vivo studies*. Compared to healthy controls, levels of *miR-1, miR-206, miR-486, and miR-*
314 *29b* were significantly upregulated in the VL specimens of non-cachectic COPD patients
315 (Figures 2A-2C, and 3A), while no differences were seen in the expression of *miR-133a, miR-*
316 *27a,* or *miR-181a* between these two groups (Figures 3B-3D). Interestingly, expression levels
317 of *miR-1, miR-206, miR-486, miR-29b, miR-133a, miR-27a*, and *miR-181a* were significantly
318 lower in cachectic COPD patients than in non-cachectic patients (Figures 2A-2C and 3A-3D,
319 respectively).

320 *In vitro studies*. Compared to in vivo condition, expression levels of *miR-1, miR-486*, and
321 *miR-133a*, were significantly reduced in the myotubes of healthy controls and in both groups

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322 of COPD patients (Figures 2A, 2C, and 3B, respectively). Moreover, in non-cachectic
323 patients, but not in cachectic ones, expression levels of *miR-206, miR-29b, miR-27a,* and *miR-*
324 *181a* were also significantly lower in the myotubes than in the corresponding in vivo muscle
325 specimens (Figures 2B, 3A, 3C, and 3D, respectively).

326 **Changes in microRNA expression between in vivo and in vitro states in the study groups**

327 Delta changes of expression between myotubes and in vivo specimens of *miR-1* and *miR-29b*
328 were significantly greater in non-cachectic COPD patients than in the controls (Figure 4).
329 Conversely, delta changes of expression between myotubes and muscle specimens of *miR-1,*
330 *miR-486, miR-29b,* and *miR-133a* were significantly lower in cachectic patients than in non-
331 cachectic COPD, while that of *miR-181a* was significantly higher in the former than in the
332 latter group (Figure 4).

333 **Profile of expression of all microRNAs in the muscle specimens and myotubes within**
334 **each study group**

335 *In vivo studies*. The pattern of expression of the microRNAs *miR-1, miR-206, miR-486, miR-*
336 *29b, miR-133a, miR-27a,* and *miR-181a* did not significantly differ in the muscle specimens
337 of each study group (control subjects, non-cachectic and cachectic COPD patients) as shown
338 in Figure 5A.

339 *In vitro studies*. In the myotubes of the control subjects, the pattern of expression of *miR-1,*
340 *miR-486,* and *miR-133a* were comparable, whereas that of *miR-206, miR-29b,* and *miR-27a*
341 followed a different pattern (Figure 5B). Additionally, in the same individuals, *miR-29b, miR-*
342 *27a* and *miR-181a* were also similarly expressed (Figure 5B). In the myotubes of non-
343 cachectic COPD patients, the expression of *miR-1, miR-486*, and *miR-133a* were comparable
344 (Figure 5B). On the other hand, the expression profile of *miR-206, miR-29b,* and *miR-27a* was
345 also comparable between them (Figure 5B). In the same patients, *miR-181a* expression
346 differed from that of the other microRNAs (Figure 5B). In the myotubes of the cachectic

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347 patients, the expression of *miR-1* and *miR-486* was similar (Figure 5B). Interestingly, in the
348 same patients, the expression of *miR-206, miR-29b, miR-27a* were similar, while that of *miR-*
349 *133a* was different (Figure 5B). Finally, the expression of *miR-29b* and *miR-181a* followed a
350 similar pattern in cachectic COPD patients (Figure 5B).
351

352 **DISCUSSION**

353 In the current investigation, the hypothesis has been confirmed to a great extent. Expression
354 profile of muscle-enriched microRNAs differed between in vivo (muscle specimens) and in
355 vitro primary cultures (differentiated myotubes) of COPD patients with and without cachexia
356 as well as of the healthy controls. Moreover, expression levels of muscle-specific microRNAs
357 showed a differential pattern in the muscle specimens of the COPD patients compared to
358 those of the healthy controls, while no significant differences were seen among the study
359 groups under in vitro conditions. These are relevant findings that may question the validity of
360 the use of in vitro models of primary cultured cells to study the expression of microRNAs
361 known to regulate muscle proliferation and differentiation during myogenesis.

362 In the study, patients with COPD exhibited a severe airway obstruction and functional
363 signs of emphysema as demonstrated by the reduced diffusion capacity observed in both
364 groups of patients. As expected, patients with cachexia exhibited a significant decrease in
365 levels of the parameters BMI and FFMI compared to both non-cachectic COPD patients and
366 the healthy controls. Moreover, all patients and particularly those with cachexia exhibited
367 signs of systemic manifestations such as increased levels of CRP, fibrinogen, and GSV, while
368 nutritional abnormalities were not seen in any of the groups of patients. Exercise capacity and
369 quadriceps muscle strength were also significantly lower in both groups of patients, especially
370 in the cachectic group than in control subjects. Smoking history was also similar in both
371 groups of patients compared to the controls. Taken together, these results reveal that patients

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372 were carefully recruited as the inclusion and exclusion criteria were strictly followed for the
373 specific objectives of the investigation and that sufficient differences were observed between
374 patients and the healthy controls.

375 In the current investigation, the implications of environmental factors directly related
376 to the muscle cell niche should be considered. Lung function impairment, hypoxia,
377 hypercapnia, acidosis, smoking status, systemic inflammation, and deconditioning are
378 probably counted among the most relevant factors that may influence gene and protein
379 expression of different biological mechanisms within the myofibers (in vivo condition) of
380 COPD patients (5; 6; 18). In the study, lung function parameters including blood gases
381 analyses, physical activity levels, and smoking history were similar between the two study
382 groups of patients, with and without cachexia. Nonetheless, cachectic COPD patients
383 exhibited clinical signs of a systemic disease (increased levels of CRP and fibrinogen) and
384 their quadriceps strength was significantly lower than in patients with normal body
385 composition. These are relevant findings that suggest that systemic inflammation is likely to
386 play a significant role in the cachectic and muscle wasting process experienced by those
387 patients, while it may also be a major driver of the differences encountered in microRNA
388 expression levels in their muscles under in vivo conditions. Interestingly, such differences
389 were not detected in the myotubes between the two study groups of patients or the controls.
390 Taken together, these results have potential clinical implications as systemic inflammation
391 should be considered as a relevant target in the prevention and treatment of body and muscle
392 weight loss in COPD. Promotion of increased physical activity and/or exercise training may
393 favor the decrease in systemic inflammation and muscle mass maintenance (18; 21; 39).

394 In addition to the classical transcription factors, muscle-enriched microRNAs also
395 regulate muscle mass maintenance and development in vivo and in vitro conditions (4; 9; 10;
396 25; 27-30). Several reports have demonstrated a differential expression profile of muscle-

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397 specific microRNAs in the vastus lateralis of patients with COPD compared to healthy
398 subjects (20; 28-30). Furthermore, in patients with severe airway obstruction and cachexia,
399 the differential profile of expression of muscle-enriched microRNAs was even more blatant
400 compared to healthy controls and patients with normal body composition (30). The
401 conclusions from that study were that epigenetic events (microRNAs and histone acetylation
402 profile) rather regulate muscle differentiation than proliferation aside from muscle growth and
403 atrophy signaling, probably as protective mechanisms to prevent additional muscle wasting in
404 cachectic COPD patients (30). In that investigation, expression levels of miR-1 and miR-206
405 were significantly greater in the lower limb muscles of patients with COPD than in the
406 control subjects (30). In the current study, compared to healthy controls, expression levels of
407 miR-1, miR-206, miR-486, and miR-29b were significantly upregulated in the VL specimens
408 of COPD patients with preserved body composition. However, in muscle specimens of non-
409 cachectic patients, expression levels of the same microRNAs along with those of miR-133,
410 miR-27a, and miR-181a were significantly lower than in muscles of the cachectic COPD
411 patients. These findings are somehow in agreement with those previously shown in the VL of
412 COPD patients with preserved body composition (20). Nevertheless, they are partly counter
413 to those previously reported in our group (30). The fact that body composition alterations
414 were more severe in patients in the current study (BMI was notably lower in the cachectic
415 COPD patients) might account for certain discrepancies encountered in the expression levels
416 of several microRNAs between this study and the previous report (30).

417 Importantly, expression levels of most of the study microRNAs were significantly
418 lower in the myotubes (in vitro conditions) than in the muscle specimens (in vivo model) in
419 each of the study groups, and particularly in the COPD patients with preserved body
420 composition. In the myotubes obtained from the cachectic COPD patients and the healthy
421 controls, expression levels of miR-1, miR-486, and miR-133a were also significantly lower

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422 than in the muscle specimens of the same study groups. Interestingly, no significant
423 differences among the three study groups were seen in the expression levels of the analyzed
424 microRNAs. These findings were also noticeable when expression levels of all the target
425 microRNAs were analyzed individually in each study group of subjects. The profile of
426 microRNA expression within the myotubes was similar in all three groups, whereas
427 significant differences were detected between patients and healthy controls in the muscle
428 specimens (Figure 5). These findings imply that cultured muscle cells express microRNAs in
429 a comparable fashion regardless of the underlying condition, whether obtained from patients
430 or healthy controls. These results also suggest that muscle progenitor cells behave similarly,
431 at least for muscle-enriched microRNA expression, in both patients and control subjects,
432 while this was not the case in the in vivo model. Taken together, these findings imply that the
433 muscle niche (systemic inflammation, blood flow, muscle metabolism, deconditioning, etc.)
434 in the cachectic COPD patients probably accounts for the differences in the microRNA
435 expression levels detected in these patients (9; 25).

436 Furthermore, when delta changes of the target microRNAs between in vitro and in
437 vivo conditions were analyzed for each of the study groups, a significant reduction was
438 observed in the cachectic COPD patients compared to non-cachectic patients for miR-1, miR-
439 1486, miR-29b, and miR-133a. These results suggest the differences in the expression of
440 these microRNAs between in vivo and in vitro conditions were significantly smaller in the
441 cachectic COPD patients, probably as a result of the lower expression levels detected in the
442 muscle specimens in this group of patients, while the largest differences between the two
443 conditions were seen in the non-cachectic patients.

444 **Conclusions**

445 We conclude from these findings that environmental factors taking place in vivo (biopsies)
446 may account for the differences observed in microRNA expression between COPD patients

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447 and controls. In the myotubes, however, the expression of the same microRNAs did not differ
448 between the study subjects as such environmental factors were not present. These findings
449 suggest that therapeutic strategies aimed to favor/promote muscle maintenance should rather
450 target environmental factors in COPD muscle weakness and cachexia as the profile of
451 microRNA expression in myotubes is similar in patients to that observed in the healthy
452 controls.
453

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461

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463 Planning of the experiments carried out in the investigation: Esther Barreiro, Ester Puig-
464 Vilanova; Acquisition of the reagents and materials required for the molecular biology
465 experiments: Ester Puig-Vilanova, Esther Barreiro; Full assessment of patients and healthy
466 controls, recruitment of the candidate patients and controls according to the inclusion and
467 exclusion criteria established in the investigation, and muscle sample collection from the
468 study subjects: Antonio Sancho-Muñoz, Sergio Pascual-Guardia, Joaquim Gea; Molecular
469 biology analyses: Ester Puig-Vilanova, Anna Salazar-Degracia, Esther Barreiro; Statistical
470 analyses, data interpretation and presentation: Ester Puig-Vilanova, Anna Salazar-Degracia,
471 Esther Barreiro, Joaquim Gea; Preparation of the manuscript figures and tables: Ester Puig-
472 Vilanova, Anna Salazar-Degracia, Esther Barreiro; Manuscript drafting and intellectual input:
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FIGURE LEGENDS

Figure 1: Representative phase-contrast images of myotubes after 5 days of myogenic induction of a muscle-wasted severe COPD patients. Black arrows indicate multinucleated myotubes. Scale bar: 50 micrometers (x10, left panel) and 30 micrometers (x20, right panel).

Figure 2

A) Mean values and standard deviation (relative expression) of *miR-1* expression in vastus lateralis (VL) specimens (in vivo) and myotubes of both non-cachectic and cachectic COPD patients and healthy controls. Definition of abbreviations: *miR*, microRNA; COPD, chronic obstructive pulmonary disease. Statistical significance is represented as follows: *:p<0.05 for comparisons between any of the two groups of COPD patients and control subjects; ###:p<0.001 for comparisons between non-cachectic and cachectic COPD patients; §:p<0.05, §§:p<0.01, and §§§:p<0.001 for comparisons between myotubes and the in vivo measurements in each study group of subjects. For the sake of clarity, the absence of statistical symbols indicates that no significant differences were encountered between groups for the different study comparisons.

B) Mean values and standard deviation (relative expression) of *miR-206* expression in VL specimens (in vivo) and myotubes of both non-cachectic and cachectic COPD patients and healthy controls. Definition of abbreviations: *miR*, microRNA; COPD, chronic obstructive pulmonary disease. Statistical significance is represented as follows: *:p<0.05 for comparisons between any of the two groups of COPD patients and control subjects; ##:p<0.01 for comparisons between non-cachectic and cachectic COPD patients; §:p<0.05 for comparisons between myotubes and the in vivo measurements in each study group of subjects. For the sake of clarity, the absence of

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652 statistical symbols indicates that no significant differences were encountered between
653 groups for the different study comparisons.

654 C) Mean values and standard deviation (relative expression) of *miR-486* expression in VL
655 specimens (in vivo) and myotubes of both non-cachectic and cachectic COPD patients
656 and healthy controls. Definition of abbreviations: *miR*, microRNA; COPD, chronic
657 obstructive pulmonary disease. Statistical significance is represented as follows:
658 *:p<0.05 for comparisons between any of the two groups of COPD patients and
659 control subjects; ###:p<0.001 for comparisons between non-cachectic and cachectic
660 COPD patients; §§:p<0.01, and §§§:p<0.001 for comparisons between myotubes and
661 the in vivo measurements in each study group of subjects. For the sake of clarity, the
662 absence of statistical symbols indicates that no significant differences were
663 encountered between groups for the different study comparisons.

664 **Figure 3**

665 A) Mean values and standard deviation (relative expression) of *miR-29b* expression in VL
666 specimens (in vivo) and myotubes of both non-cachectic and cachectic COPD patients
667 and healthy controls. Definition of abbreviations: *miR*, microRNA; COPD, chronic
668 obstructive pulmonary disease. Statistical significance is represented as follows:
669 *:p<0.05 for comparisons between any of the two groups of COPD patients and
670 control subjects; ##:p<0.01 for comparisons between non-cachectic and cachectic
671 COPD patients; §§§:p<0.001 for comparisons between myotubes and the in vivo
672 measurements in each study group of subjects. For the sake of clarity, the absence of
673 statistical symbols indicates that no significant differences were encountered between
674 groups for the different study comparisons.

675 B) Mean values and standard deviation (relative expression) of *miR-133* expression in VL
676 specimens (in vivo) and myotubes of both non-cachectic and cachectic COPD patients

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677 and healthy controls. Definition of abbreviations: *miR*, microRNA; COPD, chronic
678 obstructive pulmonary disease. Statistical significance is represented as follows:
679 ###:p<0.001 for comparisons between non-cachectic and cachectic COPD patients;
680 §:p<0.05, §§:p<0.01, and §§§:p<0.001 for comparisons between myotubes and the in
681 vivo measurements in each study group of subjects. For the sake of clarity, the
682 absence of statistical symbols indicates that no significant differences were
683 encountered between groups for the different study comparisons.

684 C) Mean values and standard deviation (relative expression) of *miR-27a* expression in VL
685 specimens (in vivo) and myotubes of both non-cachectic and cachectic COPD patients
686 and healthy controls. Definition of abbreviations: *miR*, microRNA; COPD, chronic
687 obstructive pulmonary disease. Statistical significance is represented as follows:
688 #:p<0.05 for comparisons between non-cachectic and cachectic COPD patients;
689 §:p<0.05 for comparisons between myotubes and the in vivo measurements in each
690 study group of subjects. For the sake of clarity, the absence of statistical symbols
691 indicates that no significant differences were encountered between groups for the
692 different study comparisons.

693 D) Mean values and standard deviation (relative expression) of *miR-181a* expression in
694 VL specimens (in vivo) and myotubes of both non-cachectic and cachectic COPD
695 patients and healthy controls. Definition of abbreviations: miR, microRNA; COPD,
696 chronic obstructive pulmonary disease. Statistical significance is represented as
697 follows: ###:p<0.001 for comparisons between non-cachectic and cachectic COPD
698 patients; §§:p<0.01 for comparisons between myotubes and the in vivo measurements
699 in each study group of subjects. For the sake of clarity, the absence of statistical
700 symbols indicates that no significant differences were encountered between groups for
701 the different study comparisons.

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702 **Figure 4:** miRNAs delta expression between myotubes versus their respective in vivo
703 measurements (VL specimens) in both non-cachectic and cachectic COPD patients and
704 healthy controls. Definition of abbreviations: miRNA, microRNA; miR, microRNA; COPD,
705 chronic obstructive pulmonary disease. Statistical significance is represented as follows:
706 *:p<0.05 between any of the two groups of COPD patients and control subjects; #:p<0.05,
707 ##:p<0.01, and ###:p<0.001 between non-cachectic and cachectic COPD patients. For the
708 sake of clarity, the absence of statistical symbols indicates that no significant differences were
709 encountered between groups for the different study comparisons.

710 **Figure 5**

711 A) Mean values and standard deviation (relative expression) of each studied miRNA in
712 VL muscle specimens of both non-cachectic and cachectic COPD patients and healthy
713 controls. Definition of abbreviations: miRNAs, microRNAs; miR, microRNA; COPD,
714 chronic obstructive pulmonary disease. Statistical analyses were performed separately
715 for each study group of subjects. In each study group, the expression levels of the
716 study microRNAs do not differ among them if they share the same letter. Different
717 letters reflect different levels of expression for each study group.

718 B) Mean values and standard deviation (relative expression) of each studied miRNA in
719 myotubes of both non-cachectic and cachectic COPD patients and healthy controls.
720 Definition of abbreviations: miRNAs, microRNAs; miR, microRNA; COPD, chronic
721 obstructive pulmonary disease. Statistical analyses were performed separately for each
722 study group of subjects. In each study group, the expression levels of the study
723 microRNAs do not differ among them if they share the same letter. Different letters
724 reflect different levels of expression for each study group.

725

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726 **Table 1. MicroRNA assays of quantitative RT-PCR analyses of the study genes in the vastus lateralis**
727 **specimens and myotubes**
728

Assay Name	Assay ID	miRBase accession number
Muscle-specific, myomiRs		
hsa-miR-1	002222	MIMAT0000416
hsa-miR-206	000510	MIMAT0000462
hsa-miR-133a	002246	MIMAT0000427
Other miRNAs (highly expressed in muscles)		
hsa-miR-486	001278	MIMAT0002177
hsa-miR-29b	000413	MIMAT0000100
hsa-miR-27a	000408	MIMAT0000084
hsa-miR-181a	000480	MIMAT0000256
		NCBI Accession number
U6 snRNA, housekeeping gene	001973	NR_004394

729
730
731 *Abbreviations*: ID, identification; hsa, homo sapiens; miR, microRNA; MIMAT, mature microRNA;
732 snRNA, small nuclear RNA; and NR, non-coding RNA RefSeq database category.
733
734

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735 **Table 2. Main clinical characteristics and functional variables of the study subjects**
736

	Controls	Severe COPD	
		Non-cachectic	Cachectic
Subjects	N = 10	N = 14	N = 15
Female/male	4/6	3/11	2/13
Anthropometry			
Age (years)	62 (6)	67 (10)	65 (9)
BMI (kg/m^2)	27 (2)	26 (3)	18 (2) ***, §§§
FFMI (kg/m^2)	19 (2)	18 (2)	15 (1) ***, §§§
Smoking History			
Active (N, %)	0, 0	6, 43 ***	6, 40 ***
Ex-smoker (N, %)	5, 50	8, 57 ***	9, 60 ***
Never smoker (N, %)	5, 50	0, 0 ***	0, 0 ***
Packs-year	62 (16)	69 (18)	66 (31)
Lung function			
FEV_1 (% pred)	94 (9)	35 (11) ***	31 (12) ***
FVC (% pred)	92 (8)	54 (16) ***	58 (18) ***
FEV_1/FVC (%)	75 (3)	45 (10) ***	40 (11) ***
RV (% pred)	115 (18)	195 (53) **	210 (69) ***
TLC (% pred)	104 (10)	115 (20)	119 (17)
RV/TLC (%)	43 (3)	66 (9) ***	69 (11) ***
DLco (% pred)	102 (16)	49 (21) ***	42 (18) ***
K_{CO} (% pred)	94 (14)	58 (21) ***	57 (21) ***
PaO_2 (mmHg)	85 (2.7)	68 (9.5) ***	65 (8.8) ***
$PaCO_2$ (mmHg)	42 (4.2)	46 (4.5)	45 (5.3)
Exercise capacity & muscle force			
QMVC (kg)	44 (11)	32 (8) *	23 (11) ***,§
Six-minute walking distance (m)	526 (47)	408 (111) *	380 (113) **
Blood parameters			
Albumin (g/dL)	4.5 (0.4)	4.2 (0.4)	4.6 (0.9)
Total proteins (g/dL)	6.3 (1.5)	6.9 (0.7)	6.7 (1.2)
CRP (mg/dL)	0.9 (0.6)	1.7 (0.5) *	6.0 (5.6) **,§§
Fibrinogen (mg/dL)	384 (108)	416 (90) *	535 (127) *,§
GSV (mm/h)	15 (7)	29 (13) *	28 (13) *

737
738
739 Values are expressed as mean (standard deviation).
740
741 *Abbreviations*: COPD, chronic obstructive pulmonary disease; N, number of patients; kg, kilograms; m, meters; BMI,
742 body mass index; FFMI, fat-free mass index; FEV_1, forced expiratory volume in one second; pred, predicted; FVC,
743 forced vital capacity; RV, residual volume; TLC, total lung capacity; DLco, carbon monoxide transfer; K_{CO}, *Krogh*
744 transfer factor for diffusion capacity; PaO_2, arterial oxygen partial pressure; mmHg, millimeter of mercury; $PaCO_2$,
745 arterial carbon dioxide partial pressure; QMVC, quadriceps maximal velocity contraction; g, grams; dL, deciliter;
746 CRP, C-reactive protein; mg, milligrams; GSV, globular sedimentation velocity; mm, millimeters; h, hour.
747
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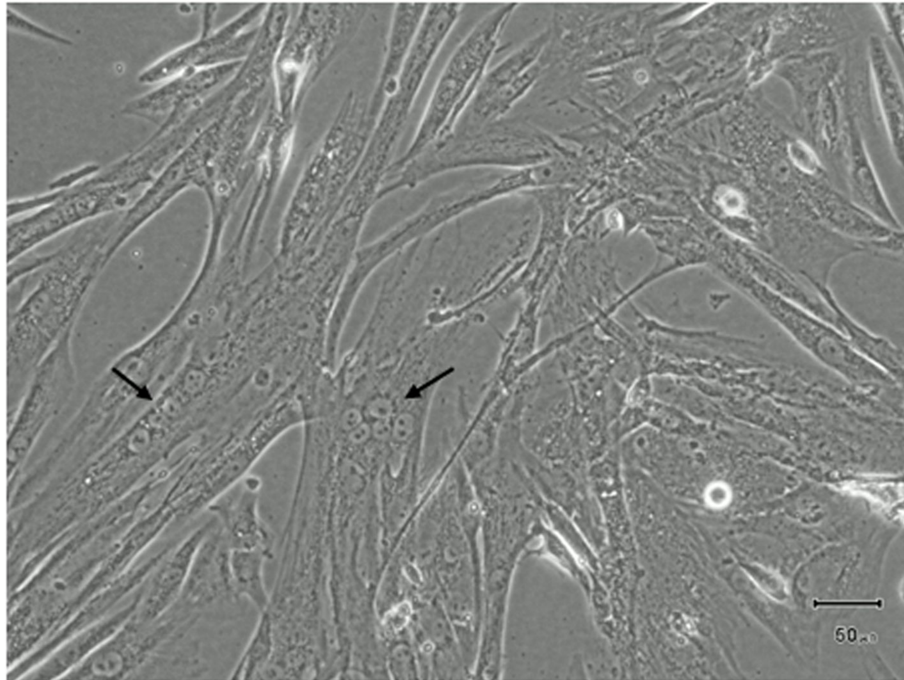
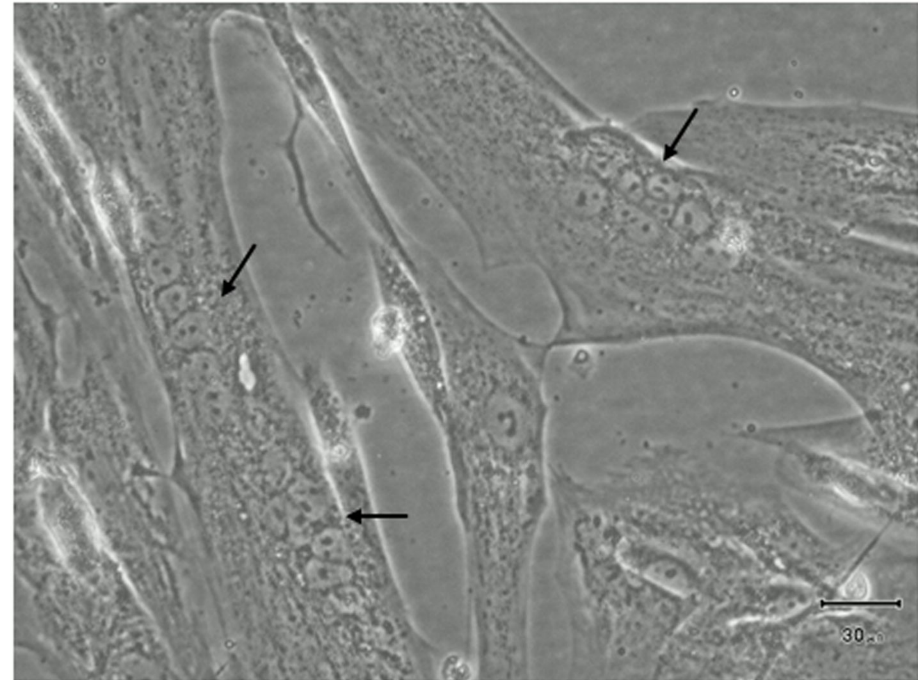
Barreiro et al. Figure 1

Myotubes





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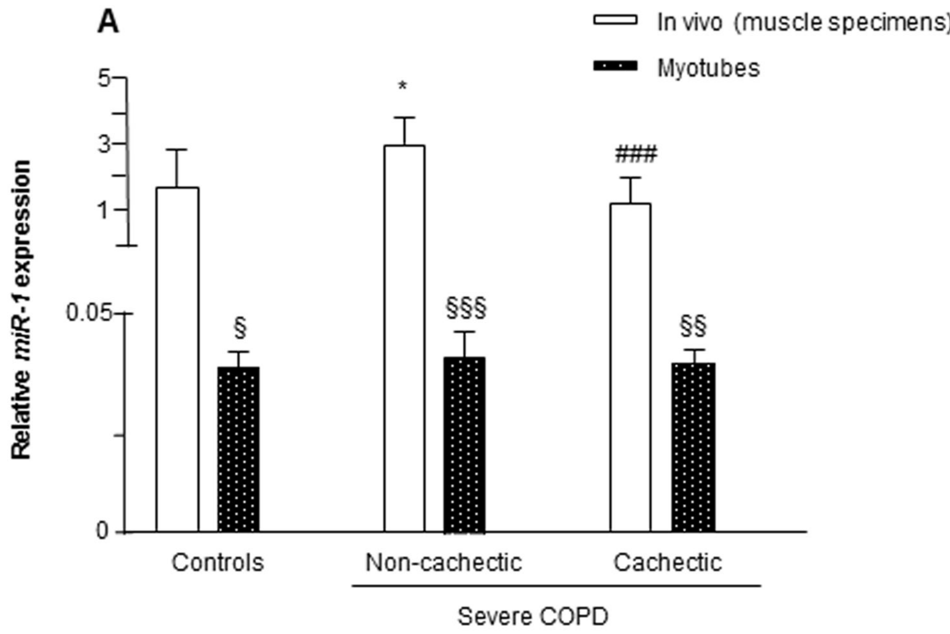
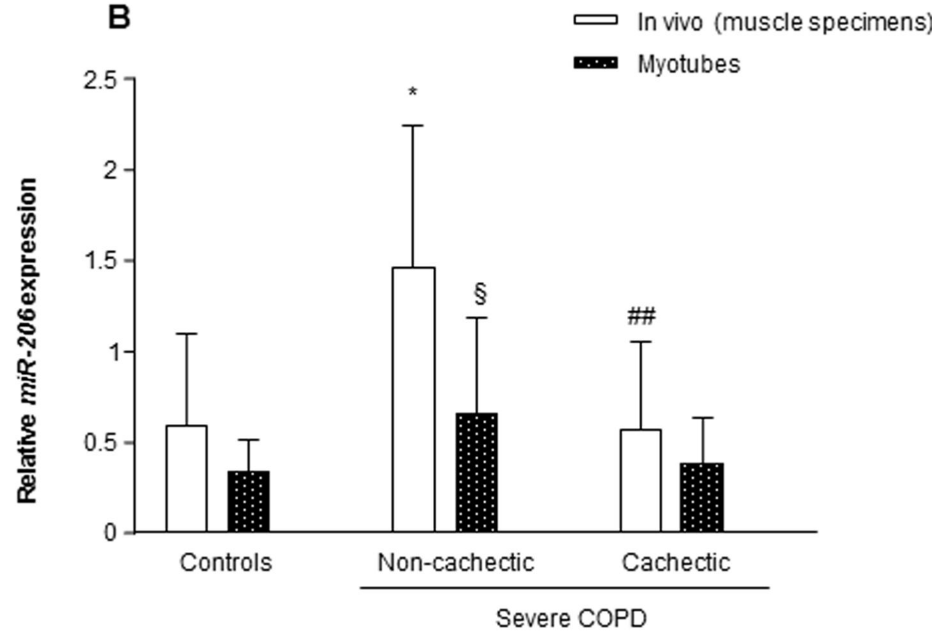
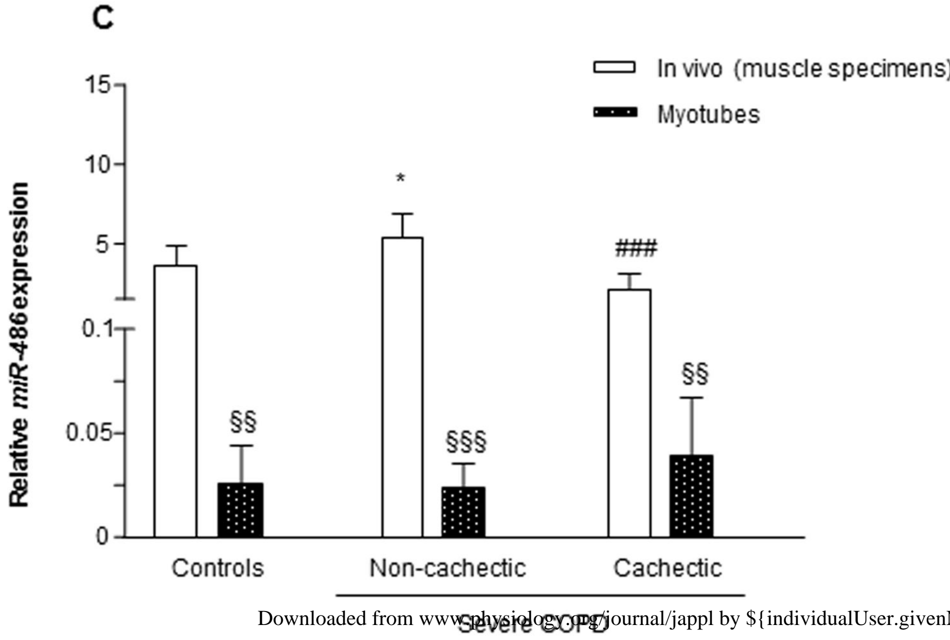
Barreiro et al. Figure 2







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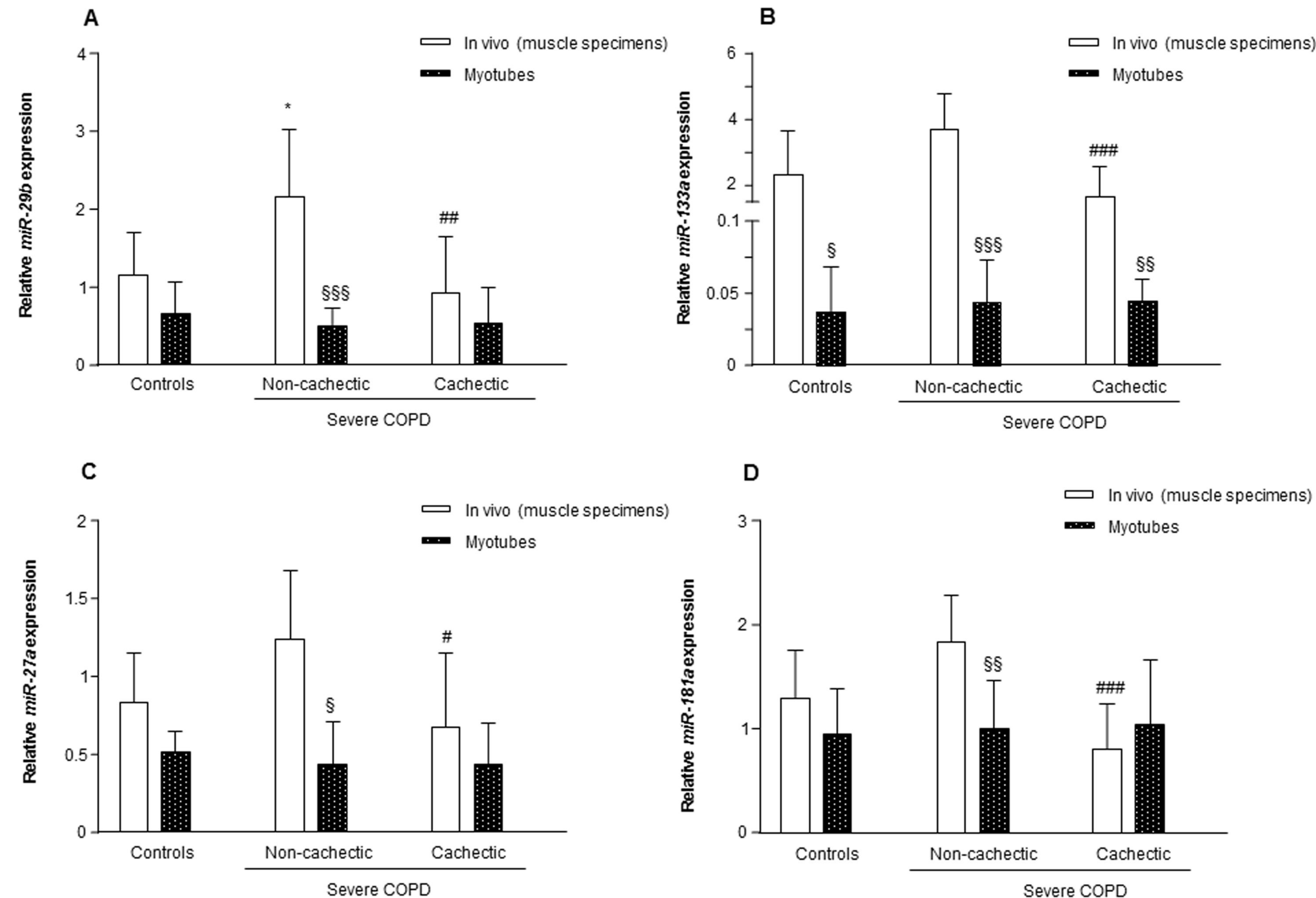
Barreiro et al. Figure 3



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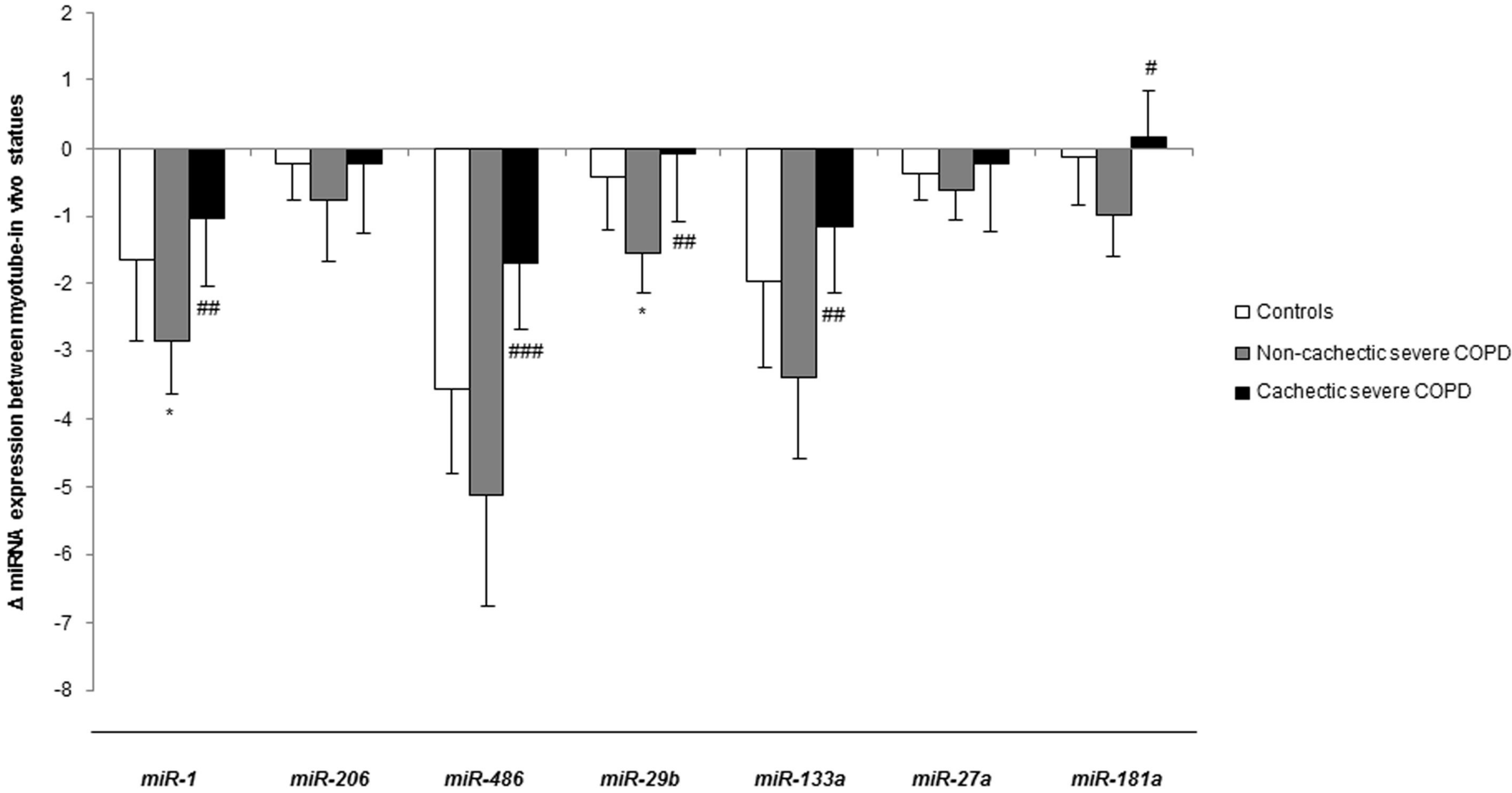
Barreiro et al. Figure 4



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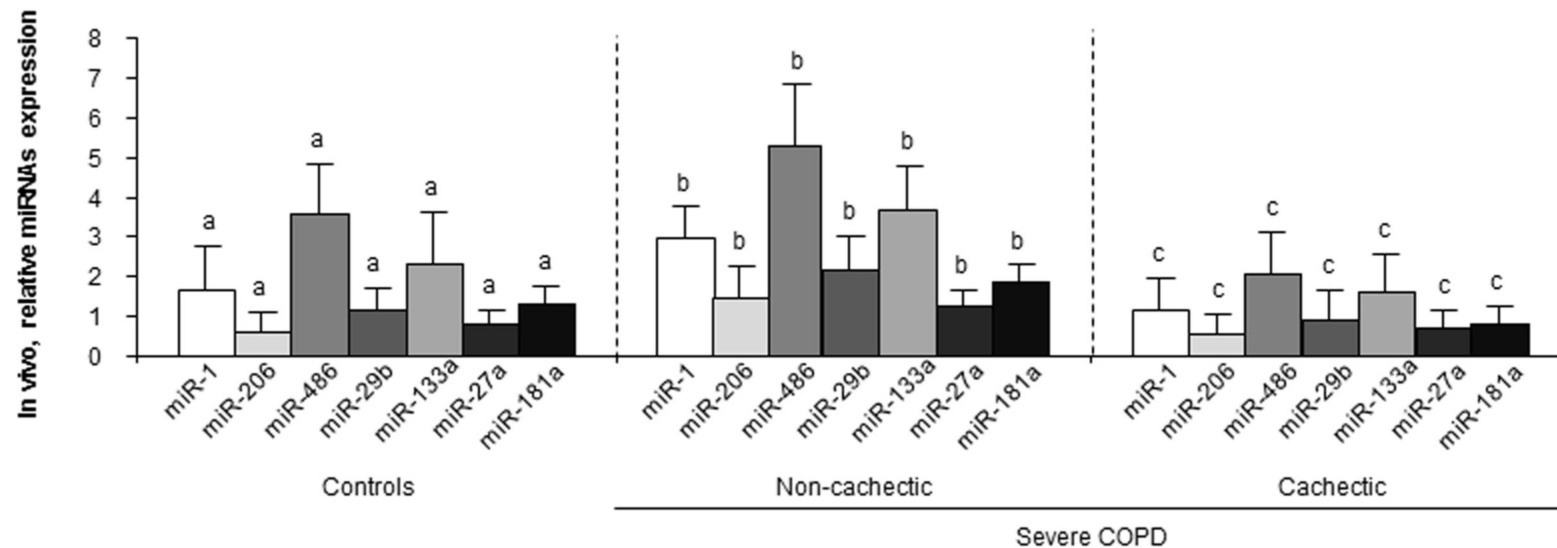
Barreiro et al. Figure 5



B

Myotubes, relative miRNAs expression

0
0.1
0.1
0.5
1
1.5
2

a b a b,c a b,c c

a b a b a b c

a b a b,c d b c

miR-1 miR-206 miR-486 miR-29b miR-133a miR-27a miR-181a

Controls

Non-cachectic

Cachectic

Severe COPD

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