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(Article begins on next page)

1 **Title:**

2 **Correlation between estrogen plasma level and miRNAs in muscle of Piedmontese cattle**

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13 Declaration of interest: none.

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17

18 **Abstract**

19 A loss-of-function mutation of the myostatin gene has a very high prevalence in the Piedmontese
20 cattle breed. The effect of such mutation is a double-muscle phenotype due to hypertrophy.
21 However, differences in muscle mass development can still be detected in individuals of this breed.
22 Such differences must be generated by other factors controlling skeletal muscle development.
23 MicroRNAs are short non-coding RNA molecules that modulate gene expression at a post-
24 transcriptional level. MicroRNAs have been demonstrated to be involved in skeletal muscle
25 development and some of them are controlled by steroid hormone signaling. Data on estrogen
26 signaling are lacking, while more studies have been carried out on the effect of androgens. We
27 aimed at identifying putative estrogen responsive miRNAs that might be involved in skeletal
28 muscle development. At a slaughterhouse, we collected both muscle samples from longissimus
29 dorsi and blood samples. Blood 17 β -estradiol concentration was assessed and RNA was extracted
30 from muscle samples. The animals we sampled were divided in groups according to estrogen blood
31 concentration and through qPCR expression levels of seven muscle related miRNAs were
32 evaluated. We found that miR-26b (p<0.01), miR-27a-5p (p<0.05), miR-27b (p<0.05) and miR-
33 199a-3p (p<0.01) were differentially expressed in experimental groups. Expression levels of miR-
34 26b were approximately 2-fold lower in samples with a low blood estrogen concentration and the
35 other miRNAs showed a tendency to increase their expression levels when blood estrogens were
36 higher. The variations of the circulating concentrations of estrogens in Piedmontese cattle might
37 influence muscle mass development through miRNAs and thus contribute to individual variability
38 in a breed with a high prevalence of a myostatin point mutation.

39 **Keywords:** 17 β -estradiol, cattle, microRNA, skeletal muscle

1. Introduction

MicroRNAs are a class of small (22 nt), noncoding RNA molecules which can regulate gene expression. MiRNAs can repress translation of mRNA by recognizing and pairing to a seed sequence at the 3'-UTR, but they can also promote complete degradation of target mRNA in case of perfect complementarity (1).

miRNAs modulate gene expression at a post-transcriptional level in many biological processes. They include, but are not limited to, cell cycle progression (2), apoptosis (3), stem cell maintenance and differentiation (4-6).

Many miRNAs play an important role in skeletal myogenesis and muscle regeneration. miR-1, miR-206 and miR-133 have been shown to have opposite effect on myoblasts: while the former two promote differentiation of satellite cells, high expression of miR-133 results in myoblast proliferation rather than differentiation (7,8).

The Piedmontese breed of the north-western part of Italy shows a muscular hypertrophy phenotype which has been attributed to a point mutation in the myostatin gene (9). This mutation has a high incidence in the Piedmontese bovine population and induces both muscular hyperplasia and hypertrophy. However phenotypic variations are still evident. Therefore, other factors must play an important role in determining muscle mass in adult individuals.

When comparing miR-206 expression in Piedmontese and Friesian cattle irrespective of sex, our group found no difference between the two breeds. However, when comparing just female Piedmontese and Friesian cattle, a statistically significant difference was found (10). This evidence points to a possible influence of sex steroids on muscle mass build up, possibly mediated by microRNAs.

The role of gonadal steroids in muscle development has been investigated in depth. Androgens are known to induce muscle hypertrophy (11). A recent study demonstrated that there is a correlation

64 between androgen stimulation and myostatin expression in satellite cells (12). However, when
65 considering female individuals there is considerably less data on the effect of estrogen and
66 progesterone on skeletal muscle growth. In post-menopausal women hormone replacement therapy
67 (HRT) has a positive effect on skeletal muscle function, as shown by maintenance or increase of
68 muscle size and performance during exercises (13). A partial explanation for this effect is that
69 estrogen might act as a proliferative stimulus for myoblasts through activation of estrogen receptor
70 α (ER- α) (14). Similarly in steers, supplementation of estrogen and trenbolone resulted in increased
71 activation of muscle satellite cells which strengthen the concept that estrogens might directly
72 influence skeletal muscle growth (14,15).

73 However, the mediators that are involved in this signaling pathways in skeletal muscle cells are not
74 well known. We chose the Piedmontese breed as our model because of the high prevalence of the
75 myostatin mutation to investigate whether estrogens, specifically 17 β -estradiol, can modulate
76 miRNAs involved in muscle differentiation and thus explaining phenotypic variations in this breed.
77

2. Materials & Methods

2.1 Animals

24 Piedmontese cows were used for this study. The age of the selected animals ranged from 4 to 13 years and were from farms belonging to Consorzio La Granda (Cuneo, Italy) located in the Piedmont region (north-western part of Italy).

The animals were slaughtered and processed for human consumption according to Italian regulations. At the time of slaughtering, blood samples and muscle samples from *Longissimus Dorsi* were taken. Muscle samples were stored in RNAlater solution (Ambion, ThermoFisher Scientific Inc., Waltham, MA, USA) for transport at the laboratory. Muscle samples were then minced, placed in fresh RNAlater, incubated overnight at 4°C and then stored at -80°C.

2.2 Health assessment of recruited animals

During slaughtering, uterus and ovaries were collected for each animal and they underwent a macroscopic post-mortem examination carried out by a veterinary clinician to assess the presence of developing follicles and their size, the presence of corpora lutea and the presence of macroscopic abnormalities (like cysts).

Only animals with no signs of aberrations and with signs of a regular reproductive activity were included in this study, that is where corpora lutea or developing follicles of normal appearance were detected.

2.3 RNA extraction and RT-qPCR

RNA extraction and reverse transcription was performed as previously described (16). Briefly, 50 to 100 mg of muscle tissue was homogenized with a TissueLyser II (QIAgen, Venlo, Netherlands) in 1 ml TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), total RNA was purified and quantified by

spectrophotometry. Residual DNA was removed with DNase I Recombinant RNase-free kit (Roche, Mannheim, Germany) as per manufacturer instruction. For estrogen receptor (ER) - α and - β analysis 0.5 μ g RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). For miRNA analysis, 100 ng of RNA was reverse transcribed with TaqMan microRNA reverse transcription kit (ThermoFisher Scientific Inc.) using miRNA specific primers from TaqMan microRNA assays (ThermoFisher Scientific Inc.).

Quantitative PCR was subsequently performed using SsoAdvanced Universal SYBR green Supermix (Bio-Rad) for ER- α and β and TaqMan Universal PCR Master Mix for miRNA analysis on a CFX Connect instrument (Bio-Rad).

Primers for ER α were: 5'-AGGGAAGCTCCTATTTGCTCC-3' (forward) and 5'-CGGTGGATGTGGTCCTTCTCT-3' (reverse), based on NCBI sequence NM_001001443.1.

Primers for ER β were: 5'-GCTTCGTGGAGCTCAGCCTG-3' (forward) and 5'-AGGATCATGGCCTTGACACAGA-3' (reverse), based on NCBI sequence NM_174051.3

Primer efficiency was calculated with CFX Manager (Version 3.0, Bio-Rad) using the linear regression slope of a sample dilution series. For ER α efficiency was 94.5%, for ER β efficiency was 95.8%.

For mature miRNA detection and quantification, primers and probes were from the respective kits of TaqMan MicroRNA assays (ThermoFisher Scientific Inc.).

Detected mature miRNA sequences were the following: miR-1 – UGGAAUGUAAAGAAGUAUGUAU; miR-26a – UUCAAGUAAUCCAGGAUAGGCU; miR-26b – UUCAAGUAAUUCAGGAUAGGUU; miR-27a-5p – AGGGCUUAGCUGCUUGUGAGCA; miR-27b – UUCACAGUGGCUAAGUUCUGC; miR-206 – UGGAAUGUAAGGAAGUGUGUGG; miR-199a-3p – ACAGUAGUCUGCACAUUGGUUA; miR-16 – UAGCAGCACGUAAAUAUUGGCG. The analyzed sequences are bovine specific as reported in miRCarta v1.1 database but with a high homology with the corresponding human

128 miRNAs (sequence homology is 100% for analyzed miR-1, miR-26a, miR-27a-5p, miR-27b, miR-
129 206 and miR-199a-3p. Sequence homology is 95.5% for miR-26b and miR-16).
130 Expression of ER- α and β was normalized using Hypoxanthine Phosphoribosyl-transferase (HPRT),
131 while microRNA expression was normalized using miR-16 as housekeeping gene.
132 Relative quantification was done using the $2^{-\Delta\Delta C_q}$ method (17) and results were expressed as fold
133 changes compared to the low estrogen group.

134

135 *2.4 Quantification of 17 β -estradiol*

136 Serum was recovered from blood samples taken at slaughtering and then frozen at -80°C. Serum
137 samples were then shipped to Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia
138 Romagna (IZSLER, Brescia, Italy) where 17 β -estradiol concentration was assessed through RIA.

139

140 *2.5 Statistical analysis*

141 Age of the animals (months) in the three experimental groups was compared using a Kruskal-
142 Wallis non parametric test.

143 Data from Q-PCR were obtained as quantification cycle values (C_q). Mean C_q values were then
144 normalized using housekeeping genes (HPRT1 for steroid hormone receptors, miR-16 for
145 microRNAs) to calculate a ΔC_q value. Statistic elaboration was performed by using ΔC_q values.
146 Values from the low estrogen group were used as reference to calculate fold changes in miRNA
147 expression among groups by applying the $2^{-\Delta\Delta C_q}$ formula. Differences among groups were assessed
148 using a Kruskal-Wallis non parametric test followed by Dunn's multiple comparison post-hoc tests.
149 All data comparison were done using IBM SPSS Statistics v24 (International Business Machine
150 Corp., New York, USA). Differences were considered statistically significant when $p \leq 0.05$.

151

152

3.Results

3.1 Estrogen signaling in sampled animals

17 β -estradiol blood concentration was determined for each animal sampled. Cows were then assigned to one of three groups according to concentrations. Groups were i) less than 10 pg/ml (low estrogen, n=10), ii) between 15 and 30 pg/ml (mid estrogen, n=9) and iii) over 40 pg/ml (high estrogen, n=5).

Age was compared among the three experimental groups and no significant differences were found [Supplementary Fig.1].

The expression levels of ER- α and - β in sampled muscle were compared by Q-PCR among the three groups and no statistically significant difference was found.

3.2 miRNAs expression levels

The expression levels of selected miRNAs were evaluated in all samples. Chosen miRNAs were miR-1, miR-26a, miR-26b, miR-27a-5p, miR-27b, miR-206 and miR-199a-3p [Fig.1].

No statistical difference was found among experimental groups for miR-1, miR-26a and miR-206.

A statistically significant difference was found in miR-26b expression among groups ($p=0.0025$) and more specifically low estrogen group had lower miR-26b levels when compared to mid (2.4 fold decrease) and high estrogen (2.2 fold decrease) groups.

MiR-199a-3p expression was found to be statistically different among groups ($p=0.005$) and had a statistically significant higher expression in the mid estrogen group than in the low estrogen group (3.4 fold increase), but no difference was found to be statistically significant between low and high estrogen groups and the mid and high estrogen groups.

176 A statistically significant difference in miR-27a-5p and miR-27b was found among groups (p=0.03
177 for both microRNAs) and post-hoc test showed a statistically significant difference only between
178 low and mid estrogen groups.

179

180 **4.Discussion**

181

182 Myostatin mutations have been widely described as responsible for muscle hypertrophy in several
183 bovine breeds. However muscle mass variations can be seen in individuals of the same breed as such
184 a phenotype must be controlled by multiple factors with a variable importance. The role of
185 microRNAs in muscle development has been explored in several species and miRNA families have
186 been identified as capable of mediating differentiation of myoblasts, activation of satellite cells and
187 promote fusion of these cells with mature myofibers (18).

188 Several miRNA candidates were chosen according to their expressions in muscle cells and to their
189 susceptibility to 17 β -estradiol stimulation as found by other research groups.

190 The candidates we examined included well known and specific regulators of muscle development
191 such as miR-1. This miRNA is involved in myoblast differentiation, in suppression of proliferation
192 of precursor cells and in promoting cell fusion (19). Since in literature no estrogen response elements
193 are reported in the promoter region of miR-1, a direct effect of 17 β -estradiol was not expected, but
194 miR-1 expression levels were evaluated nonetheless to explore the possibility that indirect
195 mechanisms of actions could take place. In MCF7 breast cancer cells a modest decrease in miR-1
196 levels was shown when cells were exposed to estradiol (20).

197 MiR-26a and miR-26b expression has been previously shown to be down-regulated by estrogen
198 stimulation in breast cancer cells. Such a modulation results in an increased proliferation (21).
199 However, these results were obtained in human cell lines derived from breast ER⁺ tumors exposed to
200 17 β -estradiol concentrations that were much higher than what we measured in the blood of the cows

201 we sampled. The effect of physiological concentration of estrogens on muscle miR-26a and miR-26b
202 might depend on differentially activated pathways. In skeletal muscle, knockdown of miR-26a
203 increases proliferation of Pax7-positive satellite cells and high expression levels of miR-26a are
204 required to promote differentiation of satellite cells (22).

205 High miR-27a and miR-27b expression levels are necessary for satellite cells differentiation: down-
206 regulations of these miRNAs by Pitx2 overexpression results in a blocked differentiation and in
207 increased proliferation of precursor cells (23). This effect is achieved through a repression of Pax3
208 by miR-27b (24). We also have previously shown that miR-27b is able to target and downregulate
209 myostatin in Piedmontese cattle (16).

210 MiR-206 belongs to the same family as miR-1 and due to a similar, but not identical sequence, they
211 share some targets. MiR-206 has been shown to promote myoblasts differentiation and to prevent
212 their proliferation in an in vitro model using C2C12 cells (25). We also recently demonstrated that
213 bovine satellite cells strongly up-regulates miR-206 during in vitro differentiation into myotubes (26).
214 Moreover in MCF7 breast cancer cells, miR-206 and ER- α are part of a regulatory feedback
215 mechanism, in which miR-206 can decrease ER- α and in turn ER- α can decrease miR-206 expression
216 levels (27). The effect of miR-206 overexpression in ER- α -positive ovarian cancer cells is a
217 proliferation inhibition.

218 MiR-199a-3p is part of the mir-199a/214 cluster. The expression levels of these miRNAs have been
219 reported to be reduced by administration of 17 β -estradiol in ovariectomized mice and to be
220 increased by progesterone (28). In a dystrophic zebrafish model, increased expression of miR-199a-
221 5p was associated with disruption of the normal myogenic differentiation during development by
222 affecting WNT signaling, pointing to an important role for this miRNA in a normal differentiation
223 pathway (29).

224 Using these data as starting point, we decided to assess in skeletal muscle of Piedmontese cattle
225 whether physiological variations in circulating 17 β -estradiol levels could modulate miRNAs known

226 to participate in muscle homeostasis. Bovines are a species in which estrogen plasma levels are low
227 (in the range of pg/ml), so we expected small variations in blood concentrations to have a detectable
228 effect on several miRNAs.

229 Estrogens have a variable plasma concentration according to the stage of the estral cycle the
230 bovines are in. More specifically their concentration increases during the proestral phase up to the
231 estrus and then abruptly declines.

232 No differences were found in the expression of both estrogen receptors (α and β) among the
233 experimental groups, suggesting that no changes in hormone sensitivity were taking place. When
234 miRNAs expression was examined, we found that increasing estrogen levels were able to slightly
235 increase the expression of miR-26b and induced a tendency to increase miR-199a-3p, miR-27a-5p
236 and miR-27b. Given the role these miRNAs play in skeletal muscle development, estrogen seems to
237 act to promote differentiation and to inhibit cell proliferation in vivo in skeletal muscle of beef
238 cattle and more specifically in the Piedmontese breed. Since the reproductive activity is cyclical the
239 skeletal muscle is exposed to fluctuating concentration of 17β -estradiol. MiRNAs might then
240 mediate a similar cyclical activity in skeletal muscle cells, by either promoting proliferation or
241 differentiation of satellite cells during the estral cycle.

242 We detected small variations in response to fluctuations in estrogen serum concentrations, which
243 could have a limited biological significance in determining muscle mass development. However, in
244 breeds such as the Piedmontese one where mutated myostatin has a prevalence above 98%, such
245 small changes might play an important role to determine differences among individuals.

246

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249

250 **References**

251

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335

336

337

338 **Figure Caption**

339 **Fig.1 miRNAs expression levels in bovines with different estrogen serum concentration**

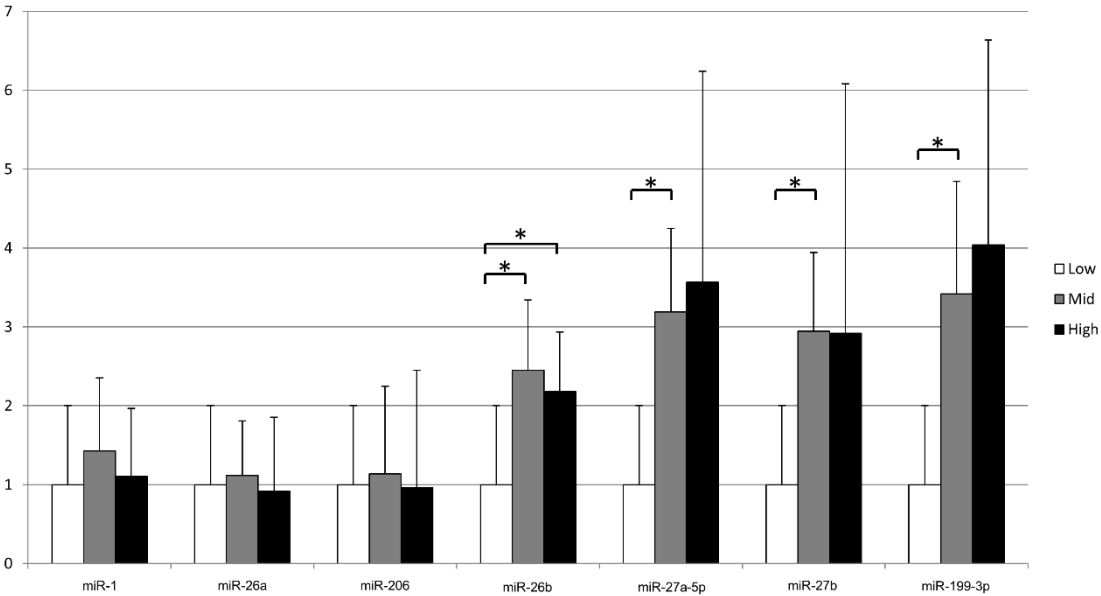
340 Graph showing the expression levels of selected miRNAs as fold changes among bovine grouped
341 according to their 17 β -estradiol serum concentration. Low, less than 10 pg/ml (n=10); mid, between
342 15 and 30 pg/ml (n=9); high, more than 40 pg/ml (n=5). Brackets indicate where the difference is
343 statistically significant (p<0.05).

344 **Supplementary Fig.1 Age comparison among experimental groups**

345 The graph shows the distribution of the age of the animals grouped by 17 β -estradiol serum
346 concentration.

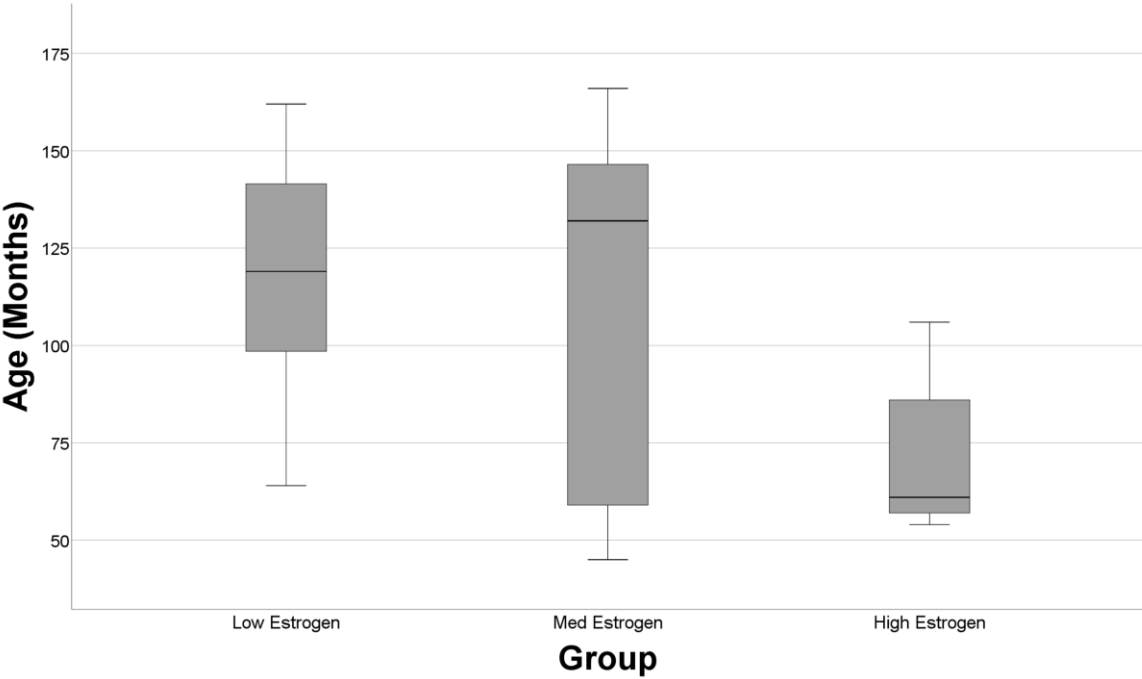
347

Fig.1



348

Supplementary Fig.1



349

350