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3 **Circulating miR-23b-3p, miR-145-5p and miR-200b-3p are potential biomarkers to**
4 **monitor acute pain associated with laminitis in horses.**

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15 Short title: Circulating miRNAs as biomarkers of pain in horses

16

17

18 **Abstract**

19 Circulating microRNAs (miRNAs) are emerging as promising biomarkers for several
20 disorders and related pain. In equine practice, acute laminitis is a common disease
21 characterised by intense pain that severely compromises horse welfare. Recently, the
22 Horse Grimace Scale (HGS), a facial-expression-based pain coding system, was shown to
23 be a valid welfare indicator to identify pain linked to acute laminitis. The present study
24 aimed to: determine whether miRNAs can be used as biomarkers for acute pain in horses
25 (*Equus caballus*) affected by laminitis; integrate miRNAs to their target genes and to
26 categorise target genes for biological processes; gather additional evidence on concurrent
27 validity of HGS by investigating how it correlates to miRNAs. Nine horses presenting acute
28 laminitis with no prior treatment were recruited. As control group, nine healthy horses were
29 further included in the experimental design. Samples were collected from horses with
30 laminitis at admission before any treatment (“pre-treatment”) and seven days after routine
31 laminitis treatment (“post-treatment”). The expression levels of nine circulating miRNAs,
32 namely hsa-miR-532-3p, hsa-miR-219-5p, mmu-miR-134-5p, mmu-miR-124a-3p, hsa-
33 miR-200b-3p, hsa-miR-146a-5p, hsa-miR-23b-3p, hsa-miR-145-5p and hsa-miR-181a-5p,
34 were detected and assessed as potential biomarkers of pain by qPCR using TaqMan®
35 probes. The area under the receiver operating curve (AUC) was then used to evaluate the
36 diagnostic performance of miRNAs. Molecular data were integrated with HGS scores
37 assessed by one trained treatment and time point blind veterinarian. The comparative
38 analysis demonstrated that the levels of miR-23b-3p ($P = 0.029$), miR-145-5p ($P = 0.015$)
39 and miR-200b-3p ($P = 0.023$) were significantly higher in pre-treatment and the AUCs
40 were 0.854, 0.859 and 0.841, respectively. MiR-200b-3p decreased after routine laminitis
41 treatment ($P = 0.043$). Combining two miRNAs in a panel, namely miR-145-5p and miR-
42 200b-3p, increased efficiency in distinguishing animals with acute pain from controls. In
43 addition, deregulated miRNAs were positively correlated to HGS scores. Computational

target prediction and functional enrichment identified common biological pathways between different miRNAs. In particular, the glutamatergic pathway was affected by all three miRNAs, suggesting a crucial role in the pathogenesis of pain. In conclusion, the dynamic expression of circulating miR-23b-3p, miR-145-5p and miR-200b-3p was detected in horses with acute laminitis and miRNAs can be considered potentially promising pain biomarkers. Further studies are needed in order to assess their relevancy in other painful conditions severely compromising horse welfare. An important implication would be the possibility to use them for the concurrent validation of non-invasive indicators of pain in horses.

Keywords : microRNA, pain, biomarkers, welfare indicator, horse.

Implications

Pain severely affects the welfare of horses and often remains underestimated. Measuring pain-related parameters in a reliable way can be challenging; however, an accurate determination and quantification of pain in horses is critical. Several physiological, endocrine and behavioural parameters have been investigated to identify pain conditions and severity in clinical studies. To date, no molecular indicators have been available to assess horse pain. Tackling the challenge of improving the welfare of horses affected by laminitis, the present study highlights the possible use of molecular biomarkers in assessing and quantifying pain in horses. Three candidate biomarkers have been identified in the serum of horses with acute laminitis. An integrated analysis between behavioural indicators (HGS) and molecular biomarkers could be used to assess and monitor painful conditions in horses.

68 **Introduction**

69 MiRNAs belong to a class of non-coding single-stranded RNA of 19–24 nucleotides with
70 the ability to modulate gene expression post-transcriptionally. They are crucial modulators
71 of cellular homeostasis and their deregulation has been associated with a wide range of
72 pathological conditions and with neuropathic, inflammatory, traumatic and cancer-
73 associated pain in humans (Bali and Kuner, 2014; Zhang and Banerjee, 2015; Pang *et al.*,
74 2016) and laboratory animals (Gong *et al.*, 2015; Pang *et al.*, 2016; Qureshi *et al.*, 2016).
75 The modulation of miRNA expression is an early event in pathogenic processes
76 (Schwarzenbach *et al.*, 2014). They act by guiding the RNA-induced silencing complex
77 (RISC) to partially complementary sequences in target mRNA to suppress gene
78 expression by a combination of translation inhibition and mRNA decay (Lagos-Quintana *et al.*, 2001). The level and composition of extracellular miRNAs in certain body fluids are
79 tightly correlated to various human pathological conditions, such as cancer, diabetes,
80 cardiovascular diseases and drug-induced organ damage (Ghai and Wang, 2016). A
81 change in the expression pattern of miRNAs targeting key regulators of pain processing
82 has been observed both in inflammatory and neuropathic pain (Zhao *et al.*, 2010; Genda
83 *et al.*, 2013). Although some investigations have already evaluated miRNAs as suitable
84 biomarkers to assess stress associated with endurance exercise in horses (Mach *et al.*,
85 2016) and with road-transportation in turkeys (Lecchi *et al.*, 2016), the diagnostic potential
86 of circulating miRNAs deserves to be further explored in veterinary medicine in relation to
87 stressful or painful conditions that might affect animal welfare.

89 Acute laminitis is a disease associated with ischaemia of digital dermal tissues whose
90 exact aetiology is still not completely understood (Wylie *et al.*, 2013). However, there are
91 evidences that support a possible link between insulin resistance, obesity, and acute
92 laminitis (Geor, 2008). Evidences suggest that the pain state associated with chronic
93 equine laminitis have a neuropathic component (Jones *et al.*, 2007) indicating that both

94 inflammatory and neuropathic pain should be considered in the management of this
95 condition. In equine practice, acute laminitis provides a valuable pain model as it severely
96 affects animal welfare causing severe, long-lasting debilitating pain in horses (Dalla Costa
97 *et al.*, 2014b; Minero and Canali, 2009). Since 2010, World Horse Welfare, an international
98 horse charity, has launched a campaign about the importance of recognising the early
99 signs of laminitis to protect horse welfare and minimise the associated pain. This disease
100 has a worldwide estimated prevalence of 7–14% and is characterised by lameness,
101 inability or reluctance to walk, frequent weight shifting, and abnormal weight distribution on
102 hind feet (Collins *et al.*, 2010; Wylie *et al.*, 2013). Albeit challenging, accurate pain
103 assessment is critical to recognise early signs associated with laminitis (e.g. strong digital
104 pulse, shortening of the stride, abnormal foot lifting (Wylie *et al.*, 2013)) and ensure
105 appropriate treatment (de Grauw and van Loon, 2016). Among commonly used systems,
106 the Composite Pain Scale (CPS), a multifactorial numerical rating scale, considers
107 physiological response to treatment and behavioural data. The CPS was originally
108 developed on an experimental model of acute orthopaedic pain in horses (Bussi res *et al.*,
109 2008) and then applied to monitor pain after surgery (e.g. castration, colic, orthopaedic
110 and soft-tissue surgery) (Van Loon *et al.*, 2010). Recently, a facial-expression-based pain
111 coding system, the Horse Grimace Scale (HGS) was developed as a welfare indicator for
112 pain assessment of horses undergoing surgical castration (Dalla Costa *et al.*, 2014a) and
113 acute laminitis (Dalla Costa *et al.*, 2016). HGS is a fast method based on behavioural
114 observation. It does not cause disturbance to the horses in their boxes, does not require
115 their walking and/or trotting as well as any palpation of their painful area. HGS has
116 potential to provide insights into the experience of pain in horses and in the assessment of
117 horse welfare, however, the authors concluded that further validation studies were needed
118 to apply the HGS in a clinical setting (Dalla Costa *et al.*, 2017, 2016, 2014a). Considering
119 the diagnostic potential of miRNAs and the evidence of their identification in the equine

120 genome, tissues and serum (Kim *et al.*, 2014; Pacholewska *et al.*, 2016), the aims of the
121 present study were to a) ascertain whether acute pain associated with laminitis may
122 modulate the expression of circulating miRNAs; b) investigate the potential use of
123 differentially expressed (DE)-miRNAs as biomarkers to measure pain in horses and c)
124 gather additional evidence on concurrent validity of HGS and CPS by investigating how
125 they correlate with miRNAs.

126

127 **Materials and methods**

128 *Ethics statement*

129 The study design was approved by the Brandenburg State Veterinary Authority (V3-2347-
130 A-42-1-2012) in compliance with German legislation on animal experiments. Individual
131 horse owner's consent was obtained for all horses participating in this study. Horses
132 involved in this study were admitted for routine veterinary treatment of acute laminitis at
133 the request of their owner on a voluntary basis. Control samples were obtained from
134 healthy stallions referred to the clinic for surgical castration.

135

136 *Animals and husbandry*

137 Between 2012 and 2014, horses admitted to the Havelland Equine Clinic presenting acute
138 laminitis with no prior treatment were included in the present study. Nine horses of
139 different breed and gender, aged between 4 and 17 years (mean=9.4±5.0) were recruited.
140 As a control group, nine healthy stallions of mixed age (mean=2.4±1) referred to the clinic
141 for routine castration were recruited. In order to be included in this study, control horses
142 had to be deemed healthy by an equine veterinarian after physical examination and

143 behavioural evaluation. All horses, after admission, were stabled in standard single boxes
144 (4 x 3 m with an outside window) on wood shavings (German Horse Span Classic,
145 German Horse Pellets, Wismar Germany), and in visual contact with conspecifics. They
146 were fed hay twice a day (approx. 3 kg/100kg body weight per day) and water was
147 provided ad libitum by automatic drinkers.

148 Each horse with laminitis underwent a routine treatment including: oral administration of
149 Phenylbutazone (initial 4.5 mg/kg bwt every 12 h for two days and subsequent 2.5 mg/kg
150 bwt every 12 h, Phenylbutarium®, Ecuphar, Germany); subcutaneous injections of heparin
151 (50 IU/kg bwt every 12 h for five days, Heparin-Natrium-25000-ratiopharm®, Ratiopharm,
152 Germany); padded hoof bandages with frog support and elevated heels (3 – 4 cm); ice
153 water applications every 2 h for the first three days of treatment and strictly restricted
154 movement in an individual box with a deep and soft bedding of wood shavings.

155 Horses with acute laminitis were assessed, as described in the following sections, at
156 admission before any treatment (“pre-treatment”) and seven days after the treatment,
157 before being discharged (“post-treatment”). Control horses (“control”) were assessed once,
158 after 2 days of acclimatisation to the clinic setting and before surgery. Assessments were
159 carried out in the morning between 8 and 12. Horses were monitored routinely at least 3
160 times a day, or more frequently if needed, by an experienced vet or nurse by collecting
161 data about HR, breath frequency, appetite, defecation, weight shifting and obvious signs of
162 pain.

163 *MiRNAs extraction and real-time quantitative PCR*

164 Blood was collected by jugular venipuncture into Monovette® tubes (Sarstedt Company,
165 Nümbrecht, Germany) and serum was stored at -80°C until RNA extraction. Total RNA
166 was extracted using miRNeasy Serum/Plasma Kit (Qiagen, catalog number 217184).

167 Serum was thawed on ice and centrifuged at 3000 g for 5 min at 4°C. An aliquot of 200 µl
168 per sample was transferred to a new tube and 1 ml of Qiazol (Qiagen) was added. The
169 *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen, catalog number 219610) was used as
170 synthetic spike-in control because of a lack of sequence homology to equine miRNAs.
171 After incubation at room temperature for 5 min, 3.75 µl (25 fmol final concentration) of
172 spike-in control was added and the samples were vortexed to ensure complete mixing.
173 RNA extraction was then carried out according to manufacturer's instruction. Reverse
174 transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit
175 (Applied Biosystems, catalog number 4366596) and using miRNA-specific stem-loop RT
176 primers, according to manufacturer's instructions. Reverse transcription reactions were
177 performed in 15 µl volume reactions containing 1.5 µl 10X miRNA RT buffer, 1 µl
178 MultiScribe reverse transcriptase (50 U/µl), 0.30 µl 100 mM dNTP mix, 0.19 µl RNase
179 Inhibitor (20 U/µl), 6 µl of custom RT primer pool and 3.01 µl of nuclease-free water. The
180 custom RT primer pool was prepared combining 10 µl of each individual 5X RT primer in a
181 final volume of 1000 µl; the final concentration of each primer in the RT primer pool was
182 0.05X each. Three µl serum RNA was added to each RT reaction. Every RT reaction
183 mixture was incubated on ice for 5 minutes, 16°C for 30 minutes, 42°C for 30 minutes and
184 then 85°C for 5 minutes.

185 The qPCR experiments were designed following the MIQE guidelines. Small RNA TaqMan
186 assays were performed according to manufacturer's instruction. MicroRNAs were selected
187 according to previous publications where they were correlated to pain in humans and mice
188 (Elramah *et al.*, 2014; Bali and Kuner, 2014; Qureshi *et al.*, 2016). The selected
189 primer/probe assays (Life Technologies) included cel-miR-39-3p (assay ID000200), hsa-
190 miR-532-3p (assay ID002355), hsa-miR-219-5p (assay ID00522), mmu-miR-134-5p
191 (assayID001186), mmu-miR-124a-3p (assay ID001182), hsa-miR-200b-3p (assay

192 ID002251), hsa-miR-146a-5p (assay ID000468), hsa-miR-23b-3p (assay ID000400), hsa-
193 miR-145-5p (assay ID002278) and hsa-miR-181a-5p (assay ID000480). Quantitative
194 reactions were performed in duplicate in scaled-down (12 μ l) reaction volumes using 6 μ l
195 TaqMAN 2X Universal Master Mix II (Applied Biosystems, catalog number 4440044), 0.6
196 μ l miRNA specific TaqMan Assay 20X and 1 μ l of the RT product per reaction on Eco Real
197 Time PCR detection System (Illumina). The standard cycling program was 50°C for 2 min,
198 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Data were
199 normalized relative to the expression of cel-miR-39. MiRNAs expression levels are
200 presented in terms of fold change normalized to cel-miR-39 expression using the formula
201 $2^{-\Delta\Delta Cq}$. To investigate the pain relevance, predicted targets of the significant DE-miRNAs
202 were computationally retrieved from the TargetScan database
203 (http://www.targetscan.org/vert_71/) and mRNA enrichment was performed using DAVID
204 bioinformatics resources (<https://david.ncifcrf.gov/>). The list of target genes was employed
205 in further analyses and biological pathways in the KEGG were examined for enrichment
206 (<http://www.genome.jp/kegg/>). Since alterations in neuronal excitability are believed to
207 contribute to the initiation and maintenance of neuropathic pain following peripheral injury,
208 an enrichment of mRNA targets that encode for ion channels - known to contribute and
209 modulate neuronal excitability - was performed. Based on knockout studies, the predicted
210 mRNA targets have already been associated with hypersensitivity in various pain models
211 (<http://www.jbldesign.com/jmogil/enter.html>) (LaCroix-Fralish *et al.*, 2007).

212

213 *Behavioural recordings*

214 An experienced veterinarian conducted the live evaluation of the Composite Pain Scale
215 (CPS) developed by Bussi res and colleagues (2008) with slight modifications, as
216 previously reported (Dalla Costa *et al.*, 2014a). Twenty-minute videos were simultaneously

217 recorded using two video cameras (Panasonic, HDC-SD99, Panasonic, Japan). In order to
218 collect videos without influencing the horse's behaviour, the video cameras were
219 positioned on the opposite sides of the box on top of their grate section. Following the
220 methods of Dalla Costa and colleagues (2014a), where images (one or two per animal)
221 per time point were included, still frames of the face of each horse were extracted from the
222 videos whenever they directly faced the video camera. The selection was based on the
223 quality of the picture. On a sample of 27 still images (9 for control horses, 9 for pre-
224 treatment and 9 for post-treatment), randomly selected by a non-participating assistant,
225 the HGS was assessed by one trained treatment and time point blind veterinarian (not
226 involved in the CPS assessment). A detailed handout with the description of the six Facial
227 Action Units (FAUs) was used as training material. FAUs are independently scored on a 3-
228 point scale, with zero indicating that the assessor is confident that the action unit is not
229 present, one indicating that the assessor is confident that the action unit is only moderately
230 present, and two indicating that the assessor is confident that the action unit is obviously
231 present. The possible maximum HGS score was 12 (2 for each FAUs).

232

233 *Statistical analysis*

234 Statistical analysis was performed using SPSS 23 (SPSS Inc., Chicago, IL, USA).
235 Statistical significance was accepted at $P \leq 0.05$. The CPS total score was calculated. The
236 HGS total score for each image consisted of the sum of the scores across the six FAUs.
237 Data were tested for normality and homogeneity of variance using the Kolmogorov-
238 Smirnov and Levene test, respectively. As data were not normally distributed, non-
239 parametric statistical tests were applied. Mann Whitney test was used to assess
240 differences in miRNAs concentrations (only for those miRNAs that had a significant
241 differential expression in the blood of horses), CPS and HGS scores between control
242 group and pre-treatment group, and control and post-treatment group. Match paired

243 Wilcoxon test was run to compare miRNA concentrations (miR-23b-3p, miR-145-5p and
244 miR-200b-3p), CPS and HGS scores pre- and post-treatment. Receiver Operating
245 Characteristic (ROC) analysis was performed to determine the diagnostic accuracy of
246 targets that statistically differed between pre-treatment and controls. The diagnostic values
247 were calculated for those miRNAs (alone and in combination) that showed a significant
248 differential expression in the blood of horses, namely miR-23b-3p, miR-145-5p and miR-
249 200b-3p. The ROC analysis was carried out by plotting the true positive (sensitivity) versus
250 the false positive (1-specificity). Linear regression was used to investigate any relationship
251 between miR-23b-3p, miR-145-5p and miR-200b-3p and age of the horses. Spearman's
252 rho test was performed to evaluate whether there was any correlation among the
253 expression levels of the various miRNAs, HGS and CPS.

254

255 **Results**

256 *Acute laminitis alters expression levels of miR-23b-3p, miR-145-5p and miR-200b-3p in*
257 *blood serum*

258 The levels of miRNAs were normalized to the levels of the artificial spike-in cel-miR-39,
259 which was used as internal control. The selected target miRNAs were detected in all
260 samples, with exception of the miRNAs has-miR-219-5p, has-miR-532-3p, mmu-miR-134-
261 5p and mmu-miR-124a-3p. Statistical analyses was performed on detected five miRNAs,
262 among which 3 circulating miRNAs, namely miR-23b-3p, miR-145-5p and miR-200b-3p,
263 had a significant differential expression in the blood of horses. Figure 1 presents an
264 overview of the results. In detail, the level of miR-23b-3p (fold change=14.6; $P = 0.029$),
265 miR-145-5p (fold change=4.4; $P = 0.015$) and miR-200b-3p (fold change=3.4; $P = 0.023$)
266 was higher in pre-treatment compared to controls. Only miR-200b-3p significantly
267 decreased from pre- to post-treatment (Match paired Wilcoxon, $P = 0.043$). The levels of

268 circulating miR-23b-3p, miR-145-5p and miR-200b-3p were not affected by the age of the
269 horse (Linear regression, $P > 0.05$).

270

271 *Diagnostic performance of miR-23b-3p, miR-145-5p and miR-200b-3p*

272 Diagnostic accuracy of miR-23b-3p, miR-145-5p and miR-200b-3p, as measured by the
273 area under the curve (AUC), was good for all targets for the discrimination of pre-treatment
274 from controls (Figure 2) and it was 0.854 (95% CI 0.5652-1; $P = 0.028$), 0.859 (95% CI
275 0.66-1; $P = 0.016$) and 0.841 (95% CI 0.643-1; $P = 0.023$), respectively.

276 Further statistical analysis was performed considering the average relative quantification
277 (RQ) values of the three pain-related miRNAs. The combination of miRNAs resulted in an
278 improved discrimination of the pre-treatment. The created model included all three
279 miRNAs (miR-23b-3p, miR-145-5p and miR-200b-3p) or two (miR-145-5p and miR-200b-
280 3p). The AUC from ROC analysis increased to 0.917 (95% CI 0.782-1; $P = 0.004$;
281 sensitivity 100% and specificity 78%, cut-off 0.1936) and 0.917 (95% CI 0.773-1; $P =$
282 0.004; sensitivity 87.5% and specificity 89%, cut-off 0.279), respectively.

283

284 *Composite Pain Scale (CPS) and Horse Grimace Scale (HGS) and their correlation to* 285 *miRNAs*

286 Both CPS and HGS scores were significantly lower in the control group (Mann Whitney
287 test, $P < 0.001$) than in horses with acute laminitis (pre-treatment) (Figure 3). Control
288 horses did not differ from post-treatment for both CPS and HGS scores (Mann Whitney
289 test, $P = 0.167$ and $P = 0.059$ respectively).

290 A positive correlation was observed among miR-145-5p, HGS ($R^2 = 0.482$; $P = 0.015$) and
291 CPS ($R^2 = 0.575$; $P = 0.003$). A positive correlation was also found between the
292 combination of miR-145-5p and miR-200b-3p with both HGS ($R^2 = 0.559$; $P = 0.004$) and
293 CPS ($R^2 = 0.595$; $P = 0.002$).

294

295 *miRNA target prediction and pathway enrichment*

296 The predicted mRNA targets were 97, three of which were regulated by all three miRNAs
297 and 15 by at least two miRNAs (Figure 4A). The target mRNA are grouped in ion channel
298 families: solute carrier family transporters (SLC), potassium voltage-gated channel (KCN),
299 voltage-gated sodium channel (SCN), gamma-aminobutyric acid (GABA) A or B receptor
300 (GABRB and GABBR), voltage-dependent calcium channel (CACN), adrenoceptor alpha
301 (ADRA), potassium channel (tetramerization domain) (KCTD), hyperpolarization activated
302 cyclic nucleotide-gated potassium channel (HCN), two pore segment channel (TPCN),
303 transient receptor potential cation channel (TRP) (Figure 4B). The KEGG pathway analysis
304 demonstrated that most of these genes code for proteins involved in the glutamatergic
305 pathway (Figure 4C).

306

307 **Discussion**

308 The present study investigated the circulating levels of nine miRNAs and assessed
309 their diagnostic value in serum of horses affected by acute laminitis. In particular, their
310 incremental diagnostic value was investigated. We report five major findings. Firstly,
311 circulating levels of miR-23b-3p, miR-145-5p and miR-200b-3p were significantly higher in
312 animals with acute laminitis than in controls. Secondly, diagnostic accuracy for acute
313 laminitis was good ($0.80 < \text{AUC} < 0.90$) for miR-23b-3p, miR-145-5p and miR-200b-3p.
314 Thirdly, diagnostic accuracy of the combination of two or three differentially expressed
315 miRNAs was excellent ($0.90 < \text{AUC} < 1$). Fourthly, the level of miR-200b-3p significantly

316 decreased from pre- to post-treatment. Lastly, the correlation between miRNAs and other
317 behavioural pain measures (HGS and CPS) was positive.

318 Accurate determination of pain is crucial, given that it is often difficult to measure
319 pain-related parameters in a quantitative way. In horses affected by acute laminitis, the
320 common challenging question for clinicians is whether their level of pain and suffering is
321 acceptable to continue their treatment. An important development in understanding pain
322 level and mechanisms is represented by miRNAs (Bali and Kuner, 2014). The use of
323 miRNAs as diagnostic and prognostic biofluid-derived markers is advancing in various
324 fields, including oncology and cardiology. The presence of an altered circulating miRNA
325 profile has been demonstrated for several painful conditions, including neuropathic,
326 inflammatory, traumatic and cancer-associated pain in humans and rodents (Andersen *et al.*,
327 2014; Qureshi *et al.*, 2016). Recent studies reported that miRNome is involved in
328 equine osteochondrosis physiopathology and quickly reacts to endurance exercise stress
329 (Desjardin *et al.*, 2014; Mach *et al.*, 2016). Our results suggest that circulating levels of
330 miR-23b-3p, miR-145-5p and miR-200b-3p were effective in accurately differentiating
331 horses affected by acute laminitis from controls. To the best of our knowledge, the effect of
332 Phenylbutazone on circulating miRNAs has never been investigated, while contradictory
333 results have been reported on heparin. Some authors indicated that heparin might have an
334 impact on the circulating level of miRNA expression in patients undergoing cardiac
335 catheterisation (Kaudewitz *et al.*, 2013), whereas others did not find any difference in
336 patients with acute myocardial infarction (Wang *et al.*, 2016). Our results showed that miR-
337 200b-3p upregulated by acute laminitis was restored by drug administration, suggesting
338 that molecular changes induced by drugs can be assessed from serum. Thus, increased
339 understanding of miRNA expression pattern in the serum of pain-affected animals would
340 improve both pre-treatment evaluation and monitoring of the disease course.

341 When facing intense and long lasting painful diseases, such as laminitis, a combination of
342 non-invasive pain measures and molecular biomarkers should be considered in the
343 attempt of increasing the overall accuracy of the former. Previous studies adopted a
344 similar approach to assess concurrent validity of new molecular pain indicators or to
345 investigate the effectiveness of pain mitigation methods in laboratory animals (Amin *et al.*,
346 2014, Tiwari *et al.* 2012) or in dairy cattle (Rialland *et al.*, 2013). The authors generally
347 reported that combining behavioural and molecular biomarkers meaningfully contributed in
348 providing a global picture of the animal's state. For the first time this paper addressed this
349 question in horses: interestingly, positive correlations were found between miRNA
350 expression levels and other non-invasive pain measures based on observation of horse
351 demeanour (HGS and CPS). These findings suggest directions for a more accurate pain
352 assessment that may help the equine clinician focus onto the peculiar issue of animal
353 welfare in the course of laminitis. This implies, for example, acceptance or refusal of long
354 lasting pain and suffering, likely to range from weeks to months, even in the presence of
355 medical treatment. Our results suggest that an integrated analysis of non-invasive pain
356 measures (CPS, HGS) and molecular biomarkers may be useful for more reliable
357 assessment and monitoring of painful conditions.

358 Pathway enrichment of the predicted targets of the differentially expressed (DE)-miRNAs
359 was identified. Despite the differences in the lists of genes targeted by DE-miRNAs, the
360 glutamatergic pathway was found to be enriched by all DE-miRNAs. Glutamate is the
361 major excitatory neurotransmitter in the mammalian central nervous system and mediates
362 many aspects of sensory function, including acute and chronic pain in both humans and
363 animals (Stephens, 2011; Brearley, 2000). After release from presynaptic nerve terminals,
364 glutamate is quickly removed from the synaptic cleft by a family of five glutamate
365 transporters, the excitatory amino acid transporters (EAATs) (Fahlke *et al.*, 2016). Our
366 analysis demonstrated that EAATs might be regulated by DE-miRNAs. EAATs belong to

367 solute carrier 1 family (SLC) and they can function both as transporters, by mediating the
368 re-uptake of synaptic released glutamate, and as anion-selective channels (Fahlke et al.,
369 2016). Since glutamate is the major excitatory neurotransmitter released in the first central
370 synapse of the pain-transmitting afferent neurons, the modulation of transporters and ion
371 channels activities, which regulate extracellular levels of glutamate, may be an important
372 target for pain management strategies both in humans and in animals (Stephens, 2011;
373 Brearley, 2000). An excessive accumulation of extracellular glutamate is implicated in the
374 pathogenesis of trauma, ischemia, and neurodegenerative diseases in humans (Kim *et al.*,
375 2011). The modulation of EAATs by de-miRNAs as identified in the present study might
376 involve decrease in glutamate re-uptake leading to excitotoxicity and neuronal damage
377 (Kim *et al.*, 2011). A previous study demonstrated that neuronal exosomal miR-124a
378 regulates the expression of EAAT2 by modulation of translation process in mice (Morel *et*
379 *al.*, 2013). Although miR-124a resulted not expressed in our model, the outcomes reported
380 by Morel and colleagues (2013) demonstrated that miRNAs are involved in the regulation
381 of EAATs and, consequently, in glutamate re-uptake. Augmentation of synaptic strength in
382 nociceptive pathways represents a cellular model of pain amplification (Clark *et al.*, 2015).
383 Ding et colleagues (2017) demonstrated that miRNAs modulate the expression levels of
384 NMDA receptor during chronic pain in rats. Our data may therefore suggest that, under
385 chronic pain formation, the activation of NMDA receptors (mediated by miR-23b-3p, miR-
386 145-5p and miR-200b-3p overexpression) may drive the facilitation of excitatory synaptic
387 transmission in the dorsal horn, which contributes to maintaining allodynic and
388 hyperalgesic pain in chronic pain states. Given that miR-200b-3p expression is restored to
389 the control group level in animals affected by acute laminitis after treatment, the predicted
390 targets of this miRNA was investigated using KEGG bioinformatics sources. Interestingly,
391 the GABAergic pathway was found to be enriched. GABA is the main inhibitory transmitter
392 of the nervous system and GABA receptors play important roles in the dampening of

393 neuropathic hyperexcitability by depolarizing neurons, thus blocking the information
394 transmission from dorsal root ganglia to the spinal cord (Gwak and Hulsebosch, 2011).
395 Naik and colleagues (2008) demonstrated that GABA receptors are down-regulated in
396 injured neurons after spinal nerve injury in rats and hypothesised that they play an
397 important role in the development of increased synaptic transmission in neuropathic pain.
398 The influence of miRNAs on GABA receptors has been previously demonstrated, i.e. miR-
399 33 regulates hippocampal extrasynaptic GABAA receptors in a mouse model of contextual
400 fear conditioning (Jovasevic *et al.*, 2015) and miR-181a down-regulates GABA(A α -1)
401 receptor in adult spinal cords from rats with neonatal cystitis (Sengupta *et al.*, 2013). We
402 speculate that high levels of miR-200b-3p might downregulate the expression of GABA
403 receptors during acute phase of laminitis, while the inhibition of miRNA expression after
404 treatment might increase receptor density and thus sensitivity of GABA receptors.

405 The present study has some limitations to be pointed out. Its sample size is hampered
406 because accessing cases of horses with acute laminitis having no prior treatment is
407 difficult. However, conducting this initial study on a limited number of animals showed the
408 advantage of testing the research design adequacy in view of future larger scale studies.
409 G-Power analysis suggests that clinical studies involving more than 20 horses per group
410 are needed to confirm the diagnostic value of DE-miRNAs for acute laminitis. Moreover,
411 future studies should elucidate whether DE-miRNAs could further discriminate between
412 pain induced by laminitis and other acute or chronic pain conditions. In addition, exosome-
413 mediated transfer of miRNAs may be involved in cell-to-cell communication (Valadi *et al.*,
414 2007). The underlying functions in intracellular communication of miRNAs identified in the
415 present study and their possible roles in the pathophysiological processes of laminitis
416 remain unknown. A possible limitation of the use of HGS is that still images taken from
417 videos may not accurately reflect the potentially changing nature of facial expressions in

418 real time. Based on methods adopted in previous publications, this study only considered
419 one image per animal per time point. It is suggested that future studies include more high
420 quality images or videos of the horse head. Tackling the challenge of improving the welfare
421 of horses affected by laminitis, for the first time our study investigated the dynamic
422 expressions of circulating miRNAs during the acute phase of the disease. ROC analysis
423 suggested that circulating miR-23b-3p, miR-200b-3p and miR-145-5p might be considered
424 novel and promising biomarkers for early diagnosis of acute pain in horses. Eventually, a
425 multi-miRNA panel including miR-200b-3p and miR-145-5p might present even greater
426 diagnostic value and be meaningfully applied to the validation of behavioural pain
427 measures.

428

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435

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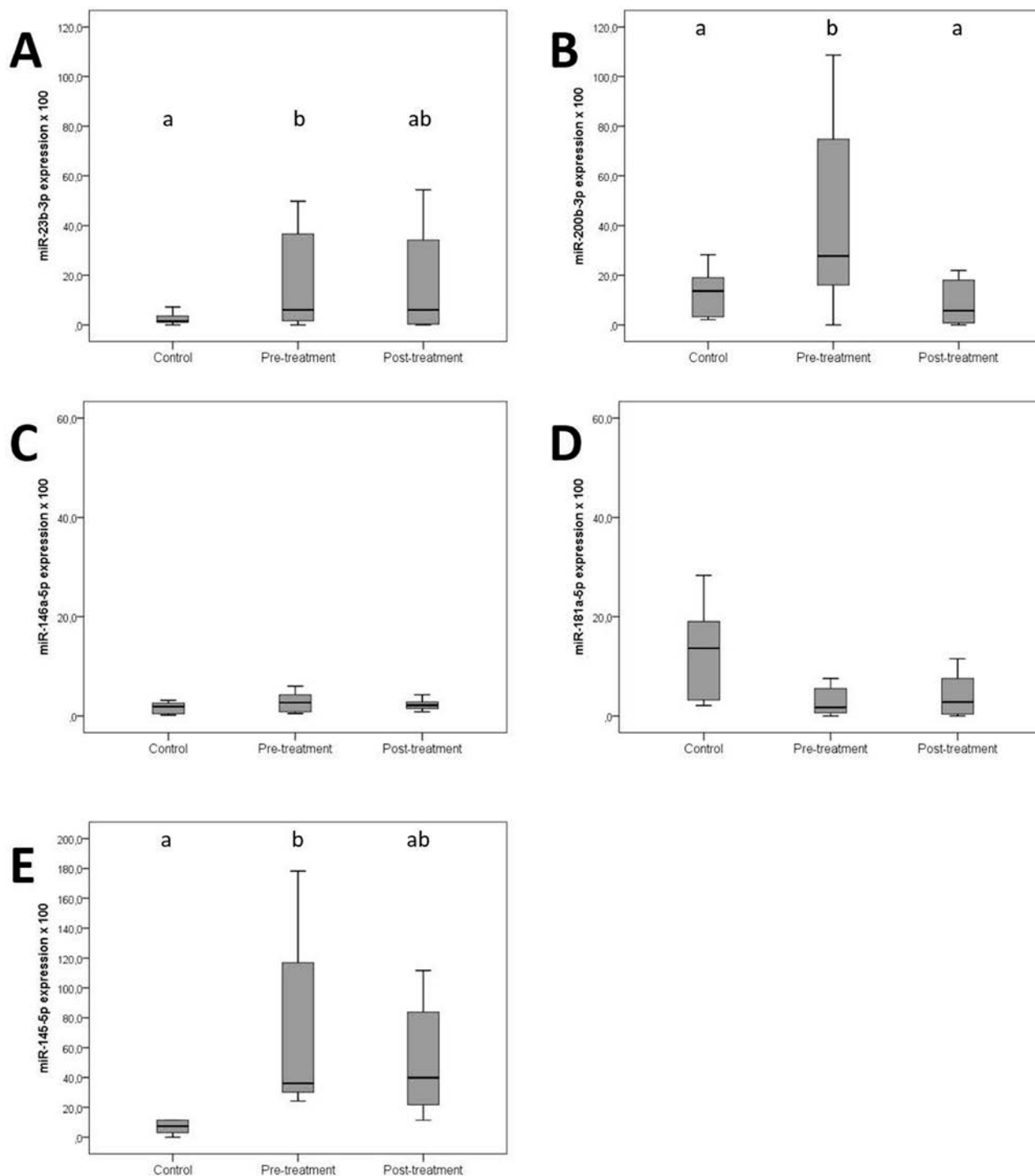
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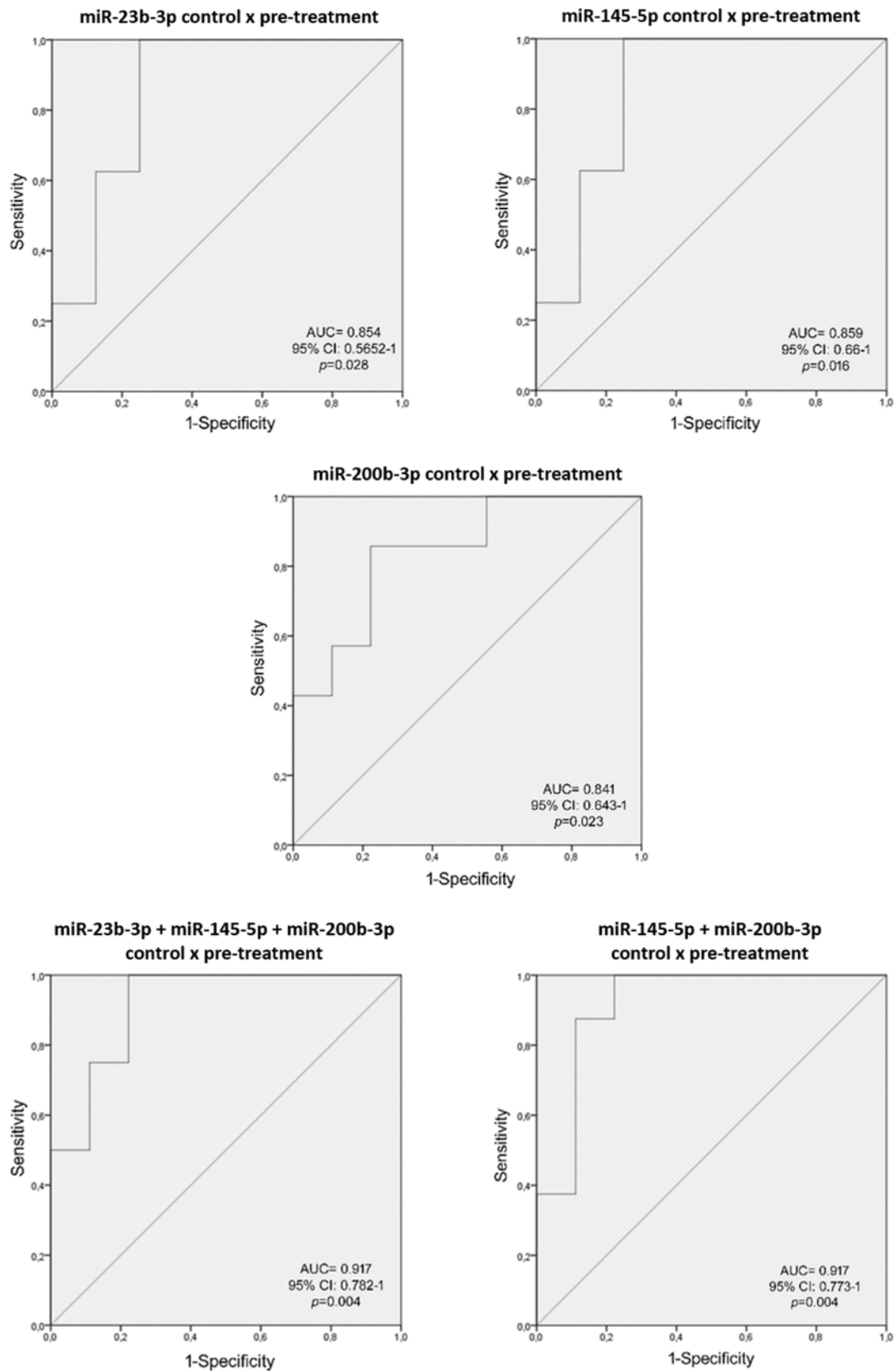
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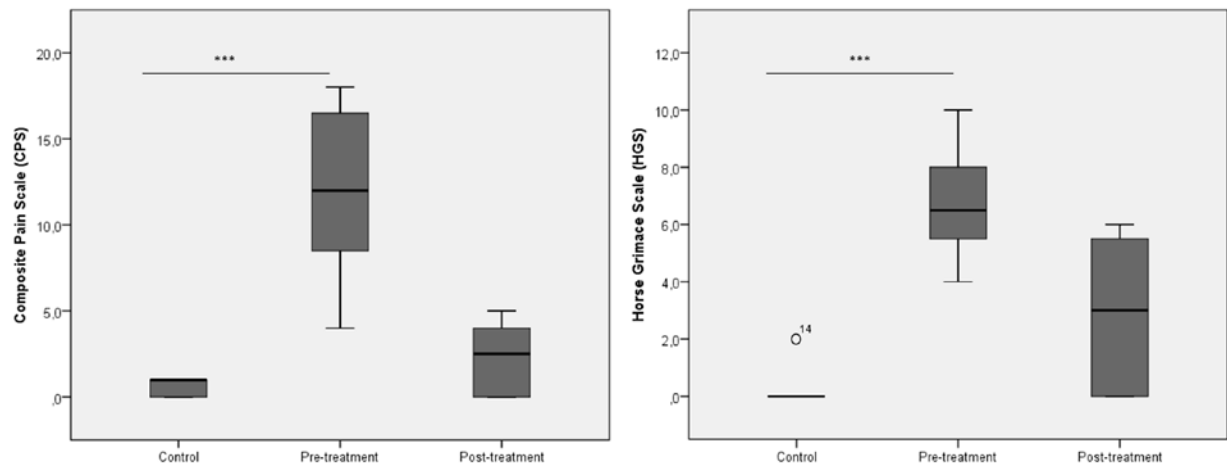
581 Figure 1. Box plots for circulating pain-related miRNAs levels: (A) miR-23b-3p, (B) miR-200b-3p,
 582 (C) miR-146a-5p, (D) miR-181a-5p and (E) miR-145-5p. Different letters (a, b, ab) indicate
 583 significant differences among the groups (Control, Pre-treatment and Post-treatment) with a
 584 significance level at $P < 0.05$. The black lines inside the boxes mark the medians. Whiskers
 585 indicate variability outside the upper and lower quartiles.

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588 Figure 2. Receiver-operator characteristics (ROC) curve analysis of candidate pain-related
589 miRNAs. AUC, area under the curve; CI, confidence interval.



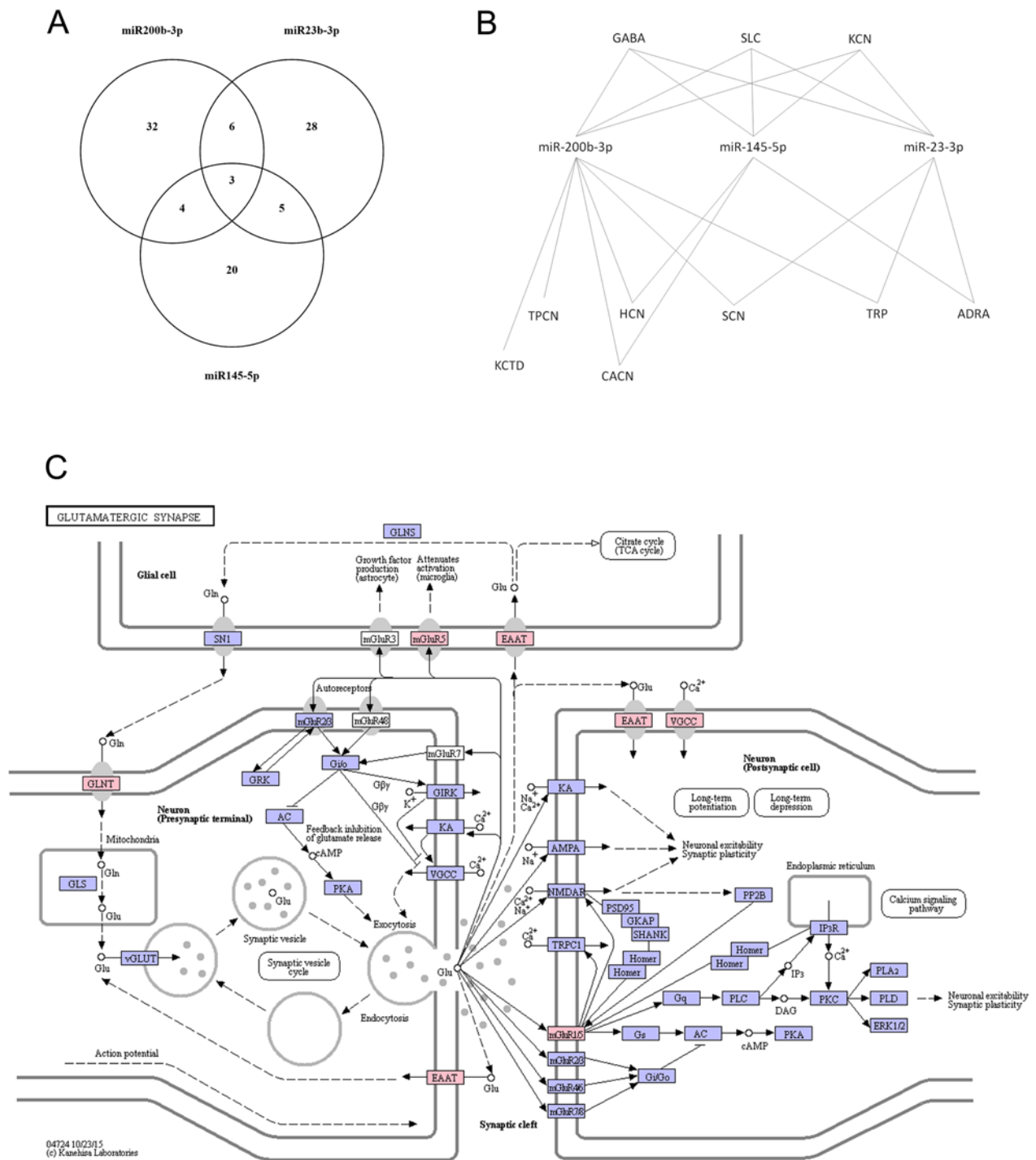
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592 Figure 3. CPS and HGS scores of the control, pre-treatment and post-treatment groups drawn in a
 593 box plot. Differences between the groups (Control, Pre-treatment and Post-treatment) are indicated
 594 as follows: *** $P < 0.000$. The black lines inside the boxes mark the medians. Whiskers indicate
 595 variability outside the upper and lower quartiles. Circles, labelled with the individual case numbers,
 596 represent outliers (1.5 to 3 times length of the box), .

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Figure 4. Predicted mRNA targets for DE-miRNAs. (A) Venn diagram showing the genes that are potential targets of DE-miRNAs; (B) TargetScan software was used to identify potential mRNA targets. The list was filtered by selecting those genes that encode for ion channels: solute carrier family transporters (SLC), potassium voltage-gated channel (KCN), voltage-gated sodium channel (SCN), gamma-aminobutyric acid (GABA) receptor, voltage-dependent calcium channel (CACN), adrenoceptor alpha (ADRA), potassium channel (tetramerization domain) (KCTD),

606 hyperpolarization activated cyclic nucleotide-gated potassium channel (HCN), two pore segment
607 channel (TPCN), transient receptor potential cation channel (TRP). (C) Glutamatergic pathway
608 identified by KEGG pathway analysis using the mRNA targets. Proteins coded by mRNA targets
609 genes are highlighted in red. GLNT and EAAT belong to solute carrier family transporters (SLC);
610 VGCC to voltage-dependent calcium channel (CACN).

611