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(Article begins on next page)

1 **Dysregulated miRNAs in a canine model of hemangiosarcoma metastatic to the brain**

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6

7

8 **Abstract**

9 Hemangiosarcoma is a highly metastatic and lethal cancer of blood vessel-forming cells that
10 commonly spreads to the brain in both humans and dogs. Dysregulations in Phosphatase and Tensin
11 Homolog (PTEN) have been identified in various types of cancers, including hemangiosarcoma.
12 MicroRNAs (miRNAs) are short noncoding single-stranded RNA molecules that play a crucial role
13 in regulating gene expression. Some miRNAs can function as oncogenes or tumor suppressors,
14 influencing important processes in cancer, such as angiogenesis. This study aimed to investigate
15 whether miRNAs targeting PTEN were disrupted in canine hemangiosarcoma and its corresponding
16 brain metastases (BM). The expression levels of miRNA-10b, miRNA-19b, miRNA-21, miRNA-141
17 and miRNA-494 were assessed in samples of primary canine cardiac hemangiosarcomas and their
18 matched BM. Furthermore, the miRNA profile of the tumors was compared to samples of adjacent
19 non-cancerous tissue and healthy control tissues.

20 In primary cardiac hemangiosarcoma, miRNA-10b showed a significant increase in expression, while
21 miRNA-494 and miRNA-141 exhibited downregulation. Moreover, the overexpression of miRNA-
22 10b was retained in metastatic brain lesions. Healthy tissues demonstrated significantly different
23 expression patterns compared to cancerous tissues. In particular, the expression of miRNA-10b was
24 nearly undetectable in both control brain tissue and perimetastatic cerebral tissue. These findings can
25 provide a rationale for the development of miRNA-based therapeutic strategies, aimed at selectively
26 treating hemangiosarcoma.

27

28 Keywords: brain metastases, dog, hemangiosarcoma, miRNA, PTEN

29

30

31 **Introduction**

32 In dogs, hemangiosarcoma is a common malignant and highly metastatic cancer of blood vessel-
33 forming cells. It shares similarities with its human counterpart in terms of biological behavior,
34 treatment response, and prognosis.¹⁻³ Metastases occur early in the disease process, with up to 80%
35 of dogs presenting with evident metastatic disease.⁴ Notably, in dogs, hemangiosarcoma is one of the
36 tumors that most often metastasize to the brain.^{5,6} Brain metastases (BM) present a challenging
37 treatment scenario due to the limited permeability of the blood-brain barrier to systemic therapies and
38 the advanced stage of the disease.^{7,8} Historically, the prognosis for patients with BM, both in human
39 and veterinary oncology, has been extremely poor.⁹ As a result, there is an unmet need to develop
40 strategies for preventing BM in both human and veterinary oncology patients.

41 The exact pathogenesis of canine hemangiosarcoma remains largely unknown, and it often remains
42 unidentified in most cases. However, there are established factors that contribute to its development,
43 including genetic predisposition, environmental factors, and the inactivation of tumor suppressor
44 genes, such as Phosphatase and Tensin Homolog (PTEN).¹⁰ Recent research has demonstrated that
45 the PTEN pathway is frequently disrupted in canine hemangiosarcoma, either through inactivating
46 mutations or epigenetic downregulation.¹¹⁻¹⁴

47 MicroRNAs (miRNAs) are small RNA molecules, typically 18-25 nucleotides in length, that are
48 highly conserved and noncoding. They play a crucial role in gene expression regulation following
49 transcription by specifically binding to complementary target messenger RNAs (mRNAs).^{15,16}

50 MiRNAs are involved in the regulation of various cellular processes, including cell differentiation,
51 angiogenesis, proliferation and apoptosis.^{15,16} Depending on the target mRNAs they interact with,
52 miRNAs can function as either oncogenes or tumor suppressors, and there is substantial evidence

53 supporting a causal relationship between dysregulation of miRNAs and the development of
54 cancer.^{15,17}

55 Among the hundreds of miRNAs implicated in cancer development, a significant contribution to its
56 pathogenesis is attributed to those that inhibit the expression of PTEN, including miRNA-494,
57 miRNA-141, miRNA-21, miRNA-10b, and miRNA-19b.¹⁸⁻²³

58 Considering this, the aforementioned miRNAs targeting PTEN were chosen for examination in
59 canine cardiac hemangiosarcoma and matched BM. To accomplish this, the miRNA profile of the
60 tumors was compared to samples of adjacent non-cancerous tissue and healthy control tissues.

61

62 **Materials and methods**

63 *Histopathology*

64 Formalin-fixed and paraffin-embedded (FFPE) post-mortem samples of primary cardiac
65 hemangiosarcoma and matched BM from companion dogs were retrieved from the tissue bank of the
66 (*masked for review*).

67 FFPE post-mortem samples of right cardiac auricle (n=6) and brain (n=6) from dogs deceased from
68 tumor-unrelated causes were included as controls.

69 Ethical approval was not required since the study was conducted on archived FFPE tissue blocks
70 collected for diagnostic purposes.

71 Four-millimeter cores of each primary tumor (PT), BM, perimetastatic brain (periBM) and control
72 samples were punched out manually from donor blocks by use of commercially available skin biopsy
73 punches and transferred to blank recipient paraffin blocks (one for each sample). The resulting blocks
74 were placed in a conventional tissue embedding machine and re-embedded to completely integrate
75 the donor tissue cores into the surrounding paraffin.

76 For PT and BM, the cores were chosen based on prior microscopic examination of the areas of interest
77 in HE-stained sections. The first section of the new blocks was histologically evaluated to confirm
78 the presence of neoplastic cells. For periBM, the perimetastatic tissue was observed histologically,

79 and the non-neoplastic area of interest was marked. Subsequently, the corresponding area of the tissue
80 block was sampled with a punch and reincluded in a new block. The first section of the new block
81 was examined histologically to exclude the accidental sampling of tumor cells.

82

83 *RNA extraction*

84 Twenty sections from each block were cut in a microtome at 10 μ m and placed in 1.5 ml tubes for
85 RNA extraction.

86 Peri-tumoral cardiac tissue was not analyzed, since hemangiosarcomas mostly occupied the whole
87 right cardiac auricle, leaving no healthy tissue around.

88 Total RNA enriched by miRNA was extracted using the miRNeasy FFPE kit (Cat. 217504, Qiagen,
89 Hilden, Germany) following the manufacturer's instructions. We performed a first step of
90 deparaffinization using xylene, followed by ethanol washing and proteinase K incubation. The total
91 RNA enriched by miRNA was then extracted using the dedicated columns and the elution step was
92 performed in RNase-free water.

93 Representative samples of the extracted RNA (BM, n = 7; periBM, n = 6; PT, n = 6; CH, n = 4; CB,
94 n = 4) was used to perform miRNA sequencing (miRNAseq) to obtain quality controls on miRNA
95 integrity and reliability in terms of phred score, identifying the overall quality, and the trimmed reads'
96 length, to evaluate the integrity of miRNAs. Sequencing quality analysis were performed at Qiagen
97 company, using the Illumina technology specific for small RNA sequence (15 – 55 nt).

98

99 *Reverse transcription*

100 For the reverse transcription step the miRCURY LNA RT kit (Cat. 339340, Qiagen) was used
101 following the manufacturer's instruction. Briefly, according to the concentration and total quantity of
102 extracted RNA, 5–10 μ l of each sample were diluted to reach a concentration of 5 ng/ μ l. For each
103 reaction, 2 μ l (10 ng total) of RNA were used in the reverse transcription mix, which also included
104 the miRCURY RT enzyme (1 μ l of the 10x solution), the miRCURY RT Reaction buffer for SYBR

105 green (2 µl of the 5x solution), the synthetic RNA spike-ins used as RT positive control template (0.5
106 µl) and 4.5 µl of RNase-free water, for a total volume of 10 µl per reaction. No-RT sample was added,
107 using the same reaction mix without the RT enzyme.

108 Samples were incubated for 60 minutes at 42°C for the reverse transcription reaction and for 5
109 minutes at 95°C for enzyme inactivation, using the thermocycler Byometra (Bio-rad, Hercules, CA,
110 USA), then stored at 4°C.

111

112 *miRNA expression analysis*

113 The miRCURY LNA SYBR Green PCR kit (Cat. 339346, Qiagen) was used for miRNA qPCR
114 analysis. The following specific miRCURY LNA PCR Assay (Cat. 339306, Qiagen) were used to
115 analyze specific miRNA expression: miRNA-10b (YP00205499 – rno-miR-10b-5p), miRNA-19b
116 (YP02105441 – cfa-miR-19b), miRNA-21 (YP00204230 – has-miR-21-5p), miRNA-141
117 (YP02110018 – cfa-miR-141) and miRNA-494 (YP00204579 – has-miR-494-3p). The U6 snRNA
118 was used as normalizer RNA (YP02119464, v2). Moreover, as positive RT control, the UniSP6 assay
119 (YP00203954) was used to amplify the synthetic RNA employed during the RT step for each reaction.
120 Following the manufacturer's instructions, all samples were diluted 1:60, adding 590 µl of RNase-
121 free water to the 10 µl of the RT sample. Each reaction was composed by the miRCURY SYBR®
122 Green Master Mix (5µl of the 2x solution), 1 µl of the specific PCR assay, 1 µl of the RNase-free
123 water, and 3 µl of the diluted RT sample, for a total reaction volume of 10 µl.

124 The reactions were performed using the CFX96 machine (Bio-rad) with the following cycles: 2
125 minutes at 95°C (PCR initial heat activation), 2-step cycling (40 cycles) of 10 seconds at 95°C
126 (denaturation) and of 60 seconds at 56°C (annealing/extension), followed by the melting curve
127 analysis (60 – 95°C). Fluorescence data collection was performed during the annealing/extension
128 step.

129

130 *Quality control*

131 Before proceeding with the qPCR data elaboration for the relative quantification of miRNA
132 expression, we conducted a series of quality controls to ensure a reliable analysis, particularly due to
133 the potential degradation induced by formalin fixation.

134 First, we assessed the RNA extraction yield using a spectrophotometer (Nanodrop 2000, Thermo-
135 fisher scientific, Waltham, MA, USA) and concurrently quantified the 260/280 nm ratio for each
136 sample. The data is included in Supplementary Table S1. The extracted RNA exhibited high
137 concentrations (>50 ng/ μ l) in 23 out of 32 samples ($\approx 72\%$) and low concentrations (<10 ng/ μ l) in
138 only 1 sample ($\approx 3\%$). Additionally, all analyzed samples displayed a 260/280 nm ratio exceeding
139 the minimum threshold for high purity (>1.7).

140 The subsequent qPCR experiments validated the consistency of Ct values for each analyzed miRNA
141 within their respective experimental groups. As outlined in the methods section, considering the
142 specified number of samples per group, we conducted a total of 32 runs (in technical duplicates) for
143 each of the 5 miRNAs of interest, resulting in a grand total of 160 runs. We categorized these runs
144 based on their Ct values, defining "expressed" as $Ct \leq 35$, "low-expressed" as $Ct = 36 - 38$, and "not-
145 expressed" as $Ct > 38$. Out of the 160 runs, the majority (104) were classified as "expressed" miRNAs,
146 while 44 were labeled as "low-expressed," and only 12 fell under the "not-expressed" category.
147 Among the five miRNAs (miRNA-10b, miRNA-19b, miRNA-21, miRNA-141, and miRNA-494),
148 only miRNA-141 and miRNA-494 exhibited a "low-expression" profile, with only 2 samples in each
149 miRNA group being marked as "not expressed" (Supplementary Table S2).

150 In the quality miRNAseq control, all samples exhibited a phred value ranging from 35 to 40. Phred
151 levels are typically categorized as poor (0 – 20), medium (20 – 28), and good (>28). Therefore, all
152 samples demonstrated high quality. This was further affirmed by the distribution of trimmed reads'
153 lengths, which exhibited a distinct peak around 21 nt for all samples, a value characteristic of typical
154 miRNA length.

155 Based on the combined assessment of RNA quantification data, Ct values and miRNAseq analysis
156 obtained from all samples, we deemed the miRNA-qPCR analysis to be reliable, allowing us to
157 proceed with the relative expression quantification.

158

159 *Data analysis*

160 The Geneglobe online software (geneglobe.qiagen.com) was used to analyze the entire experimental
161 dataset in accordance with the quality controls provided by the kit. It was also utilized for the
162 processing of miRNA expression compared to healthy tissues (Figure 2).

163 The $2^{(-\Delta\Delta Ct)}$ method was used for the calculation of gene expression in all other analyses (Figure 3
164 and 4). After the first normalization on the normalizer U6 snRNA (ΔCt), a second normalization was
165 performed on the reference group, depending on the analysis. The reference group is specified in each
166 figure legend. The average, minimum, and maximum Ct values for each group and each miRNA are
167 reported in Supplementary Table S2.

168 For miRNA-10b expression in brain-derived tissues, two different analyses were performed: i) as for
169 the other graphs, $2^{(-\Delta\Delta Ct)}$ was calculated using the average ΔCt values for each group (Figure 4A; see
170 Supplementary Table S3 for raw data and elaboration); ii) each BM sample was normalized to its
171 periBM control derived from the same animal (Figure 4B; see Supplementary Table S4 for raw data
172 and elaboration).

173 The differential expression of the evaluated miRNAs was analyzed on ΔCt with One Way ANOVA,
174 followed by Tuckey's post-test, or Student's T test. Statistical analysis was performed with Prism v.
175 5.0 (GraphPad, San Diego, CA, USA). Significance was set at $P < 0.05$.

176

177 *PTEN immunohistochemistry*

178 Automated immunohistochemistry (IHC) was performed on the Discovery ULTRA system (Roche,
179 Ventana Medical Systems Inc., Tucson, AZ, USA), using the primary antibody anti-PTEN (mouse
180 monoclonal, clone A2B1, diluted 1:50, Santa Cruz Biotechnologies, Santa Cruz, CA, USA, cod. SC-

181 7974), which has previously been described to cross-react with canine tissues.²⁴ Briefly, 3- μ m-thick
182 sections were mounted onto superfrost plus slides, deparaffinized in aqueous-based detergent solution
183 (Discovery Wash, Ventana) and underwent heat induced antigen retrieval (CC2, pH 6.0 32 min,
184 91°C). The primary antibody (dilution 1:10) was incubated for 1 h at room temperature (RT). The
185 OmniMap anti-Mouse HRP (Roche) secondary antibody was incubated for 16 min at RT and the
186 chromoMap DAB (Roche) was used as chromogen. After detection, sections were counterstained
187 with hematoxylin (Hematoxylin II, Ventana), dehydrated and mounted with Eukitt (Kalttek, Padova,
188 Italy). Positive controls from canine tissues (kidney and prostate) and sections with omission of the
189 primary antibodies (negative controls) were included in each run.

190

191 **Cell Line Validation Statement**

192 No cell lines were used in the current study.

193

194 **Results**

195

196 *Clinical, histologic and immunohistochemical data*

197 Seven cases of canine cardiac hemangiosarcoma with BM were included in the analysis. There were
198 4 intact males and 3 spayed females. Median age was 10 years (range, 6-13). Breeds included one
199 each of English setter, German Shepherd, Boxer, Akita, Cane Corso, Maremma Shepherd and mixed
200 (Table 1).

201 Only one dog underwent therapy: after the removal of the cardiac tumor, chemotherapy was
202 administered, followed by an experimental treatment. Survival time for this dog was 394 days. All
203 the other dogs were euthanized upon diagnosis.

204 At necropsy, all dogs had multiorgan metastases, with the primary tumor being located in the right
205 cardiac atrium or auricle. The heart has been hypothesized as the primary site because the other
206 metastatic organs showed widespread lesions.

207 The primary tumor was sampled during post-mortem examination in 6 dogs. In one case the primary
208 cardiac tumor had been surgically removed 13 months prior at a different veterinary clinic, and the
209 paraffin block could not be obtained.

210 BM were sampled during post-mortem examination in all 7 dogs; they were mostly located within
211 the cortex, in the parietal, frontal or temporal lobes, and consisted of 5 to 20 round multifocal
212 hemorrhagic foci, 0.1-2 cm in diameter (Fig. 1A). Histologically, neoplastic cells were spindle-
213 shaped, variably pleomorphic, arranged in irregular channels filled with erythrocytes and associated
214 with hemorrhages (Fig. 1B).

215

216 *miRNA analysis*

217 In the first subset of samples, we analyzed miRNA expression in control tissues using the GeneGlobe
218 online software for calculating the fold difference (FD) and conducting statistical analysis. We
219 utilized the preset options for miRNA analysis through the miRCURY LNA PCR Assay. When
220 comparing the expression of the five target miRNAs in control hearts and brains, the scatter plot and
221 the clustergram revealed that all miRNAs were more expressed in the heart (Fig. 2A). However, only
222 the expression of miRNA-10b resulted significantly different ($P=0.004$), with a FD of more than 300
223 times (Fig. 2B).

224 When comparing miRNA expression between cardiac hemangiosarcomas and control hearts,
225 miRNA-10b, miRNA-141 and miRNA-494 were significantly dysregulated. MiRNA-10b was
226 significantly overexpressed, with a mean FoC of 4.2 compared with control cardiac tissues ($P=0.031$;
227 Fig. 3A). MiRNA-141 and miRNA-494 were significantly underexpressed, with a mean FoC of 0.05
228 ($P<0.001$; Fig. 3B) and 0.15 ($P=0.02$; Fig. 3C), respectively. No significant differences were observed
229 in the expression levels of the remaining miRNAs.

230 Overall, miRNA-10b in BM was significantly overexpressed, with a mean FoC of 11.6 and 7.7
231 compared with periBM ($P=0.025$) and control brains ($P=0.013$), respectively (Fig. 4A). The

232 expression levels of miRNA-10b in periBM and control brains were similar and extremely low,
233 requiring a mean number of amplification cycles of 38 and 39, respectively.

234 The expression levels of miRNA-10b were significantly lower in BM compared with the primary
235 tumor ($P < 0.001$), with a mean FD of 0.009. The individual ΔC_t values utilized for the miRNA-10b
236 analysis, along with the corresponding elaboration used to generate the FD values shown in Fig. 4A,
237 are provided in Supplementary Table S3.

238 Normalizing the expression of miRNA-10b in BM to the periBM of the same animal, an upregulation
239 was observed in 4 dogs (57%), ranging from 17.12 to 174.85 folds (Fig. 4B). However, there was no
240 correlation in the expression of miRNA-10b between BM and PT. The individual ΔC_t values used
241 for the miRNA-10b single-sample normalization analysis, along with the corresponding elaboration
242 used to generate the graph shown in Fig. 4B, are included in Supplementary Table S4.

243 In BM, miRNA-494 was downregulated compared with normal brain, although not statistically
244 significant ($P = 0.074$).

245 No other differentially expressed miRNAs were observed in BM compared with periBM or normal
246 brains.

247

248 *PTEN immunohistochemistry*

249 PTEN immunohistochemical expression was assessed subsequent to miRNA analysis in 6 out of 6
250 PT and in 3 out of 7 BM due to insufficient tissue availability. Overall, moderate to strong nuclear
251 and cytoplasmic positivity was observed in 2 out of 6 (33.3%) PT and 1 out of 3 (33.3%) BM (Figs.
252 1C and 1D). Considering the two PTEN-positive PTs, the positivity was confirmed in the
253 corresponding BM in one case, while in the other case, unfortunately, there was not enough brain
254 tissue available to conduct the analysis. The PTEN-positive BM also exhibited low miRNA-10b
255 expression, whereas moderate to high miRNA-10b expression was detected in the 2 PTEN-positive
256 PT (Table 1).

257

258

259 **Discussion**

260

261 Considering the significant role of PTEN in canine hemangiosarcoma, we selected 5 miRNAs that
262 are known to be involved in its regulation. The aim was to investigate whether these miRNAs
263 exhibited dysregulation in both primary and metastatic brain tissues, and to compare their expression
264 with that in normal control tissues.

265 Among the miRNAs analyzed in this study, miRNA-10b, miRNA-141 and miRNA-494 demonstrated
266 significant dysregulation in primary cardiac hemangiosarcoma. Furthermore, miRNA-10b exhibited
267 sustained overexpression in metastatic brain lesions. MiRNA-10b showed elevated expression levels
268 in both PT and matched BM, while miRNA-494 and miRNA-141 displayed reduced expression in
269 primary cardiac hemangiosarcoma.

270 The findings of this study are consistent with previous research in human patients. The upregulation
271 of miRNA-10b and dysregulation of miRNA-141 and miRNA-494 have been shown to function by
272 inhibiting the tumor suppressor PTEN.^{20,22,25} Loss of PTEN function is commonly observed in various
273 cancers, leading to the accumulation of its activated downstream effectors, such as AKT, a Ser/Thr
274 kinase associated with proliferation, migration, and growth.^{22,26}

275 MiRNA-10b has been studied to the greatest degree in human breast cancer, and its overexpression
276 has been associated with increased invasive potential both in vitro and in vivo.²² To date, more than
277 100 studies have been carried out on miRNA-10b across 18 human cancer types, demonstrating its
278 pleiotropic effects as a driver of tumor invasiveness and metastasis.²²

279 In various types of human cancers, the expression of miRNA-494 and miRNA-141 has yielded
280 conflicting results, suggesting tissue specificity. For instance, miRNA-494 is upregulated in
281 hepatocellular, colorectal and non-small-cell lung carcinomas, while it is downregulated in gastric,
282 prostatic, esophageal, mammary, pancreatic carcinomas, as well as in chondrosarcomas.²⁷⁻³² This dual

283 role also applies to miRNA-141, as observed in various human cancers, where some show
284 overexpression while others exhibit downregulation.³³

285 In dogs, preliminary evidence of the dysregulation of miRNA-10b and miRNA-141 has been
286 provided in mammary cancer and intestinal T-cell lymphomas, respectively.^{34,35} MiRNA-494
287 dysregulation has been reported in serum between dogs with splenic masses (namely
288 hemangiosarcoma or hematoma), however, conversely to the present study, an upregulation was
289 identified.³⁶

290 The present study identified that miRNA-494 and miRNA-141 were downregulated in cardiac
291 hemangiosarcomas. It is conceivable that, similar to human medicine, in dogs, the function of
292 miRNA-494 is dependent on tumor type and tissue type. This could potentially account for the
293 variation in expression observed between the spleen, as previously reported, and the brain, as outlined
294 in this study.

295 Up to 30% of canine and human patients with hemangiosarcoma develop BM, with the highest
296 frequency observed in tumors originating from cardiac tissue.^{5,6,37,38} Unlike metastases in other
297 anatomic locations, BM are subjected to the unique biology of the brain and its microenvironment.
298 This creates significant selective pressure on cancer cells, leading to adaptations that confer resistance
299 to current cancer therapeutics.^{8,9}

300 Previous studies have shown that the manipulation of specific miRNA alterations using miRNA
301 mimics or antagomirs can restore the abnormal signaling downstream pathways, offering a promising
302 therapeutic approach for cancer treatment.³⁹ However, the therapeutic use of miRNAs has been
303 hindered by safety concerns, particularly regarding off-target biological effects.^{40,41} Indeed, due to
304 the wide range of genes they regulate, each individual miRNA can have numerous targets, potentially
305 leading to unintended changes.⁴⁰ As of now, no miRNA-based drug candidates have advanced to
306 phase 3 clinical trials in humans.

307 In the current study, the expression profiles in healthy tissues differed significantly from those
308 observed in cancerous tissues. Specifically, the expression of miRNA-10b was nearly undetectable

309 in both normal brain tissue and perimetastatic cerebral tissue. The subtly altered miRNA profiles in
310 periBM compared to control brains may be attributed to isolated tumor cells that went undetected
311 during histologic examination, resulting in brain tissue dilution. Alternatively, it may be dependent
312 on phenomena related to the tumor microenvironment.

313 Manipulating the miRNA profile observed in hemangiosarcoma could potentially involve inhibiting
314 miRNA-10b if it is overexpressed, and mimicking miRNA-141 and miRNA-494 if they are lost. This
315 approach may enable targeted attacks on cancer cells while minimizing toxicity to healthy tissues.
316 However, further studies are needed to investigate the miRNA expression profile in other canine
317 tissues to identify any potential toxicity. Also, our results demonstrate the complexity of the topic,
318 particularly referring to tissue-specificity, and add intricacy to potential therapeutic aspects, but they
319 help shed light on the function of these miRNAs.

320
321 The semi-quantitative approach used in this study to analyze miRNA expression has certain
322 limitations, particularly in accurately quantifying low-expressed genes, necessitating two different
323 normalization steps. Therefore, a quantitative real-time PCR analysis encompassing the entire
324 dysregulation of the miRNome in canine hemangiosarcoma is warranted to better understand the
325 involvement of miRNAs in the development of this tumor.

326 Furthermore, there are several challenges that contribute to the complexity of the issue. While
327 miRNA-10b demonstrated overall overexpression in BM compared to control healthy brains and
328 perimetastatic cerebral tissues, 3 dogs did not exhibit dysregulation. This finding is not unexpected,
329 as miRNA expression is a dynamic process that undergoes temporal patterns. Some miRNAs are
330 strongly induced or silenced by various biological stimuli, including chemotherapy, while others may
331 be consistently over- or under-expressed during development and potentially disease progression.^{42,43}
332 Additionally, miRNA degradation may be regulated by disease-unrelated changes, which can affect
333 miRNA turnover rates and consequently impact the interpretation of phenotypic changes.⁴⁴

334 Of note, one of the BM showing no miRNA-10b dysregulation had immunohistochemically
335 detectable PTEN, suggesting a potential negative regulatory role for miRNA-10b in PTEN
336 expression, whereas in the remaining 2 cases, PTEN expression could not be assessed. This finding
337 is not unexpected, as the over- or underexpression of miRNAs is thought to result in down- or
338 upregulation of the protein product of the target genes, thereby affecting tumorigenesis. However,
339 since we were not able to analyze PTEN gene expression and mutational status, a definitive
340 conclusion cannot be drawn, and it remains to be determined if the observed miRNA alterations are
341 truly tumor drivers.

342 Finally, all cases, except for one, were euthanized at the time of diagnosis. Therefore, it was not
343 possible to correlate outcome data with the miRNA profile.

344

345 In conclusion, the dysregulation of multiple miRNAs targeting PTEN was observed in canine cardiac
346 hemangiosarcoma and matched BM. Upon demonstration of their effects on tumor progression, these
347 findings may provide a basis for developing a therapeutic strategy that selectively targets neoplastic
348 disease. Using dogs with naturally occurring hemangiosarcoma as a preclinical model for drug testing
349 may hold promise in advancing towards a pharmaceutical breakthrough for human treatment as well.

350

351

352 **Data availability**

353 The datasets generated during and/or analyzed during the current study are available from the
354 corresponding author on reasonable request.

355

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460 **Figure legends**

461 **Figure 1.** A) Dog, brain metastasis of hemangiosarcoma. B) Histologic preparation of the metastatic
462 lesion seen in A); hematoxylin and eosin, 20x magnification. C) Primary cardiac tumor showing
463 intense PTEN positivity; hematoxylin counterstain, 20x magnification. D) Brain metastasis with
464 positive PTEN expression; hematoxylin counterstain, 10x magnification.

465 **Figure 2.** miRNA-10b expression in control tissues and expression regulation in brain metastasis and
466 cardiac hemangiosarcoma (CA). **A)** Scatter plot analysis of the target miRNAs (miRNA-10b,
467 miRNA-141, miRNA-494, miRNA-21, and miRNA-19b) in healthy control heart tissues (CH)
468 compared to healthy control brain tissues (CB). A clustergram is included in the figure, representing
469 the magnitude of expression of the different miRNA in the whole samples. Colors represent the
470 magnitude of expression (green: minimum, red: maximum) of the same miRNA within all the
471 samples. **B)** Volcano plot representation of the Fold of Difference (FD) considering the statistical
472 significance of $P < 0.05$. A table is included with all the average values of the FD of CH compared
473 to CB. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

474 **Figure 3.** Graph representation of the semiquantitative analysis of miRNA-10b (**A**), miRNA-141
475 (**B**) and miRNA-494 (**C**) expression in cardiac hemangiosarcoma (primary tumor, PT) normalized on
476 control heart tissue (CH). Bars represent mean + SEM. Asterisks represent significant differences
477 between groups. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

478 **Figure 4.** **A)** Graph representation of the semiquantitative analysis of the miRNA-10b expression in
479 brain metastasis (BM) and brain perimetastatic tissue (periBM) normalized on control brain tissue
480 (CB). Bars represent mean + SEM. Asterisks represent significant differences between groups. **B)**
481 Graph representation of the semiquantitative analysis of miRNA-10b expression in each BM
482 compared to periBM of the same subject. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

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