

# Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

Dysregulated miRNAs in a canine model of haemangiosarcoma metastatic to the brain

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Sabattini, S., Baldassarro, V.A., Zaccone, R., Calzà, L., Giardino, L., Vascellari, M., et al. (2024). Dysregulated miRNAs in a canine model of haemangiosarcoma metastatic to the brain. VETERINARY AND COMPARATIVE ONCOLOGY, 22(1), 70-77 [10.1111/vco.12949].

Availability:

This version is available at: https://hdl.handle.net/11585/954534 since: 2024-01-30

Published:

DOI: http://doi.org/10.1111/vco.12949

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (https://cris.unibo.it/). When citing, please refer to the published version.

(Article begins on next page)

- Dysregulated miRNAs in a canine model of hemangiosarcoma metastatic to the brain
   2
- 3

4 **Word count: 3672** 

- 5 Number of figures and tables: 4 figures, 1 table
- 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.

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

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.<sup>1-3</sup> Metastases occur early in the disease process, with up to 80% of dogs presenting with evident metastatic disease.<sup>4</sup> Notably, in dogs, hemangiosarcoma is one of the 35 tumors that most often metastasize to the brain.<sup>5,6</sup> Brain metastases (BM) present a challenging 36 37 treatment scenario due to the limited permeability of the blood-brain barrier to systemic therapies and the advanced stage of the disease.<sup>7,8</sup> Historically, the prognosis for patients with BM, both in human 38 and veterinary oncology, has been extremely poor.<sup>9</sup> As a result, there is an unmet need to develop 39 40 strategies for preventing BM in both human and veterinary oncology patients.

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

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).<sup>15,16</sup> 50 MiRNAs are involved in the regulation of various cellular processes, including cell differentiation, 51 angiogenesis, proliferation and apoptosis.<sup>15,16</sup> Depending on the target mRNAs they interact with, 52 miRNAs can function as either oncogenes or tumor suppressors, and there is substantial evidence supporting a causal relationship between dysregulation of miRNAs and the development of
 cancer.<sup>15,17</sup>

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

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*

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

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

Ethical approval was not required since the study was conducted on archived FFPE tissue blockscollected for diagnostic purposes.

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

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

and the non-neoplastic area of interest was marked. Subsequently, the corresponding area of the tissue
block was sampled with a punch and reincluded in a new block. The first section of the new block
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 µm and placed in 1.5 ml tubes for
85 RNA extraction.

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

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

For the reverse transcription step the miRCURY LNA RT kit (Cat. 339340, Qiagen) was used following the manufacturer's instruction. Briefly, according to the concentration and total quantity of extracted RNA, 5–10  $\mu$ l of each sample were diluted to reach a concentration of 5 ng/ $\mu$ l. For each reaction, 2  $\mu$ l (10 ng total) of RNA were used in the reverse transcription mix, which also included the miRCURY RT enzyme (1  $\mu$ l of the 10x solution), the miRCURY RT Reaction buffer for SYBR 105 green (2  $\mu$ l of the 5x solution), the synthetic RNA spike-ins used as RT positive control template (0.5 106  $\mu$ l) and 4.5  $\mu$ l of RNase-free water, for a total volume of 10  $\mu$ l per reaction. No-RT sample was added, 107 using the same reaction mix without the RT enzyme.

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

111

## 112 miRNA expression analysis

The miRCURY LNA SYBR Green PCR kit (Cat. 339346, Qiagen) was used for miRNA qPCR 113 114 analysis. The following specific miRCURY LNA PCR Assay (Cat. 339306, Qiagen) were used to analyze specific miRNA expression: miRNA-10b (YP00205499 - rno-miR-10b-5p), miRNA-19b 115 (YP02105441 - cfa-miR-19b), miRNA-21 (YP00204230 - has-miR-21-5p), miRNA-141 116 117 (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 RNasefree water to the 10 µl of the RT sample. Each reaction was composed by the miRCURY SYBR® 121 122 Green Master Mix (5 $\mu$ l of the 2x solution), 1  $\mu$ l of the specific PCR assay, 1  $\mu$ l of the RNase-free 123 water, and  $3 \mu l$  of the diluted RT sample, for a total reaction volume of  $10 \mu l$ .

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

129

130 *Quality control* 

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

First, we assessed the RNA extraction yield using a spectrophotometer (Nanodrop 2000, Thermofisher scientific, Waltham, MA, USA) and concurrently quantified the 260/280 nm ratio for each sample. The data is included in Supplementary Table S1. The extracted RNA exhibited high concentrations (>50 ng/µl) in 23 out of 32 samples ( $\approx$ 72%) and low concentrations (<10 ng/µl) in only 1 sample ( $\approx$  3%). Additionally, all analyzed samples displayed a 260/280 nm ratio exceeding 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  $Ct \le 35$ , "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, while 44 were labeled as "low-expressed," and only 12 fell under the "not-expressed" category. 146 147 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).

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

Based on the combined assessment of RNA quantification data, Ct values and miRNAseq analysis obtained from all samples, we deemed the miRNA-qPCR analysis to be reliable, allowing us to 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 ( $\Delta$ Ct), 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.

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

173 The differential expression of the evaluated miRNAs was analyzed on  $\Delta$ Ct 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. <sup>24</sup> Briefly, $3-\mu$ 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 (Kaltek, 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.

#### 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

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

At necropsy, all dogs had multiorgan metastases, with the primary tumor being located in the right cardiac atrium or auricle. The heart has been hypothesized as the primary site because the other

206 metastatic organs showed widespread lesions.

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

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

215

#### 216 miRNA analysis

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

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

expression levels of miRNA-10b in periBM and control brains were similar and extremely low,
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

tumor (P<0.001), with a mean FD of 0.009. The individual  $\Delta$ Ct values utilized for the miRNA-10b

analysis, along with the corresponding elaboration used to generate the FD values shown in Fig. 4A,

are provided in Supplementary Table S3.

Normalizing the expression of miRNA-10b in BM to the periBM of the same animal, an upregulation

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  $\Delta$ Ct values used

for the miRNA-10b single-sample normalization analysis, along with the corresponding elaboration

used to generate the graph shown in Fig. 4B, are included in Supplementary Table S4.

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

No other differentially expressed miRNAs were observed in BM compared with periBM or normalbrains.

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

## 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.

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

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

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

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

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

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

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

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

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

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

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

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

320

The semi-quantitative approach used in this study to analyze miRNA expression has certain limitations, particularly in accurately quantifying low-expressed genes, necessitating two different normalization steps. Therefore, a quantitative real-time PCR analysis encompassing the entire dysregulation of the miRNome in canine hemangiosarcoma is warranted to better understand the 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.<sup>42,43</sup> 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.<sup>44</sup>

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.

Finally, all cases, except for one, were euthanized at the time of diagnosis. Therefore, it was notpossible to correlate outcome data with the miRNA profile.

344

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

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

#### 356 **References**

Fosmire SP, Dickerson EB, Scott AM, *et al.* Canine malignant hemangiosarcoma as a model
 of primitive angiogenic endothelium. *Lab Invest* 2004;84:562–572.

359	2.	Megquier K, Turner-Maier J, Swofford, et al. Comparative genomics reveals shared
360		mutational landscape in canine hemangiosarcoma and human angiosarcoma. Mol Cancer Res
361		2019;17:2410-2421.
362	3.	Wang G, Wu M, Durham AC, et al. Molecular subtypes in canine hemangiosarcoma reveal
363		similarities with human angiosarcoma. PLoS One 2020;15:e0229728.
364	4.	Griffin MA, Culp WTN, Rebhun RB. Canine and feline haemangiosarcoma. Vet Rec
365		2021;189:e585.
366	5.	Snyder JM, Lipitz L, Skorupski KA, Shofer FS, Van Winkle TJ. Secondary intracranial
367		neoplasia in the dog: 177 cases (1986-2003). J Vet Intern Med 2008;22:172-177.
368	6.	Waters DJ, Hayden DW, Walter PA. Intracranial lesions in dogs with hemangiosarcoma. J
369		Vet Intern Med 1989;3:222–230.
370	7.	Doolittle ND, Muldoon LL, Culp AY, Neuwelt EA. Delivery of chemotherapeutics across the
371		blood-brain barrier: challenges and advances. Adv Pharmacol 2014;71:203–243.
372	8.	Kim M, Kizilbash SH, Laramy JK, et al. Barriers to effective drug treatment for brain
373		metastases: a multifactorial problem in the delivery of precision medicine. Pharm Res
374		2018;35:177.
375	9.	Achrol AS, Rennert RC, Anders C, et al. Brain metastases. Nat Rev Dis Primers 2019;5:5.
376	10	. Kim JH, Graef AJ, Dickerson EB, Modiano JF. Pathobiology of Hemangiosarcoma in Dogs:
377		Research Advances and Future Perspectives. Vet Sci 2015;2:388-405.
378	11	. Wang G, Wu M, Durham AC, et al. Molecular subtypes in canine hemangiosarcoma reveal
379		similarities with human angiosarcoma. PLoS One 2020;15:e0229728.
380	12	. Dickerson EB, Thomas R, Fosemire SP, et al. Mutations of phosphatase and tensin homolog
381		deleted from chromosome 10 in canine hemangiosarcoma. Vet Pathol 2005:42:618-632.
382	13	. Wong S, Ehrhart EJ, Stewart S et al. Genomic landscapes of canine splenic angiosarcoma
383		(hemangiosarcoma) contain extensive heterogeneity within and between patients. PLoS One
384		2022;17:e0264986.

- 385 14. Wang G, Wu M, Maloneyhuss MA, *et al.* Actionable mutations in canine hemangiosarcoma.
   386 *PLoS One* 2017;12:e0188667.
- 387 15. Bartel DP. MicroRNA target recognition and regulatory functions. *Cell* 2009;136:215–233.
- 388 16. Kane NM, Thrasher AJ, Angelini GD, Emanueli C. Concise review: microRNAs as
   389 modulators of stem cells and angiogenesis. *Stem Cells* 2014;32:1059–1066.
- 390 17. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857–
  391 866.
- 392 18. Sun HB, Chen X, Ji H, *et al.* miR 494 is an independent prognostic factor and promotes cell
  393 migration and invasion in colorectal cancer by directly targeting PTEN. *Int J Oncol.*394 2014;45:2486-2494.
- 395 19. Ghafouri-Fard S, Abak A, Shoorei H, *et al.* Regulatory role of microRNAs on PTEN
  396 signaling. *Biomed Pharmacother* 2021;133:110986.
- 397 20. Gao Y, Feng B, Han S, *et al.* The Roles of MicroRNA-141 in Human Cancers: From
  398 Diagnosis to Treatment. *Cell Physiol Biochem* 2016;38:427-448.
- Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates
   expression of the PTEN tumor suppressor gene in human hepatocellular cancer.
   *Gastroenterology* 2007;133:647-658.
- 402 22. Sheedy P, Medarova Z. The fundamental role of miR-10b in metastatic cancer. *Am J Cancer*403 *Res* 2018;8:1674-1688.
- 404 23. Liu DT, Yao HR, Li YY, Song YY, Su MY. MicroRNA-19b promotes the migration and
  405 invasion of ovarian cancer cells by inhibiting the PTEN/AKT signaling pathway. *Oncol Lett*406 2018;16:559-565.
- 407 24. Asproni P, Ressel L, Millanta F, Vannozzi I, Poli A. Co-localization of PTEN and E-cadherin
  408 in canine mammary hyperplasias and benign and malignant mammary tumors. *Res Vet Sci*409 2015;103:113-8.

410	25. Ghorbanhosseini SS, Nourbakhsh M, Zangooei M, et al. MicroRNA-494 induces breast
411	cancer cell apoptosis and reduces cell viability by inhibition of nicotinamide
412	phosphoribosyltransferase expression and activity. EXCLI J 2019;18:838-851.

- 413 26. Wan H, Zhang D, Hu W, *et al.* Aberrant PTEN, PIK3CA, pMAPK, and TP53 expression in
  414 human scalp and face angiosarcoma. *Medicine (Baltimore)* 2021;100:e26779.
- 415 27. Liu K, Liu S, Zhang W, *et al.* miR-494 promotes cell proliferation, migration and invasion
  416 and increased sorafenib resistance in hepatocellular carcinoma by targeting PTEN. *Oncol Rep*417 2015;34:1003–1010.
- 418 28. Sun HB, Chen X, Ji H, *et al.* miR-494 is an independent prognostic factor and promotes cell
  419 migration and invasion in colorectal cancer by directly targeting PTEN. *Int J Oncol*420 2014;45:2486–2494.
- 421 29. Wang J, Chen H, Liao Y, *et al.* Expression and clinical evidence of miR-494 and PTEN in
  422 non-small cell lung cancer. *Tumour Biol* 2015;36:6965–6972.
- 30. Shen PF, Chen XQ, Liao YC, *et al.* MicroRNA-494-3p targets CXCR4 to suppress the
  proliferation, invasion, and migration of prostate cancer. *Prostate* 2014;74:756–767.
- 425 31. Song L, Liu D, Wang B, *et al.* miR-494 suppresses the progression of breast cancer in vitro
  426 by targeting CXCR4 through the Wnt/β-catenin signaling pathway. *Oncol Rep* 2015;34:525–
  427 531.
- 428 32. Li J, Wang L, Liu Z, *et al.* MicroRNA-494 inhibits cell proliferation and invasion of
  429 chondrosarcoma cells in vivo and in vitro by directly targeting SOX9. *Oncotarget*430 2015;6:26216–26229.
- 431 33. Gao Y, Feng B, Han S, *et al.* The Roles of MicroRNA-141 in Human Cancers: From
  432 Diagnosis to Treatment. *Cell Physiol Biochem* 2016;38(2):427-48.
- 433 34. Bulkowska M, Rybicka A, Senses KM, *et al.* MicroRNA expression patterns in canine
  434 mammary cancer show significant differences between metastatic and non-metastatic
  435 tumours. *BMC Cancer* 2017;17:728.

- 436 35. Joos D, Leipig-Rudolph M, Weber K. Tumour-specific microRNA expression pattern in
  437 canine intestinal T-cell-lymphomas. *Vet Comp Oncol* 2020;18:502-508.
- 438 36. Grimes JA, Robinson KR, Bullington AM, Schmiedt JM. Identification of serum microRNAs
  439 with differential expression between dogs with splenic masses and healthy dogs with
  440 histologically normal spleens. *Am J Vet Res* 2021;82:659-666.
- 37. Kim JH, Megquier K, Thomas R, *et al.* Genomically complex human angiosarcoma and
  canine hemangiosarcoma establish convergent angiogenic transcriptional programs driven by
  novel gene fusions. *Mol Cancer Res* 2021;19(5):847-861.
- 444 38. Siontis BL, Zhao L, Leja M, *et al.* Primary cardiac sarcoma: a rare, aggressive malignancy
  445 with a high propensity for brain metastases. *Sarcoma* 2019;2019:1960593.
- 446 39. Forterre A, Komuro H, Aminova S, Harada MA. Comprehensive review of cancer MicroRNA
  447 therapeutic delivery strategies. *Cancers (Basel)* 2020;12:1852.
- 448 40. Zhang H, Li Y, Lai M. The microRNA network and tumor metastasis. *Oncogene*449 2010;29:937–948.
- 41. Zhang S, Cheng Z, Wang Y, Han T. The risks of miRNA therapeutics: in a drug target
  perspective. *Drug Des Devel Ther* 2021;15:721–733.
- 452 42. Pauli A, Rinn JL, Schier AF. Non-coding RNAs as regulators of embryogenesis. *Nat Rev*453 *Genet* 2011;12(2):136-49.
- 454 43. Guan D, Zhang W, Zhang W, Liu GH, Belmonte JC. Switching cell fate, ncRNAs coming to
  455 play. *Cell Death Dis* 2013;4(1):e464.
- 456 44. Großhans H, Chatterjee S. MicroRNases and the regulated degradation of mature animal
  457 mirnas. Adv Exp Med Biol 2011;700:140-55.
- 458
- 459
- 460 **Figure legends**

Figure 1. A) Dog, brain metastasis of hemangiosarcoma. B) Histologic preparation of the metastatic
lesion seen in A); hematoxylin and eosin, 20x magnification. C) Primary cardiac tumor showing
intense PTEN positivity; hematoxylin counterstain, 20x magnification. D) Brain metastasis with
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 the magnitude of expression of the different miRNA in the whole samples. Colors represent the 469 470 magnitude of expression (green: minimum, red: maximum) of the same miRNA within all the samples. B) Volcano plot representation of the Fold of Difference (FD) considering the statistical 471 472 significance of P < 0.05. A table is included with all the average values of the FD of CH compared 473 to CB. \*  $P \le 0.05$ ; \*\*  $P \le 0.01$ ; \*\*\*  $P \le 0.001$ .

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

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