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## STANDARD ARTICLE

# Sensitivity and specificity of microRNA-122 for liver disease in dogs

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**Background:** Current tests for diagnosing liver disease in dogs are sub-optimal. MicroRNA-122 (miR-122) is a sensitive and specific biomarker of liver injury in humans and rodents. Circulating miR-122 could have utility in identifying dogs with liver disease.

**Objective:** Establish the reference interval for miR-122 in healthy dogs and determine performance in a range of dog breeds with liver disease and control animals with non-liver disease.

**Animals:** Stored serum from 120 healthy dogs, 100 dogs with non-liver diseases, and 30 dogs with histologically confirmed liver disease was analyzed.

**Methods:** Retrospective study. Medical records of dogs with liver disease, non-liver disease and healthy dogs were reviewed. Serum miR-122 concentrations were measured by PCR and compared with the characteristics of the dogs and their conventional clinical measurements.

**Results:** In healthy dogs the 2.5th, 50th, and 97.5th quartiles of miR-122 were 110 (90% CI 80-114), 594 (505-682), and 3312 (2925-5144) copies/ $\mu$ L, respectively. There was no difference between healthy dogs and dogs with non-liver disease (median  $\pm$  IQR: healthy dogs 609 [327-1014] copies/ $\mu$ L; non-liver disease 607 [300-1351] copies/ $\mu$ L). miR-122 was higher in dogs with liver disease (11 332 [4418-20 520] copies/ $\mu$ L,  $P < .001$  compared to healthy dogs). miR-122 identified dogs with liver disease with high accuracy (receiver operating characteristic area under curve for comparison with healthy dogs: 0.93 [95% CI 0.86-0.99]). The upper limit of normal for healthy dogs (3312 copies/ $\mu$ L) had a sensitivity of 77% and specificity of 97% for identifying liver disease.

**Conclusion and Clinical Importance:** Liver disease can be sensitively and specifically diagnosed in dogs by measurement of miR-122.

**KEYWORDS**

biomarker, canine, hepatic, microRNA

**Abbreviations:** °C, degree celsius;  $\mu$ L, microliter; ALT, alanine aminotransferase activity; ANOVA, analysis of variance; AP, alkaline phosphatase activity; AST, aspartate aminotransferase activity; AUC, area under the curve; *C. elegans*, *Caenorhabditis elegans*; cDNA, complementary deoxyribonucleic acid; CI, confidence intervals; copies/ $\mu$ L, copies per microliter;  $C_q$ , quantification cycle; CV, coefficient of variation; DILI, drug-induced liver injury; EMA, European Medicines Agency; F, female; FDA, Food and Drug Administration; GGT, gamma-glutamyltransferase activity; IFCC, International Federations of Clinical Chemistry; IQR, interquartile range; kg, kilogram; M, male; MIQE, minimum information for publication of quantitative real-time PCR experiments; miR-122, microRNA 122; miRNAs, microRNAs; *n*, sample size; NEC, no enzyme control; ng/ $\mu$ L, nanogram per microliter; NTC, no template control; *P*, *P*-value for statistical significance; PCR, Polymerase chain reaction; R(D)SVS, Royal (Dick) School of Veterinary Studies; RNA, ribonucleic acid; ROC, receiver operator characteristic; RT-PCR, real-time polymerase chain reaction; TE, tris-EDTA; U/L, units per liter

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## 1 | INTRODUCTION

Liver disease is a major cause of morbidity and mortality in dogs.<sup>1</sup> To reach a definitive diagnosis clinicians measure a panel of biochemical blood variables to identify dogs that would benefit from subsequent imaging and histopathological evaluation of liver biopsy specimens. The most commonly used circulating biochemical indicators of liver injury are alanine aminotransferase activity (ALT), aspartate aminotransferase activity (AST), alkaline phosphatase activity (AP), and gamma-glutamyltransferase activity (GGT). However, these current biochemical assays have sub-optimal sensitivity and specificity for detection of histologically confirmed liver disease.<sup>2</sup> Consequently, only ALT, which is present in high concentrations within the cytoplasm and mitochondria of canine hepatocytes, is widely used to assess dogs for the presence of liver injury and has emerged as the gold-standard marker of hepatocellular injury. The degree of increased serum ALT activity is roughly proportional to disease severity, but ALT might not increase (“false negative”) in a number of different scenarios such as reduced hepatocyte number due to advanced fibrosis, non-inflammatory primary or secondary neoplasia, and early in the course of hepatocellular disease.<sup>3,4</sup> Other non-liver diseases (eg, diabetes mellitus and hemolysis) might also result in elevated serum ALT activity.<sup>3,4</sup> In 191 clinically healthy labradors with histopathological abnormalities in the liver, the sensitivity of ALT and AP was only 45% (95% CI: 25%-65%) and 15% (0%-35%), respectively, for reporting acute hepatitis.<sup>2</sup> In the setting of chronic hepatitis, sensitivity was 71% (47%-94%) and 13% (0%-31%), respectively, for ALT and AP.<sup>2</sup>

For most liver diseases, histopathological evaluation of liver biopsy samples is needed for a definitive diagnosis and is used as the reference standard against which the accuracy of the other tests is compared. However, biopsy remains an invasive, costly procedure with associated risks of complications from the procedure. For example, a retrospective study demonstrated that 4% of dogs undergoing liver biopsy required a post-procedure blood transfusion.<sup>5</sup> In summary, there is a clear and urgent need to develop better diagnostic tests that will allow clinicians to diagnose liver disease in dogs earlier, and more accurately target invasive liver biopsy to dogs with a high likelihood of having serious liver disease.

MicroRNAs (miRNAs) are small (~22 nucleotide-long) non-protein coding RNAs that regulate post-transcriptional gene expression.<sup>6</sup> The properties of circulating microRNAs that support their utility as biomarkers include organ specificity (for certain microRNA species), relative stability in blood and their ability to be amplified and measured by polymerase chain reaction (PCR). miR-122 is highly expressed in hepatocytes accounting for up to 70% of the total liver miRNA content.<sup>7</sup> Multiple studies have demonstrated the sensitivity and specificity of miR-122 as a circulating biomarker of liver injury in chimpanzees,<sup>8</sup> mice,<sup>9</sup> rats,<sup>10</sup> zebrafish,<sup>11</sup> and in human studies of different liver diseases including drug-induced liver injury (DILI),<sup>12</sup> hepatitis C,<sup>13</sup> and ethanol consumption.<sup>14</sup> miR-122 has received regulatory support for further qualification by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in the context of DILI.<sup>15,16</sup> Importantly, miR-122 has enhanced sensitivity and specificity when compared with standard biomarkers. For example, miR-

122 accurately reports human DILI after acetaminophen overdose at first presentation to hospital at a time when current markers, such as ALT, are still within normal ranges.<sup>17,18</sup>

In dogs there have been a limited number of studies that suggest circulating miR-122 could have utility in diagnosing liver disease. In a pre-clinical drug development study, ALT was elevated in the absence of abnormal liver histology in beagles.<sup>19</sup> In this study miR-122 was measured and had increased specificity with regard to excluding important histological liver abnormality. In a study of 66 Labrador retrievers, miR-122 was reported to be a specific and sensitive biomarker for liver injury and copper accumulation.<sup>20</sup> The same research group reported that circulating miR-122 is particularly elevated in Labrador retrievers with mucoceles.<sup>21</sup> These discovery studies investigated only small numbers of a single breed of dog, namely labradors, and were insufficiently sized to determine the normal reference interval for miR-122. Also, they did not include unwell dogs with non-liver pathology (an important control population needed to establish biomarker specificity). Building on our biomarker qualification experience in humans, the aims of this study were to establish the reference interval for serum miR-122 concentration in healthy dogs and then determine the sensitivity and specificity of miR-122 as a biomarker of liver injury across multiple dog breeds.

## 2 | METHODS

### 2.1 | Animals

All dogs were recruited to this study at the Royal (Dick) School of Veterinary Studies (R[D]SVS), Edinburgh, UK. Healthy dogs presenting to the R[D]SVS general practice for routine annual vaccination, who had a normal history and clinical examination, were invited to have a serum biochemical health screen which included measurement of ALT. miR-122 was measured in consecutive healthy dogs which had a normal serum ALT activity. Consecutive dogs who had a final diagnosis of non-primary liver disease, had ALT measured as part of their clinical evaluation and had a residual serum sample retained were also enrolled into the study. Finally, consecutive dogs that had a diagnostic assessment which included histopathological examination of a liver biopsy and serum ALT measurement leading to definitive diagnosis of a primary liver disorder were enrolled into the study. The histopathological diagnosis was classified according to World Small Animal Veterinary Association (WSAVA) criteria by a board certified pathologist.<sup>22</sup> The study was approved by The University of Edinburgh Veterinary Ethics Research Committee.

### 2.2 | RNA isolation

Serum samples were stored at  $-80^{\circ}\text{C}$  until miRNA analyses. The median time in storage between blood sampling and RNA isolation was 402 days (IQR: 207-593,  $n = 250$ ) with 1091 days being the maximum storage time. This is less time in storage compared with published studies that demonstrate miRNA stability when frozen.<sup>23,24</sup> miRNA was extracted using a miRNeasy Serum/Plasma kit (Qiagen, Venlo, Netherlands) following the manufacturer's instructions. Total

RNA was extracted from 50  $\mu\text{L}$  of serum diluted in 150  $\mu\text{L}$  nuclease free water. Briefly, RNA was extracted from the serum by lysis reagent (1000  $\mu\text{L}$ ) and chloroform (200  $\mu\text{L}$ ). After centrifugation at 12 000g for 15 min at 4°C up to 600  $\mu\text{L}$  of the aqueous phase was transferred to a new tube with 900  $\mu\text{L}$  absolute ethanol. RNA was purified on a RNeasy minElute spin column and eluted in 15  $\mu\text{L}$  RNase-free water and stored at -80°C. Extraction efficiency was monitored by adding  $5.6 \times 10^8$  copies of synthetic *C. elegans* miR-39 spike-in control after the addition of lysis reagent before the addition of chloroform and phase separation.

### 2.3 | Reverse transcription and real-time polymerase chain reaction (RT-PCR)

The miScript II Reverse Transcription kit was used to prepare cDNA according to the manufacturer's instructions. Briefly, 2.5  $\mu\text{L}$  of RNA eluate was reverse transcribed into cDNA. The synthesised cDNA was diluted and used for cDNA template in combination with the miScript SYBR Green PCR kit (Qiagen, Venlo, The Netherlands) using the specific miScript assays (Qiagen, Venlo, The Netherlands). RT-PCR was performed in duplicate on a Light Cycler 480 (Roche, Burgess Hill, UK) using the recommended miScript cycling parameters.

In the current study, miRNA was quantified as copy number per  $\mu\text{L}$  by generating a standard curve. A calibration curve of  $C_q$  as a function of miRNA was determined by performing PCR on miR-39 at  $5 \times 10^5$ ,  $5 \times 10^4$ ,  $5 \times 10^3$ , and  $5 \times 10^2$  copies/ $\mu\text{L}$  (independent from serum samples) according to manufacturer's protocol (miRNeasy Serum/Plasma Spiked-In Control, Qiagen). This calibration curve was used to determine the recovery of miR-39 from the samples based on the 10 800 copies/ $\mu\text{L}$  spiked control in the final PCR reaction. The calibration curve was also used to determine the concentration of miR-122 in each sample following the protocol reported in the miRNeasy Serum/Plasma Handbook. Concentrations of miR-122 were corrected for the recovery measured for miR-39.

Repeatability was determined by measuring the intra-assay variability of miR-122 duplicates and was deemed acceptable as per previous studies<sup>25</sup> and expressed as concentration (copies/ $\mu\text{L}$ ) per MIQE guidelines<sup>26</sup> (CV: 11.84% [3.21%-15.92% IQR]). Reproducibility was determined by measuring inter-assay variability across plates and days by measuring miR-122 concentrations (copies/ $\mu\text{L}$ ) of reference samples. Variability was acceptable (CV 18.25% [7.55%-25.92% IQR]).<sup>25</sup> A no enzyme control (NEC), omitting the reverse transcriptase enzyme during reverse transcription, and no template control (NTC) omitting the cDNA in the RT-PCR plate were also included in every run. NEC and NTC controls had  $C_q$  values of 40.  $C_q$  values less than 40 were regarded as positive amplification signals.

### 2.4 | Sample size

The International Federation of Clinical Chemistry (IFCC) recommends that samples from 120 or more individuals are required for the development of reference intervals for analytes.<sup>27</sup>

### 2.5 | Statistical analyses

Data were summarized as median and range for summary statistics of the study subjects. Serum miR-122 concentrations from healthy dogs were used to calculate the overall reference intervals following recommended approaches.<sup>28,29</sup> The following three R packages were used: Outliers,<sup>30</sup> jmuOutlier,<sup>31</sup> and Quantreg.<sup>32</sup> Firstly, the distribution of miR-122 concentrations was investigated using visual inspection of QQ plots to determine whether it required a log or square root transformation. The appropriate transformation was applied, if required, to the miR-122 concentrations to approximate a Gaussian distribution, fulfilling the normality assumption of subsequent analyses. Outliers were identified and removed according to Tukey's method.<sup>33</sup> The distribution of the miR-122 concentrations, transformed (if appropriate) and with outliers removed, was inspected for an approximate Gaussian distribution before reference intervals were calculated. The 2.5th and 97.5th quartiles were derived in order to calculate the 95% reference intervals for miR-122 concentration. The 90% confidence intervals (CI) for the reference intervals were calculated through non-parametric methods.<sup>34</sup>

Differences in miR-122 concentrations and different breeds were calculated by non-parametric, Kruskal-Wallis test by ranks. The difference in miR-122 concentration between female and male dogs was measured by non-parametric Mann-Whitney *U* test. Associations between age and body weight and miR-122 concentrations were calculated by Spearman's rank correlations and simple linear regression. Differences between miR-122 concentrations in healthy controls, non-liver disease and liver disease subjects were measured by non-parametric Kruskal-Wallis test by ranks. Associations between ALT and miR-122 in healthy controls, non-liver disease and liver disease subjects were measured by Spearman's rank correlations and simple linear regression. Receiver operating characteristic (ROC) curve analyses were used to determine the sensitivity and specificity of miR-122 and ALT for detecting the presence of liver disease in dogs. Differences between the area under the curve (AUC) between miR-122 and ALT ROC curves were measured as per Hanley and McNeils method.<sup>35</sup> Normally distributed data were presented as mean  $\pm$  standard deviation and non-normally distributed data as median and interquartile range. Nominal *P* values equal to or less than .05 were considered significant. Statistical analyses were performed using Graphpad Prism (GraphPad Software, La Jolla, California).

## 3 | RESULTS

### 3.1 | Dog characteristics

Serum samples from 250 dogs were analyzed (120 healthy, 100 with non-liver diseases, and 30 with histologically confirmed liver disease). Characteristics of the healthy dogs including sex, age, breed, and clinical chemistry results are summarized in Table 1. The diagnoses of the dogs with non-liver disease are listed in Supporting Information TableS1. All the dogs with non-liver disease had normal range serum ALT activity (median: 42 U/L. IQR: 29-53). The characteristics of the dogs with liver disease are presented in Table 2.

**TABLE 1** Characteristics of the healthy dog group (n = 120 per group)

Breed (n = 120)	Sex (F, M)	Age (years)	Weight (kg)	ALT (U/L)	miR-122 (copies/ $\mu$ L)
Labrador Retriever and cross breed (n = 31)	F20, M11	5 (3.5-9)	27.6 (25.1-31.4)	36 (31.5-44.5)	486.1 (299.5-821.7)
Collie Border and cross breed (n = 11)	F7, M4	9 (6.5-10.5)	22.5 (20.5-24.7)	40 (32.5-45)	599.2 (496.3 - 2434.1)
Cocker Spaniel (n = 11)	F7, M4	4 (3-10)	15.4 (13.5-17.8)	30 (28-42)	774.7 (247.7 - 957.2)
Labrador Poodle (n = 6)	F2, M6	5.5 (3.5-6.8)	19.9 (13.7-22.8)	30 (27.5-4.8)	487.9 (271.3-799.2)
Golden retriever (n = 6)	F2, M4	2.5 (2-5.25)	29.4 (25.5-31.3)	35 (33-46)	675.8 (644.8-812.1)
Border terrier (n = 5)	F2, M3	8 (7-8)	10.5 (8.5-12.1)	34 (25-51)	769.8 (741.1-852)
Staffordshire bull terrier (n = 4)	F4	9 (7-10.3)	20.1 (18.3-22.7)	33 (29-38)	561.7 (489.9-743.7)
English Springer Spaniel (n = 4)	F2, M2	9 (6.5-10.5)	19.6 (18.3-21.4)	28 (26-31)	320.4 (104.6-1213.1)
Jack Russel terrier (n = 4)	F3, M1	5.5 (4.3-6.5)	7 (6.8-7.4)	36 (26.8-52.5)	1314.7 (451.2-2168.6)
Lurcher (n = 3)	M3	5 (5-6.5)	25.7 (20.1-29.7)	44 (42-47.5)	467.9 (454.9-576.2)
Whippet (n = 3)	F1, M2	4 (3.5-4)	17.2 (15.1-18.55)	20 (19-31)	508.11 (494.8-1827.4)
Cross breed terrier (n = 2)	F2	8.5 (8.2-8.8)	10.6 (9.7-11.5)	32.5 (29.2-35.8)	584.7 (341.8-827.7)
Cross breed (n = 3)	F2, M1	4 (3-4.5)	22.9 (22-25)	39 (32-52)	812.4 (579.3-1574)
French bulldog (n = 2)	F2	6 (3.5-8.5)	12.4 (12-12.8)	42.5 (38.8-46.2)	1002.6 (556.6-1448.6)
Shih tzu (n = 2)	F1, M1	10.5 (10.2-10.8)	9.55 (7.9-11.18)	47 (46-48)	601.44 (497.2-705.7)
West Highland terrier (n = 2)	F1, M1	6 (5.5-6.5)	9.9 (9.63-10.23)	48 (35.5-60.5)	1335.8 (1163.9 - 1507.6)
Beagle	F1	3	17.7	22	180.7
Boxer	M1	4	26	39	182.4
Chinese crested	F1	10	8.3	47	988.9
Dachshund	M1	6	13.8	30	537.9
Doberman	F1	7	30	35	247.4
Dogue de Bordeaux	M1	4	33.4	75	2110.3
English Mastiff	M1	5	81	71	4446.1
English Pointer	F1	5	23.7	52	1067.2
German Shepherd	F1	3	28.6	37	426.8
Greyhound	F1	2	26.3	53	114.5
Hungarian Vizsla	F1	9	21.5	45	642.5
Ihasa Apso	M1	2	9.6		682.4
Miniature Schnauzer	M1	8	9	42	140.5
Newfoundland	M1	6	70.8	17	1020.8
Pomeranian	M1	5	3.2	84	1755.7
Poodle	M1	2	6.4	31	543.1
Rhodesian Ridgeback	F1	11	47.3	49	371.2
Rottweiler	F1	3	40.3	21	230.7
SBT Cross	F1	8	24.9	59	871.4
Tibetan Mastiff	M1	1	40.4	38	636.4
Utonagan	F1	2	33	47	1766.9

Continuous variables are expressed as median and inter-quartile range. F, female; M, male; kg, kilogram; U/L, units per liter.

### 3.2 | Reference intervals of miR-122 in healthy dogs

Firstly, we investigated whether breed, sex, age, and weight in healthy dogs influenced circulating miR-122 concentrations. There were no significant differences in miR-122 concentration across the different

breeds (breeds with more than three dogs per group are presented in Figure 1A). Similarly, there was no difference between female and male dogs in this healthy cohort (Figure 1B). No significant relationship was found between circulating miR-122 concentration and dog body weight or age (Figure 1C,D).

**TABLE 2** Characteristics of the dogs with histologically confirmed liver disease

Liver disease (n = 30)	Sex (F, M)	Age (years)	Weight (kg)	ALT (U/L)	miR-122 (copies/ $\mu$ L)
Fibrosis (n = 6)	F2, M4	6 (3.5-8.3)	24.8 (18.9-27.8)	452 (244-564)	13 466 (6752-22 142)
Inflammatory (n = 19)	F12, M7	7 (6-8)	25 (10.8-29.8)	207 (169-406)	11 696 (4902-20 086)
Neoplastic (n = 5)	F3, M2	11 (9-11)	10.9 (10.1-15.5)	194 (182-233)	9065 (3001-11 188)

Continuous variables are expressed as median and inter-quartile range. F, female; M, male; kg, kilogram; U/L, units per liter.

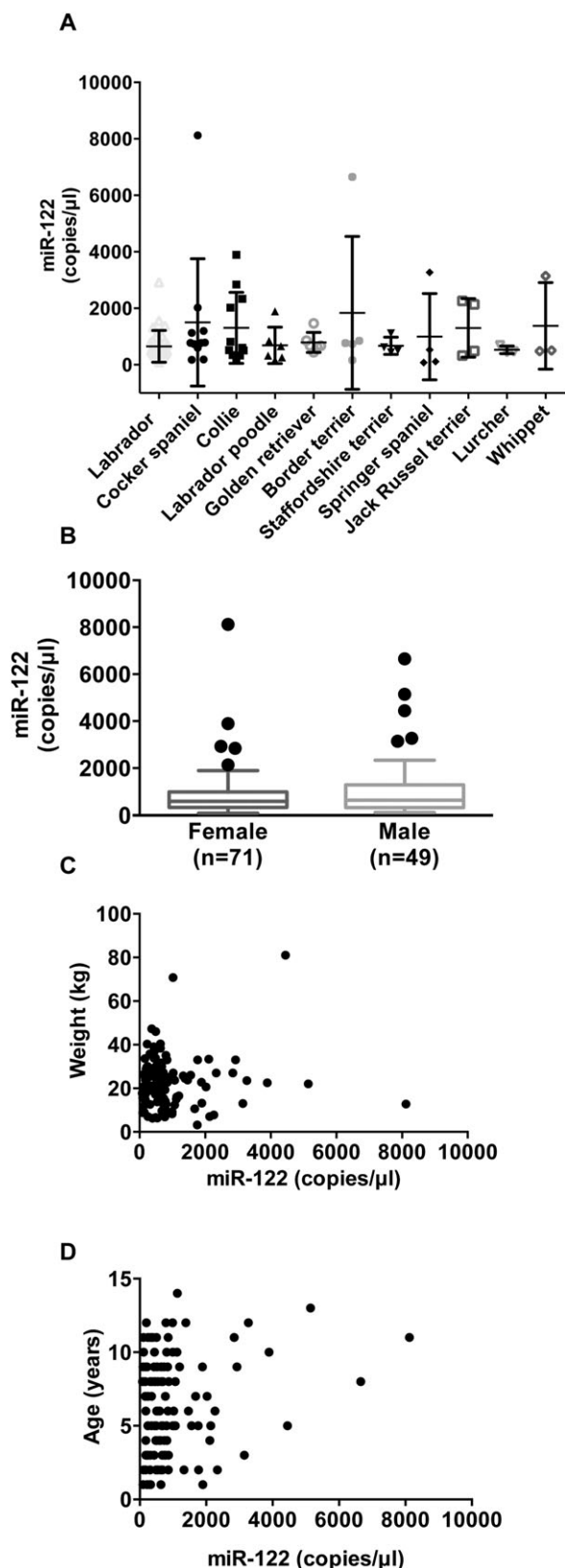
Secondly, we determined the reference interval for circulating miR-122 in healthy dogs ( $n = 120$ , as per IFCC guidelines). The data were normalized by a log transformation. After transformation, two values were removed as outliers (values: 8121 and 6651 copies/ $\mu\text{L}$ ). The distribution of miR-122 in healthy dogs is presented in Supporting Information Figure S1. The 2.5th, 50th, and 97.5th quartile of miR-

122 were 110 (80-114), 594 (505-682), and 3312 (2925-5144) copies/ $\mu\text{L}$ , respectively. The 97.5th quartile represents the upper limit of normal (ULN) for this reference population.

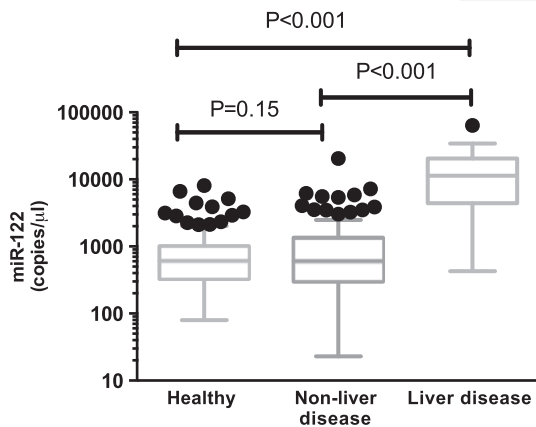
### 3.3 | miR-122 serum concentrations in dogs with disease

Circulating miR-122 was measured in the three groups of dogs (Figure 2). There was no difference between healthy dogs and dogs with non-liver disease (median  $\pm$  IQR: healthy dogs 609 [327-1014] copies/ $\mu\text{L}$ ; non-liver disease 607 [300-1351] copies/ $\mu\text{L}$ ). By contrast, miR-122 concentration was substantially higher in dogs with liver disease (11 332 [4418-20 520] copies/ $\mu\text{L}$ ). The histopathological findings in the liver were grouped into fibrosis, inflammatory, and neoplastic processes. miR-122 was significantly elevated in all these sub-groups compare with healthy dogs but did not differ across processes (Figure 3A). In those dogs with liver disease, miR-122 concentration had a significant correlation with ALT activity (Figure 3B).

We performed ROC analysis to quantify the performance of miR-122 with regard to separation of liver disease from the other two groups (Supporting Information Figure S2). miR-122 identified dogs with liver disease with high accuracy (ROC area under curve [AUC] for comparison with healthy dogs: 0.93 [95% CI 0.86-0.99]). The ULN for healthy dogs (3312 copies/ $\mu\text{L}$ ) had a sensitivity of 77% (95% CI: 58%-90%) and specificity of 97% (95% CI: 93%-99%) for identifying liver disease compared to healthy dogs (positive likelihood ratio 30). miR-122 similarly identified dogs with liver disease with high accuracy in comparison with non-liver disease dogs: ROC-AUC for comparison with non-liver disease: 0.91 (95% CI 0.84-0.98). When liver disease dogs were compared to dogs with non-liver disease the ULN for healthy dogs had a sensitivity and specificity of 77% (95% CI: 58%-90%) and 88% (95% CI: 80%-94%), respectively (positive likelihood ratio 7). The accuracy of miR-122 was similar to ALT (ROC-AUC for comparison of liver disease with healthy dogs: 0.96 (95% CI: 0.92-1).  $P = .09$  when compared with miR-122 ROC-AUC (liver disease dogs compared to healthy dogs) by Hanley and McNeil test. ROC-AUC for the ALT comparison with non-liver disease dogs: 0.95 (95% CI: 0.90-1).  $P = .06$  when compared with miR-122 ROC-AUC by Hanley and McNeil test).



**FIGURE 1** Relationship between characteristics and circulating miR-122 concentration in healthy dogs ( $n = 120$ ). (A) No significant differences between different breeds in circulating miR-122. Groups with more than 3 dogs are included and presented. Labrador ( $n = 31$ ), Cocker spaniel ( $n = 11$ ), Collie ( $n = 11$ ), Labrador poodle ( $n = 6$ ), Golden retriever ( $n = 6$ ), Border terrier ( $n = 5$ ), Staffordshire terrier ( $n = 4$ ), Springer spaniel ( $n = 4$ ), Jack Russel terrier ( $n = 4$ ), Lurcher ( $n = 3$ ), and Whippet ( $n = 3$ ) included in one-way Kruskal-Wallis ANOVA ( $P = .68$ ). (B) No significant differences between female ( $n = 71$ ) and male ( $n = 49$ ) dogs in circulating miR-122. Data are presented as a Tukey plot ( $P = .54$  by Mann-Whitney test). (C) Correlation and linear regression of circulating miR-122 and age demonstrated no significant relationship ( $P = .27$ ,  $R = 0.10$ ,  $n = 120$ ). (D) Correlation and linear regression of miR-122 and body weight demonstrated no significant relationship ( $P = .14$ , Spearman  $r = -0.19$ ,  $n = 120$ )



**FIGURE 2** Circulating miR-122 concentration in healthy dogs ( $n = 120$ ) and in dogs with non-liver diseases ( $n = 100$ ) and liver disease ( $n = 30$ ). Data are presented as Tukey plots. The significance of differences between the groups were determined by one-way Kruskal–Wallis ANOVA

#### 4 | DISCUSSION

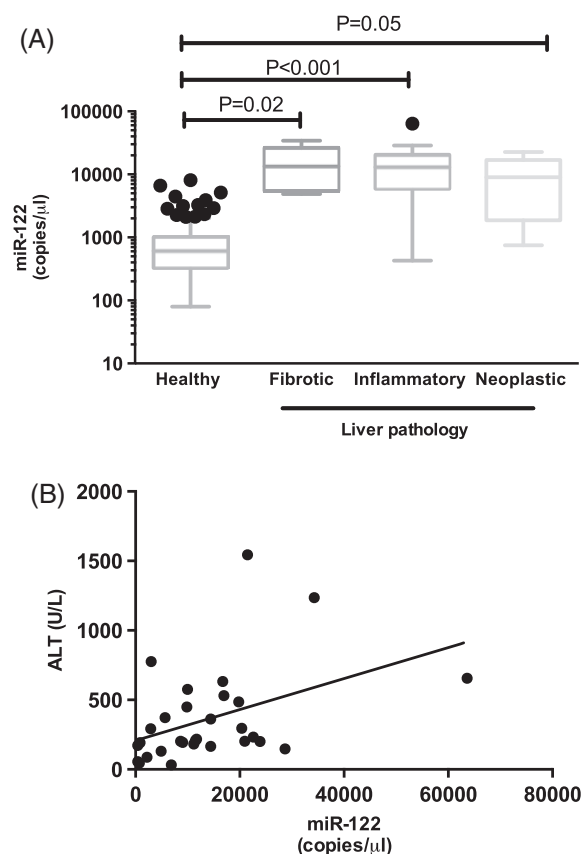
This study defines the normal reference interval for circulating miR-122 concentration in healthy dogs and demonstrates the sensitivity and specificity of circulating miR-122 concentration as a biomarker of liver disease across multiple dog breeds. Importantly, this study included dogs with non-liver disease and established the specificity of miR-122 for liver pathology.

Current biochemical blood variables used as markers of liver disease in dogs have significant limitations and the gold standard to reach a definitive diagnosis remains histopathological evaluation of liver biopsy samples. It would, therefore, be of great value to develop a non-invasive liver disease biomarker that can reliably detect liver disease with high specificity and sensitivity across different dog breeds. MicroRNAs are emerging as biomarkers for several diseases due to their organ specificity, ease of measurement, potential for amplification, and relative stability in the blood. Several multicenter studies have demonstrated the utility of circulating miR-122 as a biomarker of liver disease in humans and this has led to circulating miR-122 receiving FDA support as a biomarker of DILI. However, data in the veterinary field and especially across different dog breeds remain scarce.

In the present study, we were the first to establish a normal reference interval of circulating miR-122 concentrations in healthy dogs across several different dog breeds. Interestingly, this reference interval is comparable to the normal reference interval in humans,<sup>36</sup> which might reflect this microRNA's concentration in the circulation being under tight regulation. However, the mechanism of miR-122 release into, and clearance from, the circulation in healthy animals remains undefined. We were also able to demonstrate that there were no significant differences in miR-122 concentrations across different age-, sex-, and dog breed groups, which in turn further enhances the current literature available on circulating miR-122 concentrations in dogs. These findings build on previous studies which have reported no differences in miR-122 concentration between female and male dogs and no age-related differences.<sup>20</sup> Previously, the available literature

on microRNA concentrations in dogs could only postulate on the consistency in miR-122 concentrations between different dog breeds based on the highly conserved nature of miRNA between species with similar physiology,<sup>20</sup> a hypothesis which we were able to support in this study. In our study dogs were classified as healthy based on clinical history, examination, and routine clinical chemistry. A challenge for all biomarker studies is to have confidence that the healthy population are truly healthy and do not have members with sub-clinical disease which may be reported by the new biomarker in development (ie, a false positive result in a healthy population is actually a true positive which has been incorrectly classified due to limitations of the gold standard test). In the absence of histological analysis of liver tissue from healthy dogs at the time of blood sampling a strategy for future work is to longitudinally follow dogs for disease development and relate this to miR-122 at baseline.

Secondly, circulating miR-122 was able to discriminate between dogs with liver disease and both healthy dogs and dogs with non-liver



**FIGURE 3** miR-122 concentration in liver disease. (A) Increased miR-122 concentration in fibrotic ( $n = 6$ ), inflammatory ( $n = 19$ ) and neoplastic ( $n = 5$ ) etiologies of liver disease compared to healthy dogs ( $n = 120$ ) as determined by one-way Kruskal–Wallis ANOVA ( $P = .02$  healthy vs fibrotic,  $P = .0006$  healthy vs inflammatory and  $P = .05$  healthy vs neoplastic, respectively). There was no difference in miR-122 concentration across the liver pathology groups ( $P = .92$  fibrotic vs inflammatory,  $P = .30$  fibrotic vs neoplastic and  $P = .27$  inflammatory vs neoplastic group) as determined by one-way Kruskal–Wallis ANOVA. Data are presented as Tukey plots. (B) There was a significant relationship between miR-122 concentration and ALT as determined by correlation and linear regression ( $P = .005$ , Spearman  $r = .49$ ,  $n = 30$ )

disease with a significantly higher miR-122 concentration in the liver disease dog cohort. The sensitivity and specificity of miR-122 was comparable to our human studies of patients with acetaminophen toxicity but the dogs with liver disease in this study were heterogeneous with regard to the underlying disease etiology. This supports miR-122 having a potential context of use as a screening tool for liver disease in dogs.

The results from this study are a promising step in further developing circulating miR-122 as a qualified biomarker of liver disease in dogs. It remains to be conclusively determined as to whether miR-122 can accurately detect liver disease when current markers such as ALT are still in their respective reference interval (a specific context of use for miR-122 in humans). In the present study we could not test this as the diagnosis of liver disease was already made using ALT to indicate need for liver biopsy. Future studies with prospective, serial, blood sampling are needed to determine the clinical utility of miR-122 in dogs. However, published work in Labradors supports miR-122 reporting liver disease when ALT is still in its normal reference interval.<sup>20</sup> Furthermore, as microRNA biomarkers are translatable across species, we believe it is justifiable to cautiously extrapolate human and rodent data to veterinary practice. In humans<sup>16</sup> and mice<sup>9</sup>, it is conclusively demonstrated that miR-122 is more sensitive than ALT, at least in the context of DILI. We would predict the same performance in dogs and the present study would suggest utility across a range of liver diseases. An important hurdle to overcome is developing a point-of-care assay that clinicians could use to measure miR-122 in a timely and cost-efficient manner. The "market pull" from both human medicine and drug development has resulted in promising assays that could be applied to veterinary medicine because of the sequence conservation of miR-122.<sup>37</sup>

In summary, miR-122 is a sensitive and specific biomarker for liver disease in dogs. With further development it could become a valuable tool in the diagnostic evaluation and treatment pathway of dogs with suspected liver disease.

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## CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflicts of interest with the contents of this article.

## OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by The University of Edinburgh Veterinary Ethics Research Committee.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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