Hepatocyte-derived miR-122 as an early serum biomarker of hepatocellular injury in Labrador retrievers

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ABSTRACT

Dogs with liver disease can present with normal ALT levels, so that the disease is often not recognized until it has reached an advanced stage where treatment is less effective. Novel, more sensitive biomarkers that can detect minor levels of hepatocellular injury would allow liver disease to be diagnosed at an earlier stage where treatment may be very effective, especially in the case of copper-associated hepatitis. Recent animal and human studies have highlighted the potential of hepatocyte-derived microRNAs (HDmiRs) in serum as early, stable, sensitive and specific biomarkers of hepatocellular injury. The aim of this study was to investigate the association between miR-122 levels in serum and hepatocellular injury in dogs with different forms of liver disease.

MiR-122 levels were quantified in sera from 65 Labrador retrievers. Liver histopathology data was extracted from a database and used to categorize the animals as having either: normal livers (controls, n = 11); high hepatic copper content (n = 10); reactive hepatitis (n = 13); acute hepatitis (n = 10); chronic hepatitis (n = 17) or steroid-induced hepatopathy (n = 4). Median miR-122 levels in serum were compared between dogs with and without hepatic histological changes. Correlations were then analyzed between the miR-122 level in serum and the histological grade, histological stage, ALT level and the hepatic copper concentration. Lastly, the accuracies of both miR-122 and ALT for the detection of liver disease were determined and compared.

Compared to the controls, median miR-122 levels in serum were significantly elevated in dogs with reactive hepatitis, acute hepatitis, chronic hepatitis or steroid-induced hepatopathy. Even in dogs with normal ALT levels (n = 41), median miR-122 levels in serum were significantly elevated in dogs with acute or chronic hepatitis. Moreover, the miR-122 level in serum correlated strongly with liver histopathology and the ALT level. Finally, miR-122 was superior to ALT in discriminating controls from dogs with liver disease.

In conclusion, this study confirmed that hepatocellular injury is associated with increased miR-122 levels in serum and showed that miR-122 is a sensitive biomarker to distinguish dogs with liver disease from dogs with histologically normal livers. In a clinical setting, more sensitive biomarkers such as miR-122 may improve general testing for liver disease by identifying those dogs with liver disease, that are not identified by current screening with ALT testing. Another example of how serum miR-122 measurement may be used is in the prospective screening of dogs that are members of breeds that are genetically predisposed to developing copper-associated hepatitis.

CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Description	First mentioned (Page)
AH	Acute hepatitis	2
ALT	Alanine amino transferase	1
AST	Aspartate amino transferase	1
AUC	Area under the curve	6
CH	Chronic hepatitis	2
FIR	Fold increase range	4
HDmiR	Hepatocyte-derived microRNA	1
HC	High hepatic copper	2
miRNA	MicroRNA	1
mRNA	Messenger RNA	1
RH	Reactive hepatitis	2
ROC	Receiver operating characteristics	3
SIH	Steroid-induced hepatopathy	2
ULN	Upper limit of normal	2

INTRODUCTION

Different forms of liver disease, especially (chronic) hepatitis, are commonly recognized in many dog breeds [1,2]. Besides idiopathic forms of chronic hepatitis, hereditary copper-associated hepatitis occurs in several breeds with a prevalence of up to 10% [3]. Due to the large reserve capacity of the liver, clinical signs of liver disease are often only recognized in an advanced stage when treatment is less effective. More importantly, when the disease is diagnosed in an early, subclinical phase, treatment may be very effective, especially in the case of copper-associated hepatitis. Alanine amino transferase (ALT) activity is currently considered to be the most sensitive and specific indicator for hepatocellular injury in dogs [4]. Recently, the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University collected liver biopsies and serum samples from Labrador retrievers (n=55) that appeared clinically healthy, but were investigated because they were family members of dogs with copper-associated hepatitis. In this cohort of dogs that appeared clinically healthy, parenchymal disorders ranging from reactive hepatitis to chronic hepatitis were identified in 76 percent of the dogs [5]. In this cohort, the sensitivity and specificity of increased ALT levels for predicting histological abnormalities were only 23% and 80% respectively. The percentage of dogs with histological changes in the liver but normal ALT levels was as high as 77%. This means that if ALT were to be used to prospectively screen seemingly healthy (i.e. subclinical) dogs for the presence of liver disease, up to 77% of the diseased animals may not be identified as such. The result of this inventorial study clearly illustrates the need for new sensitive and specific biomarkers that can detect liver disease in an early stage when liver damage and fibrosis is limited and treatment is most effective.

Hepatocyte-derived microRNAs (HDmiRs) are currently considered among the most promising candidate markers. MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate post-transcriptional gene expression by targeting messenger RNA (mRNA) transcripts, resulting in cleavage or translational repression [6,7]. It has been shown that different cell types have highly specific miRNA expression patterns, suggesting a role in cellular identity. MiR-122 is an important regulator of cholesterol metabolism and iron homeostasis and is found almost exclusively in hepatocytes where it is also the most abundant miRNA, constituting about 70% of the total liver miRNA population [8–10]. Recently, miRNAs were shown to be detectable and highly stable in the circulation, which suggested the potential for miRNA based blood biomarkers [11,12]. The release of hepatocyte-derived miRNAs into the circulation was first described in rodents with drug-induced acute liver injury [13,14]. In these studies, the miR-122 level in serum was found to correlate with liver histopathology and aminotransferase (aspartate aminotransferase (AST) and ALT) levels. Interestingly, the elevation of miR-122 levels in serum appeared earlier than the increase in aminotransferase levels. Additionally, in humans chronically infected with Hepatitis C, miR-122 levels in serum were highly elevated even in patients without elevated aminotransferases, further highlighting the potentially superior sensitivity of this biomarker [15].

The aim of the current study was to investigate the association between circulating miR-122 levels and hepatocellular injury, and the potential of miR-122 to serve as an early diagnostic biomarker in the setting of canine liver disease. In order to see if miR-122 could overcome the lack in sensitivity of ALT, we gathered serum samples and analyzed miR-122 in a unique cohort of Labrador retrievers where the majority of animals had been diagnosed with a liver condition through histopathology, whilst having normal ALT levels.

MATERIALS AND METHODS

Samples

Stored serum samples from 65 Labrador retrievers were analyzed. All samples had been collected with a standard protocol as diagnostic specimens from privately owned dogs that underwent a liver biopsy in the Department of Clinical Sciences of Companion Animals, Utrecht University. Whole blood had been separated into serum and cellular fractions by centrifugation at 4,000 rpm for 5 min. The supernatant serum had been stored at –20°C or –70°C until analysis. All samples were used with full informed consent from the owners, according to the Ethics guidelines as required under Dutch legislation. Animal demographics (age, sex and ALT level) were extracted from a database and are summarized in Table 1. The upper limit of normal (ULN) serum ALT was defined as 70 U/L, consistent with the cut-off level in our clinic's laboratory.

Histopathology

Liver biopsy samples of every animal had been examined through histopathology (including rubeanic acid staining for the demonstration of copper) and quantitative hepatic copper determination [17]. Animals were divided into one of six categories based on the results of these examinations, which were extracted from a database: normal liver (controls) (NL); high hepatic copper (HC); reactive hepatitis (RH); acute hepatitis (AH); chronic hepatitis (CH) or steroid-induced hepatopathy (SIH) (Table 1). For 33 of 65 (51%) dogs, the histological grade and stage were available as determined by a single board certified veterinary pathologist.

RNA isolation

Total RNA was extracted from 100 µL serum with the miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, RNA was extracted from the serum by lysis reagent (500 µL) and chloroform (100 µL). After centrifugation at 12,000 × g for 15 minutes at 4°C, the aqueous phase was transferred to a fresh tube with 450 µL of ethanol. RNA was purified on a RNeasy minElute spin column (Qiagen) and eluted in 14 µL RNase-free water and stored at –20°C. Normalization was achieved by adding 5.6×10^8 copies of synthetic *C. elegans* miR-39 spike-in control (Qiagen).

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

The miScript II RT kit (Qiagen) was used to prepare cDNA according to the manufacturer's protocol. Every multiplex cDNA reaction consisted of 4 µL 5 × HiSpec buffer, 2 µL 10 × Nucleics mix, 2 µL Reverse Transcriptase mix and 10.5 µL of diluted template RNA. A total reaction volume of 20 µL was obtained by adding 1.5 µL RNase-free water. The obtained cDNA was diluted to a total volume of 200 µL. Quantitative real-time PCR was performed using the miScript SYBR® Green PCR kit (Qiagen). All PCRs were carried out in duplicate in a CFX-384™ (Bio-Rad, Veenendaal, the Netherlands). Each reaction consisted of 5 µL 2 × QuantiTect SYBR Green PCR mastermix, 1 µL 10 × universal primer, 1 µL 10 × miRNA-specific primer (Qiagen) and 1 µL of the previously diluted cDNA.

The total reaction volume of each PCR was adjusted to 10 µL by adding 2 µL RNase-free water. The miR-122 level was quantified using absolute quantification via a standard curve, with quantities normalized to the spike-in control as described by Kroh *et al.* [16].

Statistical analyses

The Mann-Whitney U test was used to compare median miR-122 levels in serum between dogs with and without hepatic histological changes, in both the complete cohort as well as in the subgroup of dogs with normal ALT levels. Influence of baseline characteristics (age and sex) on the miR-122 level in serum was examined by linear regression. Associations between the miR-122 level in serum and the histological grade, histological stage, ALT level and the hepatic copper concentration were analyzed using the Spearman's rank correlation. Logistic regression models and receiver operating characteristic (ROC) curve analyses were used to assess the accuracy of miR-122 and ALT to detect the presence of liver disease. Dogs from the HC group were excluded since copper accumulation without further histological abnormalities was considered a predisposing factor for hepatitis rather than a form of liver disease in itself [17]. All statistical tests were two-sided and a P-value < 0.05 was considered statistically significant. R version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses.

Table 1 Animal characteristics

	Complete cohort (n=65)	Subgroup with normal ALT (n=41)
Median (Range) age, years	6.7 (2.2–13.5)	5.7 (2.4–11.3)
Sex: male/female (n/n)	17/48	8/33
Median (Range) ALT, in U/L	60 (22–1142)	44 (22–68)
Category (n)		
Normal liver (controls) (NL)	11	11
High hepatic copper (HC)	10	10
Reactive hepatitis (RH)	13	8
Acute hepatitis (AH)	10	4
Chronic hepatitis (CH)	17	6
Steroid-induced hepatopathy (SIH)	4	2

RESULTS

Dogs with hepatic histological changes have elevated miR-122 levels in serum

MiR-122 levels were detectable in serum samples from dogs with and without hepatic histological changes. In the complete cohort, miR-122 levels in serum were significantly higher in dogs with RH, AH, CH or SIH (Fig. 1). In the subgroup of dogs with a normal ALT level, miR-122 levels in serum were significantly higher in dogs with AH or CH. In the complete cohort, the median number of miR-122 copies was 2.8 times higher in dogs with RH (fold increase range (FIR) 0.7–21.9, P = 0.041), 27.3 times higher in dogs with AH (FIR 5.4–393.4, P < 0.001), 15.7 times higher in patients with CH (FIR 1.9–756.3, P < 0.001) and 4.7 times higher in dogs with SIH (FIR 1.5–67.9, P = 0.018). In dogs with normal ALT levels, the median number of miR-122 copies was 20.2-fold higher in dogs with AH (FIR 5.4–191.5, P = 0.001), and 7.9-fold higher in dogs with CH (FIR 1.9–13.6, P = 0.003). Linear regression analyses showed a significant association between age and the circulating level of miR-122, while no association was found between circulating miR-122 levels and sex (Table 2).

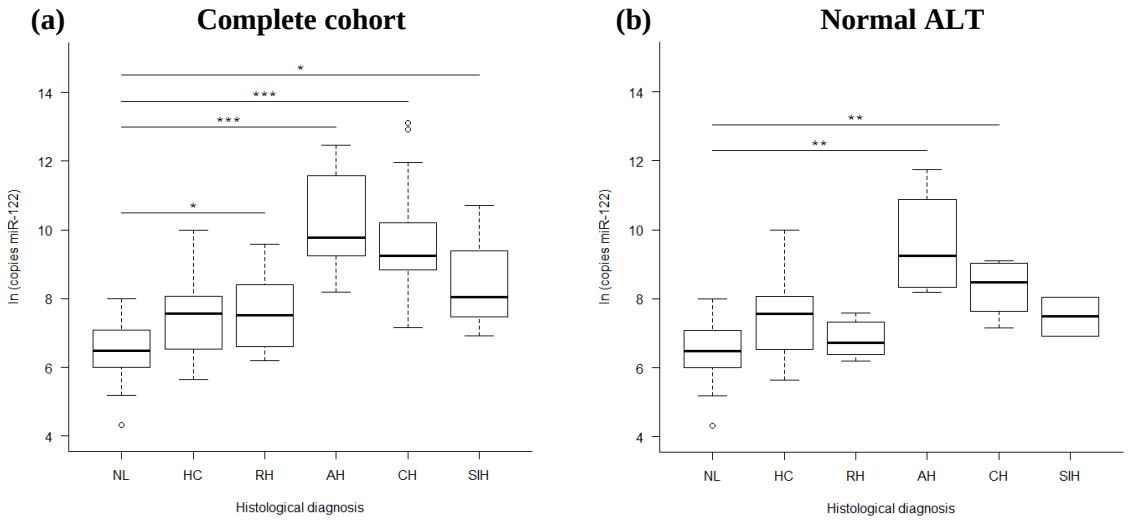

Fig. 1 MiR-122 levels in serum in dogs with and without hepatic histological changes. Results are shown for (a) the complete cohort and (b) the subgroup of dogs with a normal ALT level. The boxes represent the median and quartiles, and the whiskers represent the minimum and maximum of the number of miR-122 copies (data log-transformed). Differences between the control group and the other categories are analyzed using the Mann-Whitney U test, P-values: *P < 0.05, **P < 0.01, ***P < 0.001. *Abbreviations:* NL, normal liver (controls); HC, high hepatic copper; RH, reactive hepatitis; AH, acute hepatitis; CH, chronic hepatitis; SIH, steroid-induced hepatopathy.

Table 2 Linear regression for the influence of baseline characteristics on serum miR-122 levels

	Univariate analysis		Multivariate analysis	
	ln (β) (95% CI)	P	ln (β) (95% CI)	P
Age	9.048 (6.820–9.685)	0.029	8.956 (4.660–9.643)	0.047
Sex (F)	-10.521 (-11.358–9.352)	0.132	-10.287 (-11.257–9.837)	0.227

miR-122 levels in serum correlate with liver histopathology and ALT levels

Hepatic inflammation (grade) and fibrosis (stage) were determined in liver biopsy samples of 33 dogs. In these dogs, the miR-122 level in serum showed a significant positive correlation with both the histological grade ($R = 0.65$, $P < 0.001$) and the histological stage ($R = 0.47$, $P = 0.005$) (Fig. 2a and 2b respectively). Additionally, in the complete cohort, the miR-122 level in serum showed a strong correlation with the ALT level ($R = 0.74$, $P < 0.001$), while no correlation was found between the miR-122 level in serum and the hepatic copper concentration (Fig. 2c and 2d respectively).

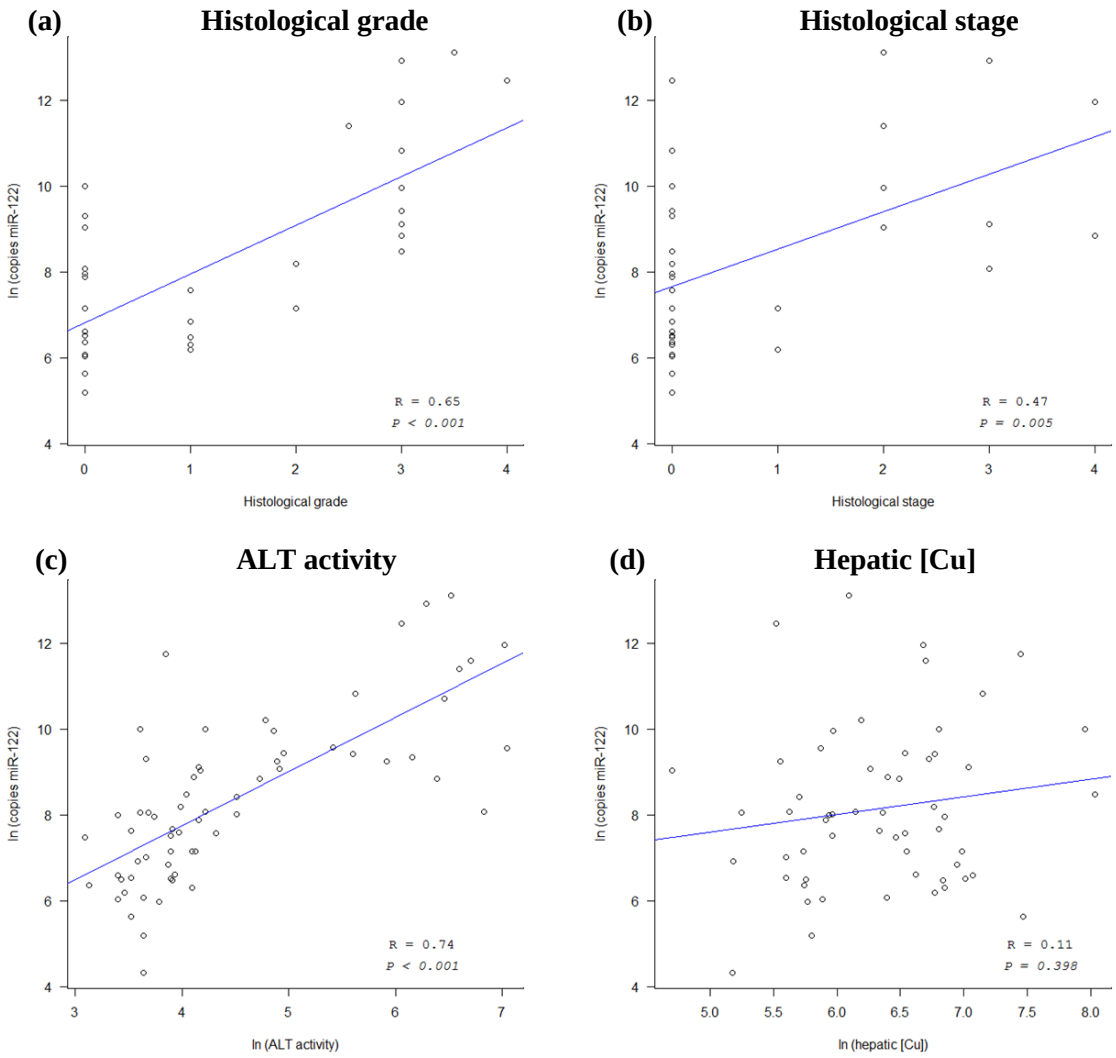

Fig. 2 MiR-122 levels in serum in relation to grade, stage, ALT and hepatic copper concentration. Correlation between the number of miR-122 copies (data log-transformed) and the histological (a) grade and (b) stage in 33 biopsies that were assessed by a veterinary pathologist. Correlation between the number of miR-122 copies and the (c) ALT activity and the (d) hepatic copper concentration in the complete cohort (all log-transformed). The correlation coefficient (R) is calculated using the Spearman's correlation test.

Serum miR-122 is superior to ALT in predicting the presence of liver disease

The odds for the presence of liver disease increased with rising ALT or miR-122 levels (Table 3). Neither marker remained significantly associated with liver disease in the multivariate model. The power to discriminate controls from dogs with liver disease was significantly higher (P = 0.036) for miR-122 (area under the curve (AUC) = 0.91, 95% CI 0.83–0.99) compared to ALT (AUC = 0.89, 95% CI 0.79–0.98). The performance of ALT declined in repeated receiver operating characteristics (ROC) curve analyses among those dogs with normal ALT (AUC = 0.75, 95% CI 0.56–0.94). A similar though slightly less pronounced decline was seen for miR-122, which retained its stronger discriminating ability (AUC = 0.80, 95% CI 0.65–0.96, P = 0.044) (Fig. 3).

Table 3 Logistic regression for predicting the presence of liver disease

	Univariate marker analysis		Multivariate marker analysis	
	Exp (β) (95% CI)	P	Exp (β) (95% CI)	P
MiR-122	1.001 (1.001–1.002)	0.030	1.001 (NA–1.002)	0.177
ALT	1.086 (1.029–1.173)	0.014	1.056 (0.947–1.078)	0.146

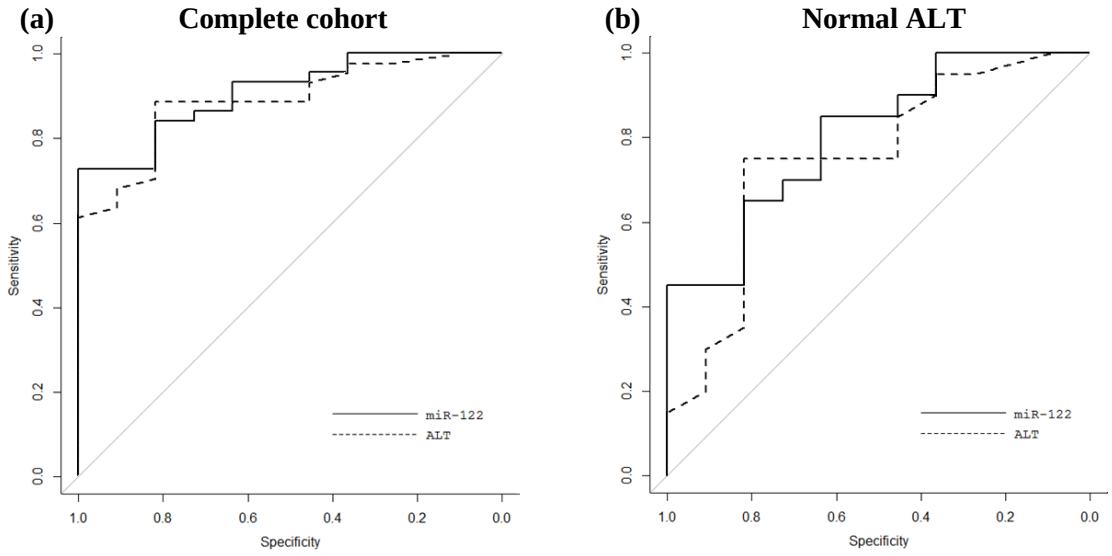

Fig. 3 Receiver operating characteristic curves. Receiver operating characteristic curves of serum miR-122 and ALT for discriminating controls from dogs with liver disease in (a) the complete cohort and in (b) the subgroup of dogs with a normal ALT level.

DISCUSSION

Due to the large reserve capacity of the liver, dogs with liver disease often remain subclinical for long periods of time. In addition, these subclinical animals often show normal ALT levels, so that they are easily missed by current screening methods [5]. New biomarkers are needed that overcome this lack in sensitivity of ALT since a more timely diagnosis can improve the effectiveness of treatment. In this study, we investigated the potential of the hepatocyte-derived miR-122 to serve as an early diagnostic or screening biomarker for the detection of liver disease in dogs.

We show that circulating levels of miR-122 are detectable and elevated in serum of dogs with different forms of liver disease. This finding is in accordance with various studies in humans where the level of miR-122 in blood has been shown to rise because of hepatocellular injury of various etiologies [15,18–21]. Elevated miR-122 levels in serum were especially pronounced in dogs with AH or CH, less pronounced in dogs with RH or SIH and absent in dogs with copper accumulation without further histological abnormalities. What separates these conditions on a morphological level, is that AH and CH are characterized by irreversible forms of hepatocellular injury (i.e. necrosis and apoptosis), while RH, SIH and copper accumulation are not [17].

Currently, little is known about the mechanism of the release of HDmiRs in response to hepatocellular injury. It seems unlikely, however, that miR-122 is simply leaked into the circulation upon uncontrolled cell death, given that serum is rich in ribonucleases. Indeed, exogenous RNAs added to serum are immediately degraded, whereas endogenous plasma RNAs are stable for hours under the same conditions [22]. Instead, it has been observed that cellular miRNAs can be released from cells by the secretion of microvesicles, including apoptotic bodies and exosomes [23]. While the biological function of circulating miRNAs is also largely unknown, Zernecke *et al.* have shown that in mice with atherosclerosis, transfer of miR-126 loaded into endothelial apoptotic bodies induced the recruitment of progenitor cells, thereby counteracting tissue damage [24]. Based on our results, it seems plausible that hepatocyte-derived microvesicles containing miR-122 may also serve to convey auto- or paracrine alarm signals to recipient cells.

This theory is supported by the correlation we found between the miR-122 level in serum and the biochemical and histopathological degree of liver damage, suggesting that more vesicles are released as the severity of hepatocellular injury and the occurrence of apoptosis both increase. Similar correlations were found in earlier studies in mice and humans with drug-induced liver injury and chronic viral hepatitis respectively [13–15]. In contrast to other studies, we also found a positive correlation between the miR-122 level in serum and the histological degree of fibrosis [15,25]. Our findings might be explained by the fact that most dogs in our cohort with a high degree of fibrosis also showed a high degree of inflammation, the latter being the more likely cause of any increases in circulating miR-122 levels.

A key finding in our study concerns the elevation of miR-122 levels in serum in dogs with AH or CH and normal ALT levels, suggesting that miR-122 is a more sensitive detector of hepatocellular injury caused by these disorders than ALT. Two separate studies in humans, one in patients that had recently undergone a liver transplantation and one in patients with chronic viral hepatitis, also showed significantly higher miR-122 levels in serum of patients with normal ALT levels compared to healthy controls [15,20].

Our ROC analyses showed that miR-122 has an improved sensitivity for the detection of liver disease over ALT, even in the subgroup of dogs with normal ALT levels. It is likely that the ROC analyses primarily reflect the difference between the sensitivities of miR-122 and ALT for the detection of liver injury caused by AH or CH, rather than all forms of liver disease. However, even if miR-122 is superior to ALT in predicting the presence of hepatocellular injury in dogs with AH or CH and not in dogs with RH or SIH, these would still be exciting results since primary hepatitis is the most common form of liver disease in dogs [1].

When we restricted our analyses to dogs with normal ALT levels, ROC analysis showed that ALT maintained a decent discriminating power for liver disease. This implies that the current cut-off level of normal ALT in our clinic's laboratory may be suboptimal and that the sensitivity of ALT to identify dogs with liver disease may be increased when lower cut-off values are used. This said, lowering the upper limit of normal (ULN) would also decrease the specificity, leading to more false-positive results. In our cohort we found that lowering the ULN from 70 U/L to 45 U/L would have a greater positive effect on the sensitivity than a negative effect the specificity (data not shown).

In conclusion, our study suggests that the circulating HDmiR miR-122 may represent an easily accessible and more sensitive biomarker than the currently used ALT to identify dogs with different forms of liver disease. Although further validation is required, the potential of miR-122 as a general test for liver disease is high. In addition, serum miR-122 measurement may improve the prospective screening of dogs that are members of breeds that are genetically predisposed to developing copper-associated hepatitis, thereby aiding in early detection and the development of effective breeding strategies.

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