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Hepatocyte-derived microRNAs as sensitive serum biomarkers of hepatocellular injury in Labrador retrievers

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ABSTRACT

Common parenchymal liver diseases in dogs include reactive hepatopathies and primary hepatitis (acute or chronic). In chronic hepatitis, there is usually a long subclinical phase. Specific clinical signs become overt only when liver damage is severe and in this phase, treatment is usually less effective. Limited data are available regarding the sensitivity of liver enzyme activity or biomarkers for early detection of subclinical hepatitis. Hepatocyte-derived microRNAs (HDmiRs) were recently identified as promising biomarkers for hepatocellular injury in multiple species. Here, the potential of the HDmiRs miR-122 and miR-148a as sensitive diagnostic biomarkers for hepatocellular injury in Labrador retrievers was investigated.

Samples from 66 Labrador retrievers with histologically normal livers, high hepatic copper, and with various forms of liver injury were evaluated for serum alanine aminotransferase (ALT) activity and microRNA values. Median values of HDmiR-122 were 34.6 times higher in dogs with liver injury and high ALT than in normal dogs (95% confidence intervals [CI], 13–95; $P < 0.001$). HDmiR-122 values were significantly increased in dogs with liver injury and normal ALT (4.2 times; 95% CI, 2–12; $P < 0.01$) and in dogs with high hepatic copper concentrations and unremarkable histopathology (2.9 times; 95% CI, 1.1–8.0; $P < 0.05$). Logistic regression analyses demonstrated that miR-122 and miR-148a were both predictors of hepatocellular injury. The sensitivity of miR-122 was 84% (95% CI, 73–93%), making it superior to ALT (55%; 95% CI, 41–68%) for the detection of hepatocellular injury in Labrador retrievers ($P < 0.001$). This study demonstrated that serum HDmiR, particularly miR-122, is a highly sensitive marker for the detection of hepatocellular injury in Labrador retrievers and is a promising new biomarker that may be used for early detection of subclinical hepatitis in dogs.

Introduction

The two most frequently recognised canine parenchymal hepatic diseases are reactive hepatopathies and primary hepatitis (Twedt, 1998; Poldervaart et al., 2009). Reactive hepatopathies result from a non-specific response to a variety of extra-hepatic diseases or endo- or exogenous steroids (Neumann and Danner, 2012). Primary hepatitis can be acute or chronic in clinical and/or histopathological classification systems. Acute hepatitis can be caused by infectious agents (i.e. canine adenovirus-1 infection or leptospirosis) or by the ingestion or administration of various drugs and/or toxins (Boomkens et al., 2004). In most chronic hepatitis cases, the aetiology remains undetermined and are therefore classified as idiopathic. More recently, hepatic copper accumulation was identified as cause of both acute and chronic hepatitis with increasing incidence (Watson, 2004; Favier, 2009; Poldervaart et al., 2009).

Serum alanine aminotransferase (ALT) activity is the most commonly used biochemical indicator for hepatocellular injury in dogs (Center, 2007; Favier, 2009). ALT is primarily and abundantly located in the hepatocyte cytosol. It is released into the bloodstream in association with minor changes in membrane integrity. High serum ALT activity suggests the presence of hepatocellular injury, especially in clinically ill dogs (Favier, 2009). The average reported sensitivity of serum ALT for detecting common parenchymal liver diseases in clinically ill dogs varies between 60% and 76% (Sevelius, 1995; Center, 2007). However, during the subclinical phase, little is known about the sensitivity of serum ALT activity. As treatment is more likely to be beneficial in early disease stage, before fibrosis occurs, there is a need for a sensitive marker of hepatocellular injury to detect subclinically affected dogs.

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MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate post-transcriptional gene expression (Bartel, 2009; Krol et al., 2010). Recent studies have shown the potential of hepatocyte-derived miRNAs (HDmiRs) as highly stable and sensitive blood-based biomarkers for hepatocellular injury in animal models and in human patients with normal and high ALT activities. Several of these studies have indicated that HDmiRs have a higher sensitivity than serum ALT (Laterza et al., 2009; Wang et al., 2009; Zhang et al., 2010; Farid et al., 2012; van der Meer et al., 2013).

The aim of the present study was to investigate the potential of two serum HDmiRs, miR-122 and miR-148a, to serve as a non-invasive diagnostic biomarker for reactive hepatopathies, hepatitis, and early stage hepatic copper accumulation in Labrador retrievers, and to compare it with plasma ALT activity.

Materials and methods

Labrador retrievers

Labrador retrievers in this study were referred to the Department of Clinical Sciences of Companion Animals, Utrecht University, between 2007 and 2014 either because of liver-related clinical signs or increased liver enzymes. In addition, client-owned clinical healthy dogs related to affected Labrador retrievers were recruited to participate in the ongoing research programme into copper associated hepatitis of the Faculty of Veterinary Medicine, Utrecht University (Fieten et al., 2012). To confirm if these dogs were clinically healthy or if they were subclinically affected, liver biopsies and blood samples were collected according to the Act on Veterinary Practice, as required under Dutch legislation. Data concerning signalment and laboratory and histopathology findings were retrospectively identified from medical records. Samples were taken with informed consent of the owners and all procedures were known and approved by the Animal Welfare Body of the University of Utrecht.

Histopathology

Liver biopsies were taken with a 14 G needle using a Tru-cut device under ultrasound guidance and processed as described previously (Fieten et al., 2013). Based on histological evaluation according to the World Small Animal Veterinary Association standards (Van den Ingh et al., 2006), dogs were assigned to the normal liver (NL) group or to the liver injury (LI) group. Dogs with liver injury (LI) were further subdivided into reactive hepatopathies (RH), acute hepatitis (AH), and chronic hepatitis (CH). Labradors with histologically normal livers but with elevated and centrolobular localised hepatic copper concentrations (>400 mg/kg dry weight liver; Puls, 1994) were assigned to the high copper (HC) group.

Blood samples

ALT and HDmiRs values were assessed in heparinised plasma and serum, respectively. Samples were obtained concurrent with the liver biopsy specimens and stored at –20 °C or –70 °C until analysis. Based on ALT activity dogs were included in normal (<70 U/L, N-ALT) or high (≥70 U/L, H-ALT) ALT groups, using the reference from our laboratory.

RNA isolation

Total RNA was extracted from 100 µL serum with the miRNeasy Serum/Plasma kit (Qiagen). RNA was extracted from the serum by lysis reagent (500 µL) and chloroform (100 µL). After centrifugation at 12,000 × g for 15 min 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. Normalisation was achieved by adding 5.6×10^8 copies of synthetic *Caenorhabditis elegans* miR-39 spike-in control to the 100 µL serum (Qiagen).

Reverse transcription and real-time quantitative polymerase chain reaction (qPCR)

The miScript II Reverse Transcription kit (Qiagen) was used to prepare cDNA. The cDNA obtained was diluted to a total volume of 200 µL. Real-time quantitative polymerase chain reaction (qPCR) was performed using the miScript SYBR Green PCR kit (Qiagen). All qPCRs were carried out in duplicate in a CFX-384 (Bio-Rad). Each reaction consisted of 5 µL 2× QuantiTect SYBR Green qPCR mastermix, 1 µL 10× universal primer, 1 µL 10× canine miRNA-specific primer (Qiagen) and 1 µL of the previously diluted cDNA. The total reaction volume of each qPCR was adjusted to 10 µL. The values of both miRs were quantified using absolute quantification via a standard curve, with quantities normalised to the spike-in control (Kroh et al., 2010).

Statistical analysis

Associations between HDmiRs and serum ALT activity or hepatic copper concentration were analysed using the Spearman's rank correlation. Comparative statistics between the NL group and the LI groups and influences of age and sex on serum HDmiR and ALT activity were examined by linear regression. The best fitting model for the data was determined with a stepwise forward model using Akaike's information criterion. Logistic regression models and receiver operating characteristic (ROC) curve analyses were used to assess the accuracy of miR-122, miR-148a and ALT to detect the presence of liver injury. The validity of all models was checked by studying the residuals on normality and constant variance. To meet these criteria, both ALT and HDmiR were ln transformed. Confidence intervals of sensitivity and specificity at a certain threshold were computed with bootstrap resampling. For the evaluation of two diagnostic tests, McNemar's test was used to compare the sensitivities and specificities, respectively. Normally distributed data were summarised as mean ± standard deviation and non-normally distributed data as median and range. All statistical tests were two-sided and a significance level of 0.05 was used. All data were analysed using R statistics version 3.1.2. ROC curves were generated using the R package 'pROC' (Robin et al., 2011).

Results

Animal characteristics

Serum samples and liver biopsy specimens from 66 Labrador retrievers (normal liver, NL, $n = 11$; high copper, HC, $n = 11$; liver injury, LI, $n = 44$) were analysed. Characteristics of the NL group, HC group, and the LI group with normal (N-ALT) and high ALT activity (H-ALT) are shown in Table 1. In the LI N-ALT group, 14/20 dogs had increased hepatic copper concentrations and in the LI H-ALT group 13/24 dogs had increased hepatic copper concentrations. Dogs with reactive hepatopathies included a dog treated with prednisolone ($n = 1$), dogs with gastrointestinal clinical signs ($n = 5$), and clinically normal dogs ($n = 11$). Mean ALT activity in the LI H-ALT group was 8.0 times higher than the NL group (95% CI, 5–13; $P < 0.001$). No difference in mean ALT activity was found between the NL group and the LI N-ALT group.

Correlation between circulating HDmiR serum values and plasma ALT activity

Correlation coefficients for combinations of miR-122, miR-148a, and plasma ALT results were determined in the 55 Labrador retrievers of the NL and LI groups (Fig. 1). The correlation coefficient between miR-122 and miR-148a was $r = 0.67$ ($P < 0.001$). Both miR-122 ($r = 0.80$; $P < 0.001$) and miR-148a ($r = 0.44$; $P < 0.01$) showed a significant positive correlation with ALT activity.

Table 1
Patient characteristics.

	NL (controls, $n = 11$)	LI N-ALT ($n = 20$)	LI H-ALT ($n = 24$)	HC ($n = 11$)
Age (years; mean ± SD)	5.4 ± 1.5	6.1 ± 2.7	8.7 ± 2.9	6.1 ± 1.6
Sex	8 F, 3 M	16 F, 4 M	15 F, 9 M	10 F, 1 M
ALT (U/L; median and range)	38 (23–64)	52 (30–68)	324 (75–1142)	40 (22–51)
Subcategory	–	RH, $n = 10$ AH, $n = 4$ CH, $n = 6$	RH, $n = 7$ AH, $n = 6$ CH, $n = 11$	Normal histology

ALT, alanine aminotransferase; AH, acute hepatitis; CH, chronic hepatitis; F, female; H-ALT, high ALT activity (≥70 U/L); HC, high copper; M, male; N-ALT, normal ALT activity (<70 U/L); NL, normal liver; LI, liver injury; RH, reactive hepatopathies; SD, standard deviation.

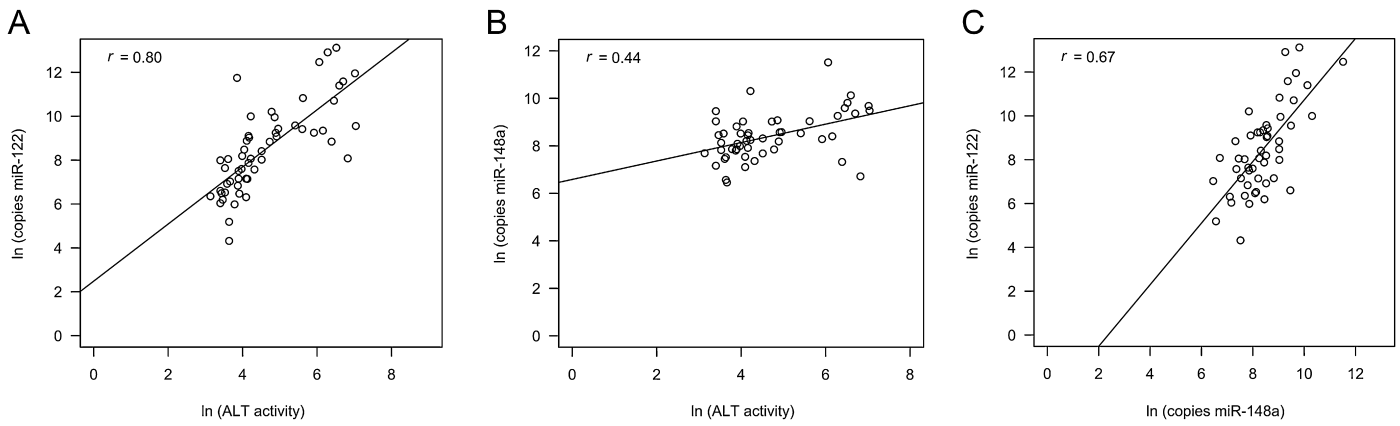

Fig. 1. Scatterplots with Spearman correlation coefficient (*r*) of serum microRNAs (HDmiRs) HDmiR-122 (A) and HDmiR-148a (B) with ALT activity. The scatterplot and correlation between both HDmiRs is depicted in (C).

Serum HDmiR values in Labrador retrievers with hepatocellular injury

Both miR-122 and miR 148a were detectable in serum samples from Labrador retrievers with liver injury (LI) and without hepatic histological changes (NL; Fig. 2). Mean serum miR-122 values in the LI H-ALT group were 34.6 times higher than in the NL group (95% CI, 13–95; $P < 0.001$). In the LI N-ALT group, mean serum miR-122 values were 4.2 times higher than in the NL group (95% CI, 2–12; $P < 0.01$). The increase in miR-122 value in the LI N-ALT group was due to dogs with acute and chronic hepatitis, with 23.4 (95% CI, 6–94; $P < 0.001$) and 6.4 (95% CI, 2–21; $P < 0.01$) times increase in miR-122 compared to control dogs. Only Labrador retrievers with RH and normal plasma ALT activity did not have a significant rise of miR-122 values (Fig. 3). Although less pronounced, miR-148a showed similar results. Compared to the NL group, miR-148a values were only significantly increased in the LI H-ALT group (Fig. 2; estimate, 3.1; 95% CI, 2–6; $P < 0.01$). In the H-ALT group, Labrador retrievers with acute and chronic hepatitis had a 6.7 (95% CI, 3–17; $P < 0.001$) and 2.7 (95% CI, 1–6; $P < 0.05$) times increase in miR-148a values compared to the control group (Fig. 3). HDmiR-148a values were not significantly increased in the LI N-ALT group, with the exception of Labradors with acute hepatitis (estimate, 5.2; 95% CI, 2–16; $P < 0.01$; Fig. 3). Linear regression analyses showed no significant association between sex, age, and the values of both HDmiRs (data not shown).

Serum HDmiR values in Labrador retrievers with high hepatic copper concentrations

In total, 11 dogs had increased hepatic copper concentrations but no hepatic injury and normal plasma ALT activity. Median hepatic copper concentrations were 836 (range, 580–1750) mg/kg/dwl in the HC group and 317 (range, 177–380) mg/kg/dwl in the NL group. In comparison with the NL group, there was a 2.9 (95% CI, 1.1–8.0; $P < 0.05$) fold increase in serum miR-122 values in Labrador retrievers with high hepatic copper concentrations (Fig. 4). This was not observed for miR-148a values.

The ability of HDmiR values and plasma ALT activity to diagnose hepatocellular injury

Univariate marker analyses showed that the odds for the presence of liver injury increased with increasing plasma ALT ($P < 0.01$), miR-122 ($P < 0.01$), and miR-148 ($P < 0.05$) values. There were no statistical significant differences between ALT (area under the curve [AUC] = 0.89; 95% CI, 0.8–1.0), miR-122 (AUC = 0.91; 95% CI, 0.8–1.0), and miR-148a (AUC = 0.78; 95% CI, 0.6–0.9) in their power to discriminate dogs with liver injury from dogs without liver injury (Fig. 5). The best thresholds for miR-122 and miR148a were 1278 and 3488 copies, respectively. As the upper limit of plasma ALT in our laboratory was 70 U/L, corresponding specificity and sensitivity were also calculated (Table 2). With a difference of 29% (95% CI,

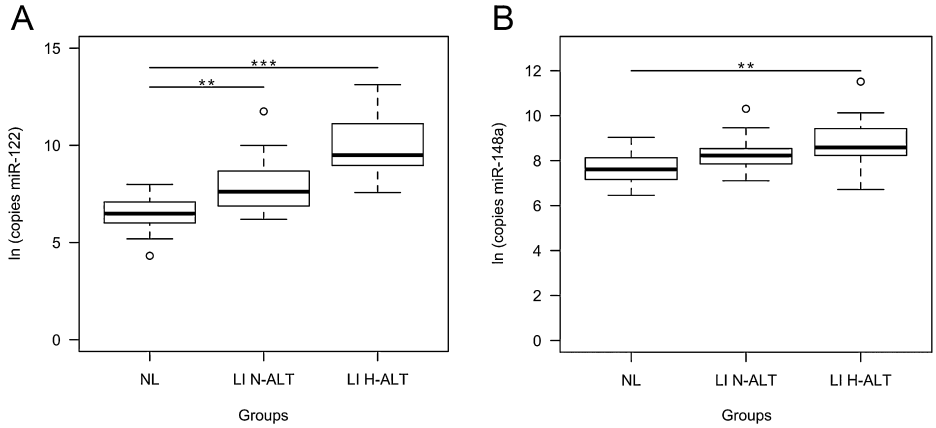

Fig. 2. Serum microRNAs (HDmiRs; ln transformed) values in dogs without histological evidence of liver injury and normal ALT activity (NL, $n = 11$) and in dogs with liver injury (LI) and normal (N-ALT, $n = 20$) or high (H-ALT, $n = 24$) activity. (A) HDmiR-122. (B) HDmiR-148a. $^{**}P < 0.01$, $^{***}P < 0.001$.

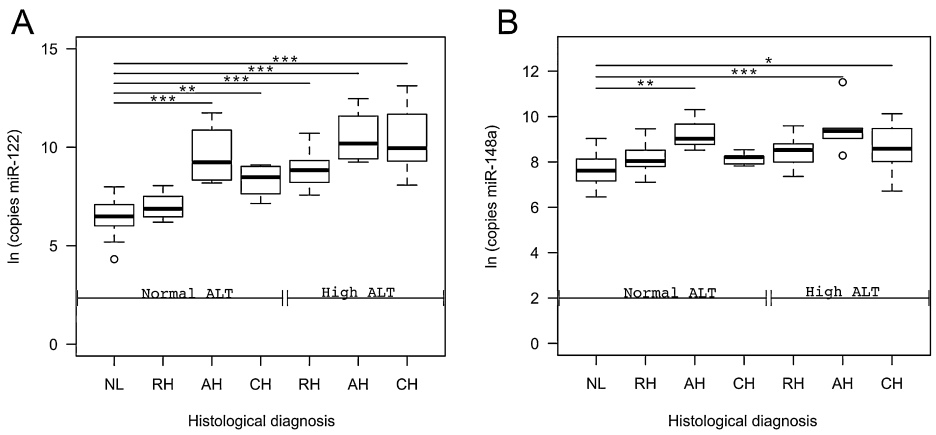

Fig. 3. Serum microRNAs (HDmiRs; ln transformed) values in dogs with normal ALT activity (RH, reactive hepatopathies, $n = 10$; AH, acute hepatitis, $n = 4$; CH, chronic hepatitis, $n = 6$) and high ALT activity (RH, reactive hepatopathies, $n = 7$; AH, acute hepatitis, $n = 6$; CH, chronic hepatitis, $n = 11$). (A) HDmiR-122. (B) HDmiR-148a. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

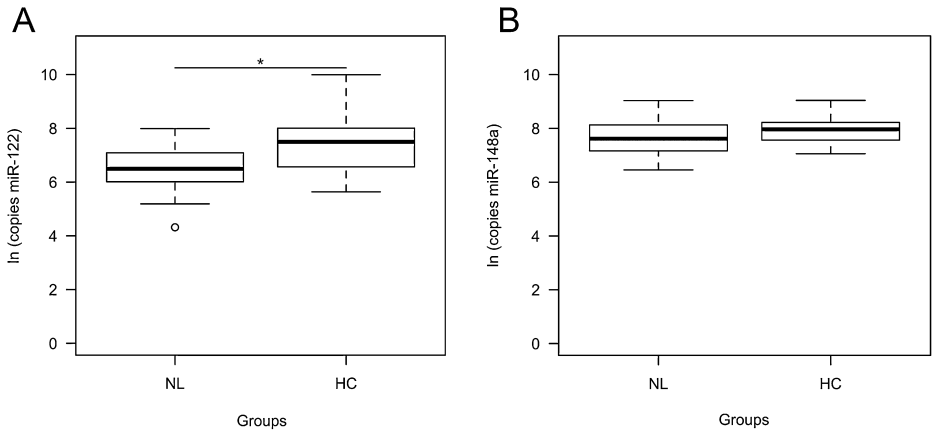

Fig. 4. Serum microRNAs (HDmiRs; ln transformed) in dogs with normal (NL, $n = 11$) and high (HC, $n = 11$) hepatic copper concentrations. (A) HDmiR-122. (B) HDmiR-148a. $^{*}P < 0.05$.

Table 2
Threshold and corresponding specificity and sensitivity for liver injury detection for each diagnostic test.

	Threshold	Specificity (95% CI)	Sensitivity (95% CI)
miR-122 (copies)	1278	0.82 (0.55–1.00)	0.84 (0.73–0.93)
miR-148a (copies)	3488	0.80 (0.50–1.00)	0.68 (0.54–0.83)
ALT (U/L)	70	1.00 (1.00–1.00)	0.55 (0.41–0.68)

ALT, alanine aminotransferase; CI, confidence interval.

16%–43%), miR-122 was significantly more able to identify Labrador retrievers with hepatocellular injury than ALT was ($P < 0.001$). There was no significant difference observed in the specificity of miR-122 and ALT. Sensitivity and specificity of miR-148a was also not significantly different from those for ALT or miR-122.

Discussion

Mature microRNAs are small non-coding RNAs that have emerged as well-conserved and important regulators of a variety of cellular processes (Krol et al., 2010; Chen and Verfaillie, 2014). Recent studies have demonstrated an important role for hepatocyte-derived miRNAs as novel biomarker for different types of liver injury in humans, rats, and mice (Laterza et al., 2009; Zhang et al., 2010; Bihrer et al., 2011; Cermelli et al., 2011; Starkey Lewis et al., 2011; Farid et al., 2012; van der Meer et al., 2013; John et al., 2014; Roderburg et al., 2014). In a recent safety study for NP260, a selective antagonist of α4-subtype $GABA_A$ receptors, performed in Beagle dogs, acute hepatocellular necrosis occurred resulting in increased miR-122 values (Harrill et al., 2014).

In the present study, we analysed the potential of HDmiR-122 and HDmiR-148a to serve as non-invasive and sensitive diagnostic biomarkers for parenchymal hepatic diseases in a cohort of Labrador retrievers. Using the Qiagen qPCR platform we were able to detect miR-122 and miR-148a in the serum of control dogs as well as dogs with hepatocellular injury. Circulating miR-122 and miR-148a values were elevated in the serum of Labradors with liver injury, and miR-122 values increased more markedly than those in the control group. These findings agree with the study of Farid et al. (2012) in humans, which also demonstrated a more pronounced increase in miR-122 values. It appears that miR-122 is a more promising biomarker for hepatocellular injury than miR-148a, and this is further supported by the fact that miR-122 accounts for 72% of all miRNAs detected in liver. Liver-specific miR-122 is known to have an important function in the maintenance of cellular homeostasis in hepatocytes by influencing gene expression, with roles in reducing hepatic inflammation, tumour suppression, and lipid metabolism (Hsu et al., 2012; Tsai et al., 2012). In addition, miR-122 regulates both plasma and liver iron values (Castoldi et al., 2011). While the expression profile of miR-148a is relatively non-organ

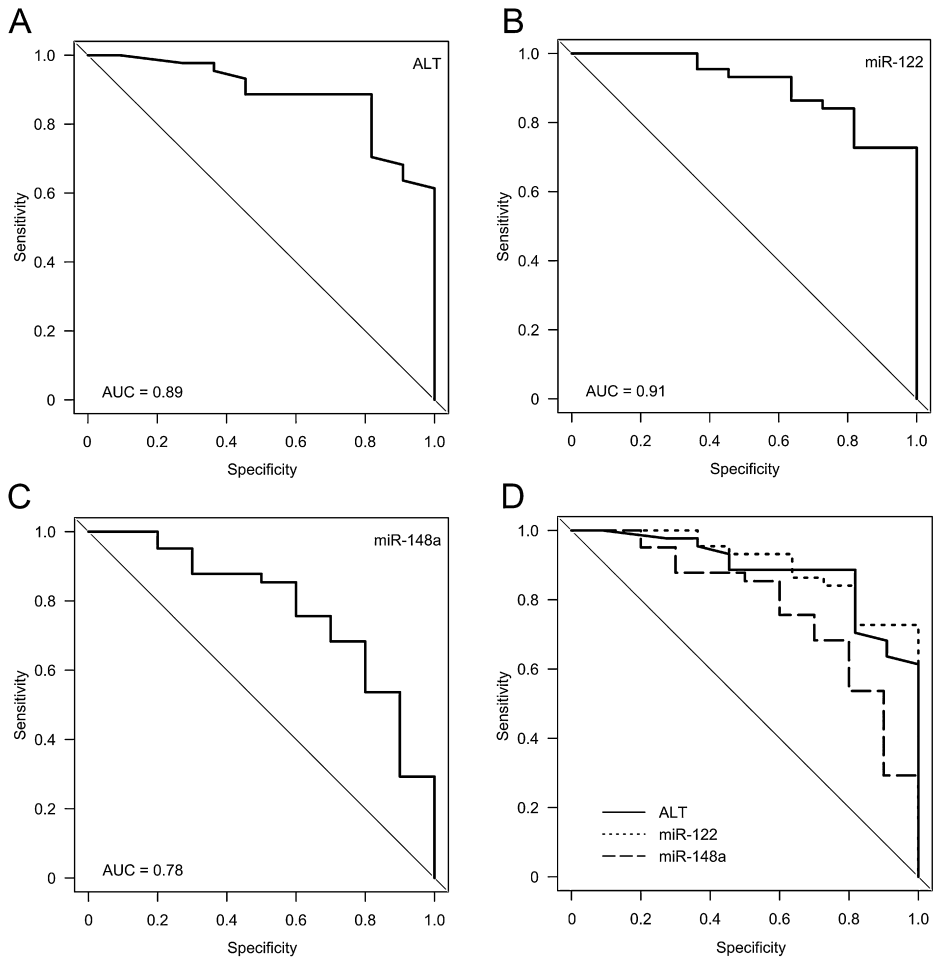

Fig. 5. Receiver operating characteristics (ROC) curves of ALT, microRNAs (HDmiRs) miR-122, and miR-148a for discriminating healthy controls from dogs with liver injury. ALT (A), HDmiR-122 (B), HDmiR148a (C), and ROC curves of ALT, HDmiR-122 and HDmiR-148a together (D). AUC, area under curve.

specific, miR-122 has almost no expression in extra-hepatic tissues (Lagos-Quintana et al., 2002; Landgraf et al., 2007).

Due to the absence of clinical signs and the large reserve capacity of the liver, canine liver injury is usually only diagnosed in end stage disease when severe liver damage is present. In this disease stage, dogs experience minimal benefit from therapeutic interventions and the prognosis in case of severe liver fibrosis is guarded (Poldervaart et al., 2009). Therefore, it is necessary to improve screening techniques for liver injury. The ideal biomarker would be accessible through a non-invasive method and must be highly sensitive. Currently, plasma ALT activity is considered the most sensitive and specific blood parameter for hepatocellular injury. Detection of high ALT activity in subclinical and clinical cases can be a reliable indicator of hepatocellular injury, but does not indicate the specific underlying disease (Fuentealba et al., 1997; Speeti et al., 1998; Neumann and Danner, 2012; Tantary et al., 2014). However, a recent study reported that among subclinical Labrador retrievers with increased copper concentration and different histological changes, only one dog had high ALT activity, which indicates a lack of sensitivity in subclinical cases of canine copper-induced hepatopathy (Fieten et al., 2015). Interestingly, human patients with chronic hepatitis C virus infection and histological signs of mild to moderate hepatitis can present with normal ALT activity (Hoofnagle, 1997). In addition, in humans and rats it has been shown that ALT activity is not specific for liver injury and can be high in muscle disorders, while miR-122 values remained unchanged (Laterza et al., 2009; Zhang et al., 2010).

One of the key findings of this study was increased serum miR-122 in Labrador retrievers with liver injury but with plasma ALT activities below the upper limit of the reference range. Similar results were found in human patients with chronic hepatitis C and in patients after liver transplantation (Farid et al., 2012; van der Meer et al., 2013). Other studies in humans and mice only reported the superior sensitivity of miR-122 by describing an earlier and/or higher increase in miR-122 values during liver injury compared to the changes in ALT activity (Wang et al., 2009; Zhang et al., 2010; Starkey Lewis et al., 2011; Thulin et al., 2014).

ROC curves were obtained to evaluate the performance of miR-122, miR-148a, and plasma ALT in identifying liver injury in Labrador retrievers. Although we did not find a statistically significant difference between the AUCs, the sensitivity of miR-122 was significantly better than that for ALT using the current threshold of 70 U/L. This emphasises the value of miR-122 as a promising new diagnostic test for detection of dogs with subclinical liver disease.

Remarkably, miR-122 values in the group with liver injury and normal plasma ALT were only increased in Labradors with acute and chronic hepatitis and not in dogs with reactive hepatopathies. As dogs with reactive hepatopathies do not generally require therapeutic intervention, the ability to discriminate between primary hepatitis and reactive hepatopathies may be of clinical importance. Although the exact mechanisms of HDmiR release from hepatocytes during different forms of hepatocellular injury remains to be elucidated, studies have been performed to determine the characterisation of extracellular microRNAs. It has been shown that the

great majority of extracellular miR-122 is present in a protein-bound form and less is transported in micro-vesicles or exosomes (Arroyo et al., 2011; Verhoeven, 2013). It has also been suggested that the release of HDmiRs is not a passive process, but is managed through a selective and active pathway. However, Bala et al. (2012) demonstrated in an experimental mouse model that there were different miR-122 associations when less severe liver disease (exosome fraction) and rapid and severe acetaminophen-induced liver injury (protein fraction) were compared. Since it is protected against degradation, miR-122 is a suitable blood-based biomarker for different forms of hepatocellular injury in dogs. Nevertheless, to further discriminate between different parenchymal hepatic disease processes, the reference standard is still histopathologic evaluation of liver biopsy specimens.

Another promising result of our study was the increased value of miR-122 in Labrador retrievers with elevated centrolobular hepatic copper accumulation but without histological evidence of liver injury (HC group). This echoes the findings of Laterza et al. (2009), who demonstrated an increase in miR-122 in rats that were treated with liver toxicants but lacked histological signs of liver injury. As HDmiRs are believed to have an important role in inter-cellular communication (Kosaka et al., 2010; Steer and Subramanian, 2012), this suggests that miR-122 values can be increased before hepatocellular injury becomes histologically evident. This reinforces the potential utility of miR-122 measurements in the early detection of hepatic copper accumulation, which is of utmost importance because early initiation of treatment might prevent clinical illness in affected dogs.

The results of this study were solely based on measurements in Labrador retriever dogs. However, Harrill et al. (2014) found similar results in miR-122 expression in Beagle dogs with hepatocellular necrosis. Although we expect the current results to be applicable in other dog breeds because of the highly conserved nature of microRNAs between species, further studies in other dog breeds are warranted to confirm these results.

Conclusion

This study reported serum analysis for HDmiRs, especially miR-122, as highly specific and sensitive biomarkers for liver injury and hepatic copper accumulation in Labrador retrievers. Determination of serum miR-122 in dogs can enable early stage diagnosis of canine hepatopathy, when treatment is more likely to be effective.

Conflict of interest statement

None of the authors of this paper has any financial or personal relationships with other people or organisations that could inappropriately influence or bias the content of the paper.

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