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Erythrocyte copper chaperone for superoxide dismutase and superoxide dismutase as biomarkers for hepatic copper concentrations in Labrador retrievers

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ABSTRACT

Hereditary hepatic copper accumulation in Labrador retrievers leads to hepatitis with fibrosis and eventually cirrhosis. The development of a non-invasive blood-based biomarker for copper status in dogs could be helpful in identifying dogs at risk and to monitor copper concentrations during treatment. In this study, two cellular copper metabolism proteins, Cu/Zn superoxide dismutase (SOD1) and its chaperone (copper chaperone for SOD1, CCS) were measured in erythrocytes and tested for association with hepatic copper concentrations in 15 Labrador retrievers with normal or increased hepatic copper concentrations.

Antibodies against CCS and SOD1 were applicable for use in canine specimens. This was demonstrated by the loss of immune-reactive bands for CCS and SOD1 in siRNA treated canine bile duct epithelial cells. Erythrocyte CCS and CCS/SOD1 ratios were decreased 2.37 ($P < 0.001$) and 3.29 ($P < 0.001$) fold in the high copper group compared to the normal copper group. Erythrocyte CCS and CCS/SOD1 ratio are potential new biomarkers for hepatic copper concentrations in Labrador retrievers and could facilitate early diagnosis and treatment monitoring for copper-associated hepatitis in dogs.

Introduction

Canine copper toxicosis is characterised by gradual copper accumulation in the centrilobular regions of the liver and is described in several dog breeds, such as the Bedlington terrier (Twedt et al., 1979), Skye terrier (Haywood et al., 1988), West Highland white terrier (Thornburg et al., 1986), Dalmatian (Cooper et al., 1997), Doberman (Mandigers et al., 2004), and Labrador retriever (Hoffmann et al., 2006). In the Bedlington terrier, the disease is due to an autosomal recessive gene which codes for the deletion of exon 2 of the *COMMD1* gene (van de Sluis et al., 2002). In Labrador retrievers, a missense mutation in the Wilson disease gene *ATP7B* (Tanzi et al., 1993) is associated with high hepatic copper concentrations, which can be attenuated by a concurrent missense mutation in the Menkes disease gene *ATP7A* (Vulpe et al., 1993; Fieten et al., 2016). In addition, dietary uptake of copper and zinc is involved in disease progression (Fieten et al., 2012). In other dog breeds, the genetic background has not yet been elucidated.

Copper concentrations in affected Labrador retrievers can be as high as 4000–5000 mg/kg dry weight liver (dwl; Smedley et al., 2009; Johnston et al., 2013), leading to hepatocellular injury and eventually fibrosis and cirrhosis. Clinical signs usually become apparent late in disease, when severe liver damage is already present. Treatment with the copper chelator D-penicillamine (Fieten et al., 2013b) and a low-copper high-zinc diet are effective in reducing hepatic copper concentrations (Fieten et al., 2014, 2015) and treatment is most effective in an early stage of disease when liver damage is limited. Follow-up liver biopsies are needed to evaluate treatment effect.

Serum liver enzymes, including alanine aminotransferase and alkaline phosphatase, can indicate the presence of hepatocellular injury, but do not correlate with hepatic copper concentrations (Favier et al., 2012; Fieten et al., 2014). Currently, the only way to confirm a diagnosis of copper toxicosis and to monitor treatment effect is by liver histology and determination of hepatic copper concentration. A biomarker in blood or urine that correlates with hepatic copper concentrations could help to identify at risk dogs, so that early treatment can be commenced to prevent clinical illness and to facilitate longitudinal monitoring. Recently, copper/zinc ratio was shown to correlate with hepatic copper concentrations in Labrador retrievers. However, there was an overlap between dogs with normal and increased hepatic copper concentrations; therefore,

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copper/zinc ratio has practical limitations as a biomarker (Fieten et al., 2013a).

Because excessive copper is toxic, intracellular copper concentrations are tightly regulated (Fieten et al., 2011). After copper uptake by hepatocytes, it is bound by specialised copper chaperones that shuttle it to its destination molecules. One of these chaperones is the copper chaperone for Cu/Zn superoxide dismutase (CCS; Culotta et al., 1997). CCS delivers copper to Cu/Zn superoxide dismutase (SOD1), a protein which protects against oxidative stress by metabolising superoxide radicals (McCord and Fridovich, 1969). SOD1 and CCS have been studied as biomarkers for hepatic copper status in animals with copper deficiency and overload. In copper deficient rodents, decreased SOD1 protein concentrations and activities and high CCS protein concentrations have been reported (Bertinato et al., 2003; West and Prohaska, 2004). Conversely, erythrocyte CCS concentrations were reduced in rats with hepatic copper accumulation (Bertinato et al., 2010).

The primary aim of our study was to evaluate the specificity of polyclonal antibodies against human SOD1 and CCS proteins in dogs. Our secondary aim was to evaluate erythrocyte SOD1 (eSOD1) and erythrocyte CCS (eCCS) protein concentrations as biomarkers for hepatic copper concentrations in 15 Labrador retrievers, to determine associations between eSOD1 and eCCS and hepatic copper concentrations.

Materials and methods

Animals

Medical records of 22 Labrador retrievers (nine males and 13 females) admitted to the Department of Clinical Sciences of Companion Animals of the University of Utrecht between 2011 and 2015 were retrospectively reviewed. Dogs with increased serum liver enzymes and/or clinical illness were referred, and clinically healthy dogs related to dogs affected with copper-associated hepatitis were actively recruited. The clinically healthy dogs participated in an ongoing research programme investigating copper-associated hepatitis at the Faculty of Veterinary Medicine, Utrecht University. Signalment, histopathology from liver biopsies, and hepatic copper concentrations were identified from the medical records. Animal care and handling was performed in accordance with the European Directive for the Protection of Vertebrate animals used for Experimental and Scientific Purpose, European Community Directive 86/609/CEE. All data were collected according to the Act on Veterinary Practice, as required under Dutch legislation. Specimens were collected with informed consent of the owners.

To determine correlations between hepatic SOD1 and CCS protein concentrations with hepatic copper concentrations and eSOD1 and eCCS, 15 clinical specimens were initially selected. To determine the correlations between hepatic copper concentrations and eSOD1 and eCCS, 15 clinical specimens were used. Eight specimens were re-used and seven new specimens were included. These dogs presented with copper-associated hepatitis during the course of the study and had a wide distribution of hepatic copper concentrations.

Specimen collection

Liver tissue was collected with a 14 G needle under ultrasound guidance. Biopsy specimens were fixed in 4% neutral buffered formalin, embedded in paraffin, and stained with rubeanic acid to evaluate if the distribution of copper was centrilobular. A separate biopsy specimen of minimally 5 mg was freeze dried prior to quantitative copper determination by instrumental neutron activation analysis (Bode et al., 2008). Dogs were considered to have normal hepatic copper concentrations if concentrations were <400 mg/kg dwl (Puls, 1994). Additional biopsies were fixed in RNAlater (Ambion) for a maximum of 24 h or snap-frozen in liquid nitrogen and stored at −70 °C. EDTA blood specimens were collected for the isolation of erythrocytes. EDTA specimens were washed three times with phosphate buffered saline and centrifuged at 460 *g* for 5 min. Pellets were stored at −70 °C until protein isolation.

Cell lines

Canine bile duct epithelial (BDE) cells were acquired from the Amsterdam Medical Center (Oda et al., 1996) and human hepatocellular carcinoma (HepG2) cells were obtained from the American Type Culture Collection. Both cell-lines were cultured as described previously (Spee et al., 2006; Muller et al., 2007). HepG2 cells were seeded at a concentration of 33,300 cells/cm^2 prior to transfection. BDE cells were seeded at a concentration of 5000 cells/cm^2 prior to transfection.

Table 1
Sequences for siRNA induced silencing experiments.

Target	Sequence 5′-3′	GenBank accession number
Human SOD1	AGGGCAUCAUCAAUUUCGAGCAGAA	NM_000454.4
Human CCS	GCAACAGCUGUGGGAAUCACUUUAA	NM_005125.1
Canine SOD1	UGUACUAGUGCAGGUCCUCACUUUA	NM_001003035.1
Canine CCS	CAGGCAUCCAGAGUGUUAAAGUGCA	NM_001194970.1

SOD1, Cu/Zn superoxide dismutase 1; CCS, copper chaperone for Cu/Zn superoxide dismutase 1.

Establishment of SOD1 and CCS knockdown

For silencing experiments, specific Stealth dsRNA molecules (Thermo Fisher Scientific) were obtained and sequences are presented in Table 1. A predesigned nonsense Stealth dsRNA sequence was used as a non-target control (NT). Forward transfection of BDE cells was performed using the Magnet Assisted Transfection (MATra) technique (IBA, BioTAGnology) in combination with Lipofectamine 2000 (Thermo Fisher Scientific), according to manufacturer's instructions. Briefly, 50 nM of siRNA molecules were transfected into the cell-lines in the presence of 1.5 μL/mL Lipofectamine 2000 and Lipofectamine MATra enhancer reagent for 20 min on the plate magnet. After transfection, cells were washed twice with Hanks' balanced salt solution and cultured in growth media including antibiotics. Transfection of HepG2 cells was established using a reverse transfection technique with Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, complexes of 10 nM siRNA and 3 μL/mL Lipofectamine RNAiMAX were prepared in the wells. After an incubation period of 20 min at room temperature, cells and antibiotic-free medium were added. Twenty four hours later, the medium was replaced by antibiotic-supplemented growth medium. Transfection was performed in triplicate in 96-well plates for RNA isolation and in duplicate in six-well plates for protein isolation. Knockdown was calculated as percentage relative to expression in the NT.

RNA isolation

Total RNA was isolated for each group (SOD1 siRNA, CCS siRNA, and NT) on days 2, 3, 4, and 7 after transfection using iScript RT-qPCR Sample Preparation Reagent (Bio-Rad), according to manufacturer's instructions. Subsequently, cDNA was synthesised with the iScript cDNA Synthesis Kit (Bio-Rad), according to manufacturer's instructions.

Quantitative measurements of mRNA concentrations after transfection

Quantitative real-time PCR was performed as described previously (Spee et al., 2005). Accurate quantification including three endogenous reference genes (ribosomal protein S19, beta-2 microglobulin, ribosomal protein S5) was based on the MIQE-precise guidelines (Bustin et al., 2010).

Western blot analysis

Proteins from BDE and HepG2 cells were isolated on days 2, 3, 4, and 7 after transfection in 350 μL ×1 RIPA buffer containing 1% v/v Igepal, 1 mM phenylmethylsulphonyl fluoride, 1 μg/mL aprotinin, and 1 mM sodium orthovanadate (Sigma). Liver tissue and erythrocyte specimens were homogenised and resuspended in equal amounts of ×2 RIPA buffer (Sigma). Protein concentrations were obtained using a Lowry-based assay (DC Protein Assay, Bio-Rad) and subsequently proteins were denatured for 2 min at 95 °C. For the detection of SOD1, 10 μg of protein for erythrocytes and 4 μg of protein for liver tissue and BDE and HepG2 cells were separated over 15% Tris-HCl polyacrylamide gels (Bio-Rad) and transferred onto Hybond-C Extra Nitrocellulose membranes (GE Healthcare). For CCS, 60 μg of protein was used for erythrocytes, BDE and HepG2 cells and 17 μg was used for liver tissue. Membranes were blocked in TBS-Tween (0.1% v/v) supplemented with 4% w/v nonfat dry milk (Bio-Rad) for 1 h at room temperature. Membranes were incubated overnight with rabbit polyclonal antibody against CCS (FL-274, Santa Cruz) and SOD1 (FL-154, Santa Cruz) at a dilution of 1:1000 in TBS-Tween with 4% w/v BSA at 4 °C. After washing with TBS-Tween (0.1% v/v), membranes were incubated for 1 h at room temperature with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (RD systems) at a 1:5000 dilution in TBS with 4% w/v BSA. As a loading control, an anti-beta-actin (Pan ab-5, Neomarkers, 1:2000 dilution) or an anti-GAPDH (Sigma, 1:1000 dilution) antibody was used. After washing with TBS-Tween (0.1% v/v), the ECL Western blot analysis system was used according to manufacturer's instructions (GE Healthcare). Images were captured with ChemiDoc XRS Chemi Luminescent Image Capture (Bio-Rad). Density of immune-reactive bands was measured using Quantity one (Version 4.6.9, Bio-Rad), corrected for background and normalised to beta-actin (BDE, HepG2) or GAPDH (liver, erythrocytes).

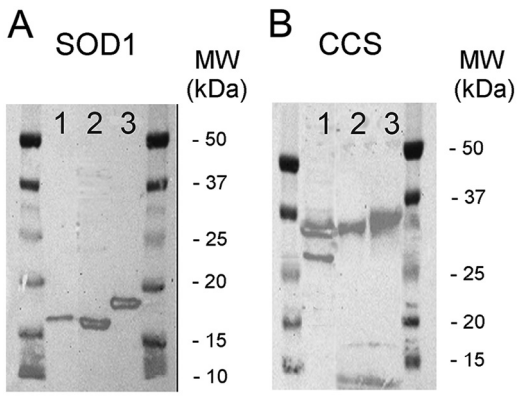

Fig. 1. Immuno-reactive bands for Cu/Zn superoxide dismutase (SOD1) (a) and the copper chaperone for SOD1 (CCS) (b) in erythrocytes, canine liver and human hepatocellular carcinoma (HepG2) cell lines. Western blot detected bands for SOD1 at 16 kDa in canine erythrocytes, liver, and canine bile duct epithelium (BDE) cells (latter not shown) and at 18 kDa in HepG2 cells. For CCS bands at 35 kDa were detected in HepG2 cells and BDE cells (not shown) and liver cells. In erythrocytes, an additional band was detected at approximately 32 kDa. 1, erythrocyte; 2, canine liver; 3, HepG2 cells. MW, molecular weight.

Statistical analyses

Associations between hepatic SOD1 and CCS protein concentrations and hepatic copper concentrations and eSOD1 and eCCS were determined using Spearman's rank correlations. Relationships between eSOD1, eCCS protein concentrations and eCCS/eSOD1 ratio with hepatic copper concentrations were examined by linear regression and by Wilcoxon rank sum test (for comparison between groups of Labrador retrievers with normal or increased hepatic copper concentration). In the linear regression model, quantitative copper was the dependent variable, either eSOD1, eCCS or eCCS/eSOD1 ratio were added as independent variables, and age at time of biopsy and sex were analysed as covariates. Quantitative copper was log transformed to guarantee the validity of the model, after studying the residuals for normality and constant variance. For all tests, a significance level of 0.05 was used. All data were analysed using R statistics version 3.1.2.

Results

SOD1 and CCS antibody specificity

Immune-reactive bands for SOD1 and CCS are shown in Fig. 1. Antibodies against SOD1 detected a single immune-reactive band of approximately 16 kDa in canine specimens (erythrocytes, BDE cells [data not shown], liver tissue) and a band of 18 kDa in HepG2 cells (Fig. 1a). For CCS, Western blot analysis yielded a 35 kDa immune-reactive band in erythrocytes, liver tissues, HepG2 cells and BDE cells (data not shown), but an extra band was identified at 32 kDa in canine erythrocytes (Fig. 1b). No immune-reactive bands were observed if the first antibodies were omitted (data not shown). Antibody specificity was proven after siRNA-mediated silencing. SOD1 mRNA expression was markedly reduced at all days in both cell lines, with the highest decrease at 4 days after transfection in HepG2 cells (Fig. 2a) and 3 days after transfection in BDE cells (Fig. 2b). At these respective time points, mRNA knockdown was 95% in both cell-lines. SOD1 protein knockdown was highest at day 4 in both HepG2 and BDE cells, with a knockdown of 88% and 77%, respectively. For CCS, the highest decrease in mRNA expression was achieved at day 3 for HepG2 cells (Fig. 2c) and at day 2 for BDE cells (Fig. 2d). At this time, mRNA knockdown was 98% in HepG2 cells and 90% in BDE cells. Seventy-four percent of protein knockdown

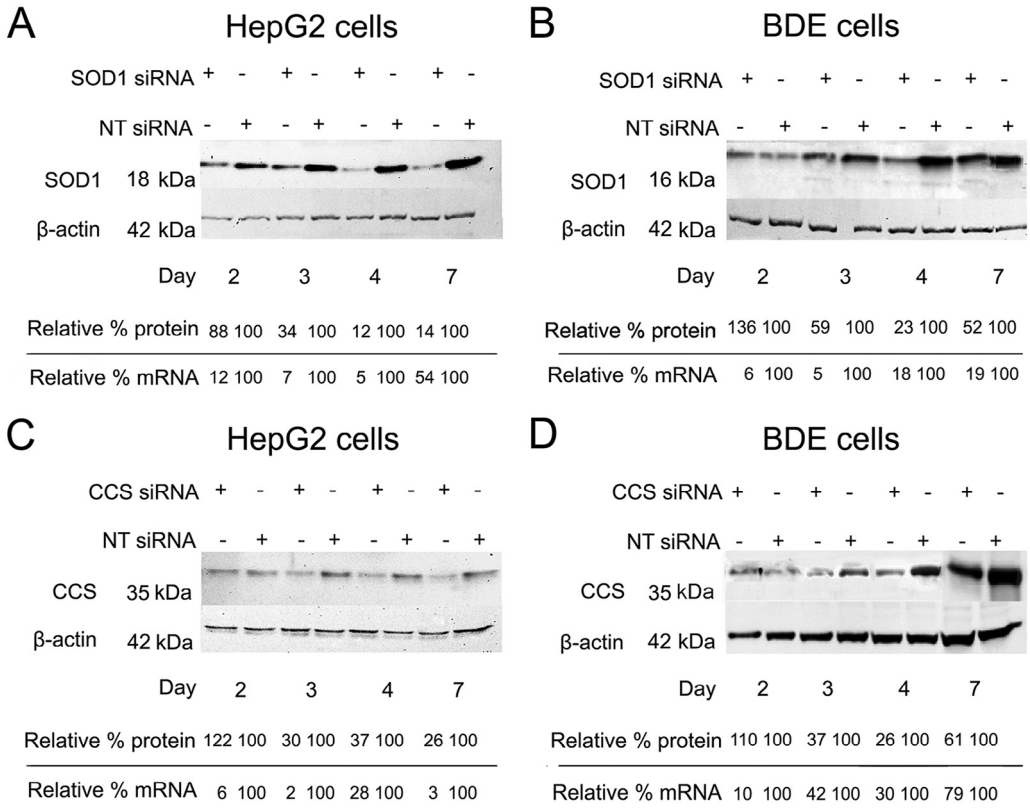

Fig. 2. siRNA mediated knockdown of Cu/Zn superoxide dismutase (SOD1) and the copper chaperone for SOD1 (CCS) in human hepatocellular carcinoma (HepG2) and canine bile duct epithelium (BDE) cells. (a) SOD1 in HepG2 cells. (b) SOD1 in BDE cells. (c) CCS in HepG2 cells. (d) CCS in BDE cells. Immune-reactive bands on Western blot at days 2, 3, 4 and 7 post transfection are shown. β-actin was used as a loading control. In the bottom line, the relative amounts of protein and mRNA (%) are depicted. NT, non-target control; MW, molecular weight.

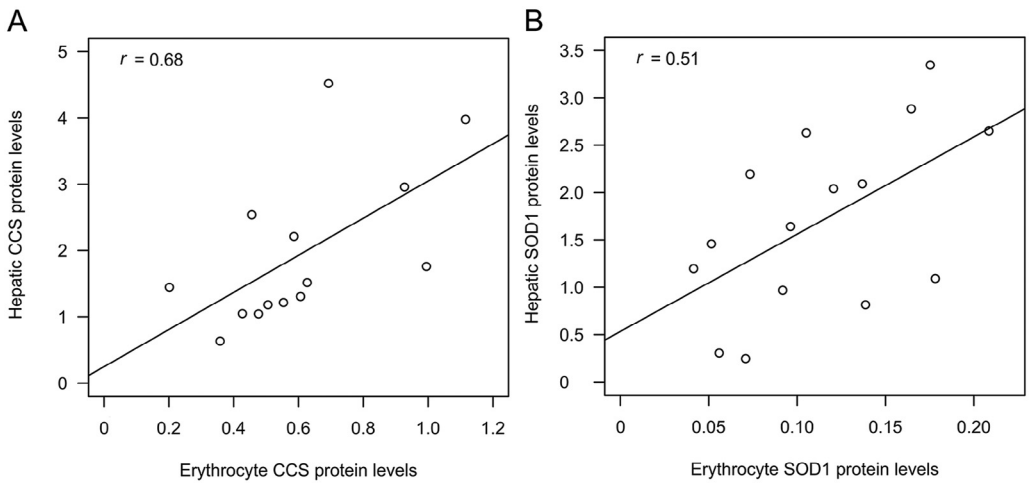

Fig. 3. The correlation between hepatic and erythrocyte copper chaperone (CCS) for Cu/Zn superoxide dismutase (SOD1) (a) and SOD1 (b) protein concentrations. There is a significant ($P = 0.0095$) correlation between hepatic CCS and erythrocyte CCS protein concentrations.

of CCS was achieved in both HepG2 cells (Fig. 2c) and BDE cells (Fig. 2d) at days 7 and 4, respectively.

Correlation of hepatic SOD1 and CCS protein concentrations with eSOD1 and eCCS and hepatic copper concentrations

Correlations were measured in 15 Labrador retrievers (eight females, seven males) with a median age of 7.9 years (range 5.3–12.3 years). These dogs had a median hepatic copper concentration of 446 mg/kg dwl (range 28–1270 mg/kg dwl). The erythrocyte copper chaperone for SOD1 concentrations was significantly and positively correlated with hepatic CCS concentrations ($r = 0.68$, $P = 0.0095$; Fig. 3a), while eSOD1 concentrations were less strongly correlated with hepatic SOD1 concentrations ($r = 0.51$, $P = 0.054$; Fig. 3b). There was a negative correlation between hepatic copper concentration and hepatic CCS protein concentrations ($r = 0.63$, $P = 0.018$). A correlation was not demonstrated between hepatic copper concentration and hepatic SOD1 protein concentrations (data not shown).

eSOD1 and eCCS concentrations in Labrador retrievers with normal and high hepatic copper concentrations

Of the 15 dogs, five dogs (three females, two males), with a median age of 7.2 years (range 6.0–11.4 years) had normal hepatic copper concentrations (median 177 mg/kg/dwl, range 28–393 mg/kg dwl). Ten dogs (six females, four males), with a median age of 7.9 years (range 5.3–12.7 years), had increased hepatic copper concentrations (median 738 mg/kg/dwl, range 482–1445 mg/kg dwl). Age and sex did not predict hepatic copper concentrations in any of the three models (eCCS, eSOD1, and eCCS/eSOD1 ratio; Table 2). Both eCCS ($P = 0.013$) and eCCS/eSOD1 ratio ($P = 0.008$) were significantly associated with hepatic copper concentrations.

In the high copper group, eCCS concentrations were 2.37 fold decreased compared to Labradors with normal hepatic copper concentrations ($P < 0.001$; Fig. 4a). The increase in eSOD1 protein concentrations in dogs with high copper concentrations did not reach statistical significance ($P = 0.099$; Fig. 4b). The eCCS/eSOD1 ratio was 3.29 fold decreased in the high hepatic copper group compared to the normal copper group ($P < 0.001$; Fig. 4c). There was no overlap in eCCS and eCCS/eSOD1 ratio between dogs with normal and increased hepatic copper concentrations.

Discussion

In the present study, we investigated whether SOD1 and CCS could serve as possible biomarkers for hepatic copper status in Labrador retrievers. The specificity of the rabbit polyclonal antibodies for human SOD1 and human CCS in canine specimens was confirmed by the loss of immune-reactive bands for SOD1 and CCS on gene silencing in canine cells. Anti-human SOD1 FL-154 antibody also detected canine SOD1 protein in a study of canine degenerative myelopathy (Crisp et al., 2013). For CCS, there was an immune-reactive band at 35 kDa and an additional smaller band at 32 kDa, only in canine erythrocytes. This additional band was also detected in bovine erythrocytes and rat erythrocytes, white blood cells and platelets, but was absent in several rat tissues (Broderius and Prohaska, 2009; Hepburn et al., 2009). Peptide blocking experiments, in which the antibody is neutralised, indicated CCS immune-specificity, but neither study examined this smaller transcript variant further; therefore its role remains undetermined.

SOD1 and CCS were previously investigated for their suitability as biomarkers for copper deficiency in mice, rats, and cattle. These

Table 2
Estimates and standard errors of the model parameters for the prediction of hepatic copper concentration in Labrador retrievers by the independent variables eCCS, eSOD1, or eCCS/eSOD1.

Predictors	Estimate	Standard error	*P* value
eCCS			
Intercept	6.83 (4.67–9.00)	1.10	<0.001
Independent variable eCCS	–0.95 (–1.57 to –0.32)	0.32	0.013
Covariate age	0.01 (–0.08–0.28)	0.09	0.314
Covariate sex	–0.69 (–1.60–0.23)	0.47	0.170
eSOD1			
Intercept	2.48 (–0.68–5.64)	1.61	0.152
Independent variable eSOD1	2.76 (0.22–5.30)	1.30	0.056
Covariate age	0.21 (0–0.42)	0.11	0.076
Covariate sex	–0.38 (–1.41–0.65)	0.53	0.489
eCCS/eSOD1			
Intercept	6.42 (4.49–8.36)	0.99	<0.001
Independent variable eCCS/eSOD1 ratio	–0.51 (–0.81 to –0.20)	0.16	0.008
Covariate age	0.12 (–0.05–0.29)	0.09	0.200
Covariate sex	–0.66 (–1.54–0.21)	0.45	0.165

eCCS, erythrocyte copper chaperone for Cu/Zn superoxide dismutase 1; eSOD1, erythrocyte Cu/Zn superoxide dismutase 1.

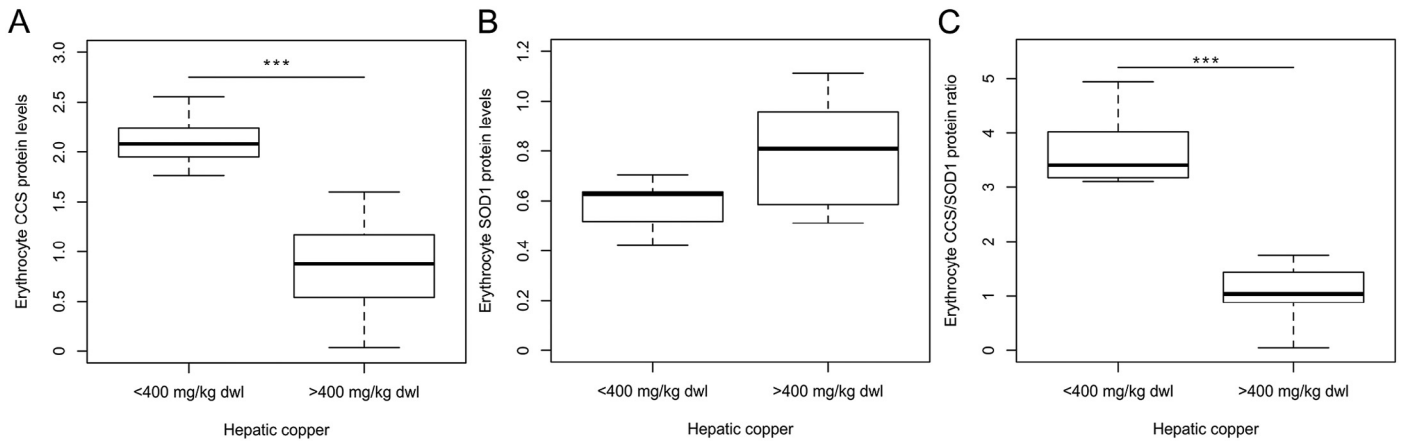

Fig. 4. Erythrocyte copper chaperone (CCS) for Cu/Zn superoxide dismutase (SOD1) (a) and SOD1 (b), and CCS/SOD1 ratio (c) in Labrador retrievers with normal (<400 mg/kg dry weight liver, dwl) and high (>400 mg/kg dwl) hepatic copper concentrations. ***$P < 0.001$.

studies consistently demonstrated significant increases in CCS protein concentrations and decreases in SOD1 protein concentrations in organs and erythrocytes following dietary induced copper deficiency (Prohaska and Brokate, 2001; Bertinato et al., 2003; West and Prohaska, 2004; Broderius and Prohaska, 2009; Hepburn et al., 2009). After copper repletion, eSOD1 and eCCS protein concentrations normalised (Lassi and Prohaska, 2011), suggesting a mutual relationship between copper and these proteins. In our study, the increase in eSOD1 protein in Labrador retrievers with increased hepatic copper concentrations was not significant. This could be explained by methodological differences in measuring SOD1 activity and SOD1 protein, which is the apoSOD1 and the copper loaded (holo) form of SOD1. Some studies recognise a larger decrease in SOD1 activity compared to SOD1 protein in copper deficiency in both organs and erythrocytes (Rossi et al., 1994; West and Prohaska, 2004). This difference was explained by inactive apoSOD1. Because protein turnover in erythrocytes is restricted and SOD1 protein concentrations did not change after copper injection in both young and old erythrocytes, the concomitant increase in SOD1 activity was explained by the activation of apoSOD1 (DiSilvestro, 1989). We did not measure SOD1 activity and our SOD1 antibody most likely detected both apoSOD1and holoSOD1 (Prohaska et al., 2003).

It has been suggested that CCS is a more sensitive biomarker for copper status than SOD1 (Bertinato and L'Abbe, 2003; Iskandar et al., 2005; Lassi and Prohaska, 2012). Additionally, CCS concentrations were not influenced by iron deficiency (Lassi and Prohaska, 2011) or inflammation (Hepburn et al., 2009; Araya et al., 2014). The most promising finding of our study was the dramatic decrease in eCCS concentrations in Labrador retrievers with high hepatic copper concentrations. To the authors' knowledge, only one study has measured eCCS in rats fed high copper diets. Rats with high hepatic copper concentrations had a 47% decrease in eCCS compared to rats with low hepatic copper concentrations (Bertinato et al., 2010). As hepatic copper concentrations were similar to those in Labrador retrievers, this corroborates our data. The mechanism of copper-mediated CCS regulation is not completely elucidated. When copper reserves are low, CCS presumably gathers stability by binding to apoSOD1, as the interaction of SOD1 and CCS is independent of copper binding (Casareno et al., 1998). Increasing concentrations of copper-scavenging CCS make copper transfer to SOD1 more rapid and efficient. When SOD1 becomes fully activated, the necessity for CCS and its expression decreases. The substantial increase of copper-loaded CCS promotes degradation of CCS by the 26S proteasome in hepatocytes (Bertinato and L'Abbe, 2003). At present, it is not clear whether these changes take place in mature enucleate erythrocytes or only in their nucleated precursors. If these changes can only take place in nucleated cells, this implies that changes in eCCS and eSOD1 are not indicative of acute changes in copper status. As the lifespan of erythrocytes is approximately 103 days (Garon et al., 2010), changes in eCCS reflect copper status over this preceding period. Because small changes in either eCCS or eSOD1 amplify the eCCS/eSOD1 ratio, this ratio can be regarded as an even more sensitive biomarker for copper status.

There was no overlap between Labrador retrievers with normal and high hepatic copper concentrations in both eCCS and eCCS/eSOD1 ratio. This emphasises the potential of eCCS and/or eCCS/eSOD1 as potential biomarkers in Labrador retrievers or other dog breeds with severe hepatic copper accumulation. Most Labrador retrievers in our study had subclinical hepatic copper toxicosis, further emphasising the potential of eCCS and eCCS/SOD1 ratio to detect early cases.

Conclusions

Our results suggest possible roles for eCCS and eCCS/eSOD1 ratio as biomarkers for screening and therapeutic monitoring of Labrador retrievers with hepatic copper toxicosis. Larger cohorts and further quantitative assays (e.g. ELISAs) are needed to validate these results. Applicability in other dog breeds with copper toxicosis has yet to be determined.

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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