Copper-associated hepatitis in dogs; pathogenesis, diagnosis and treatment
Karen Dirksen
PhD thesis, Utrecht University, Faculty of Veterinary Medicine, the Netherlands

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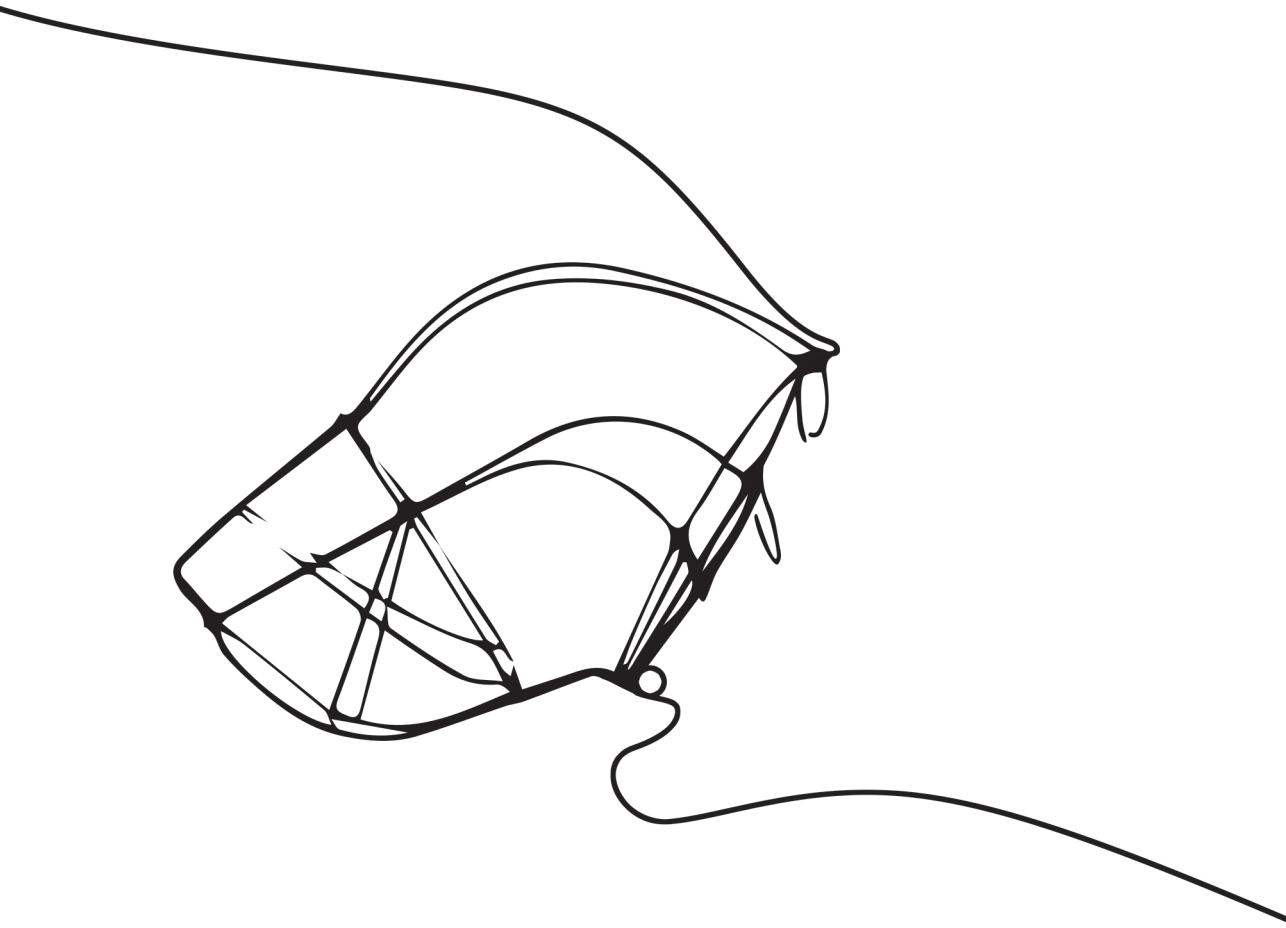

1
chapter

Scope and aims of the thesis

The importance of a tight regulation of copper homeostasis is illustrated by genetic disorders caused by mutations in copper trafficking genes. Examples include the human disorders Menkes disease [1] (causative defective gene ATP7A), leading to impaired copper absorption and copper deficiency, and Wilson disease [2] (ATP7B), characterized by copper accumulation in liver, brain and cornea. In dogs copper accumulation due to malfunctioning of copper transporters is known in the Bedlington terrier [3] (COMMD1) and the Labrador retriever [4] (ATP7A, ATP7B). Moreover, forms of copper toxicosis with an unknown mode of inheritance are known in man [5-7] and familial forms of copper-associated hepatitis are suspected in other pure bred dog breeds, including the Doberman,[8] West Highland white terrier, [9] and the Dalmatian. [10] In addition, a recent retrospective study in dogs represented at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, the Netherlands reported one-third of the primary hepatitis cases to be copper-related, [11] suggesting that copper-associated hepatitis might have a more prominent role than currently acknowledged. In both human and canine cases of copper toxicosis environmental influences (e.g. dietary) are thought to influence the phenotypic expression of the disease. In **Chapter 2** a detailed overview of cellular copper metabolism and copper-associated hepatitis is provided.

The main focus of this thesis is on the Labrador retriever. The Labrador retriever is one of the most popular and largest dog breeds in the Netherlands and has a high frequency of coppers-associated hepatitis. [12-15] The Labrador retriever is also the first dog breed in which hepatic copper levels are associated with the Wilson disease gene ATP7B and the Menkes disease gene ATP7A.[4] The ATP7B mutation (ATP7B:p.Arg1453Gln) is associated with hepatic copper accumulation due to retention of ATP7B in the endoplasmatic reticulum and failure to reach the canalicular surface in high copper conditions. In contrast, the ATP7A mutation (ATP7A:p.Thr327Ile) has a protective effect by preventing intestinal copper uptake. Both mutations only explain part of the total heritability. Besides a genetic background, there is a large influence of dietary copper and zinc intake on the expression of the disease phenotype. The relatively high disease frequency in the Labrador retriever, the long life span and a body mass that allows comparable procedures as in humans (including multiple liver biopsies over time), fulfils essential prerequisites for being a non-rodent mammalian model for human copper storage diseases.

Parallels can be made between copper-associated hepatitis in the Labrador retriever and Wilson disease or non-Wilsonian forms of copper toxicosis in humans. As the liver is the main organ involved in copper metabolism, dogs and humans suffer

from hepatic copper accumulation leading to liver cirrhosis. However, histopathological differences are also present; in dogs copper accumulates in the centrolobular region of the liver, while periportal or a more diffuse distribution of copper granules is present in humans.[16-18] Furthermore, steatosis is also frequently observed in Wilson disease patients but not in dogs.[18]

An increase of hepatic copper concentration can be anticipated in the case of cholestatic disease, since excess copper is secreted into the bile. In humans, primary biliary cirrhosis and long-standing large bile duct obstruction can cause copper concentrations in the same range as seen in Wilson disease, but clinical presentation is different.[19] In dogs however, no periportal copper accumulation is evident in cholestatic disease[20] and although it was believed that high hepatic copper concentrations in some breeds were secondary to cholestasis,[21,22] nowadays it is assumed that copper is the primary event triggering hepatocellular injury. The aim of **Part I (Chapter 3)** of this thesis was to clarify the primary role of copper in the development of hepatitis in Labrador retrievers. The second aim was to unravel the pathways involved in disease progression towards chronic hepatitis. Increasing knowledge on pathways that become deregulated during hepatic copper accumulation and the development of hepatocellular injury and liver fibrosis, will not only help to shed light on copper associated disease in other dog breeds but, as a natural non-rodent model, also on human copper storage disorders. To this end, transcriptomic alterations in Labrador retrievers in different stages of the disease were investigated.

In **Part II (Chapters 4, 5, and 6)** of this thesis we aim on optimizing the diagnosis of copper-associated hepatitis and of hepatocellular injury/hepatobiliary disease in general. Clinical signs of liver disease are non-specific and, with some exceptions, occur often late in disease, due to considerable reserve capacity of the liver. As a first step towards a presumptive diagnosis, biochemical indicators of liver injury and function (i.e. alanine aminotransferase, ALT; alkaline phosphatase, ALP; bile acids, BA) can help in detecting the presence of hepatobiliary disease.[23, 24] In clinically ill dogs these parameters are useful, however data considering sensitivity and specificity of ALT, ALP, and BA in subclinically affected dogs are lacking. The recognition of dogs in the subclinical phase of disease is of utmost importance because treatment, if necessary, can already be initiated. Therefore, the major aims of **Chapter 4** were to (1) determine the sensitivity (and specificity) of ALT, ALP, and BA for detecting hepatitis in clinically healthy Labrador retrievers; and (2) if ALT and ALP could aid in the discrimination between dogs with a primary hepatitis and a non-specific reactive hepatitis. As shown in **Chapter 4**, there is a need for new sensitive biomarkers for the early detection of hepatocellular injury.

MicroRNAs are small non-coding naturally occurring RNAs that regulate gene expression by destabilizing or repressing translation of target mRNAs.[25, 26] They exert critical functions in virtually all physiological cellular processes and are therefore also involved in the pathogeneses of many diseases. MicroRNAs have been shown to have potential as a biomarker for a variety hepatobiliary diseases in humans, including chronic hepatitis C,[27] non-alcoholic fatty liver disease,[28] rejection after liver transplantation,[29] drug- or alcohol- induced liver injury,[30, 31] intrahepatic cholangiocarcinoma,[32] and hepatocellular carcinoma.[32, 33] Because microRNAs are well conserved across species and highly stable in blood, they are good candidate biomarkers for liver diseases in dogs. **Chapter 5** covers the first study that evaluates two hepatocyte-derived microRNAs as new serum biomarkers of hepatocellular injury in Labrador retrievers. Moreover, it compares its diagnostic utility to that of plasma ALT activity. The results described in **Chapter 5** prompted us to further explore the possibilities of microRNAs as biomarkers for hepatobiliary diseases in dogs, which is the focus of **Chapter 6**.

Besides lacking sensitivity, the second major drawback of current biochemical indicators is, with the exception of vascular disorders (i.e portosystemic shunts), that they are not able to point towards the underlying disease itself. An extensive work-up with multiple techniques including imaging, cytology, and in most cases histopathology, is necessary to establish a definitive diagnosis.[34] The aim of the study presented in **Chapter 6** was to evaluate if a panel of six serum microRNAs were able to differentiate between common parenchymal, biliary, vascular, or neoplastic hepatobiliary diseases in dogs. In contrast to the rest of the studies presented in this thesis, this study was conducted in 57 dogs of different breeds.

Currently, the only way to establish the diagnosis of copper accumulating disease is by a relatively invasive liver biopsy procedure and the subsequent assessment of copper localization and copper quantification. In addition, there is also a delay between the accumulation of copper and the development of appreciable histological signs in the liver. Moreover, copper accumulation is not correlated to liver enzymes or to the existence of hepatocellular injury (**Chapter 4, Chapter 8**). Thus, to identify dogs at risk in an early stage of disease and to monitor copper levels during treatment a blood-based biomarker for copper status would be helpful. In humans with Wilson disease, the serum concentrations of copper, ceruloplasmin and the calculated non-ceruloplasmin bound copper and the urinary copper excretion can al be used to diagnose high hepatic copper concentrations in these patients.[35] In dogs, serum copper and ceruloplasmin levels do not correlate with hepatic copper concentrations. In addition the urinary copper/zinc ratio was only of limited diagnostic value,[36] stimulating us to look for alternatives.

CCS, the copper chaperone for Cu/Zn superoxide dismutase (SOD1) and SOD1 are two proteins in copper metabolism that are extensively studied in animals with copper deficiency and, to a lesser extent, with copper overload.[37-40] The aim of the study in **Chapter 7** was to evaluate erythrocyte SOD1 and CCS for their usefulness as biomarker for hepatic copper accumulation in Labrador retrievers.

The last part of this thesis (**Part III, Chapter 8**) was aimed to optimize the current treatment protocol for dogs with copper-associated hepatitis. D-penicillamine is the most frequently used drug in the treatment of copper associated hepatitis.[13, 41, 42] Because of their extremely high hepatic copper concentrations, *COMMD1* deficient dogs are usually receiving life-long chelating therapy. However, in dogs with complex forms of copper-associated disease, including the Labrador retriever, copper concentrations are generally lower and life-long therapy is not recommended. It is believed that prolonged therapy in these dogs might lead to copper and zinc deficiency due to enhanced urinary excretion.[36] Besides copper chelating properties, immunomodulatory and anti-fibrotic effects of D-penicillamine are described.[43, 44] The study presented in **Chapter 8** addresses all these aspects in order to constitute the best treatment plan. The main goals of the study were (1) to establish a model that can predict the necessary duration of treatment with D-penicillamine to reach normal hepatic copper concentrations in Labrador retrievers; (2) to evaluate the effect of D-penicillamine treatment on the activity and stage of copper-associated hepatitis; and (3) since D-penicillamine is able to chelate other biologically active trace metals,[45] to determine the effect of D-penicillamine treatment on hepatic iron and zinc concentrations.

In **Chapter 9** the results of the studies are summarized and discussed. In addition, recommendations and suggestions for future studies are made that could further aid in the understanding of copper-associated hepatitis, and in improving current diagnostics and treatment opportunities.

Aims of the thesis

I

Pathogenesis (Chapter 3)

- Establish the presence of copper as primary initiating factor
- Unravel pathways in disease progression
- Dog as a non-rodent model for (copper-associated) hepatitis

II

Diagnosis: Biomarkers (Chapter 4, 5, 6, and 7)

- Sensitivity and specificity of ALT, ALP and BA in clinically healthy Labrador retrievers
- ALT and ALP to differentiate between primary hepatitis and non-specific reactive hepatitis
- MicroRNAs as biomarker for hepatocellular injury and different hepatobiliary diseases
- CCS and SOD1 as biomarker for hepatic copper accumulation

III

Treatment (Chapter 8)

- Necessary duration of DPA treatment to normalize hepatic copper concentrations
- DPA and its effect on activity and stage of hepatitis
- Influence of DPA treatment on hepatic iron and zinc concentrations

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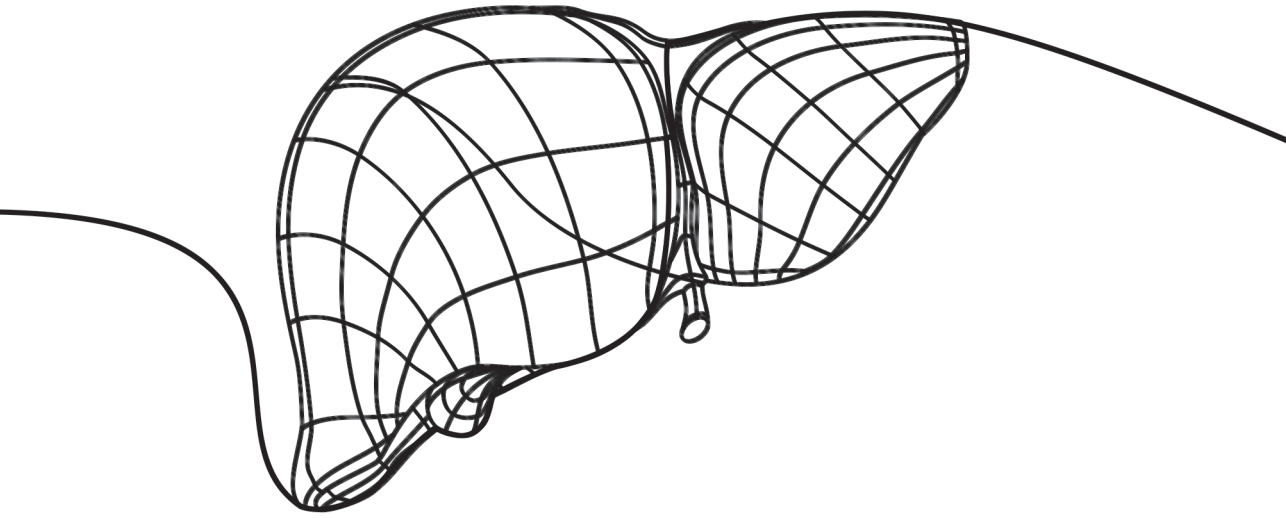

2
chapter

Introduction: Canine copper-associated hepatitis

Karen Dirksen[a]
Hille Fieten[a]

Adapted from Vet Clin North Am Small Anim Pract

[a] *Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands*

Synopsis

Copper-associated hepatitis is recognised with increasing frequency in many dog breeds. The disease is characterized by centrolobular copper accumulation, leading to hepatitis and eventually cirrhosis. The only way to establish the diagnosis is by histological assessment of copper distribution and copper quantification. Treatment with the copper chelator D-penicillamine is the most commonly used treatment. In addition, a low copper, high zinc diet can help to prevent (re-) accumulation of hepatic copper. Mutations in the copper metabolism genes COMMD1, ATP7A, and ATP7B have been associated with hepatic copper concentrations. In some breeds, dietary copper intake contributes strongly to the disease phenotype

Introduction: pathophysiology of copper homeostasis and cellular copper metabolism

Copper homeostasis

Copper is an essential trace element necessary for many vital functions in the body. However, free copper is toxic due to the potential to create reactive oxygen species. Therefore, copper uptake, distribution, and excretion are tightly regulated. [1] Dietary copper is predominantly absorbed in the small intestine. Copper uptake by the enterocyte is mainly mediated by CTR1, a high affinity copper transporter. The copper transporting P-type ATPase A (ATP7A) is located at the basal membrane of the enterocytes and facilitates copper transport into the portal circulation. In the portal blood, copper is predominantly bound to albumin and is delivered to the hepatocellular cytosol via apically located CTR1. The liver is the most important organ in copper metabolism and is responsible for copper storage, redistribution to other tissues and organs and excretion of excess copper via the biliary system. The kidneys excrete a small proportion of excess body copper.

Cellular copper metabolism

After copper enters the hepatocytes it is immediately bound by proteins to prevent oxidative damage (Fig 1). Copper scavengers, including small proteins like metallothionein (MT) and glutathione (GSH), are the first to bind and store copper. Special delivery proteins, the copper chaperones, ensure safe handover of copper to their destination molecules. [2] Cox17 is the copper chaperone for cytochrome C oxidase (CCO), which resides in the inner mitochondrial membrane. Cytochrome C oxidase is the terminal enzyme in the mitochondrial respiratorychain and thus plays a crucial role in aerobic energy metabolism. Copper chaperone for superoxide dismutase (CCS) shuttles copper to Cu,Zn superoxide dismutase (SOD1), which is an important protein in the defense against oxidative stress. ATOX1 is the copper chaperone for the copper transporting ATP-ases, ATP7A and ATP7B. Both ATP-ases reside in the Trans-Golgi Network (TGN) under normal copper conditions. When intracellular copper concentrations are rising, they move away from the TGN to their respective destinations. In the TGN, APT7B loads six copper atoms onto the ferroxidase ceruloplasmin (CP), which is secreted into the circulation. [3] Ceruloplasmin is the main copper transport protein in the blood. Under elevated copper conditions, ATP7B traffics to a lysosomal or apical membrane associated cellular component and facilitates excretion of excess copper into the bile. [4] Previously, the main role of ATP7A was presumed to be copper uptake in the intestines, but recently hepatocellular ATP7A was demonstrated to have an important role in mobilizing and redistributing hepatic copper stores in case of peripheral copper deficiency. [5] The Copper Metabolism (Murr1) Domain Containing

1 (COMMD1) protein interacts with the amino terminus of ATP7B, and presumably facilitates biliary excretion of copper. In addition, COMMD1 has a role in the stability and quality control of both ATPases. [6]

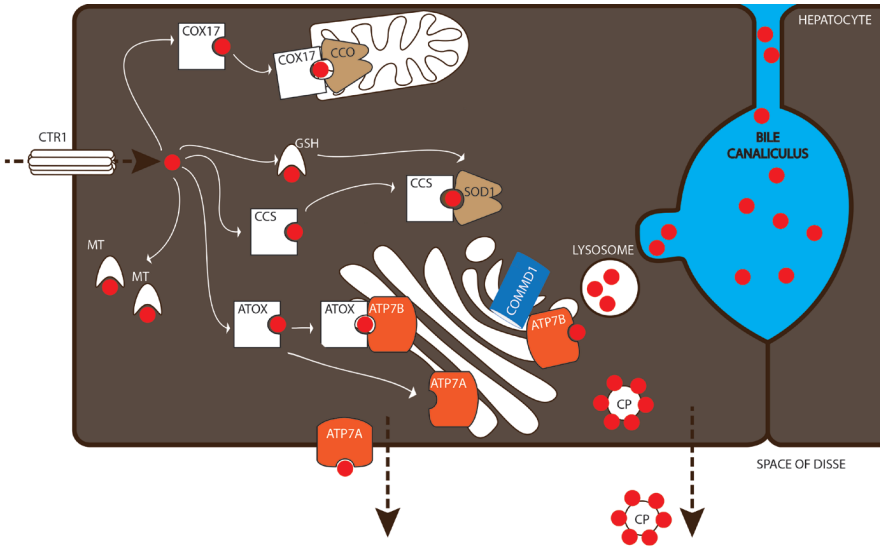

Figure 1 Hepatocellular copper metabolism.

Copper enters the cell via CTR1 and is immediately bound by metallothioneins (MT) and/or GSH to prevent oxidative stress. The chaperones COX17, CCS, and ATOX1 transfer copper to their respective destination molecules CCO, SOD1, and ATP7A/ATP7B. ATP7A and ATP7B function in the export of copper to the blood (ATP7A and ATP7B) or to the bile (ATP7B). COMMD1 interacts with both ATPases. ATOX1, antioxidant 1 copper chaperone; ATP7A, ATPase, Cu++ transporting, alpha polypeptide; ATP7B, ATPase, Cu++ transporting, beta polypeptide; CCO, cytochrome C oxidase; CCS, copper chaperone for superoxide dismutase; COMMD1, copper metabolism (Murr1) domain containing 1; COX17, cytochrome C oxidase copper chaperone; CP, ceruloplasmin; CTR1, copper transporter 1; GSH, glutathione; MT, metallothionein; SOD1, Cu,Zn superoxide dismutase 1

Copper metabolism disorders in humans

Wilson disease

Wilson disease is an autosomal recessive disorder in humans in which copper accumulates in liver and neuronal tissues. The disease manifests as hepatopathy and/or neurological or psychiatric symptoms. Wilson disease can result from a

number of mutations in the copper transporter ATP7B. Because of its role in the incorporation of copper into ceruloplasmin, this may lead to low serum ceruloplasmin concentrations, which is one of the diagnostic criteria. Further, urinary copper excretion may be increased in patients with Wilson disease. Conventional treatment consists of lifelong copper chelation with D-penicillamine.[7]

Non-Wilsonian forms of copper toxicosis

Other copper storage disorders in humans in which the causative genes have not yet been identified include Indian childhood cirrhosis[8], endemic Tyrolean infantile cirrhosis[9], and idiopathic copper toxicosis.[10] In these diseases, a predominant hepatic presentation is observed. A hereditary predisposition in combination to an increased (dietary) exposure to copper is thought to be responsible for the observed disease symptoms.

Menke's disease

Mutations in the copper transporter ATP7A result in X-linked, recessive copper deficiency, due to impaired dietary intestinal copper uptake. Patients suffer from severe neurological impairment and failure to thrive in early childhood, and the disease is often lethal despite parenteral copper supplementation.[11]

Hereditary copper-associated hepatitis in dogs

Bedlington terrier

Historically, the Bedlington terrier was the first dog breed in which canine copper-associated hepatitis was studied extensively and where a causal mutation was identified. The disease is characterized by liver cirrhosis induced by massive, centrolobular copper accumulation (Fig 2A,D). Hepatic copper may be as high as 10,000 mg copper per kg dry weight liver (dwl). The causal mutation is a large deletion in the second exon of the *COMMD1* gene, leading to autosomal recessive copper toxicosis.[12] Due to the development of a DNA test, the disease frequency in the Bedlington terrier population has been drastically reduced. Recently, cases of non-*COMMD1* related copper toxicosis were observed in Bedlington terriers.[13] Variations in the metal transporter ABCA12 were found to be associated to non-*COMMD1* copper toxicosis, however convincing functional data proving involvement of this gene was lacking.

Labrador retriever

The Labrador retriever was the second breed in which part of the hereditary background of copper-associated hepatitis was elucidated. In this breed, the

disease follows a complex inheritance pattern, and genetic as well as dietary factors [14-18] play a role in pathogenesis. A recently performed genome wide association study showed a significant association of increased hepatic copper concentrations with a mutation in the Wilson disease gene (*ATP7B*). Interestingly, concurrent presence of a mutation in the Menkes disease gene (*ATP7A*) seemed to attenuate hepatic copper accumulation without resulting in a copper deficient phenotype. Approximately 12% of total heritability can be explained by the two identified mutations. Missing heritability may be explained by environmental factors or yet unidentified genetic mutations. Functional assays in cell lines showed that the *ATP7B* mutation in the conserved arginine resulted in an aberrant retention of the protein in the endoplasmic reticulum in high copper circumstances. The *ATP7A* mutation, located in a conserved phosphorylation site, did not affect trafficking of the protein, yet lead to a decrease of copper efflux in dermal fibroblasts, indicating a functional impairment of the protein. [19]

Other breeds

Copper-associated hepatitis with a suspected hereditary background has been described in several other dog breeds including the Dobermann, [20] the West Highland white terrier, [21] and the Dalmatian. [22] Case-reports of dogs diagnosed with copper-associated hepatitis include the Skye terrier, [23] Anatolian shepherd, [24] the Pembroke and Cardigan Welsh Corgi, [25, 26] and the Clumber spaniel. [26] More extensive reviews of hepatic copper concentrations in dogs diagnosed with primary hepatitis suggest that there are many more breeds, including crossbreeds, in which copper-associated hepatitis is present. [27, 28] We think that it is very unlikely that environmental factors, such as dietary composition could explain copper accumulation and associated hepatitis in genetically healthy dogs. We anticipate that most of the reported breeds will have some form of hereditary dysfunction in their copper metabolism. Genetic studies are needed to elucidate the affected genes in these dogs.

Diagnosis

Signalment

The rate of hepatic copper accumulation and development of associated clinical signs depends on genetic predisposition and dietary copper intake and varies between breeds and between individuals within a breed. In Labrador retrievers, the age at which dogs present with clinical signs can range from 2-12 years old, but most dogs are middle-aged (median age of 6 years). Bitches in the post-partum period may be at increased risk for development of clinical signs. A strong female

predisposition is noted in the Labrador retriever [29] and the Doberman [30], whereas in other dog breeds both sexes are usually represented equally.

Clinical signs

The subclinical phase in dogs with inherited copper-associated hepatitis is usually long for two reasons. First, hepatic copper accumulation precedes the development of histological changes in the liver. In Bedlington terriers it has been shown that copper starts accumulating between six and twelve months of age without overt histological signs of hepatitis. [31] Second, clinical signs only develop when a large portion of liver parenchyma is affected. Because the liver has an enormous reserve capacity, this is usually in the end stage of the disease when chronic hepatitis or cirrhosis becomes overt. Initially, clinical signs are nonspecific and may include anorexia, lethargy, nausea, vomiting, and weight loss. When the disease becomes more progressive, more specific signs, pointing towards hepatic failure, such as ascites, hepatic encephalopathy, polyuria/polydipsia, and icterus can be noticed. Although rare, in the Bedlington terrier an acute hemolytic crisis due to the massive release of copper into the bloodstream has been reported. [32]

Clinical pathology

The most commonly used biochemical indicators for hepatocellular injury are alanine aminotransferase (ALT) and alkaline phosphatase (ALP). [33] However, in the subclinical stage of copper accumulation, extensive hepatocellular injury is not necessarily present. In affected Bedlington terriers, hepatocellular injury became visible between 12 and 18 months of age, while an increase in ALT and ALP was only detected at 24 and 18 months of age, respectively. [31] This observation underlines that ALT and ALP are not useful for screening for subclinical copper-associated hepatitis. In a more advanced stage of the disease, an increase of ALT and ALP can be noticed, and a slight decrease in albumin concentration may be observed as well, although concentrations still may be within the reference range. [22, 29, 34] Other laboratory indicators include an increase in bile acids (BA), ammonia, bilirubin, prothrombin time, and activated partial thromboplastin time, and a decrease in fibrinogen and packed cell volume. [29, 34] Serum/plasma ALT and ALP activity, as well as BA concentration are useful parameters for detecting liver disease but neither specific for copper-associated liver disease. In dogs, serum copper concentrations do not correlate with hepatic copper concentrations. Decreased ceruloplasmin is a diagnostic hallmark for human Wilson disease. It would be interesting to investigate serum ceruloplasmin concentrations in Labrador retrievers with copper-associated hepatitis due to *ATP7B* mutations.

Fanconi syndrome

Comparable to observations in humans with Wilson disease, Fanconi syndrome has been recognized in dogs with copper-associated hepatitis.[26, 35, 36] In these dogs proximal tubular dysfunction was present due to copper accumulation in the proximal tubular epithelium. Dogs presented with low urinary specific gravity, proteinuria, and normoglycemic glucosuria. The observed abnormalities were reversed by D-penicillamine chelation therapy.

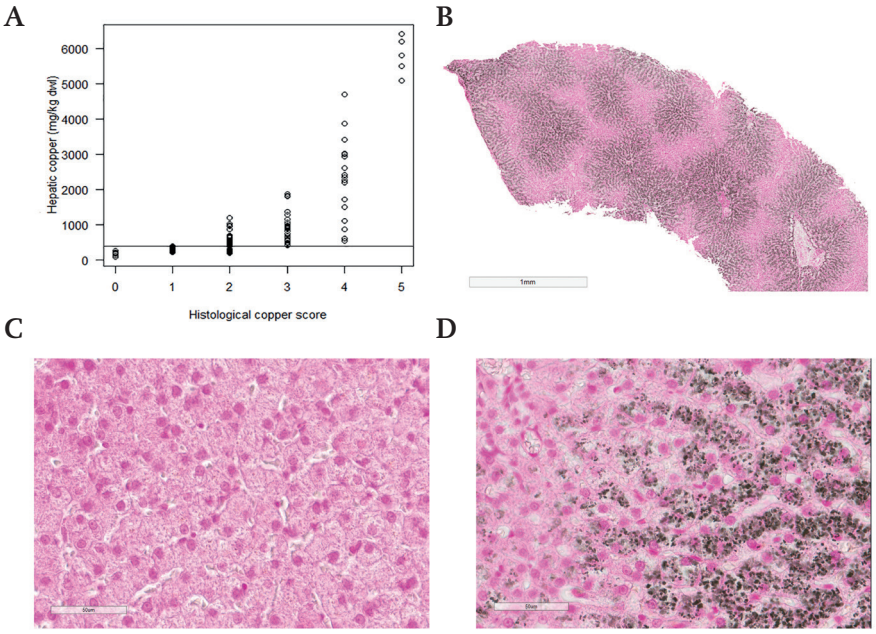

Figure 2 Rubeanic acid stain for copper.

A relation between hepatic copper scores (x-axis) and quantitative copper measurements (y-axis) in 109 canine liver samples collected at the Faculty of Veterinary Medicine, Utrecht University between 2010 and 2016. Horizontal black line indicates cut-off level for normal hepatic copper (400 mg/kg dwl). **B** Centrolobular copper distribution is clearly visible in a liver biopsy of a *COMMD1*-deficient dog. **C** Copper score 0 (rubeanic acid stain) in a Labrador retriever (quantitative copper concentration 146 mg/kg dwl). **D** Copper score 5 (rubeanic acid stain) in a Bedlington terrier (quantitative copper concentration 6540 mg/kg dwl).

Cytology

Fine needle aspiration and cytology of hepatocytes stained with a specific copper stain (i.e. rubeanic acid) may be used as a relatively non-invasive way to get an indication of the presence of copper in individual hepatocytes. [20, 37] Obvious limitations of this technique are the impossibility to evaluate zonal copper distribution, degree of hepatocellular injury and the determination of the exact amount of copper (needed for determination of treatment duration). Further studies into the negative predictive value are necessary, as copper distribution in the liver lobules is zonal and theoretically a negative sample could be obtained from a dog affected with copper-associated hepatitis.

Liver histopathology

Histological distribution of copper

A histological biopsy remains the gold standard for diagnosing copper-associated hepatitis. Samples can be obtained via laparotomy, laparoscopy or, percutaneously with a needle (14-16 gauge) using ultrasound guidance. As copper is not visible on routine hematoxylin and eosin stains, additional staining for copper (rubeanic acid [38] or rhodanine [39]) is necessary for the diagnosis. In the case of primary copper-associated hepatitis copper typically starts to accumulate in the centrolobular regions of the hepatic lobule (zone 3; Fig 2B). [28, 29, 40] Copper-loaded hepatocytes trigger the emergence of an inflammatory infiltrate, which can be mononuclear or mixed. Since excess copper is excreted into bile, an increase of hepatic copper, especially in the periportal areas of the liver lobules could be anticipated in dogs with cholestatic diseases. However, in many cases of cholestatic liver disease, no periportal copper accumulation is detectable and the interpretation of rare cases with periportal copper accumulation is not totally clear. [41] In end-stage liver disease, where severe cirrhosis, massive necrosis and lobular collapse are present, it may be difficult to distinguish the different zones of the liver lobule. Furthermore, in an advanced stage of the disease necrotic hepatocytes have released their copper burden and newly formed hepatocytes, which arise during the regeneration process, do initially not contain copper. [21, 42] Neither does scar tissue, further diluting total copper content in the transition to end-stage disease. Mainly because of these unevenly distributed histological changes, one always has to consider the results of both the histological examination (distribution and scoring) and quantitative copper determination in hepatic copper assessment.

Histological scoring of copper

A semi-quantitative grading system to determine hepatic copper content can be applied on copper stained sections of liver tissue (Fig 2A). [40] Scoring is based on zonal location and number of hepatocytes and macrophages containing copper

granules. In a grading scale of 0 to 5, copper scores of 2 or higher are considered to be abnormal (Fig 2A,C,D). However, each semi-quantitative score includes a wide range of quantitative copper concentrations, with overlap between scores (Fig 2A).

Quantitative copper determination

Hepatic copper concentrations can also be assessed quantitatively by the irradiation of small pieces of liver and the measurement of the induced copper radioactivity. [43] Therefore an additional biopsy specimen of at least 5 mg is needed, which is freeze dried before analysis to determine dry weight copper. Other methods for quantification include spectrophotometric methods, including atomic absorption spectrometry, or inductively coupled plasma emission spectrometry. Normal hepatic copper concentrations in dogs are considered to be below 400 mg/kg dwl. [44] In dogs affected with copper-associated hepatitis, hepatic copper concentrations are usually above 800 mg/kg dwl, but can reach 10,000 mg/kg dwl. In this respect, dogs are markedly different from humans where normal copper concentrations lie in the range of 50 mg/kg dwl and patients with Wilson disease usually have hepatic copper concentrations in the range of 500 mg/kg dwl.

Digital calculation of copper concentrations

Digital microscopic scanning of copper stained liver sections has shown to be more accurate than qualitative copper scoring, but still allows assessment of zonal histological lesions. [45] This technique can be applied for histological slides of liver biopsies where no additional sample for copper quantification is available.

Biomarkers

As currently the only way to diagnose and monitor copper-associated hepatitis is by (repeated) histological assessment of liver biopsies, the development of a non-invasive biomarker for copper status from samples of blood or urine is warranted. Such a biomarker could help to identify at risk dogs in order to prevent clinical illness by intuition of early treatment and to prevent breeding of affected individuals. Moreover, it would be easier to monitor copper concentrations during treatment using a serum or urine biomarker. The urinary copper/zinc ratio was significantly associated with hepatic copper concentration in Labrador retrievers, but the diagnostic value was limited due to overlap between normal and affected dogs. [46] Cu,Zn superoxide dismutase (SOD1) and its chaperone (CCS) have both been studied as biomarkers in humans and animals with copper deficiency and overload. Other biomarkers under investigation include microRNAs, which are small non-coding RNAs that regulate gene expression. [47] Ideally, new copper-specific microRNAs should be identified.

Treatment

General recommendations

The goal of treatment in dogs with copper toxicosis is to create a negative copper balance. This can be achieved by restricting copper intake and by increasing urinary copper excretion using copper chelators (Table 1). Because treatment has the best outcome early in the disease when hepatocellular injury is limited, it is important that treatment is initiated as soon as possible and ideally in the subclinical phase.

Increased hepatic copper concentrations may induce oxidative stress and in this way contribute to progression of hepatocellular injury. It is currently unknown at exactly which levels of copper this process starts and when chelation therapy should be initiated. For dogs, usually an hepatic copper level of > 400 mg/kg dwl is considered increased, which is already quite high when, for example, compared to normal hepatic copper levels in humans (50 mg/kg dwl). In general, clinically ill dogs or dogs with overt hepatocellular injury and increased hepatic copper levels should be treated with a copper chelator and treatment should be monitored by follow-up liver biopsies. In dogs without clinical signs, with normal hepatic enzymes and moderately increased hepatic copper levels (i.e. 400-600 mg/kg dwl), changing to a low copper/high zinc diet may be sufficient in normalization of hepatic copper levels. However, individual variation in response to diet was noted and continuing copper accumulation may occur despite feeding a low copper high zinc diet. [48] Because it is currently not possible to predict response to diet in individual dogs, a control biopsy is always necessary within 6 months after initial diagnosis and dietary change.

D-penicillamine

D-penicillamine (DPA) is a highly soluble degradation product of penicillin that is excreted by the kidneys. It binds one copper atom at its sulphydryl-group and facilitates excretion of copper into the urine. [49] D-penicillamine is one of the most potent copper chelators, and is also able to form lower avidity complexes with other metals like zinc and iron. [50, 51] Besides chelating properties, DPA may have additional favorable immunomodulatory, and anti-fibrotic activities. [52, 53] It has shown to be effective in the treatment of canine copper-copper associated hepatitis and is the most commonly used chelator. [29, 54-56] The recommended dose for use in dogs is 10 to 15 mg/kg *PO* twice daily. To increase bioavailability and to maximize plasma concentrations, DPA should not be given with food. [57] Side effects in dogs are usually limited to gastrointestinal signs, such as anorexia and vomiting. [56, 57] Gastro-intestinal side effects are easy manageable by temporarily decreasing the dose or by giving anti-emetics 1 hour before DPA administration. [56, 57]

Table 1 Overview of drugs used in the treatment of copper-associated hepatitis.

Drug	Dose	Adverse effects	Remarks
D-penicillamine (DPA)	10-15 mg/kg *PO* BID, separate from meals	Anorexia, vomiting, and possibly immune-mediated reactions	Most commonly used Possibly immunomodulatory and anti-fibrotic properties Prediction model for treatment duration available for Labrador retrievers
Trientene (2,2,2-tetramine)	15 mg/kg *PO* BID	None reported in dogs	
2,3,2-tetramine	15 mg/kg *PO* BID	None reported in dogs	Not commercially available
Ammonium Tetrathiomolybdate	Unknown	Anorexia, vomiting	High risk of severe copper deficiency resulting in bone marrow depression
Zinc salts; - zinc acetate - zinc gluconate - zinc sulphate	5-10 mg/kg elemental zinc *PO* BID	Generally well tolerated, but gastrointestinal side effects may occur	Should not be the sole therapy in clinical cases Slow onset of action Monitoring of plasma zinc concentrations necessary

In humans DPA may induce immunological side effects, but these have not been regularly reported in dogs. The authors and editor are aware of two cases in which immunological effects were presumed to be related to DPA administration. A 4 year old, female neutered English Springer Spaniel developed severe protein-loosing glomerulonephropathy resulting in hypoalbuminemia and ascites approximately 4 months after initiation of DPA therapy. Proteinuria and hypoalbuminemia resolved completely within 2 weeks after cessation of DPA. Further, a West Highland White Terrier presented with severe dermatological lesions shortly after starting DPA, which quickly and completely resolved after DPA cessation. While in both cases a causal link remains difficult to prove, there was a suspicion of DPA related immunological side effects.

Treatment should be continued until normal hepatic copper concentrations are achieved. Continuous DPA therapy may lead to copper and zinc deficiency due to enhanced urinary excretion of these metals.[46, 56] Despite one case-report of an affected Bedlington terrier developing DPA induced copper deficiency[58] affected Bedlington terriers usually need lifelong continuous chelation therapy. In many of these dogs, normalization of hepatic copper concentrations does not occur despite therapy[54], but progression of disease is precluded. In other dog breeds lifelong

therapy is not recommended. An intermittent treatment regime with recheck biopsies every 1-2 years will prevent copper (re)accumulation and concurrently avoid copper- and zinc deficiencies.

Trientene (2,2,2-tetramine)

Trientine is a tetramine chelator that was originally introduced for humans that developed adverse reactions to DPA. Like DPA, trientene is an effective promoter of urinary copper excretion although it may act through a different pool of body copper.[59] In humans fewer side effects are reported than for DPA[60], whereas in dogs no side effects have been reported.[61] However, studies in dogs are limited. The recommended dose in dogs is 15 mg/kg twice daily. At the time of writing the cost of trientine in the United States makes it prohibitively expensive to use in veterinary patients.

2,3,2-Tetramine

2,3,2- Tetramine is another tetramine chelator, but was reported to produce a 4-9 fold greater urinary copper excretion than trientene.[61] 2,3,2-Tetramine therapy was studied in five Bedlington terriers with copper toxicosis.[62] After 200 days of treatment hepatic copper concentrations decreased 55%, without the development of adverse reactions. Beside this study, limited data is available and 2,3,2-tetramine is not commercially available.

Ammonium Tetrathiomolybdate

Ammonium tetrathiomolybdate (TTM) is a very strong copper chelator that forms a tripartite complex with copper and protein in the intestines, plasma and liver tissue. It decreases metallothionein-bound hepatic copper by excretion of copper-tetrathiomolybdate complexes into the bile and blood.[63, 64] Due to its extensive de-coppering effects it has anti-angiogenic properties, which makes it also a candidate for cancer treatment in humans and dogs.[65, 66] To date, TTM has not been used for the treatment of copper-associated hepatitis in dogs. One study, conducted in healthy dogs, showed that TTM administration (1 mg/kg) resulted in a significant increase in serum copper concentration underlining the possible potential as future therapeutic agent.[67]

Zinc

The oral administration of zinc salts (-acetate, -gluconate, -sulphate), interferes with copper uptake in the enterocytes. Zinc-oxide has a limited bio-availability. Increased intestinal zinc concentrations are believed to induce upregulation of intestinal metallothionein, which has a high affinity for copper. With high levels of copper bound to metallothionein, less copper is available for serosal transfer

and passage into the portal circulation is blocked.[68, 69] Presumably, zinc may also attenuate the toxicity of copper by inducing metallothionein upregulation in hepatocytes.[70] In humans copper storage diseases, long-term effectivity is similar to that of DPA, but zinc is generally tolerated better.[71, 72] Zinc –acetate, -gluconate, and -sulfate have all been used in dogs with copper toxicosis.[14, 73, 74] Acetate and gluconate salts may be better tolerated than sulfate, but individual differences in response exist. Normal plasma zinc concentration range from 90 to 120 μg/dL. To suppress gastrointestinal copper uptake, a minimal plasma zinc concentration of 200 μg/dL is needed.[73] The recommended dose to achieve a plasma concentration between 200 to 300 μg/dL, is 5 to 10 mg/kg elemental zinc twice daily or 200 mg of elemental zinc per day, in divided doses. To be effective, zinc salts should not be given with any food. Plasma zinc concentrations exceeding 1,000 μg/dL may result in hemolysis. Therefore plasma zinc concentrations should be monitored during treatment. Because a minimum of 3 months of administration is required to obtain a therapeutic response, zinc therapy is not recommended as the sole treatment in clinical cases. Those cases require more aggressive therapy with copper chelators.

Dietary management

Dietary intake has a significant impact on hepatic copper accumulation[15, 18] and an adjusted diet (low-copper, high-zinc) may be valuable in the management dogs with copper-associated hepatitis. A low-copper, high-zinc diet may be beneficial to prevent or postpone re-accumulation of copper in dogs that were initially treated with a copper chelator.[14, 17] Another role for dietary management could be in subclinical dogs with moderate copper accumulation. In one study, hepatic copper concentrations could be normalized with dietary intervention alone in approximately 50% of subclinical Labrador retrievers with increased hepatic copper.[16] However, some individuals continued to accumulate copper, even when fed a low copper/high-zinc diet. Individual variation in response to diet may be influenced by hereditary factors[19]. As variation in response to low copper-high zinc diets occurs, hepatic copper concentrations should be evaluated by repeated biopsies with copper staining and quantification.

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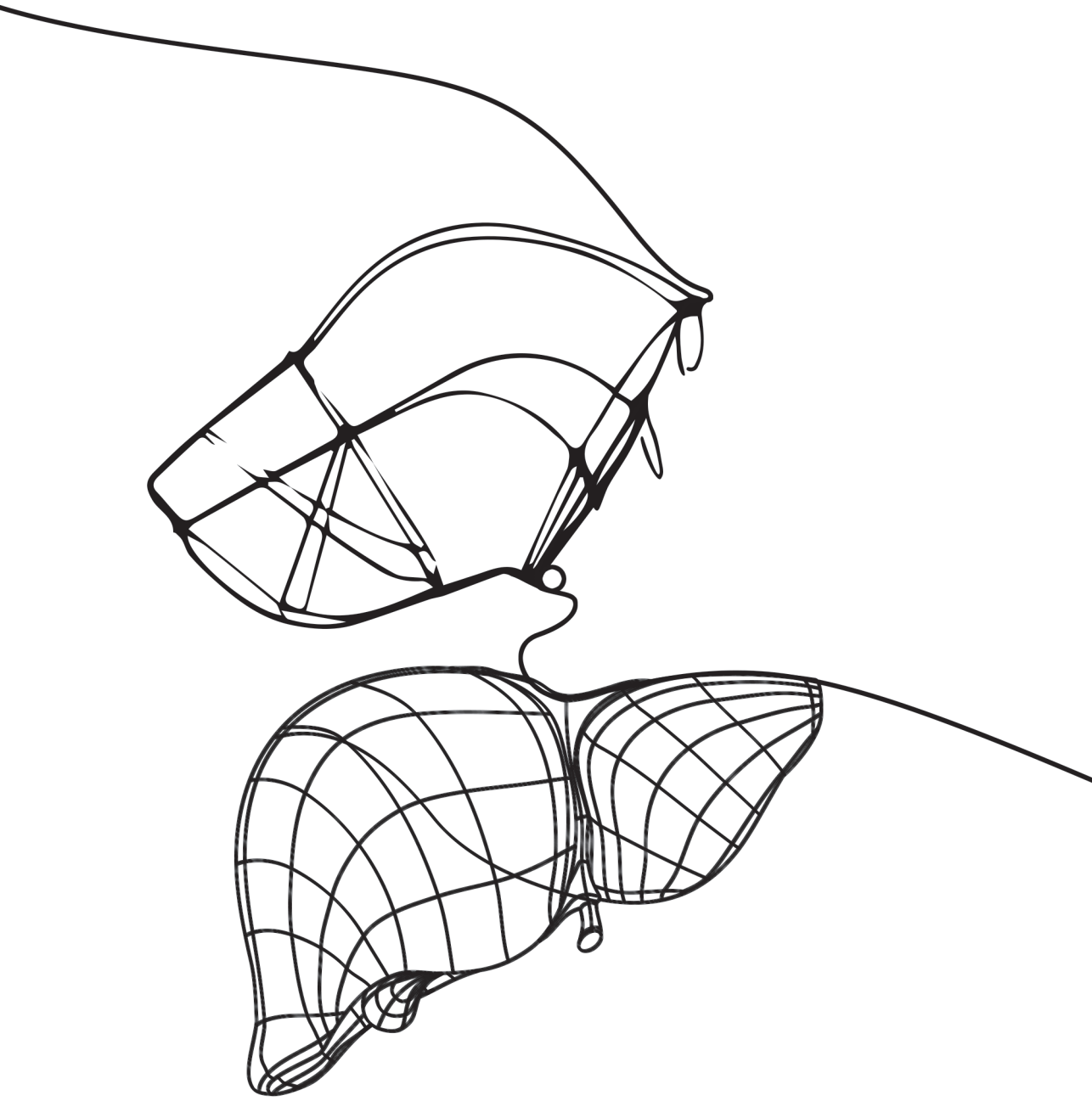

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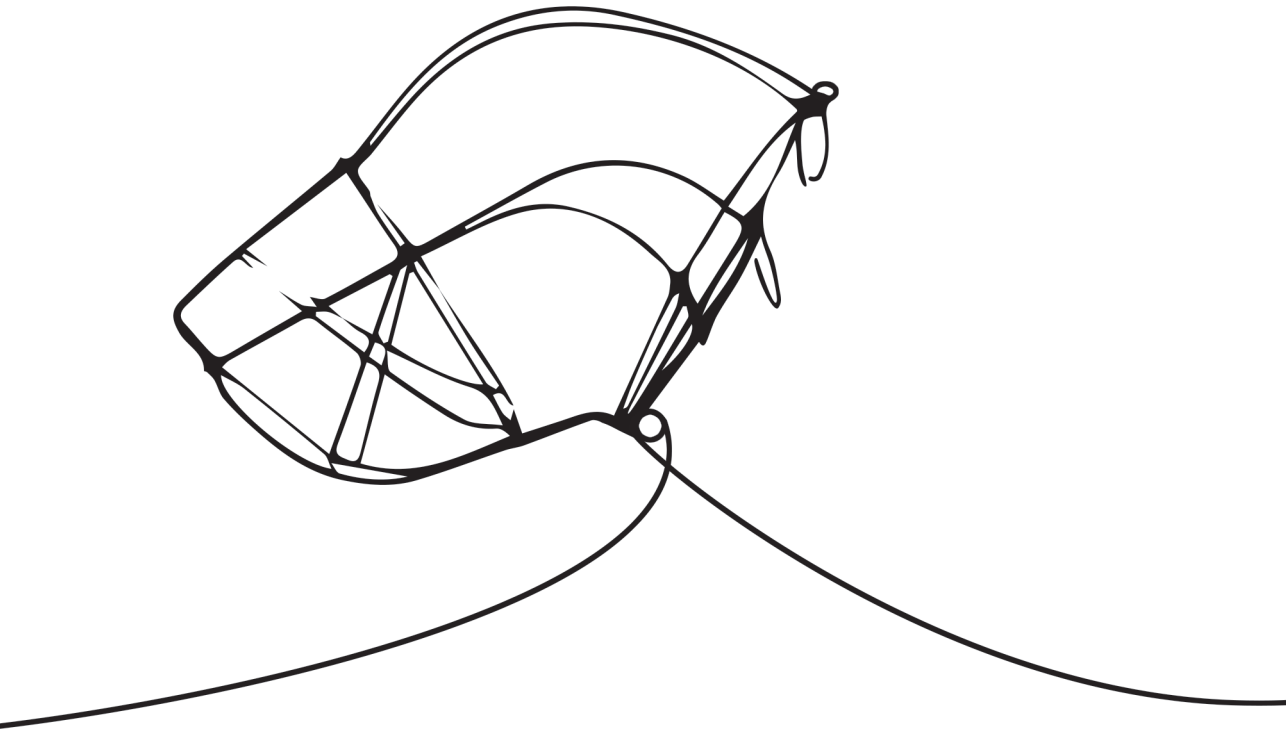

3
chapter

Gene expression patterns in the progression towards canine copper-associated hepatitis

Karen Dirksen[a]
Bart Spee[a]
Louis C. Penning[a]
Ted S.G.A.M van den Ingh[b]
Iwan A. Burgener[a,c]
Adrian L. Watson[d]
Marian Groot-Koerkamp[e]
Jan Rothuizen[a]
Frank G. van Steenbeek[a]
Hille Fieten[a]

Submitted

[a]*Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands*
[b]*TCCI Consultancy BV, Utrecht, The Netherlands*
[c]*Department für Kleintiere und Pferde, Veterinärmedizinische Universität Wien, Vienna, Austria*
[d]*The Royal Canin Research Center, Aimargues, France.*
[e]*The Princess Maxima Center, Utrecht, The Netherlands*

Abstract

Copper is an essential trace element, but can become toxic when present in abundance. The severe effects of copper-metabolism imbalance are illustrated by the inherited disorders Wilson disease and Menkes disease. The Labrador retriever dog breed is a novel non-rodent model for copper-storage defects displaying identical phenotypic alterations and carrying mutations in genes known to be involved in copper transport. Besides disease initiation and progression of copper accumulation, the molecular mechanisms and pathways involved in progression towards copper associated chronic hepatitis still remains unclear. Using liver tissue of Labrador retrievers in different stages of copper-associated hepatitis, expression levels targeted at candidate genes as well as transcriptome microarrays, have shed light on involved molecular pathways. At the initial phase, *i.e.* increased hepatic copper levels, transcriptomic alterations in livers revealed enrichment for cell adhesion, developmental, inflammatory, and cytoskeleton pathways. Upregulation of targeted *MT1A* and *COMMD1* mRNA shows the livers first response to rising intrahepatic copper concentrations. In livers with copper-associated hepatitis mainly an activation of inflammatory pathways is detected. Once the hepatitis is in the chronic stage, transcriptional differences are found in cell adhesion adaptations and cytoskeleton remodelling. In view of the high similarities in hepatopathies between men and dog, extrapolation of these dog data into human biomedicine seems feasible.

Introduction

Copper is a trace element in living organisms and functions as a catalytic and structural cofactor essential for several important biological processes in life.[1] Dietary copper is absorbed via enterocytes in the small intestines and transported to the liver via the portal circulation.[2] The liver is the main organ responsible for copper storage, distribution throughout the body, and copper excretion via the biliary system. When in excess, copper can be highly toxic and can induce oxidative stress by the formation of reactive oxygen species (ROS).[3-5] Copper induced hydroxyl radicals can lead to DNA damage, oxidation of bases, and lipid peroxidation. Therefore copper uptake, distribution, and excretion are tightly regulated and mediated by several copper binding proteins [6] (Fig 1).

Copper uptake by the enterocyte and hepatocyte is mediated by CTR1.[20] Intracellular copper is immediately bound and transported by glutathione, which has an important role in the cellular defence against oxidative stress, or stored and incorporated into metallothioneins (MT).[4] Specific copper chaperones escort copper to their destination molecules. The chaperone COX17 directs copper to cytochrome C oxidase in the mitochondria. [21, 22] CCS is the chaperone for Cu/Zn superoxide dismutase (SOD1), which plays an important role in the defence against oxidative stress. [23] ATOX1 delivers copper to the copper transporting ATPases, ATP7A and ATP7B. ATP7B is predominantly expressed in the liver and facilitates incorporation of copper in the ferroxidase ceruloplasmin (CP).[10] Further, ATP7B mediates excretion of excess copper via the apical membrane into bile canaliculi.[9] The biliary excretion of copper also depends on COMMD1, which interacts with the amino terminus of ATP7B and is a presumed regulator of ATP7B stability. [11, 13]

The importance of the tight regulation of copper homeostasis is shown by diseases caused by mutations in copper trafficking genes. Mutations in ATP7A, result in the X-linked recessive disorder Menkes disease. [24] Mutations in ATP7B are responsible for the autosomal recessive Wilson disease. [25] Familial copper toxicosis is also common in several dog breeds. [26-31] Due to the limited genetic variability within inbred dog populations, [32] dogs are used as large animal model to dissect genetics basis of (complex) inherited diseases. [33-36] A deletion of exon 2 of the COMMD1 gene was found in affected Bedlington terriers [31] and recently two missense mutations in copper transporters ATP7B (Wilson disease gene) and ATP7A (Menkes disease gene) were identified in Labrador retrievers suffering from copper toxicosis. [35] Besides a genetic background, hepatic copper concentrations in Labrador retrievers are also influenced by dietary copper intake,[37] exemplifying the similarities with both Wilson disease and non-Wilsonian ecogenetic forms of human copper

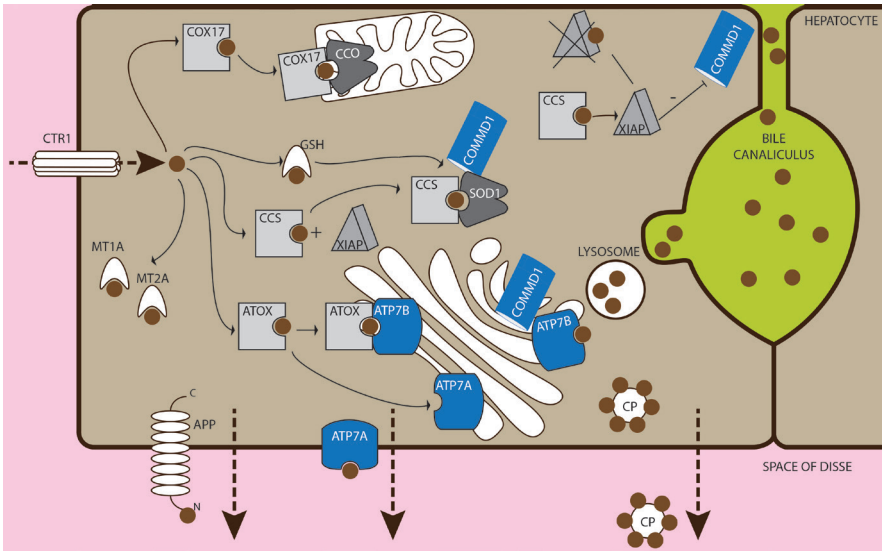

Figure 1 Cellular copper metabolism.

Copper enters the cell via CTR1 and is immediately bound by metallothioneins (MT) and/or GSH to prevent cellular damage. COX17, CCS, and ATOX1 transfer copper to its destination molecules CCO, SOD1, and ATP7A/ATP7B respectively. In the enterocyte ATP7A facilitates copper transport over the basolateral membrane into the portal circulation, [2, 7] while in the hepatocyte it mobilizes hepatic copper stores in the case of peripheral copper deficiency. [8] ATP7B functions in the export of copper to the blood bound to ceruloplasmin (CP) or to the bile when copper levels are high. [9, 10] The biliary excretion of copper also depends on COMMD1, which interacts with the amino terminus of ATP7B. In addition COMMD1 may be involved in quality control of ATP7A and ATP7B. [11-13] COMMD1 interacts with also with other proteins, including SOD1 and CCS, in the regulation of intracellular copper levels. XIAP inhibits COMMD1 functioning by promoting its degradation, resulting in rising cellular copper levels. [14] In turn, XIAP is regulated by intracellular copper levels. Under basal copper conditions XIAP-mediated ubiquitination of CCS leads to enhanced copper acquisition and positively regulates SOD1 activation by CCS.[15] When copper levels are elevated, CCS delivers copper to XIAP, resulting in degradation of CCS and XIAP and decrease in caspase inhibition, which may result in enhanced apoptosis. [15, 16] APP is proposed to have a role in the copper efflux pathway, and intracellular copper levels have shown to modulate cellular APP trafficking in neuronal cells. [17-19] APP, amyloid beta (A4) precursor protein; ATOX1, antioxidant 1 copper chaperone; ATP7A, ATPase, Cu++ transporting, alpha polypeptide; ATP7B, ATPase, Cu++ transporting, beta polypeptide; CCO, cytochrome C oxidase; CCS, copper chaperone for superoxide dismutase; COMMD1, copper metabolism (Murr1) domain containing 1; COX17, cytochrome C oxidase copper chaperone; CP, ceruloplasmin; CTR1, copper transporter 1; GSH, glutathione; MT1A, metallothionein 1A; MT2A, metallothionein 2A; SOD1, Cu,Zn superoxide dismutase 1; XIAP, X-linked inhibitor of apoptosis.

toxicosis. Affected Labrador retrievers accumulate copper in their livers and can reach copper levels of over 4,000 mg/kg dry weight liver, [38, 39] whereas normal copper levels in dog liver are < 400 mg/kg dwl. [40] In both humans and dogs, hepatic copper accumulation leads to hepatitis and eventually cirrhosis. Although it is assumed that copper is the primary event triggering hepatocellular injury, good supporting evidence is still lacking. When the disease progresses, regeneration, apoptosis and fibrosis pathways appear to dominate. [36]

Although some concepts in the disease initiation and progression of copper accumulating diseases have been shared, the exact molecular mechanisms and pathways leading to copper accumulation, hepatocellular injury and disease progression toward chronic hepatitis still remain unclear. To gain more insights in the disease initiation and pathogenesis of hereditary copper-associated hepatitis in Labrador retrievers, we investigated transcriptomic alterations in liver tissue of affected Labrador retrievers in various stages of copper-associated hepatitis. In addition, by including dogs with normal histology but increased hepatic copper concentrations we can explore if copper accumulation is indeed a primary event triggering subsequent inflammatory processes.

The results of this study represent a targeted candidate gene approach as well as an unbiased transcriptome analysis. It describes the range of events in copper metabolism, oxidative stress, inflammation, and cell adaptations towards chronic hepatitis and fibrosis in the Labrador retriever. Since Labrador retrievers are a natural non-rodent model for Wilson and non-Wilson copper toxicosis the results of this study can aid in improved management of human copper storage disorders and on human chronic hepatitis cases in general.

Materials and Methods

Animals

All dogs (n=31) were referred to the Department of Clinical Sciences of Companion Animals, Utrecht University and underwent physical examination. Blood was collected to check coagulation parameters prior to the biopsy procedure. No treatment was initiated prior to tissue collection. Liver biopsies were taken under ultrasound guidance with a 14 G needle using a Tru-cut device as described previously.[41] One biopsy specimen was fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Five micron thick slides were cut and stained with haematoxylin and eosin for routine evaluation, with rubeanic acid [42] for semi-quantitative copper scoring and for fibrosis according to Gordon and Sweet's

staining protocol [43] based on reticulin expression. All histological evaluations were performed by one board certified pathologist. An adjacent sample of at least 5 mg was collected in a metal free container and freeze-dried prior to the determination of quantitative copper content by instrumental neutron activation analysis. [44] The third tissue sample was stored in RNAlater (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) for 24 hours at 4 °C and after removing supernatant subsequently stored at -80 °C until analysis. All samples were collected according to the Act on Veterinary Practice, as required under Dutch legislation. 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. Dogs were considered to have normal hepatic copper when copper concentrations were <400 mg/kg dry weight liver (dwl).[40] Based on hepatic quantitative copper concentrations and histopathologic evaluation of liver biopsy specimens, dogs were divided in four groups (characteristics in Supplementary Table 1). Dogs (n=7) without histologic abnormalities and normal hepatic copper concentrations were included into the control group (N, normal liver, median copper 260 mg/kg dwl (dry weight liver), range 195-335 mg/kg dwl). The high copper group (HC, median copper 1,035 mg/kg/dw, range 745-2,050 mg/kg dwl) consisted of dogs (n=8) without histologic abnormalities but with increased hepatic copper concentrations. Dogs with histological evidence of hepatitis and increased hepatic copper concentrations (n=8) were included into the high copper hepatitis group (HCH, median copper 2,380 mg/kg/dw, range 530-3,870 mg/kg dwl), and dogs with chronic hepatitis and high copper concentrations (n=8) were included into the high copper chronic hepatitis group (HCCH, median copper 1,435 mg/kg/dwl, range 1,080-2,210 mg/kg dwl). Liver tissue with normal liver histology and normal hepatic copper concentrations were taken from 10 healthy Beagle dogs euthanized for other unrelated research (University 3R policy) and used as controls.

RNA isolation and reverse transcription

Total cellular RNA was isolated from liver tissue using RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions. An on-column DNase-I (QIAFEB, RNase-free DNase kit) treatment was used to digest residual genomic DNA. RNA concentrations and quality were measured spectrophotometrically using the Nanodrop ND-1000 (Isogen Life Science BV, IJsselstein, The Netherlands). RNA integrity was checked on a Bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). The RNA integrity number of all samples was above a value of 7. Common reference RNA for microarray analysis consisted of mixed RNA isolated from liver samples from 10 Beagle dogs (controls). Per sample 3 μg of RNA was used for further workup. From all RNA samples cDNA was synthesized with the iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, The

Netherlands) containing both oligo-dT and random hexamer primers. A total of 600 ng of RNA was incubated with iScript reaction mix, iScript reverse transcriptase and nuclease free water at 42°C for 30 min, in a 60 µl reaction volume.

Targeted gene approach

mRNA expression of copper metabolism related genes (*APP, ATOX1, ATP7A, ATP7B, COMMD1, COX17, CP, CTR1, MT1A, MT2A,* and *XIAP*) and genes with a role in the protection against oxidative stress (*GCLC, GPX1, GSHS, GSHR, GSTP1, MAT1A, MAT2A*) or both (*CCS* and *SOD1*) were measured with quantitative real-time polymerase chain reaction (qPCR) in 28 dogs (four groups, n=7 for each group). RNA expression of six endogenous references genes; ribosomal protein S19 (*RPS19*), beta-2 microglobulin (*B2M*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), ribosomal protein L8 (*RPL8*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and ribosomal protein S5 (*RPS5*) was performed in order to normalize expression. Primer sequences for specific sequence-confirmed amplicons (Supplementary Table 2) and qPCR conditions were as described previously.[45] The qPCR reactions were performed in duplicate using Bio-Rad detection system. Amplifications were carried out in a volume of 25 µl containing 12.5 µl of SYBR green supermix (BioRad), 0.4 µM of forward and reverse primer and 1 µl cDNA in milliQ water. Cycling conditions included: denaturation at 95 °C for 3 minutes, followed by 45 cycles of denaturation (95 °C for 10 s) and annealing/elongation (temperatures in Supplementary Table 2) for 30 s. A melt curve analysis was performed for every reaction to verify amplicon specificity. IQ5 Real-Time PCR detection system software (BioRad) was used for data analysis. A no template control was also run in duplicate with each plate as a negative control. Expression levels were normalized by using the average expression levels of the reference genes, taking into account the qPCR efficiencies per gene product.

Transcriptome analysis

Canine Gene Expression Microarrays V1 (Agilent Technologies, Belgium) representing 42,034 canine 60-mer probes in a 4x44K layout were used. The experiment was carried out in dye swap set-up in 18 Labrador retriever dogs (N: n=4, HC: n=5, HCH: n=4, HCCH: n=5, random samples per group). Double round RNA amplifications and labeling were performed[46] on an automated system (Caliper Life Sciences NV/SA, Belgium) with 10-50 ng total RNA from each sample. Hybridizations were done on a HS4800PRO system supplemented with QuadChambers (Tecan Benelux B.V.B.A., Giessen, The Netherlands) using 300-500 ng labeled cRNA per channel.[47] Hybridized slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 100% PMT. After automated data extraction using Imagene 8.0 (BioDiscovery), printtip Loess normalization was performed[48] on mean spot-intensities. Dye-bias

was corrected based on a within-set estimate [49]. Gene enrichment based on biological profiles and pathways was performed using the MetaCore program (GeneGo, Thomson Reuters, St. Joseph, MI, USA) by ranking the significant ontology and pathways dominant in each stage of disease progression. All data are currently deposited in NCBI's Gene Expression Omnibus [50] and will be accessible through GEO Series.

Statistical analysis

All qPCR data were analyzed using R statistics version 3.1.2 (R Core Team 2014). Relative gene expression of each gene product was used as the basis of all mRNA comparisons. A Mann-Whitney U test was used to determine statistical differences between each successive phenotype in disease progression. P values were adjusted for multiple comparisons using the Bonferroni correction. Microarray data was analyzed using ANOVA. In a fixed effect analysis, sample, array and dye effects were modeled. *P* values were determined by a permutation F2-test, in which residuals were shuffled 5,000 times globally. Genes with $P<0.05$ after either family wise error correction (FWER) or determination of false discovery rate (FDR) were considered significantly changed. Resulting gene-lists from indirect comparisons between the diseased groups normal vs high copper, high copper vs high copper hepatitis, high copper hepatitis vs high copper chronic hepatitis were used for subsequent gene set enrichment analysis.

Results

Gene expression of copper metabolism related gene products

Relative mRNA expression of genes encoding proteins important in copper trafficking and metabolism was measured to gain insight into the pathogenesis of copper-associated hepatitis in Labrador retrievers. After Bonferroni correction for multiple testing (Supplementary Table 3), no significant differential mRNA expression was found in *ATOX, ATP7A, ATP7B, COX17, CP, CTR1, XIAP,* and *SOD1*. *APP, CCS, COMMD1, MT1A,* and *MT2A*, were differentially expressed in one or more groups compared to the preceding disease stage (Fig 2 and Fig 3). In the HC group *COMMD1* and *MT1A* were both upregulated compared to the N group. *COMMD1* mRNA levels were increased 1.8 times (range 1.6-2.0, $P<0.01$), while *MT1A* levels were increased 1.9 fold (range 1.4-2.1, $P=0.02$). In the HCCH group, *APP* levels were increased 2.5 times (range 1.8-4.1, $P<0.01$) compared to the HCH group, while levels of *CCS* (1.8, range 1.4-2.4, $P<0.01$), *MT1A* (1.4, range 1.1-1.8, $P<0.01$), and *MT2A* (1.5, range 1.1-2.8, $P<0.01$) were all decreased.

Gene expression of oxidative stress related gene products

To gain insight in the occurrence of oxidative stress during the initiation and progression of hepatic copper accumulation, mRNA levels of *MAT1A, MAT2A, S0D1*, *GCLC*, *GPX1*, *GSHR*, *GSHS*, and *GSTP1* were measured. After correction for multiple testing the only significant differences were found in the genes coding for *MAT1A* and *GSTP1*, which is part of the GST family (Fig 2 and Fig 3). In the HC group, *GSTP1* was decreased 3.6 times (range 1.5-4.5, P=0.04) compared to the N group. Compared to the HC group, *MAT1A* levels were decreased 1.3 times in the HCH group (range 1.1-2.0, *P*=0.04).

Whole transcriptome microarray

Expression differences correlated with disease progression were detected using the gene expression microarray (Fig 4). Clear clustering was detected based on phenotypic differences. Phenotype specific expression was detected in the different stages HC group (293 genes), HCH group (99) and HCCH group (1,079) compared to the normal Labrador retriever group (Fig 5). Ninety-five genes were only expressed in the HC and HCH group, compared to the N group. In the HCH and HCCH group, 130 genes were specifically differentially expressed compared to the normal liver group.

Gene enrichment based on biological functional level was assessed using Metacore (Thomson Reuters). Most networks fitted into eight types of biological functions: inflammation, development, cell adhesion, cytoskeleton, protein folding, blood coagulation, proteolysis, and apoptosis. In the HC group an enrichment was found for pathways involved in cell adhesion, developmental, inflammatory, and cytoskeleton networks (Fig 6A). The encoded proteins in these networks mainly include members of the NF-κB family, signal transducers like mitogen-activated protein kinases (MAPK) and Syk, interleukins (IL-12, IL-2), TGF-alfa, and cell adhesion and cytoskeleton elements such as plectin, cadherins, and talin. In the HCH group, most of the detected pathways involved in this stage of disease include inflammatory pathways (Fig 6B). Genes in the networks of the HCH group mostly involved those of the kinin-kallikrein system, complement factors (C9, factor B), and chemokines. For the chronic form of hepatitis (HCCH), the most significant transcriptional differences were found in cell adhesion adaptations and cytoskeleton remodelling (Fig 6C). Apoptosis, development and proteolysis were other important biological processes with multiple pathways involved. Differentially expressed genes in the cell adhesion and cytoskeleton remodelling pathways include extracellular matrix (ECM) constitutors (collagens, galectins, laminins), ECM remodelling enzymes (ADAM metallopeptidases, TIMP), cytoskeleton components (tubulin, vimentin, myosin, actin), integrins, and amyloid proteins

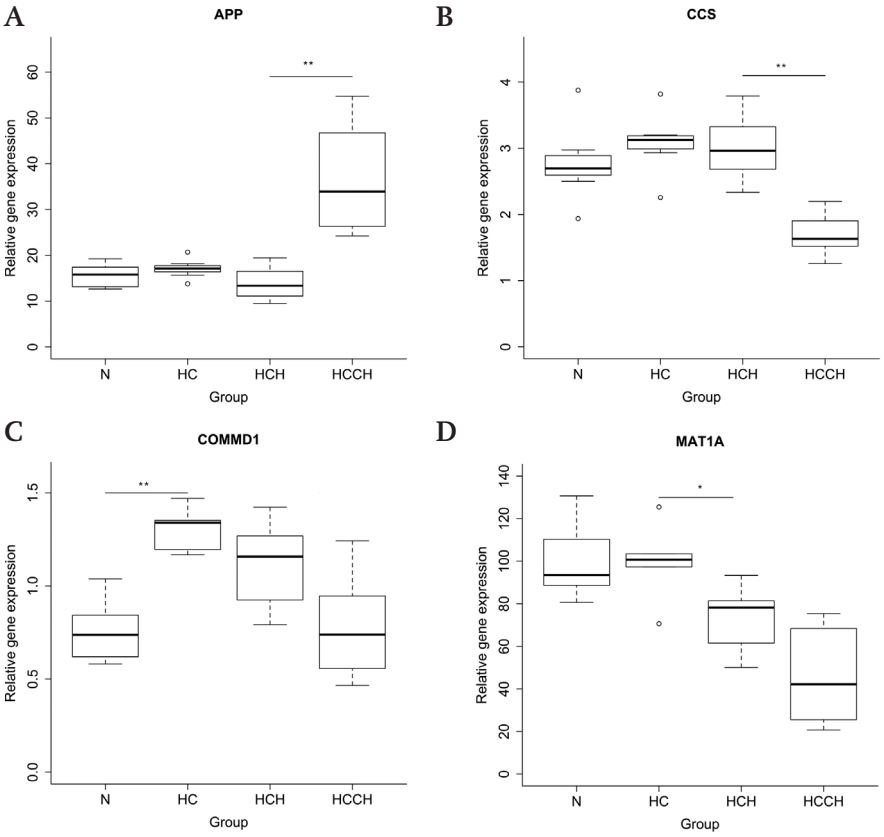

Figure 2 qPCR results.

Liver tissue of Labrador retrievers with normal copper (N; n=7), high copper (HC; n=8), high copper hepatitis (HCH, n=8), and high copper chronic hepatitis (HCCH; n=8) was used for mRNA quantification of genes involved in copper metabolism. Gene expression of APP (**A**), CCS (**B**), COMMD1 (**C**), MAT1A (**D**), MT1A (**E**), MT2A (**F**), and GSTP1 (**G**) was significantly changed between two successive stages of the disease. The thick black line represents the median (50th percentile), also the first and third quartile (25th and 75th percentile respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the interquartile range. APP, amyloid beta (A4) precursor protein; CCS, copper chaperone for superoxide dismutase; COMMD1, copper metabolism (Murr1) domain containing 1; GSTP1, glutathione s-transferase pi 1; MAT1A, methionine adenosyltransferase I alpha; MT1A, metallothionein 1A; MT2A, metallothionein 2A. $^{*}P<0.05$, $^{**}P<0.01$

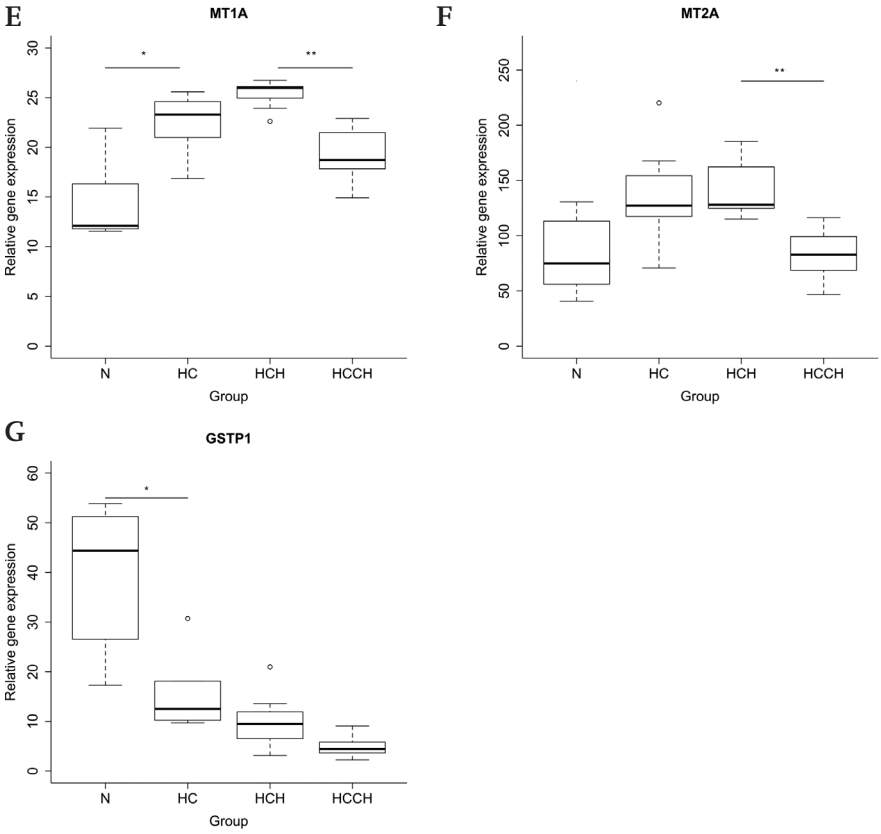

Figure 2 Continued.

(APP, APLP2). Numerous genes encoding for proteins involved inflammation, coagulation and immune response are present. Examples include cytokines, chemokines, growth factors, coagulation components, signal transducers, and transcription factors.

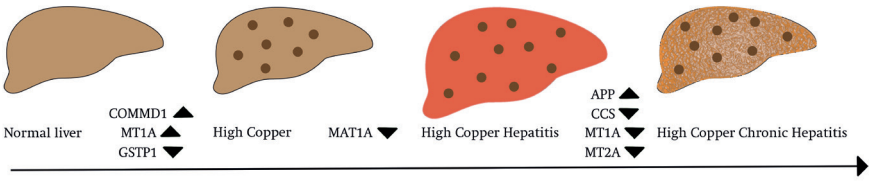

Figure 3 Copper metabolism adaptation in disease progression.

A schematic overview of up- or downregulated of genes, involved in copper metabolism and oxidative stress within the different stages of disease progression. APP, amyloid beta (A4) precursor protein; CCS, copper chaperone for superoxide dismutase; COMMD1, copper metabolism (Murr1) domain containing 1; GSTP1, glutathione s-transferase pi 1; MAT1A, methionine adenosyltransferase I alpha; MT1A, metallothionein 1A; MT2A, metallothionein 2A.

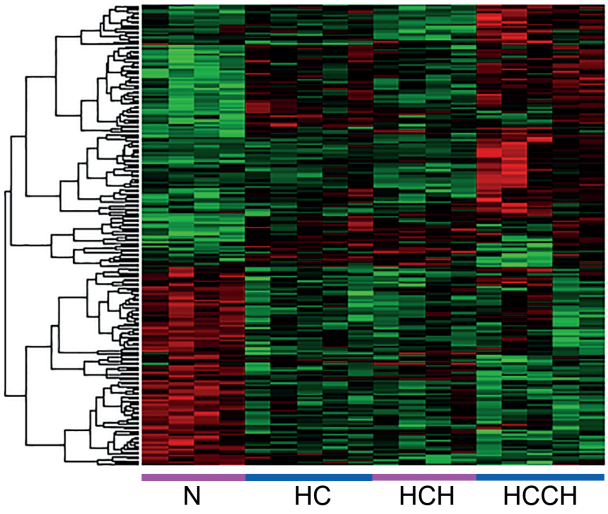

Figure 4 Heatmap of genes regulated through disease progression.

216 probes (listed in rows) were expressed significantly ($P<0.001$) different samples in the four different stages (listed in columns) compared with the common reference pool (Beagles). HC, high copper; HCH, high copper hepatitis; HCCH, high copper chronic hepatitis; N, normal liver.

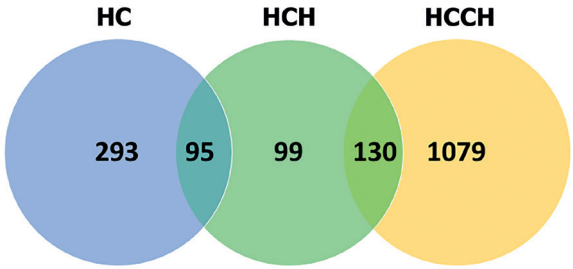

Figure 5 Total number of genes involved in specific stages of disease progression.

The Venn-diagram depicts the number of genes that are differentially expressed compared to the normal liver group (N) within the specific phenotypes. Selected genes were filtered on log2 Fold Change under -0.5 or over 0.5, and a *P* value of <0.001. HC, high copper; HCH, high copper hepatitis; HCCH, high copper chronic hepatitis.

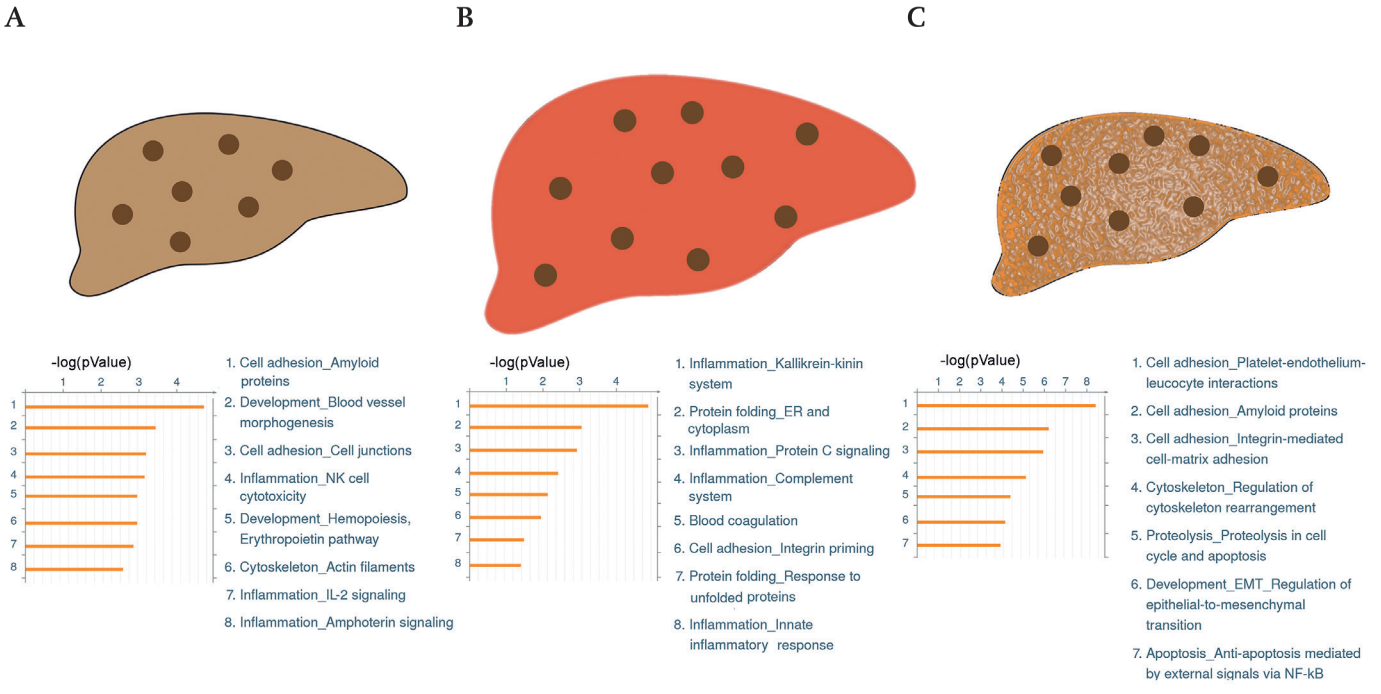

Figure 6 Process networks enriched in the different stages of disease.

The unique genes involved in the different stages of disease were used to determine functional enrichment using Metacore. The most involved and significant process networks are depicted for high copper (**A**), high copper hepatitis (**B**), and high copper chronic hepatitis (**C**).

Discussion

This study shows transcriptomic alterations in four well-defined groups of Labrador retrievers, representing one normal group and three successive stages of copper-associated hepatitis. Targeted gene approach showed the relatively minor effect of transcriptional regulation of cellular copper metabolism during disease, whereas unbiased micro-array study highlighted the immediate involvement of inflammatory pathways, followed by matrix remodeling pathways. Importantly these results elucidate aspects of copper as an initiating factor, introduce APP as candidate gene for copper-associated chronic hepatitis, and lastly shed light on the molecular background of (chronic) hepatitis.

In this study we show that Labrador retrievers in the HC group already had changes in copper metabolism genes and multiple cellular pathways, including inflammatory, cell structural and developmental pathways, while no appreciable histopathological signs of hepatocellular injury were visible at histology. The upregulation of the copper scavenger *MT1A* with increasing copper levels is the cells first response to maintain homeostasis by protecting the cell from copper toxicity. These findings are in line with previous studies in Wilson disease patients,[51] LEC rats, [52, 53] and COMMD1-deficient dogs [54] with high copper concentrations. The copper induced MT transcription is initiated through both metal- and oxidative stress responsive signal transduction pathways. [55] *COMMD1* mRNA levels were also increased in the HC group. COMMD1 specifically binds copper (II) [56] and is believed to be involved in the quality control of both copper transporting ATPases and in the biliary excretion of copper in case of high hepatic copper concentrations. [11,13,57] As both the incorporation of copper into ceruloplasmin [58, 59] and ATP7B trafficking form the Trans Golgi Network (TGN) is not affected by COMMD1, [13, 60] COMMD1 is thought to act in the final step of biliary copper excretion.[61] Therefore, the combined increase of *MT1A* and *COMMD1* mRNA levels could be an effective way of lowering hepatic copper.

Multiple human and animal studies advocate that oxidative stress is one of the most important deleterious effects of excess copper, leading to hepatocellular injury.[62-66] However, recent studies in fibroblasts from ATP7A mouse mutants and liver samples from ATP7B knock out mice, did not detect convincing evidence of oxidative stress caused by excess copper. [67, 68] In our data only modest evidence for oxidative stress in the HC group exists; differential mRNA expression of oxidative stress-responsive transcription factors and genes was identified with qPCR and microarray analysis, including *MT*, *Syk*, MAP kinases, NF-κB family, *MAT1A* and *GSTP1*. NF-κB is a transcription factor involved in hepatocyte survival during liver

disease and orchestrates the livers inflammatory response. [69] Increasing hepatic copper (II) concentrations promote the formation of hydroxyl radicals, therewith increasing oxygen consumption, which is paralleled by the activation of numerous signaling pathways, such as MAPK and Syk, leading to increased NF-κB DNA binding activity.[70-73] It has been shown that nuclear COMMD1 is able to inhibit NF-κB activity.[74, 75] In a previous study *COMMD1* mRNA and protein levels were decreased as a late response to copper overload and it was suggested that prolonged activation of NF-κB activity was at least partly mediated to reduced COMMD1 protein levels.[76] In our study *COMMD1* levels in the HCH and HCCH tended to decrease again but levels were only compared with the previous stage and this decrease did not reach statistical significance.

In the HC group, 11/32 differentially expressed pathways are associated with inflammation although this was not yet macroscopically visible. Two important key effectors in the liver's inflammatory and immune response are the Kupffer cell and the hepatic stellate cell (HSC).[77, 78] The HSC is normally a quiescent cell, located in the peri-sinusoidal space of Disse. Upon activation HSCs undergo myofibroblastic transformation to stimulate hepatic fibrosis by producing extracellular matrix (ECM) components.[79] The amphoterin signalling pathway, which was one of the enriched pathways in the HC group, has shown to activate HSCs. [80] In addition, oxidative stress and TGF-α can also induce HSC activation through a NF-κB mediated pathway.[81] Another remarkable finding was the differential expression of cytoskeletal and cell adhesion related genes in the HC compared to the NL group. Similar results were found in a study into copper overload in fibroblast cells from two mouse mutants.[68] Altogether, the results in the HC group comprise changes concerning various aspects of cellular homeostasis, indicating the primary role of copper in the development of hepatocellular injury. When homeostatic defense mechanisms start to fail, progression towards the next disease stage occurs.

In the transition to the HCH stage of the disease, inflammatory and blood coagulation pathways, which are known to strongly interact and influence each other, [82] are enriched and play an important role in the disease progress. Similar results were found during disease progression in LEC rats, were genes related to inflammation and acute phase proteins were upregulated when the disease progresses to hepatitis.[53] As shown with qPCR analysis, *MAT1A* in the HCH group was significantly decreased compared to the HC group. *MAT1A* encodes for the isoenzymes MATI and MATIII, responsible for the synthesis of s-adenosylmethionine (SAM), the key methyl donor involved in numerous methylation reactions. The decrease in *MAT1A* expression is in conjunction with the results found in LEC rats as well as in cirrhotic livers of Wilson disease patients. [83, 84] It was demonstrated

that the mRNA reduction or knockout of MAT1A also caused a decrease in protein levels, and subsequently a decrease of SAM and glutathione levels. [84, 85] As a consequence, MAT1A knockout mice showed an induction of many acute phase proteins and inflammatory markers and were shown to be more susceptible to develop liver injury. The measured decrease in *MAT1A* mRNA, leading to a decrease in SAM, may contribute to the pathogenesis of liver injury in the HCH group and the progression towards HCCH. Two studies in humans with alcoholic liver diseases showed no convincing evidence to support or refute the benefit of SAM treatment. [86, 87] However two studies in dogs suggests beneficial effects of SAM treatment in dogs with hepatic diseases, [88, 89] and therefore further studies of this agent in the treatment of dogs with chronic hepatitis are warranted.

In the HCH group, hepatic copper concentrations reach a maximum level at which *MT1A* and *MT2A* are maximally upregulated. In the last stage of the disease (HCCH) both copper concentrations and metallothionein (*MT1A* and *MT2A*) expression decreased compared to the HCH group. This decrease in hepatic copper has been described previously and might be due to necrotic hepatocytes that release their copper burden and regenerative nodules that initially do not contain copper.[90, 91] When copper concentrations in the HCCH group decrease, MT levels decrease as well. These results are in accordance with findings of chronic copper overload in Dobermans [65] and Bedlington terriers [66] and a longitudinal study of copper toxicosis in five COMMD1-deficient dogs. [54] In this stage of the disease, copper-loaded MT has shown to gradually displace towards the lysosomes, where it is subjected to (incomplete) degradation, rendering a possible reactive degradation product which could potentially further amplify liver damage. [92, 93]

A remarkable finding was the strong upregulation of *APP* in the HCCH group compared to the HCH group. In addition, amyloid proteins was also one of the enriched process networks in the HCCH group. The APP protein products are known to be involved in the pathogenesis of Alzheimer disease, and seem to have an important role in copper homeostasis as well. [94] APP is a transmembrane protein that is able to bind and reduce copper at the extracellular domain at a cysteine-rich region of the N-terminus. [95] Several studies propose that APP has a role in cellular efflux of copper as overexpression of APP resulted in decreased copper concentrations.[96, 97] Conversely, mutant APP lacking copper binding domain resulted in increased cellular copper concentrations. [96] In addition, APP knockout mice showed to have increased copper concentrations in their livers and brain. [19] It was recently demonstrated that high cellular copper concentrations promote the trafficking of APP from the TGN to the plasma membrane in epithelial and neuronal cells. [17, 18] Copper depleted human fibroblasts due to overexpression of the

Menkes disease protein, presented with a downregulation of APP gene expression and decreased APP protein concentrations.[98] Our findings are similar to a study of chronic copper overload in fibroblast cells form two mouse mutants that found up-regulation of *APP* and prion protein (*PRNP*).[68] These findings indicate that the relative abundance of *APP* transcripts in our study is an adaptive response to prolonged high intracellular copper levels. Therefore, APP might be considered as candidate gene for chronic copper associated disease.

Histological changes in HCCH are predominantly characterized by hepatocellular apoptosis and necrosis, a mononuclear or mixed inflammatory infiltrate, regeneration and the presence of fibrosis and cirrhosis.[99] Two studies in humans with chronic hepatitis C also showed changes in genes encoding cytoskeleton organization, ECM production and remodeling, cytokines, growth factors, cell junction, and cell proliferation.[100, 101] In dogs with chronic hepatitis, regulation of fibrosis-related genes (e.g. collagens, matrix metalloproteinases, TGFβ) correlates with the degree of fibrosis and disease progression.[102] It is therefore not surprising that process networks in the HCCH group show a strong enrichment for cell adhesion, cytoskeleton rearrangement, apoptosis, development, and inflammation.

One of the limitations of this study is the lack of longitudinal biopsies of the same dogs. The increase in statistical power by a longitudinal study would be at the cost of a severe reduction in number of samples. Therefore, liver biopsies of different dogs in successive disease stages were used. In addition, in the current study two approaches were used; a targeted qPCR approach for copper metabolism and oxidative stress genes and an unbiased genome-wide expression approach, and the results presented and discussed are an overlap between these two techniques. Except for APP, which was also part of the enriched networks in the microarray, no copper metabolism or oxidative stress pathways were enriched in the microarray analysis. Corroborating on a previous study,[76] no differential expression in most of the copper genes was found with the more sensitive qPCR technique. This might implicate that transcriptional regulation of copper metabolism is not the most important mechanism for regulating copper homeostasis. It is known that upon changing intracellular copper concentrations altered trafficking or posttranslational modifications, such as ubiquitination, of proteins are ways to maintain copper homeostasis.[15, 16, 103] Interestingly, two other studies in our group were able to detect differential expression of some copper- and oxidative stress related genes in Dobermans and COMMD1-deficient dogs with qPCR.[54, 65] This difference might be due to at least higher copper concentrations in COMMD1-deficient dogs or to the fact that we only looked for differential expression between two successive stages of diseases.

Conclusions

This is the first study clearly describing transcriptomic alterations in livers of Labrador retrievers, representing initial copper accumulation, copper-induced hepatitis, and lastly copper-associated chronic hepatitis. Our results show that prior to appreciable histological signs in the liver, copper is a primary event leading to changes in several cellular pathways. The upregulation of *MT1A* and *COMMD1* shows the livers first adaptive response to rising intracellular copper concentrations. Increased expression of *APP* in the chronic hepatitis phase is presumed to be specific for chronic copper-accumulating disease as this is thought to be an adaptive response to prolonged high intracellular copper levels. Transcriptomic alterations in the liver, histologically characterized by fibrosis, are mainly dominated by changes in cell structure and arrangements.

Supplementary material

Supplementary Table 1 Animal characteristics.

Group	Labrador identification number	Used in study	Hepatic CuQ (mg/kg dwl)	Sex	Age (years)
N	184	qPCR	195	Male	1,8
N	123	qPCR, MA	207	Male	4,3
N	193	qPCR, MA	250	Male	5,5
N	166	qPCR, MA	260	Female	9,2
N	162	qPCR, MA	270	Male	5,0
N	62	qPCR	290	Male	1,4
N	177	qPCR	335	Male	1,8
HC	117	MA	745	Male	5,1
HC	106	qPCR, MA	840	Female	9,1
HC	115	qPCR, MA	1729	Female	5,1
HC	59	qPCR, MA	774	Male	5,7
HC	197	qPCR, MA	830	Female	6,0
HC	44	qPCR	1230	Male	6,7
HC	80	qPCR	1980	Female	1,2
HC	49	qPCR	2050	Female	5,8
HCH	156	MA	530	Female	4,9
HCH	122	qPCR, MA	1150	Female	6,5
HCH	113	qPCR, MA	1900	Male	4,9
HCH	105	qPCR	2330	Female	4,7
HCH	186	qPCR	2430	Female	4,1
HCH	164	qPCR, MA	2620	Female	11,0
HCH	160	qPCR	2950	Female	5,6
HCH	275	qPCR	3870	Female	4,2
HCCH	33	MA	1080	Female	8,2
HCCH	70	qPCR, MA	1180	Female	8,0
HCCH	114	qPCR, MA	1194	Female	3,3
HCCH	185	qPCR	1380	Female	6,3
HCCH	103	qPCR, MA	1490	Female	2,7
HCCH	247	qPCR	1720	Female	9,2
HCCH	77	qPCR, MA	2060	Female	6,7
HCCH	277	qPCR	2210	Female	9,4

CuQ, quantitative copper concentrations; HC, high copper; HCH, high copper hepatitis; HCCH, high copper chronic hepatitis; MA, microarray; N, normal liver; qPCR, quantitative real-time polymerase chain reaction

Supplementary Table 2 Primers and qPCR conditions.

Gene			Sequence (5'-3')	Tm (°C)	Product size (bp)	Accession number
B2M	Reference	F	TCCTCATCCTCCTCGCT	61	85 bp	ENSCAFG00000013633
	gene	R	TTCTCTGCTGGGTGTCG			
GAPDH	Reference	F	TGTCCCCACCCCCAATGTATC	58	100 bp	AB038240.1
	gene	R	CTCCGATGCCTGCTTCACTACCTT			
HPRT	Reference	F	AGCTTGCTGGTGAAAAGGAC	56	104 bp	AY283372.1
	gene	R	TTATAGTCAAGGGCATATCC			
RPL8	Reference	F	CCATGAATCCTGTGGAGC	55	64 bp	XM_853403
	gene	R	GTAGAGGGTTTGCCGATG			
RPS5	Reference	F	TCACTGGTGAGAACCCCCT	62.5	141 bp	XM_533568
	gene	R	CCTGATTCACACGGCGTAG			
RPS19	Reference	F	CCTTCCTCAAAAAGTCTGGG	61	95 bp	XM_005616513
	gene	R	GTTCTCATCGTAGGGAGCAAG			
APP	Copper	F	TGCCGAGTTCCGACATGAC	64	120 bp	AY498706.1
	metabolism	R	TATGACAACACCGCCCACC			
ATOX1	Copper	F	ACGCGGTCAGTCGGGTGCTC	67	137 bp	AF179715.2
	metabolism	R	AACGGCCTTTCCTGTTTTCTCCAG			
ATP7A	Copper	F	AAACATCAAAGGCTCCTATCC	57	198 bp	AY603040
	metabolism	R	GGAAAGCAAAGCGTATTATCG			
ATP7B	Copper	F	GGTGGCCATCGACGGTGTGC	56	136 bp	AY603039
	metabolism	R	CGTCTTGCGGTTGTCTCCTGTGAT			
CCS	Copper	F	GACTCCATGTCCATCAGTTTGG	63	77 bp	AY572228.1
	metabolism	R	ATGCTCCATCAGGGTTAAAGTG			
COMMD1	Copper	F	GACCAAGCTGCTGTCATTTCCAA	60	122 bp	AY047597
	metabolism	R	TTGCCGTCAACTCTCCAACTCA			
COX17	Copper	F	ATCATTGAGAAAGGAGAGGAGCAC	60	127 bp	AY603041.1
	metabolism	R	TTCATTCTTCAAGGATTATTCATTTACA			
CP	Copper	F	AATTCTCCCTTCTGTTTTTGGTT	62	97 bp	AY572227
	metabolism	R	TTGTTTACTTTCTCAGGGTGGTTA			

CTR1	Copper	F	CAGTACCTTCTCACCATCACC	60	175 bp	XM_538800
	metabolism	R	AAACACTGCCACGAAAGC			
MT1A	Copper	F	AGCTGCTGTGCCTGATGTG	61	130 bp	D84397
	metabolism	R	TATACAAACGGGAATGTAGAAAAC			
MT2A	Copper	F	ATGGATCCCAACTGCTCCT	58	78 bp	AB028042.1
	metabolism	R	TGCATCTGCACTCTTTGCA			
XIAP	Copper	F	ACTATGTATCACTTGAGGCTCTGGTTTC	54	80 bp	AY603038
	metabolism	R	AGTCTGGCTTGATTCATCTTGTGTATG			
SOD1	Copper	F	TGGTGGTCCACGAGAAACGAGATG	64	99 bp	AF346417.1
	metabolism & oxidative stress	R	CAATGACACCACAAGCCAAACGACT			
GCLC	Oxidative	F	GATGATGCCAATGAATCTGACC	64	170 bp	XM_847752
	stress	R	CACCACAAACACCACATATGC			
GPX1	Oxidative	F	GCAACCAGTTCGGGCATCAG	62	123 bp	NM_001115119
	stress	R	CGTTCACCTCGCACTTCTCAAAA			
GSHR	Oxidative	F	TTCAACCACCTTTACCCCAATGTATC	61	103 bp	XM_532813
	stress	R	GATCCCAACCACCTTTTCCTCCA			
GSHS	Oxidative	F	CTGGAGCGGCTGAAGGACA	62	131 bp	AY572226
	stress	R	AGCTCTGAGATGCACTGGACA			
GSTP1	Oxidative	F	AATGCCATCCTGAGACACCT	65	88 bp	ENSCAFG00000025332
	stress	R	CCGTCATTCACCATATCCACC			
MAT1A	Oxidative	F	CACTGTCCATTTCCATCTTCACCT	63	128 bp	XM_014112924
	stress	R	GGGCTTCTTCAAATCCAAATCC			
MAT2A	Oxidative	F	TGCTTTTGGCGGGGAGGAG	67	121 bp	NM_001287067
	stress	R	TTTAAAAGCTGCCATCTGAGGTGA			

APP, amyloid beta (A4) precursor protein; ATOX1, antioxidant 1 copper chaperone; ATP7A, ATPase, Cu++ transporting, alpha polypeptide; ATP7B, ATPase, Cu++ transporting, beta polypeptide; B2M, beta-2 microglobulin; bp, base pairs; CCS, copper chaperone for superoxide dismutase; COMMD1, copper metabolism (Murr1) domain containing 1; COX17, cytochrome C oxidase copper chaperone; CP, ceruloplasmin; CTR1, copper transporter 1; F, forward primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCLC, glutamate-cysteine ligase, catalytic subunit; GPX1, glutathione peroxidase 1; GSHR, glutathione reductase; GSHS, glutathione synthetase; GSTP1, glutathione s-transferase pi 1; HPRT, hypoxanthine-guanine phosphoribosyltransferase; MAT1A, methionine adenosyltransferase I alpha; MAT2A, methionine adenosyltransferase II alpha; MT1A, metallothionein 1A; MT2A, metallothionein 2A; R, reversed primer; RPL8, ribosomal protein L8; RPS5, ribosomal protein S5; RPS19, ribosomal protein S19; SOD1, Cu,Zn superoxide dismutase 1; XIAP, X-linked inhibitor of apoptosis.

Supplementary Table 3 P values qPCR data.

Gene	Group comparison	Fold change	Fold range	Uncorrected P value	Corrected P value
APP	N-HC	-	-	ns	ns
	HC-HCH	-	-	ns	ns
	HCH-HCCH	↑2.5	1.8-4.1	< 0,001	<0,01
ATOX1	N-HC	-	-	ns	ns
	HC-HCH	↓1.2	1.1-1.8	0,05	ns
	HCH-HCCH	-	-	ns	ns
ATP7A	N-HC	-	-	ns	ns
	HC-HCH	↓2.0	0.9-3.7	0,05	ns
	HCH-HCCH	-	-	ns	ns
ATP7B	N-HC	-	-	ns	ns
	HC-HCH	↓1.3	1.2-4.2	0,04	ns
	HCH-HCCH	-	-	ns	ns
CCS	N-HC	-	-	ns	ns
	HC-HCH	-	-	ns	ns
	HCH-HCCH	↓1.8	1.4-2.4	< 0,001	<0,01
COMMD1	N-HC	↑1.8	1.6-2.0	0,001	<0,01
	HC-HCH	-	-	ns	ns
	HCH-HCCH	↓1.6	0.9-2.5	0,04	ns
COX17	N-HC	-	-	ns	ns
	HC-HCH	↑1.1	1.1-1.3	0,04	ns
	HCH-HCCH	-	-	ns	ns
CP		-	-	ns	ns
CTR1		-	-	ns	ns
MAT1A	N-HC	-	-	ns	ns
	HC-HCH	↓1.3	1.1-2	0,01	0,04
	HCH-HCCH	↓1.9	1.0-3.8	0,05	ns
MAT2A	N-HC	↑1.6	1.2-3.0	0,05	ns
	HC-HCH	-	-	ns	ns
	HCH-HCCH	-	-	ns	ns
MT1A	N-HC	↑1.9	1.4- 2.1	< 0,01	0,02
	HC-HCH	↑1.1	1.0-1.2	0,04	ns
	HCH-HCCH	↓1.4	1.2-1.8	< 0,01	<0,01
MT2A	N-HC	↑1.7	1.0-2.9	0,05	ns
	HC-HCH	-	-	ns	ns
	HCH-HCCH	↓1.5	1.1-2.8	0,001	<0,01

Supplementary Table 3 Continued.

Gene	Group comparison	Fold change	Fold range	Uncorrected P value	Corrected P value
XIAP	N-HC	↑1.5	1.0-2.2	0,03	ns
	HC-HCH	↓1.5	1.0-2.2	0,04	ns
	HCH-HCCH	-	-	ns	ns
SOD1		-	-	ns	ns
GCLC		-	-	ns	ns
GPX1		-	-	ns	ns
GSHS		-	-	ns	ns
GSHR		-	-	ns	ns
GSTP1	N-HC	↓3.6	1.5-4.5	0,01	0,04
	HC-HCH	-	-	ns	ns
	HCH-HCCH	↓2.2	1.0-4.3	0,05	ns

APP, amyloid beta (A4) precursor protein; ATOX1, antioxidant 1 copper chaperone; ATP7A, ATPase, Cu++ transporting, alpha polypeptide; ATP7B, ATPase, Cu++ transporting, beta polypeptide; CCS, copper chaperone for superoxide dismutase; COMMD1, copper metabolism (Murr1) domain containing 1; COX17, cytochrome C oxidase copper chaperone; CP, ceruloplasmin; CTR1, copper transporter 1; GCLC, glutamate-cysteine ligase, catalytic subunit; GPX1, glutathione peroxidase 1; GSHR, glutathione reductase; GSHS, glutathione synthetase; GSTP1, glutathione s-transferase pi 1; HC, high copper; HCH, high copper hepatitis; HCCH, high copper chronic hepatitis; MAT1A, methionine adenosyltransferase I alpha; MAT2A, methionine adenosyltransferase II alpha; MT1A, metallothionein 1A; MT2A, metallothionein 2A; N, normal liver; ns, not significant; SOD1, Cu,Zn superoxide dismutase 1; XIAP, X-linked inhibitor of apoptosis.

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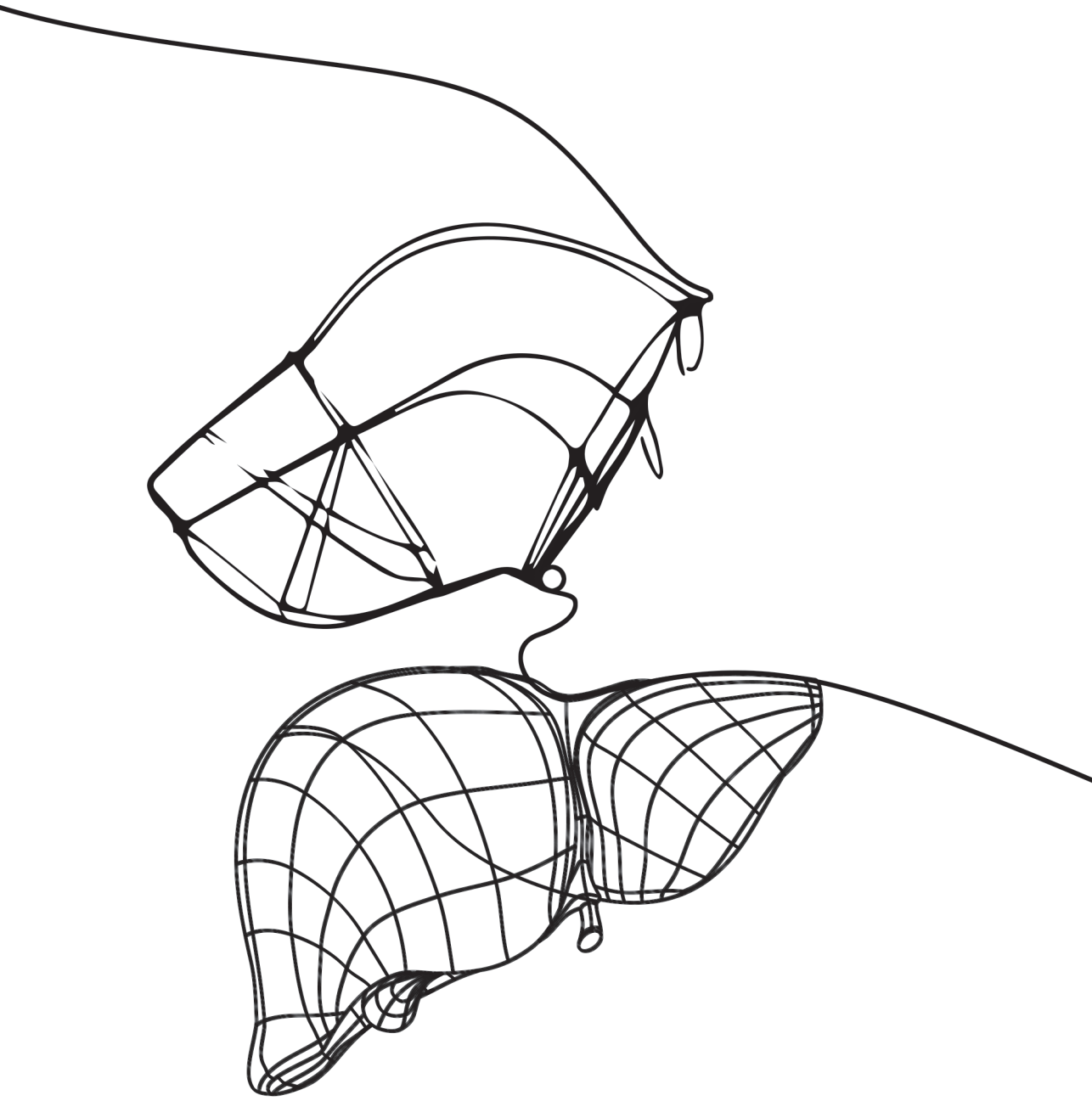

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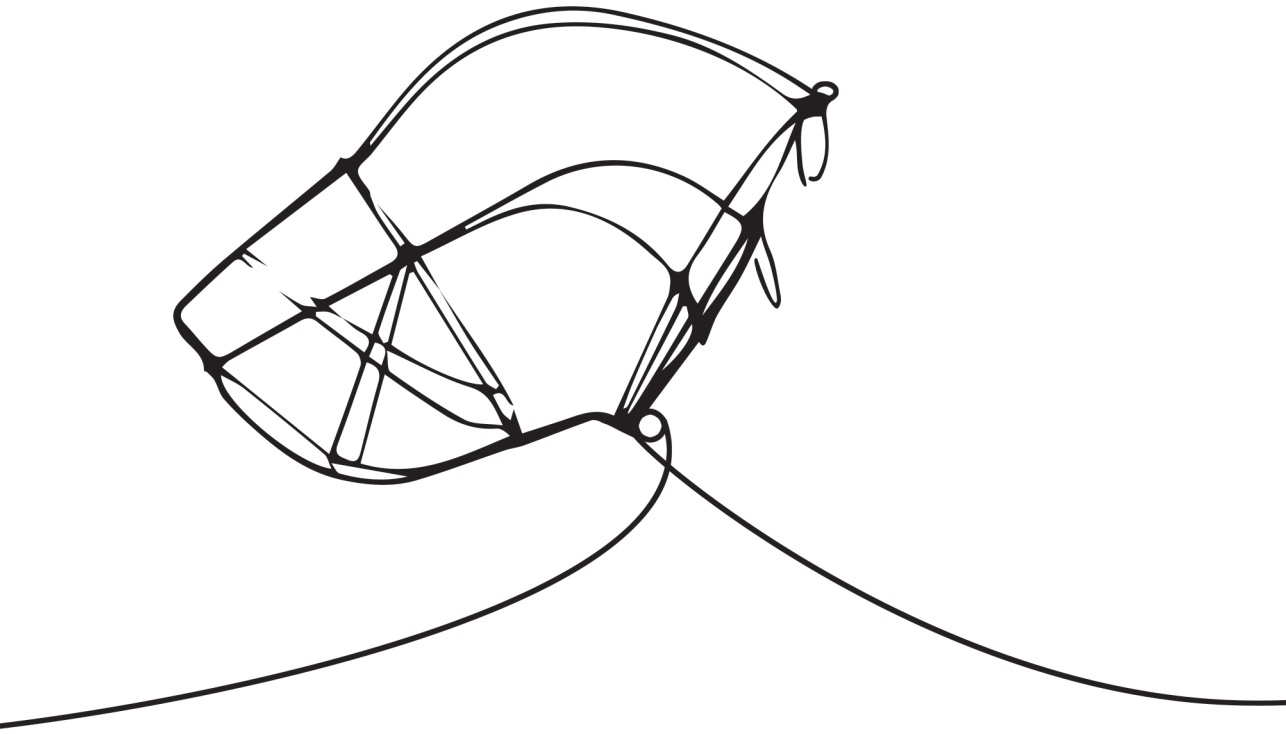


4
chapter

Sensitivity and specificity of ALT, ALP, and bile acids for hepatitis in Labrador retrievers

Karen Dirksen[a]
Iwan A. Burgener[a]
Jan Rothuizen[a]
Ted S.G.A.M. van den Ingh[b]
Louis C. Penning[a]
Bart Spee[a]
Hille Fieten[a]

Provisionally accepted, JVIM

[a]*Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands*
[b]*TCCI Consultancy BV, Utrecht, The Netherlands*

Abstract

Background: The most commonly used biochemical indicators of liver disease are plasma alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bile acids (BA). Different forms of primary hepatitis are present in dogs. Hereditary forms of hepatitis, including copper-associated hepatitis, are recognised with increased frequency in certain dog breeds, including the Labrador retriever.
Objectives: To determine (1) the sensitivity and specificity of ALT, ALP and BA for detecting primary hepatitis (PH) in clinically healthy Labrador retrievers, and (2) if ALT and ALP can discriminate between dogs with a PH and non-specific reactive hepatitis (RH).
Animals: 191 clinically healthy and 51 clinically ill Labrador retrievers with known hepatic copper concentrations and hepatic histopathology were included.
Methods: Retrospective study. Medical records were reviewed for ALT, ALP, BA, liver histopathology, and quantitative copper concentrations.
Results: In 64% (122/191) of the clinically healthy Labrador retrievers hepatic histology revealed abnormalities. Sensitivity of ALT, ALP, and BA in this population in detecting acute hepatitis was 45%, 15%, and 15% respectively. For chronic hepatitis sensitivity was 71%, 35%, and 13% respectively. In Labradors with increased liver enzymes, median ALT was significantly higher (312 U/L, range 38 – 1,369) in dogs with a PH compared to dogs with a RH (91 U/L, range 39 – 139) $P<0.001$. There was no difference in ALP between dogs with a PH and a RH.
Conclusions and Clinical Importance: ALT, ALP, and BA have a low sensitivity for detecting hepatitis and new more sensitive biomarkers for inflammatory liver disease are needed.

Introduction

Primary hepatitis (PH) is one of the most frequently occurring group of parenchymal liver diseases in the dog.[1] Examples of PH include acute hepatitis (AH), chronic hepatitis (CH), lobular dissecting hepatitis, and granulomatous hepatitis.[2] CH is by far the most common observed form of PH.[1, 3] In the majority of dogs with CH, the etiology remains undetermined and is considered to be of idiopathic origin.[4] AH can be induced by a variety of stimuli, including toxins, adverse drug reactions, infectious disease (*i.e.* canine adenovirus-1 infection, leptospirosis), or is considered idiopathic.[1, 2, 5-7] In the last few decades, copper toxicosis is recognized more as an etiologic factor in the development of both AH and CH in several dog breeds,[8-12] including the Labrador retriever.[13-16] In the Labrador retriever[17] and the Bedlington terrier[8, 18] a hereditary background has been established, while in other dog breeds a form of hereditary dysfunction is only suspected.

Clinical signs of liver disease are usually not very specific, except in end-stage liver disease, where icteric mucous membranes and presence of ascites may indicate advanced liver disease. Unfortunately, cases of CH are often only recognized in an advanced stage when there is extensive hepatocellular injury and/or loss of liver function. At this stage, treatment is less likely to be effective and prognosis guarded.[1] For successful management of the disease, early identification of (subclinical) dogs with PH is of great importance.

The most commonly used indicators of hepatobiliary disease are liver enzymes and bile acids (BA).[19, 20] Alanine aminotransferase (ALT) is a liver specific cytosolic enzyme and a sensitive indicator for hepatocellular necrosis and inflammation.[19] Alkaline phosphatase (ALP) is believed to have a good sensitivity for liver diseases, with higher activities in cholestatic disease and chronic hepatitis/cirrhosis, although being less liver specific.[19, 21-23] Elevations in liver enzymes are, however, also possible findings in dogs wherein the liver parenchyma reacts to a variety of extra-hepatic diseases (*i.e.* inflammatory bowel disease, pancreatitis) or other stimuli.[24, 25] This results in a non-specific reactive hepatitis (RH). The concomitant increase in ALT and/or ALP can make it difficult to distinguish RH from true primary liver disease. Whereas liver enzymes establish the presence of liver injury, bile acids are often used in the identification of hepatobiliary dysfunction due to cholestasis or portosystemic shunting.[20, 26] Measuring BA is a sensitive method to establish liver dysfunction,[23] but similar to liver enzymes these measurements are not sufficient in specifying the underlying liver disease. Therefore, definitive diagnosis relies on an extensive clinical work-up, usually including histopathologic evaluation of a liver biopsy specimen.

Although biochemical indicators with high sensitivity for liver disease (*i.e.* ALT and ALP) are essential to identify affected dogs in an early stage of disease, robust data considering sensitivity and specificity of ALT, ALP, and BA in dogs with subclinical hepatitis is currently missing. The main aims of the current study were 1) to calculate the sensitivity and specificity of ALT, ALP and BA for detecting hepatitis in clinically healthy Labrador retrievers and 2) to evaluate whether increased ALT and ALP aid in the discrimination between dogs with PH and RH.

Material and methods

Labrador retrievers

All Labrador retrievers in this study were referred to the Department of Clinical Sciences of Companion Animals, Utrecht University, between 2003 and 2015. Dogs were referred because of possible liver-related clinical signs or because of increased liver enzymes. In addition, client-owned clinically healthy Labrador retrievers were used that participated in the ongoing research program into copper-associated hepatitis. These dogs were either first-line relatives of dogs diagnosed with copper associated hepatitis or admitted for screening for copper-associated hepatitis prior to breeding. Data of 299 Labrador retrievers, concerning signalment, medical history, clinical, laboratory, and histopathologic findings were retrospectively identified from medical records. Only data from dogs that were admitted for the first time were included. At time of admission, liver biopsies and blood samples were collected from all dogs according to the Act on Veterinary Practice, as required under Dutch legislation and all procedures were known and approved by the Animal Welfare Body of the University of Utrecht.

Histopathology was considered as the gold standard for diagnosing dogs with parenchymal liver disease and was reviewed as described below. Five dogs had malignant lymphoma, nine dogs had steroid induced hepatopathy, one dog had granulomatous hepatitis, and one dog had lobular dissecting hepatitis on liver histology and were all excluded of the study. Dogs defined as normal in this study had no clinical signs of disease and absence of liver disease on histopathologic evaluation of liver biopsy specimen. Based on the localization and concentration of hepatic copper, these dogs were further subdivided in dogs with normal or high hepatic copper concentrations. Dogs were considered to be subclinically affected when they had no clinical signs of disease but histopathology revealed hepatitis, regardless of copper or biochemical blood parameter results. Normal or subclinically affected dogs with non-systemic concurrent disease (*e.g.* arthrosis, incontinence) were included in the study provided that they were not receiving any medication.

Twenty-six normal or subclinical dogs were excluded from the study because they were receiving medications for extra-hepatic disease. Another five dogs were excluded due to concurrent suffering from (extra-hepatic) cancer. Clinically ill dogs were also included in the study and had one or more of the following clinical signs: weight loss, anorexia, vomiting, diarrhea, lethargy, icterus, polyuria and polydipsia, ascites, fever, abdominal pain, or exercise intolerance. Nine clinically ill dogs and one clinically healthy dog were excluded since they received corticosteroids for more than three weeks or for unknown duration or in an unknown dose. In total, 242 Labrador retrievers were selected for this study.

Blood analyses

Immediately prior to the biopsy procedure plasma ALT, ALP, and fasting BA concentrations were measured from heparinized plasma and determined using a DXC-600 Beckman analyzer[a] at the Veterinary Diagnostic Laboratory of Utrecht University (UVDL). Reference ranges for ALT, ALP, and fasting BA were established by the UVDL and are <70 U/L, <89 U/L, and <10 μmol/L, respectively. Results of ALT, ALP, BA, and quantitative copper (CuQ) measurements were available for 236, 238, 233, and 222 dogs, respectively. In addition, citrate plasma was taken for analysis of the coagulation profile, including prothrombin time, activated partial thromboplastin time, and fibrinogen concentration and thrombocytes were determined in an EDTA blood sample. Only when coagulation parameters were not within reference range, the liver biopsy procedure was postponed. In that case dogs were treated with 1 mg/kg prednisone daily during 1 week to normalize coagulation parameters. [27]

Histopathology of liver specimens

At least three liver biopsies from the left lateral liver lobe were collected with a 14 G needle using a Tru-cut device under ultrasound guidance as described previously.[28] Two biopsy specimens were fixed in 4% neutral buffered formalin for 3 hours and transferred to 70% ethanol and embedded in paraffin. Paraffin sections of biopsies were stained with hematoxylin and eosin and reticulin according to Gordon and Sweet.[29] After histopathological evaluation, liver biopsy results were categorized into PH or RH. PH included dogs with AH or CH (including dogs with cirrhosis). Dogs with normal liver histology on hematoxylin and eosin and reticulin were included in the normal liver (NL) group. A rubeanic acid stain was used to assess the presence and distribution of copper within the liver lobule. All samples were evaluated by a board certified pathologist (TSGAMvdI, diplomat ECVP) according to the standards of the WSAVA. [2] A third biopsy specimen was collected in a copper free container and dry freezed prior to quantitative copper determination by Instrumental Neutron Activation Analysis. [30] Quantitative

copper (CuQ) concentrations > 400 mg/kg dry weight liver (dwl) were considered to be abnormally elevated.[31]

Statistical analysis

A Wilcoxon rank sum test was used to compare ALT, ALP, and BA between dogs with low and high hepatic copper concentrations. Associations between ALT and ALP and histological grade and stage were analysed using the Spearman's rank correlation. ALT, ALP, BA, and CuQ were analysed by linear regression where histological diagnosis, age at time of biopsy, and sex were entered as fixed variables. The best fitting model for the data was determined with a stepwise forward model using Akaike's information criterion. The validity of all models was checked by studying the residuals on normality and constant variance. To ensure validity of the model ALT, ALP, and CuQ were ln transformed, after studying the residuals on normality and constant variance. For BA the transformation ln(BA+0.1) was used, as some dogs had BA concentrations of 0. A logit model was used to calculate the odds ratio for PH, with ALT as co-factor, corrected for sex and age. NL and RH were used as reference category. Receiver operating characteristic (ROC) curve analyses were used to determine the sensitivity and specificity of ALT, ALP, and BA for detecting the presence of hepatitis in clinically healthy dogs. Confidence intervals of sensitivity and specificity at a certain threshold were computed with bootstrap resampling. Normally distributed data were presented as mean ± standard deviation and non-normally distributed data as median and range. P values were adjusted for multiple testing using the Bonferroni correction. All data were analyzed using R statistics[b]. ROC curves were generated using the R package 'pROC'.[32]

Results

Animal characteristics

Of the 242 Labrador retrievers, 155 dogs were female and 87 were male. The mean age at time of liver biopsy was 6.1±2.9 years. Of all dogs 81 dogs were single admitted cases and 161 dogs were family members of at least one other dog included in the study, representing 22 different families. Of the 51 clinically ill dogs, 12 dogs received 1 mg/kg body weight prednisolone for a median of seven days to normalize coagulation. These twelve dogs had no histopathological signs of steroid induced hepatopathy. Of the 191 clinically healthy dogs, 17 dogs had concurrent non-systemic disease that did not require therapy. Twelve dogs had primary epilepsy, with seizure frequency too low to require therapy and no seizures in the previous months. The other dogs suffered from ectopic ureters

(n=2), deafness (n=1), arthrosis (n=1), and partial cranial cruciate rupture (n=1). Beside these diseases there were no indications for other diseases in participating dogs. Fifty-one Labradors were clinically ill. Most common clinical signs were vomiting (n=36), lethargy (n=32), anorexia (n=31), and icterus (n=27). Other clinical signs at presentation were polyuria and polydipsia (n=18), weight loss (n=15), diarrhea (n=7), fever (n=5), ascites (n=6), and exercise intolerance (n=3). Forty-nine dogs had more than one clinical sign of disease.

Hepatic copper assessment

Hepatic copper granules in Labrador retrievers with increased hepatic copper concentrations were located in the centrolobular region of the liver lobule on rubeanic acid stain, indicating primary copper accumulating disease. Compared to normal liver (NL), acute hepatitis (AH) and chronic hepatitis (CH) had increased hepatic copper concentrations (Fig 1). The highest copper concentrations were identified in dogs with AH (median 964 mg/kg dwl, range 243-3,030). In these dogs, copper concentrations were 1.7 times higher compared to those dogs with normal liver histology (95% CI: 1.2-2.5; *P*=0.002; Fig 1). Dogs with CH had a median copper concentration of 685 mg/kg dwl (range 85-5,084), which was also significantly increased compared to the NL group (estimate, 1.3; 95% CI: 1.0-1.8; *P*=0.035; Fig 1). There were no statistically significant differences in ALT, ALP and BA concentrations in dogs with normal (<400 mg/kg dwl) or increased (>400 mg/kg dwl) hepatic copper concentrations. This was measured in all dogs and in dogs with normal liver histology (Supplementary Figs 1& 2). Because there was not any difference in ALT, ALP, and BA level, no differentiation between normal and high copper groups were made for the rest of the study.

Biochemical indicators and liver histology

In Table 1 the results of ALT, ALP, and BA measurements in 242 Labrador retrievers with different clinical presentations and liver histopathology are summarized. Of the 242 dogs, 28% (69/242) dogs had NL, 11% (27/242) AH, 23% (55/242) CH, and 38% (91/242) RH. In these 242 dogs, ALT, ALP, and BA concentrations were increased in dogs with AH and CH compared to dogs with NL or RH (*P*<0.001; Fig 2). Dogs with CH had higher ALT and ALP concentrations compared to dogs with AH (*P*<0.001; Fig 2A,B). In 64% (122/191) of the clinically healthy Labrador retrievers histology revealed abnormalities. Of these dogs, 30% (37/122) were diagnosed with PH and 70% (85/122) showed RH.

In the 191 clinically healthy dogs (69 “Normal” and 122 “Subclinically affected”), 146 dogs had ALT, ALP, and BA within reference range, and one dog had an increase of all three parameters (Fig 3). In all other dogs at least one parameter was

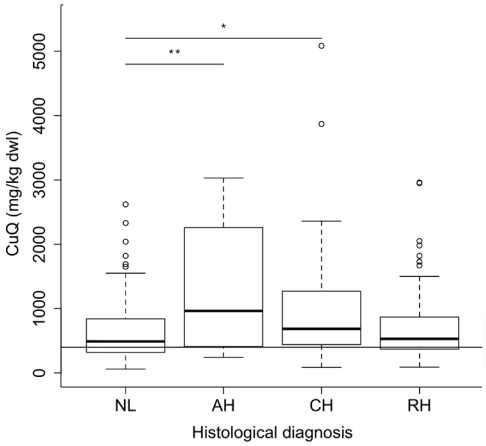


Figure 1 Quantitative copper concentrations in Labrador retrievers with different histological diagnosis.

All Labrador retrievers with known hepatic quantitative copper concentrations (n=222) were included. Horizontal black line indicates upper reference limit of normal hepatic copper concentration (400 mg/kg dwl). AH, acute hepatitis; CH, chronic hepatitis; CuQ, quantitative copper concentration; dwl, dry weight liver; NL, normal liver; RH, non-specific reactive hepatitis. The thick black line represents the median (50th percentile), also the first and third quartile (25th and 75th percentile respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the interquartile range. * P< 0.05, ** P< 0.01.

increased. Clinically healthy dogs with CH had a significantly increased ALP compared to dogs with NL (P<0.001), AH (P=0.029), and RH (P<0.001) (Fig 3B). ALT concentrations were increased in dogs with AH and CH compared to dogs with NL or RH (P<0.001; Fig 3A). There were no significant differences in BA concentrations between histology groups in the clinically healthy dogs (Fig 3C). In clinically ill dogs, 88% (45/51) had a PH and 12% (6/51) a RH. Only two dogs had ALT, ALP, and BA within reference range (Fig 4). ALT and BA concentrations were significantly increased in dogs with AH and CH compared to dogs with RH, while ALP concentrations did not differ between groups (Fig 4). In the group of 173 (122 "Subclinically affected" and 51 "Clinically ill") dogs with hepatitis, 98 dogs had ALT and ALP activity within reference range and 93 dogs had ALT, ALP, and BA within reference range. In the 69 dogs with normal liver histology, 13 dogs showed increase in either ALT, ALP, or BA.

Table 1 Biochemical indicators in 242 Labrador retrievers with different clinical presentation and liver histopathology.

	Clinically healthy		**Clinically ill**
	Normal n=69	**Subclinically affected n=122**	**n=51**
	Normal histology NL, n=69	Abnormal histology PH, n=37 (AH, n=20, CH, n=17) RH, n=85	Abnormal histology PH, n=45 (AH, n=7, CH, n=38) RH, n=6
ALT (U/L, median and range)	33 (17 - 197) n=68	40 (5 - 575) n=122	376 (14 - 1369) n=46
ALP (U/L, median and range)	23 (8 -109) n=68	27 (12 - 478) n=121	381 (20 - 1500) n=49
BA (μmol/L, median and range)	2 (0 - 16) n=69	2 (0 - 146) n=120	36 (1 - 325) n=44

AH, acute hepatitis; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BA, bile acids; CH, chronic hepatitis; NL, normal liver; PH, primary hepatitis; RH, non-specific reactive hepatitis.

Liver enzymes and grade and stage of the hepatitis

Grading of the necro-inflammatory activity of the hepatitis and staging of the fibrosis were assessed in 95 liver biopsies specimens (NL, n=18; AH, n=12; CH, n=25; RH, n=40) of both clinically healthy and clinically ill dogs. Median grading scores (scores 0-5) were 0 (NL), 1 (RH), 2 (AH), and 3 (CH). Median staging scores (scores 0-4) were 0 (NL), 0 (RH), 0 (AH), and 3 (CH). A significant positive correlation between ALT and grade (r=0.63, P<0.001) or stage (r=0.43, P<0.001) of the hepatitis was present (Supplementary Fig 3A,B). For ALP there was a significant positive correlation with grade (r=0.38, P<0.001) and stage (r=0.42, P<0.001) of the hepatitis (Supplementary Fig 3C,D).

Differentiation between PH and RH using liver enzymes

One goal of this study was to see whether or not it was possible to discriminate PH from RH, based on the increase in ALT and ALP in dogs in which ALT, ALP or both were increased. In 80 clinically healthy and clinically ill dogs at least one of the liver enzymes was increased above reference range. Most of these dogs (65/80; 81%) had a PH, whereas 11% (9/80) had a RH, and 8% (6/80) had no abnormalities on histology. Median ALT activity of dogs with PH (312 U/L, range 38 – 1,369) was 4.3 times increased (95% CI 2.1 – 8.9; P<0.001) compared to dogs with NL (median ALT

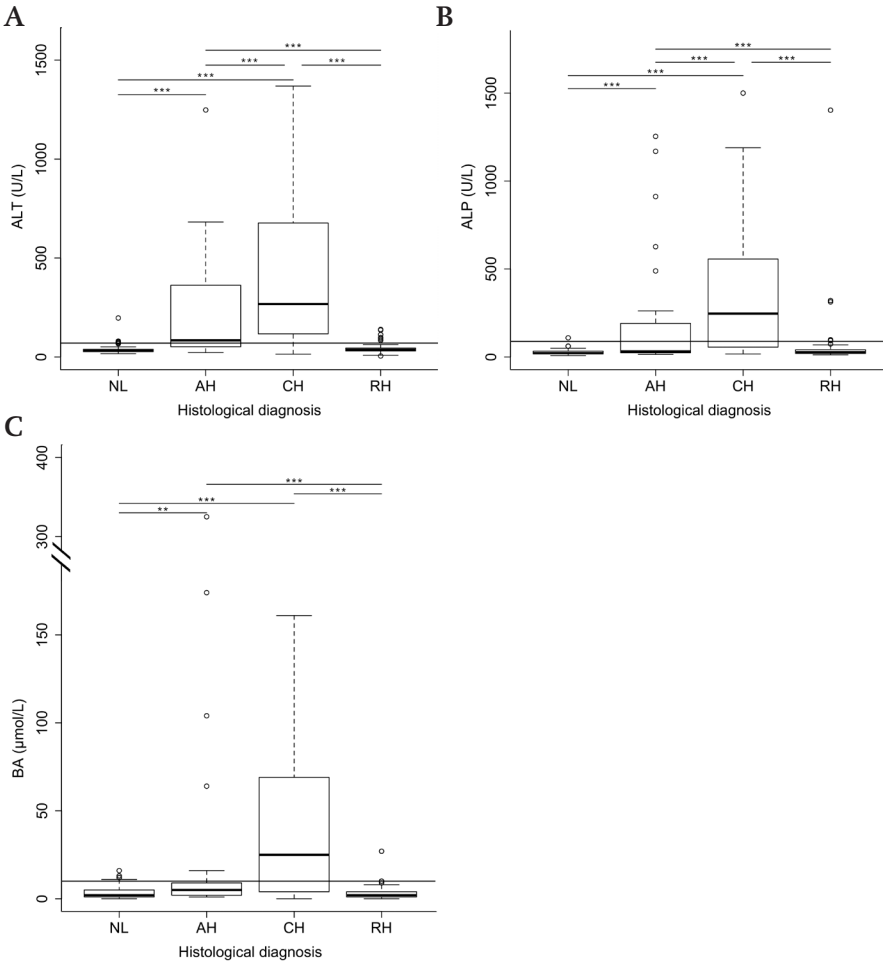


Figure 2 Concentrations of liver enzymes and bile acids in all Labrador retrievers.

Concentrations of ALT (**A**), ALP (**B**) and BA (**C**) in all (*n*=242) Labrador retrievers. AH, acute hepatitis; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BA, bile acids; CH, chronic hepatitis; NL, normal liver; RH, non-specific reactive hepatitis. Horizontal black lines indicate upper reference limits for ALT (<70 U/L), ALP (<89 U/L), and BA (<10 µmol/L). The thick black line represents the median (50th percentile), also the first and third quartile (25th and 75th percentile respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the interquartile range. ** $P< 0.01$, *** $P< 0.001$

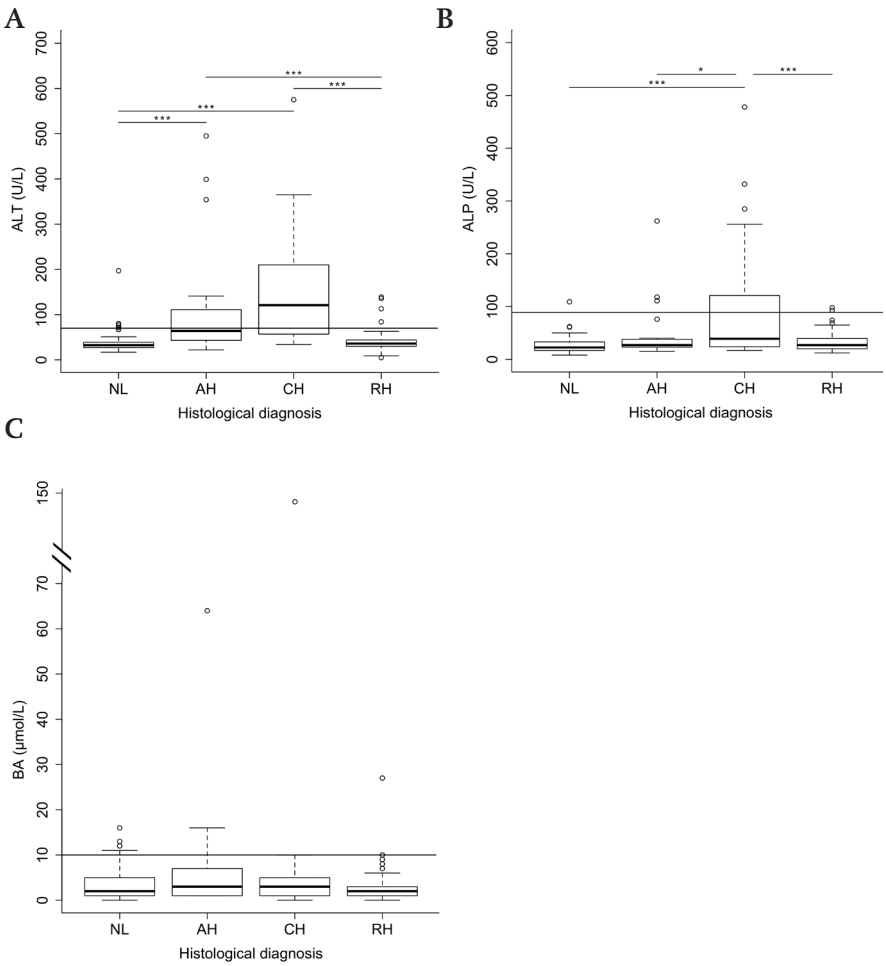


Figure 3 Concentrations of liver enzymes and bile acids in clinically healthy Labrador retrievers.

Concentrations of ALT (**A**), ALP (**B**) and BA (**C**) in 191 clinically healthy Labrador retrievers. This includes 69 dogs with normal histology and 122 dogs with abnormal histology. AH, acute hepatitis; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BA, bile acids; CH, chronic hepatitis; NL, normal liver; RH, non-specific reactive hepatitis. Horizontal black lines indicate upper reference limits for ALT (<70 U/L), ALP (<89 U/L), and BA (<10 µmol/L). The thick black line represents the median (50th percentile), also the first and third quartile (25th and 75th percentile respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the interquartile range. * $P < 0.05$, *** $P < 0.001$

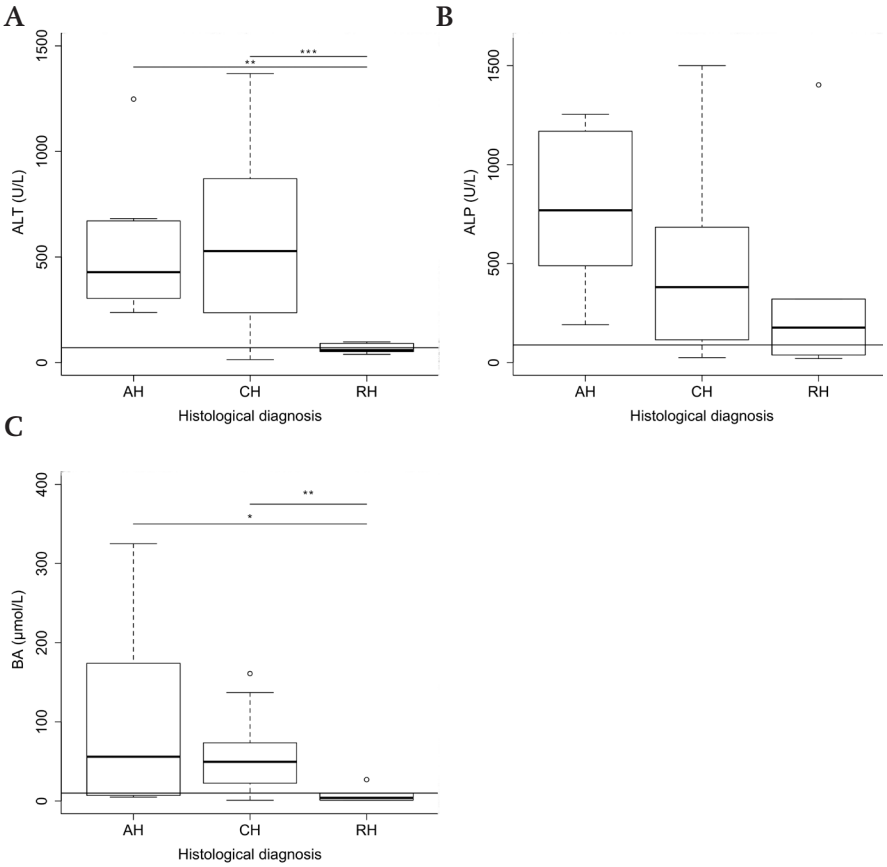


Figure 4 Concentrations of liver enzymes and bile acids in clinically ill Labrador retrievers.

Concentrations of ALT (**A**), ALP (**B**) and BA (**C**) in 51 clinically ill Labrador retrievers. AH, acute hepatitis; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BA, bile acids; CH, chronic hepatitis; RH, non-specific reactive hepatitis. Horizontal black lines indicate upper reference limits for ALT (<70 U/L), ALP (<89 U/L), and BA (<10 µmol/L). The thick black line represents the median (50th percentile), also the first and third quartile (25th and 75th percentile respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the interquartile range. * $P < 0.05$,** $P < 0.01$,*** $P < 0.001$

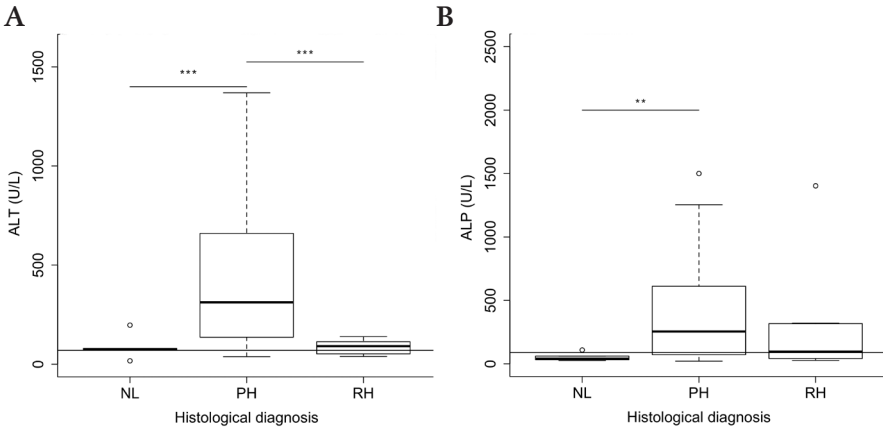


Figure 5 ALT and ALP activity in dogs with normal liver histology, primary hepatitis, or non-specific reactive hepatitis.

This group included 80 normal, subclinically affected or clinically ill dogs of which at least one of the liver enzymes was increased above reference range. (**A**) ALT. Horizontal black line indicates upper reference limit of ALT (70 U/L). (**B**) ALP. Horizontal red line indicates upper reference limit of ALP (89 U/L). The thick black line represents the median (50th percentile), also the first and third quartile (25th and 75th percentile respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the inter-quartile range. Significant differences between groups are marked with stars (** P< 0.01, ***P<0.001). ALT, alanine aminotransferase; ALP, alkaline phosphatase; NL, normal liver; PH, primary hepatitis; RH, non-specific reactive hepatitis.

of 75 U/L, range 17 - 197) and 3.6 times increased (95% CI 2.0 – 6.7; P<0.001) compared to dogs with RH (median ALT of 91 U/L, range 39 -139) (Fig 5A). There was no significant difference in ALT between dogs with NL and RH. To measure the association between ALT and the presence of a PH, odds ratios were calculated. For every 1 U/L increase in ALT activity a two percent increase in the odds of having a PH (versus having NL or a RH) is expected (95% CI: 1.01 – 1.04; P=0.007). Median ALP activity in dogs with PH (255 U/L, range 21 - 1,500) was significantly increased (estimate, 4.3; 95% CI: 1.5-12.4; P=0.009) compared to dogs with NL (median ALP of 41 U/L, range 26 -109) (Fig 5B). No difference was found in ALP activity between dogs with PH and RH (median ALP of 96 U/L, range 27 - 1,403) or between dogs with RH and NL.

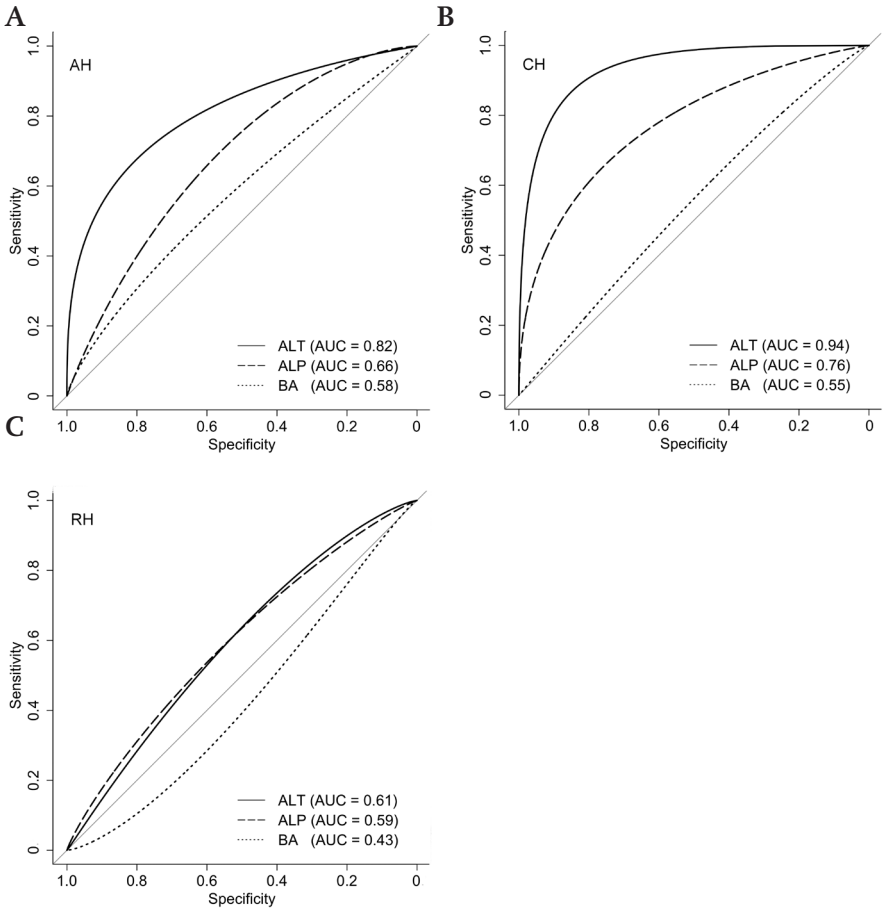


Figure 6 Receiver operator characteristic curves.

Receiver operator characteristic (ROC) curves for ALT (solid line), ALP (dashed line), and BA (dotted line) in clinically healthy Labrador retrievers (*n*=191). ROC curves for discriminating dogs with normal liver (*n*=69) from dogs with acute hepatitis (*n*=20) (**A**), dogs with chronic hepatitis (*n*=17) (**B**), and dogs with non-specific reactive hepatitis (*n*=85) (**C**) ALT, alanine aminotransferase; ALP, alkaline phosphatase; AUC, area under curve; BA, bile acids.

Table 2 Sensitivity and specificity for detecting acute hepatitis, chronic hepatitis and non-specific reactive hepatitis in clinically healthy Labrador retrievers.

	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)
Acute hepatitis			
ALT 70 U/L	0.45 (0.25-0.65)	0.93 (0.85-0.99)	0.82 (0.69-0.94)
ALP 89 U/L	0.15 (0.00-0.35)	0.99 (0.96-1.00)	0.66 (0.53-0.79)
BA 10 μmol/L	0.15 (0.00-0.30)	0.90 (0.83-0.97)	0.58 (0.43-0.72)
Chronic hepatitis			
ALT 70 U/L	0.71 (0.47-0.94)	0.93 (0.85-0.99)	0.94 (0.87-1.00)
ALP 89 U/L	0.35 (0.12-0.59)	0.99 (0.96-1.00)	0.76 (0.63-0.89)
BA 10 μmol/L	0.13 (0.00-0.31)	0.90 (0.81-0.96)	0.55 (0.39-0.71)
Non-specific reactive hepatitis			
ALT 70 U/L	0.05 (0.01-0.09)	0.93 (0.85-0.99)	0.61 (0.52-0.70)
ALP 89 U/L	0.02 (0.00-0.06)	0.99 (0.96-1.00)	0.59 (0.50-0.68)
BA 10 μmol/L	0.04 (0-0.08)	0.90 (0.83-0.97)	0.43 (0.34-0.52)

ALT, alanine aminotransferase; ALP, alkaline phosphatase; AUC, area under the curve; BA, bile acids; CI, confidence interval.

Sensitivity and specificity of ALT, ALP and BA in clinically healthy Labrador retrievers

One hundred and ninety-one clinically healthy Labrador retrievers were included of which 69 "Normal" and 122 "Subclinically affected" (AH, n=20; CH, n=17; RH, n=85) dogs. ROC curves of ALT, ALP, and BA for discriminating normal dogs from dogs with AH, CH, or RH were made (Fig 6). Using our laboratory's threshold values, corresponding sensitivity and specificity of these parameters were determined (Table 2). Based on the area under the curves (AUC, Table 2), the power to discriminate dogs with AH from NL was significantly higher for ALT compared to ALP (P=0.023) and BA (P=0.016). For dogs with CH, ALT had also a significant better ability to discriminate dogs with CH from dogs with NL than ALP (P=0.005) and BA (P<0.001). The discriminating ability of ALP was also significantly better compared to BA (P=0.040).

Discussion

Biochemical parameters including ALT, ALP, and BA are often useful to make a presumptive diagnosis of liver disease in dogs with clinical signs that may be related to liver dysfunction. However, especially in cases of CH, there is a long subclinical phase in which dogs do not show clinical signs but hepatitis is already present. As our data set also included a large number of clinically healthy dogs with biopsy-confirmed liver status, one of the aims of this study was to determine the sensitivity and specificity of ALT, ALP, and BA for detecting inflammatory liver disease in clinically healthy Labrador retrievers. In the present study we showed that the sensitivity ALP and BA for primary inflammatory lesions in these clinically healthy dogs is low and these parameters are therefore not appropriate to use as screening parameters to detect early primary hepatic disease. ALT, on the other hand, has shown to have a reasonable sensitivity to identify dogs with CH. As expected, ALT, ALP and BA are also not able to detect hepatic injury in the face of excess copper accumulation. We also showed that ALT can aid in the discrimination between dogs with a PH and a RH.

Copper-associated hepatitis is one of the most common forms of primary hepatitis in dogs as shown by a study conducted in 101 purebred and crossbred dogs in The Netherlands, of which up to 36% of the CH and 24% of the AH cases were copper associated. [1] Familiar dog breeds known to develop copper-associated hepatitis are the Bedlington terrier, [18] West Highland white terrier, [33] Dalmatian, [34] Dobermann, [12] and the Labrador retriever. [17] In the Bedlington terrier hereditary copper-associated hepatitis is due to deletion of exon 2 of the *COMMD1* gene and hepatic copper concentrations can easily reach 5,000 mg/kg dwl or higher.[35, 36] Copper associated hepatitis in the Labrador retriever is one of the best-studied examples of copper toxicosis with a complex molecular background. Besides the involvement of mutations in the copper transporters ATP7A and ATP7B [17] the expression of the disease phenotype also relies on environmental factors such as dietary copper intake. [14, 37] In the current study, increased hepatic copper concentrations were present in Labrador retrievers with different histological diagnosis, including histologically normal liver, but concentrations were highest in dogs with AH. In contrast to dogs with AH, dogs with CH have fibrosis with or without cirrhosis. Neither fibrotic nor regenerative nodules contain accumulated copper granules, diluting total copper content of the original hepatocyte population. [33, 38, 39] Liver biopsies might not be representative due to the sampling of fibrotic tissue only, but this was likely compensated by taking three different 14G samples. There was no difference between dogs with normal and high hepatic copper concentrations in ALT, ALP, and BA, which was measured in dogs with and

without concurrent presence of inflammatory liver disease. Although this result was not surprising, the current data provides evidence that ALT, ALP, and BA are not sensitive enough to test for hepatic copper accumulation. Liver histology, including copper staining and copper quantification remains necessary to obtain a diagnosis. This is also illustrated by a study in *COMMD1* deficient crossbred dogs, which provides a longitudinal analysis of hepatic copper accumulation. [40] *COMMD-1* deficient dogs started to accumulate copper at the age of six months but without evidence of hepatitis or increase of liver enzymes. Only after there were signs of hepatitis on histology, liver enzymes were increased. [40]

ALT, ALP, and BA were measured in dogs with NL, RH, AH, and CH. Dogs with CH generally had higher ALT and ALP concentrations compared to dogs with AH. This can be explained by the higher necro-inflammatory grade in dogs with CH and the positive correlation of ALT and ALP with grade of the hepatitis. A previous study also reported a significant positive association of ALT with fibrosis stage. [41] Hepatic changes in Labrador retrievers in the present study were only mild, which might be a reason for the relatively poor sensitivity of biochemical indicators to detect the presence of CH or AH. Another reason could be that the current reference values are set too high, at least for this population. In our laboratory, the upper limit of the reference range is used as cut-off to discriminate healthy animals from diseased animals. Reference ranges are usually determined in a group of clinically healthy dogs, but in most cases no histopathology results are present to confirm the health status of the dog. With this study we provide evidence that a considerable amount of clinical healthy dogs can have inflammatory lesions in the liver. At least in Labrador retrievers, which are known to suffer from (hereditary) subclinical hepatitis, it is possible to increase sensitivity by decreasing the current thresholds. In this way a more acceptable sensitivity for ALT can be obtained. The power to discriminate dogs with CH and AH from dogs with NL was higher for ALT compared to ALP and BA, indicating lack of discriminating ability of ALP and BA. As all dogs were clinically healthy dogs, the degree of liver dysfunction was assumed to be minimal reflecting low BA concentrations. In contrast to clinically ill dogs which had increased BA concentrations.

In the present study, the specificity of ALT and ALP in clinically healthy Labrador retrievers was found to be 93% and 99%, respectively. However, Center et al (2007) reported much lower specificities for both enzymes, especially ALP. This difference can be explained because our study did not include dogs with known extra-hepatic disease, and therefore specificity may be overestimated compared to the general population. ALP is a membrane-bound enzyme found in hepatocytes and bile canaliculi, and is cleaved from its anchors by phospholipases. This process is

facilitated by the presence of increased bile acid concentrations during cholestasis.[42] However, ALP is the liver enzyme with the lowest specificity in the dog.[19] Other tissues with high quantities of ALP are the intestinal mucosa, kidney, placenta, and bone. Due to their much longer half-lives, mainly liver, bone, and glucocorticoid induced isoenzymes of ALP are found in canine serum.[43] In addition, it has been shown that dogs with mammary tumors and dogs treated with medications such as phenobarbital can have increased serum ALP concentrations.[44, 45] ALT can also have an extra-hepatic origin, which includes mainly muscle.[46, 47] Considering the possibility of extra-hepatic sources of ALT and ALP, lower specificities than reported in the present study could be more realistic when studied in another population of dogs. Six dogs in the present study had high liver enzymes but liver histology detected no abnormalities. Besides false positive results due to iso-enzyme activity/ extra-hepatic origin, possibilities include decreased serum enzyme clearance upon resolution of the initial injury or a not representative liver biopsy sample.

Another goal of our study was to evaluate the possibility to discriminate between a PH and a RH in dogs with increased ALT, ALP or both. In both RH and PH, dogs might present with elevated ALT and/or ALP. In dogs with RH, it is the underlying disease that requires attention and usually the hepatic changes resolve once the extra-hepatic diseases or stimuli are treated or removed. Interestingly, we found that ALT was significantly higher in dogs with a PH compared to dogs with a RH. ALT is present in the hepatocyte cytosol and leaks upon altered hepatocyte membrane integrity, and is reported to be the first and most sensitive parameter to increase in dogs with hepatocellular inflammation and necrosis.[19, 48] As RH may be less characterized by hepatocellular damage than intrahepatic cholestasis due to circulating endotoxins,[2] leakage of ALT might be limited and of ALP more prominent in these cases. Although there is still overlap in ALT levels between dogs with a PH and with a RH, the increase of ALT can be used to help predict if a PH can be present.

One of the limitations of this study was that this study was conducted in Labrador retrievers only. The majority of dogs were also related to one or more other dogs included in the study. Due to the complex nature of the disease it is not known what the odds of disease is for related Labradors that were included in the NL group compared to Labradors included in the NL group that do not descend from high risk pedigrees. In addition, pedigree information of the single admitted cases is not known. It is known that some pedigrees have higher disease frequency and higher hepatic copper concentrations than others.[49] High hepatic copper concentrations in this breed are believed to be the cause of primary hepatitis in this breed, consequently increasing biochemical indicators of liver disease. Due to

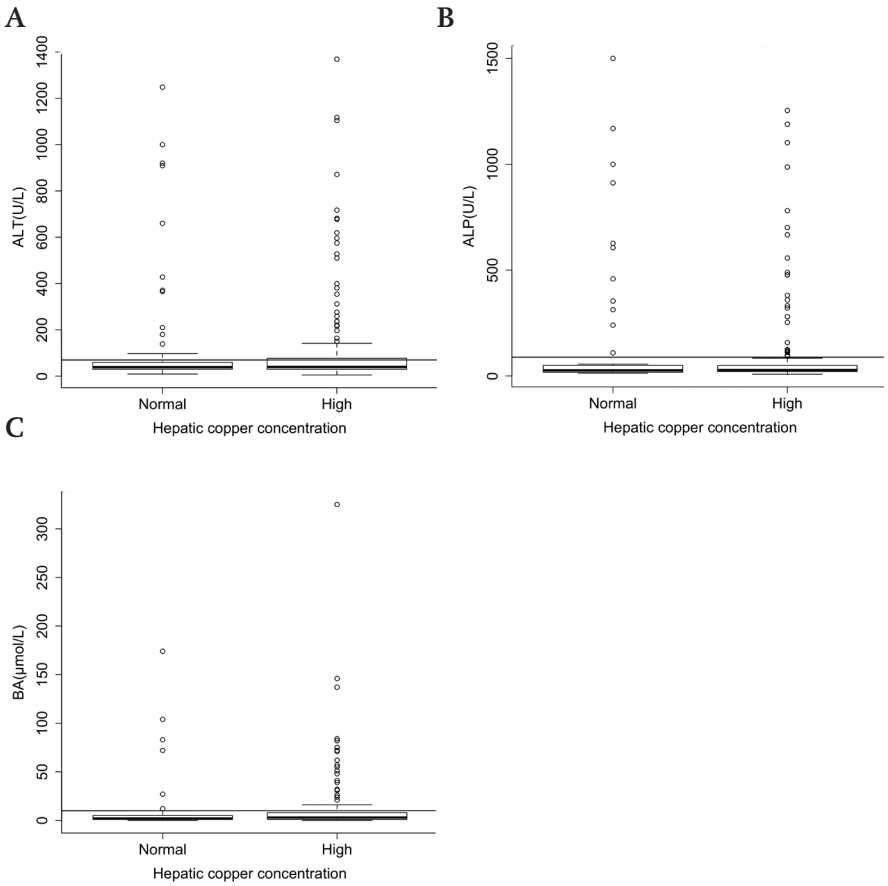
the comparative nature of parenchymal liver disease between dog breeds, it is likely that the results of this study can still be extrapolated to other dog breeds. To confirm these observations additional studies in mixed dog populations are necessary, but these are limited due to the lack of histologic sampling in clinically healthy dogs with normal liver enzymes. Another limitation is that this study did not include dogs with extra-hepatic disease influencing enzyme levels (*e.g.,* hyperadrenocorticism, or bone disease). This is mainly due to the fact that there is no indication for liver histopathology in these dogs. If these dogs were included in the study, true specificity of biochemical indicators would likely be lower. In the present study, twelve dogs received 1 mg/kg prednisone treatment for one week to normalize coagulation parameters [1, 27], and liver biopsy was therefore precluded. It is known that prednisone treatment can lead to glycogen accumulation in the hepatocytes. [50] In addition, prednisone has beneficial effects on inflammation and in some dogs might even reduce or limit the progression of fibrotic changes. [27] In our study, no vacuolar changes were present on histopathology but it cannot be excluded that prednisone treatment ameliorated necro-inflammatory activity in these dogs.

In conclusion, results of this study indicate that the ALP and BA are not sensitive enough to detect hepatitis in clinically healthy Labrador retrievers. Although ALT is not sensitive in detecting dogs with AH, sensitivity for detecting dogs with CH is reasonable. At least for Labrador retrievers, we propose that decreasing the current thresholds can increase the sensitivity further. Using the increase in ALT, it is possible to assess the odds for having a PH. Even though hepatic copper accumulation is a well-described problem in de Labrador retriever, it is not correlated to ALT, ALP, BA or to the existence of hepatocellular injury. As indicators for subclinical PH are scarce, there is still need for more sensitive and specific biomarkers for the early detection of inflammatory liver disease.

Acknowledgments

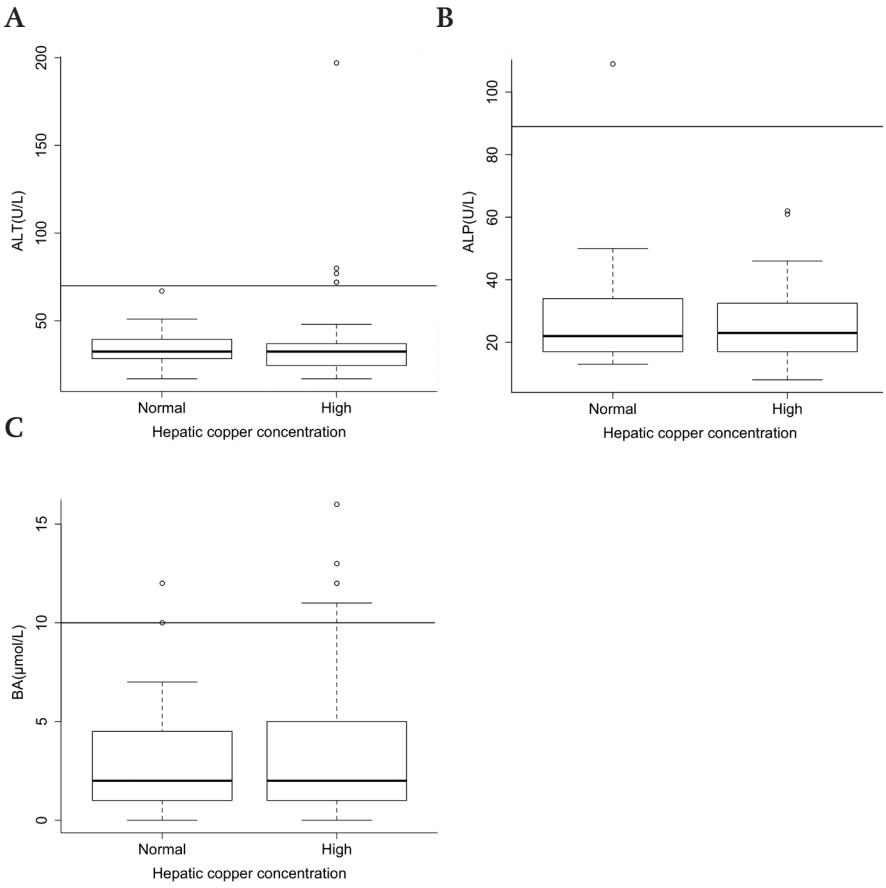
The authors thank Hans Vernooij for providing statistical advice.

Supplementary material



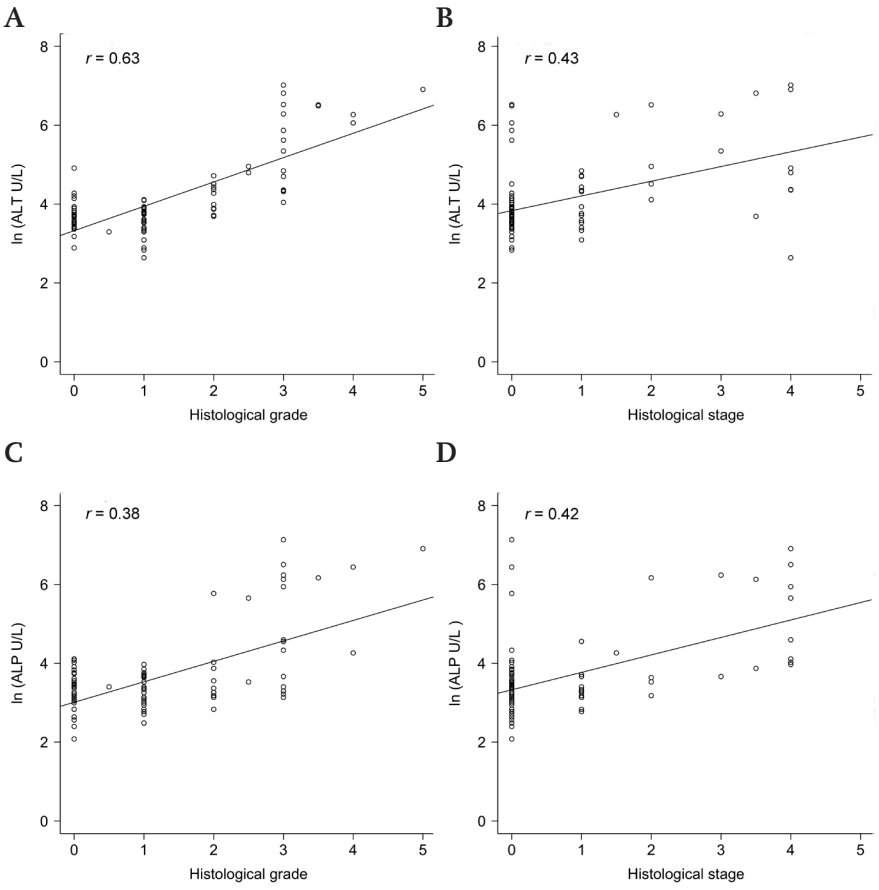
Supplementary Figure 1 Concentrations of liver enzymes and bile acids in all Labrador retrievers.

Concentrations of ALT (**A**), ALP (**B**) and BA (**C**) in all Labrador retrievers with known hepatic quantitative copper concentrations (n=222). Horizontal black lines indicate upper reference limits for ALT (<70 U/L), ALP (<89 U/L), and BA (<10 µmol/L). There were no statistically significant differences in ALT, ALP and BA concentrations in dogs with normal (<400 mg/kg/dwl) or increased (>400 mg/kg/dwl) hepatic copper concentrations. The thick black line represents the median (50th percentile), also the first and third quartile (25th and 75th percentile respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the interquartile range. ALT, alanine aminotransferase; ALP, alkaline phosphatase; BA, bile acids



Supplementary Figure 2 Concentrations of liver enzymes and bile acids in Labrador retrievers with normal liver histology.

Concentrations of ALT (**A**), ALP (**B**) and BA (**C**) in Labrador retrievers with normal liver histology (n=69). Horizontal black lines indicate upper reference limits for ALT (<70 U/L), ALP (<89 U/L), and BA (<10 µmol/L). There were no statistically significant differences in ALT, ALP and BA concentrations in dogs with normal (<400 mg/kg/dwl) or increased (>400 mg/kg/dwl) hepatic copper concentrations. The thick black line represents the median (50th percentile), also the first and third quartile (25th and 75th percentile respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the interquartile range. ALT, alanine aminotransferase; ALP, alkaline phosphatase; BA, bile acids



Supplementary Figure 3 Correlation of liver enzymes with grade and stage of hepatitis.

Correlation between histological grade (**A**) and stage (**B**) with ALT. In the bottom row the correlation between histological grade (**C**) and stage (**D**) with ALP is depicted. ALT, alanine aminotransferase; ALP, alkaline phosphatase; r, Spearman correlation coefficient

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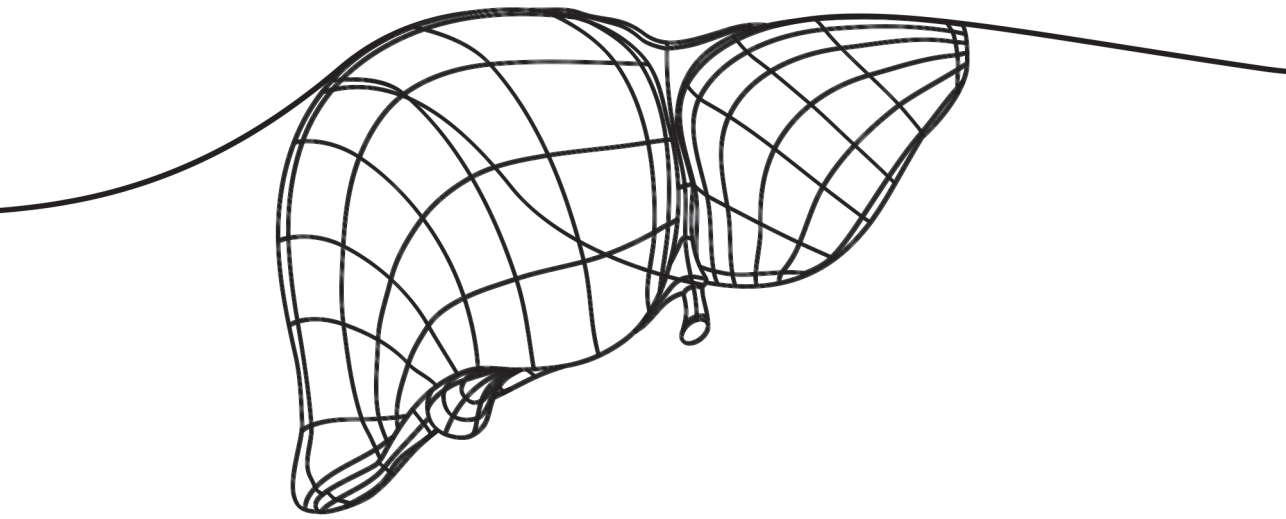
Footnotes

[a] Beckman Coulter, Woerden, The Netherlands
[b] R Foundation for Statistical Computing, Vienna, Austria 2014



5
chapter

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

Karen Dirksen[a]
Tim Verzijl[a]
Ted S.G.A.M. van den Ingh[b]
J.C.M. (Hans) Vernooij[c]
Luc J.W. van der Laan[d]
Iwan A. Burgener[a]
Bart Spee[a]
Hille Fieten[a]

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[a]*Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands*
[b]*TCCI Consultancy BV, Utrecht, The Netherlands*
[c]*Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands*
[d]*Department of Gastroenterology and Hepatology, Erasmus MC: University Medical Center Rotterdam, the Netherlands*

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 analyzes 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 recognized canine parenchymal hepatic diseases are reactive hepatopathies and primary hepatitis. [1, 2] Reactive hepatopathies result from a non-specific response to a variety of extra-hepatic diseases or endo- or exogenous steroids. [3] Primary hepatitis can be acute or chronic in clinical and/or histopathological classification systems. Acute hepatitis can be caused by infectious causes (*i.e.* canine adenovirus-1 infection or leptospirosis) or by the ingestion or administration of various drugs and/or toxins. [4] In most chronic hepatitis cases, the etiology 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. [2, 5, 6]

Alanine aminotransferase (ALT) activity is the most commonly used biochemical indicator for hepatocellular injury in dogs. [5, 7] ALT is primarily and abundantly located in the hepatocyte cytosol. It is released into the bloodstream upon minor changes in membrane integrity. High ALT activity suggests the presence of hepatocellular injury, especially in clinically ill dogs. [5] The average reported sensitivity of ALT for detecting common parenchymal liver diseases in clinically ill dogs varies between 60% and 76%.[7, 8] However, during the subclinical phase little is known about the sensitivity of 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 subclinical affected dogs.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate post-transcriptional gene expression. [9, 10] 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 human patients with normal and high ALT activities. Several of these studies have indicated that HDmiRs have a higher sensitivity of HDmiRs than serum ALT. [11-18]

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 program into copper associated hepatitis of the Faculty of Veterinary Medicine, Utrecht University.[19] 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.[20] Based on histological evaluation according to the World Small Animal Veterinary Association standards [21], dogs were assigned to the normal liver group (NL) 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 localized hepatic copper concentrations (>400 mg/kg dry weight liver [22]) were assigned to the high copper (HC) group.

Blood samples

ALT and HDmiRs levels were assessed in heparinized 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. Normalization 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 obtained cDNA 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 normalized to the spike-in control. [23]

Statistical analysis

Associations between HDmiRs and ALT activity or hepatic copper concentration were analyzed 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 summarized 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 analyzed using R statistics version 3.1.2. ROC curves were generated using the R package 'pROC'. [24]

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 analyzed. 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 coefficient for combinations of miR-122, miR-148a, and plasma ALT activity 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	8F, 3M	16F, 4M	15F, 9M	10F, 1M
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.

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 of 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

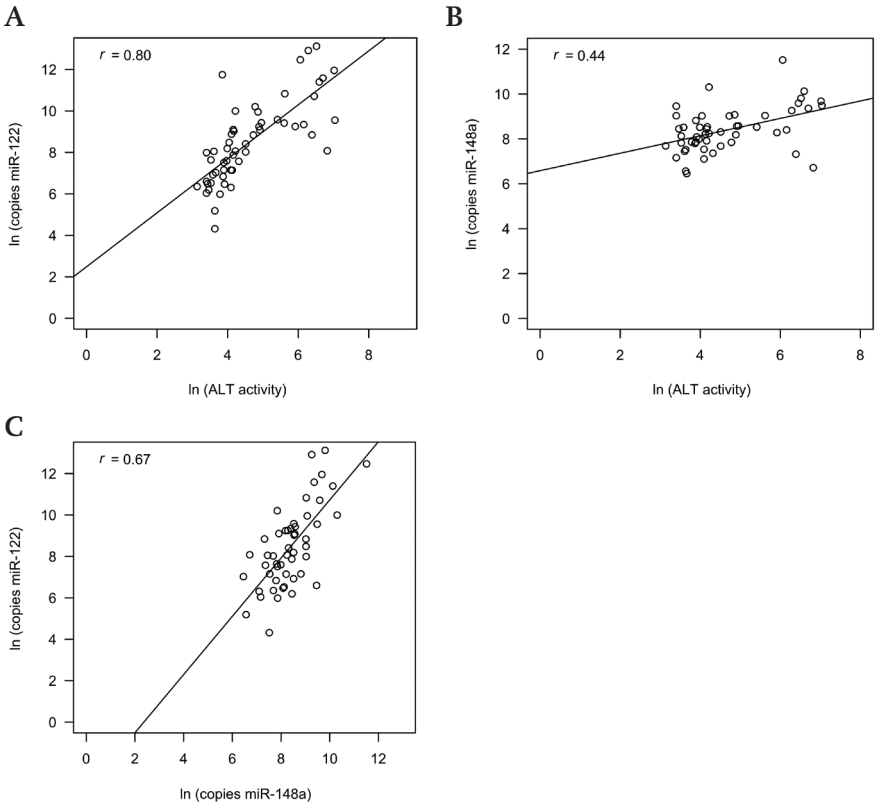

Figure 1

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

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 of 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 levels of both HDmiRs (data not shown).

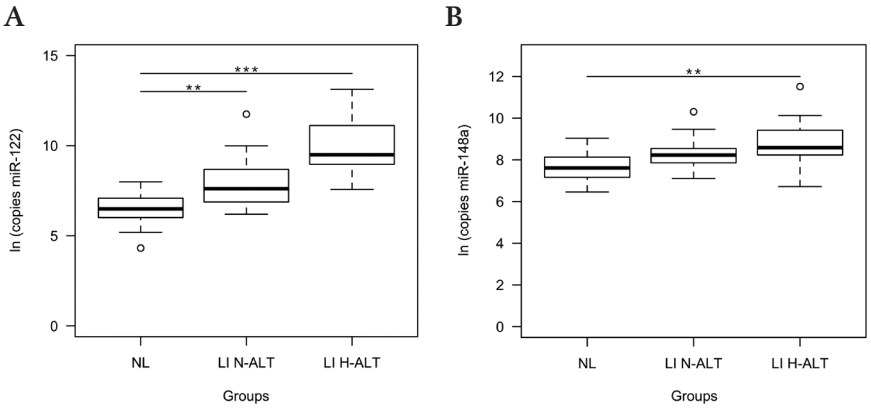

Figure 2

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. $^{**}P<0.01$, $^{***}P<0.001$.

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, 580–1,750) 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 of 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 was no statistical significant difference 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 1,278 and 3,488 copies, respectively. As the upper limit of plasma ALT in our laboratory is 70 U/L, corresponding specificity and sensitivity were also calculated (Table 2). With a difference of 29% (95% CI, 16%–43%), miR-122

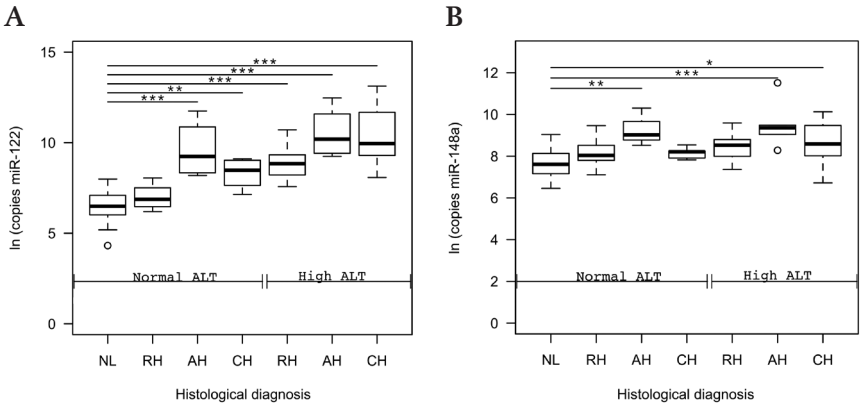

Figure 3

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. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

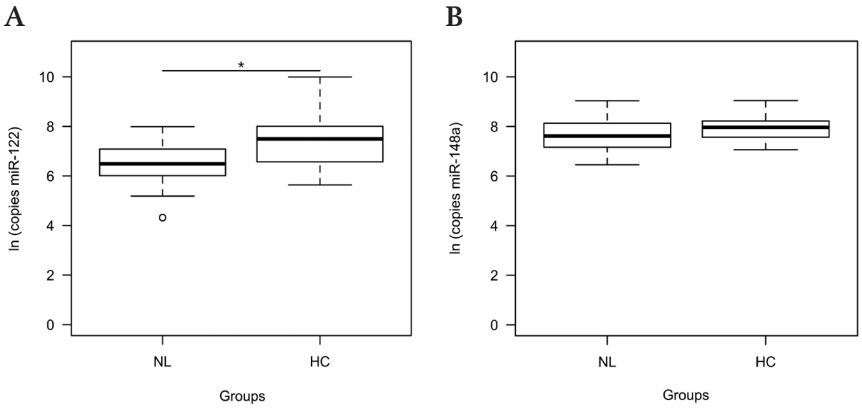

Figure 4

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

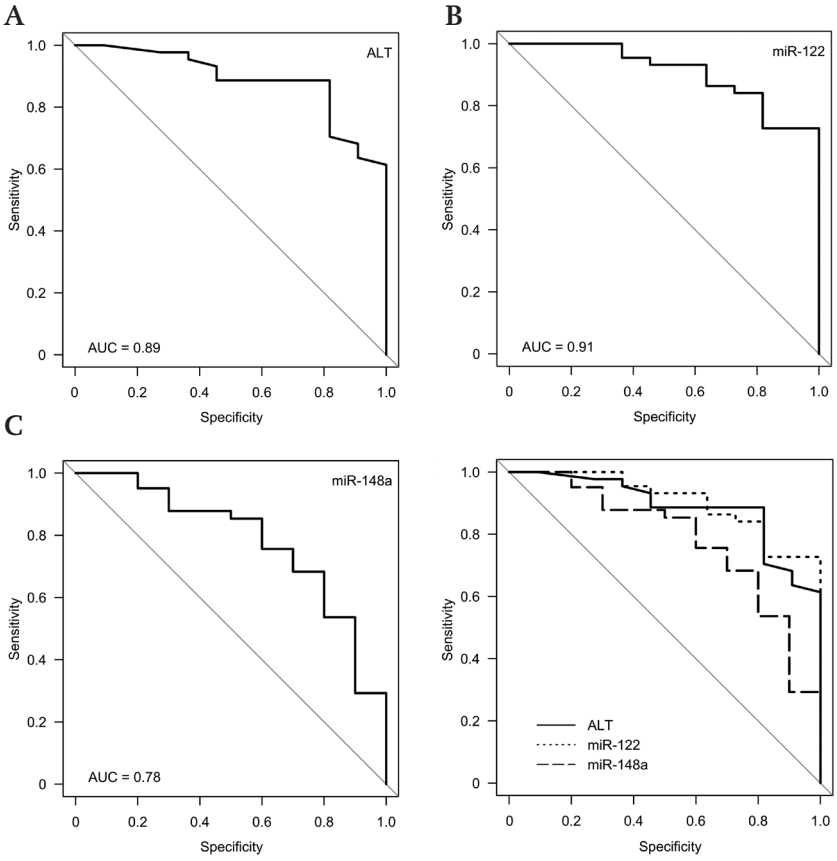

Figure 5

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.

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 important regulators of a variety of cellular processes.[10,25] 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.[13-17, 26-29] In a recent safety study for NP260, a selective antagonist of 4-subtype $GABA_A$ receptors, performed in Beagle dogs, acute hepatocellular necrosis occurred resulting in increased miR-122.[30]

In the present study, we analyzed 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 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, tumor suppression, and lipid metabolism.[31, 32] In addition, miR-122 regulates both plasma and liver iron values.[33] While the expression profile of miR148a is relatively non-organ specific, miR-122 has almost no expression in extra-hepatic tissues.[34, 35]

Due to the absence of clinical signs and the large reserve capacity of the liver, canine liver injury is usually diagnosed in the end stage of disease when severe liver damage is present. In this disease stage, dogs experience minimal benefit of therapeutic interventions and the prognosis in case of severe liver fibrosis is guarded.[2] Therefore, it is necessary to improve screening techniques for liver injury. The ideal biomarker should 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. [3, 36-38] 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 copper-induced hepatopathy.[39] Interestingly, human patients with chronic Hepatitis C virus infection and histological signs of mild to moderate hepatitis can present with normal ALT activity [40]. 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 levels remained unchanged. [13, 14]

One of the key findings of this study was the 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.[16,17] 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. [12, 14, 26, 41]

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 emphasizes 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 generally not 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 upon different forms of hepatocellular injury remains to be elucidated, studies have been performed to determine the characterization of extracellular mircoRNAs. 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. [42, 43] 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.[44] 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 this 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 apparent histological signs of liver injury. As HDmiRs are believed to have an important role in inter-cellular communication, [45, 46] 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 organizations that could inappropriately influence or bias the content of the paper.

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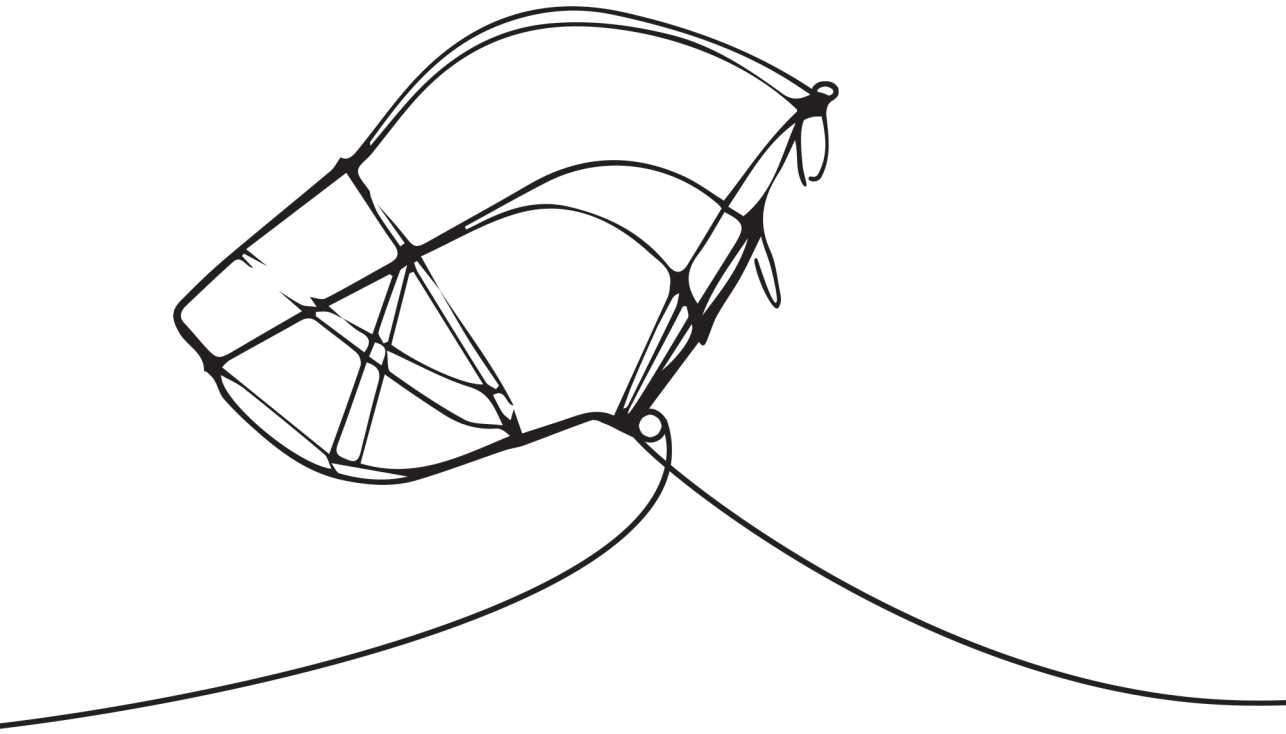

6
chapter

The potential of serum microRNAs as biomarker for various types of canine hepatobiliary diseases

Karen Dirksen[a]
Tim Verzijl[a]
Guy C.M. Grinwis[b]
Robert P. Favier[a]
Louis C. Penning[a]
Iwan A. Burgener[a]
Luc J.W. van der Laan[d]
Hille Fieten[a]
Bart Spee[a]

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[a]Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
[b]Department of Pathobiology, Utrecht University, Utrecht, The Netherlands
[c]Department of Gastroenterology and Hepatology, Erasmus MC: University Medical Center Rotterdam, the Netherlands

Abstract

Background: Current biochemical indicators cannot discriminate between parenchymal, biliary, vascular, or neoplastic hepatobiliary disease. MicroRNAs have been identified as promising new biomarkers for hepatobiliary disease in humans and dogs.

Objective: To measure serum levels of an established group of microRNAs in dogs and to investigate their levels in various types of hepatobiliary diseases.

Animals: Forty-six client-owned dogs with an established diagnosis of hepatobiliary disease and stored serum samples and eleven client-owned healthy control Labrador retrievers.

Methods: Retrospective study. Medical records of dogs with parenchymal, biliary, vascular, or neoplastic hepatobiliary diseases and control dogs from 1999 to 2014 were reviewed. Levels of miR-21, miR-122, miR-126, miR-148a, miR-200c, and miR-222 were quantified in serum using reverse transcriptase and real-time polymerase chain reaction.

Results: miR-122 and miR-21 were both associated with hepatobiliary disease in general, while miR-126 was uniquely upregulated in chronic hepatitis and miR-200c in hepatocellular carcinomas. No different microRNA levels were found in the adenoma and congenital portosystemic shunt groups. With a microRNA panel consisting of miR-21, miR-122, miR-126, miR-200c, and miR-222 it was possible to distinguish between parenchymal, biliary, and neoplastic hepatobiliary diseases. Within these classes, a differentiation between the subclasses acute/chronic hepatitis, adenomas/carcinomas/lymphomas, and mucoceles/other biliary diseases could be made.

Conclusions and Clinical Importance: Serum microRNA profiling is a promising new tool that might be a valuable addition to conventional diagnostics to help diagnose various hepatobiliary diseases in dogs.

Introduction

Hepatobiliary diseases are commonly encountered in dogs and can be divided into four main groups: parenchymal, biliary, vascular, or neoplastic diseases.[1] In many cases, clinical signs of hepatobiliary diseases are non-specific. To establish a tentative diagnosis, biochemical blood parameters are used as a first step in most cases. Several laboratory tests can be used to evaluate hepatocellular damage and hepatic function. The most commonly used biochemical indicators of hepatobiliary injury are alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT).[2] To confirm the existence of significant hepatic impairment, liver function tests can be performed, including measurements of bilirubin, bile acids (BA), and ammonia levels in blood.[3,4] With the exception of some vascular hepatic diseases (*i.e.* congenital portosystemic shunts),[5] biochemical indicators function at best establishing the presence of hepatobiliary disease but are usually not sufficient to specify the underlying disease.

A thorough and extensive diagnostic work-up including imaging techniques, cytology and culture of bile, and cytologic and histopathologic evaluation of liver biopsies is usually necessary to establish a definitive diagnosis.[1] A relatively non-invasive sensitive and specific blood-based biomarker profile that can differentiate between various types of hepatobiliary disease, can potentially restrict or specify follow-up tests and be a valuable addition in current diagnostic work-up protocols.

Mature microRNAs are a class of small non-coding RNAs that are important regulators of post-transcriptional gene expression.[6] With critical functions in the regulation of multiple aspects of hepatic development, microRNAs have recently emerged as promising and stable candidate biomarkers for a variety of hepatobiliary diseases in humans.[7-9] Several hepatocyte-derived microRNAs (HDmiRs) and cholangiocyte-derived microRNAs (CDmiRs) showed to be sensitive and stable candidate biomarkers in human patients with acute or chronic liver injury due to various etiologies, for example drug- or hepatitis C virus-induced liver injury.[10-12] Studies focusing on neoplastic diseases demonstrated different serum levels of several microRNAs in human patients with intrahepatic cholangiocarcinoma or hepatocellular carcinoma versus normal patients.[13-15] The first aim of the present exploratory study was to evaluate if the levels of a selected group of hepatocyte-derived, cholangiocyte-derived, and oncogenic microRNAs, based on the current human literature, was measurable in serum of dogs with parenchymal, biliary, vascular, or neoplastic hepatobiliary diseases. Our second aim was to explore the first steps in the use of a microRNA profile as biomarker for canine hepatobiliary diseases by providing an overview of the differences in their serum levels.

Materials and Methods

Animals

Medical records of dogs referred to the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, between 1999 and 2014 were reviewed. Data concerning signalment, physical examination, biochemical, ultrasonographic, cytologic, and histopathologic findings were retrieved from the medical records. At time of admission all dogs underwent physical examination, a biochemistry panel (including at least ALT, ALP and BA), and ultrasonographic examination of the hepatobiliary system. Of the four main groups of hepatic diseases, *i.e.* parenchymal, biliary, vascular, or neoplastic, eight subgroups were distinguished in this study. Parenchymal diseases included dogs with acute hepatitis (AH) or chronic hepatitis (CH). Biliary diseases were divided into mucoceles (MU) and dogs with cholangitis (lymphocytic or destructive) or extra-hepatic bile duct obstruction, the latter two termed "other biliary diseases" (BI). The vascular disease group solely contained dogs with congenital portosystemic shunts (CPSS). The neoplastic diseases group included dogs with hepatocellular adenomas (HCA), hepatocellular carcinomas (HCC), and hepatic lymphoma (L). The final diagnosis of AH, CH, BI, HCA, and HCC was established by the evaluation of a histological liver biopsy. The presence of L was established by histology or cytology. Diagnostic criteria for MU included the typical appearance of an enlarged gallbladder with immobile, echogenic bile with a striated or stellate pattern during ultrasonographic examination [16] and/or cases that were histologically confirmed after surgical removal of the gall bladder. The presence of CPSS was confirmed either on surgery or a CT-contrast study. All subgroups contained at least five confirmed cases. Control dogs (normal liver group, NL) were Labrador retrievers, selected from the database from an ongoing research program about copper-associated hepatitis of the Faculty of Veterinary Medicine, Utrecht University. [17] All control dogs were clinically healthy Labrador retrievers that underwent a liver biopsy for screening for copper-associated hepatitis. The control dogs had a normal biochemistry panel, unremarkable abdominal ultrasound, and absence of hepatic disease on histopathologic evaluation of a liver biopsy. Of all dogs included in the study clinical data as well as biological material (i.e serum samples) were stored until further analysis was done. Abdominal ultrasounds were performed by board certified radiologists. Histology was evaluated by a board certified veterinary pathologist (GCMG) according to the World Small Animal Veterinary Association standards and who was unaware of the results of the microRNA analysis at the time of histopathological evaluation. [1] All data were collected according to the Act on Veterinary Practice, as required under Dutch legislation and all procedures were known and approved by the Animal Welfare Body of the University of Utrecht.

RNA isolation

Serum samples obtained at the time of diagnostic work-up of the patients were stored at −20°*C* or −70°*C* until microRNA analysis. Total RNA was extracted from 100 µL serum with a miRNeasy Serum/Plasma kit,[a] following the manufacturer's instructions. 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[a] 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[a] after the addition of lysis reagent, prior to addition of chloroform and the phase separation.

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

The miScript II Reverse Transcription kit[a] was used to prepare cDNA according to the manufacturer's instructions. The obtained cDNA was diluted to a total volume of 200 µL. Based on the current human literature the serum levels of miR-122 and miR148a (HDmiRs in humans), miR-21 and miR-126 (oncogenic miRs in human), and miR-200c and miR-222 (CDmiRs in humans) were chosen to be measured in patients with various hepatic diseases. [18-23] RT-PCR was performed using the miScript SYBR Green PCR kit.[a] All PCRs were carried out in duplicate in a CFX-384 Real-Time PCR detection system.[b] Each reaction consisted of 5 µL 2 × QuantiTect SYBR Green PCR mastermix, 1 µL 10 × universal primer, 1 µL 10 × miR-specific primer[a] 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 levels of all microRNAs were quantified using absolute quantification via a standard curve, with quantities normalized to the spike-in control. [24]

Statistical analysis

Data were summarized as median and range for summary statistics of the study subjects. Linear regression was used with miRNA serum levels as dependent variables and diagnostic group as independent variables . Eight diseased groups (AH, CH, BI, MU, CPSS, HCA, HCC, and L) were included and the normal liver group was used as the reference category. The natural logarithm of the different microRNAs was taken to ensure validity of all models, which was checked by studying the residuals on normality and constant variance. P values were adjusted for multiple comparisons using the Benjamini-Hochberg correction. Data were analyzed using R statistics (version 3.1.2).[c]

Results

Patient characteristics

Serum samples of 57 dogs were analyzed (11 healthy, 46 with hepatic disease). Patient characteristics including sex, age, BA, and liver transaminases are summarized in Table 1. Dogs from the hepatobiliary disease groups consisted of crossbreeds (n=9), Labrador retrievers (n=6), English cocker spaniels (n=6), Golden retrievers (n=4), Cairn terriers (n=3), Maltese dogs (n=2), miniature pinschers (n=2), Scottish terriers (n=2), and one of each of the following breeds: Beagle, Bernese mountain dog, Bouvier des Flandres, Cavalier king Charles spaniel, Dobermann, German shepherd, Fox terrier, Munsterlander, Polish lowland sheepdog, Shetland sheepdog, Shih Tzu, and Tibetan terrier.

Table 1 Patient characteristics.

	Age (years), median and range	**Sex (F, M)**	**ALT (U/L), median and range (ref < 70 U/L)**	**ALP (U/L), median and range (ref < 89 U/L)**	**BA (μmol/L), median and range (ref < 10 μmol/L)**
NL (n=11)	5.4 (3.6 – 7.3)	8F, 3M	38 (23 - 64)	34 (14 - 94)	1 (0 – 6)
AH (n=6)	7.3 (5.5 – 14.5)	5F, 1M	233 (54 – 845)	178 (29 – 1,920)	9 (1 – 153)
CH (n=6)	6.4 (3.3 – 11.7)	5F, 1M	268 (29 – 2,258)	494 (23 – 1,200)	15 (2 – 112)
MU (n=5)	8.2 (2.6 – 13.0)	4F, 1M	2,000 (823 – 4,590)	3,094 (1,019 – 8,305)	528 (62 – 660)
BI (n=6)	9.2 (5.8 – 13.2)	4F, 2M	933 (408 – 2,700)	2,995 (208 – 3,850)	432 (20 – 1,605)
CPSS (n=5)	0.7 (0.3 -1.6)	2F, 3M	83 (22 – 225)	112 (103 – 183)	79 (19 – 221)
HCA (n=6)	11.8 (6.7 – 15.2)	3F, 3M	837 (85 – 1,556)	1,548 (354 – 7,390)	24 (6 – 118)
HCC (n=6)	9.2 (4.8 - 11.1)	3F, 3M	467 (42 – 1,300)	943 (29 – 4,175)	55 (5 – 415)
L (n=6)	8.5 (5.4 – 9.9)	2F, 4M	338 (179 – 894)	1,375 (325 – 4,625)	67 (11 – 555)

AH, acute hepatitis; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BA, bile acids; BI, Other biliary diseases (cholangitis or extra-hepatic bile duct obstruction); CH, chronic hepatitis; CPSS, congenital portosystemic shunts; F, female; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma; L, lymphoma; M, male; MU, mucoceles; NL, normal liver; ref, reference.

MicroRNA serum levels in dogs with hepatic disease

Fold changes, confidence intervals and P values of microRNA levels in dogs with various hepatobiliary diseases relative to microRNA levels in the normal liver group are summarized in Supplementary Table 1. Except for miR-148a, all microRNAs were higher in one or more disease groups (Fig 1, Fig 2) compared to the normal group. In dogs with HCA and CPSS, none of the tested microRNAs were higher compared to the normal liver group. MicroRNA-122 levels were increased in all parenchymal diseases (AH, CH), biliary diseases (MU, BI), and two neoplastic diseases (HCC, L) compared to controls. Except for the HCC group, miR-122 was the highest microRNA in each disease group, with a 267-fold upregulation in the MU group compared to control.

The second most often increased microRNA was miR-21. Compared to dogs with normal livers, miR-21 levels were increased 26, 19, 21, and 10-fold in the MU, CH, HCC, and L groups respectively. In dogs with biliary diseases miR-21 was only increased in dogs with MU, as dogs with BI did not have increased miR-21 levels. In dogs with parenchymal diseases miR-21 was only increased in dogs with CH and not in dogs with AH. MicroRNA-222 was increased 13 and 9-fold in dogs with MU and HCC compared to normal dogs. Both miR-200c and miR-126 were uniquely increased in one disease group. From all microRNAs increased in the HCC group, miR-200c had the highest levels, with a 35-fold increase compared to the normal liver group. With a 22-fold increase, miR-126 was only significantly increased in the CH group.

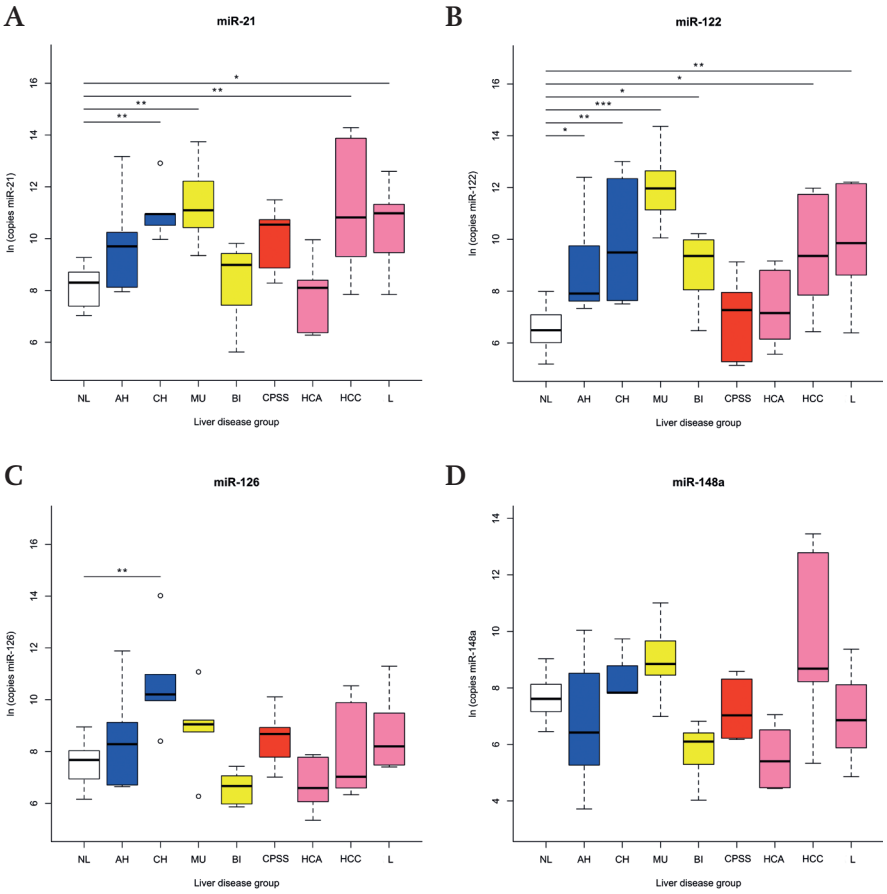
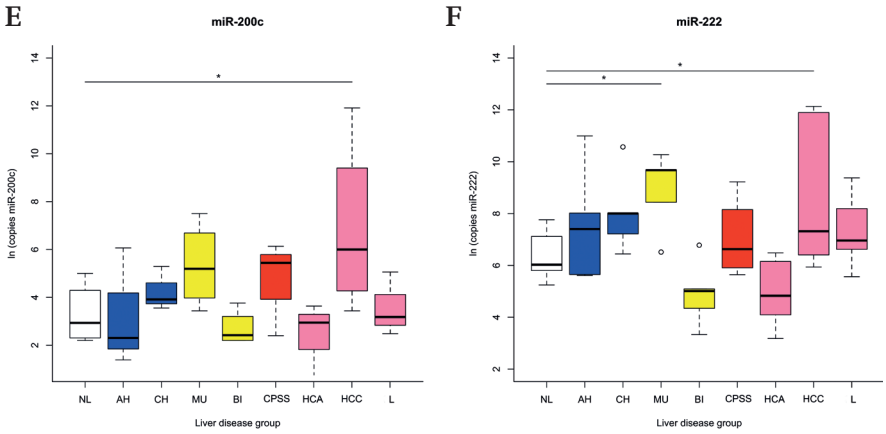


Figure 1 MicroRNA levels in dogs with various hepatobiliary diseases.

MicroRNA levels in dogs with normal livers (white) and in dogs with parenchymal (blue), biliary (yellow), vascular (red), or neoplastic (pink) hepatobiliary disease.(**A**) miR-21. (**B**) miR-122. (**C**) miR-126. (**D**) miR-148a (**E**) miR-200c. (**F**) miR-222. Significant differences between groups of hepatobiliary diseases and the normal liver group are marked with stars (*P<0.05, ** P< 0.01, ***P<0.001). AH, acute hepatitis (n=6); BI, Other biliary diseases (cholangitis or extra-hepatic bile duct obstruction, n=6); CH, chronic hepatitis (n=6); CPSS, congenital portosystemic shunts (n=5); HCA, hepatocellular adenoma (n=6); HCC, hepatocellular carcinoma (n=6); L, lymphoma (n=6); Ln, natural logarithm; MU, mucoceles (n=5); NL, normal liver (n=11).



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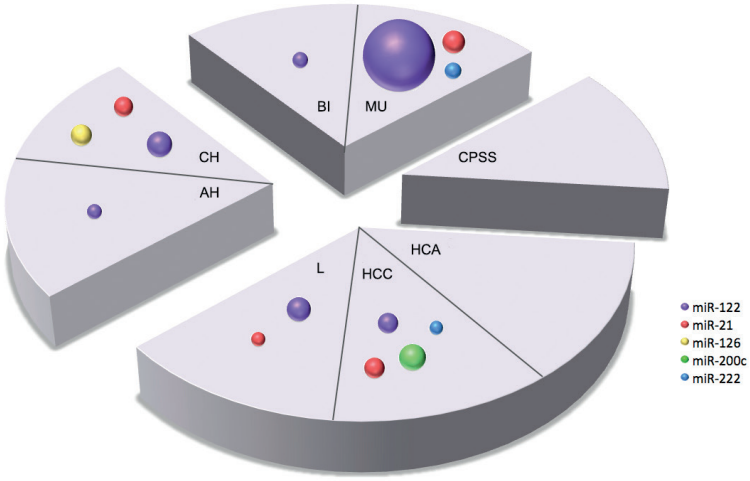

Figure 2 Diseased-based microRNA profile.

Disease-based microRNA profile. Colored circles indicate relative increase in microRNA levels compared to the normal liver group (n=11). Pie charts indicate main groups of hepatic disease (parenchymal, biliary, vascular, or neoplastic diseases). Dogs with mucoceles (MU, n=5), hepatocellular carcinomas (HCC, n=6), lymphomas (L, n=6) and chronic hepatitis (CH, n=6) all have their own unique microRNA profile. No microRNA increase is seen in dogs with adenomas (HCA, n=6) and congenital portosystemic shunts (CPSS, n=5). AH, acute hepatitis (n=6); BI, Other biliary diseases (cholangitis or extra-hepatic bile duct obstruction, n=6).

Discussion

Results of this study demonstrate that a panel of different microRNAs in dogs with hepatobiliary disease could be possibly used in the future as a diagnostic marker for different hepatobiliary diseases in dogs. Current biochemical blood parameters of hepatobiliary injury and dysfunction cannot discriminate between most hepatobiliary diseases. [4, 25] The gold standard to differentiate between these diseases is histopathologic evaluation of liver biopsy specimens after ultrasonographic examination of the liver and biliary tract system. [1] It would be of great value to use a relative non-invasive biomarker to characterize the type of hepatobiliary disease. MicroRNAs are emerging as biomarkers for hepatic disease because they play critical roles in liver development and metabolism and are measurable in blood even after prolonged storage. [7, 21, 26] MicroRNAs are already evaluated as promising biomarkers for hepatobiliary disease, including primary hepatic tumors, in humans.[8, 9] In dogs, serum HDmiR-122 and HDmiR-148a have shown to be increased upon hepatocellular injury.[27, 28] In the present study, we demonstrated different levels of our selected microRNA panel in dogs with several parenchymal, biliary, and neoplastic diseases. This is the first evidence that a microRNA panel might be a valuable extra diagnostic tool, helping in diagnosing and differentiating several common hepatobiliary diseases in dogs.

One of the diseases evaluated in this study was congenital portosystemic shunts. None of the microRNA showed significantly increased levels in this group. Several studies in man and dogs demonstrated an increased in serum miR-122 upon hepatocellular injury. [18, 21, 27, 28] One can presume that in the case of congenital portosystemic shunting there is not enough hepatocellular injury to give a rise in microRNA levels. Of all common hepatic diseases, this is the only disease of which a presumptive diagnosis can be made based on signalment (age, breed), clinical signs and an ammonia tolerance test, fasting plasma ammonia, fasting plasma bile acids, or bile acid stimulation test. [5, 29] Therefore, microRNA profiling seems to be of no added value in this group of diseases.

In dogs with hepatobiliary disease, the liver parenchyma, gallbladder, and biliary tree can be further evaluated with ultrasonography. However, in 20% of dogs with a primary hepatitis no abnormalities are found on abdominal ultrasound.[30] Our previous study reported miR-122 to have a sensitivity of 84% and a specificity of 82% in detecting dogs with hepatocellular injury. [28] Results of this study indeed demonstrate an increase of miR-122 in both dogs with acute and chronic hepatitis. Although recent studies in humans [21] and dogs [28] also showed an increase of miR-148a after hepatocellular injury, no significant difference in serum levels was seen in the present study after correction for multiple testing.

In dogs with neoplastic disease, ultrasonographic examination of the liver can identify nodules or mass lesions. However, it is often not possible to distinguish between hepatocellular adenoma or carcinoma or between neoplasms and nodular hyperplasia or cirrhosis.[31] In case of hepatic lymphoma, the liver can appear ultrasonographically unremarkable or hypoechoic and diffusely enlarged. [31, 32] This emphasizes the importance of a non-invasive microRNA panel that is able to differentiate between causes of hepatic lesions. Several studies in human patients state that single serum measurements of miR-21, miR-122, and miR-222 could not differ between hepatocellular carcinoma and hepatitis, [33-35] and only one study reported that miR-21 was higher in human patients with hepatocellular carcinoma compared to patients with hepatitis.[13] However in our study we showed that different microRNA levels were increased in dogs with hepatitis and hepatocellular lymphomas and carcinomas. This suggests that a panel consisting of miR-21, miR-122, miR-126, miR-200c, and miR-222 are possible candidates for a biomarker panel in the discrimination of AH, CH, HCA, HCC, and L in dogs.

One of the most important findings of this study were the increased serum levels of miR-200c, miR-21, miR-222, and miR-122 in dogs with HCC. All of these microRNAs are associated with several (human) cancer types, especially miR-21 and miR-200c. The miR-200 family is known to be a powerful regulator in epithelial-to-mesenchymal transition as occurs in embryogenesis, carcinogenesis, and remodeling responses of adult (liver) tissue after damage.[36-38] In our study, miR-200c was only found to be increased in the HCC group compared to controls. MicroRNA-21 is suggested to contribute to a malignant phenotype by exerting both anti-apoptotic [39] and tumor disseminating properties.[19, 40] Although tissue miR-126 is thought to play critical roles in several human cancers as well,[23] we did not see any difference in serum miR-126 between dogs with liver cancer and control dogs. This might be due to a difference between tissue expression and serum presence of miR-126, as serum microRNA release does not necessarily correlate to tissue microRNA expression.[41] Several studies examined serum levels of miR-21, miR-122, and miR-222 in human patients with HCC, with similar results as our study in dogs with HCC. Four studies identified an increase in serum miR-21 in patients with HCC, [13, 33, 35, 42] which is also consistent with our results, as dogs with HCC and L had an increase in miR-21. In addition, human patients with HCC were found to have an increase in serum miR-122 [33, 34] and miR-222[34, 42] as well. Interestingly, in our study, none of these microRNAs were differentially expressed in dogs with HCA, and only miR-21 and miR-122 were increased in dogs with L. The upregulation of miR-21, miR-122, miR-222, and miR-200c in dogs with L and/or HCC compared to controls suggests that these microRNAs can be used as a potential biomarker for neoplastic liver disease in dogs.

Dogs with biliary diseases usually have a marked increase in liver enzymes, especially ALP, GGT, and high BA (Table 1).[4] A future microRNA panel could help in further differentiating underlying disease, making oriented ultrasonographic examination of the hepatobiliary tree possible. In our study, all dogs with biliary tract diseases had high serum miR-122. This can be explained by hepatocellular damage as a result of cholestasis, and the subsequent release of miR-122 into serum. Furthermore, we selected miR-200c and miR-222 as CDmiRs and therefore expected them to be increased in dogs with biliary diseases. Only one of them, miR-222, was increased in serum of dogs with MU. An interesting theory that could explain this difference, might be the polarized release of CDmiRs and HDmiRs by cholangiocytes and hepatocytes. This was recently investigated in bile and serum samples of human patients.[18] Upon impaired liver function and liver injury, cholangiocytes release their CDmiRs into bile rather than blood, resulting in decreased serum concentrations. Concurrently, serum HDmiR-148a and HDmiR-122 levels were observed to increase upon liver cell injury and their secretion into bile decreased. That there are such striking differences between the MU and BI groups is intriguing. A gallbladder mucocele is an inappropriate accumulation of bile-laden mucus material occupying the gallbladder lumen. One explanation of higher microRNA levels in dogs with mucoceles can be the higher amount of necrosis of the gallbladder wall and intrahepatic bile duct epithelium and thus increased release of microRNAs into serum in dogs with mucoceles.[16, 43] Another assumption of the increased miR-21, miR-122, and miR-222 serum levels, is based on one of the most important histologic features of gallbladder mucoceles: hyperplasia of the mucus-secreting glands.[16, 44, 45] All three microRNAs are known to influence cellular proliferation, cell growth, cell cycle progression, and apoptosis, which are common features of hyperplasia.[19, 46, 47] Different levels of miR-21, miR-122, and miR-222 in the two biliary disease groups compared to the control group, again indicate that further investigations into these microRNAs in dogs holds potential for future biomarker development.

The results of this study are a promising new step in the development of a biomarker profile for the evaluation of hepatobiliary disease in dogs. Despite the unarguable potential of circulating microRNAs to act as biomarker, several points need to be addressed. To date, even in the human literature there is no consensus about the quantification and normalization (reference microRNAs or spike-in controls) of circulating microRNA levels.[48] In the veterinary field, microRNA expression studies are scarce and further studies into detection and quantification of microRNAs have to be conducted. In the present study all control dogs were Labrador retrievers. As the research of microRNAs in dogs is limited, there is nothing known about breed, sex or age related differences of microRNA expression

in dogs. Because of the highly conserved nature of microRNAs between species with similar physiology [49] we do not expect microRNA levels to differ between breeds. In humans, microRNA levels have proven to be stable under low storage conditions [50, 51] and over a long period of time [52], resistant and reproducible and for most microRNAs there is no difference in expression between female or male individuals. [53] Although age related differences in humans are described, these are mainly found comparing healthy adults with healthy octogenarians, nonagenarians or centenarians. [54, 55] Dogs in the present study are, with exception of dogs with congenital portosystemic shunting, of comparable age and age-related effects in microRNA levels are therefore considered negligible. In addition, group size in the present study was small and to determine test performance of this microRNA profile, this study should be extended with larger cohorts, and possibly more microRNA markers. Furthermore, only HDmiR-122 has shown to be liver specific as it accounts for 72% of all liver microRNAs with almost no extra-hepatic expression. [22, 56] Because all other microRNAs lack specificity for the hepatobiliary tree, dogs suffering from extra-hepatic diseases, including metastatic liver disease and dogs with non-specific reactive hepatitis, should be included in subsequent studies to indicate specificity of this microRNA panel. In the present study, corresponding tissue microRNA expression was not measured. At the moment the relationship between tissue and serum microRNAs remains unclear, justifying the need for further investigations into tissue microRNA expression and release. Based on the study of Verhoeven et al (2016), which indicates notable amounts of microRNAs in bile, [18] more research into the combination of serum and biliary microRNAs as biomarker for hepatic disease is warranted.

In conclusion, we propose that a serum microRNA might be used as a diagnostic marker for hepatobiliary diseases in dogs and therefore can be a promising and valuable addition to the currently available diagnostic tools. Therefore, further studies are needed to confirm these findings and to determine corresponding sensitivity and specificity. In addition, further studies into the release of microRNAs in serum and bile and the correlation with expression in liver tissue are warranted to shed light on the role of microRNAs in hepatic disease and their usefulness as relative non-invasive biomarkers in dogs.

Supplementary material

Supplementary Table 1 Increase in microRNA levels in dogs with different hepatobiliary diseases compared to control dogs.

	miR-21	miR-122	miR-126	miR-148a	miR-200c	miR-222
AH (n=6)	6 (CI: 1-27) *P*=0.095	**11 (CI: 2–63) *P*=0.041**	3 (CI: 0.6-11) *P*=0.329	0.4 (CI: 0-2) *P*=0.419	1 (CI: 0.1-13) *P*=0.985	3 (CI: 0.6-16) *P*=0.285
CH (n=6)	**19 (CI: 4-103) *P*=0.008**	**32 (CI: 5-190) *P*=0.006**	**22 (CI: 5-91) *P*=0.002**	2 (CI: 0.3-20) *P*=0.609	3 (CI: 0.2-36) *P*=0.610	5 (CI: 1-29) *P*=0.133
MU (n=5)	**26 (CI: 5-141) *P*=0.005**	**267 (CI: 40-1,768) *P*<0.001**	4 (CI: 0.8-17) *P*=0.182	4 (CI: 0.6-23) *P*=0.268	8 (CI: 0.7-85) *P*=0.208	**13 (CI: 2-70) *P*=0.025**
BI (n=6)	1 (CI: 0.3-6) *P*=0.776	**12 (CI: 2-69) *P*=0.037**	0.4 (CI:0.1-2) *P*=0.336	0.2 (CI: 0.0.9) *P*=0.099	0.6 (CI: 0-6) *P*=0.713	0.2 (CI: 0-1) *P*=0.184
CPSS (n=5)	7 (CI: 1-35) *P*=0.095	2 (CI: 0.3-11) *P*=0.706	3 (CI: 0.6-12) *P*=0.329	0.7 (CI: 0.1-4) *P*=0.745	5 (CI: 0.4-53) *P*=0.342	2 (CI: 0.3-12) *P*=0.598
HCA (n=6)	0.8 (CI: 0.2-4) P=0.811	2 (CI: 0.4-14) *P*=0.460	0.4 (CI: 0.1-2) *P*=0.380	0.1 (CI: 0-0.7) *P*=0.061	0.4 (CI: 0-6) *P*=0.635	0.2 (CI: 0-1) *P*=0.193
HCC (n=6)	**21 (CI: 4-104) *P*=0.004**	**20 (CI: 3-120) *P*=0.011**	1 (CI: 0.4-6) *P*=0.670	7 (CI: 1-36) *P*=0.095	**35 (CI: 3-382) *P*=0.035**	**9 (CI: 2-42) *P*=0.041**
L (n=6)	**10 (CI: 2-56) *P*=0.038**	**30 (CI: 5-177) *P*=0.004**	3 (CI: 0.8-13) *P*=0.230	0.5 (CI: 0-3) *P*=0.621	1 (CI: 0.1-13) *P*=0.843	3 (CI: 0.5-14) *P*=0.388

Statistical significant results after correction for multiple testing ($P<0.05$) are depicted in bold. AH, acute hepatitis; BI, Other biliary diseases (cholangitis or extra-hepatic bile duct obstruction); CH, chronic hepatitis; CI, 95% confidence interval; CPSS, congenital portosystemic shunts; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma; L, lymphoma; MU, mucoceles

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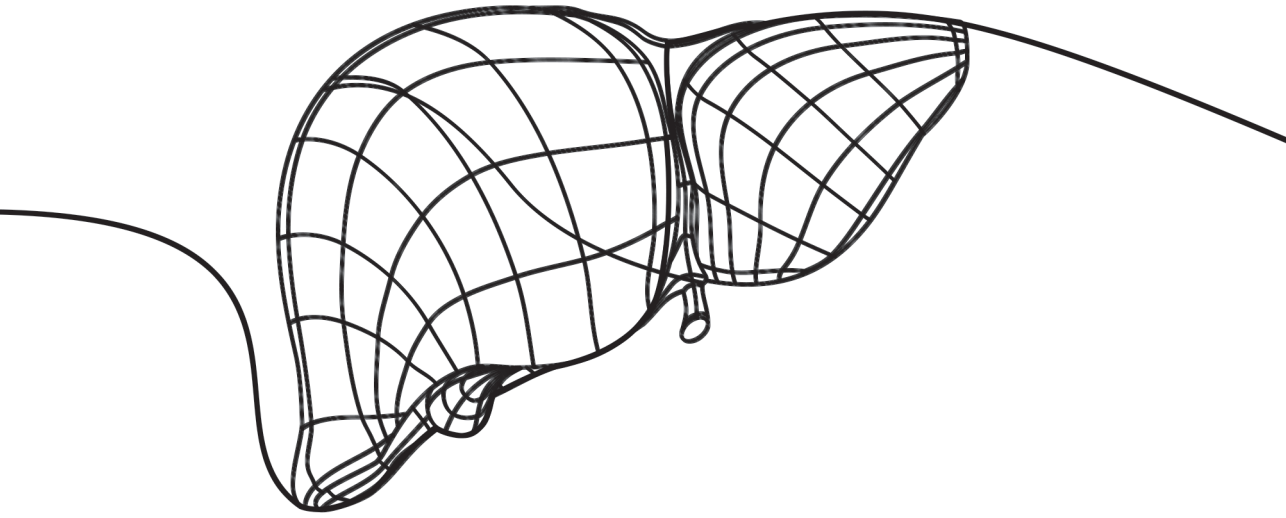
Footnotes

[a] Qiagen, Valencia, CA, USA
[b] Bio-Rad, Veenendaal, The Netherlands
[c] R Foundation for Statistical Computing, Vienna, Austria 2014



7
chapter

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

Karen Dirksen [a]
Yara S. Roelen [a]
Monique E. van Wolferen [a]
Hedwig S. Kruitwagen [a]
Louis C. Penning [a]
Iwan A. Burgener [a]
Bart Spee [a]
Hille Fieten [a]

Accepted, Vet J

[a]*Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands*

Abstract

Copper toxicosis is a hereditary disease in several dog breeds, including the Labrador retriever. It is characterized by hepatic copper accumulation leading to hepatitis with fibrosis and eventually cirrhosis. Currently, the only way to establish the diagnosis is by means of an invasive liver biopsy procedure followed by histologic assessment and additional quantitative determination of hepatic copper concentrations. 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 levels during treatment. Two cellular copper metabolism proteins, Cu/Zn superoxide dismutase (SOD1) and its chaperone CCS (Copper Chaperone for SOD1) are both biomarkers for hepatic copper status in humans and rodents with copper deficiency or copper overload.

In the present study, erythrocyte derived CCS and SOD1 protein levels were tested for association with hepatic copper concentrations in 15 Labrador retrievers with normal or increased hepatic copper concentration. Antibodies against CCS and SOD1 proved to be applicable for use in canine samples. This was demonstrated by loss of immune-reactive bands for CCS and SOD1 in siRNA treated canine bile duct epithelial cells. Erythrocyte CCS and CCS/SOD1 ratio were 2.37 ($P<0.001$) and 3.29 ($P<0.001$) fold decreased in the high copper group compared to the normal copper group. Erythrocyte CCS and CCS/SOD1 ratio showed to be potential new biomarkers for hepatic copper concentrations in the Labrador retriever and could facilitate early diagnosis and treatment monitoring for copper-associated hepatitis in dogs.

Introduction

Canine copper toxicosis is characterized by gradual copper accumulation in the centrolobular regions of the liver and is described in several dog breeds such as the Bedlington terrier[1], Skye terrier[2], West Highland White terrier[3], Dalmatian[4], Doberman[5], and Labrador retriever[6]. In the Bedlington terrier, the disease is autosomal recessive and caused by a deletion of exon 2 of the *COMMD1* gene.[7] In the Labrador retriever, a missense mutation in the Wilson disease gene *ATP7B*[8] is associated with high hepatic copper concentrations, which can be attenuated by a concurrent missense mutation in the Menkes disease gene *ATP7A*.[9, 10] In addition, dietary uptake of copper and zinc are involved in progression of the disease.[11] In other dog breeds, the genetic background has not yet been elucidated.

Copper concentrations in affected Labrador retrievers can be as high as 4,000-5,000 mg/kg dry weight liver (dwl)[12, 13], leading to hepatocellular injury and eventually fibrosis and cirrhosis. Clinical symptoms usually become apparent late in disease, when severe liver damage is already present. Treatment with copper chelator D-penicillamine[14] and a low-copper high-zinc diet have shown to be effective in reducing hepatic copper concentrations.[15, 16] Treatment is most effective in an early stage of disease when liver damage is limited. In both treatment strategies, follow-up liver biopsies are needed to evaluate treatment effect.

Serum levels of liver enzymes, including alanine aminotransferase and alkaline phosphatase, can indicate the presence of hepatocellular injury but do not correlate with hepatic copper concentrations.[15, 17] Currently, the only way to establish the diagnosis of copper toxicosis and to monitor treatment effect is by liver histology and additional determination of hepatic copper concentration. A biomarker in blood or urine that correlates with hepatic copper concentration could help to identify dogs at risk in order to institute early treatment and possibly prevent clinical illness. Furthermore, such a biomarker would facilitate longitudinal monitoring. Recently, copper/zinc ratio were shown to correlate with hepatic copper concentrations in Labrador retrievers. However, there was an overlap between dogs with normal and increased hepatic copper concentrations and copper/zinc ratio is therefore less useful as a biomarker.[18]

Because excessive copper is toxic, intracellular copper concentrations are tightly regulated.[19] After copper uptake in the hepatocyte, copper is bound by specialized copper chaperones that shuttle copper to its destination molecules. One of these chaperones is the copper chaperone for Cu/Zn superoxide dismutase (CCS).[20] It delivers copper to Cu/Zn superoxide dismutase (SOD1), a protein protecting

against oxidative stress by metabolizing superoxide radicals.[21] SOD1 and CCS have both been studied as biomarkers for hepatic copper status in animals with copper deficiency and overload. In copper deficient rodents, a decrease of SOD1 protein levels and activity and high CCS protein levels were found.[22, 23] Conversely, erythrocyte CCS concentrations were reduced in rats with hepatic copper accumulation.[24]

The first aim of the present study was to evaluate canine specificity of polyclonal antibodies against human SOD1 and CCS proteins. The second aim of this study was to evaluate erythrocyte SOD1 (eSOD1) and erythrocyte CCS (eCCS) protein levels as biomarker for hepatic copper concentrations in Labrador retrievers. Hereto, the association between eSOD1 and eCCS and hepatic copper concentrations in 15 Labrador retrievers was investigated.

Materials and Methods

Animals

Medical records of 22 Labrador retrievers (nine males, 13 females) admitted to the Department of Clinical Sciences of Companion Animals of the University of Utrecht between 2011 and 2015 were retrospectively reviewed. They were either referred, because of increased liver enzymes or clinical illness, or they were actively recruited clinically healthy family members of dogs affected with copper-associated hepatitis. Clinically healthy family members participated in the ongoing research program into copper-associated hepatitis of the Faculty of Veterinary Medicine, Utrecht University. Data concerning signalment, histopathologic findings of liver biopsies, and hepatic quantitative copper concentrations were identified from the medical records. All data was collected according to the Act on Veterinary Practice, as required under Dutch legislation. 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.

To determine the correlation between hepatic SOD1 and CCS protein levels with hepatic copper concentrations and eSOD1 and eCCS, initially 15 patient samples were selected. To determine the correlation between hepatic copper concentrations and eSOD1 and eCCS, again 15 patient samples were used. Seven of these were new samples of dogs that were presented with copper-associated hepatitis during the course of the study. These dogs had a wider distribution of hepatic copper concentrations and were included as independent replication cohort to validate the correlation between hepatic copper and SOD and CCS as biomarkers.

Sample collection

Liver tissue was collected with a 14G needle under ultrasound guidance. Biopsy specimen were fixed in 4% neutral buffered formalin, embedded in paraffin, and stained with rubeanic acid to evaluate if the distribution of copper was centrolobular. A separate biopsy specimen of minimally 5 mg was freeze dried prior to quantitative copper determination by instrumental neutron activation analysis.[25] Dogs were considered to have normal hepatic copper concentrations if concentrations were below 400mg/kg dwl.[26] 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 samples were taken for the isolation of erythrocytes. EDTA samples were washed three times with PBS and centrifuged at 2,500 rpm for 5 min and the pellet was stored at -70 °C until protein isolation.

Cell-lines

Canine bile duct epithelial (BDE) cells were acquired from the Amsterdam Medical Center [27] and human hepatocellular carcinoma (HepG2) cells were derived from American Type Culture Collection. Both cell-lines were cultured as described previously[28, 29]. HepG2 cells were seeded at a concentration of 33,300 cells/cm^2 prior to transfection. BDE cells were seeded at a concentration of 5,000 cells/cm^2 prior to transfection.

Establishment of SOD1 and CCS knockdown

For silencing experiments, specific Stealth dsRNA molecules (Invitrogen) 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 with the Magnet Assisted Transfection (MATra) technique (IBA, BioTAGnology) in combination with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. In short, 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 (Invitrogen) according to the manufacturer's instructions. In short, complexes of 10 nM siRNA and 3 μL/mL Lipofectamine RNAiMAX were prepared inside the wells. After an incubation period of 20 min at room temperature, cells and antibiotic-free medium were added. 24 h 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 6-well plates for protein isolation. Knockdown was calculated as percentage relative to expression in the NT.

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

CCS, Copper Chaperone for Cu/Zn superoxide dismutase; SOD1, Cu/Zn superoxide dismutase 1.

RNA isolation

Total RNA was isolated for each group (SOD1 siRNA, CCS siRNA, and NT) at day two, three, four, and seven after transfection using iScript RT-qPCR Sample Preparation Reagent (Bio-Rad) according to the manufacturer's instructions. Subsequently, cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

Quantitative measurements of mRNA levels after transfection

Quantitative real-time polymerase chain reaction was performed as described previously.[30] Accurate quantification including three endogenous reference genes (ribosomal protein S19, beta-2 microglobulin, ribosomal protein S5) was based on the MIQE-precise guidelines.[31]

Western blot analysis

Proteins from BDE and HepG2 cells were isolated at day two, three, four, and seven after transfection in 350 μL 1x RIPA buffer containing 1% v/v Igepal, 1 mM Phenyl-MethylSulfonyl Fluoride, 1 μg/mL aprotinine, and 1 mM sodium orthovanadate (Sigma). Liver tissue and erythrocyte samples were homogenized and re-suspended in equal amounts of 2x 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 as well as BDE and HepG2 cells were separated over 15% Tris-HCl polyacrylamide gels (Bio-Rad) and transferred onto Hybond-C Extra Nitrocellulose membranes (Amersham Biosciences). 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 with a rabbit polyclonal antibody against CCS (FL-274, Santa Cruz) and SOD1 (FL-154, Santa Cruz) in a dilution of 1:1,000 in TBS-Tween with 4% w/v BSA overnight at 4°C. After washing with TBS-Tween (0.1% v/v), membranes were

incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (RD systems) at a 1:5,000 dilution in TBS with 4% w/v BSA for 1 h at room temperature. As a loading control, an anti-beta-actin (Pan ab-5, Neomarkers, 1:2,000 dilution) or an anti-GAPDH (Sigma, 1:1,000 dilution) antibody was used. After washing with TBS-Tween (0.1% v/v), the ECL Western blot analysis system was used according to the manufacturer's instructions (Amersham Biosciences Europe). 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 normalized to beta-actin (BDE, HepG2) or GAPDH (liver, erythrocytes).

Statistical analyses

Associations between hepatic SOD1 and CCS protein levels and hepatic copper concentrations and eSOD1 and eCCS were analyzed using the Spearman's rank correlation. Relation between eSOD1, eCCS protein levels 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 analyzed as covariates. Quantitative copper was in transformed to guarantee validity of the model, after studying the residuals on normality and constant variance. For all tests, a significance level of 0.05 was used. All data were analyzed 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. Antibody against SOD1 detected a single immune-reactive band of approximately 16 kDa in canine samples (erythrocytes, BDE cells (data not shown), liver tissue) and a band of 18 kDa in HepG2 cells (Fig 1A). For CCS, Western blotting 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 four days after transfection in HepG2 cells (Fig 2A) and three 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 four 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 three for HepG2 cells (Fig 2C) and at day two 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 of CCS was achieved in both HepG2 cells (Fig 2C) and BDE cells (Fig 2D) at day seven and four, respectively.

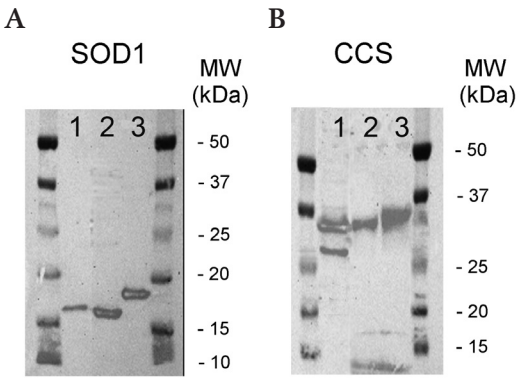


Figure 1 Immune-reactive bands for SOD1 and CCS.

Immune-reactive bands for SOD1 (**A**) and CCS (**B**) in erythrocytes, canine liver and HepG2 cell lines. Western blot detected bands for SOD1 at 16 kDa in canine erythrocytes, liver, and BDE cells (latter not shown) and at 18 kDa in HepG2 cells. For CCS bands at 35 kDa were detected in HepG2 cells and canine 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. BDE, bile duct epithelial cells; CCS, Copper Chaperone for Cu/Zn superoxide dismutase; HepG2, human hepatocellular carcinoma cells; MW, molecular weight; SOD1, Cu/Zn superoxide dismutase 1.

Correlation of hepatic SOD1 and CCS protein levels 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). These dogs had a median hepatic copper concentration of 446 mg/kg dwl (range 28-1270 mg/kg dwl). eCCS levels were significantly positively correlated with hepatic CCS levels (r=0.68, P=0.0095; Fig 3A), while eSOD1 levels were less strongly correlated with hepatic SOD1 levels (r=0.51, P=0.054; Fig 3B). There was a negative correlation between hepatic copper concentration and hepatic CCS protein levels (r=0.63, P=0.018). No correlation was found between hepatic copper concentration and hepatic SOD1 protein levels (data not shown).

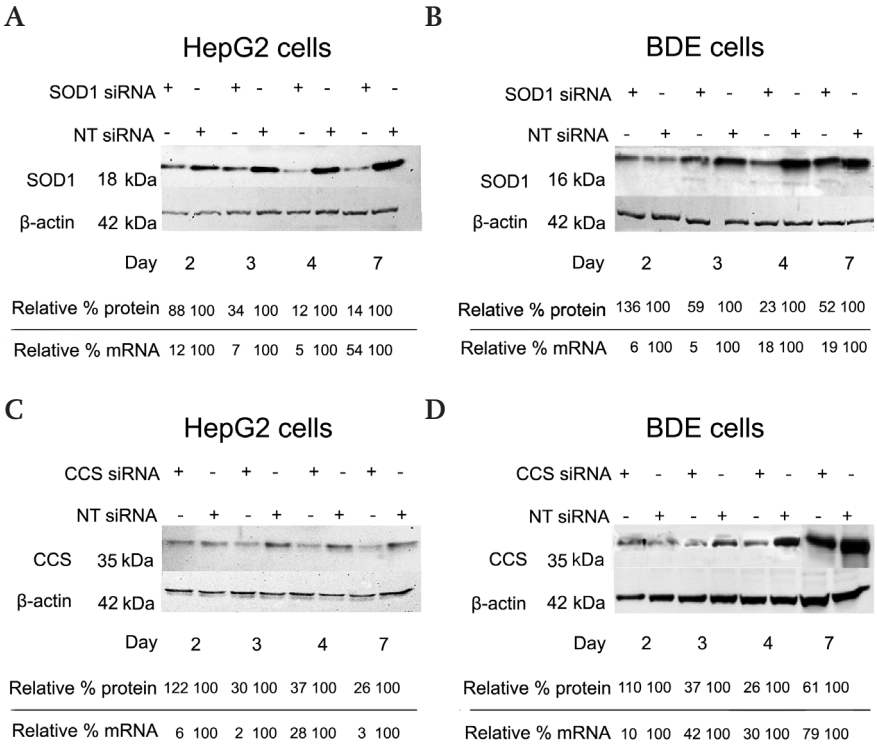


Figure 2 siRNA mediated knockdown of SOD1 and CCS in HepG2 and BDE cells.

(**A**) SOD1 in HepG2 cells. (**B**) SOD1 in BDE cells. (**C**) CCS in HepG2 cells. (**D**) CCS in BDE cells. Immune-reactive bands on Westernblot at day two, three, four, and seven post transfection are shown. β-actin was used as a loading control. In the bottom line, the relative protein and mRNA levels (in percentage) are depicted. BDE, bile duct epithelial cells; CCS, Copper Chaperone for Cu/Zn superoxide dismutase; HepG2, hepatocellular carcinoma cells; NT, non-target control; MW, molecular weight; SOD1, Cu/Zn superoxide dismutase 1.

eSOD1 and eCCS levels 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) 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), had increased hepatic copper concentrations (median 738 mg/kg/dwl, range 482 – 1,445 mg/kg dwl). For neither of the three models with eCCS, eSOD1, and eCCS/eSOD1 ratio as independent variables, sex and age had a significant

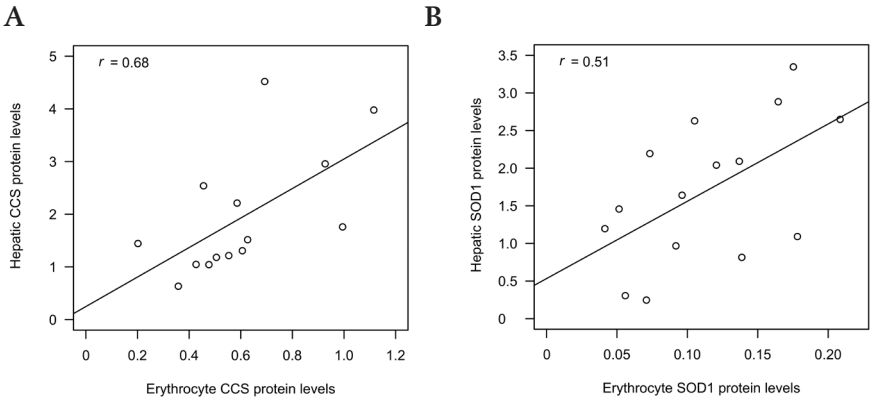


Figure 3 Correlation between hepatic and erythrocyte protein levels.

The correlation between hepatic and erythrocyte CCS (**A**) and SOD1 (**B**) protein levels. There is a significant (*P*=0.0095) correlation between hepatic CCS and erythrocyte CCS protein levels. Hepatic SOD1 levels were less strongly correlated with eSOD1 (*P*=0.054). CCS, Copper Chaperone for Cu/Zn superoxide dismutase; SOD1, Cu/Zn superoxide dismutase 1.

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 - -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 - -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; eSOD1, erythrocyte Cu/Zn superoxide dismutase.

effect on hepatic copper concentrations (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 levels were 2.37 (P<0.001) fold decreased compared to Labradors with normal hepatic copper concentrations (Fig 4A). The increase in eSOD1 protein levels in dogs with high copper concentrations did not reach statistical significance (P=0.099; Fig 4B). The eCCS/eSOD1 ratio was 3.29 (P<0.001)

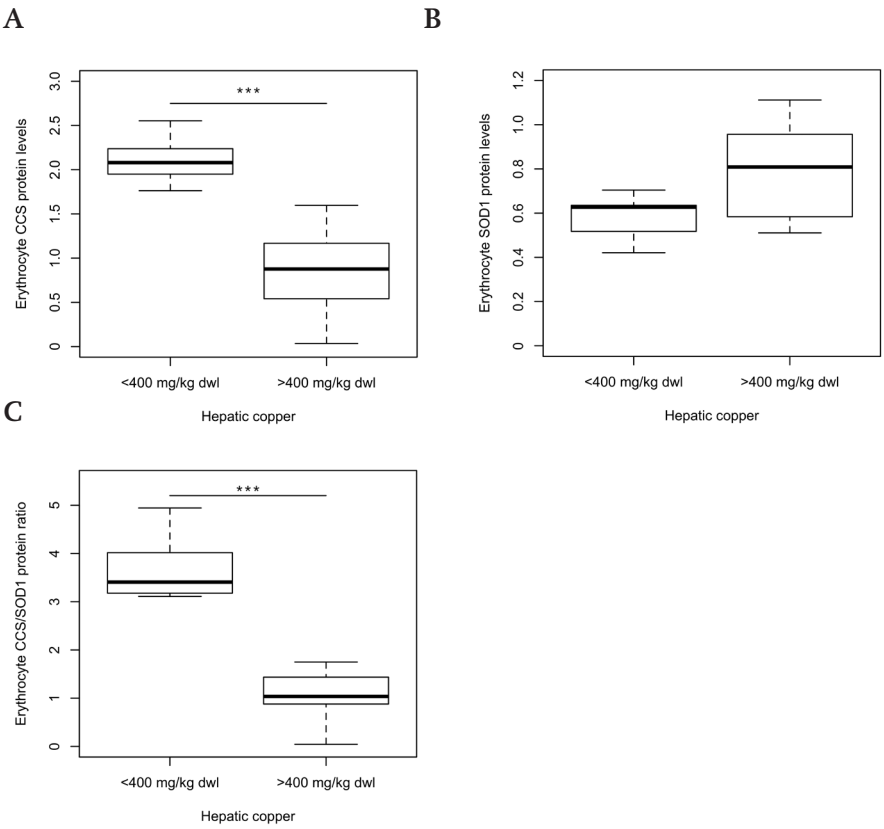


Figure 4 Erythrocyte protein levels in Labrador retrievers with normal (<400 mg/kg dwl) and high (> 400 mg/kg dwl) hepatic copper concentrations.

eCCS (**A**), eSOD1 (**B**), and eCCS/eSOD1 ratio (**C**). ***P < 0.001. dwl, dry weight liver. CCS, Copper Chaperone for Cu/Zn superoxide dismutase; SOD1, Cu/Zn superoxide dismutase 1.

fold decreased in the high hepatic copper group compared to the normal copper group (Fig 4C). There was no overlap in eCCS and eCCS/eSOD1 ratio between dogs with normal and increased hepatic copper levels.

Discussion

In the present study, we investigated whether SOD1 and CCS could serve as possible biomarkers for hepatic copper status in Labrador retrievers.

In our study, the specificity of the rabbit polyclonal antibodies for human SOD1 and human CCS in canine samples was confirmed by the loss of immune-reactive bands for SOD1 and CCS upon gene silencing in canine cells. The anti-human SOD1 FL-154 antibody was also shown to detect the canine SOD1 protein in a study into canine degenerative myelopathy.[32] For CCS, besides an immune-reactive band at 35 kDa, an additional smaller band was detected 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.[33, 34] Peptide blocking experiments, in which the antibody is neutralised, indicated CCS immune-specificity, but neither study further examined this smaller transcript variant and its role therefore remains undetermined.

SOD1 and CCS were previously studied for their suitability as biomarkers for copper deficiency in mice, rats, and cattle. These studies consistently demonstrated significant increases in CCS protein levels and decreases in SOD1 protein levels in organs and erythrocytes following dietary induced copper deficiency.[22, 23, 33-35] Upon copper repletion, eSOD1 and eCCS protein levels normalized[36] suggesting a mutual relationship between copper and these proteins. The increase in eSOD1 protein in Labrador retrievers with increased hepatic copper concentrations in our study was not significant. An explanation can be found in the difference between measuring SOD1 activity and SOD1 protein, which consists of the apoSOD1 and the copper loaded (holo) form of SOD1. Some studies recognize a larger decrease in SOD1 activity compared to SOD1 protein upon copper deficiency in both organs and erythrocytes.[23, 37] This difference was explained by inactive apoSOD1. Because protein turnover in erythrocytes is restricted and SOD1 protein levels did not change upon copper injection in both young and old erythrocytes, the concomitant increase in SOD1 activity was explained by the activation of apoSOD1.[38] We did not measure SOD1 activity and our SOD1 antibody most likely detects both apoSOD1 and holoSOD1.[39]

CCS is suggested as a more sensitive biomarker for copper status compared to SOD1.[22, 40, 41] In addition, CCS levels have shown not to be influenced by iron deficiency[36] and inflammation.[33, 42] The most promising finding of the current study was the drastic decrease of eCCS levels in Labrador retrievers with high hepatic copper concentrations. At present, only one study measured eCCS in rats fed high copper diets. Rats with high hepatic copper concentrations had a 47% decrease of eCCS compared to rats with low hepatic copper concentrations.[24] 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 yet. In cases of low copper, CCS presumably gathers stability by binding to apoSOD1, as the interaction of SOD1 and CCS is independent of copper binding.[43] Increasing levels of copper-scavenging CCS make copper transfer to SOD1 more rapid and efficient. When SOD1 becomes fully activated, CCS necessity and expression decreases. The substantial increase of copper-loaded CCS promotes degradation of CCS by the 26S proteasome in hepatocytes.[44] At present, it is not clear whether these changes take place in mature enucleated erythrocytes or only in their nucleated precursors. Supposing that these changes can only take place in nucleated cells, this implies that changes in eCCS and eSOD1 are not indicative for acute changes in copper status. As the life span of erythrocytes is generally around 103 days[45], changes in eCCS will reflect copper status over this preceding period of time. 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.

Despite copper concentrations in the high copper group were only moderately increased, both eCCS and eCCS/eSOD1 ratio showed no overlap between Labrador retrievers with normal and high hepatic copper concentrations. This emphasises the potential of eCCS or eCCS/eSOD1 as biomarker in Labrador retrievers or other dog breeds with severe hepatic copper accumulation. The majority of Labrador retrievers in our study were subclinical cases of hepatic copper toxicosis, further underlining the possible potential of eCCS and eCCS/SOD1 ratio in an early stage of disease.

Conclusion

Our results implicate a possible future role of eCCS and eCCS/eSOD1 ratio as biomarkers for screening and treatment follow-up of Labrador retrievers suffering from hepatic copper toxicosis. Larger cohorts and more quantitative assays (*e.g.* ELISAs) are needed for validation of the results. Applicability for 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 organizations that could inappropriately influence or bias the content of the paper.

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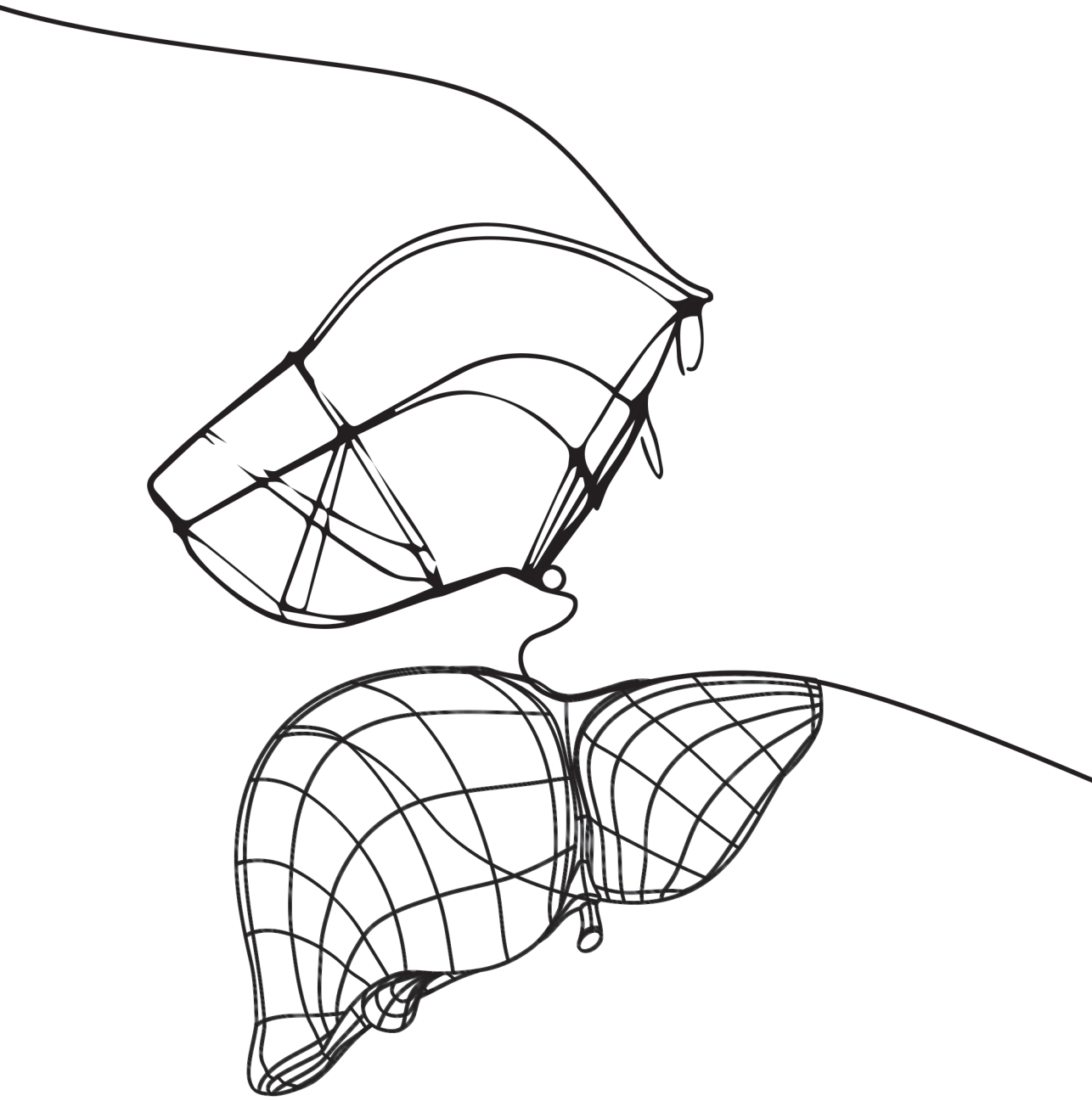

III

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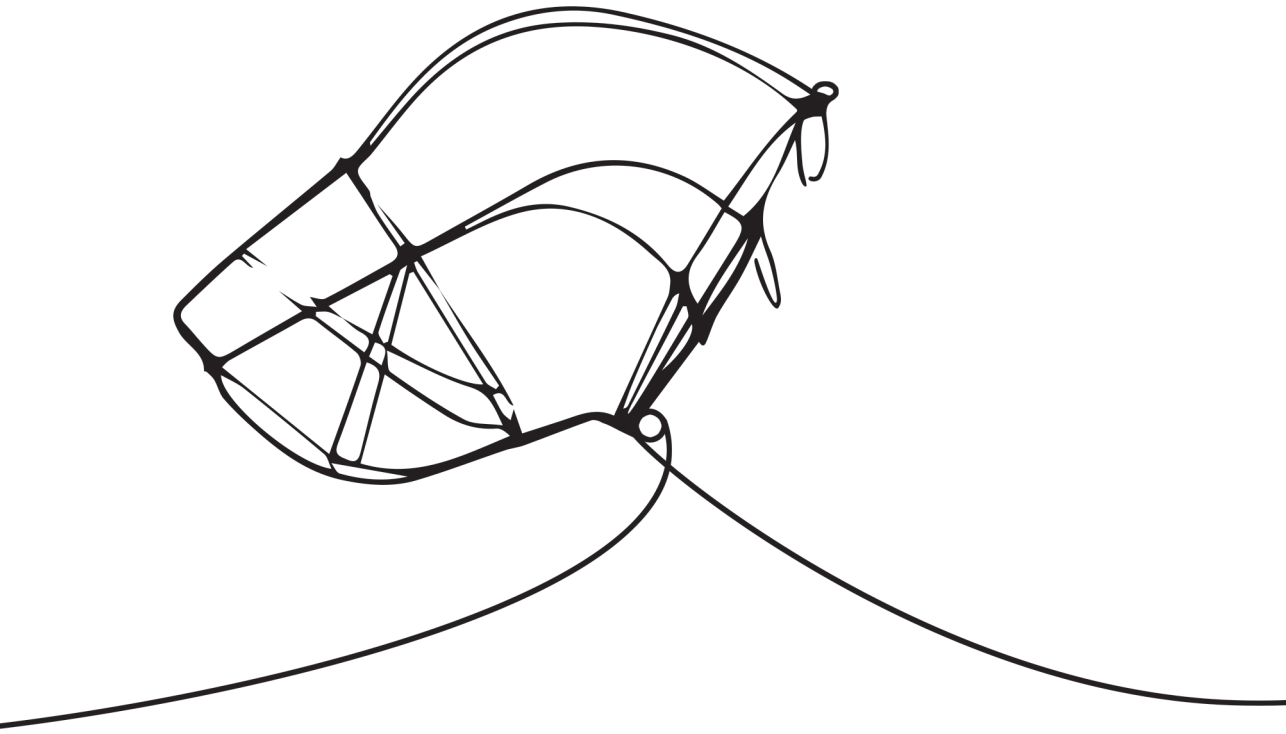
Treatment



8
chapter

D-penicillamine treatment of copper-associated hepatitis in Labrador retrievers

Karen Dirksen [a]
Hille Fieten [a]
Ted S.G.A.M. van den Ingh [b]
Esther Winter [c]
Adrian L. Watson [d]
Peter A.J. Leegwater [a]
Jan Rothuizen [a]

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[a] *Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*
[b] *TCCI Consultancy BV, Utrecht, The Netherlands*
[c] *Pharmacy of the Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*
[d] *The Waltham Centre for Pet Nutrition, Waltham-on-the-Wolds, Melton Mowbray, Leicestershire, UK*

Abstract

D-penicillamine is effectively used in the lifelong treatment of copper toxicosis in Bedlington terriers and Wilson disease in humans. A complex form of copper-associated hepatitis has recently been characterized in the Labrador retriever. The aims of this study were to evaluate the effectiveness of D-penicillamine treatment for copper-associated hepatitis in this breed, to study the effects on hepatic copper, iron and zinc concentration, and to estimate parameters to predict optimal duration of treatment. Forty-three client-owned Labrador retrievers that were diagnosed with copper-associated hepatitis were treated with D-penicillamine and underwent at least 1 follow-up examination including a liver biopsy. Inflammatory lesions were scored histologically. Hepatic copper, iron and zinc concentrations were determined in the initial and follow-up biopsies by instrumental neutron activation analysis. The influence of initial hepatic copper concentration, sex, age, D-penicillamine formulation and the occurrence of side effects were investigated for their influence on hepatic copper concentration after a certain period of treatment by generalized mixed modeling. D-penicillamine proved to be effective in reducing hepatic copper concentration and associated inflammatory lesions. Parameters derived from our model can be used to estimate necessary D-penicillamine treatment duration for Labrador retrievers with increased hepatic copper concentration. Continuous, lifelong D-penicillamine treatment is not recommended in this breed as there may be a risk for hepatic copper and zinc deficiency.

Introduction

Hereditary copper toxicosis in dogs has been identified in a number of purebred dog populations including the Bedlington terrier,[1] West Highland white terrier,[2] Skye terrier,[3] Dalmatian,[4] Dobermann[5] and most recently the Labrador retriever.[6] In Bedlington terriers a deletion of exon 2 of the *COMMD1* gene leads to extreme accumulation of copper in the liver.[7] In the other breeds, hepatic copper accumulation does not reach the very high concentrations reported in the Bedlington terrier and appears to have a more complex genetic background with an important role for environmental factors in the pathogenesis.[8] The Labrador retriever is a breed with such a complex form of copper-associated hepatitis. There is a strong female predisposition and the disease is often characterized by a long subclinical phase. Nutrition seems to be an important environmental factor in the etiology.[6; 9-11] In affected Labrador retrievers, copper accumulation in the liver continues over time[10] and without treatment, eventually causes hepatitis. The best studied human form of copper toxicosis is Wilson disease. This autosomal recessive disease is caused by mutations in the gene coding for the copper transporter ATP7B[12;13] and is characterized by accumulation of copper in the liver, brain and cornea.[14]

The overall therapeutic approach for copper toxicosis in man and animals is creating a negative copper balance. This can be achieved by using the copper chelator D-penicillamine. This highly soluble degradation product of penicillin, binds copper at its SH-group and promotes urinary copper excretion.[15] Since its discovery, D-penicillamine has become the most widely used copper chelator in the treatment of Wilson disease in humans[16] and proved to be effective for treatment of copper toxicosis in dogs as well.[1;6;17] Treatment monitoring relies on evaluation of repeated liver biopsies. However, no evidence based data exists on the rate of decrease in hepatic copper content nor is there information available on an optimal interval for recheck biopsies in dogs with complex forms of copper toxicosis. Also, no information is available about factors that may influence the rate of copper decrease including: sex, age, hepatic copper concentration before start of therapy, occurrence of side effects and D-penicillamine formulation. D-penicillamine forms relatively stable chelates with all biologically active trace metals including iron and zinc[18] and could promote urinary excretion of these metals as well. The influence of D-penicillamine treatment on hepatic iron and zinc concentrations in dogs has not yet been studied. Besides evaluation of metal chelation, the effects of D-penicillamine treatment on the activity and stage of copper-associated hepatitis are important aims for successful treatment.

The present study investigated the effects of D-penicillamine in Labrador retrievers with increased hepatic copper: (1) to establish a model to predict the necessary duration of treatment to reach a normal hepatic copper concentration; (2) to evaluate the effect of D-penicillamine treatment on the activity and stage of copper-associated hepatitis; and (3) to determine the effect of D-penicillamine treatment on hepatic iron and zinc concentrations. The results of this study contribute to the development of an evidence based treatment protocol for Labrador retrievers with copper toxicosis.

Materials and Methods

Animals

The Labrador retrievers used in this retrospective study were referred to the Department of Clinical Sciences of Companion Animals, Utrecht University, the Netherlands between 2003 and 2010. Diagnosis of copper-associated hepatitis was established through histological assessment of liver biopsy specimens and quantitative copper determination in liver tissue. All affected dogs were treated with D-penicillamine capsules produced at our Veterinary Pharmacy or with Metalcaptase® (Heyl) (enteric coated D-penicillamine tablets intended for human use). All dogs were evaluated using at least one follow-up biopsy. Data on signalment, type and duration of treatment, plasma levels of alanine amino-transferase (ALT) and alkaline phosphatase (ALP) and the occurrence of side effects during treatment were collected from the medical records. Side effects were scored on a 0-2 scale (0 = no side effects; 1 = mild side effects with no necessity to cease therapy (decrease in appetite, occasional vomiting); 2 = severe side effects with necessity to temporarily cease therapy (anorexia, severe vomiting). All dogs were client-owned and data were collected after obtaining the informed consent of their owners. Procedures were approved by the University of Utrecht's Ethical Committee on Animal Experiments as required under Dutch legislation.

Assessment of liver biopsies

At least 3 liver biopsies were collected from the left lateral liver lobe with a 14G needle using a Tru-cut device under ultrasound guidance. Two biopsy specimens were fixed in 4% neutral buffered formalin and embedded in paraffin. Paraffin sections of liver biopsies were stained with rubeanic acid, hematoxylin and eosin and according to Gordon and Sweet for reticulin and histology was evaluated by one board-certified veterinary pathologist (TSGAM vd Ingh). Grading (necro-inflammatory activity) and staging (fibrosis/nodular transformation) of hepatitis was based on the system of Ishak et al.[19;20]

A separate biopsy specimen of minimally 5 mg was collected in a metal free container and freeze dried prior to quantitative metal determination by instrumental neutron activation analysis.[21] Dogs were considered to have normal hepatic copper when copper concentrations were below 400 mg/kg dry weight liver (dwl).[22] Hepatic metal concentrations are reported in mg/kg dwl.

Statistics

All data were analyzed using R statistics package 2.14.0.[23] A mixed model was used to assess the duration of treatment required for a decrease in hepatic copper concentration and to investigate the factors influencing this process. The analysis was performed with the R-package "nlme". Restricted maximum likelihood was used to estimate the best fitting model. Maximum likelihood estimation was used to estimate the fixed effects of the parameters in the model. Dog ID was added as random effect to take into account the correlation between observations within a dog. The outcome was defined as hepatic copper concentration at a certain time of treatment in months (CuQy), wherein CuQ is the hepatic copper concentration in mg/kg dwl and y is the time of the control biopsy in months after initiation of therapy. Explaining parameters under investigation were sex (male, female), age at start of therapy (years), time of therapy until control biopsy (months), occurrence of side effects (0-1-2) and therapy type (compounded D-penicillamine or Metalcaptase® (Heyl)). The best fitting model for the data was determined with a stepwise forward model using Akaike's information criterion. The validity of the final model was checked by studying the residuals on normality and constance of variance.

A Wilcoxon signed rank test was used to compare hepatic grading and staging scores, and ALT and ALP activity before and after therapy. The association between ALT and ALP and hepatic copper and grade of hepatitis before and after treatment was tested with Pearson's product moment correlation. A paired *t*-test was used to compare hepatic copper, iron and zinc concentrations before and after D-penicillamine therapy. $P < 0.05$ was considered significant. Normally distributed data were summarized as mean ± standard deviation (SD) and non-normally distributed continuous data or count data was presented as median and range.

Results

Animals

The study population consisted of 43 Labrador retrievers of which 12 were male and 31 were female. The mean age at the start of therapy was 6.4 ± 2.2 years. Labrador retrievers were treated for a median duration of 4.8 months (range, 1.8 – 15.7). One dog was treated with compounded D-penicillamine capsules at age 4.7 years for 6.8 months. At age 8.7 years hepatic copper had re-accumulated and this dog was therefore treated again with Metalcaptase® (Heyl) for 6.3 months. A decrease in hepatic copper below 400 mg/kg dwl occurred in 21 dogs, while 23 dogs still had hepatic copper values above 400 mg/kg dwl (Fig 1). Twenty-eight dogs were treated with compounded D-penicillamine capsules and 16 dogs with enteric coated tablets of Metalcaptase® (Heyl). All dogs received their medication in a dose of 10 mg/kg orally twice daily, 30 minutes before a meal. Side effects were scored as 0, 1, and 2 in respectively 12, 16 and 3 dogs. For 12 dogs no data regarding side effects was available from the medical records.

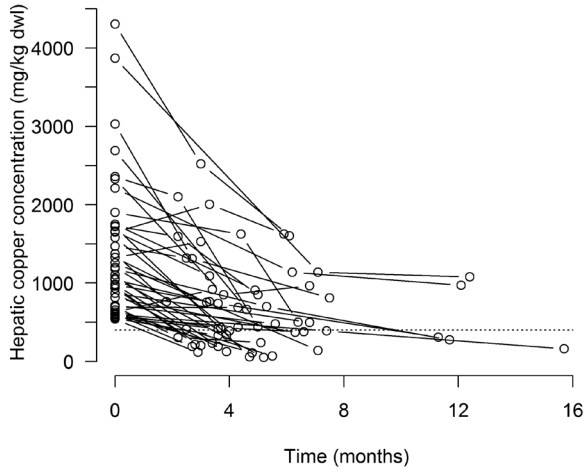

Figure 1 Decline of hepatic copper concentrations (mg/kg dwl) in individual Labrador retrievers during D-penicillamine treatment.

Factors influencing necessary treatment duration and modeling of copper decrease

Sex, age, therapy type and side effects did not significantly influence CuQy. A shorter therapy duration and higher hepatic copper concentrations before initiation of therapy both resulted in higher copper concentrations at time y. The relation between hepatic copper concentration and therapy duration was non-linear due to an interaction between copper concentrations before therapy onset and therapy duration and followed the equation:

$$CuQy = -81.85 + 0.99\ CuQ0 + 51.0\ T1 - 3.92\ T1^2 - 0.16\ CuQ0{*}T1 + 0.92{*}10^{-2}\ CuQ0{*}T1^2$$

Using estimates from this model (Table 1) we could predict hepatic copper concentration after a certain period of therapy (CuQy) for dogs for which hepatic copper concentration before onset of therapy (CuQ0) and indicated treatment duration (T1) are known (Fig 2).

Table 1 Estimates and standard errors of the model parameters for the prediction of hepatic copper concentration in Labrador retrievers during D-penicillamine treatment.

Covariate	Estimate	Standard error	P-value
(Intercept)	-81.85	370.51	0.82
CuQ0	0.99	0.21	≤0.01
T1	51.0	111.13	0.65
$T1^2$	-3.92	6.54	0.56
CuQ0*T1	-0.16	$0.62{*}10^{-1}$	≤0.05
CuQ0*$T1^2$	$0.92{*}10^{-2}$	$0.38{*}10^{-2}$	≤0.05

CuQ0: Hepatic copper concentration at the start of therapy
T1: Time of treatment in months

Histopathology

Localization and the extent of hepatic copper accumulation, grading of the inflammatory activity and staging of fibrosis were assessed before and after therapy. Copper was localized in the centrolobular zone of the liver lobules in hepatocytes and macrophages and was often accompanied by an inflammatory infiltrate. Before therapy the grading scores for necro-inflammatory activity ranged from 0 (absent) to 3 (moderate), with a median grading score of 1 (slight activity of the hepatitis). The median grading scores decreased significantly to 0

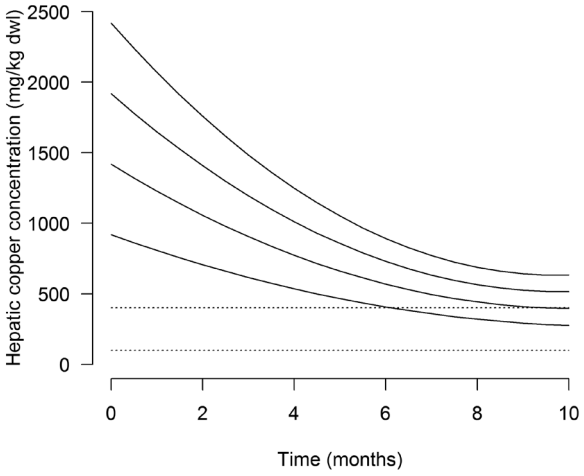

Figure 2 Prediction of hepatic copper decrease in time based on the model parameters.

Solid lines indicate predicted lines for dogs with different initial hepatic copper concentration. Horizontal dotted lines indicate the lower and upper range for normal hepatic copper[22].

(range, 0-3) after therapy ($P \leq 0.01$) (Fig 3). Before and after D-penicillamine therapy, fibrosis staging scores ranged from 0 (absent) to 4 (very marked), with a median score of 0 (absence of fibrosis) and there was no significant difference (Fig 4).

Plasma ALT and ALP

For 41 dogs ALT or ALP values were available for testing the correlation with grade of hepatitis and hepatic copper levels before and after treatment. In 37 dogs, both ALT and ALP values before and after treatment in the same dogs were available for paired tests.

The percentage of dogs with an increased ALT before or after treatment was respectively 45% and 25%. For ALP these percentages were 24.4% before treatment and 12.5% after treatment. There was no significant difference in ALT and ALP levels before and after treatment ($P=0.057$ and $P=0.94$ for ALT and ALP, respectively). ALT and ALP were not correlated with hepatic copper concentration. A significant correlation between ALT and grade of hepatitis was present before ($r^2 = 0.26$, $P \leq 0.01$) and after ($r^2 = 0.26$, $P \leq 0.01$) treatment. ALP was also significantly correlated with grade of hepatitis before ($r^2 = 0.10$, $P=0.04$) and after ($r^2 = 0.14$, $P=0.017$) treatment.

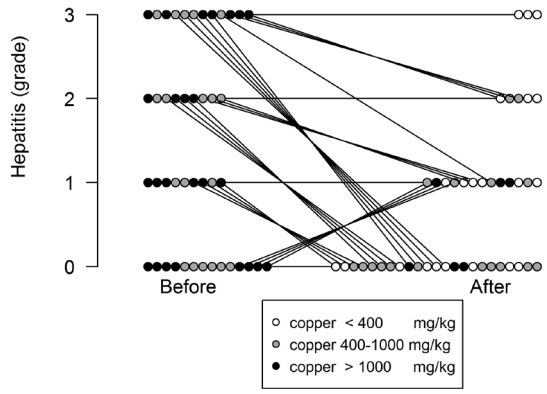

Figure 3 Grade of hepatitis before and after D-penicillamine treatment.

Before treatment dogs with hepatic copper between 400 and 1,000 mg/kg dwl and dogs with hepatic copper exceeding 1,000 mg/kg dwl are evenly distributed among grade category. The majority of dogs show an improvement of hepatitis after D-penicillamine treatment.

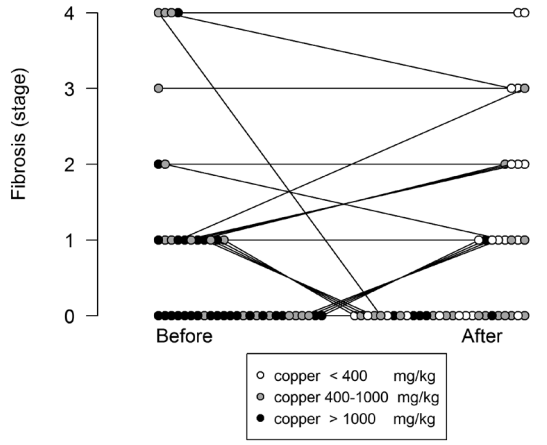

Figure 4 Stage of fibrosis before and after D-penicillamine treatment.

The majority of dogs in this dataset did not have fibrosis in their liver. Increase as well as decrease in fibrosis scores is noted after D-penicillamine treatment.

Hepatic iron and zinc

Hepatic iron and zinc concentrations before and after D-penicillamine therapy were available for 42 Labradors (Fig 5). Copper concentrations decreased significantly from 1,354 ± 864 mg/kg dwl before treatment to a mean copper concentration of 556 ± 433 mg/kg dwl after treatment ($P \leq 0.01$). Zinc concentrations decreased significantly from 181 ± 71 mg/kg dwl before treatment to a mean zinc concentration of 138 ± 49 mg/kg dwl after treatment ($P \leq 0.01$). Iron concentrations did not significantly differ before (mean hepatic iron of 2,320 ± 1,790 mg/kg dwl) or after (mean hepatic iron of 1,973 ± 1,168 mg/kg dwl) therapy (Fig 5).

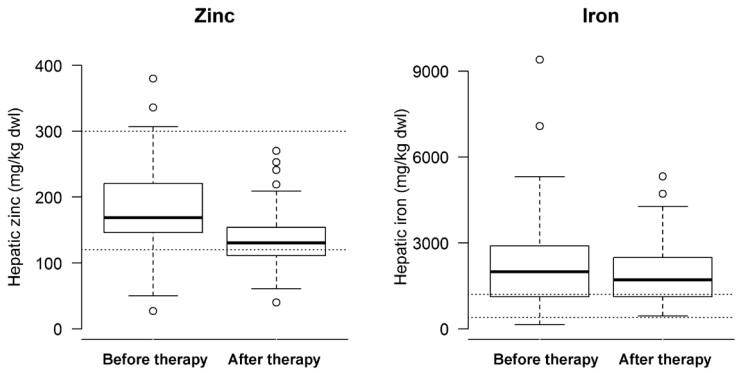

Figure 5 Hepatic zinc and iron concentrations before and after treatment with D-penicillamine.

Horizontal dotted lines indicate lower and upper range of the metal reference values[22].

Discussion

The first aim of our study was to investigate the factors that may influence the necessary treatment duration for Labrador retrievers with increased hepatic copper and to provide a guideline for the period of D-penicillamine treatment required to reach a normal hepatic copper concentration. Bedlington terriers with copper toxicosis can have hepatic copper concentrations as high as 10,000 mg/kg dwl[1] and lifelong chelation therapy is often indicated. In contrast to the Bedlington terrier, copper concentrations in Labrador retrievers[6] and other affected dog breeds[2-5] generally range from 600 to 5,000 mg/kg dwl. Continuous administration

of chelating agents may lead to the risk of overtreatment. This phenomenon is reported in humans with Wilson disease[16] and in a case report of a Bedlington terrier.[24] In the lifelong management of hepatic copper storage disorders it is important to monitor treatment efficacy throughout life by evaluation of repeated liver biopsies.

In the current study we were able to provide estimates for treatment duration necessary to obtain normalization of hepatic copper concentration in Labrador retrievers by generalized mixed modeling, which can aid in the decision when to re-biopsy after initiation of treatment. Labrador retrievers with hepatic copper concentration exceeding 1,500 mg/kg dwl need treatment for at least 10 months to reach a hepatic copper concentration of 400 mg/kg dwl. Since the majority of our data was collected from dogs that were treated for a shorter period of time, a reliable estimation for necessary treatment time beyond 10 months of treatment was not possible. Our study was based on histological evaluation and metal quantification of hepatic biopsies obtained with a 14G needle. Collection of biopsies in this way is less invasive compared to collection of biopsies via laparoscopy or laparotomy. Discordance between results from 18G needle core biopsies and wedge biopsies was reported previously.[25] In the current study we used 14G needle biopsies that result in sample volume almost three times higher than obtained with 18G needles and comparable with the volume of a laparoscopic wedge biopsy. However, results from liver biopsies can be influenced by sampling errors, especially in cases of liver cirrhosis, where newly formed regenerative nodules and fibrotic septa contain less copper. In the current study, a total of 103 hepatic biopsies were evaluated, from which the large majority showed no fibrosis (n = 63) or mild fibrosis (n = 22). Seven biopsies had a fibrosis score of 2 (moderate), 5 biopsies had a score of 3 (marked) and in 6 biopsies very marked fibrosis (score 4) was identified. In biopsies with a fibrosis score ≥ 2, metal quantification by instrumental neutron activation analysis may have been less reliable.

Hepatic copper concentration upon D-penicillamine treatment declined in a non-linear fashion, with a steeper decline in dogs with high initial hepatic copper concentrations. This observation is in concordance with results from studies of urinary copper excretion in Wilson disease patients, in which there is an initially high excretion of urinary copper upon institution of D-penicillamine treatment that later stabilizes gradually.[26;27] There was no significant effect of sex, age, D-penicillamine formulation and the occurrence of side effects on D-penicillamine induced decrease of hepatic copper over time. We hypothesised that side effects could have led to a decrease in therapy compliance and therefore a decreased reduction of hepatic copper concentration, but this was not confirmed in the

current data set. Side effects that were commonly noticed in dogs treated with Metalcaptase® or compounded D-penicillamine were restricted to gastro-intestinal signs including anorexia and vomiting and were almost always manageable by increasing the therapy dose gradually. D-penicillamine in human enteric coated tablets (Metalcaptase®, Heyl), are preferred compared to compounded D-penicillamine capsules implemented in the Dutch Veterinary Medicines Decree. However Metalcaptase® was not available from our veterinary pharmacy until 2008. Before this date compounded D-penicillamine was prescribed. In our clinical experience enteric coated tablets were not always as well tolerated as the compounded capsules. Medication was typically administered 30 min before a meal and several owners reported that their dogs vomited up to several hours after their meal, with the vomitus containing just the enteric coated tablet, without any food. This phenomenon has been reported before for enteric coated aspirin tablets in Beagle dogs.[28] Grinding the enteric coated tablets before administration or switching therapy to compounded D-penicillamine capsules may diminish vomiting in dogs that suffer from retention of the tablets in the stomach.

We detected inter-individual variation in the response to treatment in the Labrador retrievers. One of the reasons may have been variation in dietary copper intake during D-penicillamine treatment. The effect of diet on hepatic copper concentration was reported previously.[10;11] However, no information was available about diet composition of the dogs in this study. We assessed hepatic histology before and after treatment with D-penicillamine. Scoring of the necro-inflammatory activity (grade) and the degree of fibrosis/nodular transformation (stage) gives an indication of the severity and progression of the hepatitis.[19] A decrease in grade of hepatitis was noted after treatment, which was reflected in a decrease in the number of dogs with increased ALT or ALP. This is consistent with the results from other studies in which the effect of D-penicillamine was evaluated in the Dobermann[17] and humans with Wilson disease.[27;29] We observed increases as well as decreases in fibrosis scores after D-penicillamine treatment. Based on the present findings it is not possible to draw conclusions whether improvement of fibrosis did occur in certain dogs, or was a result of differences in samples due to heterogeneity of the liver.

Remarkably, the majority of dogs had a hepatic iron concentration above the range that is considered normal.[22] Increased hepatic iron concentrations in conjunction with inflammatory lesions[30;31] and associated with increased hepatic copper concentrations was reported before in dogs.[31] In Wilson disease patients increased hepatic iron has been reported as well. Interestingly, in humans that are treated with D-penicillamine for several years, marked hepatic iron accumulation

occurred.[32] In our group of D-penicillamine treated Labrador retrievers there were no indications for additional iron accumulation. In contrast, hepatic zinc concentrations significantly decreased during the D-penicillamine treatment regimen. Zinc is an essential trace element with a number of important functions in the body and is necessary for proper liver function.[33] The decrease of zinc in the liver upon D-penicillamine treatment can therefore be considered as an unwanted side effect. It is known that D-penicillamine does not bind copper specifically, but that it has affinity for other metals as well, including zinc and iron. The dissociation constants for metal-penicillamine complexes shows decreasing stability in the order Hg>Cu>Ag>Pb>Ni>Cd>Zn>Co>Fe> Mn.[18;34;35] Therefore it may well be that in the beginning of treatment, urinary copper excretion exceeds zinc excretion and that when copper has decreased, relatively more zinc will be excreted, which may lead to an enhancement of zinc depletion.[26] Iron-D-penicillamine complexes are far less stable, which may in part explain that hepatic iron did not change significantly during D-penicillamine treatment.

Conclusions

D-penicillamine treatment is effective in Labrador retrievers with copper toxicosis in decreasing hepatic copper with a concomitant improvement of inflammatory lesions. We estimated model parameters for a decrease in hepatic copper upon D-penicillamine treatment that can be used as a guideline for determining treatment duration in the Labrador retriever. Copper and zinc deficiency may be a risk with prolonged D-penicillamine treatment and measurement of these metals in follow-up hepatic biopsies can be useful to monitor treatment effectiveness and risk of overtreatment.

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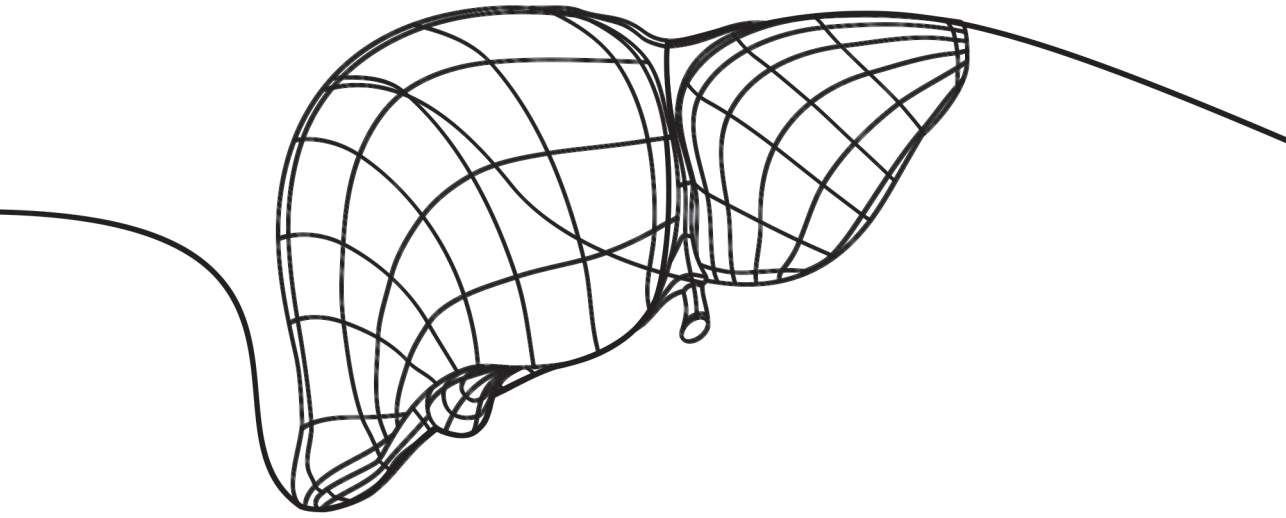

9
chapter

Summarizing discussion and concluding remarks

Copper is an essential trace element for living organisms, but can have deleterious consequences when present in excess. Because the liver has a central role in copper metabolism, this is the predominant organ affected. Hepatic copper accumulation results in hepatocellular inflammation and necrosis, eventually leading to severe liver dysfunction. Copper-accumulating disorders are recognized in man[1-4] and other mammals, including dogs. In dogs, copper-associated hepatitis is one of the most common causes of primary hepatitis.[5]

Studies presented in this thesis were focused on the Labrador retriever. In the Labrador retriever hepatic copper accumulation arises form an inherited defect in copper metabolism[6] in combination with excessive copper intake via the diet or drinking water.[7, 8] In this chapter (**Chapter 9**) the main findings of all studies, together with recommendations and future perspectives, are summarized and discussed. This summary is visually illustrated in the figure at the end of this chapter.

In **Chapter 1** the aims and scopes of this thesis were presented. Together they were aimed at 1) obtaining insight in the pathogenesis of copper-associated hepatitis, 2) improving the diagnosis in dogs with (copper-associated) liver disease, 3) optimize the current medical treatment regime of copper-associated hepatitis in the Labrador retriever. **Chapter 2** starts with an introduction in copper metabolism and copper-associated hepatitis in dogs. Clinical signs and current diagnostic and treatment possibilities are discussed.

Part I Pathogenesis

To gain insights in the molecular mechanisms underlying the disease progression and to establish the primary involvement of copper in copper-associated hepatitis we performed a gene expression study in liver tissue of Labrador retrievers (**Chapter 3**). Samples were collected from dogs with different stages of the disease; normal copper concentrations and no histological changes (N); high copper concentrations, no histological changes (HC); high copper, hepatitis (HCH), and high copper chronic hepatitis (HCCH). Every stage was compared with the preceding stage, resulting in the following comparisons: N-HC, HC-HCH, and HCH-HCCH. A target quantitative real-time polymerase chain reaction (qPCR) approach was used to examine the involvement of copper transporter and oxidative stress responsive genes.

Results of the target qPCR show that, after correction for multiple testing, only a small number of genes were differentially expressed. This suggests that, perhaps, posttranscriptional regulation of copper metabolism in disease[9-12] is more important to regulate copper homeostasis, although this is in contrast with other studies in Doberman pinschers [13], Bedlington terriers [4], and COMMD1-deficient dogs[15] (Beagle x Bedlington terrier crossbreeds), that showed differential expression of more copper transporters including the copper transporters ATP7A and ATP7B. Possible explanations include the higher copper concentrations found in Bedlington terriers and COMMD1 deficient crossbreeds, a compensatory mechanism for the loss of functional COMMD1 protein, or the gradual comparisons between successive stages in our study. In addition, the influence of the mutations in ATP7A and ATP7B6, associated with the disease phenotype in Labrador retrievers, on gene expression is not known.

Upregulation of metallothioneins (MT) and COMMD1 in the HC group show a first adaptive response to high intracellular copper concentrations. Both MT1A and MT2A, which are copper scavengers, follow the increase and decrease in hepatic copper concentrations. CCS expression was significantly decreased in the HCCH group compared to the HCH group. As shown in Chapter 7, high hepatic copper concentrations result in low hepatic and erythrocyte CCS protein concentrations. It is presumed that, when copper levels are elevated, CCS delivers copper to XIAP, resulting in degradation of CCS and XIAP, eventually resulting in apoptosis.[9, 16] Under basal copper conditions XIAP-mediated ubiquitination of CCS leads to enhanced copper acquisition and positively regulates SOD1 activation by CCS. [9] We also studied gene-expression of amyloid-β precursor protein (*APP*), which is involved in the pathology of at least two neurodegenerative diseases, Alzheimer's disease and prion disease. APP is a transmembrane protein with copper binding properties at the extracellular domain. In neuronal cells, copper stimulates APP trafficking to the plasma membrane.[17,18] APP is believed to be a copper transporter, which maintains copper levels in liver and brain, by regulating copper efflux.[19-21] Gene expression profiling in a genetic model of chronic copper overload revealed upregulation of APP. In line with these findings, we found a 2.5 times increase of APP mRNA expression in the HCCH group compared to the HCH group ($P<0.01$).

Oxidative stress, generated by excess copper, has shown to be an important contributor copper accumulating in disease in man and animals.[13,14, 22-24] Glutathione (GSH), the main hepatocellular antioxidant, is one of the proteins to bind copper upon entering the cell. [25] GSH antioxidant activity involves oxidation to GSH-disulfide (GSSG), and hepatic GSH concentrations and GSH/GSSG ratio are believed to reflect oxidant injury. Previous studies corroborated their gene expression measurements

with decreased total GSH [13]or GSH/GSSG ratios [14] in dogs with copper-associated liver disease. Although a decreased GSH/GSSG ratio is not specific for copper associated disease,[14, 26] additional GSH and GSSG measurements can confirm the presence of oxidative injury in the HC group. The GSH precursor S-adenosylmethionine (SAM) is synthesized by methionine adenosyltransferases (MAT). Previously it has been shown that decreased MAT1A mRNA expression, leads to a decrease in total MAT activity and a decrease in SAM.[27] In our study, MAT1A mRNA expression was significantly decreased in the HCH group compared to the HC group. If this is indeed paralleled by a decreased in SAM and GSH, this may further aggravate liver damage.

Although histologic changes of hepatitis were not yet visible in the HC group, results of this study clearly showed transcriptomic alterations in the HC group compared to the N group. Pathways involved in predominantly cell adhesion, development, inflammation, and cytoskeleton were already enriched, indicating the primary involvement of copper. When progression towards hepatitis (HCH) was made, the most significantly enriched networks were those involved in inflammation. Inflammation and the immune response promote coagulation by complement factors, inflammatory cytokines, neutrophil activation, downregulation of the protein C pathway, and inhibition of fibrinolysis.[28,29] Kinins (e.g. bradykinin) are inflammatory mediators that can also be formed via the initiating mechanism by which the coagulation pathway is activated.[30] The strong relationship between inflammation, immune response, and coagulation, explains why in the present study, changes in chemoattractants, complement factors, protein S, bradykinin, kallidin, and kininogen were found. In the HCCH group, pathways with functions in mainly the following biological processes were enriched: cell adhesion, cytoskeleton, proteolysis, development and apoptosis. Multiple pathways involved in inflammation and the immune response were also enriched, but these were less significant than those involved the other biological processes.

Histological changes seen in chronic hepatitis include apoptosis, necrosis, inflammatory infiltrate, regeneration and the presence of fibrosis and cirrhosis.[31] Hepatic fibrosis is the extensive deposition of extracellular matrix (ECM) proteins, especially collagens. Epithelial-mesenchymal transitions (EMT), one of the affected processes in our study, is known to occur in injured adult hepatic tissue as a repair-associated event.[32] In the case of ongoing inflammation, EMT leads to fibrosis. EMT generates activated mesenchymal cells (i.e. the hepatic stellate cell, HSC) that produce large amounts of collagen-rich ECM, upon, predominantly, TGFβ stimulation.[33] Our results show that in the last phase of the disease (HCCH), EMT might have a prominent role in disease progression as shown by the differential

expression of ECM components, ECM remodeling proteins, cytoskeleton constitutors, and signal transducers. HSCs are also postulated to activate the hepatic progenitor cell (HPC). The HPC is a bipotential cell, capable to differentiate towards hepatocytes or cholangiocytes, and becomes activated when mature hepatocytes are damaged and replication is insufficient. Earlier studies in man and dogs with different degrees of hepatic fibrosis and disease activity have already shown the involvement of HSC and HPC activation.[34, 35] The fibrotic and regenerative reaction patterns have shown to be comparable in human and canine liver disease.[34, 36] In addition, both fibrotic and regenerative pathways in man and dog are highly comparable irrespective of the differences in origin of fibrosis and cirrhosis.[37, 38] Therefore, besides being a dog model for human forms of copper storage diseases[39], we propose that the Labrador retriever can also be a non-rodent mammalian model for human chronic hepatitis.

Recommendations and future perspectives

Wilson disease, Menkes disease and Alzheimer disease are al characterized by mutations in genes encoding copper transporters (*ATP7B*, *ATP7A*, and *APP*, respectively). All three proteins reside in the trans Golgi network and relocalize to the vesicular compartment and eventually to the plasma membrane under elevating copper conditions where they (propose) to function in the cellular efflux of copper.[17, 18, 40, 41]It is intriguing that recent studies have shown involvement of ATP7B in the pathogenesis of Alzheimer disease,[42-44]suggesting that all diseases can be explained by similar mechanisms related to copper. Therefore it would be interesting to study the APP gene in dogs more closely. In addition, the ABCA12 gene, which encodes a membrane bound transport protein, is another candidate gene to evaluate more closely in Labrador retrievers. Recently two SNPs were found to be significantly associated with copper-associated hepatitis in Bedlington terriers, lacking the homozygous *COMMD1* deletion.[45]

The presumed decrease in SAM, may provide an opportunity to implement new treatment strategies. In humans several studies into SAM treatment haven been performed although with controversial results.[46-49] Basically only one study reports that long-term SAM treatment may improve survival in patients with alcoholic liver disease.[46] The main issue in these human studies is the lack of large high quality clinical trials and therefore they are not able to support or refute SAM treatment. Second, SAM is mainly used in the treatment of advanced liver cirrhosis. One can speculate that, when it is used in earlier disease stages (i.e. HC, HCH) it might have more pronounced effects on prognosis. A few studies conducted in dogs and cats showed increased plasma SAM, increased GSH concentrations and a more favorable red blood cell and hepatic redox status upon SAM treatment,

encouraging further studies into the beneficial effects of SAM treatment in dogs (and cats) with hepatobiliary disease.[50-52]

Part II Diagnosis

Especially dogs with copper-associated hepatitis or dogs with idiopathic forms of chronic hepatitis can have a long subclinical stage of disease. Clinical signs only develop when most of the liver parenchyma is affected and the reserve capacity of the liver is exceeded. For dogs affected with copper-associated hepatitis, there is another reason for the long subclinical stage of disease; as shown in **Chapter 3**, copper starts to accumulate in hepatocytes without appreciable histological signs, but transcriptomic alterations are already present. When cellular defense mechanisms are exhausted hepatocellular injury becomes increasingly evident. The road from hepatocellular injury to liver fibrosis, and eventually cirrhosis has already been described in **Chapter 3**. To interfere with this process early diagnosis of parenchymal (inflammatory) liver disease in general, and of copper-accumulating disease, is essential. Therefore, easily accessible blood- or urine-based biomarkers for both copper status and hepatocellular injury are necessary.

To date, the only way to diagnose copper-associated liver disease in dogs is through an invasive liver biopsy procedure with subsequent assessment of liver histology, copper distribution, and copper quantification. This is not an ideal procedure for screening dogs at risk and for treatment monitoring. In case of liver disease in general, liver enzymes and liver function tests are generally performed as first steps in the diagnostic work-up.

Liver enzymes include alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and γ-glutamyltransferase (GGT). According to the literature, all liver enzymes have considerable sensitivity for hepatobiliary diseases, but interpretation can be hampered by their lack of specificity and their concomitant increase in secondary (reactive) liver disease. ALT is one of the liver transaminases and is located in the hepatocyte cytosol where it leaks upon altered membrane integrity. The largest increases can be seen with hepatocellular necrosis and inflammation.[53, 54] Compared to AST, ALT has a higher specificity. [54]ALP is believed to have a good sensitivity for liver diseases, with higher activities in cholestatic disease (including intrahepatic cholestasis), chronic hepatitis/cirrhosis, corticosteroid-induced hepatopathy, and hepatic necrosis.[55] Due to iso-enzyme activity, ALP lacks specificity.[54, 55] Serum GGT is mainly derived from the liver and GGT is located on the hepatocyte canalicular membrane. Therefore an increase

is most common with cholestatic disorders.[54] Whereas liver enzymes establish the presence of injury, bile acids (BA) are often used in the identification of hepatobiliary dysfunction due to cholestasis or portosystemic shunting. [56, 57]

Our extensive dataset (n=242), including a large number of clinically healthy Labrador retrievers with biopsy confirmed liver status, prompted us to study ALT, ALP, and BA levels and hepatic copper concentrations more closely (**Chapter 4**). Labrador retrievers studied in this chapter were clinically healthy or clinically ill and had normal livers (NL), primary hepatitis (PH; acute hepatitis [AH] or chronic hepatitis [CH]), or a non-specific reactive hepatitis (RH). Increased hepatic copper concentrations were present in all groups, including dogs with NL, but significantly higher copper concentrations were found in dogs with AH (median 964 mg/kg dwl, range 243-3,030, *P*=0.002) and CH (median 685 mg/kg dwl, range 85-5,084, *P*=0.035) compared to NL. As shown in **Chapter 3**, hepatic copper accumulation leads to inflammation, explaining the higher copper concentrations in dogs with AH and CH.

One of the major aims of the study presented in **Chapter 4** was to evaluate whether increased ALT and ALP aid in the discrimination between dogs with PH and RH. General (health) profiles frequently include liver enzymes. It is known that dogs with RH can also present with increased liver enzymes because the liver parenchyma reacts to extra-hepatic diseases or stimuli (e.g. pancreatitis, inflammatory bowel disease). In this case, however, it is the underlying disease that requires attention and the hepatic changes resolve once the underlying stimuli are treated or removed. We showed that dogs with PH have a significantly higher ALT compared do dogs with RH (*P*<0.001). And for every 1 U/L increase in ALT activity a two percent increase in the odds of having a PH (versus having NL or a RH) is expected (95% CI: 1.01–1.04; *P*=0.007). There was no significant difference in ALP between dogs with PH and RH.

The second aim of **Chapter 4** was to determine the sensitivity and specificity of ALT, ALP, and BA to detect dogs with AH, CH, and RH in subclinically affected Labrador retrievers. Currently, there are no studies reporting sensitivity and specificity of liver enzymes and bile acids for detecting hepatitis in subclinically affected dogs. As detecting liver disease in an early disease stage is of utmost important to increase therapy efficacy, biomarkers with high sensitivity are needed. In 19% (37/191) of the clinically healthy Labradors histology revealed a PH (20 AH, 17 CH) and in 45% (85/191) a RH. And of all dogs with liver pathology (both subclinical and clinically ill dogs, n=173), 98 dogs had ALT and ALP levels within reference range. In clinically healthy dogs, ALT was the test with the best sensitivity: 71% for CH and 45% for AH. Sensitivity of ALP for CH was 35%. All other

sensitivities for AH, CH, and RH were 15% or even lower. Specificities of ALT, ALP, and BA were 0.93, 0.99, and 0.90 respectively (similar for CH, AH, and RH). Given the fact that our study did not include liver biopsies of dogs with extra-hepatic disease, true specificity would be even lower as described in other studies. [54, 55]In conclusion, ALP and BA are not adequate indicators of subclinically AH and CH. Sensitivity of ALT to detect subclinical dogs with AH is low. It has been shown that AH may progress toward CH, which may progress to cirrhosis. [5]Therefore a higher sensitivity to detect these dogs is necessary. On the other hand, ALT can be used to detect the presence of subclinically CH. In contrast to clinically healthy dogs, most of the clinically ill dogs had a PH (45/51). Only two clinically ill dogs had ALT, ALP, and BA levels within reference range. Moreover, BA concentrations were significantly increased in dogs with AH and CH compared to dogs with RH and NL, indicating a substantial impairment of liver function.

In the search for more sensitive and specific biomarkers for hepatocellular injury serum microRNAs were evaluated as first candidates. MicroRNAs are well conserved small non-coding RNAs (~22 nt) with a critical function in post-transcriptional regulation of gene expression. [58]Circulatory microRNAs are protected against degradation by nucleases, rendering stability through transportation in a protein-bound form, in association with lipo-proteins, or as exosomes or micro vesicles. Their stability under different storage conditions[59-61] and over a long period of time[62] makes them ideal candidate biomarkers. Over the last decades microRNAs have shown to be important candidate biomarkers in human disease, including hepatobiliary diseases.[63-67] In **Chapter 5**, Hepatocyte-derived microRNA (HDmiR) 122 and miR-148a were evaluated in 66 Labrador retrievers with different histopathology and ALT levels. In the liver, miR-122 accounts for 72% of all microRNAs and miR-122 has virtually no extra-hepatic expression.[68,69] MicroRNA-122 ($r = 0.80$; $P < 0.001$) and miR 148a ($r = 0.44$; $P < 0.01$) both showed significant positive correlation with ALT levels. Only mir-122 was significantly increased in both Labradors with liver injury and normal (< 70 U/L) ALT levels (4.2 fold increase, $P < 0.01$) and Labradors with liver injury and increased (≥ 70 U/L) ALT levels (34.6 fold increase, $P < 0.001$) compared to dogs with normal liver. The increase in miR-122 levels in the Labradors with normal ALT levels was due to dogs with AH and CH, as dogs with RH did not have increased miR-122 levels. Basically, miR-148a showed similar results, but less pronounced. As shown in **Chapter 4**, ALT itself shows similar performance in detecting liver injury, because it is also better in discriminating AH (AUC 0.82) and CH (AUC 0.94) than RH (AUC 0.61) from dogs with normal liver.

The sensitivity and specificity of miR-122 to detect hepatocellular injury (RH=17, AH=10, CH=17) was 0.84% and 0.82% respectively (**Chapter 5**). The sensitivity of

miR-122 (threshold 1,278 copies) was significantly better than the sensitivity of ALT (threshold 70 U/L) to detect hepatocellular injury, which was only 55% ($P < 0.001$). However, there were no significant differences in AUC between miR-122 (AUC=0.91) and ALT (AUC=0.89), which means that both are fairly good in discriminating dogs with and without hepatocellular injury. This is in line with the results of **Chapter 4**, where the AUC of ALT was also 0.82 and 0.94 for AH and CH respectively. The reason that the sensitivity of ALT in **Chapter 5** was only found to be 55% was probably because dogs with RH and AH were also included in the liver injury group.

Together the results of **Chapter 4 and Chapter 5** (ROC curves) indicate that the current threshold for ALT (70 U/L), which is also the upper limit of the reference range, is set too high. This is true for the Labrador retriever and its breed-specific hepatopathy, which is known to be subclinically affected for a long time. In case of our studies, ROC curves show that sensitivity of ALT can be increased by decreasing its current threshold. Calculations on the data retrieved from this study indicate that sensitivities can be increased to about 90% (CH) and 75% (AH) by decreasing the threshold to 40 U/L and 47 U/L respectively (data not shown). However, decreasing the current threshold would be at the expense of the specificity. In addition, the reference range describes the range of a value in healthy individuals and is determined in a population of normal (clinically) non-diseased dogs. [70] For ALT, ALP, and BA the Veterinary Diagnostic Laboratory of Utrecht University (UVDL) determined the reference range in plasma of 79 normal non-diseased dogs of 32 different breeds. However, no histopathology results of these dogs were available. Although the Labrador retrievers used in **Chapter 4**, might not be not a good reflection of the general population, results of this study show that 122 out of 191 clinically healthy Labradors have abnormal liver histology.

We showed that miR-122 can be used as promising new biomarker of hepatocellular injury (**Chapter 5**). This is, however, not specific for the underlying disease. Therefore, the second step is to characterize a microRNA profile that can also distinguish between several canine hepatobiliary diseases. Hereto, six microRNAs (miR-21, miR-122, miR-126, miR-148a, miR-200c, and miR-222 were investigated in 46 dogs of different breeds with parenchymal (acute and chronic hepatitis), biliary (mucoceles and other biliary diseases), vascular (congenital portosystemic shunts), or neoplastic (adenomas, carcinomas, lymphomas) hepatobiliary diseases compared to 11 healthy control dogs (**Chapter 6**). These microRNAs were chosen based on the recent human literature and suggested by experts in the field that published several papers about microRNAs as biomarkers in human liver disease.[64, 65, 67, 71-74] In Figure 2 of **Chapter 6** a visual summary is provided of the different microRNA

levels per disease. As expected miR-122 levels were increased in all disease groups (acute and chronic hepatitis, mucoceles, other biliary diseases, carcinomas and lymphomas) except for congenital portosystemic shunts and adenomas. MicroRNA-21 was also increased in multiple groups, while miR-126 was uniquely increased in chronic hepatitis and miR-200c in hepatocellular carcinomas compared to the normal group. In dogs with adenomas and congenital portosystemic shunts no microRNAs were increased. Dogs with chronic hepatitis, mucoceles, carcinomas, and lymphomas all had different patterns of increased microRNAs. Remarkably, there was no group with significantly increased miR-148a levels, while the study in **Chapter 5** showed increased miR-148a levels in Labradors with acute and chronic hepatitis. Because microRNAs have shown to be well conserved between species with similar physiology,[75] these differences are more likely to be group size related than breed, sex, or age related. Concluding, the results of the study in **Chapter 6** show that there are indeed different microRNA levels in disease. This gives us the hope that it might be possible to develop a serum microRNA profile that is able to discriminate between different hepatobiliary diseases. Such a profile would be a valuable addition to conventional diagnostics. However, in the authors' opinion it would not completely replace conventional diagnostics such as ultrasonography or histological assessment of liver biopsies.

The main focus of **Chapter 7** was to find a biomarker for hepatic copper concentrations in Labrador retrievers. As illustrated by **Chapter 4** and **Chapter 8** of this thesis ALT and ALP were not correlated with hepatic copper concentrations. Consequently, normal ALT or ALP levels do not exclude copper accumulating disease. The urinary copper/zinc ratio was found to be of limited use as non-invasive biomarker of hepatic copper concentrations.[76] In addition, pilot studies showed that both ceruloplasmin and serum copper were not suitable as biomarker for hepatic copper concentrations (unpublished data). In **Chapter 5** HDmiR-122 values were 2.9 (95%CI, 1.1-8.0; $P<0.05$) times increased in Labradors with high hepatic copper concentrations (median 836 mg/kg dwl) compared to dogs with normal hepatic copper concentrations (median 317 mg/kg dwl). Although ALT levels were within reference range and there were no signs of hepatocellular injury in both groups, miR-122 values have not proven to be specific for copper-accumulating disease (**Chapter 5, Chapter 6**). Thus, the search for biomarkers for copper-accumulating disease was continued.

Several studies consistently describe the respective negative and positive correlation of erythrocyte CCS (eCCS) and SOD1 (eSOD1) protein with hepatic copper concentrations in cattle and rodents.[77-84] Although some studies[12,85,86] suggests a posttranscriptional regulation of CCS and SOD1 accompanying changing

cellular copper concentrations, the few studies conducted in humans, however, assessed CCS mRNA transcript.[87-89] In **Chapter 7** of this thesis, erythrocyte CCS (eCCS) and SOD1 (eSOD1) levels were evaluated in 15 Labrador retrievers for the association with hepatic copper concentrations. Antibodies against CCS and SOD1 proved to be applicable for use in canine samples, as demonstrated by loss of immune-reactive bands for CCS and SOD1 in siRNA treated canine bile duct epithelial cells. In the high copper group eCCS and eCCS/eSOD1 ratio were 2.37 (P<0.001) and 3.29 (P<0.001) fold decreased compared to Labrador retrievers with normal copper concentrations. Although not reaching statistical significance, eSOD1 levels tended to be positively correlated with copper concentrations (P=0.056) and to be higher (P=0.099) in the high copper group. Given the function of SOD1 in oxidative stress response,[90, 91] SOD1 levels might be influenced by inflammatory status of the patient,[79, 92] and therefore be less suitable as biomarker. Results of this pilot study suggest that eCCS and eCCS/eSOD1 ratio are potential new biomarkers for copper-associated hepatitis in the Labrador retriever.

Recommendations and future perspectives

As shown in **Chapter 4** it is important to realize that a substantial amount of clinically healthy Labrador retrievers can have abnormal liver histology. In addition, dogs with abnormal liver histology can also have normal liver enzymes (ALT, ALP) and BA, especially when they are in the subclinically stage of disease. At least this was true for the Labrador retrievers in this study. In another study conducted in Doberman pinschers, a breed also known to develop copper-associated hepatitis, 21% of the dogs were found to have a subclinical hepatitis.[93] It would be interesting to know if this is also true for the general dog population and for breeds known to have an increased prevalence of idiopathic CH, for example the American cocker spaniel, the English cocker spaniel, and the Springer spaniel.[5,94-96]

Basically, ALT was only a moderate sensitive indicator for Labrador retrievers with CH, but sensitivity could be increased by decreasing the current threshold. ROC curves for ALT for detecting subclinical primary hepatitis in the general dog population should indicate whether current thresholds can also increase sensitivity in this population. In contrast to subclinically affected dogs, in clinical ill dogs ALT, ALP, and/or BA are useful parameters that point towards a liver disease and corroborate the need for further investigations into the underlying nature of this liver disease.

Clinicians should also be aware that normal liver enzymes do not exclude hepatic copper accumulation. Since hepatic copper accumulation is also common in clinically healthy Labradors (also with normal liver histology), taking a liver

biopsy for screening purposes should be considered in clinically healthy dogs, especially when they are family members of affected dogs.

The studies in **Chapter 5 and 6** are, to the authors' knowledge, the first studies evaluating targeted microRNAs using qPCR in veterinary medicine. Moreover, these are the first exploratory studies testing serum microRNAs in dogs with hepatobiliary disease. Our future studies will further explore the possibilities of microRNAs in hepatobiliary disease. Future studies need to include more markers, more dogs, and dogs with extra-hepatic disease, since miR-122 is the only liver specific microRNA. In addition, with an untargeted microarray analysis approach, in which the simultaneous screening of a complete microRNA set is possible, we aim to look for copper-specific microRNAs as well. A major disadvantage of this approach compared to a targeted approach, is the complexity of analyzing the large data set that is created with this technique. However, before microRNAs can be used as biomarkers in a clinical setting, there are more challenges to overcome: at the moment there is no clear consensus about quantification and normalization of microRNA data in plasma or serum. In tissue, other small non-coding RNAs, for example small nuclear RNAs (snRNAs) or small nucleolar RNAs (snoRNAs), are used for data normalization.[97] The expression of such endogenous RNAs should be relatively constant and highly abundant in the target tissue, cell, or fluid. In addition they should have the same features as microRNAs (stable and small). Alternatively, the most stably expressed microRNAs for a specific experimental condition can be used to normalize the data. A major difference is that the amount of cell-free (micro)RNA in the circulation is relatively minute compared to the amount in tissue or cells. And at the moment no well-developed normalization strategy for biological variability is present.[98] Even in humans, the consistency of circulatory reference microRNAs is questionable.[99] Some studies use non-liver abundant microRNAs (but muscle- or blood-abundant microRNAs instead),[71] miR-16 [100] or miR-142-3p,[101] snRNAs,[102] one or multiple spike-in controls,[61] or a not detectable mouse microRNA [103] to normalize data. Also recent studies in veterinary medicine used human snRNAs and snoRNAs for data normalization.[104-107]However, multiple (human) studies now advocate that snRNAs, snoRNAs, or `universal' endogenous microRNAs should not be used to normalize serum/plasma data.[108-111] One study even reports that the presence of snRNAs and snoRNAs can indicate cellular contamination. [112] Recent studies involved in the discussion about normalizing microRNA conclude that, although it is tempting to use universal endogenous reference microRNA, one cannot use them in new studies without validation.[110, 113] Instead, they suggest that after the use of a spike-in control, a case-specific endogenous reference should be used. That can be achieved either by taking the mean of all expressed microRNAs or to determine the most stable

microRNAs of that particular study with algorithms including geNorm or Normfinder.[110] This is, however, only suitable for large data-sets and not in the targeted approach described in chapter 5 and 6.[111] Since even in human medicine there is still no consensus at this point, we used the synthetic spike-in control (*C. elegans*) to adjust for differences in the efficiency of RNA recovery between samples. Absolute quantification of circulating microRNAs might be the best (temporary) solution.[113] As this remains an interesting discussion with major impact on results and data presentation, future studies should therefore also focus on sample handling and preparation, quantification and normalization.

The pilot study into biomarkers for copper-associated hepatitis in **Chapter 7** shows promising results, which are currently validated in new Labrador retrievers and other dog breeds known to be affected with copper-associated hepatitis. In addition, a more precise quantification is necessary and therefore an ELISA is designed. When a commercial applicable ELISA becomes available dogs at risk for copper-associated hepatitis can be screened for the presence of high hepatic copper concentrations. This includes breeds known for hepatic copper accumulation (i.e. Labrador retriever, Doberman, West Highland white terriers, Skye terriers, Dalmatians) or dogs from high-risk pedigrees. When results indicate the presence of high hepatic copper, a liver biopsy can be advised to assess hepatic histology and distribution of copper. In addition, monitoring of copper concentrations during treatment will be facilitated.

Recently the amino acid substitution ATP7B:p.Arg1453Gln was found to be associated with hepatic copper accumulation, since functional studies of this mutant demonstrated partial mis-localization of this protein at the endosplasmatic reticulum. Under normal circumstances ATP7B is known to incorporate copper into ceruloplasmin (CP) in the trans Golgi network.[114, 115] Although earlier pilot studies did not show associations between serum CP levels and hepatic copper concentrations (unpublished data), it would be interesting to measure if there are differences in CP levels in dogs with and without the ATP7B mutation. If so, one would expect dogs with the mutations to have lower CP activity, similar to some Wilson disease patients with distinct ATP7B mutations.[116, 117] However, CP is also an acute phase protein and levels are also elevated in inflammation.[118]

Part III Treatment

Once dogs at risk or with established hepatic copper accumulation have been diagnosed, treatment is aimed to lower intrahepatic copper levels. At present, the most frequently prescribed drug in the treatment of copper-associated hepatitis is D-penicillamine (DPA). DPA promotes urinary copper excretion by binding copper at its free sulfhydryl group.[119] It has been successfully used in dogs affected with copper-associated hepatitis, including multiple different breeds.[15, 120-122] However, guidelines describing treatment duration are lacking. Since COMMD1-deficient dogs accumulate hepatic copper to a very high extent, usually around 5,000 mg/kg dwl (with excessive cases above 10,000 mg/kg dwl),[15, 123] lifelong DPA therapy is recommended. Despite one case-report of an affected Bedlington terrier developing DPA induced copper deficiency,[124] in many of these dogs normalization of hepatic copper concentrations does not occur.[15] Therapy, however, precludes progression of the disease. In breeds with complex forms of copper-associated hepatitis, for example the Labrador retriever, hepatic copper concentrations are generally not extremely high and continuous DPA treatment is not recommended. In **Chapter 8**, 43 Labrador retrievers with a hepatic copper concentration of on average 1,354±864 mg/kg dwl were treated with 10mg/kg DPA BID, 30 minutes before a meal. Median treatment duration was 4.8 months (range 1.8-15.7 month). We developed a prediction model for the necessary treatment duration in Labrador retrievers, based on initial hepatic copper concentrations. There was no evidence that age, sex, the occurrence of side effects, or medication type (compounded capsules or enteric coated tablets) influenced hepatic decoppering rates.

Even though only 50% of the dogs in **Chapter 8** reached normal hepatic copper concentrations (on average 556±433 mg/kg dwl, $P \leq 0.01$), hepatic zinc concentrations also decreased significantly (181±71 mg/kg dwl before, 138±49 mg/kg dwl after, $P \leq 0.01$). It is known that DPA also forms complexes with other metals including zinc [125] accounting for the increased urinary zinc excretion that has been found upon DPA treatment in humans and dogs.[76,126] Although DPA-zinc complexes are considered less stable, when copper concentrations decrease upon treatment relatively more zinc becomes available for excretion, potentiating the risk of zinc deficiency.[126] Clinical zinc deficiency due to DPA treatment has been described in humans but not in dogs.[127] Clinical signs of (mild) zinc deficiency in dogs are mainly of gastro-intestinal and/or dermatological origin and may include anorexia, poor wound healing, alopecia, or cutaneous lesions.[128] Whether clinical signs of zinc deficiency in dogs are underestimated, not recognized as zinc-related, or simply not present is not clear. However, the results of this study show that zinc deficiency during DPA treatment is a realistic risk of which clinicians should be aware.

DPA treatment also resulted in a significantly improvement of the hepatitis; median grading scores for necro-inflammatory activity decreased from 1 to 0 ($P \leq 0.01$). First, this can be explained by the decrease in hepatic copper. As shown by the results in **Chapter 3**, copper activates multiple cellular pathways leading to inflammation, which became only appreciable in the next stage of the disease. When the trigger for this inflammation is removed, necro-inflammatory activity will decrease subsequently. A second explanation may be found in the immuno-modulating activity of DPA itself.[129]

We established two minor drawbacks with the use of DPA. First, side effects (anorexia and vomiting) are relatively common encountered side effects, especially when DPA is administered without food. However, administration with food markedly reduces bioavailability and maximum plasma drug concentrations, leading to decreased efficacy of DPA.[130] Second, to normalize hepatic copper concentration the necessary treatment period is long. This combination can prevent owners to pursue treatment.

Recommendations and future perspectives

Treatment of high (>400 mg/kg dwl) hepatic copper concentrations with DPA is advised when there is clinical illness and/or histological evidence of hepatocellular injury. Treatment should be continued until normal hepatic copper concentrations are achieved. In Labrador retrievers, the model can be used to determine treatment duration and optimal time of recheck biopsies; for example dogs with copper concentrations of 1,000 or 1,500 mg/kg dwl need treatment for at least 6 or 10 months respectively. Most likely, this model can also be used for other dog breeds with copper concentrations in a similar range. It is important to recheck both quantitative copper and zinc concentrations as well. After normalization, a low copper high zinc diet[131] alone or in combination with intermittent DPA chelating, can prevent copper (re)accumulation and concurrently avoid copper- and zinc deficiencies. Recheck biopsies every 1-2 years are advised.

The management of copper-associated hepatitis comprises multiple invasive liver biopsies for the assessment of hepatic histology, copper distribution and copper quantification. ALT and ALP levels only correlate with grade of the hepatitis but do not correlate with hepatic copper concentrations (**Chapter 4**, **Chapter 8**) and can therefore not replace these procedures. Owner compliance and willingness will considerable increase if part of these biopsies can be replaced by blood sampling for eCCS/eSOD1 ratio and treatment monitoring can be facilitated by measuring eCCS/eSOD1 ratio. (**Chapter 7**).

To overcome the two drawbacks of DPA treatment, side effects and treatment duration, future studies should also focus on new copper chelators. In humans ammonium tetrathiomolybdate (TTM) is used in patients with neurological presentation of Wilson disease.[132] TTM removes copper from metallothioneins and excretes copper via the urine and bile.[133] Besides its extensive decoppering effects, TTM also has major anti-inflammatory properties and anti-angiogenic effects.[134] In healthy dogs, TTM administration resulted in increased serum copper concentrations.[135] Other possible therapeutics worthwhile investigating are melatonin and its metabolites,[136] the glycoconjugate Chel2,[137] and especially the peptide methanobactin. Methanobactin is recently described by Lichtmannegger and colleagues (2016) as possible alternative for current copper chelators in humans (DPA, trientine, TTM).[138] It has a very high affinity for copper and was able to deplete accumulated mitochondrial copper via excretion into the bile without significant cellular toxicity.

As additional treatment, future studies should evaluate beneficial effects of SAM treatment in dogs with (copper-associated) liver disease, as described previously (**Part I, Chapter 3**).

Conclusions

We have made great progress in the understanding of the pathogenesis, diagnosis and treatment of Labrador retrievers suffering from copper toxicosis. Although several challenges exist, the combined results in this thesis will provide a valuable basis for clinicians to diagnose and treat Labradors in an early stage of the disease.

Key findings

I

Pathogenesis (Chapter 3)

- Hepatic copper accumulation results in transcriptomic alterations and can be considered as a primary initiating factor
- Pathways in progression towards copper-associated chronic hepatitis involve inflammation, coagulation, cell adhesion, cytoskeleton, development, and apoptosis
- The Alzheimer disease protein APP, which is proposed to function in copper efflux, was upregulated in the HCCH group
- The Labrador as a non-rodent model for (copper-associated) hepatitis

II

Diagnosis: Biomarkers (Chapter 4, 5, 6, and 7)

- Normal ALT, ALP and BA do not preclude hepatic copper accumulation
- Labradors with PH have significantly higher ALT levels than dogs with RH
- For every 1 U/L increase in ALT, there is a two percent increase in the odds of having a PH
- ALP and BA are no good indicators for AH and CH in subclinical dogs, whereas ALT is only moderate sensitive (71%) in detecting dogs with CH
- HDmiR-122 and miR-148a correlate with ALT levels
- The HDmiR-122 increase in dogs with liver injury and normal ALT was due to dogs with acute and chronic hepatitis
- HDmiR-122 has superior sensitivity (84%) over ALT (55%) in detecting liver injury and is a liver specific microRNA
- miR-21, miR-122, miR-126, miR-200c, and miR-222 have different levels in dogs with parenchymal, biliary, and neoplastic disease
- eCCS and eCCS/eSOD1 ratio are promising new biomarkers for hepatic copper accumulation

III

Treatment (Chapter 8)

- The relation between hepatic copper concentration and therapy duration is non-linear
- A predictor model for hepatic copper decrease upon D-penicillamine therapy in Larbador retrievers is now available
- DPA treatment results in improvement of necro-inflammatory activity
- Hepatic zinc deficiency is a potential risk of DPA treatment

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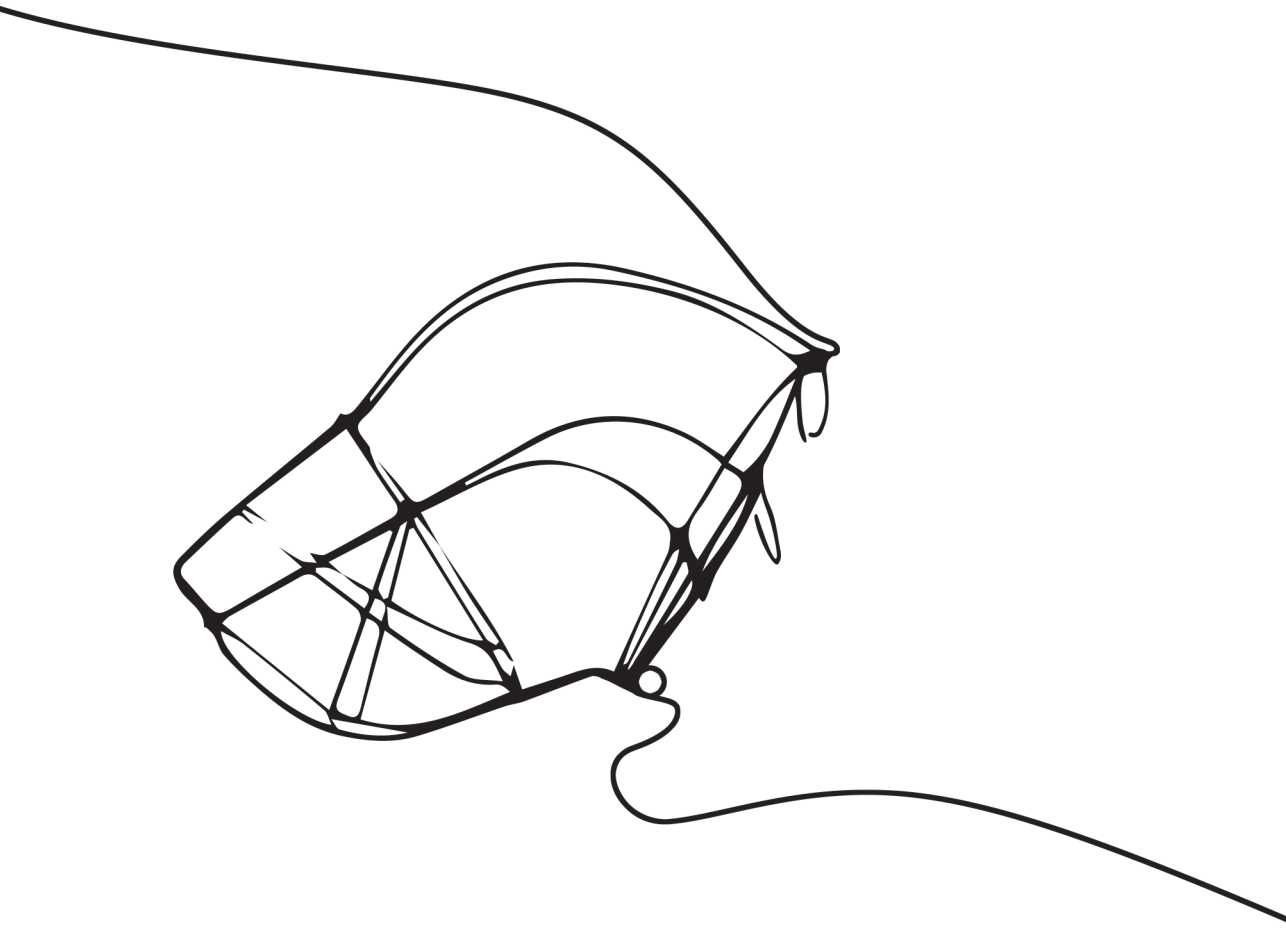
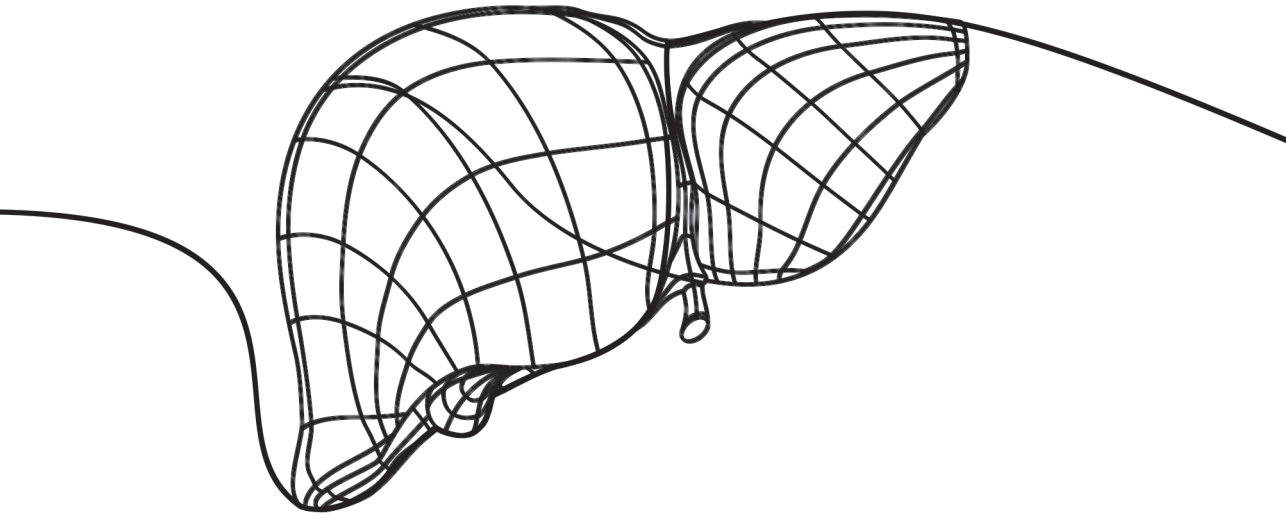


Addendum



Addendum

Nederlandse samenvatting
voor niet-ingewijden

Koper is een essentieel element dat vele vitale functies in het lichaam vervult. Wanneer dit element echter in overmaat aanwezig is, kan er ernstige celschade ontstaan door de vorming van zuurstofradicalen. Daarom is het kopermetabolisme in het lichaam zeer zorgvuldig gereguleerd. Het belang van deze zorgvuldige regulatie wordt geïllustreerd door de ziektes die ontstaan wanneer kopertransport-eiwitten niet goed functioneren.

Erfelijke kopergeassocieerde leverontsteking (hepatitis) is beschreven in mensen en honden, maar ook in andere zoogdieren zoals knaagdieren en herkauwers. In dit proefschrift ligt de focus vooral op de Labrador retriever, een van de populairste hondenrassen in Nederland.

In **Hoofdstuk 1** worden de belangrijkste doelstellingen van dit proefschrift beschreven. Ten eerste een beschrijving van het ziekteproces en de rol van koper in het ontstaan van kopergeassocieerde hepatitis (**Deel I, Hoofdstuk 3**). Ten tweede het vereenvoudigen van de diagnose van zowel kopergeassocieerde hepatitis als leverziekten in honden in het algemeen (**Deel II, Hoofdstuk 4, 5, 6** en **7**). Het laatste was gericht op de verbetering van de huidige behandeling van honden met kopergeassocieerde hepatitis (**Deel III, Hoofdstuk 8**). Tenslotte wordt afgesloten met een (Engelse) samenvatting van de belangrijkste resultaten van dit proefschrift, waarbij deze resultaten ook worden bediscussieerd (**Hoofdstuk 9**).

In **Hoofdstuk 2** wordt een overzicht gegeven van de koperstofwisseling op celniveau en de ziekten die in mens en dier kunnen ontstaan wanneer er iets misgaat in deze regulatie. Omdat losse koperionen schadelijk zijn voor de cel en zijn componenten, worden koperionen gebonden aan eiwitten. Koper uit drinkwater en voedsel wordt met name opgenomen in de dunne darm. Opname door de darmcel vindt plaats middels het eiwit CTR1. Vanuit de darmcel wordt koper doorgesluisd naar de bloedbaan via ATP7A, waar het gebonden aan andere eiwitten via de poortader de lever bereikt. Wanneer koper is opgenomen (via CTR1) door de levercel, moet het ook weer meteen gebonden worden aan eiwitten om schade aan de levercel te voorkomen. De lever heeft een centrale rol in de koperstofwisseling en is betrokken bij de opslag, herverdeling en uitscheiding van koper via de gal. Gespecialiseerde koperchaperone-eiwitten (ATOX1, CCS, COX17) transporteren koper naar hun eindgebruikers in de levercel (ATP7B, SOD1, CCO). Een groot deel van het koper verlaat de lever (ATP7B, CP) en wordt via de bloedbaan weer aangeboden aan andere organen. Een teveel aan koper wordt voornamelijk uitgescheiden via de gal (ATP7B, COMMD1).

Wanneer deze kopertransporteiwitten niet goed functioneren ontstaat er een overmaat of een tekort aan koper. Mensen met de ziekte van Menkes hebben een tekort aan koper omdat de kopertransporter ATP7A niet goed functioneert. De best beschreven (erfelijke) koperstapelingsziekte bij de mens is de ziekte van Wilson. Deze wordt veroorzaakt door mutaties in het gen dat codeert voor het kopertransporteiwit ATP7B. Door het niet goed kunnen uitscheiden van koper, stapelt koper zich op in, onder andere, de lever en in zenuwweefsel. Dit leidt tot ernstige leverschade en overmatige aanmaak van bindweefsel (levercirrose) en neurologische verschijnselen. De ziekte van Wilson is een zeldzame stofwisselingsziekte en onderzoek is daarom slechts beperkt mogelijk. Koperstapelingsziekten in honden komen daarentegen vaker voor en zijn een van de meest voorkomende oorzaken van hepatitis in honden. De Bedlington terriër is het eerste ras waarin kopergeassocieerde hepatitis goed onderzocht is. Een mutatie in het gen dat codeert voor het kopertransporteiwit COMMD1, zorgt voor extreem hoge koperconcentraties in de lever. Door de ontwikkeling van een DNA-test, komt de ziekte in Bedlington terriërs gelukkig bijna niet meer voor. De Labrador retriever is het tweede ras waar een deel van de genetische achtergrond is opgehelderd. Mutaties in zowel het ATP7B-gen (ziekte van Wilson) en het ATP7A-gen (ziekte van Menkes) zijn van invloed op de koperconcentraties in de lever. In andere hondenrassen (onder andere de Doberman, West Highland white terriër en Dalmatiër) wordt tot op heden een genetische oorzaak vermoed, maar deze is nog niet aangetoond. Koperstapeling in andere rassen dan de Bedlington terriër, is vaak minder extreem en lijkt daarbij ook te zijn gerelateerd aan koperopname via de voeding.

Omdat de koperstofwisseling in mensen en honden voor een groot deel hetzelfde is en er veel overeenkomsten zijn tussen de koperstapelingsziektes in mensen en honden, kunnen honden ook als model voor de mens gebruikt worden. Er zijn echter ook een aantal verschillen te noemen tussen humane koperstapelingsziekten en hun equivalenten in honden. Zo wordt er in honden met kopergeassocieerde hepatitis, in tegenstelling tot mensen met de ziekte van Wilson, geen koperstapeling gezien in zenuwweefsel en is er dus ook geen sprake van neurologische verschijnselen. Ook is de plaats van koperstapeling in de lever in honden juist heel specifiek (rond de centrale vene) in tegenstelling tot die in mensen met de ziekte van Wilson. Omdat een teveel aan koper wordt uitgescheiden door de galwegen, is het voor de hand liggend dat hoge koperconcentraties in de lever ook zouden kunnen ontstaan ten gevolge van galwegziekten, zoals beschreven is bij mensen. Op dit moment wordt echter aangenomen dat dergelijke hoge leverkoperconcentraties in honden niet worden veroorzaakt door galstuwing, maar door een primair defect in de koperstofwisseling.

Het eerste doel van de studie - beschreven in **Deel I, Hoofdstuk 3** - was om de primaire rol van koper in het ontstaan van de kopergeassocieerde hepatitis in Labrador retrievers te bevestigen. Daarnaast werd beoogd meer informatie te verkrijgen over het verloop van het ziekteproces. Om het ziekteproces weer te geven is leverweefsel gebruikt van 31 verschillende Labrador retrievers. Deze zijn onderverdeeld in vier groepen, waarvan de laatste drie groepen kunnen worden gezien als drie opeenvolgende stadia van de ziekte: honden met een normale lever en een normale hoeveelheid koper (N), honden met een normale lever maar een verhoogde concentratie koper in de lever (HC), honden met een hepatitis en een verhoogde concentratie koper in de lever (HCH) en honden met een chronische hepatitis en een verhoogde concentratie koper in de lever (HCCH). Elke groep werd vergeleken met de voorgaande, wat resulteerde in de volgende vergelijkingen: N-HC, HC-HCH en HCH-HCCH.

Een goede manier om te inventariseren welke biochemische signaleringspaden belangrijk zijn in het ziekteproces, is het meten van de hoeveelheid RNA in de cel. Dit RNA speelt een belangrijke rol in het tot expressie brengen van de genetische informatie (DNA). Door middel van RNA worden de juiste eiwitten in de juiste hoeveelheden op het juiste tijdstip aangemaakt en worden de celprocessen aangestuurd. De hoeveelheid RNA van vooraf bepaalde genen met een functie in de koperstofwisseling en oxidatieve stress is gemeten met een hele gevoelige techniek (qPCR). Een andere methode die in deze studie is gebruikt, is microarray-analyse. Hiermee is de expressie van alle bekende genen in de cel gemeten, waardoor een compleet genprofiel van het onderzochte leverweefsel is verkregen.

Uit de resultaten van de studie is gebleken dat koper inderdaad een primaire rol heeft in het ontstaan van de ziekte. Er is namelijk aangetoond dat in de initiatiefase van de ziekte (N-HC), waarin de lever nog geen histopathologische afwijkingen laat zien, er al wel verandering is in verschillende genen (waaronder MT en COMMD1) en signaleringspaden. De meeste signaalwegen in de HC-groep zijn geassocieerd met ontsteking, maar er treden ook al veranderingen op in celadhesie en celontwikkelingspaden. Naarmate het ziekteproces vordert (HC-HCH en HCH-HCCH) zijn er ook histopathologische aanwijzingen voor hepatitis zonder of met bindweefselvorming (fibrose). Deze studie toont inderdaad aan dat genen betrokken bij ontsteking, immuunrespons en de stollingscascade vooral van belang zijn in de Labradors met hoog kopergehalte en hepatitis (HCH). In de laatste fase van de ziekte, waarin duidelijke bindweefselvorming is opgetreden (HCCH), wordt het belang van celadhesie, apoptose (geprogrammeerde celdood), verbind-weefseling en herschikking van het celskelet bevestigd. Ontstekingssignaalpaden en de immuunrespons spelen dan een meer ondergeschikte rol. In dit stadium van

de ziekte zien we ook een verhoging van het gen dat codeert voor het amyloid precursor protein (APP). APP is bekend vanwege zijn betrokkenheid bij de ziekte van Alzheimer. Het idee is dat APP zorgt voor het transport van koper uit lever- en zenuwcellen. De verhoogde expressie van het APP-gen in Labrador retrievers met chronische koperstapeling zou daarom een adaptieve reactie kunnen zijn op langdurige hoge koperconcentraties. Eerdere studies hebben al laten zien dat oxidatieve stress door een teveel aan koper bijdraagt aan leverschade in zowel mens als dier. Ook in deze studie is er een verandering van genen en signaalpaden, betrokken bij oxidatieve stress, te zien in de verschillende opeenvolgende stadia.

Omdat we uit andere studies weten dat het reactiepatroon van de lever vergelijkbaar is tussen mens en hond, kan de Labrador retriever fungeren als een model voor de mens met kopergeassocieerde hepatitis. Omdat deze studie ook laat zien dat het reactiepatroon van de lever vanaf het hepatitisstadium niet heel koperspecifiek meer is, kan de Labrador mogelijk ook model staan voor mensen met chronische hepatitis door andere oorzaken. De resultaten van deze studie kunnen daarom in een breder perspectief geplaatst worden.

In **Deel II (Hoofdstuk 4 tot en met 7**) van dit proefschrift werd gekeken naar het optimaliseren van de diagnose van zowel kopergeassocieerde hepatitis als leverziekten in het algemeen.

Wanneer koper zich opstapelt in de lever, leidt dit tot hepatitis en uiteindelijk levercirrose. Met name honden met kopergeassocieerde hepatitis en honden met chronische hepatitis door onbekende oorzaak (idiopathische chronische hepatitis) lijken lang klinisch gezond. Dit komt omdat de lever een grote reservecapaciteit heeft, waardoor ziekteverschijnselen vaak pas optreden als al veel leverweefsel is aangetast. Initieel zijn klinische verschijnselen erg aspecifiek (niet eten, lusteloosheid, overgeven) en kunnen pas later gaan wijzen op betrokkenheid van de lever (geelzucht, waterbuik, veel drinken en veel plassen). Omdat ziekteverschijnselen pas in een laat stadium van de ziekte optreden, wordt een behandeling pas laat ingezet en zal deze in de meeste gevallen ook minder toereikend zijn. Een tijdige diagnose is dan ook essentieel.

Op dit moment kan de diagnose van (kopergeassocieerde) hepatitis alleen met zekerheid gesteld worden door het laten onderzoeken van een histologisch leverbiopt (stukje leverweefsel dat met een dikke naald weg wordt genomen). De hoeveelheid koper wordt geschat door het leverweefsel te kleuren met een speciale koperkleuring en kan worden gekwantificeerd door middel van neutronenactiveringsanalyse. Een eenvoudigere manier om de overmaat koper in de lever vast te stellen

is bij honden nog niet gevonden. Daarnaast wordt in het leverbiopt ook de ernst van de ontsteking (hepatitis) en de aanwezigheid van bindweefsel (fibrose) beoordeeld.

Voor het vaststellen van leverziekten in het algemeen wordt in de praktijk veel gebruik gemaakt van leverenzymen en leverfunctietesten. Voorbeelden van twee belangrijke leverenzymen zijn alanine aminotransferase (ALT) en alkaline phosphatase (ALP). Beiden hebben een verschillende sensitiviteit (kans op een positieve test als een dier ziek is) en specificiteit (kans op een negatieve test als een dier niet ziek is) en worden gebruikt voor het vaststellen van schade aan de lever. De functie van de lever kan worden beoordeeld door zijn producten of stoffen die door de lever uit het bloed worden verwijderd te meten, bijvoorbeeld galzuren, bilirubine, ammoniak of albumine. Omdat het juist belangrijk is dieren met leverziekten in een zo vroeg mogelijk ziektestadium op te kunnen sporen (wanneer ze nog geen ziekteverschijnselen hebben), is het belangrijk om te weten hoe betrouwbaar deze levertesten in die gevallen zijn.

In **Hoofdstuk 4** hebben we data van 242 Labrador retrievers bekeken waarvan ALT, ALP, galzuren, koperwaarden en leverhistologie bekend waren. Als eerste hebben we gekeken naar de koperwaarden in deze honden. Hoge koperconcentraties in de lever kwamen ook voor bij alle soorten leve histologie, maar waren het hoogste bij honden met acute en chronische hepatitis. Het was niet mogelijk om met behulp van de leverwaarden (ALT, ALP of galzuren) te voorspellen of dieren hoge concentraties koper in hun lever hadden. Omdat er geen verschil zat tussen de dieren met hoge en normale koperconcentraties, is er voor de rest van de studie geen onderscheid meer gemaakt tussen honden met hoog of normaal kopergehalte.

Dieren met hoge concentraties leverenzymen kunnen ook een reactielever hebben. Dit is een reactie van het leverweefsel op ontstekingsprikkels elders in het lichaam en is in wezen geen primaire leverziekte. Een belangrijke vraagstelling van **Hoofdstuk 4** is of het mogelijk is onderscheid te maken tussen een primaire leverontsteking (acute en chronische hepatitis) en een reactielever met behulp van de leverenzymen ALT en ALP. Tachtig honden (zowel ziek als klinisch gezond) hadden een verhoging van een of beide leverenzymen. We hebben gevonden dat ALT significant hoger is in honden met een primaire hepatitis dan in honden met een reactielever of een normale lever. Voor elke eenheid toename in ALT, is er twee procent meer kans op het hebben van een primaire hepatitis.

Het tweede belangrijke doel van deze studie was het bepalen van de sensitiviteit en specificiteit van ALT, ALP en galzuren in klinisch gezonde Labrador retrievers. Van de 242 honden hadden 51 honden ziekteverschijnselen. In slechts twee zieke

dieren waren ALT, ALP en galzuren binnen normale waarden. Van de 191 klinisch gezonde honden had 19% een primaire hepatitis, 45% een reactielever en 36% een normale lever. Van de klinisch gezonde honden hadden 146 honden ALT, ALP en galzuren binnen normale waarden. De sensitiviteit en specificiteit voor het aantonen van chronische hepatitis, acute hepatitis en reactielever in klinisch gezonde Labrador retrievers zijn berekend middels ROC-curves. Dit is een grafiek waarbij de fractie positieven (sensitiviteit) wordt uitgezet tegen de fractie vals positieven (1-specificiteit) voor verschillende drempelwaarden. De specificiteit van ALT, ALP en galzuren was respectievelijk 93%, 99% en 90% (hetzelfde voor chronische, acute hepatitis en reactielever). In de werkelijkheid zal de specificiteit echter lager zijn omdat de Labrador retrievers geselecteerd voor deze studie bijvoorbeeld geen medicijnen kregen, wat deze uitslagen nog kan beïnvloeden. Zowel ALP als galzuren blijken in Labrador retrievers een erg lage sensitiviteit te hebben om chronische hepatitis, acute hepatitis en reactielever te detecteren. ALT is de test met de beste sensitiviteit: 71% voor een chronische hepatitis en 45% voor een acute hepatitis. Echter, ook dit is een matige sensitiviteit. Door de huidige drempelwaarde van ALT te verlagen, kan de sensitiviteit van ALT worden verhoogd. Dit gaat echter ten koste van de specificiteit. In het kader van deze bevindingen is het ook belangrijk om te realiseren dat de huidige referentiewaarden (Universitair Veterinair Diagnostisch Laboratorium, Utrecht) bepaald zijn in een groep van 79 gezonde honden van 32 verschillende rassen. Echter, van deze dieren was geen leverhistopathologie beschikbaar, terwijl onze studie juist aantoont dat een substantieel deel van de klinisch gezonde dieren abnormale leverhistologie heeft (en dus eigenlijk niet gezond is).

Omdat uit deze studie is gebleken dat meer sensitieve biomarkers voor leverschade nodig zijn, is gezocht naar nieuwe kandidaten. **Hoofdstuk 5** is het eerste hoofdstuk in dit proefschrift waarin nieuwe kandidaat biomarkers worden geëvalueerd: de microRNAs. MicroRNAs zijn hele kleine stukjes goed geconserveerd, niet-coderend (er wordt geen eiwit gevormd) RNA. Ze kunnen de expressie van andere stukken RNA beïnvloeden door te interfereren met hun translate naar eiwit en bepalen zo het verloop van allerlei processen in de cel. Ze hebben dus een belangrijke rol in de regulering van genetische programma's die de cel aansturen en zijn betrokken bij verschillende ziekteprocessen, waaronder kanker. MicroRNAs zijn terug te vinden in weefsels, maar ook in de circulatie, hoewel in lagere concentraties. Omdat ze stabiel zijn in bloed, zijn het ideale biomarkers. Humaan zijn er al veel studies gepubliceerd over microRNAs in patiënten met een variëteit aan leverziekten.

In **Hoofdstuk 5** is onderzocht of twee leverspecifieke microRNAs, miR-122 en miR-148a, geschikt zijn als serum biomarker voor leverschade in 66 Labrador retrievers. Van miR-122 is bekend dat deze bijna geen expressie heeft in andere organen dan de lever. Beiden worden ook vergeleken met het leverenzym ALT. Zowel miR-122 als miR-148a hebben een significant positieve correlatie met ALT. MicroRNA-122 is significant hoger in Labradors met leverschade en hoog ALT, maar is ook significant hoger in Labradors met leverschade en normaal ALT dan in Labradors met normale levers. MicroRNA-148a was alleen verhoogd in Labradors met leverschade en een verhoogd ALT. De sensitiviteit van miR-122 om leverschade te detecteren was met 84% significant beter dan de sensitiviteit van ALT (55%). MicroRNA-122 waarden waren ook hoger in Labradors met hoge leverkoper-concentraties (normale leverhistologie, normaal ALT) dan in Labradors met normale leverkoperconcentraties (normale leverhistologie, normaal ALT).

Deze resultaten laten zien dat met name microRNA-122 gebruikt kan worden als een nieuwe biomarker voor leverschade. Het nadeel van de tot op heden gebruikte levertesten is dat ze alleen de aanwezigheid van leverziekte kunnen vast stellen, maar niet kunnen onderscheiden wat voor leverziekte er precies speelt. Om dit te weten te komen, zullen de resultaten van verschillende onderzoeken (bijvoorbeeld echografie, histopathologie) gecombineerd moeten worden. In de praktijk zou het een stuk eenvoudiger zijn als op basis van bloedonderzoek al duidelijk kan worden wat voor type leverziekte er speelt. De dierenarts kan dan een gerichte follow-up test inzetten of wellicht al starten met een behandeling.

In **Hoofdstuk 6** zijn zes verschillende microRNAs gemeten in 57 honden (46 met een leverziekte en 11 gezonde honden) van verschillende rassen, met als doel het karakteriseren van een microRNA-profiel voor verschillende leverziekten. Volgens de richtlijnen van de World Small Animal Veterinary Association kunnen leverziekten worden onderverdeeld in vier hoofdgroepen: parenchymziekten, galwegziekten, vasculaire (bloedvaten) ziekten en neoplastische (tumoren) ziekten. Deze hoofdgroepen zijn in deze studie nog onderverdeeld in een of meerdere subgroepen, zodat er in totaal acht subgroepen zijn. De zes microRNAs (miR-21, miR-122, miR-126, miR-148a, miR-200c en miR-222) zijn uitgekozen op basis van de beschikbare humane literatuur of aanbevolen door experts op het gebied van microRNAs. Figuur 2 van **Hoofdstuk 6** is een visuele samenvatting van de verschillende microRNAs per leverziekte in verhouding tot de normale groep. In vergelijking met de normale groep, zijn de serum waarden van microRNAs niet verhoogd in honden met een levershunt (vasculaire ziekte) of een adenoom (neoplastische ziekte). De leverspecifieke miR-122 is verhoogd in de zes overige subgroepen. De andere microRNAs zijn verhoogd in minder (sub)groepen waardoor

de (sub)groepen een verschillend microRNA-profiel hebben. Zo hebben bijvoorbeeld de drie neoplastische ziekten (adenomen, carcinomen en lymfomen) elk hun eigen microRNA-profiel, welke ook weer verschilt van dat van honden met acute of chronische hepatitis. Dit zijn de eerste veelbelovende resultaten, die in de toekomst hopelijk zullen leiden tot het vereenvoudigen van de diagnostiek van leverziekten in honden.

Zoals in **Hoofdstuk 4** is beschreven, correleren de leverenzymen ALT en ALP niet met leverkoperconcentraties. Sterker nog: leverenzymen zijn een maat voor leverschade en in **Hoofdstuk 3** is beschreven dat hoge koperconcentraties in de lever al tot veranderingen in de levercel kunnen leiden zonder dat er op weefselniveau tekenen van schade zichtbaar zijn. Omdat met de huidige biomarkerstudies van **Hoofdstuk 5** en **Hoofdstuk 6** nog geen biomarker is gevonden die specifiek is voor (alleen) kopergeassocieerde hepatitis, is verder gezocht naar nieuwe biomarkers.

Sinds het begin van de 21e eeuw is er met name bij knaagdieren veel onderzoek gedaan naar de eiwitten CCS en SOD1. CCS is een koperchaperone dat koper bindt en transporteert naar SOD1, welke de cel beschermt tegen oxidatieve stress. De meeste studies beschrijven de veranderingen van CCS en SOD1 in rode bloedcellen van knaagdieren met een geïnduceerd kopertekort. Een enkele studie bij ratten beschrijft echter ook een (omgekeerde) relatie van CCS in ratten met koperstapeling in de lever. In het eerste deel van de studie, beschreven in **Hoofdstuk 7**, wordt bevestigd dat CCS en SOD1 ook in verschillende typen hondencellen meetbaar zijn. Nadat bevestigd is dat de concentratie van beide eiwitten in rode bloedcellen van honden gemeten kan worden, is gekeken of deze eiwitten ook in Labrador retrievers in relatie staan tot de concentratie koper in de lever. Zowel CCS als de ratio tussen CCS en SOD1 (CCS/SOD1 ratio) zijn significant geassocieerd met de concentratie koper in de lever. SOD1, leeftijd en geslacht zijn dit niet. In de tien honden met een hoge concentratie koper in de lever zijn zowel CCS als CCS/SOD1-ratio significant lager dan in de vijf honden met een normale concentratie koper in de lever. SOD1 alleen is net niet significant hoger in honden met hogere koperconcentraties in de lever, maar wanneer SOD1 wordt meegenomen in de ratio wordt het verschil tussen beide groepen nog duidelijker. De waarden van CCS en CCS/SOD1-ratio in beide groepen zijn zodanig verschillend dat er geen overlap tussen de twee groepen zit. Daarmee lijken CCS en SOD1 veelbelovende biomarkers te zijn.

Wanneer honden met kopergeassocieerde hepatitis zijn geïdentificeerd, zal een behandeling worden ingesteld. Deze is er op gericht het kopergehalte in de lever te verlagen door de koperuitscheiding te bevorderen en de koperinname met het

dieet te beperken. Het meest gebruikte medicijn in honden om de koperuitscheiding te bevorderen is D-penicillamine. Tot op heden zijn er geen duidelijke richtlijnen voor het gebruik van dit middel. We weten dat Bedlington terriërs door hun extreem hoge koperconcentraties levenslang dit medicijn nodig hebben. We denken echter dat dit niet per se nodig is voor andere rassen, zoals de Labrador retriever.

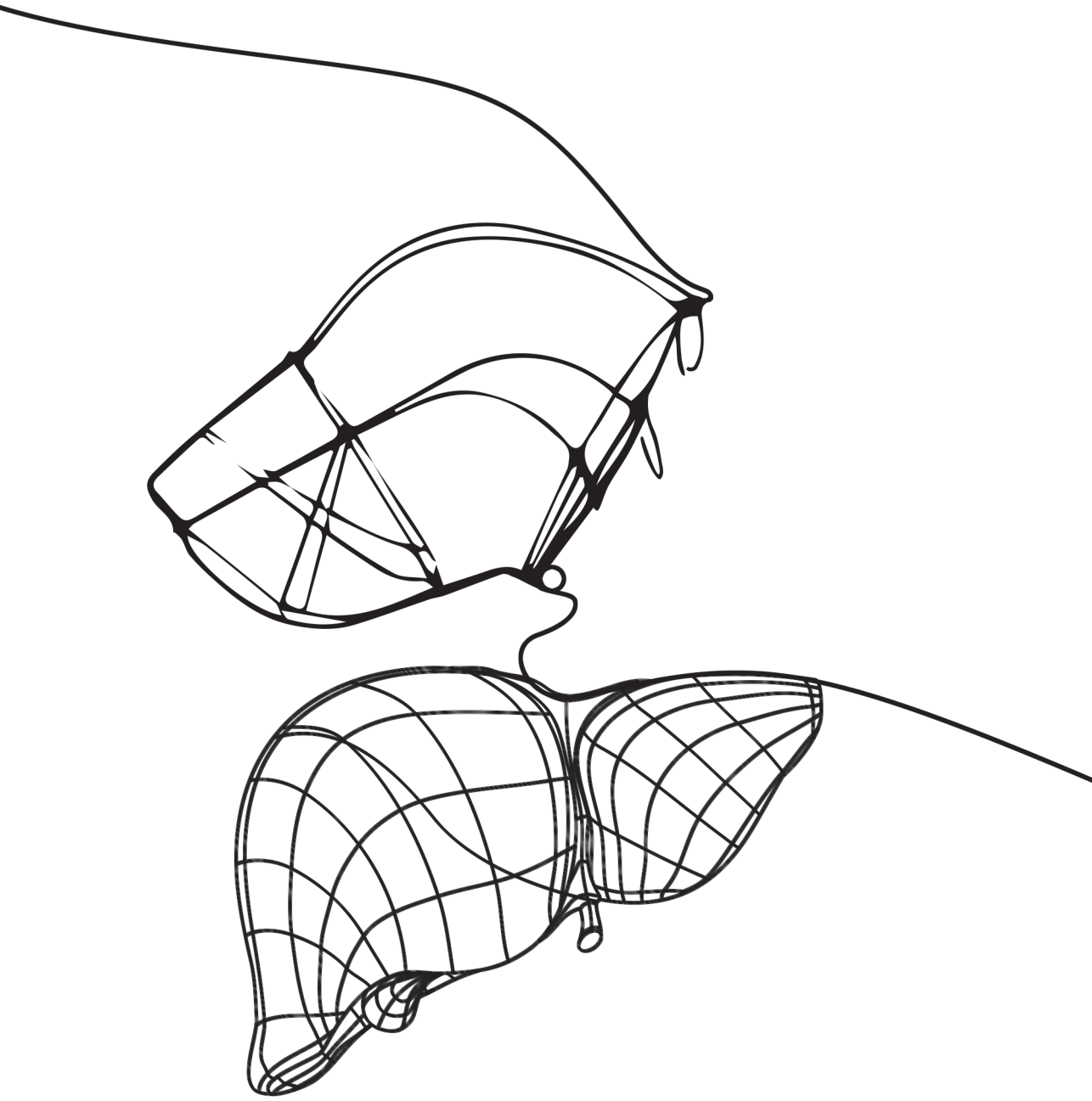
In het laatste deel, **Deel III, Hoofdstuk 8**, hebben we geprobeerd het behandelingsprotocol met D-penicillamine verder te optimaliseren. De eerste vraag die we beantwoord wilden hebben was hoe lang de individuele hond behandeld moet worden met D-penicillamine om de leverkoperconcentraties te normaliseren. Met behulp van de parameters die zijn geschat op basis van de 43 Labrador retrievers, die in deze studie behandeld zijn met D-penicillamine, is deze vraag nu te beantwoorden. De benodigde behandelingsperiode is afhankelijk van de hoogte van de koperwaarden voorafgaand aan de therapie en is niet afhankelijk van leeftijd, geslacht, bijwerkingen of formulering van het medicijn. Zo dienen honden met een koperwaarde van 1000 (milligram/kilogram droog gewicht lever) ongeveer zes maanden behandeld te worden. Eerder hoeft er dus geen controle leverbiopt genomen te worden.

We hebben gezien dat niet alleen de leverkoperconcentratie daalt door behandeling met D-penicillamine, maar ook de zink concentratie in de lever daalt significant. De ijzerconcentraties in de lever blijven onveranderd. In Labrador retrievers is een levenslange behandeling met D-penicillamine, vanwege het risico op zowel een koper- als een zinktekort, daarom niet geïndiceerd. Tenslotte is gekeken naar het effect van D-penicillamine op de ernst van de ontsteking en de aanwezigheid van bindweefselvorming. We hebben gevonden dat er na de behandeling met D-penicillamine een verbetering op is getreden van de ontstekingsveranderingen in de leverbiopten. Dit kan komen omdat de koperconcentraties lager zijn of omdat D-penicillamine zelf ook de ontsteking heeft geremd.

Behandeling met D-penicillamine zal moeten worden doorgezet totdat de koperconcentraties in de lever zijn genormaliseerd. Daarna kan met een dieet dat arm is aan koper en rijk is aan zink worden gekeken of de koperconcentraties in de lever laag kunnen worden gehouden. In sommige gevallen zal een intermitterende behandeling met D-penicillamine toch weer nodig zijn. Omdat er nu nog geen biomarkers beschikbaar zijn die de hoogte van het gehalte aan koper in de lever weerspiegelen of aan kunnen geven hoe erg de ontsteking en de bindweefselvorming in de lever zijn, is het aan te bevelen ook na normalisatie regelmatig controle-leverbiopten te nemen (elke 1-2 jaar).

In **Hoofdstuk 9** van dit proefschrift worden alle behaalde resultaten samengevat en bediscussieerd. Daarnaast worden aanbevelingen gedaan en suggesties gegeven voor toekomstig onderzoek.

Concluderend kan worden gezegd dat de resultaten, die in dit proefschrift zijn beschreven, een grote bijdrage leveren aan de kennis van kopergeassocieerde hepatitis. Er worden verschillende nieuwe biomarkers geïntroduceerd die er mogelijk in de toekomst voor zorgen dat honden met (kopergeassocieerde) leverontsteking niet alleen in een eerder stadium, maar ook op een minder belastende en eenvoudigere manier kunnen worden gediagnosticeerd. Het geoptimaliseerde behandelingsprotocol geeft clinici een goede basis voor de behandeling van kopergeassocieerde leverontsteking.



Addendum

About the author

Curriculum Vitae

Karen Dirksen was born on December 24, 1986 in Kaatsheuvel, the Netherlands. She started her veterinary education in 2006 at the Faculty of Veterinary Medicine, Utrecht University. After graduating cum laude for the first four years of the curriculum she was invited to participate in the Honours Program of the Faculty of Veterinary Medicine in 2010, granting her the opportunity to perform one year of research. Under supervision of Prof. Dr. Jan Rothuizen, Dr. Peter A.J. Leegwater, Dr. Bart Spee and Dr. Hille Fieten she investigated multiple aspects of copper-associated hepatitis in Labrador retrievers. With her project entitled: 'pathways in progression of copper associated chronic hepatitis in the Labrador retriever' she was granted a Veterinary Students Scholarship from the Morris Animal Foundation. In 2012 she was invited to present her research on the Morris Animal Foundation meeting in Denver, Colorado, which was rewarded with the second prize. During her clinical rotations (2011-2013) she continued with this research project and after graduating with honours in 2013 she enrolled as a PhD student. Her PhD studies into the pathogenesis, diagnosis and treatment of copper-associated hepatitis in dogs was supervised by Prof. Dr. Jan Willem Hesselink, Prof. Dr. Iwan Burgener, Dr. Bart Spee, and Dr. Hille Fieten. Concurrently she started working at the after hours emergency clinic and intensive care unit of the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine (2013-2016). In September 2016 she started her Residency Program in Veterinary Internal Medicine of companion animals at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, the Netherlands.

Presentations and publications

Presentations

Oral presentation **Dirksen, K.**, Verzijl, T., Grinwis, G. C.M, Favier, R.P., Penning, L.C., Burgener, I.A, van der Laan, L.J.W, Fieten, H., Spee, B. "*A panel of serum microRNAs differentiates between various types of canine hepatobiliairy diseases"* 26th ECVIM congress, Gothenburg, Sweden	*1st prize*	Sept 2016
Oral presentation **Dirksen, K.**, Burgener, I.A., Penning, L.C., van den Ingh, T.S.G.A.M., Rothuizen, J., Spee, B., Fieten, H. "*Low sensitivity and specificity of alanine aminotransferase and alkaline phosphatase for detection of hepatocellular injury in 198 Labrador retrievers without clinical signs of liver disease"* 26th ECVIM congress, Gothenburg, Sweden		Sept 2016
Oral presentation **Dirksen, K.**, Verzijl, T., Burgener, I.A, van den Ingh, T.S.G.A.M., Spee, B., Fieten, H. *"Hepatocyte-derived microRNA-122 as an early serum biomarker of hepatocellular injury in dogs"* 25th ECVIM congress, Lisbon, Portugal	*1st prize*	Sept 2015
Abstract Voorjaarsdagen Roelen, Y.S., **Dirksen, K.**, Fieten, H., Spee, B. *A biomarker for copper associated hepatitis in Labrador retrievers.*		April 2013
Poster presentation, Morris Animal Foundation Meeting Veterinary Student Scholars Program Dirksen, K., Fieten, H., Leegwater, P.A.J., Rothuizen, J. *"Pathways in progression of copper associated chronic hepatitis in the Labrador retriever"* Morris Animal Foundation, Small Companion Animal Meeting, Denver, Colorado, USA	*grant & 2nd prize*	June 2012
Abstract ECVIM Fieten, H., Dirksen K., van den Ingh, T., Waalwijk, T., Rothuizen, J. *Optimizing diagnosis and treatment of copper associated hepatitis in the Labrador retriever* 21th ECVIM congress, Sevilla, Spain		Sep 2011
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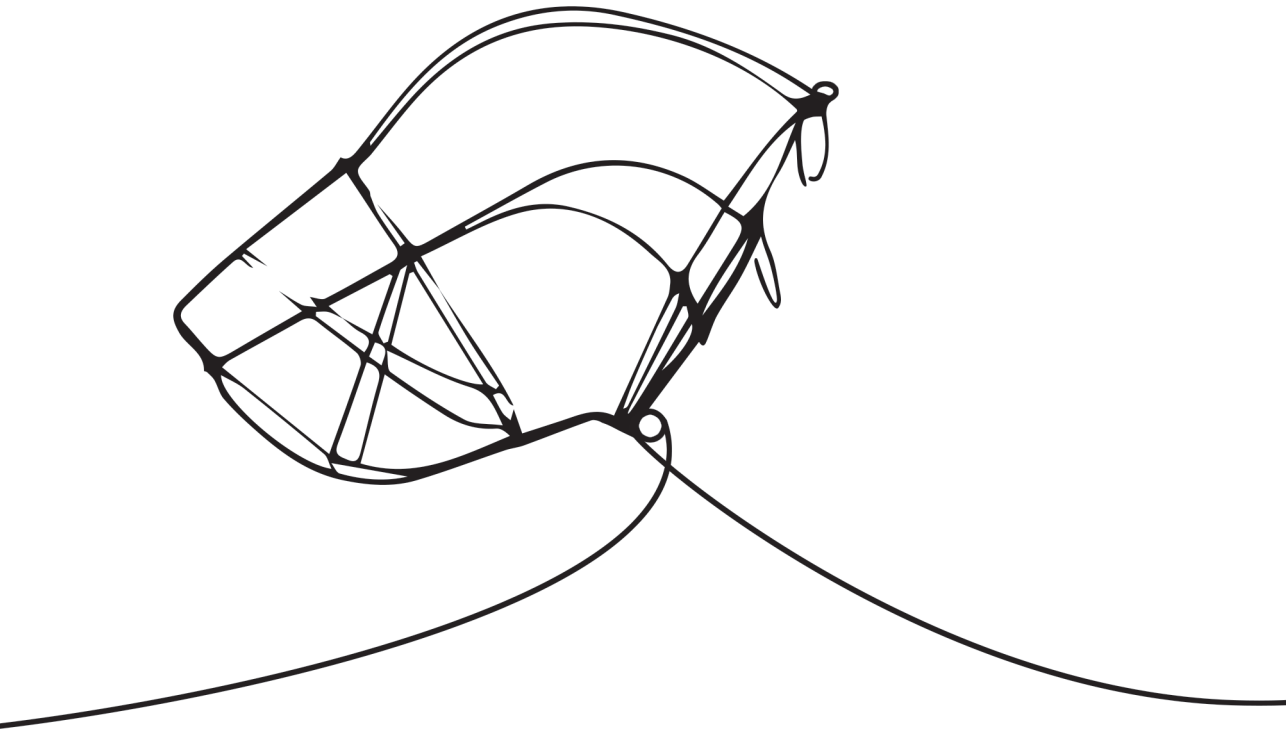

Addendum

Acknowledgements | Dankwoord

Alone we can do so little; together we can do so much
(Helen Keller)

This is it! Mijn proefschrift is klaar; versie zeshonderdzevenenzeventig zit gebundeld in dit boekwerk. De reden voor het hoge aantal versies komt mede doordat er veel mensen zijn geweest die een waardevolle bijdrage hebben geleverd aan een of meerdere onderdelen van dit proefschrift. Een proefschrift schrijven doe je namelijk niet alleen. Een dankwoord schrijven wel. Desondanks is het dankwoord vaak het meest gelezen hoofdstuk uit een proefschrift. Ik realiseer me dat het onmogelijk is om iedereen, die mij heeft geholpen tijdens dit promotietraject, persoonlijk te bedanken. Een aantal mensen wil ik wel graag in het bijzonder benoemen.

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The hardest arithmetic to master is that which enables us to count our blessings
(*Eric Hoffer*)

Proost!