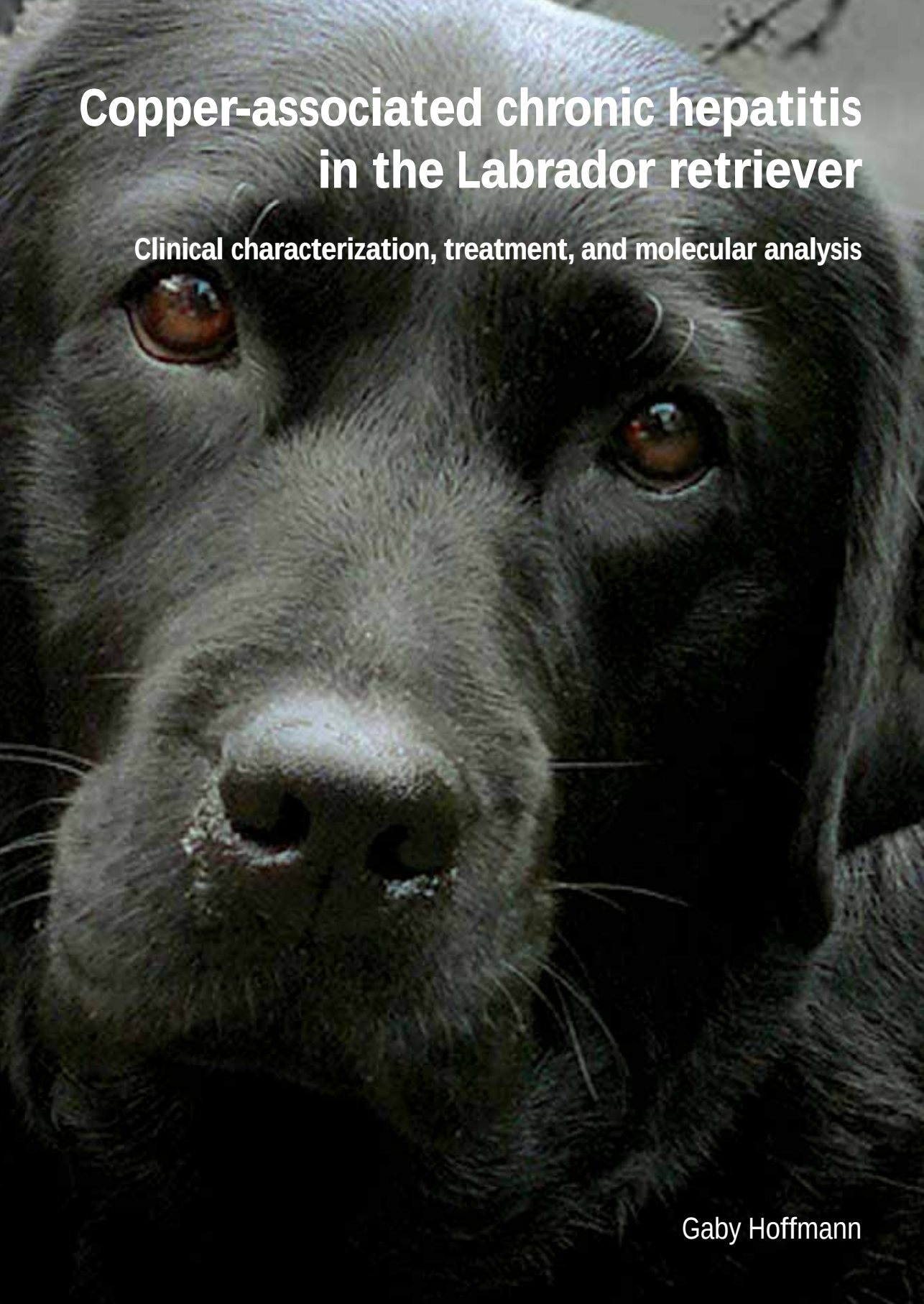

Copper-associated chronic hepatitis in the Labrador retriever

Clinical characterization, treatment, and molecular analysis

Gaby Hoffmann

Copper-associated chronic hepatitis in the Labrador retriever

Clinical characterization, treatment, and molecular analysis of the disease

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Copper-associated chronic hepatitis in the Labrador retriever

Clinical characterization, treatment, and molecular analysis
(with a summary in English)

Chronische leverontsteking als gevolg van koperstapeling bij de Labrador retriever
(Met een samenvatting in het Nederlands)

Kupferspeicherkrankheit beim Labrador retriever
(*Mit einer Zusammenfassung in deutscher Sprache)*

La toxicité du cuivre sur le foie du Labrador
(Avec un résumé en francais)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 26 juni 2008 des middags te 2.30 uur

door

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geboren op 30 september 1970
te Neuss, Duitsland

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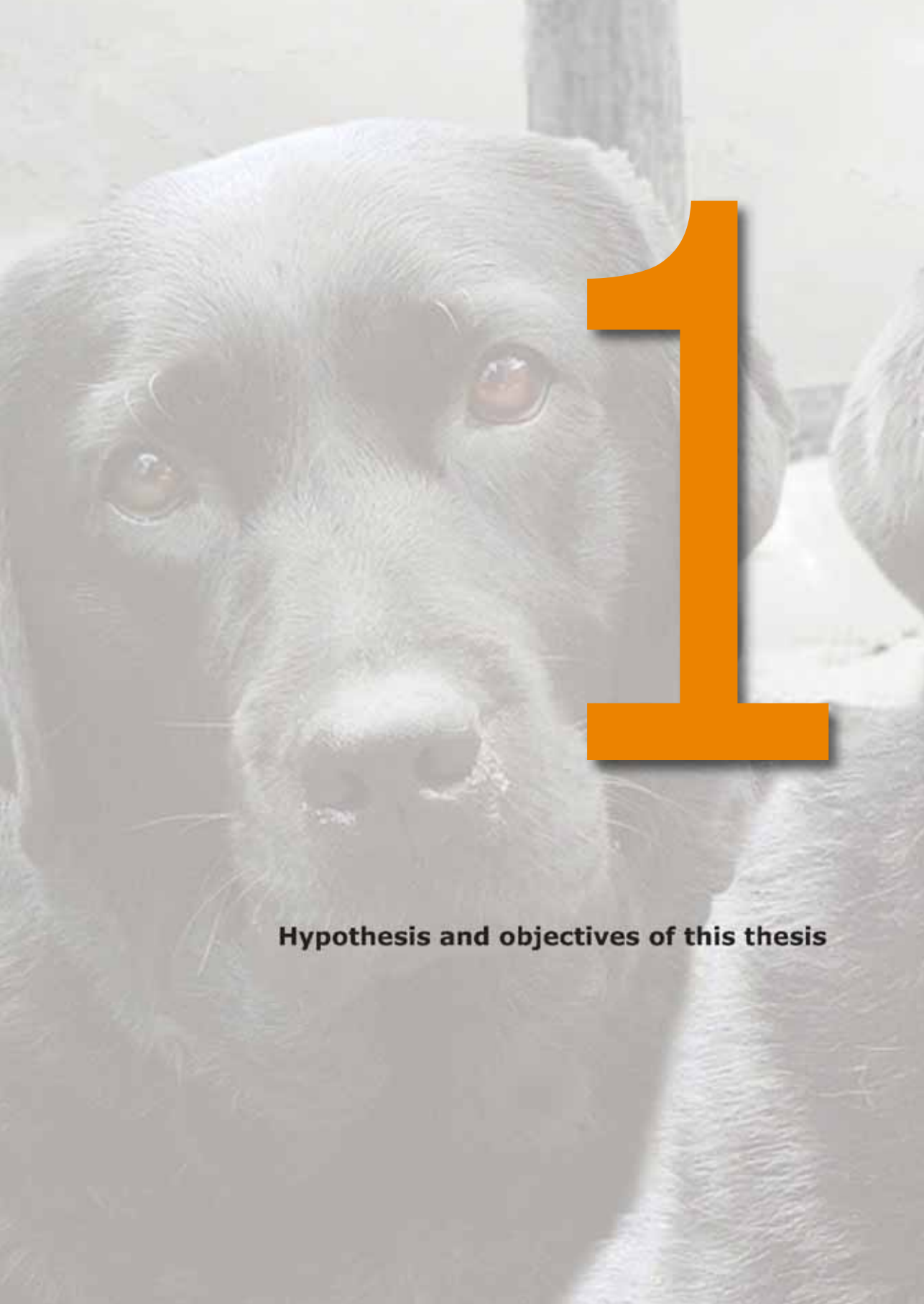
CONTENTS page



1

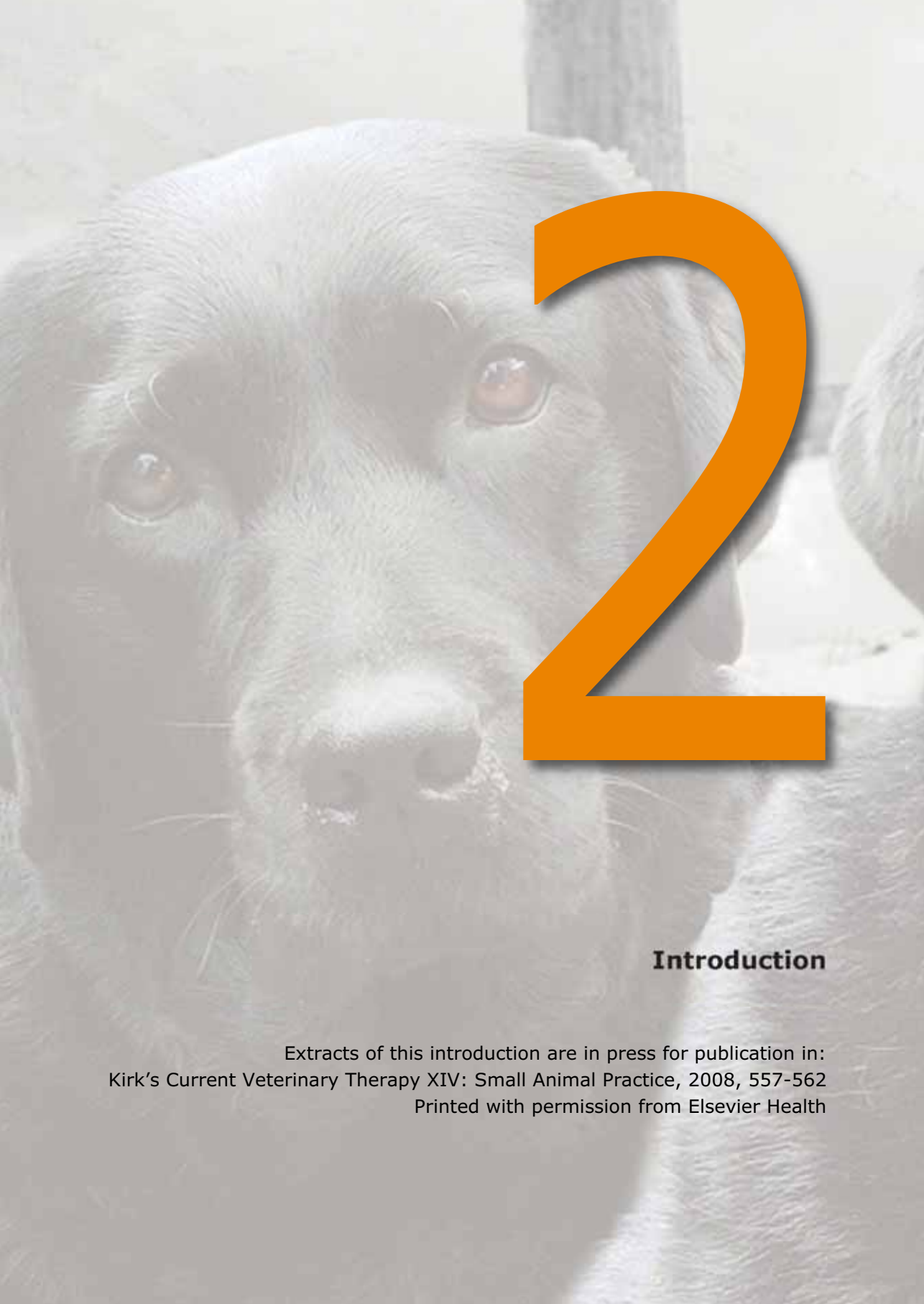
Hypothesis and objectives of this thesis

Hypothesis and objectives of this thesis

According to international kennel clubs, the Labrador retriever is the most common dog breed in Europe and in the United States, and one of the prime breeds selected as working dogs.[1,2] It is commonly recognized that Labrador retrievers are predisposed to the development of chronic hepatitis.[3,4] From experience of the past 20 years of clinical, pathobiological and molecular biologic research of a comparable disease in another dog breed (the Bedlington terrier) at the Veterinary Faculty of the University of Utrecht, it appears that the histological characteristics and clinical disease in Labradors is comparable to that observed in Bedlington terriers with copper toxicosis. Therefore, liver biopsies of Labrador patients with chronic hepatitis are routinely stained for copper with rubeanic acid. This allows the detection of copper accumulation in liver tissue in the majority of patients with chronic hepatitis. Based on these results, Labradors with copper-associated chronic hepatitis are treated with the copper-chelating drug, penicillamine, which allows patients that had previously been poorly responsive to the recommended standard treatment for idiopathic chronic hepatitis (prednisone) to recover from the disease. These clinical observations were the basis of the first **hypothesis of this thesis: that copper-associated chronic hepatitis is a new metabolic disease in the Labrador retriever, which is caused by a defect in hepatic copper metabolism.** Appendant on this assumption, the second hypothesis is **that copper accumulation in the liver of Labradors originates from an inherited genetic defect.**

The objective of this work is to investigate and describe this new breed-related hepatic copper storage disease both clinically and histopathologically, and to demonstrate heritability of copper accumulation in the Labrador retriever. These investigations are described in **part 1** of this thesis (chapters 3-5). Furthermore, different treatment strategies have been tested by randomized, double-blind, placebo-controlled treatment studies, which are described in **part 2** of the thesis (chapters 6 and 7). In addition, molecular genetic studies have been performed to investigate the genetic defect underlying hepatic copper accumulation in Labradors. These studies are reported in **part 3** of this thesis (chapters 8-12).

1. American Kennel Club. AKC Dog Registration Statistics In: 2007.
2. The Kennel Club. Registration statistics for all recognised dog breeds - 2005 and 2006. In: 2007.
3. Andersson M. and Sevelius E. Breed, sex, and age distribution in dogs with chronic liver disease: A demographic study. JSAP 1991;32:1-5.



2

Introduction

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Introduction

Copper-associated liver diseases

Copper metabolism

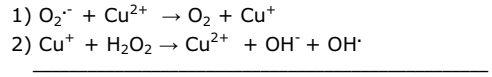
Copper (Cu) is an essential trace element, belonging to the first transition series of elements. Other members of this series include zinc, manganese, cobalt, iron, and chromium. The atomic weight of naturally occurring copper is 63.546.

The liver is essential for copper metabolism because it is the principal recipient of absorbed copper, has the highest stored copper content, delivers copper in protein-bound form to other tissues, and is the principal organ of excessive copper elimination by biliary excretion.[1,2]

Copper transport between organelles and across membranes is much the same for animals, bacteria, fungi and plants due to the highly conserved cellular copper transport elements.[2]

Trace elements, in general, function as cofactors for antioxidant enzymes. Copper is a transition metal able to cycle between two redox states: oxidized Cu^{2+} (cupric ion, stable) and reduced Cu^{+}(cuprous ion, unstable). Copper can therefore funtion as an electron acceptor/donor for different enzymes.[3] It plays a role as a cofactor in hydrolytic, electron transfer and oxygen-utilization enzymes in the generation of cellular energy (cytochrome-c-oxidase), detoxification of oxygen-derived radicals (superoxide dismutase), iron metabolism (ceruloplasmin), blood coagulation, neuropeptide modification (dopamine-B-hydroxylase), melanin synthesis (tyrosinase), and connective tissue cross-linking (lysyl-oxidase).[1,4-10]

Free copper ions are able to catalyze the formation of hydroxyl radicals via the *Haber-Weiss reaction:*



1) $O_2^{\cdot -} + Cu^{2+} \rightarrow O_2 + Cu^{+}$

2) $Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot}$

$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^{-} + \mathbf{OH}^{\cdot}$

The final outcome of this reaction is the toxic hydroxyl radical (**OH**·). This radical can directly damage lipids, proteins and nucleic acids. Oxidative damage can induce inflammation, which ultimately can lead to liver damage. Oxidative stress affects transcription factors, resulting in deregulated gene expressions. In addition oxidative stress is a major inducer of cytokine production in macrophages and other cells, of which profibrotic cytokines favor the production of collagen.[3,5,11,12]

Normal liver copper concentrations in dogs are higher than in people, mice and rats.

The daily food intake of copper is about 14-15 mg/kg dry weight food in dogs, but considerable variations can be found between brands. Copper is present in vegetables, fruits, grains, nuts, meat, seafood and drinking water but in order to obtain copper concentrations in the above range, copper is commonly added to commercial dog food. Forty to 60% of ingested copper is absorbed across the apical membrane of the mucosa of the upper small intestine. The remaining copper leaves the body unabsorbed in faeces.[11]

Two proteins are thought to be responsible for the absorption of dietary copper: the divalent metal transporter 1 (DMT1) and the copper transporter 1 (Ctr1). DMT1 transports copper (Cu^{2+}) directly from copper in the diet. Ctr1 is a transporter of Cu^{+}, which is reduced by endogenous plasma membrane reductases and dietary components such as ascorbate.[3] In the bloodstream, copper is bound to albumin (not specific binding), ceruloplasmin or transcuprein (specific binding). Within two to six hours of absorption, copper from blood enters the liver and the kidneys. In the liver, copper is immediately bound by intracellular chaperones, which are target-specific transporter proteins. These chaperones deliver copper to specific intracellular target molecules. In a second step, after 4 hours or more, copper is exported from the liver cell by the copper-transporting ATPase, ATP7A, re-enters the blood stream and is delivered to other organs.[1,3,13-16]

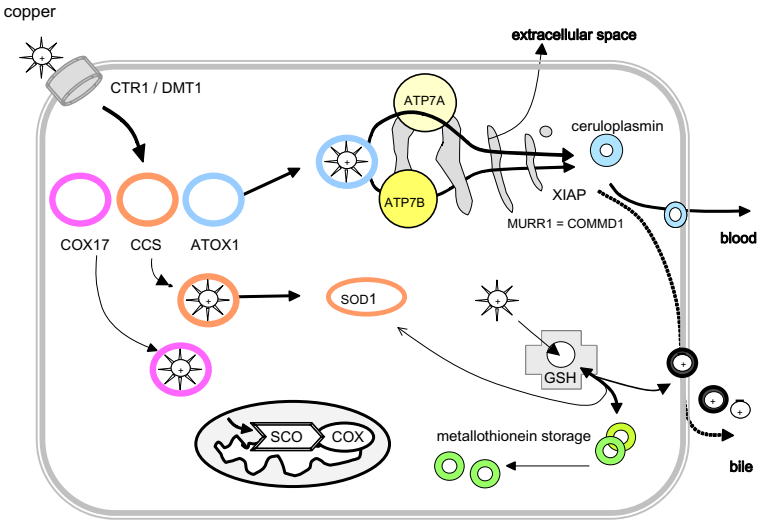


Figure 1: Copper trafficking within the cell

Normal hepatic copper metabolism involves several intracellular pathways. As free copper has a high potential for oxidative damage, there is basically no free copper present in the cell. Copper is excreted into blood after incorporation in ceruloplasmin. Excessive copper is excreted into bile. CTR1: copper transporter 1, COX17, CCS, ATOX1, SCO: target-specific copper transporters, ATP7A: Menkes disease protein, ATP7B: Wilson's disease protein, SOD1: superoxide-dismutase 1, COX: cytochrome c oxidase, MURR1 = COMMD1: copper metabolism murr1 domain-containing protein 1, associated with copper toxicosis in Bedlington terriers. DMT1: Divalent Metal Transporter 1, XIAP: X-linked inhibitor of apoptosis, GSH: Glutathion.

Extracellular transport of copper

Ceruloplasmin
Ninety-five percent of copper in plasma is bound to ceruloplasmin, a glycoprotein synthesized in hepatocytes. Subsequent to the incorporation of copper into ceruloplasmin in the liver cell, ceruloplasmin is excreted into the blood.[9,15,16]

Transcuprein
Transcuprein is a macroglobulin with strong copper and zinc-binding properties in the mouse, rat, cattle, man and dog with the greatest plasma concentration in dogs. The expression of transcuprein has recently been suggested to be modulated by copper and iron availability.[15,17] Furthermore, it was suggested that transcuprein plays a major role in the transport of copper to cells in the dog, because binding of the metal to transcuprein was superior when compared to albumin and ceruloplasmin.[18]

Copper transporter 1 (CTR1)
CTR1 is a high-affinity membrane-spanning transport protein, highly expressed in the liver and kidney. In addition, it is expressed at high concentration in the small intestine, where CTR1 is localized on the apical plasma membrane.
Recent studies using knockout-mice showed that Ctr1 has a key role in the copper uptake pathway. In addition to the uptake of copper, the transporter is involved in cellular uptake of chemotherapeutic agents, such as cisplatin.[3,6,9,13,19]

Divalent Metal Transporter (DMT1)
DMT1 is predominantly an iron transporter with a lower affinity for other selected metals, including copper. DMT1 is located in enterocytes, and can transport Cu^{2+} directly from the ingesta. High copper levels can modify the expression of DMT1, which led to the suggestion that DMT1 acts as a major intestinal copper transporter.[3,9,13]

Intracellular copper metabolism

Copper chaperones

Excess or free intracellular copper is highly toxic to most organisms. Therefore, the transport and sequestration of copper is tightly controlled. Indeed, less than one atom of free copper is present in the cytoplasm of a eukaryotic cell (essentially no free copper). This apparent absence of free copper can be attributed to a wide variety of moderate and tight binding sites in the cell, including non-specific small molecular interactions, vesicular storage sites, and specific copper-binding proteins. Target-specific soluble transport proteins, called chaperones, escort copper Cu ions to specific Cu-dependent proteins and catalyze copper loading of these proteins. The structural feature of these chaperones is a metal-binding motif in the N-terminal region, which contains two cysteine-sulfur-groups for transient complex formation with copper. Target specificity of chaperones is conferred by specific intermolecular forces between the metal-binding loop and a complementary copper-binding protein fold of a receiving enzyme. COX17 (cytochrome oxidase 17) is a shuttle protein for delivering copper (Cu^+) to SCO1 and SCO2 on the inner mitochondrial membrane. SCO1 and SCO2 (synthesis of cytochrome c oxidase) are assembly proteins involved in the incorporation of copper in cytochrome–c oxidase. ATOX1 (antioxidant protein 1) specifically traffics copper from CTR1 to the secretory pathway for incorporation into ATP7A and ATP7B, the copper-transporting P-type-ATPases of the trans-Golgi network. CCS (copper chaperone for SOD1) delivers copper to the cytosolic superoxide dismutase (SOD1).[6,7,13,20]

Glutathione (GSH)

One of the earliest copper transport proteins to be identified was glutathione (GSH). GSH is ubiquitously present in the body and a rapid turnover of GSH-Cu^+-complexes occurs within hepatocytes. GSH is functional in the cytosol where it forms stable Cu^+-GSH complexes. GSH has a protective function against copper toxicity by mediation of copper binding to different proteins, such as ceruloplasmin, SOD1 and metallothionein. Therefore, low cellular GSH concentrations increase the potential for oxidative damage from reactive copper.[6,7,21,22]

ATP7A

ATP7A receives copper from ATOX1 and metabolizes it further from the Trans-Golgi-Network where it catalyzes an ATP-dependent transfer of copper to SOD1. ATP7A also has an important role in exporting excess copper ions directly or via incorporation into apo-ceruloplasmin. The ATP7A-gene is mutated in human Menkes disease. In this disease accumulation of copper occurs in the intestinal mucosa.[2,6,9,23-27]

ATP7B

The ATP7B-gene is mutated in Wilson's disease. Human patients suffer from a lack of release of hepatic copper into bile , and defective incorporation of copper into ceruloplasmin in plasma. ATP7B is located at the Trans-Golgi Network, where it catalyzes an ATP-dependent transfer of Cu for further binding by SOD1, incorporation into ceruloplasmin, or biliary excretion via lysosomes and COMMD1. In the presence of copper excess, copper-induced trafficking of ATP7B from the Trans-Golgi Network to cytoplasmic vesicular compartments near the canalicular membrane occurs to increase copper excretion into bile. The ATP-ase ATP7B contains a large N-terminal domain with six repeats of a copper-binding-motif. Copper is bound to this domain in its cuprous form (Cu^+). Similar to ATP7A, ATP7B receives copper from the chaperone ATOX1.[23,24,28-32]

Cytochrome-c-oxidase

Cytochrome-c-oxidase is a key enzyme in the respiratory chain. It is synthesized in the liver and functional in the mitochondria as an enzyme involved in oxidative phosphorylation, by catalyzing the reduction of molecular oxygen to water. The metallochaperones COX17 and SCO1 are important for delivery and incorporation of copper ions into this enzyme.[33]

Superoxide-dismutase (SOD1)
SOD1 acts as a cellular anti-oxidant because it provides a defence against oxidative stress by catalizing the dismutation of superoxide ($O_2^{-\bullet}$) to form hydrogen peroxide and oxygen (OMIM 147450). SOD1 is synthesized in the liver and localized to the cytosol. Incorporation of copper into this enzyme is dependant upon the chaperone CCS.[34,35]

Metallothionein
Metallothionein is a small intracellular protein capable of chelating several metal ions, including copper for non-toxic storage. Metallothionein can donate copper to other proteins, either following degradation in lysosomes or by exchange via GSH-complex formation (OMIM 156350).[36-38]

MURR1 (Mouse U2af1 rs1 region 1) **= COMMD1** (COpper Metabolism Murr1 Domain - containing protein-1)
COMMD1 is the founding member of the COMMD gene family.[39,40] The COMMD1 gene is mutated in copper toxicosis of Bedlington terriers, where exon 2 is deleted.[41,42] Affected dogs do not have any detectable COMMD1 protein. The molecular function of COMMD1 remains unknown. Recent studies suggest that COMMD1 does not regulate copper-induced trafficking of ATP7B directly, but rather functions as a general negative regulator of protein stability, involved in the quality control of proteins such as ATP7B, hypoxia-inducible factor 1, and nuclear factor κB signalling.[23,39,42,43] The regulation of protein stability of key steps in copper metabolism is a likely mechanism of action of COMMD1 alternative to the proposed mechanisms of transport into bile canaliculi, or facilitated degranulation of lysosomes into bile.[8,23,44] Under in vitro conditions no interaction was detected between COMMD1 and ATOX1.[44]
The human COMMD1 protein directly interacts with the amino-terminal domain of the Wilson's disease protein in vitro. Therefore it was expected that the COMMD1 gene would affect copper metabolism in humans, especially those suffering from Wilson's disease. Although no amino acid changing sequence variation was found in the human COMMD1 gene, a GAT/GAC heterozygous state at codon Asn-164 of COMMD-1 was described in patients with early-onset Wilson's disease. Moreover 4 WD patient-derived mutations of ATP7B were described that increased binding of ATP7B to COMMD1. Two of these mutations also resulted in mislocalization and increased degradation rate of ATP7B.[23]

XIAP (X-linked inhibitor of apoptosis)
XIAP is a 57 kDa anti-apoptotic protein, that has recently been found to be involved in intracellular copper homeostasis via copper binding and the regulation of COMMD1.[45,46] (OMIM 30079)

Dynactin
Subunit p62 of dynactin (dynactin 4) was recently described to regulate trafficking of copper-loaded ATP7B for removal of excess copper into bile.[47]

Copper storage disorders in mice, rat, and sheep

The toxic milk mouse and the Long-Evans Cinnamon rat (LEC-rat) were the first animal models used to study Wilson's disease with both models having many features in common with their human counterpart. In these animals, mutations in the ATP7B gene lead to copper accumulation in the liver and progressive inflammation and cirrhosis.[12,15].
North Ronaldsay sheep, with an unknown abnormality of copper metabolism, develop liver cirrhosis comparable to idiopatic copper toxicosis in people due to copper-induced increased lysosomal activity and hepatic stellate cell activation.[48]

Copper storage disorders in man

Wilson's disease (OMIM 277900) and Menkes disease (OMIM 309400) are autosomal recessive inherited copper storage disorders. Wilson's Disease is the most completely characterized disorder of copper toxicity in humans. Patients with this disorder accumulate copper in various tissues, particularly the liver and brain and, in small amounts, in the cornea and kidney. Reduction or absence of ATP7B-gene expression in these patients reduces the rate of incorporation of copper into ceruloplasmin, and reduces biliary excretion of copper. Progressive hepatic copper accumulation, liver cirrhosis and basal ganglia degeneration ensue. Ocular accumulation of copper leads to a typical circumferential corneal pigmentation, known as Kayser-Fleisher rings. In the blood, ceruloplasmin concentrations are reduced and non-ceruloplasmin-copper is greatly increased.
Other disorders of copper metabolism in man include Indian childhood cirrhosis and non-indian childhood cirrhosis (Endemic Tyrolean infantile cirrhosis (OMIM 215600) and idiopathic copper toxicosis (OMIM 215600). These disorders of copper toxicity resemble Wilson's disease phenotypically. However, their genetic background is still unsolved, although a complex ethiology is suggested, with influencing factors from the environment, such as high copper intake.[1,2,6,9]

Furthermore, copper is involved in a number of diseases without major impact on the pathogenesis, including Parkinson's disease, Alzheimer's disease and Prion diseases.[49-53]

Copper-associated chronic hepatitis

Hepatic copper accumulation can result from increased uptake of copper, primary metabolic defects in hepatic copper metabolism or from altered biliary excretion of copper. Toxicity of copper is dependent upon the molecular association and subcellular localization of molecules as well as their total concentration in liver tissue. In inherited copper storage disorders, copper accumulation is always localized centrolobularly. This is the case in Bedlington terrier copper toxicosis, Wilson's disease in humans, and liver disease in LEC-rats. This differs from secondary copper loading of liver cells during cholestasis or cholatestasis, where copper is mainly restricted to the periportal parenchyma.[16,54]

Copper-associated chronic hepatitis in dogs

Inherited copper toxicosis is a well-described disease in the Bedlington terrier, where a deletion of exon 2 in the COMMD1 gene (previously called MURR1) causes accumulation of copper in hepatocytes, resulting in chronic hepatitis.[55-57] Moreover, hepatic copper storage and associated hepatitis are breed-associated in the West Highland white terrier, Skye terrier, Doberman pincher, Dalmatian, and Labrador retriever.[12,58-63]

The average canine liver copper concentration is 200-400 ppm (ppm= µg/g = mg/kg) per dry weight (dw) of liver tissue.[62,64,65] Hepatic copper levels in breeds with primary copper storage disease vary between individual animals and between breeds from 600 to above 2,200 ppm.

Table 1: Normal ranges of hepatic copper concentrations in liver tissue of dogs

range [ppm dw]	**normal range**	**breed**	**method**	**dogs**	**reference**
91-358	**206+/- 56**	Bedlington Terriers	sp	22	J.Am.Vet.Med.Assoc. 1979;175:269-275
94-270	**190+/- 56**	mixed breed dogs	sp	15	J.Am.Vet.Med.Assoc. 1979;175:269-275
60-270	**155+/- 66**	mixed breed dogs	sp	13	Vet Pathol. 1986; 23:148-154.
38-650	**156+/-119**	5 mixed breed dogs + 32 pure breeds	sp	37	Vet Pathol. 1990; 27: 81-88
100-700	**197 ± 113**	Doberman pinschers	NAA	13	J Vet Intern Med. 2004:18(5):647-50
120-304	**<400**	Labrador retrievers	NAA	6	J Vet Intern Med. 2006;20(4):856-61.

sp: spectroscopy, dw: dry weight, ppm: parts per million, note that ppm equals ug/g, as well as mg/kg dry matter,
NAA: neutron activation analysis

Clinical signs and laboratory results in dogs with copper-associated chronic hepatitis

Dogs with hepatic copper accumulation can appear normal over years before developing clinical signs late in disease, although copper may begin to accumulate by 5-6 months of age. One investigator followed dogs with the COMMD1 deletion from birth to 3 years of age, and found excessive copper accumulated in the liver by 1 year of age although histologic evidence of hepatitis did not occur before affected dogs were 2 years old (R.Favier, personal communication). Therefore, dogs with inherited copper storage disorders may be subject to a prolonged period of several years between severe accumulation of copper and development of histologic signs of inflammation, as well as between the consolidation of histologic signs of inflammation and recognition of clinical signs of disease.
With the exception of hemolysis from copper release into blood, which is only described for Bedlington terriers, symptoms of the disease are all non-specific, resulting from liver dysfunction. The clinical signs may start with a mild decrease in activity or appetite. In most cases, owners will only recognize these intermittent signs with retrospect. Over weeks to months, dogs may vacillate between periods of decreased activity and periods of normal behavior. After months to years, symptoms become more prominent, and may include salivation with intermittent vomiting and nausea. Polyuria and polydipsia, icterus, diarrhea, and ascites may develop in advanced disease.
Findings on routine serum biochemical analyses include a greater relative increase in ALT (alanine aminotransferase) activity than ALP (alkaline phosphatase), suggesting primary hepatocellular liver disease.

Table 2: Clinical signs of copper-associated chronic hepatitis in dogs

Reduced endurance / Depression	Polyuria/Polydipsia
Loss of appetite / Anorexia	Diarrhea
Weight loss	Icterus
Nausea	Ascites
Vomiting	Seizures

Non-specific clinical signs of copper-associated chronic hepatitis

Diagnosis

Histopathologic evaluation of liver tissue is currently the only means of diagnosis of copper-associated hepatitis. Two or more liver biopsies, taken with a large-core needle (14 gauge), are a required minimum to evaluate liver tissue and determine copper toxicosis quantitatively or semi-quantitatively. Liver biopsy samples containing more than 6-8 portal triads are considered adequate for histologic diagnosis of human liver disease.[66] From reports comparing different biopsy techniques in dogs, relatively large sized biopsies of the liver are required for accurate diagnosis (14 gauge, 1.8-mm diameter, 1cm length).[66-70] In order to avoid puncture of adjacent organs, such as the gallbladder, stomach or intestine, the patient should be fasted for 12 hours prior to the procedure. In people with liver disease, significant hemorrhage after biopsy occurs in approximately 0.2% of patients.[16,66]
The typical magnitude and localization of copper within zone 3 in the liver lobule are characteristics of primary copper storage disease.[46,71,72] Copper accumulates in hepatocytes, and results in hepatocellular inflammation with copper-laden macrophages, and chronic hepatitis. The chronic hepatitis is characterized by hepatocellular apoptosis, necrosis, regeneration, and fibrosis, as well as an inflammatory infiltrate, which can be mononuclear or mixed. Cirrhosis results as the end-stage of the disease.[73]

Copper assessment

Copper concentrations in liver tissue can be measured quantitatively by irradiation of small biopsies and measurement of the induced Cu radioactivity in small amounts of tissue, or by spectrophotometric methods on fresh frozen liver (1-2 grams of tissue needed). For the latter method, formalin fixed tissue can be submitted, but measurement of copper concentrations in wet weight liver tissue is not recommended, especially in marginally elevated copper concentrations, because the reference ranges for copper are established on dry tissue basis. Alternatively, histochemical stains, such as rubeanic acid and rhodanine, are recommended to evaluate liver tissue semiquantitatively for copper. These stains consistently detect copper in liver biopsy specimens when amounts exceed the normal limit of 400 ug/g dw. It has been suggested that rhodanine demonstrates the protein to which copper binds rather than the copper itself.[74]

A histochemical grading system for evaluation of liver tissue stained with rhodanine was developed by Johnson et al. for semiquantitative evaluation of hepatic copper concentrations in Bedlington terriers.[75] The same grading system was applied for assessment of semiquantitative copper scores in rubeanic acid (dithio-oxamide) stained liver tissue of Bedlington terriers, Doberman pinchers, and Labrador retrievers.[20,72,75,76]

Table 3: A histochemical grading system for evaluation of canine liver tissue stained with rhodanine or rubeanic acid: Copper scores above 2 are considered abnormal.

0	no copper,
1	solitary liver cells contain some copper positive granules
2	small groups or area of liver cells contain small to moderate amounts of copper positive granules
3	larger groups or areas of liver cells contain moderate amounts of copper positive granules, sometimes associated with copper containing macrophages
4	large areas of liver cells with many copper positive granules, usually associated with copper containing macrophages
5	diffuse panlobular presence of liver cells with many copper positive granules, usually associated with copper containing macrophages

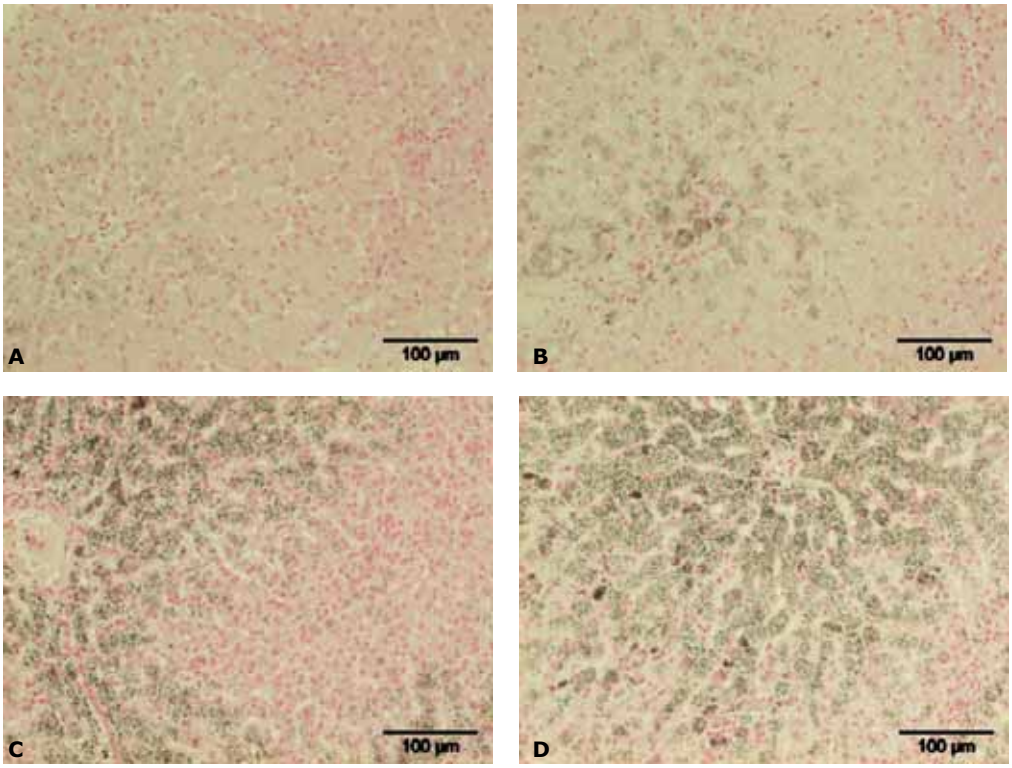


Figure 2: Rubeanic acid staining of liver tissue
Histology slides (3µm) of liver tissue from dogs stained with rubeanic acid for copper. A: score 2+, B: score 3+, C: score 4+, D: score 5+ according to table 3. By courtesy of Dr. T.S.G.A.M van den Ingh, TCCI Consultancy BV, Utrecht, The Netherlands

Further staining methods, which have been applied for detection of copper include Timm's silver stain, cresyl-violet, dithizone, and orcein for copper-associated protein.[77] These staining methods have not been established for detection of copper in pets, and no grading system is available for veterinary use.

Table 4: staining methods for copper in liver tissue

Staining method	**Grading system for veterinary use**	**Copper color**
Rhodanine	yes	red to red-yellow
Rubeanic acid (dithiooxamide)	yes	deep blue to black
Timms silver stain	no	black
Orcein	no	black

Copper accumulation secondary to cholestasis in dogs

Copper may accumulate in the liver secondary to cholestatic liver diseases. Due to defective copper excretion in the bile, cholestatic liver diseases often result in copper accumulation in the periportal areas. The accumulation occurs in hepatocytes. The magnitude of copper accumulation from cholestasis is not as high as that found in dogs with inherited copper storage disorders. In a review of 17 liver biopsies from breeds not identified to be affected by inherited copper-associated liver disease, the mean copper concentration was 984 µg/g dry weight liver.[65] Another study revealed that 3+ or higher histochemical detection of copper in the central area of the liver lobule indicates a primary copper storage disease.[20,78] In their study, Spee et al were able to find clear distinction criteria to determine whether copper accumulation is primary or secondary to hepatitis by comparison of liver biopsies from Bedlington terriers with copper toxicosis with those harvested from non-copper-associated breeds diagnosed with severe chronic hepatitis, and dogs with chronic extrahepatic cholestasis. Copper metabolism was analyzed using histochemical staining and quantitative RT-PCR by comparison of the gene expressions of *ATOX1, COX17, ATP7A, ATP7B, CP, MT1A, COMMD1, XIAP*. Oxidative stress was measured by determining GSH/GSSG ratios and gene-expression (*SOD1, CAT, GSHS, GPX1, CCS, p27KIP, Bcl-2*).

Bedlington terrier

Hepatic copper toxicity was first identified in Bedlington terriers in 1975.[79] It was subsequently shown that affected Bedlington terriers have an inherited autosomal recessive defect of the *MURR1* gene, which was renamed to *COMMD1* (copper metabolism murr1 domain–containing protein 1). The extent of hepatic damage tends to parallel the increasing hepatic copper concentrations, which occur from decreased copper excretion into bile in *COMMD1*-deficient liver cells. The accumulated copper in liver tissue is seen as dense granules in lysosomes and occurs mainly in the centrolobular region of the liver. The histologic changes extend from focal necrosis to chronic hepatitis that may ultimately lead to cirrhosis. In some cases, acute hepatic necrosis, copper-associated hemolytic anemia and acute liver failure may occur. Female and male dogs are equally affected.
Copper toxicosis in Bedlington terriers can clinically be divided into three stages. In the first stage, hepatic copper concentrations increase from 400 to 1500 ppm dw. Copper accumulation initially occurs in the centrolobular hepatocytes (zone 3). This stage remains clinically silent. A liver biopsy will reveal increased concentrations of copper but the histologic structure of the liver appears normal.
In the second stage, copper concentrations increase further into a range of 1500-2000 ppm dw. Histologically, copper accumulation is now also found in the midzonal and periportal hepatocytes (zones 2 and 1). A liver biopsy will reveal inflammation with centrilobular mixed cell foci, containing necrotic hepatocytes, lymphocytes, plasma cells, neutrophils and copper-laden macrophages. In the most advanced stage, dogs become clinically ill. Copper concentrations may exceed 2000 ppm dw and histology reveals hepatitis and cirrhosis. Cholestasis and bile duct proliferation occur along with fibrosis possibly because of compression exerted on bile ducts in a distorted fibrotic liver and/or a cytokine-induced proliferation of bile ducts.[62,79-89]

Figure 3: Bedlington terrier with Copper toxicosis
By courtesy of Prof. Dr. Jan Rothuizen, Department of Clinical Sciences of Companion Animals, University of Utrecht, The Netherlands

Table 5: Stages of copper toxicosis in Bedlington terriers

stage	clinics	copper	liver histology
1	symptom free	copper in zone 3 (centrolobular) from 400-1500 ppm	normal liver structure
2	symptom free	copper in all zones 1500 – 2000 ppm	inflammation
3	clinical illness	copper in all zones >2000 ppm dw	inflammation + cirrhosis

Homozygous affected dogs have the highest copper concentrations. Heterozygous carrier dogs generally have an increase in copper concentrations until the age of 6-9 months before concentrations fall back to within the normal range.
The disease can be diagnosed by copper measurement in liver biopsies, as well as with genetic testing. Estimates of the incidence of copper toxicosis in Bedlington terriers varied from 34% to 66% between countries before genetic testing became available. Genetic assays investigate the presence of a particular microsatellite marker, which is in linkage disequilibrium with the COMMD1 mutation, or they detect the deletion of exon 2 of *COMMD1* directly.

Doberman pinscher

The disease almost exclusively affects female Dobermans, beginning in young dogs (1-3 years) with increased serum ALT, centrolobular copper accumulation, and sub-clinical hepatitis. Clinical evidence of liver disease usually begins around 4-7 years of age with chronic hepatitis and cirrhosis. Copper appears to be associated with the disease, because recent studies suggest that copper is often increased prior to the development of clinical hepatitis. Furthermore, copper excretion studies reveal decreased biliary Cu excretion in affected Doberman pinschers. Moreover, copper chelator (penicillamine) therapy in sub-clinical dogs normalized copper concentrations with improvement in the grade of histological damage.[90]

Dalmatian

In a retrospective study of 10 Dalmatians with copper-associated chronic hepatitis, two of the dogs were related and all presented for gastrointestinal clinical signs.[63] Males were equally affected as females and all dogs had elevated liver enzymes and necro-inflammatory liver changes, as well as centrolobular copper accumulation. In 5 dogs, hepatic copper concentrations exceeded 2,000 µg/d dw liver, with several dogs having copper levels as high as those observed in Bedlington terriers.[63]

West Highland white terrier

Affected dogs of this breed were 3-7 years of age. Some dogs had elevated hepatic copper concentrations (centrolobular) but no evidence of liver disease, which led to the suspicion that copper was a cause of subsequent chronic hepatitis/cirrhosis. Copper accumulation does not appear to increase with age in the West Highland white terrier, and there is no gender predilection.[65,91] Biliary excretion studies revealed a decreased excretion of radioactive copper in affected dogs.[92]

Skye terriers

Cholestasis was the suspected etiology of copper-associated chronic hepatitis/cirrhosis in Skye terriers. The 10 described dogs were 1-10 years old. Female and male dogs were equally affected, and presented with intermittend anorexia, vomiting, and ascites. At a terminal stage of the disease, the animals developed jaundice and died.[58]

Labrador retriever

Chronic hepatitis is reported to be common in this breed and copper accumulation is associated with about 75%, but not all cases of chronic hepatitis. Females are more commonly affected, and generally are presented at around 7 years of age (range 2-10 years). Clinical signs are non-specific and include anorexia, vomiting, and weight loss. Hepatic copper concentrations generally range from 650 to 3000 µg/g dw (histologically above 2+ with rubeanic acid staining). The histological localization of copper in the centrolobular region of the liver lobule is an indicator for primary copper accumulation.[54,72,93]

Other Breeds

Publications of other breeds with liver disease associated with copper accumulation include reports of an Anatolian shepherd dog, 6 German shepherd dogs, 11 Keeshonden, and a Boxer.[10,65,94-96]

Table 6a: Literature review of copper-associated hepatitis in different dog breeds

breed	no dogs	age	gender	symptoms	blood	**copper** [ppm dw]
Bedlington T.	**21**	8mo-14y	female = male	partial anorexia, depression, weight loss, vomiting	ALT + ALP elevation	assessed in wet weight
	149	1mo-17y	female = male	no symptoms, family of high copper dog	not assessed	not assessed
	68	6mo-15y	female = male	19 dogs: 3 syndromes: 1. acute (6y): anorexia, vomiting, weakness, 2. Chronic: (5-12y) 13 dogs: anorexia, weight loss, intermittend vomiting, diarrhea, unthriftiness, 3. Hemolytic/jaundice	ALT increased	850-10600
	24	1-14y	female = male	no symptoms	not assessed	numbers not given
	18	1.7-11y	female = male	no symptoms, anorexia, vomiting, weight loss, hemolytic crisis	ALT>AST elevation	2638 (1443-3373)
	5	3-10y	female = male	no symptoms, 1 dog hemolysis	ALT increased	3000-11000
	4	unknown	unknown	not assessed	not assessed	>471
	2	3+ 5y	female = male	anorexia, weight loss > vomiting, PU/PD	ALTx10, ASTx 10	1027 + 10728
Doberman Pinscher	**30**	N/A	female >> male	no sympt., routine blood screen, ascites, weight loss, jaundice	not assessed	650-4700
	26	1.5-10y	female >> male	anorexia, weight loss, PU/PD, icterus, ascites, bleeding, seizures vomiting	ALPx10, ALTx11, high bilirubin	509 (88-722)
	22	3y	female >> male	no symptoms	ALT > ALP elevation, bile acids elevated	419 ± 414
	20	1mo-17y	N/A	no symptoms, family of high copper dog	not assessed	140-1500
	18	2.5-7y	female >> male	no symptoms	ALT elevated in 2 dogs	histology: elevated
	11	2.5-11y	female >> male	PU/PD, weight loss, decreased activity, poor appetite, vomiting, diarrhea	ALT + ALP > bilirubin elevated	404-1700
	8	2-8y	female	anorexia, weight loss, apathy, exercise intolerance, vomiting, PD	ALT x 20, AST x 7, ALP x 4.5,	histology: 3 +
	5	6 – 8y	female	no symptoms	ALT x 5, ALP x 2-3	1036 (630–1330)
	3	N/A	unknown	not assessed	not assessed	>471
	2	3 + 4y	female	not assessed	not assessed	600 + 804
	2	3y (f) + 6y (m)	male=female	partial anorexia, weight loss, vomiting	ALT x10- 20, ALP normal	1465 + 2500

breed	copper [ppm dw]	copper location	histology	therapy & outcome	literature
Bedlington T.	assessed in wet weight	no assessment in intact lobuli	chronic hepatitis, cirrhosis, acute hepatic necrosis, acute liver failure	not assessed	Minnesota Veterinarian 1975: 15: 13-24
	not assessed	begin centrolobular, later all zones	hepatitis	not assessed	Vet Pathol 1990; 27: 81-88
	850-10600	begin centrolobular (stage 1) later all zones	focal hepatitis - cirrhosis	d-penicillamine => improvement	JAVMA 1979, 175:269-275.
	numbers not given	not assessed	study compared cytologic versus histologic staining results	not assessed	Vet Rec 1992; 131:30-32
	2638 (1443-3373)	centrolobular	necrosis, inflammation, fibrosis, extramedullary hematopoiesis	preventative feeding of low-copper diet	J Vet Sci; 2004:19-28
	3000-11000		necrosis, chronic hepatitis, cirrhosis	2,3,2-tetramine => effective chelating drug	JAVMA 1988; 192: 52-56
	>471	not assessed	not assessed	not assessed	Can Vet J. 1998, 39(1): 39–43
	1027 + 10728	not assessed	chronic hepatitis/cirrhosis	penicillamine => died	JSAP 1984;25:293-298
Doberman Pinscher	650-4700	centrolobular	chronic hepatitis in zone 3	not assessed	Vet Pathol. 1998; 35:380-305
	509 (88-722)	not assessed	chronic hepatitis	prednisolone => moderate - poor response	JAVMA 1985; 187:1343-1349
	419 ± 414	centrolobular	hepatitis	not assessed	JVIM 2002, 16:665-668.
	140-1500	begin centrolobular	hepatitis	not assessed	Vet Pathol 1990; 27: 81-88
	histology: elevated	multifocal & portal	inflammation, necrosis, fibrosis	not assessed	Vet Pathol. 1998 Sep;35(5):361-9.
	404-1700	centrolobular	degeneration, inflammation, necrosis, fibrosis, cirrhosis	diuretics, antibiotics, penicillamine => 6 dogs died within 9 months	JAVMA 1982; 180:1438-1442
	histology: 3 +	no assessment in intact lobuli	cirrhosis / cholestasis	not assessed	Veterinary Quarterly 1988; 10: 84-89.
	1036 (630–1330)	centrolobular	subclinical hepatitis	200 mg d-penicillaminea PO q12h for 4 months => improvement	JVIM 2005;19:40–43
	>471	not assessed	not assessed	not assessed	Can Vet J. 1998, 39(1): 39–43
	600 + 804	no assessment in intact lobuli	cirrhosis	not assessed	Vet Med 1985; 50-54
	1465 + 2500	centrolobular	focal hepatitis	died	JAAHA 1983; 1003-1005

Table 6b: Literature review of copper-associated hepatitis in different dog breeds

breed	no dogs	age	gender	signs	blood	copper [ppm dw]
Dalmatian	**10**	2-10y	male=female	inappetence, vomiting	ALT x6 (2-12x), AST x7 (2-22x), ALP x 2,7 (07-10x)	3197 (754-8390)
	1	2y	female	vomiting, PU/PD, diarrhea, lseizures	AST, ALT, ALP elevated	1916
	1	1.5y	male	vomiting, anorexia, weight loss, lethargy	ALT x 10+ AST x 4, ALP x 1.3	2356 ug/g wet weight
	1	2y	female	lethargy, vomiting, paleness, icterus	ALT x 25, ALP x3, bili x 15, leucocytosis,	7940
Skye Terrier	**9**	18months-15y	male=female	intermittend anorexia, vomiting, ascites >> terminal jaundice	not assessed	358-2257
	2	7mo-1,5y	male=female	lethargy, inappetence, ascites	ALP x3, bile acids fastedx 5, bile acids pp:x11	not assessed
	1	1y	female	anorexia, vomiting, melaena, seizures, aggression	bile acids x 36 fasted, x 46 pp, alb (-30%), glob -6%, ALP x1.5, target cells	462
West Highland W. T.	**44**	3-7 years	female>male	not assessed	not assessed	normal - 3500
	395	1mo-17y	female = male	no symptoms	not assessed	20-6800
	7	unknown	unknown	not assessed	not assessed	>1100
	2	unknown	unknown	not assessed	not assessed	>471
Labrador Retriever	**23**	7 y (2-10)	female > male	anorexia > vomiting	ALTx10, ALPx4.5	1317 (402-2576)
	1	unknown	unknown	not assessed	not assessed	>471
other breeds & cats:						
	3	1.5-3y	male=female	ascites, icterus	ALP 4x elevated (1-6x), ALAT 4x elevated (2-12x)	1441-2921
German Shepherd	3	4mo, 8+ 9y	male=female	not assessed	not assessed	570,1352,2202
Anatolian shepherd	1	7y	male	intermittend inappetence, weight loss, decreased endurance, vomiting	ALTx3, ALPx1.5	4+
Keeshond	11	1mo-17y	female = male	no symptoms, family of high copper dog	not assessed	90-2400
Boxer	1	6y	female	PU/PD	ALT and ALP increased	1101
European Shorthair cat	1	2y	male	inappetence, vomiting, fever	not assessed	4170
Siamese Cat	1	2y	female	anorxia, depression	ALTx15, ASTx6	4074

f: female, m: male, dw: dry weight liver, ALT:alanine amino transferase, ALP: alkaline phosphatase, bili: bilirubin, y: years, T.: terrier

breed	**copper** [ppm dw]	**copper location**	**histology**	**therapy & outcome**	**literature**
Dalmatian	3197 (754-8390)	centrolobular	necrosis, fibrosis, inflammation	penicillamine, trientine, zinc => died/euthanized	JVIM 2002, 16:665-668.
	1916	not assessed	hepatic necrosis/cirrhosis	antibiotics, fluid, lactulose, penicillamine => died	Can Vet J. 1996 Jan; 37(1): 45.
	2356 ug/g wet weight	centrolobular	hepatocellular necrosis & inflammation	manifold => died	JAVMA1999:214:1502 1506
	7940	centrolobular - midzonal	hepatocellular necrosis, inflammation & fibrosis	not assessed	J Vet Diagn Invest. 1997 Apr;9(2):201-3
Skye Terrier	358-2257	centrolobular	cirrhosis, chronic hepatitis	not assessed	Vet Pathol 1988, 25:408-414.
	not assessed	not assessed	chronic hepatitis, bile stasis	died	Dansk veterinærtidsskrift 1991; 74: 14-15
	462	not assessed	micronodular cirrhosis, uneven distribution of inflammation	antibiotics, lactulose, ursodeoxycholic acid, colchicine, zinc, waltham hepatic support diet for 12 months	JSAP 2003 (44) 85-89.
West Highland W. T.	normal - 3500	not assessed	29 dogs: high Cu + normal histology, 15x high copper and hepatitis or cirrhosis		Vet Pathol 1986, 23:148-154.
	20-6800	begin centrolobular, later all zones	hepatitis	not assessed	Vet Pathol 1990; 27: 81-88
	>1100	copper excretion study	not assessed	not assessed	JVIM 1992 6: 41-43
	>471	not assessed	not assessed	not assessed	39(1): 39–43.
Labrador Retriever	1317 (402-2576)	centrolobular	chronic hepatitis, cirrhosis	penicillamine & prednisolone => improvement	JVIM 2006: 20:856–861.
	>471	not assessed	not assessed	not assessed	Can Vet J. 1998, 39(1): 39–43.
other breeds & cats:					
	1441-2921	not assessed	macronodular cirrhosis and high Cu	not assessed	Der Prakt. Tierarzt 1999: 170-175
German Shepherd	570,1352,2202	no assessment in intact lobuli	cirrhosis	not assessed	Vet Med 1985; 50-54
Anatolian shepherd	4+	centrolobular > all zones	chronic hepatitis	penicillamine + prednisolone, improvement	Vet.Rec, 2003; 152 : 84-85
Keeshond	90-2400	begin centrolobular, later all zones	hepatitis	not assessed	Vet Pathol 1990; 27: 81-88
Boxer	1101	centrolobular (zone 3+2)	pigment granulomas, normal architecture	not assessed	Tijd.v. Diergeneesk. 1002:suppl1S16
European Shorthair cat	4170	centrolobular	cirrhosis, chronic hepatitis	not assessed	Vet Pathol.2005: 42: 97-100
Siamese Cat	4074	centrolobular	hepatocellular necrosis & inflammation	died	Vet. Pathol. 1995, 32: 427-429.

f: female, m: male, dw: dry weight liver, ALT:alanine amino transferase, ALP: alkaline phosphatase, bili: bilirubin, y: years, T.: terrier

Therapy

The goal of medical therapy is to reduce the absorption of copper and to enhance its excretion. Therefore, diets heavily supplemented with copper and copper-containing vitamin/mineral supplements should be avoided. Foods containing large amounts of copper, such as eggs, liver, shellfish, organ meats, beans/legumes, mushrooms, chocolate, nuts and cereals should be excluded from the diet.
For enhanced copper excretion, chelating agents are commonly used. Chelators compete with binding sites for metals and produce a water-soluble complex with copper, which is then excreted into urine or bile. The standard chelating agent for the treatment of copper storage disorders in people and dogs is penicillamine. Another accepted treatment in people is the use of zinc.[86,97-100]

Penicillamine

Recommended dosage: 10-15mg/kg BID PO

Penicillamine can chelate a variety of metals including copper. The drug leads to mobilization of copper from tissues and promotes copper excretion in urine. Penicillamine also may increase the synthesis of metallothionein, and has anti-inflammatory, immunosuppressive and antifibrotic effects.[90,101-106] Life-long therapy might be required. The drug is effective for the treatment of chronic hepatitis due to copper accumulation. Adverse effects, such as inappatence, vomiting, and diarrhea frequently occur in dogs. These adverse effects can generally be adverted by mixing the drug with food, and dividing the daily dosage into frequent applications. Side effects reported in people include vitamin-B deficiency from increased urinary loss of pyridoxine, fever, cutaneous eruptions, lupus like symptoms, lymphadenopathy, cytopenias, and proteinuria. Penicillamine is potentially teratogenic and its use during pregnancy is not recommended. Pet owners should be informed about the potential risks of handling the drug for pregnant women.
Clinical improvement from penicillamine treatment might take weeks to months, and large interindividual variations are observed with respect to the effectiveness of the drug in people, as well as in dogs. Follow-up liver biopsies are generally required to determine if a patient will need long-term therapy or just some months of treatment. One author described an average detoxification rate of around 900 ppm copper decrease per year during penicillamine treatment in Bedlington terriers.[86,100]
Penicillamine was effective for treatment of Doberman pinschers with copper-associated subclinical hepatitis.[90] We have tested copper chelation therapy with penicillamine (10-15mg/kg twice daily PO for 3-6 months) in Labrador retrievers in a randomized, double blind, placebo-controlled study and found the drug to be effective for the treatment of hepatic copper accumulation in this breed (chapter 6).

Zinc

Recommended dosage: 200mg of elemental zinc daily per dog (in devided doses)

Oral zinc is given to reduce copper absorption from the diet. Zinc induces the production of metallothionein in intestinal mucosal cells. Metallothionein is a cysteine-rich protein, which acts as an endogenous chelator of metals with high affinity for copper. Metallothionein binds copper from the diet, preventing its transport into the circulation. Most of the bound copper is lost in the feces when intestinal cells are shed from the villi. Zinc might also induce hepatic metallothionein for nontoxic storage of copper. Since the rate of removal of hepatic copper is relatively slow, dogs with severe or fulminant copper-induced hepatitis should not be treated with zinc alone. Theoretically, zinc given orally together with penicillamine may decrease the effectiveness of both drugs.
The type of zinc salt used does not influence efficacy of the drug in people, but may affect tolerability. Acetate and gluconate salts may be more tolerable than sulphate. Theoretically, zinc should be given apart from feeding, because some food constituents (such as phytates) can bind zinc and diminish its efficacy. However, the salts might be irritant to the gastric mucosa and lead to nausea and vomiting. Therefore, mixing of the drug with small amounts of food has been recommended. The plasma zinc concentration of dogs normally ranges from about 90 to 120 µg/dl. As plasma zinc concentration increases above 200 µg/dl, copper uptake may be suppressed. Zinc is a relatively safe drug, but large doses may cause gastrointestinal disturbances. At plasma zinc concentrations above 1000 µg/dl, hemolysis may occur. In a study of 3 Bedlington terriers and 3 West Highland White terriers with copper toxicosis, 200mg of elemental zinc was given daily to each dog in order to achieve therapeutic plasma concentrations of zinc above 200 µg/dl. The effectiveness of zinc in the prevention of copper uptake from the intestine was assessed by measurement of peak plasma concentrations of radioactive copper after oral application. A minimum of 3 months of zinc treatment was necessary before copper uptake from the intestine was blocked.[92] Although zinc is currently reserved for maintenance treatment, is has been used as first-line therapy in people, most commenly for asymtomatic or presymtomatic patients. For this indication, the drug appears to be equally effective to penicillamine and is much better tolerated.[92,97-99]

Trientine (2-2-2-tetramine tetrahydrochloride)

Recommended dosage: 10-15mg/kg q 12h (66)

Trientine is a chelator, which enhances the urinary excretion of copper. Trientine is poorly absorbed from the gastrointestinal tract. The drug is described for treatment of Wilson's disease in people, where it is used in patients who are intolerant to penicillamine. Symptoms of toxicity in people include bone marrow suppression, proteinuria, and autoimmune disorders, such as systemic lupus erythematosus. In addition trientine has teratogenic effects.[86,98-100,107]
Another tetramine salt, 2,3,2-tetramine (=tetramine) was studied in 5 Bedlington terriers with copper toxicosis. The drug was very potent and without adverse effects. Hepatic copper concentrations decreased more than 50% during treatment with tetramine for 6 months, and histologic changes were improved (150mg trientine salt in capsules BID PO per dog, 10kg average weight, range 6.8-13.6kg). The authors of the study recommended serial copper assessment during long-term treatment with the drug in order to avoid copper depletion of liver tissue and blood.[86]

Tetrathiomolybdate

Ammonium tetrathiomolybdate forms a tripartite complex with copper, which is stable. Given with food, tetrathiomolybdate can form complexes between copper and food proteins, and therefore prevents the absorption of copper. When given between meals, tetrathiomolybdate forms complexes with available serum copper (free copper) and albumin, rendering cellular uptake of copper ineffective. The drug is described for intravenous use in sheep with copper toxicosis, as well as a possible emergency approach in patients with acute hemolytic crisis from hepatic copper release. No studies have been performed in dogs. Tetrathiomolybdate is toxic, and copper deficiency can occur with use of this drug, which can lead to anemia due to copper depletion of bone marrow. Tetrathiomolybdate is not commercial available.[15,48,97,98]

Molecular genetic research

Why dogs?

Specific advantages for genetic studies in dogs are the population structure of registered breeds with excellent genealogical records. Registered dog breeds are genetic isolates with minimized gene flow, comparable to geographically isolated human populations of minimized genetic heterogeneity. Dog breeds are groups of animals with similar behavioural and physical characteristics, which originate from human selection and preference. Genetic isolation of a breed occurs, because by breeding policy registration of offspring as breed members is only possible when both parents are registered to the same breed. Therefore genetic uniformity (linkage disequilibrium) is generally large within a breed. Depending on the breed under investigation linkage disequilibrium extends over 20-100 times longer genomic regions compared to humans.[108] In contrast large differences exist between different breeds.[109,110] Phenotypic diversity is so extreme between dog breeds that in some instances differences of morphologic features between breeds exceed the differences between dogs and other members of the family canidae (figure 4). The high degree of genetic differentiation between different breeds is mainly explained by human selection. A high degree of inbreeding from use of popular sires and narrow bottlenecks has resulted in more than 360 genetic diseases in dogs. The fact that many of these disorders in dogs are similar to genetic diseases in man, and that both share the same environment makes the dog an interesting genetic model.[111-117]

Figure 4: Skull of an adult Pug and an adult Pyrenean Mountain Dog

The average body height of Pugs is 30 cm, and the average body weight is 6 kg. Pyrenean Mountain dogs are between 65 and 80 cm tall and weigh between 40 and 60 kg. By courtesy of Skulls Unlimited International, Inc.

Phenotyping

Advances in molecular genetic techniques and access to the complete dog genome enable relatively easy and quick assessment of major gene disorders. The remaining difficulties facing a researching clinician investigating a new genetic disorder are threefold and consist in the precise characterization of the phenotype in order to correctly distinguish affected dogs and normal individuals, the identification or estimation of the inheritance pattern, and attaining the study population size necessary for reliable statistical testing. These difficulties have been reviewed by others.[118,119]

One goal of genetic studies is to identify one or more genes that cause a specific phenotype. A phenotype is described by observable and measurable characteristics of a disease. For practical reasons, the definition of the phenotype should be as precise and as simple as possible, allow for correct and easy distinction between affected and normal individuals, in order to avoid the inclusion of phenocopies (fig. 5). A phenocopy is an imitation of a phenotype, appearing identical but coded by a different genotype. Another potential hurdle for phenotyping can be a late onset disease. For disorders of slow, gradual, and late onset precise classification of normal can be difficult when disease is not (yet) apparent. The probands may be classified as "unknown phenotype", and analysis of affected probands only can be performed in this case. Alternatively a model with liability classes based on age can be applied.

The term "disease trait" is sometimes used interchangeably with phenotype. However, a trait is one characteristic of an individual or a disease and, strictly speaking, a trait can be expressed in different phenotypes. For example, haircolor may be a trait, whereas black or yellow may be a phenotype.[120,121] [120,121]

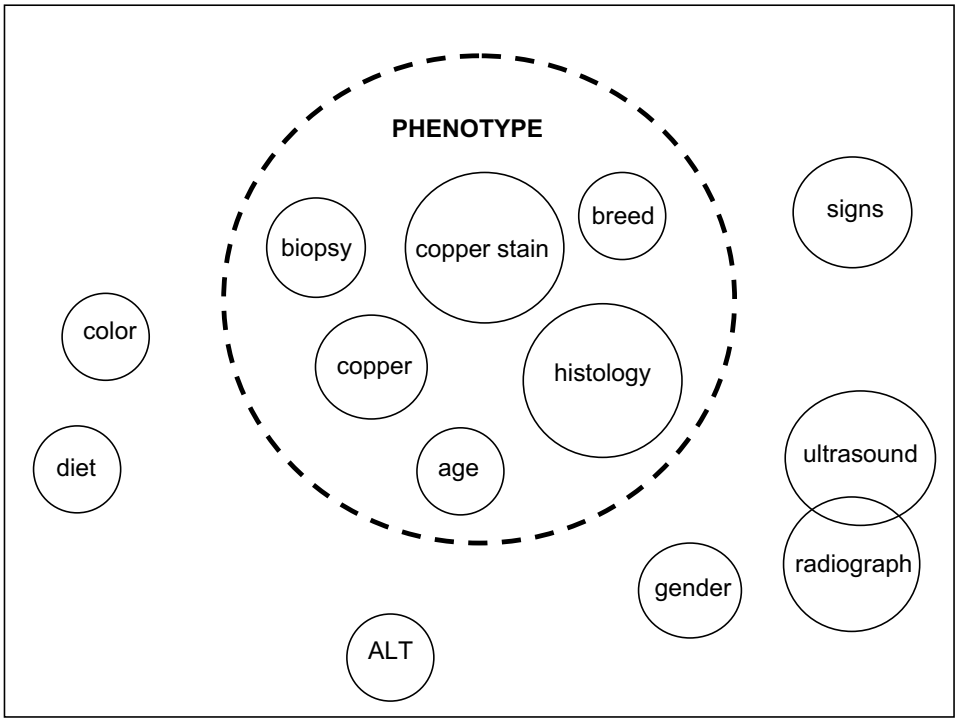


Figure 5: Phenotyping

Example of the phenotype definition for copper-associated chronic hepatitis in Labrador retrievers. Definition of the phenotype requires a clear description of the disease under investigation. A phenotype should be as precise as possible, allow for correct and easy distinction between affected and normal individuals, and avoid the inclusion of phenocopies

Genotyping

For genetic analyses, patients may be assumed to share a particular allele haplotype of a DNA marker, which is in linkage disequilibrium with the causative gene mutation and the phenotype of the investigated disease, or which is itself functional and causal for the phenotype. Linkage disequilibrium describes a correlation between two loci that occurs more often than expected by chance in very distantly related individuals within a population.[122] The underlying hypothesis is that patients of a homogeneous population inherit an identical disease mutation with the same surrounding DNA sequence from a common ancestor. The common allele haplotype of the patients is identical by descent (IBD). Genetic analysis should therefore allow for the identification of chromosomal segments that are shared by the patients. The identical chromosomal segments will be longer if they derive from a recent common ancestor, and they will be longest within a family, since fewer meiosis steps occurred which have the potential to separate the disease haplotype from it's surrounding DNA sequence during recombination.[123,124]

The identification of a shared allele can be attempted by genotyping of polymorphic DNA-markers, such as microsatellite markers, as well as single nucleotide polymorphisms (SNPs). Apart from the analysis of known regions of interest during a candidate gene approach it is possible to screen the entire genome of a test population for identification of new genes involved in the disease of interest by use of a whole genome genotyping approach. For such analysis, a large set of DNA-markers regularly spaced throughout the genome is analyzed for each individual dog, and statistical testing is performed in order to find differences between groups, or commonality within the affected group. Both methods of analysis (candidate gene approach and genome-wide genotyping) can be performed in groups of unrelated animals as well as in groups of related animals.

Linkage Analysis

Linkage analysis can be used for the assessment of genotyping results within families. The approach is very succesful for analysis of single and major gene disorders, and especially promising for analysis of diseases with inheritance in Mendelian pedigree patterns. Nevertheless, this method has also been used for analysis of complex disorders. Linkage is present if two loci are transmitted from parents to offspring more often than expected by chance (figure 6). Linkage within a family occurs over larger regions of the genome than linkage disequilibrium within a population. Therefore assessment for linkage within a family requires genotyping of fewer DNA markers than population based studies. Linkage analysis is a good first screening method for the identification of probable locations of genes.[120,125]

For disorders of a known mode of inheritance, parametric analysis using logarithm of odds (LOD) scores is the statistical method of choice to assess linkage because this method has more power to detect linkage than nonparametric tests. However, in order to acchieve reliable results, the allele frequencies for the disease allele and the marker alleles have to be accurately estimated and the mode of inheritance should be known. Furthermore, LOD scores will vary with the estimated recombination fraction between loci, theta (θ). Nonparametric linkage analysis (NPL) is a model-free calculation, which is used to assess for allele sharing among affected individuals without assumptions of the inheritance pattern of a disorder. In this analysis, genotypes of related individuals with a positive phenotype are expected to have an increased IBD sharing of the disease-associated haplotype[123,125,126]

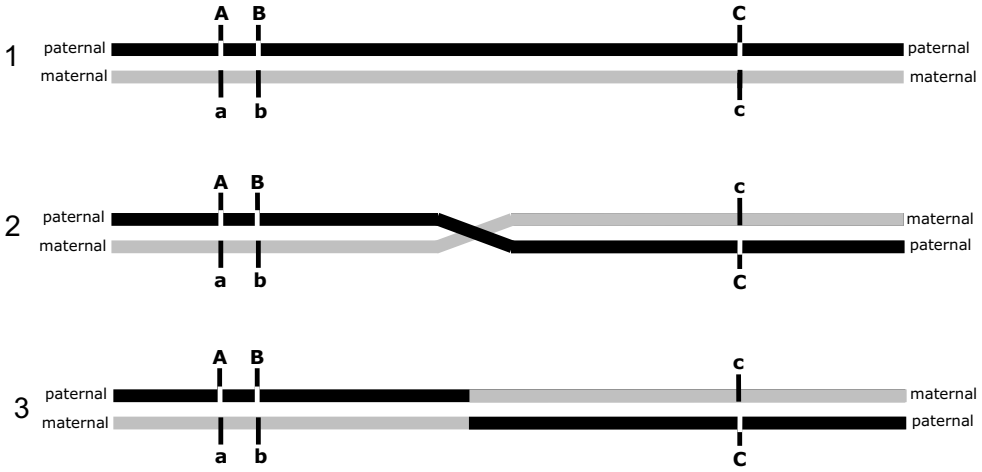


Figure 6: Principle of linkage analysis

1: paternal (black) and maternal (grey) chromosomes. Three DNA sequences are labelled with A, B and C (capital letters = paternal alleles, lower case letters = maternal alleles). 2: recombination with crossing over of DNA strands between the chromosomes. 3: Maternal and paternal alleles are recombined (mixed). If A is the disease gene and B and C are genetic markers, recombination is likely to occur much more frequently between A and C than it is between A and B.

Association studies

Association studies analyze the results of genotyping in unrelated individuals. Association can be found when comparison of genotyping results between affected and normal dogs reveals a difference in allele frequencies of a polymorphic marker accross a whole population. Association studies are succesful for investigating small effects and, therefore, are ideal for the assessment of complex disorders, as well as the fine-mapping of disease loci. Since individuals are unrelated or only distantly related, shared haplotypes are shorter and therefore the analysis requires more DNA-markers and larger test populations.[127,128] Linkage disequilibrium extends over much longer genomic regions in dog breeds compared to humans.[108] Although fine-mapping and identification of causal mutations requires less DNA-markers compared to humans, once association is detected it can be more complicated to identify causal mutations in the dog. High throughput sequencing, or comparison to other dog breeds, as well as other breeding lines within the same breed might be considered in these cases. Assortative mating leading to population fragmentation within the poodle breed has recently been reported.[115] Strong intra-breed differentiation was found by the authors, who described that one of the investigated poodle groups appeared genetically as different from the other groups of poodles as recognized dog breeds are different from each other.[115]

Our study of copper-associated chronic hepatitis (CACH) in Labradors was planned to follow a step-wise investigation of the following questions, which are an adaptation of the approach proposed by others.[120]

1. What is the phenotype underlying CACH in Labrador retrievers?
2. Is there evidence for aggregation/segregation of the phenotype within families?
3. Is the pattern of aggregation/segregation consistent with the effect of genes?
4. Are haplotypes of known genes involved in the phenotype?
5. Where in the genome is a causative gene most likely located?
6. Can we be more precise about the location?
7. Is the mRNA expression of genes in this region affected?

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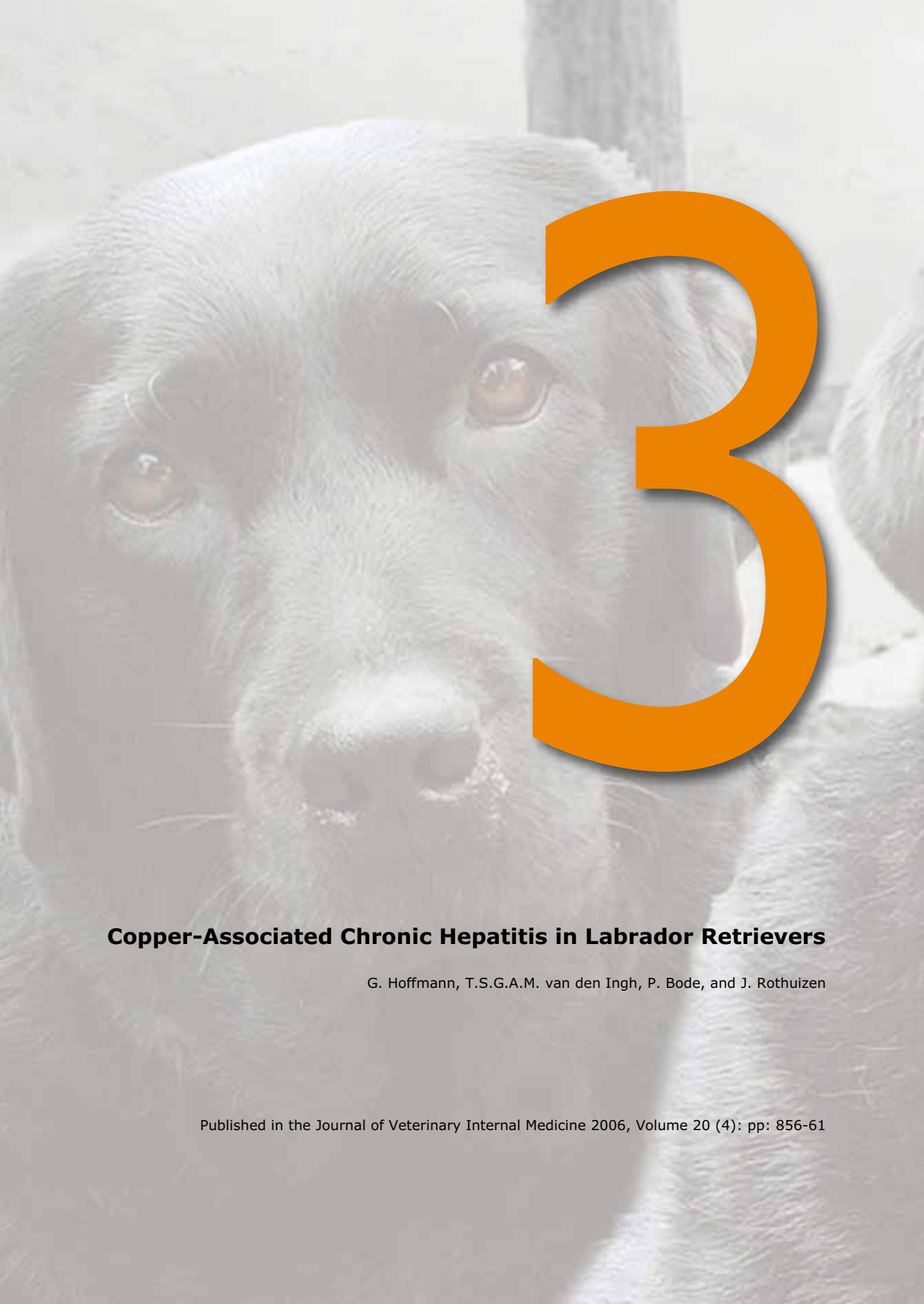
Part 1

A new breed-related disease in the Labrador retriever

Chapter 3-5



3

Copper-Associated Chronic Hepatitis in Labrador Retrievers

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Abstract

This study summarizes the clinical and pathologic findings in 15 Labrador retrievers with copper-associated chronic hepatitis (CACH). Our hypothesis was that this form of hepatitis is caused by a defect in hepatic copper metabolism, which most likely originates from a genetic defect. Affected Labradors consisted of 11 female and 4 male dogs. Eight family members of 2 of these patients were examined prospectively, as were 6 unrelated healthy Labrador retrievers. All dogs were registered at the breed club. The average age at clinical presentation was 7 years (range, 2.5–10.5 years). All dogs were presented for anorexia, which was associated with vomiting in 8 patients. The diagnosis of CACH was based on histologic examination of liver biopsy specimens in all dogs, including semiquantitation of copper. A disproportionate increase in alanine aminotransferase (ALT) activity relative to alkaline phosphatase (ALP) activity, as well as the centrolobular localization of copper and the association of copper accumulation with hepatic lesions, suggested a primary copper storage disease rather than primary cholestatic liver disease causing copper accumulation. Mean hepatic copper concentration measured in related Labradors was 1,317 µg/g dry weight liver (range, 402–2,576 µg/g). Mean hepatic copper concentration of unrelated normal Labradors was 233 µg/g dry weight liver (range, 120–304 µg/g). Our findings support the hypothesis that a hereditary form of hepatitis occurs in Labrador retrievers and is caused by a defect in hepatic copper metabolism.

Introduction

Chronic hepatitis (CH) is a histologic diagnosis, characterized by the presence of fibrosis, inflammation, and hepatocellular apoptosis and necrosis. Cirrhosis can result as the end stage of the disease.[1] The term chronic hepatitis is used regardless of the cause of the disease, which usually is unknown in naturally occurring canine chronic hepatitis, although some cases have been associated with infections[2], toxins[3-6], and hepatic copper accumulation[1,7-9]. Hepatic copper accumulation can result from increased uptake of copper, a primary metabolic defect in hepatic copper metabolism, or from altered biliary excretion of copper. Inherited copper toxicosis is a well-described disease in the Bedlington terrier in which a deletion in the COMMD1 gene (previously called MURR1) causes accumulation of copper in hepatocytes, resulting in chronic hepatitis.[8-11] Moreover, hepatic copper storage and associated hepatitis seems to be breed-associated in the West Highland White Terrier[11,12], Skye Terrier[13], Doberman Pinscher[14-16],and Dalmatians[17].

The Labrador retriever often is affected by chronic hepatitis.[18] Our objective was to summarize the clinical and pathologic findings in 15 Labrador retrievers with copper-associated chronic hepatitis (CACH) and to investigate the possibility of a genetic basis of the disease by examination of family members from a line of Labradors with copper-associated chronic hepatitis.

Materials and Methods

Affected Labrador retrievers

The study population consisted of 15 consecutively identified Labrador Retrievers that had been diagnosed with chronic hepatitis or cirrhosis associated with increased copper concentrations and were registered at the Dutch Labrador Retriever Breed Club[a]. The dogs were identified from the records of the diagnostic veterinary pathology service at the Faculty of Veterinary Medicine, University of Utrecht. All biopsy samples were evaluated by one of the authors (TvdI). Biopsy specimens from 11 of 15 dogs were obtained by our hepatology service at the University Clinic. Biopsy specimens from 4 dogs were obtained from private clinics. Clinical and clinicopathologic data were obtained from the medical records, as well as by telephone conversations with the veterinarians who managed the latter 4 dogs. Signalment, medical history, clinical examination findings, laboratory findings, histopathology, hepatic copper assessment, and the extraction method used for each liver biopsy specimen, as well as disease progression, were recorded. For comparative purposes, liver enzyme activities were reported as relative increase above the upper limit of the reference range. Pedigrees were obtained from the Dutch Labrador Retriever Breed Club.

Hepatic tissue was stained with rubeanic acid for evaluation of copper distribution and semiquantitation. A histochemical grading system previously applied to liver tissue from Bedlington terriers with copper toxicosis and Doberman pinschers with copper-associated hepatitis was used for semiquantitative evaluation of hepatic copper concentrations in all liver biopsy specimens.[19] According to this grading system, copper scores >2 are considered abnormal[b].[20]

Related Labradors and normal unrelated Labrador retrievers

For 2 dogs with CACH, 8 of 17 identified family members from 3 different litters participated in the study. In order to obtain a healthy control group for comparison of hepatic copper concentrations, owners of 6 unrelated Labrador retrievers agreed to participate in this study.

A medical history was obtained from all dogs (n = 14), and physical examinations were performed. Blood samples were collected in sodium citrate for analysis of a coagulation profile, including prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen concentration. Blood was collected in heparin and EDTA for analysis of alkaline phosphatase (ALP), alanine aminotransferase (ALT), bile acids (BA), and platelet count. Liver biopsy specimens were collected according to the Menghini technique described by Rothuizen.[21] At least 3 liver biopsy specimens were taken from each dog. Two specimens were fixed in 10% neutral buffered formalin, and 1 biopsy was stored in a copper-free container for quantitative copper determination. Quantitative assay for copper in liver tissue was performed by neutron activation analysis according to a protocol described by Teske et al.[22] using the facilities described by Bode.[23] Quantitative copper concentrations were measured in lyophilized liver and reported in µg/g dry weight liver (dwl).

The study was approved by the Utrecht University Institutional Animal Care and Use Committee. Owner consent was obtained for all dogs participating in this study.

Statistical Analysis

Statistical analysis was performed by use of commercially available software[c]. A normal reference range was established from mean hepatic copper concentrations of 6 healthy dogs ± 2 standard deviations. Because of small group sizes, a nonparametric statistical test was used for comparison between groups (Mann-Whitney test).

Results

Affected Labradors

Of 15 Labrador retrievers with copper-associated chronic hepatitis, 2 dogs were related (dog 7 was the mother of dog 9). All dogs were registered at the breed club. The mean age at clinical presentation was 7 years (range, 2.5–10.5 years). Eleven dogs were females, including 3 spayed and 8 intact dogs, and 4 dogs were males, including 3 intact and 1 neutered Labrador. All dogs were evaluated between 1998 and 2002.

All dogs were presented for gastrointestinal signs, including anorexia in all and vomiting in 8 dogs. Clinical signs were acute in onset and progressive over several days (n = 9 dogs) to weeks (n = 6 dogs) before presentation to a veterinarian. Two dogs (dogs 2 and 9) presented 21 and 12 days after whelping. Three patients had a history of weight loss (dogs 3, 13, and 14), and 2 were reported as thin (dogs 3 and 14). Five dogs developed jaundice during the course of the disease (dogs 1, 5, 9, 12, and 14). Ascites was diagnosed in 2 patients (dogs 9 and 11), which had serum albumin concentrations of 1.8 and 2.3 g/dL. One patient (dog 4) had concurrent diabetes mellitus which was diagnosed 13 months before presentation and since that time was regulated with Caninsulin[d]. IgG and IgM titers for Leptospira spp. were negative in 4 dogs. No exposure to potentially hepatotoxic drugs or exaggerated copper intake was reported.

The mean relative increase in ALT activity was 10.4 times the upper limit of the reference range (1–20.7 times above the upper reference value). The mean relative increase in ALP activity was 4.6 times the upper limit of the reference range (1.6–10.9 times above the upper normal value). In all but 1 patient (dog 10), the relative increase in ALT activity was greater than the relative increase in ALP activity. Five dogs had increased serum total bilirubin concentrations at initial presentation. The mean serum albumin concentration was 2.8 g/dL (range, 1.8–3.4; normal range, 2.6–3.7 g/dL). Four patients had decreased serum albumin concentrations, <2.0 g/dL in 1 dog (dog 9: albumin, 1.8 g/dL).

In 2 dogs serum urea nitrogen concentrations were below the normal range of 8.4 to 35 mg/dL (dogs 2 and 9). Blood ammonia concentrations were normal in both of these dogs. The serum glucose concentration was mildly increased in 1 dog (dog 9) and normalized during hospitalization. Five of 12 dogs evaluated by coagulation profile had normal PT, aPTT, and fibrinogen concentrations. Isolated increases in aPTT were identified in 2 dogs (dogs 1 and 5), and an isolated increase in PT was identified in 1 patient (dog 8). Plasma fibrinogen concentrations were decreased in 5 patients (mean, 1.47 g/L; range, 0.5–3.3 g/L). These decreases were associated with prolonged PT (2 dogs), aPTT (1 dog), or prolonged PT and aPTT (2 dogs).

Red blood cell counts were within normal reference range in all 14 dogs evaluated. Five dogs had mild to moderate neutrophilic leukocytosis (mean white blood cell count, 14,000/µL; range 6,100–28,900/µL).

Abdominal ultrasonography was performed in 12 dogs. The liver was of normal size in 5 of 12 dogs. In 6 dogs, the liver was small and located entirely within the rib cage. In 1 dog, the liver was mildly enlarged. Hepatic echogenicity and structure were normal in 9 of 12 patients. A hyperechoic liver and diffuse irregularities were seen in 3 dogs. In 4 patients, the liver was found to be normal for all 3 variables (ie, size, echogenicity, and structure). Ascites was present in 2 patients (dogs 9 and 11).

Liver tissue was obtained at postmortem examination (dogs 9, 12, 15), during laparotomy (dog 13), by ultrasound guidance (dogs 1, 2, 5, 7), or by the Menghini technique (dogs 3, 4, 6, 8, 10, 11, 14). Chronic hepatitis (n = 11), or CH with cirrhosis (n = 4) were associated with histologic evidence of copper accumulation in liver tissue of all patients. In all biopsy samples CH was characterized by varying degrees of hepatocellular apoptosis and necrosis, mononuclear inflammation, regeneration, and fibrosis. The severity of the hepatic inflammation was determined by the quantity of inflammation and extent of hepatocellular apoptosis and necrosis. Hepatitis was mild in 1 dog, moderate in 6 dogs, moderate to severe in 3 dogs, and severe in 5 dogs.

The disease stage was determined by the extent and pattern of fibrosis and the presence of cirrhosis. Liver architecture was normal in 6 dogs. Architectural distortion in liver tissue of 9 dogs included focal portal fibrosis (5), porto-central fibrosis (2), centro-central fibrosis (5), and dissecting fibrosis (2). Cirrhosis, characterized by regenerative nodules <3 mm in diameter, was diagnosed in 4 dogs (dogs 6,

9–11). Histologic features of cholestasis were present in biopsy specimens of 5 patients and graded as mild to moderate (dogs 6, 8, 9, 15) or severe (dog 1). Results of semiquantitative assessment of copper ranged from 2–3+ to 5+. Copper accumulated in zone 3 of the liver lobule (Fig. 1) and was associated with inflammation in all biopsy samples.

Follow-up

Two dogs died and 7 affected Labradors were euthanized for signs attributable to their hepatic disease. The average time from initial presentation to death in these 9 dogs was 10.4 days (range, 1–50 days). None of these dogs had received copper-chelating medication, because early in the course of data collection Labrador retrievers with chronic hepatitis were treated with a standard therapy consisting of prednisolone.[24] Because of histopathologic evidence of copper-related disease, penicillamine was added to the treatment regimen and finally applied as the sole medication. Clinical and histopathologic signs resolved with penicillamine therapy in 5 patients. Four of these 5 dogs received penicillamine exclusively (15 mg/kg PO q12h), and 1 dog received a combination of prednisolone (1 mg/kg PO q24h) and penicillamine (15 mg/kg PO q12h). One patient developed CACH again 1 year after histologic cure and cessation of penicillamine treatment. The disease in this dog was again successfully controlled by reinstitution of penicillamine monotherapy.

Related Labradors

Five dogs were female and 3 were male. The average age was 4 years and ranged from 2 to 5 years. All dogs were clinically healthy and had no previous signs of hepatic disease. There was no evidence of exposure to drugs or possible exaggerated copper intake, and all affected dogs were fed commercial dog food. Serum activities of ALT, ALP, bile acid concentrations, coagulation profiles, and platelet counts were normal in all dogs. Histopathologic examination of liver biopsy samples identified the presence of copper localized in centrolobular hepatocytes. In 6 dogs, the liver was normal histologically. In 2 dogs, mild hepatitis was present, with periportal and central pigment-laden Kupffer cells and macrophages, and moderate numbers of lymphocytes and plasma cells, as well as moderate numbers of copper granules. Results of semiquantitative assessment of copper were 1–2+ in the dog with a quantitative copper concentration of 402 µg/g dwl, and ranged between 2–3+ and 3+ in the remaining dogs.

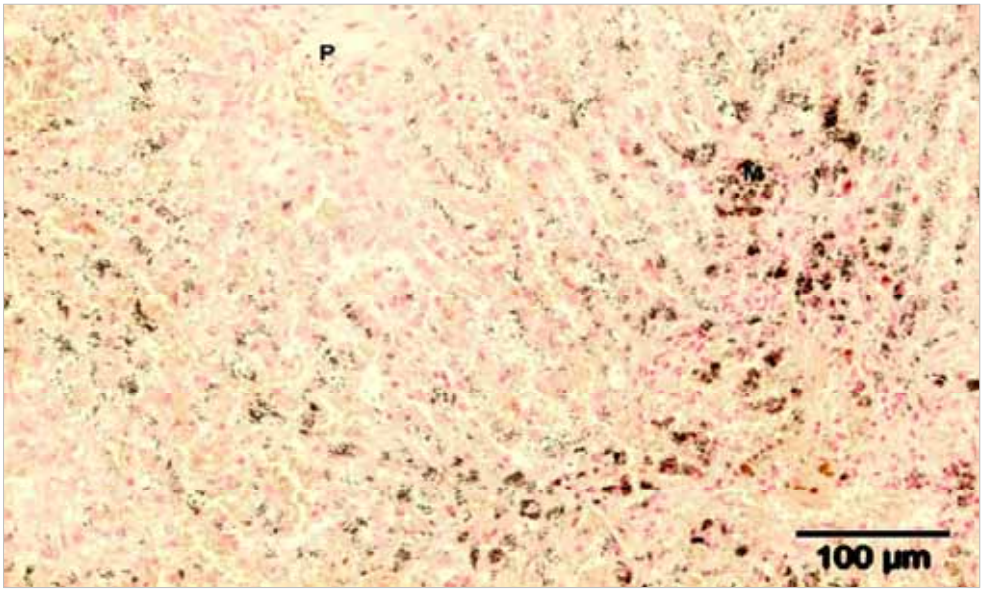

Figure 1: 3µm thick histology slide of liver tissue from a dog stained with rubeanic acid for copper. Score 2-3+. By courtesy of Dr. T.S.G.A.M van den Ingh, Department of Pathobiology, Utrecht University

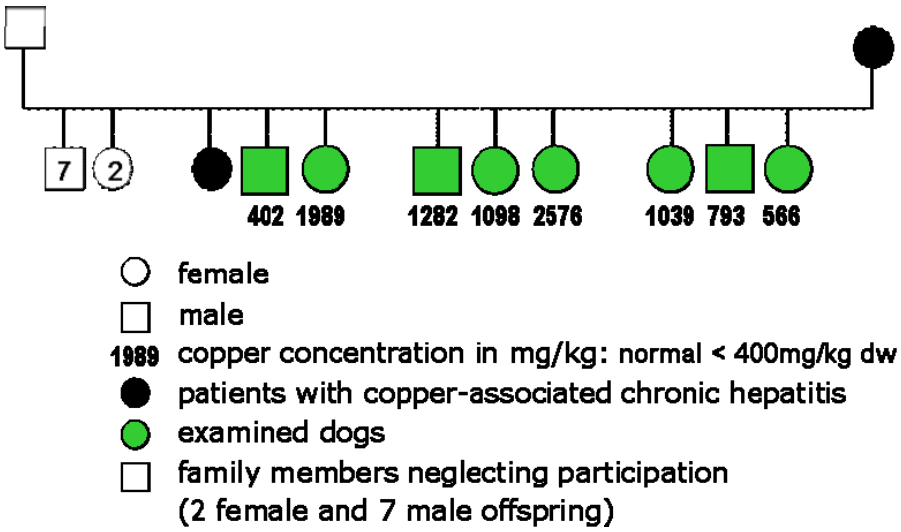

Figure 2A. Hepatic copper concentrations of 8 family members from 2 Labrador retrievers with copper-associated chronic hepatitis.

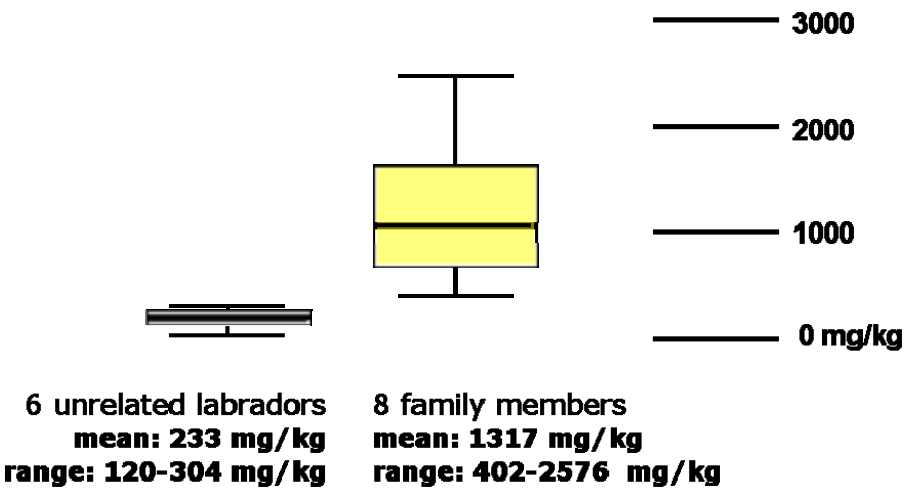

Figure 2B. Box plots depicting the copper concentrations in liver tissue of 6 unrelated Labrador retrievers (left) versus the 8 family members of patients with copper-associated chronic hepatitis (right)

The mean quantitative hepatic copper concentration measured in the clinically healthy siblings and offspring was 1,317 µg/g dry weight liver (range, 402–2,576 µg/g dwl), as shown in Figures 2A and 2B. There was good agreement between quantitative hepatic copper concentrations and semiquantitative copper measurements in this group of dogs, which ranged from 1–2+ to 3+ (mean 2–3+; Fig. 3). No difference was found between semiquantitative copper assessments in affected and related Labradors (P = .084).

Unrelated normal Labradors

All dogs were clinically healthy and had no previous signs of hepatic disease. Three dogs were female, and 3 were male. The average age was 7.8 years and ranged from 5 to 10 years. Blood biochemistry and hematology were normal in all dogs. Results of histologic examination of liver were normal in 4 dogs. In 2 Labradors, mild nonspecific reactive changes of the liver were diagnosed. In these biopsy samples, the liver architecture was normal, but scattered pigment-laden macrophages and a few polymorphonuclear leukocytes were found in the liver parenchyma. Results of histologic assessment of copper ranged from zero to 1+. Quantitative hepatic copper concentration in this group of Labradors was 233 µg/g dwl (range, 120–304 µg/g). A reference range for hepatic copper of <400 µg/g dwl was established for these Labrador retrievers (mean ± 2 SD).

A Mann-Whitney test detected a difference between hepatic copper concentrations from normal dogs and related Labradors (P = .001).

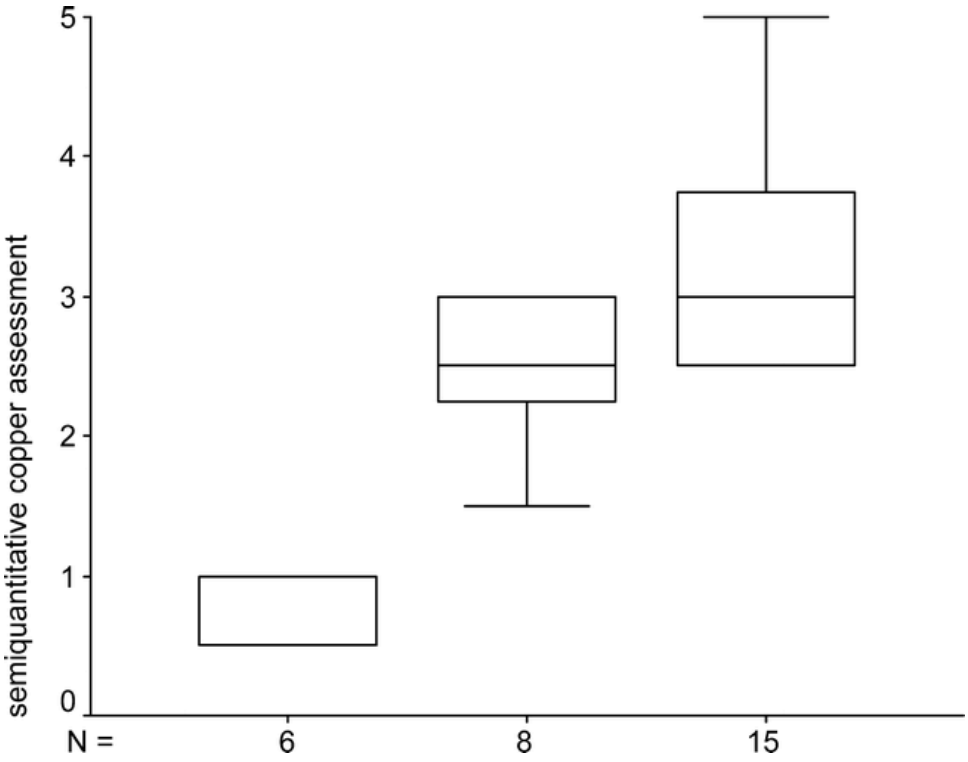

Figure 3. Box plots depicting the results from semiquantitative assessment of hepatic copper in 6 normal unrelated Labradors (left box), 8 related Labradors (middle box), and 15 affected Labradors with CACH (right box)

Discussion

Inherited copper toxicosis is an autosomal recessive disease in the Bedlington terrier in which a genetic mutation causes severe accumulation of copper in hepatocytes, resulting in chronic hepatitis. In addition to the Bedlington terrier, hepatic copper storage seems to be breed-associated in the West Highland white terrier, Dalmatian, Doberman pinscher, and Skye terrier. In the first 3 breeds, liver copper concentrations >2,000 µg/g dwl have been reported.[11,12,16,17] Copper concentrations in affected Skye terriers range from 800 to 2,200 µg/g dwl.[25]

This report describes 15 Labrador retrievers with chronic hepatitis associated with increased copper concentrations. Prospective examination of family members of these affected dogs identified subclinical hepatic copper accumulation (mean hepatic copper concentration of 1,317 µg/g dwl; range, 402–2,576 µg/g dwl).
In all but 1 of the affected dogs, serum ALT activity was increased, and ALP was above the upper limit of the reference range in all 13 dogs evaluated for ALP. Because the relative increase in ALT activity was greater than that of ALP (10.4 and 4.6 times normal, respectively), a primary hepatocellular liver disease was considered most likely. Serum total bilirubin concentration was increased in only 5 dogs. In addition, histopathology identified primary hepatitis and copper accumulation. Therefore, the cholestasis seen in some affected Labrador retrievers was considered secondary.

The reference range for hepatic copper concentration in dogs is reported to be 100–450 µg/g dwl.[11,26,27] This is in agreement with the copper concentrations measured in liver biopsy specimens of normal Labradors in this study (range, 120–304 µg/g). In our study, there was no overlap between copper concentrations of normal unrelated Labradors and related Labrador retrievers (P = .001). On the basis of the established normal range for hepatic copper in Labradors, some of the dogs in a previous study most likely had subclinical hepatic copper accumulation.[27] In that study, copper concentrations between 140 and 915 µg/g dwl were measured in 3 Labrador retrievers without hepatitis (mean, 448 µg/g dwl).[27] The dogs of that study were identified because a related Labrador retriever had liver disease. Because of a lack of additional detail on the hepatic disease of the related dog, it is not possible to compare those Labradors directly to our related Labradors. However, our finding of isolated accumulation of copper without development of hepatitis is in agreement with the previous results. Until the genetic defect underlying copper accumulation in Labradors is identified, we will not be able to explain why copper can accumulate without causing hepatitis in the Labrador retriever. One investigator had followed dogs with the COMMD1 deletion from birth to 3 years of age, and copper accumulated in the liver by 1 year of age, whereas histologic signs of hepatitis did not occur before affected dogs were 2 years old (R. Favier, personal communication). Dogs with inherited copper storage disorders may therefore be subject to a prolonged delay period of several years between severe accumulation of copper and development of histologic signs of inflammation.
The storage of copper in its nontoxic form may occur in the Labrador, allowing copper to accumulate in the liver of related and affected dogs. Exhaustion of cellular systems that protect against oxidative damage by occasional free reactive copper, or an additional stress factor that affects other protective mechanisms of the liver cell, may be necessary for copper to cause hepatitis in affected dogs.
In our group of 15 affected Labradors, hepatic copper was assessed by a semiquantitative histochemical grading system. Semiquantitative copper concentrations were previously correlated to quantitative measurements in other dog breeds, in which semiquantitative hepatic copper scores of 2–3+ corresponded to a concentration of 300–2,000 µg/g dwl in Doberman pinschers, and semiquantitative scores between 3+ and 5+ corresponded to quantitative copper concentrations between 1,500 and 4,000 mg/kg dwl in Bedlington terriers.[28,29] Our present results show a similar correlation of histochemical staining and quantitative copper concentrations in the related Labradors. This finding is in agreement with previous studies, and there was no statistical difference between semiquantitations of copper in related and affected dogs (P = .084). Nevertheless, semiquantitation of copper remains a subjective assessment and lack of quantitative measurement of copper in the affected Labradors is a limitation of our study. We did not measure quantitative copper concentrations retrospectively because liver biopsy

samples were taken by different techniques and not specifically handled and stored for quantitative analysis of copper. Quantitation of copper by neutron activation analysis is susceptible to interference from metals as well as electrolytes. Therefore, biopsy specimen handling and storage should be standardized in order to allow comparison of results to a normal range. Our normal reference range for copper was established for biopsy samples that were taken by means of the Menghini technique, using sterile water (not 0.9% NaCl) as well as copper-free plastic containers for storage. In our opinion, interpretation of quantitative copper concentrations from paraffin-embedded liver tissue, taken by different techniques and stored under nonstandardized conditions, could be difficult and potentially misleading.

Copper accumulation is always localized centrolobularly in inherited copper storage disorders, such as Bedlington terrier copper toxicosis, Wilson's disease in humans, and liver disease in Long-Evans Cinnamon rats. Therefore, localization of copper in the centrolobular region of the liver lobule in our Labrador retrievers may be considered an indicator for primary copper accumulation. This disease is unlike secondary copper loading of liver cells during cholestasis and cholatestasis, where copper is mainly restricted to the periportal parenchyma. In all biopsy samples of the investigated family members, hepatocytes that stained positive for copper were always localized in zone 3 (ie, centrolobular), and semiquantitative hepatic copper concentrations in this group of dogs ranged from 1+ to 3+ (mean, 2–3+). Although we were unable to investigate all members of the family of interest, the participating dogs represent a random sample of all 3 matings. Hepatic copper concentrations of all investigated members of this family were abnormal.

Our findings suggest that copper-associated chronic hepatitis is familial and most likely caused by an inherited genetic defect in the Labrador retriever. The results of this study support prospective studies in larger populations, as well as molecular biologic studies concerning copper metabolism, to identify the possible genetic basis of the disorder.

Acknowledgments

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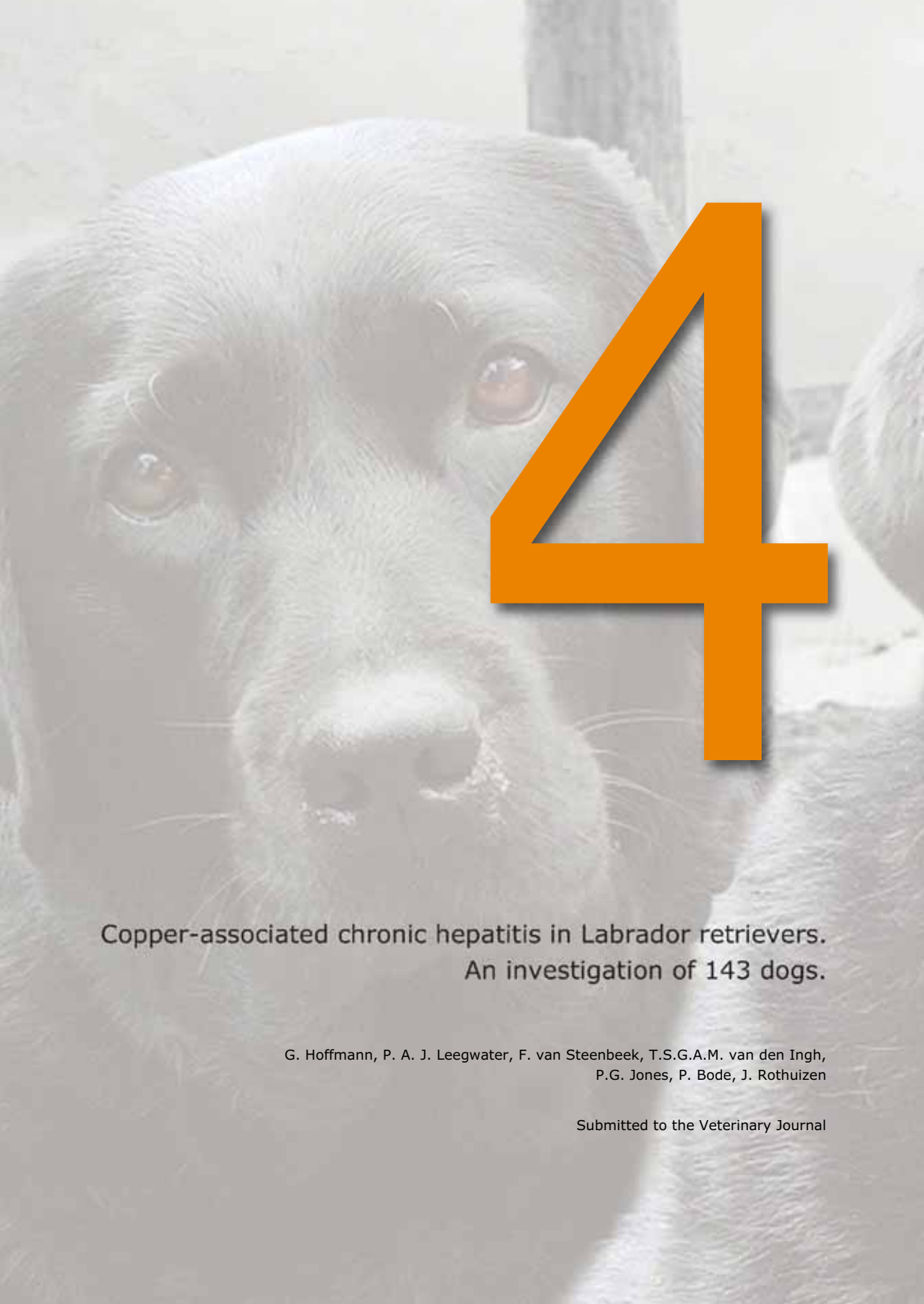
[a] Nederlandse Labrador Vereniging, Oosterbeek, Netherlands

[b] 0: no copper, 1: solitary liver cells and/or reticulohistiocytosis (RHS) cells containing some copper positive granules, 2: small groups of liver cells and/or RHS cells containing small to moderate amounts of copper positive granules, 3: larger groups or areas of liver cells and/or RHS cells with many copper positive granules, 5: diffuse presence of liver cells and/or RHS cells with many copper positive granules.

c SPSS 11.0 for windows, 2001, SPSS Inc., Chicago, IL

c Caninsulin, Intervet, Boxmeer, Netherlands



4

Copper-associated chronic hepatitis in Labrador retrievers. An investigation of 143 dogs.

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Abstract

Background: Based on the discovery of subclinical hepatic copper accumulation in a family of Labradors, a genetic background was previously suspected for copper-associated chronic hepatitis.
Hypothesis: Family members of patients with Copper-associated chronic hepatitis are at increased risk of the disease because accumulation of copper in the liver is an inherited trait in Labradors that can lead to chronic hepatitis.
Animals: One hundred and fourty-three client-owned Labrador retrievers.
Methods: A prospective study of 143 family members of patients with copper-associated chronic hepatitis. Hepatic copper concentrations and histopathology of liver biopsies were assessed along with clinical examinations and blood analyses in all dogs. Statistical testing was performed to detect differences between dogs with elevated hepatic copper and normal dogs.
Results: All dogs were registered at the Dutch Kennel Club. The average age was 5.7 years (range, 10 months-12 years). Eighty-nine dogs were female (36 intact, 53 spayed), and 54 dogs were male (38 intact, 16 neutered). The mean hepatic copper concentration was 821 mg/kg dry weight liver (range, 31–3,030 mg/kg). Ninety-eight dogs had elevated hepatic copper concentrations (above 400 mg/kg). Histologic staining for copper revealed elevated scores in 57 dogs, results in the high normal range in 23 dogs, and normal scores in 63 dogs. Histologic assessment for inflammation revealed reactive changes in 43 dogs, and chronic hepatitis in biopsies of 41 dogs (25=mild, 2=mild to moderate, 8=moderate, 6=cirrhosis). A protective effect was found for black coat color (p=0.004) and male gender (p=0.002).
Conclusions and clinical relevance: Our findings support that copper-associated chronic hepatitis of Labradors is caused by an inherited defect of hepatic copper metabolism. Gender and coat color interact with the phenotype of the disease.

Keywords: Heritability, Liver disease, hepatic zone 3, Bedlington Terrier, Wilson’s disease

Introduction

Inherited disorders of copper metabolism occur in man, dogs, mice, and other species. Well-characterized single gene disorders of copper metabolism include Wilson's disease in people and copper toxicosis in Bedlington terriers.[1,2] In these disorders, mutated genes lead to severely impaired biliary excretion of copper (ATP7B in Wilson's disease, and COMMD1 in copper toxicosis of Bedlington terriers) with subsequent copper accumulation in hepatocytes, resulting in chronic hepatitis.

Other copper accumulating liver diseases, such as Indian childhood cirrhosis and non-Indian childhood cirrhosis in people lack the simple inheritance patterns of single gene diseases. The characterization of these complex disorders is more difficult because the phenotype may be caused by the interaction of multiple genes that concur with environmental risk factors, like high copper intake.

Copper-associated chronic hepatitis (CACH) was recently described as a metabolic disease in the Labrador retriever in Europe, and was suspected in a report from the U.S.A.[3,4] The average age of Labradors with CACH is 7 years (range 2.5-10.5 years), and female dogs are more frequently affected than male dogs. Patients present with non-specific clinical signs, such as anorexia, vomiting, and weight loss. These signs occur late in the disease process, and often reflect the end-stage of a fatal cirrhotic liver disorder. A relative increase in alanine aminotransferase (ALT) activity above that of alkaline phosphatise (ALP) activity, the centrolobular localization of hepatic copper, and the association with hepatic lesions are typical findings described for this disease. Diagnosis is dependant upon histologic examination of liver biopsies, including assessment of copper. Retrieval of liver biopsies is an invasive procedure, which requires specialized veterinary practice, and diagnosis is therefore often missed at an early, treatable stage of the disease when liver pathology is still potentially reversible with appropriate medical care.[3]

The heritability of hepatic copper accumulation is moderate to high, with estimates ranging between 0.39 and 0.85 for Labrador retrievers.[5] Therefore, family members of affected dogs may be at increased risk for development of the disease.

The objective of this study was to investigate early preclinical stages of CACH in Labradors, and to reveal possible risk factors for the disease. To achieve this aim we examined 143 dogs that were related to clinical patients.

Materials and methods

More than four hundred dogs, related to patients with clinical copper-associated chronic hepatitis, were identified based on the registration records of the Labrador retriever breed club. The breed club invited owners of these dogs to participate in this prospective study. Of the invited dogs, 143 Labradors participated in the study. Written consent was obtained from each dog owner prior to the approach by the researching clinician.
A medical history was obtained from all dogs, and a physical examination was performed by one of the authors (GH). Na-citrated blood samples were taken for analysis of a coagulation profile, including prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen. Heparin-, and EDTA-blood was sampled for analysis of the hepatobiliary enzymes, alkaline phosphatase (ALP) and alanine aminotransferase (ALT), bile acids (BA), and for measurement of the platelet count. Liver biopsies were taken according to the Menghini technique described by Rothuizen.[6,7] At least three liver biopsies were taken from each dog. Two biopsies were fixed in 10 percent neutral buffered formalin, and one biopsy was stored in a copper-free container for quantitative copper determinations.
All biopsies were histologically assessed by one of the authors (TvdI). Hepatic tissue was stained with rubeanic acid for evaluation of copper distribution and semiquantitation as previously described.[7] According to the applied grading system, copper scores above 2 were considered abnormal.
A quantitative copper assay in liver tissue was performed by neutron activation analysis, according to a protocol described by Teske et al[8] using the facilities described by Bode.[9] Quantitative copper concentrations were measured in lyophilised liver biopsies and reported in mg/kg dry weight liver (dwl). Comparisons were made between dogs with elevated hepatic copper concentrations above 400mg/kg dwl and dogs with concentrations below 400mg/kg dwl for signalment, occurrence of the clinical signs vomiting, diarrhea, decreased appetite, weight loss, subjective observation of polyuria/polyduipsia, decreased endurance, and listlesness, as well as blood concentrations of ALT, AP, bile acids, platelet count, PT, aPTT, fibrinogen, zinc, and copper. In addition dietary concentrations of copper and zinc were compared between both groups. For this comparison, information provided by the feed manufacturers was used.
The study was approved by the Utrecht University Institutional Animal Care and Use Committee. Owner consent was obtained for all dogs participating in this study.

Statistical analysis
Statistical analysis was performed by use of commercially available software[a]. A Mann-Whitney test was used for comparison between groups in order to detect differences between dogs with elevated hepatic copper and normal dogs for variations in signalment, clinical parameters, and laboratory results, including histopathology (significance level $P < 0.05$).

Results

All patients were recruited between 2003 and 2006. The dogs belonged to 24 families. The average age was 5.7 years (range, 10 months-12 years). Eighty-nine dogs were female (36 intact, 53 spayed), and fifty-four dogs were male (38 intact, 16 neutered). The coat color was black in 71 dogs, chocolate in 26 dogs, and yellow in 46 dogs. All dogs were fed a commercial diet, and home addresses of dogs with high copper and normal copper were equally distributed throughout the Netherlands.

Quantitative measurement of copper
Hepatic copper concentrations of 45 dogs were within the normal range (< 400mg/kg dwl). Ninety-eight dogs had elevated hepatic copper concentrations. The mean hepatic copper concentration of all dogs was 821 mg/kg dwl (range, 31–3,030 mg/kg). The mean hepatic copper concentration of the female dogs was 921 mg/kg dwl (range, 31–3,030 mg/kg), and the mean hepatic copper concentration of the male dogs was 662 mg/kg dwl (range, 157–2,040 mg/kg). This difference was significant (p=0.042). There was no significant difference in hepatic copper concentrations between intact and spayed/neutered dogs within each group (P=0.1). The mean hepatic copper concentration was 646 mg/kg dwl (range, 31–2,620 mg/kg) in the black dogs, 1085 mg/kg dwl (range, 157–3,030 mg/kg) in the chocolate dogs, and 942 mg/kg dwl (range, 65–2,960 mg/kg) in the yellow dogs. The difference of copper concentrations between the black dogs and the other two coat colors was significant (black compared to chocolate: p=0.003, black compared to yellow: p=0.004). No difference was found between hepatic copper concentrations in chocolate and yellow dogs (p=0.5). The results are shown in Figure 1.

Hitologic staining for copper
Histologic staining for copper revealed elevated scores (above 2+) in 57 dogs, results in the high normal range (2+) in 23 dogs, and normal scores in 63 dogs (below 2+). Figure 2 depicts the correlation between hepatic copper concentrations and semi-quantitative scoring results for copper in our study population.
A significant difference was found in copper scores between female and male dogs (P=0.002). These results are shown in Figure 3. There was no difference in copper scores between intact and spayed/neutered dogs within each group (P=0.06). A difference in copper scores was found between black dogs and the other two coat colors (black compared to chocolate: P=0.002, black compared to yellow: P=0.006). No difference was found between copper concentrations of chocolate and yellow dogs (p=0.51).

Histologic assessment for inflammation revealed normal liver tissue in 59 dogs, reactive changes in 43 dogs, and chronic hepatitis in biopsies of 41 dogs (25=mild, 2=mild to moderate, 8=moderate, 6=cirrhosis). Inflammation was associated with elevated hepatic copper concentrations (p=0.005).
Eighty-five dogs had shown previous clinical signs, including intermittent vomiting in 66 dogs, decreased endurance in 32 dogs, subjectively observed increased water intake in 23 dogs, diarrhea in 17 dogs, decreased appetite in 16 dogs, unattempted weight loss in 13 dogs, and listlessness in 13 dogs. Fourty-five dogs had shown one single sign, 21 had developed 2 signs, and 19 dogs had developed three or more signs in the past. The signs had either appeared self-limiting to the owner or they had resolved under symptomatic therapy. The mean ALT activity was 52 U/L (range, 0-1104), the mean ALP activity was 51 U/L (range, 14-1615), and the mean concentration of fasted bile acids was 6.8 µmol/L (range, 1-55). The mean PT was 8.15 seconds (range, 6-16.7), the mean PTT was 15.8 seconds (range, 9.6-24.4), the mean fibrinogen concentration was 1.4 g/L (range, 0.6-3.1), and the mean platelet count was 223 $x10^9$/L (range, 179-449). Comparisons between dogs with elevated hepatic copper concentrations and normal dogs did not reveal a significant difference for the occurrence of the clinical signs, vomiting (P=0.2), diarrhea (P=0.7), subjective impression of PU/PD (P=0.4), decreased endurance (P=0.4), and listlessness (P=0.9), or for the blood concentrations of ALT (P=0.8), AP (P=0.8), bile acids (P=0.15), platelet count (P=0.25), PT (P=0.6), PTT (P=0.1), zinc (P=0.9), and copper (P=0.3). In addition dietary concentrations of copper and zinc were comparable between both groups (P=0.6-0.7).

Thirty-seven dogs were re-examined after a mean of 9 months (range 6-15 months). According to the owners, no dietary changes were made, and no copper-reducing medications were given between both examinations. In these dogs, hepatic copper concentrations had increased from a mean of 819mg/kg dwl (range 233-2960) to a mean of 998mg/kg dwl (range 181-4010).

Follow-up:
All owners of dogs with elevated hepatic copper concentrations were recommended to treat their dogs with penicillamine (10-15mg kg BID PO) directly or to participate in a placebo-controlled treatment trial with penicillamine at the same dose for 3 months.

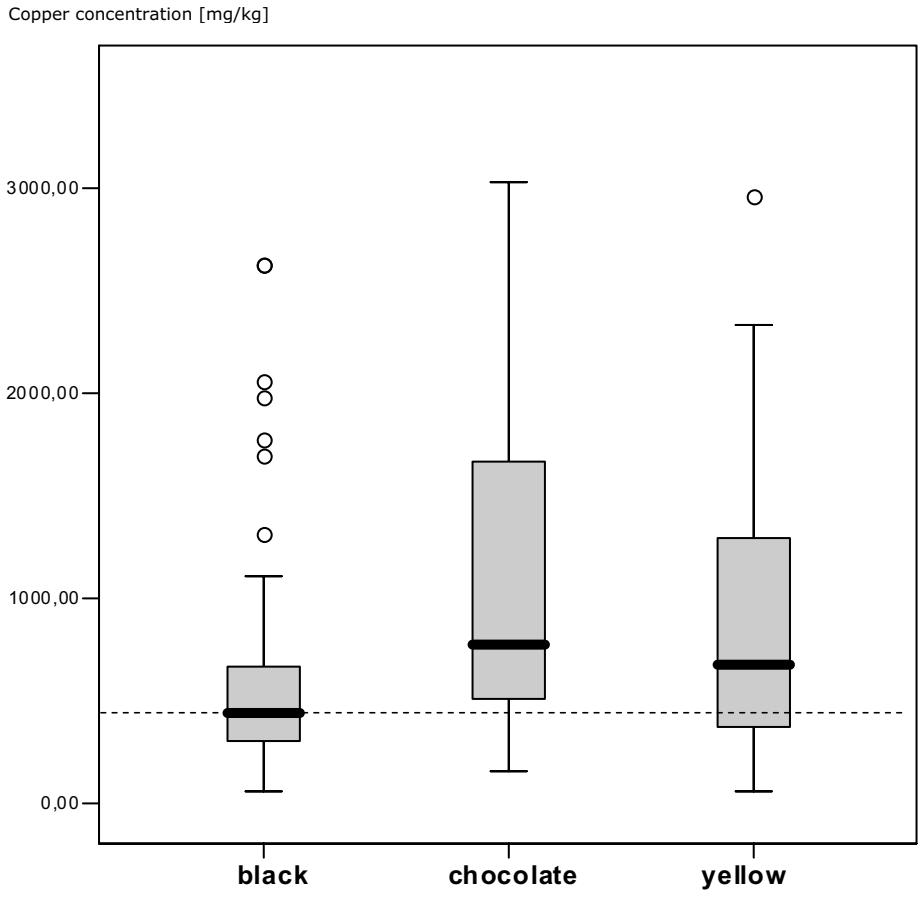

Figure 1: Quantitative copper concentrations of Labradors with different coat color

------ Normal range of copper < 400mg/kg dry weight liver. ○: outliers

67

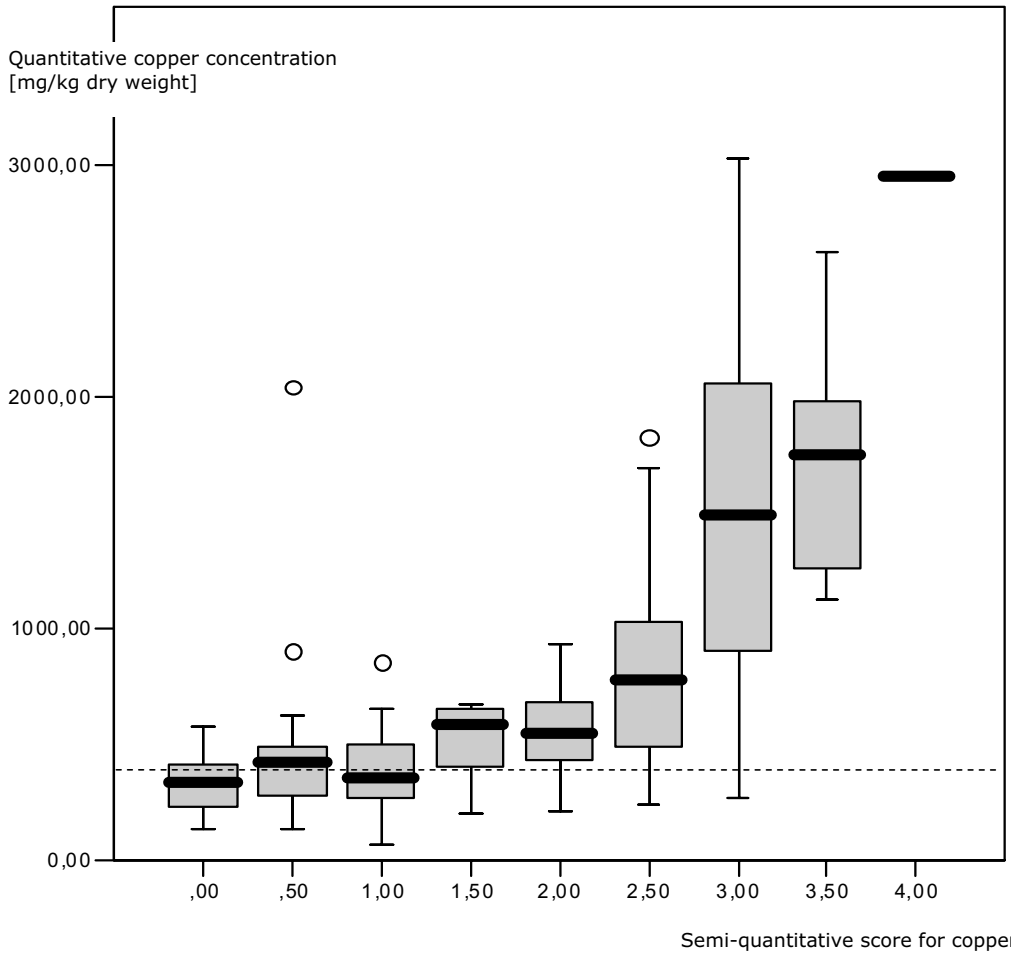

Figure 2: Correlation between quantitative copper concentrations and semi-quantitative assessment for copper

------ Normal range of copper < 400mg/kg dry weight liver. ○: outliers

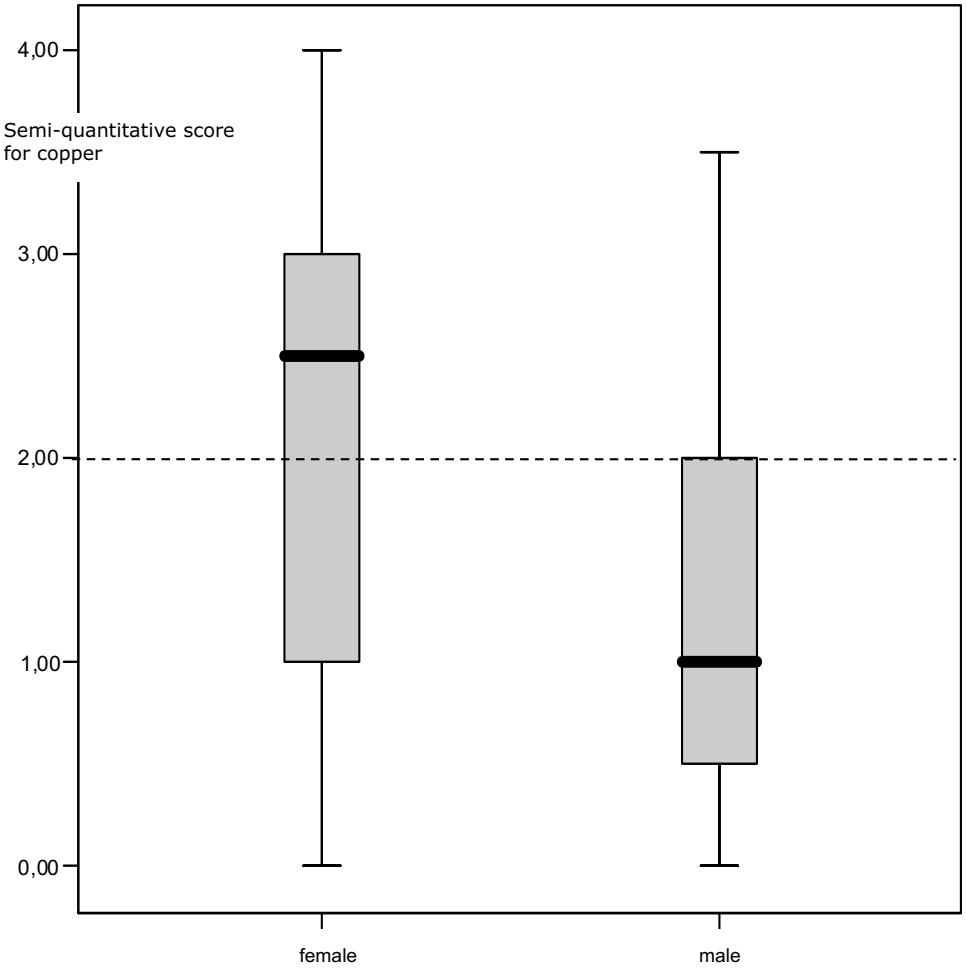

Figure 3: A significant difference was found in copper scores between female and male dogs

Discussion

The results of this study revealed that more than two thirds of family members of dogs with copper-associated chronic hepatitis have liver copper concentrations above the normal reference range. In addition, almost half of these dogs had chronic hepatitis. Environmental factors for the accumulation of copper were excluded, because affected and unaffected dogs had an equal geographic distribution throughout the country, and all dogs were fed commercial diets containing comparable concentrations of copper and zinc. These findings support the idea that CACH in Labradors is caused by an inherited defect of hepatic copper metabolism. Due to the fact that accumulating copper can lead to inflammation of the liver, we recommend examination of the liver and retrieval of liver biopsies in siblings of patients with copper-associated chronic hepatitis in order to diagnose the disease at an early and treatable stage, when liver pathology is still reversible with appropriate medical care.

We have previously described that female dogs are more frequently affected by CACH than male dogs.[3] However, whether the female predisposition for hepatitis was due to higher copper concentrations in liver tissue or a lower threshold for copper to provoke hepatitis was not clear. The results of the current study help to distinguish between the two etiologies because female dogs had higher hepatic copper concentrations compared to their male counterparts, independent of the presence of inflammation. Sex hormones can affect gene expression in somatic tissues. In the liver, growth hormone-induced activation of "male-biased genes," such as glutathione S-transferase for cellular detoxification, has been described.[10,11] However, a pulsatile secretion pattern was responsible for the gender difference in gene expression, which does not occur in dogs.[12] Moreover, spaying or neutering did not have an effect on the difference between groups in our study. Therefore, it seems unlikely that sex hormones are involved in the pathogenesis of hepatic copper accumulation; It appears more likely that the explanation for higher copper concentrations in female dogs may be found at the level of DNA, gene expression or protein function. Sexual dimorphism in genetic expression could occur and it has recently been shown to develop in liver tissue. Tissue-specific transcription factor binding sites can be responsible for differences in gene expression between different organs. Such regions were found to be enriched in sexually dimorphic genes. Moreover, there is growing evidence that whole sets of the sexually dimorphic genes are controlled by hotspots of quantitative trait loci that exhibit tissue-specific control.[13] Therefore, changes in such regulating hotspots could explain the gender difference of copper accumulation in Labradors. Future molecular research might help to reveal the cause of the female disposition for copper accumulation in this breed.

Hepatic copper concentrations in black dogs were significantly lower than in chocolate or yellow Labradors. However, this result was not corroborated when dogs within a single family were examined. The analysis of co-seggregation of the high copper phenotype and coat color within families did not confirm a protective effect of black coat color. We think that the relatively small group size of this study and unequal distribution of dogs to different families account for this result. Preference of a breeder for certain coat colors in the examined families probably has biased our results. Therefore, the investigation of more dogs from more families is necessary to confirm or reject our results. Should further investigations confirm a protective effect of black coat color against hepatic copper accumulation, possible explanations could be co-seggregation of the genes responsible for black coat color with a protective gene against copper accumulation, or co-seggregation of genes responsible for yellow coat color with the gene responsible for hepatic copper accumulation. The 3 genes involved in the expression of coat color in Labradors are the melanocortin-1 receptor (Mc1r) on chromosome 5, an Agouti-like gene on chromosome 24, and a newly described K-region on chromosome 16.[14] Mc1r is expressed on the cell surface of melanocytes. Activation of the receptor stimulates proliferation of melanocytes and melanin synthesis, resulting in a black or chocolate-colored coat. Agouti acts epistatic to Mc1r by antagonizing melanocortin signaling, resulting in a yellow coat color. The K-gene activates Mc1r, either indirectly by inhibiting the inhibitor Agouti, or directly by binding as an alternative Mc1r ligand that activates melanocortin signaling, leading to a black coat color. In addition, numerous further proteins are involved in melanocyte function (pMel-17, Mitf-M, Sox10, tyrosinase related proteins-1, and -2).

Alternatively, one of the copper excretory pathways might be compensating more effectively in black Labradors to prevent copper from accumulating. In mammals, the Menkes protein (ATP7A) has been

shown to supply copper to the pigment-forming enzyme tyrosinase within the secretory pathway.[15] Tyrosinase is a copper-containing enzyme, which catalyzes the conversion of tyrosine to melanin. Hypothetically, high activities of tyrosinase in black-colored Labradors might indirectly shuttle more copper away from the liver cell. In yellow Labradors, on the other hand, copper excretion from the liver could be prevented in a way comparable to the regulation of the yellow coat color, where black pigment is present, but prevented from deposition (epistatic allele).

The findings of this study suggest that copper-associated chronic hepatitis in Labradors is caused by an inherited defect of hepatic copper metabolism. Male gender appears to protect dogs from hepatic copper accumulation.

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[a] SPSS 12.0, Chicago, Illinois, USA

5

Heritabilities of copper accumulating traits in Labrador retrievers

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Introduction:

Recently a new copper accumulating liver disease has been described in Labrador retrievers.[1] The disorder shares morphological, and clinical features with inherited copper toxicosis in Bedlington Terriers, and with Wilson's disease in people.
The objective of this study was to determine the heritability of traits related to copper toxicosis in Labradors.

Materials & Methods:

In total 176 client owned Labradors participated in the study. Families from affected dogs were included. Three-generation pedigrees were obtained from the breed-club. The total pedigree included 350 individuals. The study was approved by the Utrecht University Institutional Animal Care and Use Committee. Informed owner consent was obtained for all dogs.
Liver biopsies were taken from 146 dogs and the concentration of copper (**Cu**) was measured by neutron activation analysis, according to a protocol described by Teske et al.[2] Quantitative copper concentrations were measured in lyophilised liver biopsies and reported in mg/kg dry weight. Furthermore hepatic tissue was stained with rubeanic acid stain for semiquantitative assessment of copper in all liver biopsies. According to this grading system copper scores above 2 are abnormal[3] (**CuHist**). Logarithmic transformation was performed for Cu to normalize a skewed distribution that was due to some extremely elevated concentrations.
The linear model used to estimate variance components included an effect of sex (male/female) and age as covariate. Both the animal and the residual effect were included as random variables which were assumed to be normally distributed with zero mean and variances σ_a^2 and σ_e^2 respectively.

Results:

Means, significance of sex (F_sex) and age (F_age), residual degrees of freedom, variance components (σ_a and σ_e), heritability (h^2**)** and according standard errors (se(h^2)) are shown in Table1.

Table1: Heritability estimates for copper accumulation

trait	units	mean	df_resid	F_sex	p-value	F_age	p-value	σ_a^2	σ_e^2	h^2	se(h^2)
Cu	mg/kg	829	140	7.99	0.005	0.1	0.752	162	259032	0.39	0.19
ln(Cu)		6.41	140	5.65	0.019	0.12	0.73	0.35	0.317	0.52	0.21
Cu_Hist	score	1.67	142	15.61	0	0.07	0.792	0.97	0.177	0.85	0.16

Heritability estimates for copper accumulation ranged between 0.39 and 0.85. Histological staining for copper revealed a stronger phenotype-genotype relation (h^2=0.85), when compared to quantitative measurement of copper (h^2=0.39-0.52).
A limitation of our results is the size of our data set. Because of a small sample size, the standard errors of heritability estimates were high. An additional limitation is the ascertained sample of dogs from families of former patients with hepatic copper accumulation. Therefore heritability is probably biased upwards.

Conclusion:

This study revealed a strong phenotype-genotype relation when histological staining is used for assessment of copper accumulation in Labradors. Our results should be confirmed in further studies of larger populations.

Acknowledgements:

We thank the dog owners and the dutch Labrador retriever club for their contribution, and the students for their assistance.
The study was supported by Waltham®

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Part 2

Randomized, double-blinded, placebo-controlled treatment studies of hepatic copper accumulation

Chapter 6-7

6

Randomized, double-blind, placebo-controlled treatment of hepatic copper accumulation in Labrador retrievers with penicillamine

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Abstract

This paper summarizes a prospective, randomized, double-blind, placebo-controlled study with D-penicillamine for the treatment of hepatic copper accumulation in Labrador retrievers.
The aim of the study was to investigate whether penicillamine is an effective drug to decrease hepatic copper concentrations in Labrador retrievers.
Our group of 43 dogs consisted of 29 female and 14 male client-owned Labradors. The dogs were family members of former patients with copper-associated chronic hepatitis, and all had hepatic copper concentrations above the normal range of 400mg/kg dry weight (mean: 1310, range: 470-3280). Treatment consisted of penicillamine (15mg/kg PO BID, group 1) or a placebo (group 2). The pharmacist was the only person aware of group allocations until completion of the study. Hepatic copper concentrations and histopathology were assessed along with clinical, and blood examinations before and after treatment.
Fourty dogs completed the study. The mean age of dogs in group 1 (n = 21) was 6.7 years (range 2.5-12). Eighteen dogs were female (10 spayed, 8 intact), and three were male (2 neutered, 1 intact). The mean age of dogs in group 2 (n = 19) was 6.2 years (range 3-11). Nine dogs were female (7 spayed, 2 intact), and 10 dogs were male (2 neutered, 8 intact). There was no difference in hepatic copper concentrations between both groups prior to treatment (P = 0.06). Isolated incidents of adverse effects were observed in both groups of dogs, and vomiting and anorexia were ongoing in 3 dogs from group 1. A difference in hepatic copper concentrations was measured after treatment with penicillamine (P=0.006), but not with placebo (P=0.9).
The results of this study show that penicillamine is an effective drug to decrease hepatic copper concentrations in Labrador retrievers.

Introduction

The Labrador retriever is the most common dog breed in Europe and the United States, and one of the prime breeds selected as guide and rescue dogs. Copper-associated chronic hepatitis (CACH) is a new metabolic disease in this breed, which was recently reported in dogs from Europe and the United States.[1,2]

The diagnosis of CACH is based on histological examination of liver biopsies, including assessment of copper. The average age of Labradors with CACH is 7 years (range 2.5-10.5 years). Female dogs are predisposed for the disease. Patients present with non-specific clinical, including anorexia, vomiting, and weight loss. A relative increase in ALT activity far above that of ALP activity, the centrolobular localization of copper, and the association of copper accumulation with hepatic lesions are typical findings described for this disease, which is most likely caused by an inherited genetic defect.[1]

In copper storage disorders, copper-rich particles accumulate in the lysosomes of hepatocytes and subsequent to hepatocytic necrosis in Kupffer cells. These particles are solubilized by D-penicillamine, which is a chelating agent that acts by reductive chelation of metals. The drug produces a large initial negative copper balance from increased urinary excretion of copper.[3,4]

D-Penicillamine is a recommended treatment for copper-accumulating liver disorders in dogs. The recommended dosages in veterinary literature vary between 200mg per dog q12h to 10-15 mg/kg q12h.[3-9] In human medicine, penicillamine treatment is recommended for preclinical copper accumulation in children with Wilson's disease.

To the authors' knowledge, this is the first placebo-controlled treatment study to evaluate the use of penicillamine for the treatment of a hepatic copper storage disorder.

Materials and methods

Labrador retrievers

The study population consisted of 43 Labrador retrievers that were family members of dogs previously described with copper-associated chronic hepatitis (CACH).[1] Due to an increased risk of these family members for the development of this potentially fatal disease, the dog owners were invited by their breed club to present their pets for a prophylactic examination of the liver at the Hepatology Service of the Faculty of Veterinary Medicine, University of Utrecht. All dogs were registered at the Dutch Labrador Retriever Breed Club.

All dogs were examined by one of the authors (GH). A medical history was obtained and a physical examination was performed. Na-citrated blood samples were taken for analysis of a coagulation profile, including prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen. Heparin-, and EDTA-blood was sampled for analysis of the hepatobiliary enzymes, alkaline phosphatase (ALP) and alanine aminotransferase (ALT), bile acids (BA), and for measurement of platelet counts. Liver biopsies were taken according to the Menghini technique, described by Rothuizen.[10] At least three liver biopsies were taken from each dog. Two biopsies were fixed in 10 percent neutral buffered formalin, and one biopsy was stored in a copper-free container for quantitative copper determination.

All biopsies were histologically assessed by one of the authors (TvdI). Hepatic tissue was stained with rubeanic acid for evaluation of copper distribution and semiquantitation. A histochemical grading system, which was previously applied on liver tissue of Bedlington terriers with copper toxicosis, Doberman pinchers, and Labradors with CACH, was used for semiquantitative assessment of hepatic copper in all liver biopsies.[1,11,12] According to this grading system, copper scores above 2 are considered abnormal.

A quantitative assay for copper in liver tissue was performed by neutron activation analysis, according to a protocol described by Teske et al, using the facilities described by Bode.[13,14] Quantitative copper concentrations were measured in lyophilised liver biopsies and reported in mg/kg dry weight liver (dwl).

Apart from the copper content, which was increased in all dogs, further histological changes were graded on a scale between 0 and 5 in order to allow statistical testing (0 = no histologic signs of inflammation, 1 = reactive hepatitis, 2 = mild chronic hepatitis, 3 = moderate chronic hepatitis, 4 = severe chronic hepatitis, 5 = cirrhosis).

The study was approved by the Utrecht University Institutional Animal Care and Use Committee. Informed owner consent was obtained for all dogs.

Drug preparation

The capsules were produced by the Veterinary Pharmacy of the Faculty of Veterinary Medicine, University of Utrecht. Microcrystalline cellulose was used as a diluent and colloidal silica as a glidant. All ingredients were analysed and approved in conformity with the European Pharmacopoeia.

The hard shell gelatine capsules size 0 were identical in both groups (placebo and penicillamine). Placebo capsules contained microcrystalline cellulose and colloidal silica exclusively but no active ingredient. Penicillamine capsules contained 200mg penicillamine, as well as the diluent and the glidant. Capsules from both groups had an identical appearance.

Randomization and blinding

The Pharmacist (CJM) randomized group allocations prospectively for sets of six dogs by flipping a coin. Three dogs received the placebo, and three dogs were treated with penicillamine. The pharmacisct was informed about dogs that were excluded from the study. The group assignments of these dogs (placebo or penicillamine) were filled by new patients.

On prescription of the clinician, the capsules (placebo or penicillamine) were dispensed according to the randomisation table.
Dosage of the prescription was according to the following scheme:

Bodyweight	Dosage
<28 kg (<61.6lbs):	3 capsules per day (1 capsule in the morning, 2 capsules in the evening).
28 – 32.9kg (61.6-72.4lbs):	4 capsules per day were prescribed (2 capsules twice daily).
33 – 37.9kg (72.6-83.4lbs):	5 capsules per day (2 capsules in the morning, 3 capsules in the evening).
> 38 kg (> 83.6lbs):	6 capsules per day (3 capsules twice daily).

Owners were instructed to give the capsules 30 minutes before feeding.
Neither the owner nor the clinician was informed about which treatment each dog received. The randomization table remained in possession of the Pharmacy during the trial and was only disclosed on completion of the trail.

Follow-up
After completion of the study period of three months, all dogs were treated with penicillamine in order to assure support for all pets, under protection of concealment of group allocations.
The treatment period was chosen based on the available literature, as well as personal clinical experience.

Statistical analysis
Statistical analysis was performed by use of commercially available software[a]. Due to small group sizes, a non-parametric statistical test was used for comparison between groups. A Mann-Whitney test was used to detect difference in hepatic copper concentrations before and after the 3-month treatment period with penicillamine or placebo (significance level $p \leq 0.05$).

Results

Histopathological examination of liver biopsies from 35 dogs revealed an elevated copper content of liver tissue, which was localized to centrolobular hepatocytes. Staining for copper was normal in 5 dogs with elevated hepatic copper from quantitative analysis. In twenty-four dogs, chronic hepatitis was present and characterized by varying degrees of hepatocellular apoptosis and necrosis, mononuclear inflammation, regeneration and fibrosis. The degree of hepatic inflammation was assessed based on the quantity of inflammatory cells and the extent of hepatocellular apoptosis and necrosis. Hepatitis was considered mild in 13 patients, moderate in 5 dogs, and severe in 6 patients. The stage of the disease was determined by the extent and pattern of fibrosis, and the presence or absence of cirrhosis. Cirrhosis, characterized by regenerative nodules of less than 3 mm diameter of regular size, was diagnosed in 4 dogs. Results from semiquantitative assessment of copper ranged from 0 to 5. Copper accumulated in zone 3 of the liver lobule and was associated with an inflammatory infiltrate in all biopsies. In eleven dogs there were no histological signs of inflammation present in the liver tissue.

The mean hepatic copper concentration of 21 dogs in group 1 was 1511 mg/kg dw (range 470-3030); mean age was 6.7 years (range 2.5-12); average body weight was 31.8kg (range 24-39), 18 dogs were female (10 spayed, 8 intact), and 3 were male (2 neutered, 1 intact). The mean hepatic copper concentration of dogs in group 2 was 1096 mg/kg dw (range 470-3280); mean age was 6.2 years (range 3-11); average body weight was 33.1kg (range 25-41.9), 9 dogs were female (7 spayed, 2 intact), and 10 dogs were male (2 neutered, 8 intact). There was no difference in hepatic copper concentrations between groups 1 and 2 before treatment (P=0.06). After treatment with D-penicillamine, hepatic copper concentrations had decreased significantly in group 1 (mean 863mg/kg, range 116-2640, P=0.006). However, no differences were observed after placebo in group 2 (P=0.9) (Fig.1 and Fig. 2).

Strong individual differences were observed between dogs with respect to the de-coppering effects of penicillamine. The hepatic copper concentration declined in all dogs, but the downward slope of copper showed large variations between individual pets (Fig. 3).

Histological changes did improve during treatment with penicillamine (P=0.048, Fig.4), and histological staining for copper revealed decreased positive staining with treatment (P=0.03).

Adverse effects observed by the owners included vomiting in five dogs from group 1 and three dogs from group 2. Anorexia was observed in 3 dogs from each group. Diarrhea occured in one dog from group 1. Adverse effects were severe in 3 dogs that were treated with penicillamine (2 females, 1 male dog). These dogs required a change in the medication protocol, and were therefore excluded from the study. For the three excluded patients, penicillamine treatment was devided into three applications per day, and the drug was given with food. With this adaptation, adverse effects did not recur.

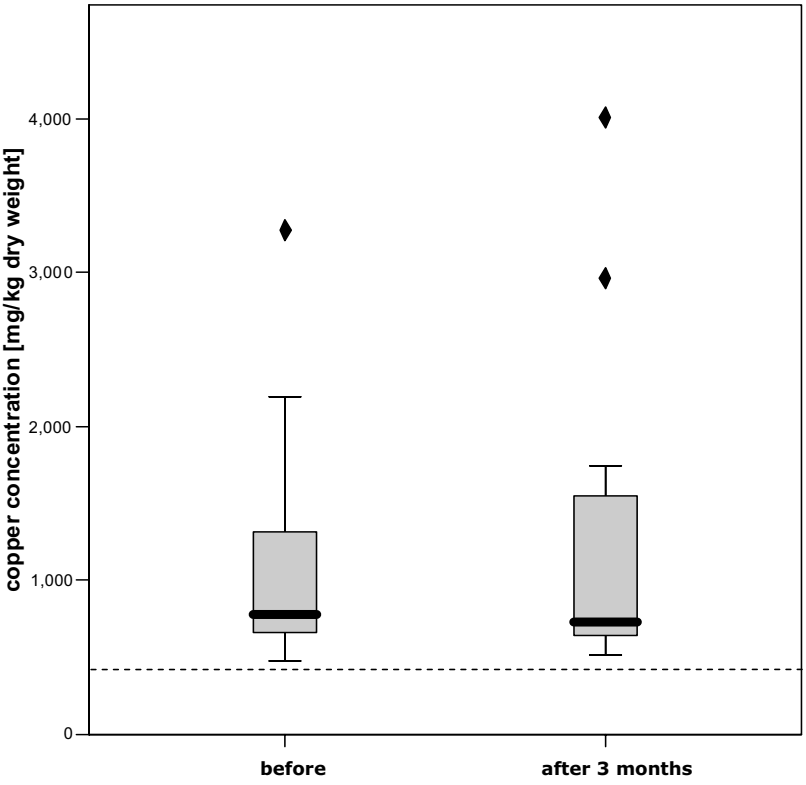

Figure 1: Hepatic copper concentrations in Labrador retrievers before and after 3 month treatment with a placebo (group 2). Normal range below 400 mg/kg dw.

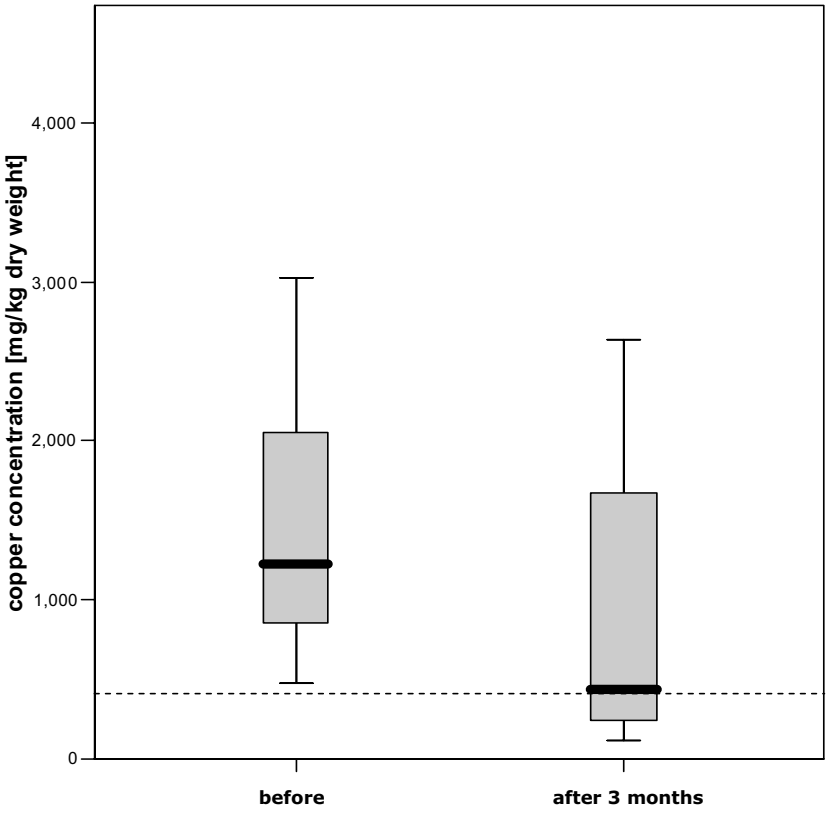

Figure 2: Hepatic copper concentrations in Labrador retrievers before and after 3 month treatment with penicillamine (group 1). Normal range below 400 mg/kg dw.

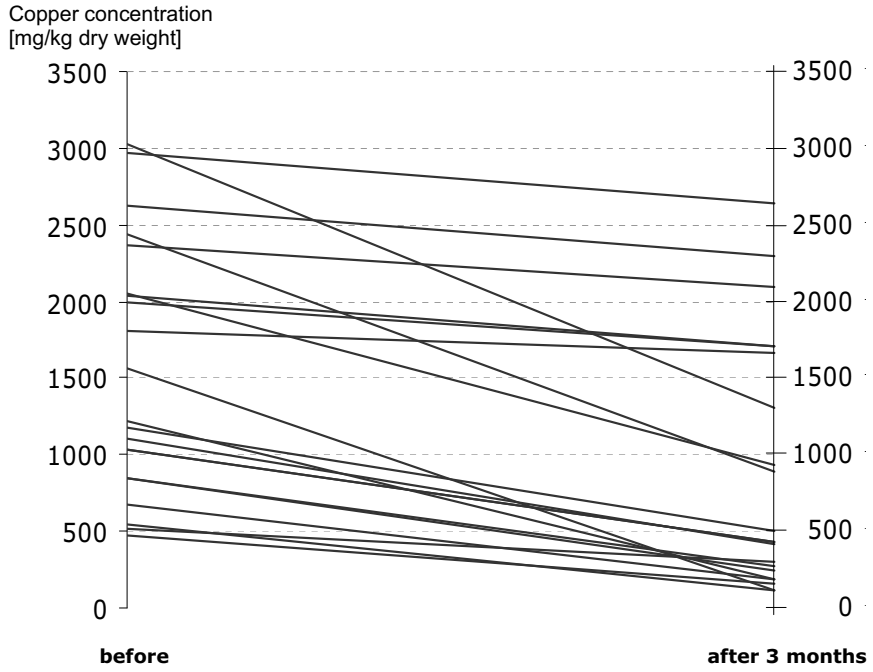

Figure 3: Hepatic copper concentrations of 21 Labrador retrievers before and after 3 months treatment with penicillamine (group 1): individual treatment responses. The difference between the mean hepatic copper concentration before and after treatment with penicillamine was 648 mg/kg.

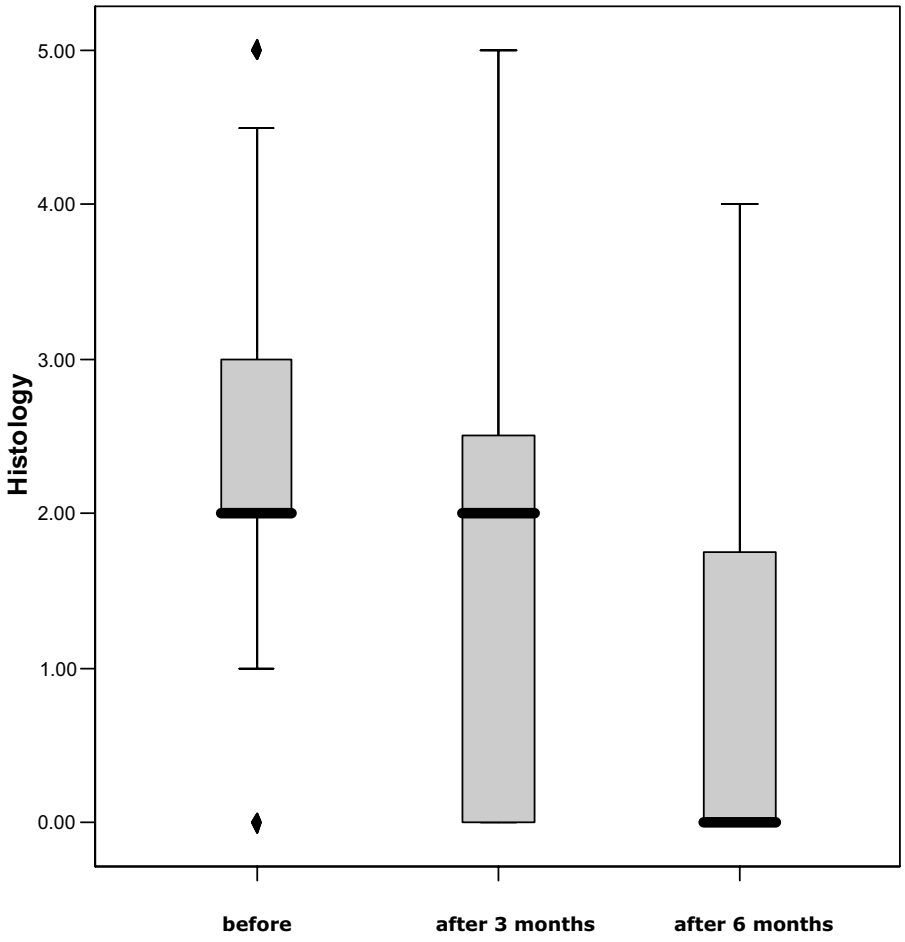

Figure 4: Development of histological changes after 3 months and after 6 months of treatment with penicillamine. 0 = no histologic signs of inflammation, 1 = reactive hepatitis, 2 = mild chronic hepatitis, 3 = moderate chronic hepatitis, 4 = severe chronic hepatitis, 5 = cirrhosis. Diamond = outliner.

Discussion

Dogs with copper-associated chronic hepatitis develop clinical signs late in the disease although copper may begin to accumulate at 5-6 months of age. Isolated accumulation of copper without histological signs of chronic hepatitis was also observed in some Labradors in this study. This is consistent with the observation that copper-associated chronic hepatitis in Labradors occurs in 3 stages with a potentially prolonged initial delay between severe accumulation of hepatic copper and histological evidence of inflammation, as well as a prolonged secondary delay between histological evidence of inflammation and the occurrence of clinical signs.[1] Besides the observation that copper accumulation precedes the development of hepatitis, our decision to treat hepatic copper accumulation prior to the occurrence of hepatic inflammation was based on the knowledge that related dogs had succumb to fatal CACH. Moreover, the findings of Spee and colleagues[15] indicated that zonal copper concentrations in the order of magnitude observed in the dogs in this study resulted exclusively from a primary copper storage disease. The dogs presented in our study were therefore considered to be at high risk for potentially irreversible liver damage and treatment with a copper chelator was considered judicious. Penicillamine is a metal chelating amino acid derivative, which is recommended as a first choice treatment for preclinical copper accumulation in children with Wilson's disease.[16]
A shortcoming of the present study was possibly that the decision to treat dogs prior to the onset of clinical signs resulted in a lack of ability to assess the usefulness of penicillamine for the treatment of symptoms arising from CACH. However, a placebo-controlled study would have been less ethically sound if it had been applied to patients already suffering clinical signs of disease. A further limitation of our study was the lack of standardization of the animals' diet during treatment. Strong individual differences in the degree of decoppering by penicillamine were observed between dogs and one possible reason for this is the variation in diet. Indeed, concentrations of copper and zinc vary widely between pet foods and a high copper diet could potentially diminish the decoppering effects of the drug. Zinc in the diet may also affect the degree of copper-reduction during treatment because zinc induces metallothionein in the gastrointestinal mucosa and inhibits the uptake of copper from the diet. Although zinc and copper concentrations in the diet were similar in good and poor treatment responders, metal concentrations in treats given in-between meals were not taken into consideration.
Given the silent nature of CACH in early stages, repeated histologic examinations of liver tissue should be considered in all patients treated with penicillamine in order to insure that therapy is continued until all histologic signs of chronic hepatitis have completely resolved.

Urine concentrations of copper were not measured during this study for logistical reasons. Penicillamine treatment causes a marked initial negative copper balance due to increased urinary excretion of copper in the initial phase of therapy. Measurement of 24-hour urinary copper excretion might therefore have allowed us to assess the optimum treatment period. Despite these facts, adverse effects of penicillamine therapy in people generally occur after prolonged treatment periods and are due to immunologic reactions. Although immunologic adverse effects were not identified in this study, minimizing the treatment period could potentially limit the occurrence of potential adverse reactions.
Penicillamine is considered to have anti-inflammatory and immunosuppressive effects, and has been used for the treatment of immune-mediated disorders.[17-22] Therefore, previous reports on penicillamine treatment for advanced copper-accumulating liver diseases raised the question of whether beneficial effects of penicillamine were due to its decoppering action or its anti-inflammatory properties. In our study, a large number of dogs had only minor histologic evidence of inflammation at the initiation of penicillamine treatment despite having increased copper accumulations. Following treatment both histologic inflammation and copper accumulation was diminished. Given that copper accumulation appears to precede inflammation, any beneficial effects of penicillamine therapy are likely to be due primarily to a decoppering effect.
Adverse effects occured in 6 of 22 dogs treated with penicillamine (27%). This is similar to findings in people treated for Wilson's disease, but discordant with a previous report on penicillamine treatment in 5 Doberman dogs with hepatic copper accumulation.[6] However, in the latter report, only a very small numbers of dogs was assessed and dogs were treated with a lower dosage of penicillamine (200mg/dog q

12h, median body weight 30kg, range 29-36kg), which may have resulted in fewer adverse reactions. A lower dosage than that used in our study may prove sufficient to achieve a decoppering effect on the liver but further studies are necessary to assess optimal dosage.
We expect dogs with elevated hepatic copper concentrations to be at high risk for the development of chronic hepatitis and, finally, cirrhosis. The results of this study show that penicillamine is effective in reducing hepatic copper concentrations in Labrador retrievers. Based on these results, we recommend treatment with penicillamine for hepatic copper accumulation in this breed before the onset of clinical signs. As we recognized mild copper accumulation without evidence of hepatitis, a staged development of copper-associated chronic hepatitis seems most likely, with exhaustion of cellular protection systems against oxidative damage as pre-requisite for copper to cause inflammation.

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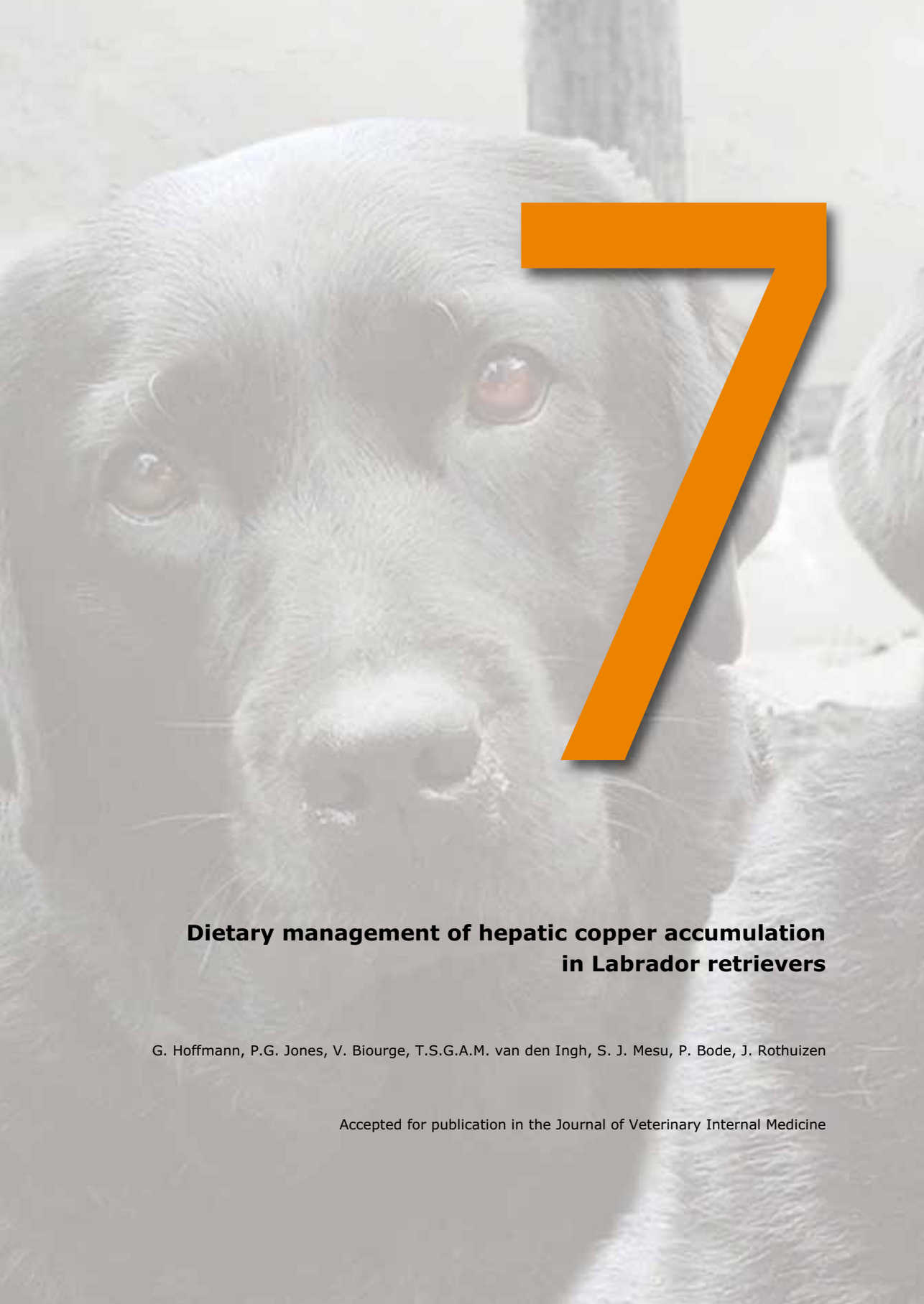
[a] SPSS, 12.0, Illinois, USA



7

Dietary management of hepatic copper accumulation in Labrador retrievers

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Abstract

Background: Copper-associated chronic hepatitis (CACH) has recently been recognized in the Labrador retriever as an inherited disorder with a late onset of clinical signs. Although treatment with penicillamine is effective in reducing hepatic copper concentrations and clinical signs in affected dogs, no studies have investigated dietary management for the long-term treatment of this disease or for its potential in delaying the onset of clinical signs in subclinical Labradors.
Objectives: To investigate the effects of a low-copper diet and zinc gluconate on hepatic copper accumulation in Labradors.
Animals: Twenty-four client-owned Labradors that were related to former dogs affected with CACH and that had previously been diagnosed with elevated hepatic copper concentrations.
Methods: Hepatic copper concentrations were assessed prior to and following an average of 8 months (recheck 1) and 16 months (recheck 2) treatment. During this time, all dogs were fed exclusively on a low copper diet. In addition, the dogs were assigned to one of two groups in a randomized double-blind manner to receive a supplement of zinc gluconate or a placebo.
Results: Twenty-one dogs completed the study. Hepatic copper concentrations decreased in both groups at recheck 1 (group 1, $P < 0.001$; group 2, $P = 0.001$) and at recheck 2 (group 1, $P = 0.03$; group 2, $P = 0.04$). No difference in hepatic copper concentrations was found between the two groups prior to treatment ($P = 0.65$), at recheck-1 ($P = 0.52$) or at recheck-2 ($P = 0.79$).
Conclusions and clinical relevance: Feeding of low copper diets to Labradors at risk for CACH is effective in reducing hepatic copper concentrations. Adjunctive treatment with zinc does not appear to increase the copper-lowering effects of dietary management.

Introduction

Copper-associated chronic hepatitis (CACH) is a newly recognized metabolic disease in the Labrador retriever, reported in dogs from both Europe and the United States.[1,2]
The average age of Labradors diagnosed with CACH is 7 years (range 2.5-10.5 years) and female dogs may be predisposed to the disease. Affected dogs generally present with nonspecific clinical signs, such as anorexia, vomiting, and weight loss. Typical clinicopathologic findings are a relative increase in alanine aminotransferase (ALT) activity far above that of alkaline phosphatase (ALP) activity, and centrolobular accumulation of copper associated with hepatic lesions.[1,3]

Penicillamine has recently been described as an effective first-line treatment for hepatic copper accumulation in Labradors.[4] However, the disorder is most likely caused by an inherited genetic defect and, therefore, lifelong support is necessary since a definitive cure is impossible. As oral zinc can reduce copper absorption from the diet, it can be used for the long-term management of hepatic copper storage disorders. Zinc induces the production of metallothionein in the intestinal mucosa. Metallothionein, a cysteine-rich chelator of metals, binds copper to the intestinal cell, preventing transport of copper into the circulation. Metallothionein-bound copper is excreted during natural sloughing of the intestinal epithelium. Since endogenous copper circulates through saliva and gastric secretions in the gastrointestinal tract, zinc treatment can generate a negative copper balance and indirectly remove free copper from the liver. In addition, zinc can induce metallothionein in hepatocytes, thereby binding free copper and protecting the cells against oxidative stress induced by free copper.[5] By minimizing oxidative damage to cellular organelles, zinc has a moderating effect on major signaling pathways of caspase activation and apoptosis.[6] The effectiveness of zinc in the prevention of copper uptake from the intestinal tract in dogs has previously been assessed by peak plasma concentrations of radioactive copper.[7] In this study, a minimum of 3 months treatment with zinc was necessary before copper uptake from the intestinal tract was blocked. Since the resulting rate of copper removal from the liver is relatively slow, zinc is currently reserved for maintenance therapy and preventive treatment regimens.

In people with Wilson's disease, a diet low in copper may delay the onset of the disease and copper-restricted diets are generally recommended at all stages in all human copper-storage disorders. Copper-restricted diets are commercially available for dogs. These diets are generally also enriched in zinc. To the authors' knowledge, this is the first study to assess the effectiveness of dietary management as well as supplemental zinc in reducing hepatic copper concentrations in dogs with copper storage disorders.

Materials and methods

The study was approved by the Utrecht University Institutional Animal Care and Use Committee, and informed owner consent was obtained for all dogs.

Labrador retrievers
The study population consisted of 24 client-owned Labrador retrievers that were family members of dogs previously diagnosed with CACH.[1] All dogs were registered at the Dutch Labrador Retriever Breed Club. All dogs had been found to have elevated hepatic copper concentrations and had been previously treated with penicillamine (10-15mg/kg PO q12h) for three to six months. This resulted in a significant reduction of hepatic copper concentrations, which nonetheless remained above the reference limit (400 mg/kg dry weight) in 20 dogs.

Progression of hepatic copper accumulation without treatment
Prior to enrollment in the study, hepatic copper concentrations were re-evaluated after a mean of 8.7 months (range, 6-15 months) in 11 dogs. During this period, all animals were fed their usual maintenance diet, which contained between 12-25 ppm dry matter of copper and between 80-270 ppm dry matter zinc according to the manufacturers.

Pharmacokinetic study of oral zinc gluconate
Two unrelated nine-year-old healthy Labrador retrievers (1 female and 1 male) were used in a preliminary pharmacokinetic study to assess the absorption of the zinc gluconate preparation used in this study. In these two dogs, food was withheld for a 12-hour period prior to oral administration of zinc gluconate and during the initial 6-hour testing period. Water was freely available at all times. Oral zinc gluconate was administered at a dose of 10 mg/kg in dog 1 and 5 mg/kg in dog 2. Heparinized blood samples were collected from the jugular vein before (time 0) and 15, 30, 45, 60, 90, and 120 minutes, and 4, 8, 12 and 24 hours after application of the drug. The plasma was separated immediately and samples were stored at -20°C pending zinc analysis by atomic absorption spectrometry. Pharmacokinetic parameters calculated were the apparent volume of distribution of oral zinc (V/F), the absorption rate constant (K_a), the elimination rate constant (K_{el}), the area under the plasma zinc concentration-time curve (AUC), the clearance as a function of bioavailability (Cl/F), the time to peak plasma concentration (T_{max}) and the maximum zinc concentration (C_{max}). Finally, the half-life (t½) was calculated and an appropriate dosage was estimated.

Diet treatment of copper accumulation
The owners of all 24 dogs were provided with the same commercial canned diet, which originated from a single, analyzed production batch. The diet contained 2 ppm of copper and 43 ppm of zinc as fed (4.8 ppm Cu and 102 ppm Zn on a dry matter basis). Owners were instructed to feed their dogs exclusively with the provided diet and to avoid any further dietary supplements and treats. The Labradors were given between 420-840g diet/day.

Zinc supplementation, study randomization and blinding
The dogs were randomly allocated into two groups to receive zinc tablets or placebo. The zinc tablets contained 25 mg elemental zinc as a zinc gluconate salt and were provided by the Veterinary Pharmacy of the Faculty of Veterinary Medicine, University of Utrecht. Placebo tablets contained 160 mg lactose. Placebo and zinc tablets had an identical appearance. The pharmacist randomized sets of six dogs prospectively by flipping a coin, each time allocating 3 dogs to receive placebo and 3 to receive zinc. On prescription by the clinician, tablets (placebo or zinc) were dispensed according to the randomization table. Dogs weighing <28 kg (<61.6lbs) were given 8 tablets q12h, dogs weighing between 28 and 35kg (61.6-77lbs) were given 9 tablets q12h, and dogs weighing >35 (>77lbs) were given 10 tablets q12h. Owners were instructed to give the tablets mixed with a small amount of their diet 30 minutes before feeding. Both the owners and the clinician were blinded to treatment allocation until completion of the trail.

Blood examinations and biopsy procedures

A complete medical history, physical examination, blood analyses and liver biopsies were performed prior to the study and after an average of 8 months (recheck 1) and 16 months (recheck 2). The physical examinations were all performed by one of the authors (GH). Sodium-citrated blood samples were taken for analysis of a coagulation profile, including a one-stage prothrombin time (OSPT), activated partial thromboplastin time (aPTT) and fibrinogen. Heparin- and EDTA-blood was sampled from fasted dogs for analysis of the hepatobiliary enzymes, ALP and ALT, bile acids, and for platelet counts and plasma zinc concentrations. In addition plasma zinc concentrations were measured after the initial month of treatment. Liver biopsies were taken according to the Menghini technique described by Rothuizen.[8] At least three biopsies were taken from each dog. Two biopsies were fixed in 10% neutral buffered formalin, and one biopsy was stored in a copper-free container for quantitative copper measurements. The histologic assessment of all biopsies was performed by one of the authors (TvdI), blinded to treatment allocation. Hepatic tissue was stained with rubeanic acid for evaluation of copper distribution and semiquantitave copper scoring as previously described, with scores above 2 considered abnormal. Histologic changes were graded on a scale between 0 and 5 to allow statistical testing. (0 = no signs of inflammation, 1 = reactive hepatitis, 2 = mild chronic hepatitis, 3 = moderate chronic hepatitis, 4 = severe chronic hepatitis, 5 = cirrhosis). A quantitative assay for copper in liver tissue was performed in lyophilized liver biopsies by neutron activation analysis, according to the protocol described by Teske et al., and using the facilities described by Bode.[9,10]

Statistical analysis

Pharmacokinetic parameters and statistical analyses were calculated using a commercially available software package.[i] Due to the small group sizes, the non-parametric Mann-Whitney test was used to detect a difference between hepatic copper concentrations before and after treatment in the two groups and to detect a difference between groups 1 and 2 before and after treatment. *P* values < 0.05 were considered statistically significant.

Results

Progression of hepatic copper accumulation without treatment
In all but one of the eleven dogs for which hepatic copper accumulation was measured whilst being fed their usual maintenance diet, hepatic copper concentrations increased significantly from a mean of 1000 mg/kg dry weight (range, 290-2370) to a mean of 1626 mg/kg dry weight (range, 630-3610; P = 0.04). This corresponded to a mean increase of 18 mg copper per kg bodyweight (Fig. 1).

Pharmacokinetic study of oral zinc gluconate
Pharmacokinetic parameters calculated after oral application of 10 mg/kg in dog 1 were as follows: volume of distribution (V/F), 2937.872 ml/kg; absorption rate constant (K_a), 0.567212 $hour^{-1}$; elimination rate constant (K_{el}), 0.053728 $hour^{-1}$; area under the plasma zinc concentration-time curve (AUC), 63.35272 µg·h/mL; clearance as a function of bioavailability (Cl/F), 157.8464 ml/hr/kg; time to maximum concentration (T_{max}), 4.589813 hours; and maximum plasma concentration (C_{max}), 2.659924 µg/ml. The parameters calculated after oral application of 5 mg/kg in dog 2 were as follows: volume of distribution, 2538.689 ml/kg; K_a, 1.160647 $hour^{-1}$; K_{el}, 0.037886 $hour^{-1}$; AUC, 51.98513 µg·h/mL; Cl/F, 96.18135 ml/hr/kg; time to T_{max}, 3.047974 hours; C_{max}, 1.754728 µg/mL. The calculated half-life (t½) of zinc was 15.1 hours. The calculated accumulation rate was 1.52. The dose interval was chosen to be 12 hours. A dose estimate was 127.0139 ml/hr/kg, calculated from the mean Cl/F of both dogs. An estimate of an appropriate dosage was based on an intended maximum plasma zinc concentration of 5µg/ml and calculated as Cl/F x (intended blood concentration) x (dose interval) to be 7.62 mg/kg q12h.

Group allocation
The mean age of dogs in group 1 was 4.1 years (range, 2.7-8.3). Six dogs were spayed females, and six were male (2 neutered, 4 intact). The average bodyweight was 33kg (range 26.2-37.5). The mean age of dogs in group 2 was 4.8 years (range, 3.6-11.2). Six dogs were female (4 spayed, 2 intact), and 6 dogs were male (2 neutered, 4 intact). The average bodyweight was 32.3kg (range 25-41.9). Three dogs in group 1 did not complete the study for reasons unrelated to the treatment trial (one owner felt his dog was getting too old, one owner had personal reasons, and one dog developed a mast cell tumor). Twenty-one dogs completed the study with only the first recheck examination and 16 dogs completed the study with both recheck examinations.

Blood examinations
The concentration of fasted bile acids in all dogs decreased from a mean of 14 µmol/l (range, 3-101) to a mean of 7.8 (range, 1-39) at recheck-1, and 7.1 (range, 0-21) at recheck-2. The mean activities of ALP and ALT, OSPT, aPTT, fibrinogen, and platelet counts remained within the normal ranges at all examinations.

Hepatic copper concentrations
Hepatic copper concentrations were above the normal range of 400mg/kg dry weight (dw) in 20 dogs prior to treatment (mean, 894 mg/kg dw; range, 70-2810). There was no statistical difference (P = 0.73) in the mean hepatic copper concentration prior to treatment in group 1 (mean, 961 mg/kg dw; range, 340-2810) and group 2 (mean, 861 mg/kg dw; range, 70-1680).

Compared to initial values, hepatic copper concentrations decreased significantly in both groups at recheck 1 (goup 1: mean, 286 mg/kg dw; range 84-700; P<0.001; and group 2: mean, 277 mg/kg dw; range 80-450; P = 0.001) as well as at recheck 2 (group 1: mean, 421 mg/kg dw; range, 220-790: P = 0.03; and group 2: mean, 401 mg/kg dw; range, 118-850; P = 0.04) (Fig. 2). However, there was no significant differences within the groups in copper concentrations between recheck 1 and recheck 2 (group 1, P = 0.44; group 2, P = 0.25). In addition, there was no significant difference in hepatic copper concentrations between the groups at recheck 1 or recheck 2 (P = 0.52 and 0.79, respectively).

Liver histology

Histological examination of liver biopsies from 17 dogs revealed elevated hepatic copper scores (greater than 2), which was localized to centrolobular hepatocytes. Staining for copper was considered normal in 5 dogs, and high normal in 2 dogs with elevated hepatic copper from quantitative analyses. The initial mean semi-quantitative assessment of copper from rubeanic acid stained biopsies was 2.5+ (range, 0-4.5) in group 1, and 2.5+ (range, 0.5-3) in group 2. The scores decreased in both groups at recheck 1 (group-1, $P = 0.031$; group 2, $P = 0.01$) and recheck 2 (group 1, $P = 0.01$; group 2, $P = 0.001$). There was no difference between the groups at any time ($P = 0.16$-0.75).

No histologic signs of inflammation were present in thirteen dogs, and non-specific reactive inflammatory changes were found in four dogs. In seven dogs, chronic hepatitis (CACH) was present and characterized by varying degrees of hepatocellular apoptosis and necrosis, mononuclear inflammation, regeneration and fibrosis. The activity of the hepatic inflammation was determined by the quantity of inflammation and extent of hepatocellular apoptosis and necrosis. The stage of the disease was determined by the extent and pattern of fibrosis and the presence of cirrhosis. Hepatitis was mild in 4 patients, moderate in 2 dogs, and cirrhosis was diagnosed in 1 dog. Histological scoring for severity of inflammation remained unchanged throughout all examinations in both groups ($P = 0.25$-0.45).

Plasma zinc concentrations

The mean plasma concentrations of zinc prior to treatment were 95 µg/dl and 96 µg/dl in groups 1 and 2, respectively. There was no significant change in plasma zinc concentrations in either group at recheck 1 or recheck 2 (P values between 0.11 and 0.79). In addition, no difference in plasma zinc concentration was found between the groups at any of the three examinations (P values between 0.34 and 0.5). The only time-point at which a difference in plasma zinc concentrations could be found was a blood examination after the initial month of treatment with zinc in group 1. Blood sampling was performed 2-6 hours after zinc application at this time point. The mean plasma zinc concentration had increased to 165 µg/dl (range 117-249, $p=0.02$).

Adverse reactions

Vomiting and anorexia were observed in 3 dogs in group 1. One of these dogs also suffered transient small bowel diarrhea. The adverse effects occurred shortly after oral application of the tablets and resolved when the tablets were mixed with larger amounts of the diet.

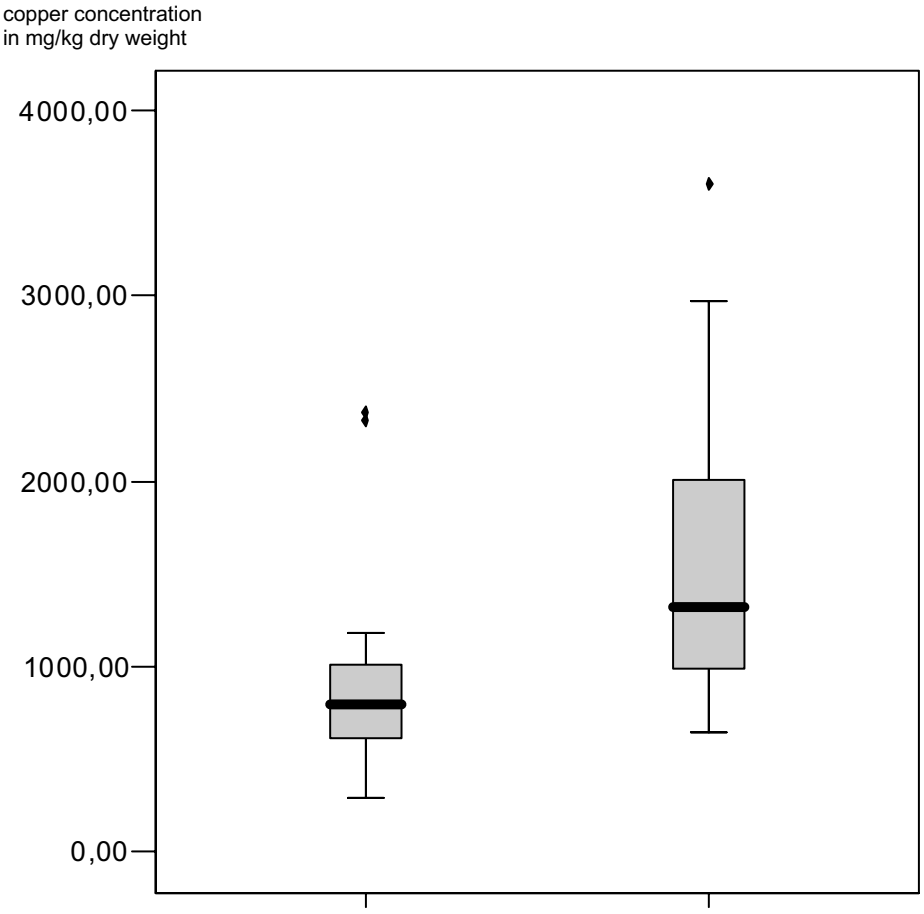

Figure 1: Progression of hepatic copper accumulation without treatment in 11 dogs.

Measurement of hepatic copper concentrations was repeated before any treatment was given, and while the dogs were fed their usual maintenance diet. In all but one dog hepatic copper concentrations increased during the time interval of 8.7 months between both measurements from a mean of 1000mg/kg dry weight (range 290-2370) to a mean of 1626 mg/kg dry weight (range 630-3610, p=0.04).

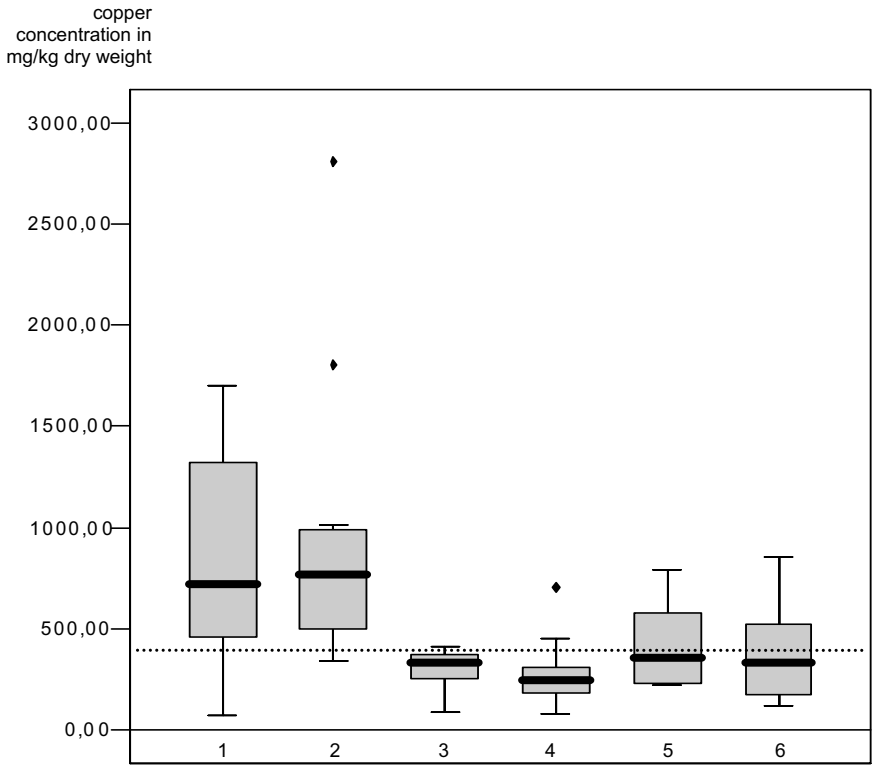

Figure 2: Hepatic copper measurements

Quantitative measurement of hepatic copper improved during treatment in both groups. At recheck-1, and at recheck-2 hepatic copper concentrations had decreased significantly in both groups of dogs, compared to the starting point 1: group-1 at starting point, 2: group-2 at starting point, 3: group-1 at recheck-1: mean 286mg/kg, range 84-700, p<0.001; 4: group-2 at recheck-1: mean 277, range 80-450, p= 0.001, 5: group-1 at recheck-2: mean 421, range 220-790, p=0.03, 6: group-2 at recheck-2: mean 401, range 118-850, p=0.04). There was no difference in hepatic copper concentrations between both groups at recheck-1 (p=0.52), and there was no difference between groups at recheck-2 (p=0.79). There was no further decrease of hepatic copper concentrations between recheck-1, and recheck-2 (group-1 p=0.44, group-2 p=0.25). Diamond: outliner.

Discussion

Although copper accumulation may begin at 5-6 months of age in dogs with CACH, clinical signs develop late in the course of the disease. Indeed, CACH in Labradors appears to occur in 3 stages with a prolonged delay period of several years between an initial accumulation of hepatic copper and subsequent histologic evidence of inflammation, as well as a prolonged second delay until the onset of clinical signs. The finding of many Labradors in the present study with isolated accumulations of hepatic copper but without histologic signs of chronic hepatitis was therefore not surprising. Besides the awareness of the late onset of signs, the decision to treat the subclinical animals in this study was based on the consideration that they may be at risk because they were related to dogs previously diagnosed with fatal CACH. In addition, hepatic copper concentrations had increased significantly over a period of 8 to 9 months from a mean of 1000 mg/kg dw to a mean of 1626 mg/kg dw in 11 dogs. Progression to a more advanced disease had occurred, or was very likely to occur in these dogs. The dogs were initially treated with penicillamine, which effectively reduced hepatic copper concentrations, but these were still above normal reference ranges in many dogs after treatment. Moreover, CACH is most likely caused by an inherited genetic defect and resurgence of copper accumulation was therefore likely. Indeed, in the authors' clinical practice, bi-annual liver biopsies are recommended after successful treatment of CACH for the early detection of resurgence of excessive hepatic copper, and reinstitution of treatment is often necessary within a year for dogs fed a regular commercial diet. Dietary management was investigated in this study in the hope of providing Labradors with CACH with a long-term means of limiting hepatic copper accumulation.

In people with Wilson's disease, a diet low in copper may delay the onset of the disease, and copper-restricted diets are generally recommended at all stages in human copper-storage disorders. Low-copper diets have recently become commercially available for dogs. These are also enriched in zinc, which is described to suppress copper uptake at plasma concentrations above 200 µg/dl.[7] In one study of 3 Bedlington terriers and 3 West Highland White terriers with copper toxicosis, 200 mg of elemental zinc was given daily to each dog in order to achieve plasma zinc concentrations above this level.[7] The concentration of zinc in the diet tested in our study (43 ppm as fed) was comparable to the concentration of zinc in the different diets the dogs received before the study. In proportion to the quantity of diet fed to the animals (420-840 g per day), dogs in the present study received 18-36mg/day of dietary zinc. Any copper-reducing effects of the diet therefore seems more likely due to the low copper content than due to an increased zinc concentration, although a concomitant protective effect of zinc is possible. Zinc has been shown to induce metallothionein in hepatocytes, thereby binding free copper, which otherwise can induce oxidative stress by the formation of hydroxyl radicals.[5] Although hepatic copper concentrations would not decrease directly by this action of zinc, it is possible that normal liver cell turnover led to the regeneration of liver tissue that did not accumulate copper because this was low in the diet.

Although zinc is a relatively safe drug, large doses may cause gastrointestinal disturbances and abdominal pain, and, at zinc plasma concentrations above 1000 µg/dl, hemolysis may occur.[11] Pancreatitis has also been described in one case report but clinical pancreatitis has been questioned by some authors who found long-term elevations in serum amylase, lipase, and ALP in people and rats with supplemental zinc without pancreatic changes.[12] In addition, zinc supplementation could theoretically cause iron depletion by reducing the intestinal absorption of the metal.[6] Plasma concentrations of zinc above 200 µg/dl were only measured in one patient in the present study. However, given that blood sampling was not timed to coincide with expected peak levels for logistical reasons, and based on the results of our pharmacokinetic study, it is fair to assume that peak concentrations in patients in this study were higher than the levels measured. It is generally recommended that oral zinc is given separately to feeding because some food constituents (phytates) can bind zinc and diminish efficacy. However, it is our clinical experience that the salt can be an irritant to the gastric mucosa, causing nausea and vomiting in our patients. Therefore, mixing of the drug with small amounts of food was recommended. This adaptation of general recommendations could theoretically also account for the lower plasma concentrations of zinc measured. The choice gluconate as a salt was based on our clinical observations of fewer adverse reactions than acetate or sulphate salts.

The results of this study indicate that dietary management of hepatic copper accumulation in Labradors reduces hepatic copper concentrations, which appear to stabilize in the high normal range after 8-16 months. This may be because the diet provided did contain some copper, considered by the manufacturer to be an adequate maintenance amount. Furthermore, owner compliance to a strict diet with no other treats might have decreased after the initial recheck examination, which showed hepatic copper concentrations within the normal range.

One limitation of the present study was that dogs with clinical signs of CACH were not included in the study. That dietary management would improve clinical signs in clinically affected animals is therefore only speculative. In addition, histologic changes were absent or too mild to adequately assess an effect on histologic scores.

The copper-reducing effect demonstrated by our results was most likely due to the low copper content of the diet because previous treatment with penicillamine is unlikely to have a long-lasting action[13] and dogs treated with penicillamine and fed on regular diets with comparable zinc concentrations to the diet used in this study were affected by increasingly elevated hepatic copper levels.

We expect dogs with elevated hepatic copper concentrations to be at high risk for the development of chronic hepatitis and finally cirrhosis, which are potentially irreversible disorders. Previous results showed that D-penicillamine is an effective first-line treatment to decrease hepatic copper concentrations in Labrador retrievers. Based on the results of this study, we recommend long-term dietary management with a low-copper diet to prevent recurrence of excessive copper accumulation. Further studies are necessary to predict if such treatment can prevent copper accumulation completely, and if copper or iron deficiency may result from long-term treatment. Follow-up examinations in patients on long-term treatment would appear prudent to address this issue.

Acknowledgments:
We thank the dog owners for their participation, the Dutch Labrador retriever club for their contribution, and the technicians and students for their assistance.
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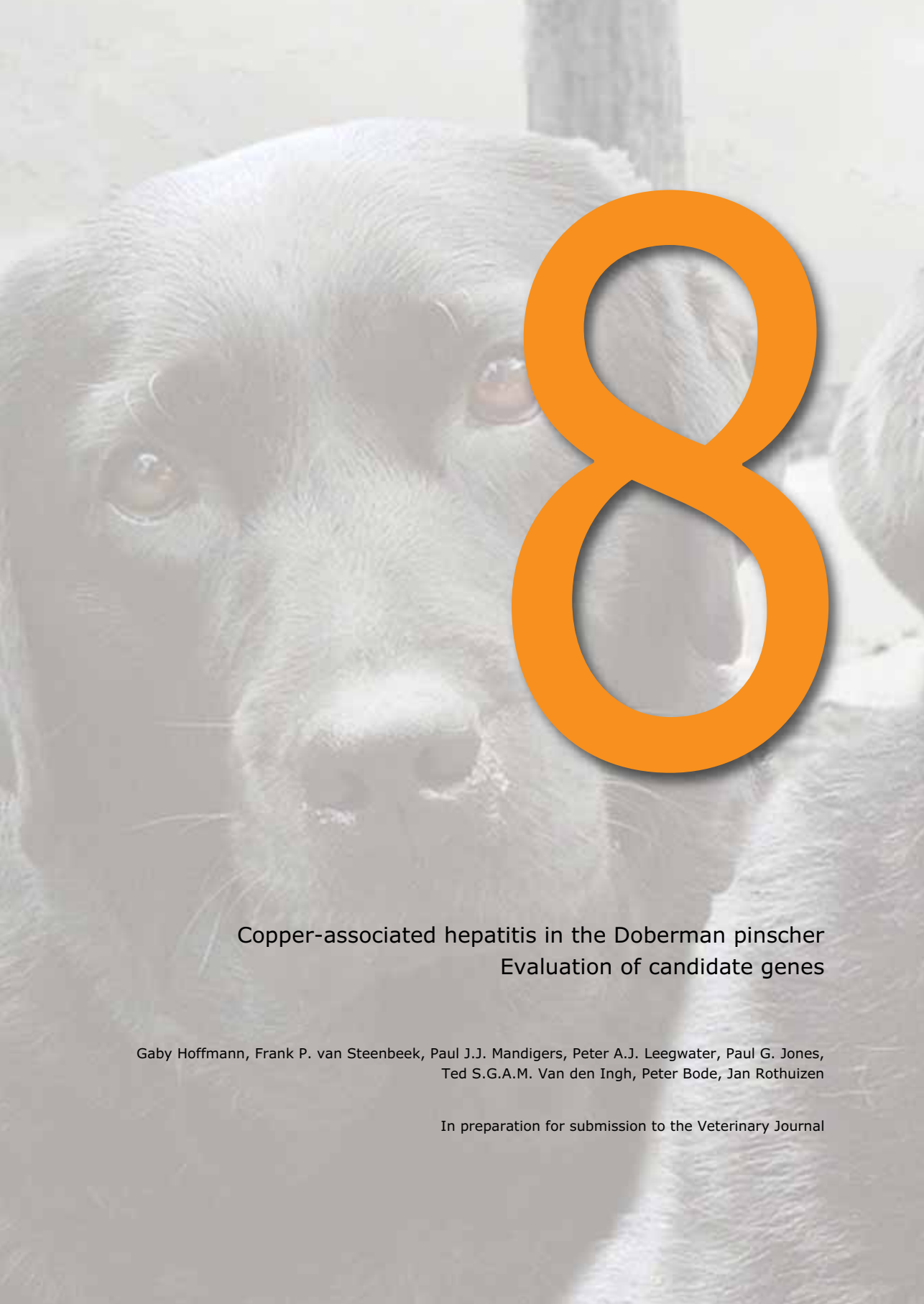
[i] SPSS 12.0.1 for Windows, Chicago, Illinois, USA

Part 3

Molecular genetic assessment of hepatic copper accumulation

Chapter 8-12



8

Copper-associated hepatitis in the Doberman pinscher
Evaluation of candidate genes

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Ted S.G.A.M. Van den Ingh, Peter Bode, Jan Rothuizen

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Abstract

Copper retention in the liver has recently been shown to be most likely a cause of hepatitis in Doberman pinschers. The objective of this study was to evaluate genes known to be involved in copper metabolism or copper storage diseases. We investigated these genes, that could be involved in copper-associated hepatitis (CAH) by an association analysis in 32 phenotyped Doberman pinschers. All dogs underwent physical examination, blood sampling for retrieval of a coagulation profile (PTT, PT, fibrinogen), liver enzyme activities, and isolation of genomic DNA. Liver biopsies for histologic examination, copper staining, and quantitative copper measurements were retrieved from all dogs. DNA was extracted from EDTA blood samples of Doberman pinschers with subclinical hepatitis and associated elevated copper concentrations and from 16 normal Doberman pinschers. The phenotype of the disease was defined as the association of subclinical hepatitis with hepatic copper concentrations higher than 400mg/kg dry matter. The candidate genes CTR1, ceruloplasmin, metallothionein gene cluster, ATP7A, ATP7B, COMMD1, CCS, SOD1, ATOX1, DYNACTIN, COX17, and XIAP were investigated. Genotyping of 47 polymorphic microsatellite markers, and 133 single nucleotide polymorphisms within or in close vicinity to the genes was performed, as well as sequence analysis of genomic DNA. There was no allele of any of the polymorphic markers associated with Doberman hepatitis. Hepatitis specific mutations were not found in the genes that were analyzed by DNA sequencing.
All investigated candidate genes were excluded as genes responsible for copper-associated hepatitis in Doberman pinschers.

Introduction

Chronic hepatitis in Doberman pinschers is often characterized by elevated hepatic copper concentrations.[1-6] Copper retention has recently been shown to be most likely a cause of hepatitis in the breed.[3,5] Normal copper concentrations in dog liver range between 150 to 400 mg/kg dry matter.[3,7-14] The elevated hepatic copper concentrations in Dobermans, up to 2000 mg/kg, have been suggested by some authors to be caused by cholestasis or cholatestasis.[1,2,5] However, increased hepatic copper concentrations were found recently in Dobermans which displayed subclinical hepatitis but were free of cholestasis or cholatestasis. In some of these animals, hepatic copper concentrations continued to rise over a period of 3 years. Copper accumulation in these dogs was associated with apoptotic hepatocytes and copper-laden Kupffer cells in centrilobular regions, suggestive for an intracellular metabolic defect of copper homeostasis.[3,15,16] A defective copper metabolism was further indicated by recent findings of copper excretion studies in which Doberman pinschers with suspected copper-associated hepatitis were compared to normal Doberman pinschers using intravenously administered radioactive copper isotopes. A hepatobiliary disorder was excluded by 99mTC-Bis-IDA-scintigraphy in these dogs, and excretion of 64Cu into bile was significantly decreased in the dogs with subclinical hepatitis.[7]
The breed predisposition for copper-associated hepatitis in the Doberman pinscher makes a genetic background likely. Copper-associated hepatitis in the Doberman pinscher has phenotypical similarities to known copper storage disease in both man, as well as dog.[11,17-20]
The investigated genes included the Wilson disease gene (ATB7B)[21-23], Bedlington terrier copper toxicosis gene (COMMD1)[24-26], the copper transporter protein gene CTR1[27,28] which is responsible for cellular uptake of copper into mammalian cells, the genes for the intracellular copper transport proteins COX17, ATOX1, and CCS[29], and the Menkes disease gene ATP7A[30],which encodes a P-type ATPase for copper transport across cellular membranes. Due to an interaction of ATP7B with dynactin[31] and XIAP[32,33], these proteins were also considered as candidates. Furthermore, the metallothionein gene cluster and ceruloplasmin[34], that are involved in copper storage and transport, were considered candidates, as well as the gene for the copper containing enzyme superoxide dismutase 1 (SOD1)[27,35]which is dependent on copper for appropriate function. We tested the hypothesis that one of these copper transporter or storage genes is mutated in Doberman pinschers with copper-associated hepatitis. For the genetic analyses, patients were assumed to share a particular allele haplotype of a DNA marker, which is in linkage disequilibrium with the causative gene mutation and the phenotype of the disease. Linkage disequilibrium describes a correlation between two loci that occurs more often than expected by chance in very distantly related individuals within a population.[36] Genetic analysis should therefore allow for the identification of chromosomal segments that are shared by the patients. For the identification of a shared allele genotyping of polymorphic microsatellite markers and single nucleotide polymorphisms was performed.

Materials and methods

Dogs included in the study

All samples were obtained from Doberman pinschers privately kept as companion animals with informed consent of the owner. The dogs were presented to the Department of Clinical Sciences of Companion Animals of the University of Utrecht for a survey investigating the prevalence of Doberman hepatitis, as described earlier.[3] The procedures were approved by the veterinary ethical committee of the University of Utrecht as required under Dutch legislation. From all animals blood was sampled for measurement of bile acids and liver enzymes (ALT, Alkaline phosphatase), a coagulation profile (PTT, PT, and fibrinogen) and isolation of DNA. Liver biopsies were taken according to the Menghini technique given by Rothuizen[37,38]. Two biopsies were fixed in 10 percent neutral buffered formalin for histological examination and two other biopsies were stored in a copper-free container for quantitative copper determination. For the genetic analysis, the phenotype of the disease was defined as the association of hepatitis with elevated hepatic copper concentrations above 400 mg/kg dry matter, and a centrolobular localization of accumulating copper. The pedigrees of all dogs included in this study were collected and the familial relationships between these dogs were established. Genomic DNA was isolated using a salt extraction method, and frozen at –20ºC until use.[39]

DNA marker retrieval and analysis

Internet search in the integrated dog map (http://www-recomgen.univ-rennes1.fr/Dogs/maquette-1800.html) as well as the ENSEMBL system http://www.ensembl.org/index.html) allowed retrieval of microsatellite markers. In order to identify new microsatellite markers a sequence of approx 500 kb up- and downstream of candidate genes was selected and screened for interspersed repeats and low complexity DNA sequences (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). The masked sequence was used as input for http://tandem.bu.edu/trf/trf.basic.submit.html to find tandem repeats. Primers were designed using PRIMER DESIGNER for windows version 2. Forward-primers were ordered with a M13-tail (GTTTTCCCAGTCACGAC). Primer sequences and PCR product lengths are given in Table 1. The forward primers were directly labelled with HEX, or FAM fluorescent dyes, or they were tailed with M13 sequencing tags, and indirectly labelled with FAM, which allowed analysis on an automated ABI PRISM 3100 Genetic Analyser. PCR reactions were performed in a PTC-100TM PCR system in a 15 µl volume containing 50ng template DNA, 50ng of each oligonucleotide primer, 200 mM dNTPb, and 0.5 unit Amplitaq Gold in 1 x PCR buffer II with 2.5mM $MgCl_2$. For the M13-tailed primers a mix was used containing 50ng template DNA, 0.03 µM forward primer, 0.3 µM reverse and labelled-M13 primer, 200 mM dNTP, and 0.5 unit Amplitaq Gold, in 1x PCR buffer II with 2.5mM $MgCl_2$. DNA was initially denaturated at 95°C for 5 min and was than subjected to 10 cycles of 95°C for 30 s, annealing for 15 s at 55°C and at 72°C for 30 s, followed by 25 cycles of 92°C for 30 s, annealing for 15 s at annealing temperature according to table 1, and at 72°C for 30 s, followed by a final extension step for 10 min at 72°C. PCR products were run with GS500 size standards on an automated ABI3100 (later 3130XL) DNA Analyzer. Genescan 3.1 (later 4.0) software was used for genotype assessment. All microsatellite markers were evaluated for heterozygosity and polymorphism, and typed in 32 Doberman pinschers. To evaluate whether a gene is involved in copper accumulation, the results of all dogs were tested statistically by chi-square testing, as well as by examination of haplotypes by grouping maternally and paternally inherited alleles (significance level $p \leq 0.01$).

Single nucleotide polymorphisms (SNPs) were selected from a set of 4608 SNPs that were chosen from the public databases where SNPs had been observed in more than one breed. SNP assays were designed optimal to the golden gate assay in the Illumina system and genotyped at Illumina Inc.

Canine DNA sequencing

All exons of CCS, COMMD1 and SOD1 were analyzed by genomic DNA sequencing of 4 Doberman pinschers with clinically active copper-associated hepatitis, and one healthy dog.

PCR primer pairs were retrieved from the ENSEMBL system. Obtained sequences were aligned using Seqman of the package.

Results

The dogs were unrelated to each other in the pedigree of three generations. The dogs were chosen from a larger survey investigating the prevalence of Doberman hepatitis in the Netherlands.[3] The affected group consisted of 16 Doberman pinschers with subclinical hepatitis. Ten dogs were female, and six dogs were male. Liver enzymes and bile acids were within reference values. At histopathology, all dogs showed centrolobular copper-laden hepatocytes, occasional apoptotic hepatocytes associated with copper-laden Kupffer cells, lymphocytes, plasma cells and scattered neutrophils. Based on these lesions subclinical hepatitis associated with copper accumulation was diagnosed.[10,13,14] A quantitative assay for copper in liver tissue was performed by neutron activation analysis, according to a protocol described by Teske et al, using the facilities described by Bode.[40,41] Quantitative copper concentrations were measured in lyophilised liver biopsies and reported in mg/kg dry weight liver. Hepatic copper concentrations were above 400 mg/kg dry matter in all affected dogs (mean 804, range 433 – 1590 mg/kg).
The control group consisted of Doberman pinschers (n = 16) which were clinically healthy with normal liver enzymes and normal bile acids. Histopathology revealed normal liver. Eight dogs were male and 8 dogs were female. Liver copper concentrations were below 250 mg/kg dry matter (mean 173 mg/kg, range 104 – 245 mg/kg) in all normal dogs.

Thirty nine microsatellite markers were polymorphic in the study population, and were found to be sufficiently informative. For each patient both alleles of each microsatellite marker are depicted. None of the deduced haplotypes showed association with the phenotype.
One hundred and thirty three SNPs were polymorphic in the investigated group of dogs. The results from chi square testing of the microsatellite markers and the SNPs are described in Table 2. Chi square testing approached significance for three markers around CCS. Therefore the gene was further analyzed by direct sequencing. The genotyping results for CTR1 were considered insufficiently informative, because of the limited variation in the corresponding microsatellite markers. Therefore, the coding exons of this gene was analyzed by genomic DNA sequencing. In addition SOD1, and COMMD1 were sequenced. There was no variation in any coding DNA sequence of the examined dogs.

Table 1: Polymorphic microsatellite markers used to investigate 32 Doberman pinschers for hepatic copper accumulation

marker	chromosom	position on chromosome	STS	product	temp	label	forward primer	reverse primer
METALLOTHIONEIN	2	62520852 - 62523438	62.5					
		62488003 - 62488902	62.4					
MT1-CA1	2	62398688 - 62399024	62.3	336	62	FAM	GCATTTCCTATGAGATAACCCCA	GTGGGGCAGAGAGATAATTGC
MT1-CA2	2	62416666 - 62417034	62.4	368	62	HEX	CAGAGGTGCTACTCCAGGCT	CGATCCTGAGTCTCCAGGATG
MT1-CA5	2	62462220 - 62462458	62.4	238	62	FAM	CTGACCCATCTCATCATGGATC	GATGAAAACCAGTTGGCTGTCTTG
MT1-CA3	2	62443288 - 62443626	62.4	339	62	HEX	GTCAGGCAGCCCAGGTG	CCAAGTCTCTTTTAGTTAGTCTCACAG
MT1-CA4	2	62501194 - 62501590	62.5	396	55-62	FAM	GCCAAACCGGTGGGCAA	GCTGAAGCCTGAACCCAC
FH2608	2	63337494 - 63337746	63.3	246	55	NED	CTACACCCTGCTTGTGCTCT	TCCTCATATAATGTGCTGACCA
ATOX1	4	60803400 - 60812280	60.8					
ATOX1-CA1	4	60707850 - 60798163	60.7	313	62	HEX	GAGCCTGACATGGGCCTCAAT	TGGGTGGCTCAACAGATGGG
ATOX1	4	60925953 - 60916325	60.9	360	55	FAM	GTCTCATCGTGGAGGTCCTG	GCTGAGAGCTTACCTTGTGC
REN195B08	4	60340457 - 60340592	60.3	145	55	M13	GCTTTCCCATTGTGTCCTCA	TGATTGATGCCCTTTCAACA
DYNACTIN	4	61626928 - 61654167	61.6					
DYNACTIN-CA2	4	61764595 - 61764989	61.7	397	55	M13	CCAGGAATCCTTGTGACTGC	CTGCCTTGTCTGGAGCTTCT
COMMD1	10	65037469 - 65210789	65					
C04107	10	65295622 - 65067965	65.2	165	55	FAM	TCAGCAACTATACATTTAAGAGCA	CTGTCCCATCTAAAGGATAGG
CF10B19	10	65375160 - 65473907	65.3	286	55	FAM	CAAGGATGCCTGGATGCCAG	CTCCTGAGTCAGTTGTCACAG
CTR1	11	70555208 - 70687734	70.5					
DGN13	11	71157310 - 71157641	71.1	320	55	M13	GGAACCCTCAGCTGTTATATGC	GATCTAAGGGCTCCAGAACACC
CTR1-CA1	11	70655367 - 70655725	70.6	524	62	FAM	CATGAAGATTTCAGTGTCATACATCACA	TCCTGTCTTTCAGTCTTGAACTGCTTC
CCS	18	53773585 - 53784729	53.7					
CCS-CA1	18	53895640 - 53896082	53.8	468	62	FAM	TCATTCTTGCAAAAACTCGGATAG	GATAGTGGTAGAGGGTAAGACAGCC
CCS-CA2	18	53989134 - 53989440	53.9	283	62	HEX	CTGATTGAGCTGTGAAAGTACCTG	GTAGTGCGGTGTCTGATCACTG
CCs-CA3	18	53989530 - 53989255	54	295	55	M13	AACTGGAACTCTGGCTGATG	CTGTATATGTTGGCCGGTGT
CCs-CA4	18	53331401 - 53331096	53.3	325	55	M13	CGGAGTTCTTGGTCACAAGC	TCCTCCACCTCATCTGATGC
REN195J11	18	55321824 - 55322030	55.3	207	55	M13	CCCCGAAGCAAAATTAAACA	CTGGGGTTTAACAGCTTTGC
ATP7B	22	3134310 - 3167688	3.1					
REN68D20F	22	5.447027 - 5468042	5.4	227	55	FAM	CAGGTGTCCCATTCAGGTAGTC	GAGCTAAGATATCAAATGGGGAAAG
REN128H16	22	7331505 - 7331744	7.2	239	55	FAM	CTGCCATTGCCACCAAACA	CAGCCAATTTCCTAAGAGTCC
REN49F22	22	3906420 - 3906567	3.9	157	55	M13	GGGGCTCTGTTATTAGGTG	TCATAAGGCAAAGAAAACC
ATP7B-CA1	22	3253107 - 3253525	3.2	413	62	FAM	GAAGAGTAAAGAGGTGACATCTGAAG	AGGTCATACTGCATGTGCGG
CERULOPLASMIN	23	47038809 - 47083165	47					
CERULO-CA3	23	47059068 - 47059600	47	605	62	FAM	CACTGCTGGTGGTAATGCAAG	GGTGCACCTTCAGACCACTACA
CERULOPLASMIN	23	47068680 - 47069084	47	405	55	HEX	AATCAAATGCATCAACCAGTCTAC	CGCTGCTGAGGAAATCATCTG
SOD1	31	29559416 - 29563336	29.5					
CAH3129	31	29460493 - 29460512	29.4	316	55	M13	CTCACTCGACCACATCAAGG	CCTTGACTGGATACAGGAGG
SOD-CA1	31	29504942 - 29505162	29.5	240	55	M13	GTGACCATGAGTTAGAGAGC	CTCACAGCCTCATTAGACAC
SOD1-CA2	31	29460492 - 29460786	29.5	314	55	M13	TCTCACTCGACCACATCAAG	TGACTGGATACAGGAGGTTC
SOD1-CA3	31	29936304 - 29936523	30	239	55	M13	CCACCATCCTTCAGGAGTTG	CCTGCGGTCCTAAGTTCTTG
COX17	33	26345268 - 26353171	26.3					
REN147E03	33	25037664 - 25037800	25	139	55	FAM	TGTTGTCAAACAGCAAGGGA	CCTACATGTCCACCAATGGAT
REN98D17	33	24584948 - 24585156	24.5	209	55	FAM	AGGGTGGCCAGAAGGTC	GAAGGAAGGTATAGGGGTGTG
COX17-CA2	33	25939374 - 25939653	25.9	299	55	M13	CGCAGGAACTCAGAGTAGAA	GGCTAGTTGTATGCTACGGA
COX17-CA3	33	25922163 - 25921834	25.9	349	55	M13	GGATCCTGGTACACTGCCTT	CCATGGACACTGACTGCAAC
ATP7A	X	63307660 - 63411694	63.3					
ATP7A-CA3	X	63445366 - 63445640	63.4	271	57-65	HEX	CGTGAGGTACTACTTCCAGACCTAG	ACCACTAGCAGTATAAGAGGGCC
D04614	X	59910734-59910893	59.9	154	55	M13	ATCAAGTCCCACATCGGGCT	GTGGTTCTTATCCTTTCTCTTATC
REN130F03	X	70133620 - 70133790	70.1	181	49	FAM	ATCGAGTCCCACATCAA	TTCTACTTCAGCCCCTACC
REN185C11	X	60643426 - 60643715	60.6	289	55	M13	CTCAACTGACTGAGCCACCA	ATTAGCAGGTCCAGGGAAGG
XIAP	X	98368886 - 98420162	98.3					
XIAP-CA3	X	98206588 - 98206015	98.2	427	62	HEX	GCTGAGTGTAGTCCTCATTTGG	CTCTAGGTTTCTAAGCTTCATCTGTG

Table 2: Results from chi-square testing of 39 microsatellite markers, and 133 SNPs

Gene	marker	number of informative markers	chromosome	position on chromosome	Chi square
METALLOTHIONEIN			2	62488003 - 62523438	**p value**
	microsatellites	6x		62.39 - 63.33	
	SNPs	7x		62.25 - 62.50	0.16 - 0.3
ATOX1 & DYNACTIN			4	60803400 - 61654167	
	microsatellites	4x		60.3 - 61.7	
	SNPs	26x		60.56 - 61.86	0.09 - 0.3
COMMD1			10	65037469 - 65210789	
	microsatellites	2x		65.29 - 65.47	
	SNPs	7x		64.98 - 65.21	0.3 - 1
CTR1			11	70555208 - 70687734	
	microsatellites	2x		70.65 - 71.15	
	SNPs	15x		70.3 - 70.8	0.17 - 0.96
CCS			18	53773585 - 53784729	
	microsatellites	5x		53.32 - 53.98	
	SNPs	13x		53.5 - 53.99	0.02 - 0.56
ATP7B			22	3134310 - 3167688	
	microsatellites	4x		3.2 - 7.3	
	SNPs	14x		3.0 - 3.2	0.08 - 0.38
CERULOPLASMIN			23	47038809 - 47083165	
	microsatellites	2x		47.05 - 47.06	
	SNPs	8x		46.8 - 47.1	0.08 - 0.32
SOD1			31	29559416 - 29563336	
	microsatellites	4x		29.46 - 29.93	
	SNPs	12x		29.4 - 31.7	0.06 - 0.58
COX17			33	26345268 - 26353171	
	microsatellites	4x		24.58 - 25.92	
	SNPs	11x		26.1 - 26.56	0.36 - 0.93
ATP7A			X	63307660 - 63411694	
	microsatellites	4x		59.91 - 70.13	
	SNPs	6x		63.1 - 63.5	0.33 - 0.97
XIAP			X	98368886 - 98420162	
	microsatellites	1x		98.2	
	SNPs	14x		98.1 - 98.4	0.06 - 0.6

Discussion

Dogs of a single breed that are affected by a hereditary disease can be expected to share the causative gene and alleles of closely situated markers. This will lead to an uneven allele distribution between groups of affected and unaffected dogs. This is the principle of association studies.
We have evaluated 12 candidate genes for copper-associated hepatitis in Doberman pinschers in an association study. Comparison of the phenotype of the disorder in the Doberman with Wilson disease and copper toxicosis in Bedlington terriers showed similarities in terms of hepatic copper concentrations and histopathological presentation.[11,19-21] The genes that cause these disorders were therefore included as candidate genes (ATP7B and COMMD1). Furthermore genes encoding proteins known to be involved in cellular copper uptake (CTR1), intracellular copper transport (ATOX1, COX17, CCS, ATP7A, XIAP, dynactin), copper storage (MT cluster) and transport in blood (CP), were included, as well as the gene for the copper containing enzyme superoxide dismutase (SOD1) which protects against oxidative stress.[21,26,29,34,35,42] Microsatellite markers in close vicinity of the candidate genes were investigated in 32 Dobermans, in order to identify chromosomal segments that are possibly shared by the patients and are identical by descent (IBD). The underlying hypothesis is that patients of a homogeneous population inherited the same disease mutation with the same surrounding DNA sequence from a common ancestor. The presence of a shared allele in a group of patients could indicate IBD and reflect a linkage between a disease and a particular genetic marker allele. The linkage should lead to a significant difference of allele frequencies between groups of affected and healthy dogs. The limitation of this molecular genetic approach is the possibility of a polygenic or complex etiology of the disorder. In such a scenario, a molecular genetic analysis would require larger groups of healthy and affected dogs and preferably inclusion of all factors in the analysis.[43]
The actual mode of inheritance could not be determined due to a lack of information regarding the relatives of the analyzed dogs. However, our genetic analysis is solely based on the assumption that affected dogs share the causative allele which is not present in healthy dogs. For this reason, dogs were extensively phenotyped and strict criteria were kept to classify individuals that were included in the analysis. As a result, all investigated candidate genes could be excluded as genes responsible for copper-associated hepatitis in the Dobermans with the condition that one fully penetrant disease gene exists.
Genotyping results for CTR1 were considered of restricted informative value because the level of heterozygosity of the corresponding microsatellite marker was limited. Therefore, coding exons of the CTR1 gene were included in the DNA sequence analysis. COMMD1 was sequenced because of it's importance in copper toxicosis of Bedlington terriers. Furthermore CCS, and SOD1 were sequenced, because chi-square testing approached significance in some of the investigated SNPs, but no hepatitis specific mutations were found.

A genetic background for copper accumulation and the associated hepatitis in the Doberman pinscher is highly likely because of the predisposition of the breed for the disease. We investigated copper-associated hepatitis in a group of 32 Doberman pinschers by means of a candidate gene approach and we excluded many genes as candidates for mutations in an assumed monogenic aetiology. Further molecular biologic investigation is needed to solve the genetic basis and mechanism of copper-associated hepatitis in Doberman pinschers.

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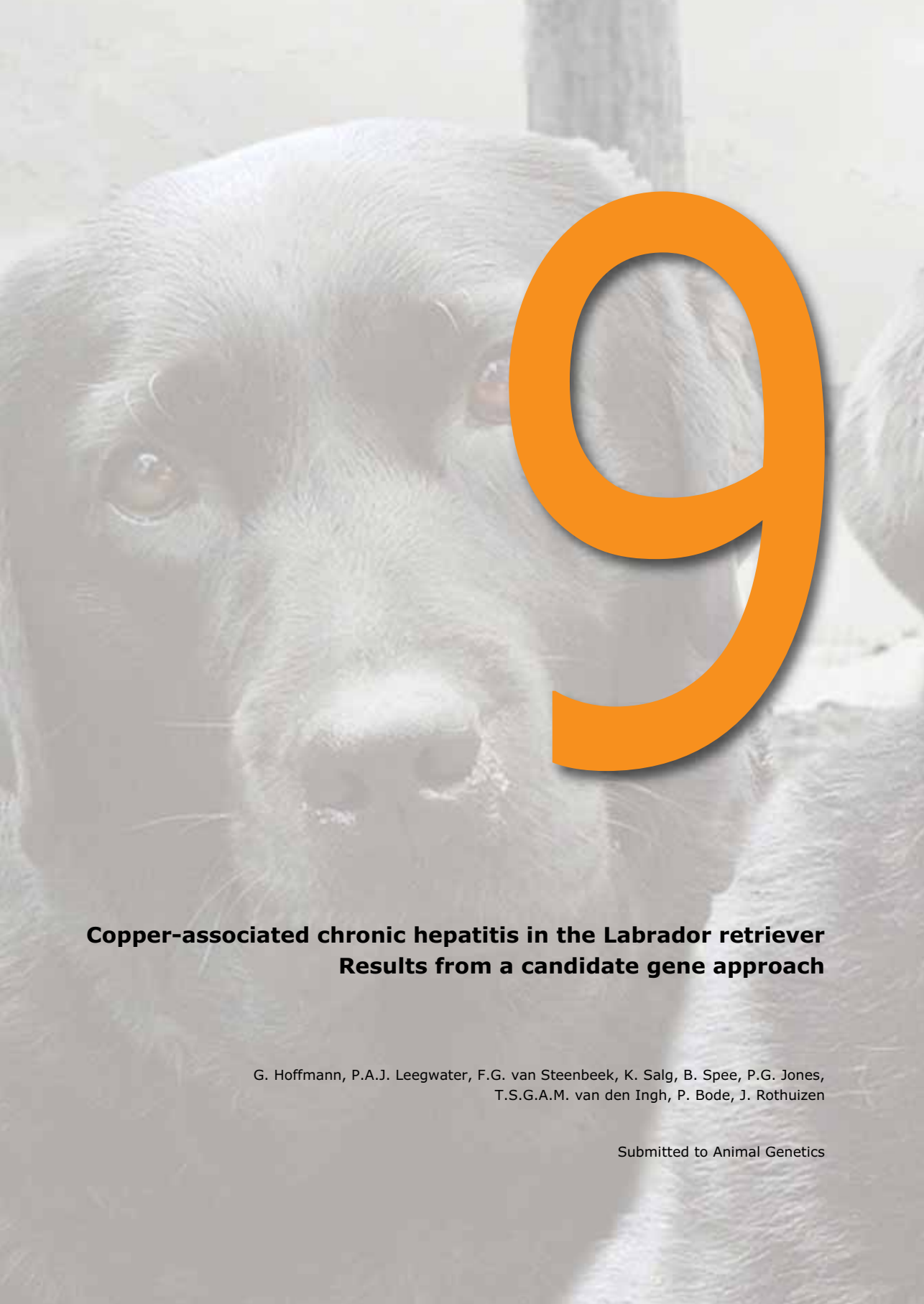

9

Copper-associated chronic hepatitis in the Labrador retriever
Results from a candidate gene approach

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Submitted to Animal Genetics

Abstract

Chronic hepatitis from copper accumulation was recently reported in Labrador retrievers from Europe as well as from the United States. Elevated hepatic copper concentrations of family members of dogs with copper-associated chronic hepatitis, as well as a characteristic localization of copper within the liver lobule suggest a primary copper storage disease.

We investigated the possible role of 14 candidate genes in hepatic copper accumulation in 60 Labrador retrievers. The investigated genes were *ATP7B, COMMD1, CTR1, COX17, ATOX1, CCS, ATP7A, SOD1 and SOD2, XIAP, DYNACTIN (p62), MT-1* and *-2,* and *CP*. The phenotype of the trait was defined as an elevated hepatic copper concentration above 600µg/g dry weight liver with localization of copper in zone 3 of the liver lobule. Thirty-seven dogs were affected, and 23 dogs were healthy, and had a normal liver on histologic examination.

Fourty-eight polymorphic microsatellite markers were genotyped. These markers were tested for association in 54 dogs, and linkage in a family of 16 dogs. Genes for which genotyping of one microsatellite marker approached a difference between groups, were further investigated. These genes were sequenced, and gene expression was measured by quantitative PCR. Our study excluded all investigated candidate genes from involvement in hepatic copper accumulation in Labrador retrievers, under the condition that a single disease gene exists.

Introduction

The Labrador retriever is the most common dog breed in Europe and in the United States, and one of the prime breeds selected as guide and rescue dogs. Copper-associated chronic hepatitis of the Labrador retriever is a metabolic disease, which was recently described for the first time. The disorder is most likely caused by an inherited genetic defect.[1,2] Clinical signs often reflect the end-stage of a fatal cirrhotic liver disease. Diagnosis is dependent upon histologic examination of liver biopsies. Retrieval of liver biopsies is an invasive procedure, which requires specialized veterinary practice, and therefore diagnosis is often missed at an early, treatable stage of the disease.

Results from previous gene expression profiling showed that at zonal copper concentrations of the magnitude of the affected dogs in this study, a primary metabolic defect of copper homeostasis is always the etiology for copper accumulation.[3]

The disease in Labradors is comparable to copper toxicosis of other dog breeds, as well as the human copper storage disorders. Results obtained from the study of the canine disease could therefore help to reveal the molecular genetic background of the disease in children.[4]

This is the first molecular genetic investigation of a hepatic copper storage disorder in the Labrador retriever.

Materials and methods

Our aim was to evaluate fourteen candidate genes, which are known to be involved in copper metabolism and associated disorders (fig. 1). The investigated genes were *ATP7B, COMMD1, CTR1, COX17, ATOX1, CCS, ATP7A, SOD1* and *SOD2, XIAP, DYNACTIN (p62), MT*-1 and -2, and *CP*. For each candidate gene a set of closely located polymorphic microsatellite markers was investigated for association with the phenotype by comparing allele frequencies between affected and normal dogs by chi-square testing, and assessment of haplotypes by grouping maternally and paternally inherited alleles. In addition, the markers were investigated by model free linkage analysis in a family of 16 dogs. Four genes that could not be excluded by these methods were investigated by DNA-sequencing, and gene expression was measurement by qPCR on RNA isolated out of liver tissue. RNA isolation and QPCR were performed as described before.[5]

Phenotyping

The phenotype of the trait was defined as elevated hepatic copper concentrations above 600 µg/g dry weight liver (dwl) with a predominant localization of copper in zone 3 of the liver lobule. Healthy dogs had hepatic copper concentrations below 400 µg/g dwl.[1] In order to clearly confine two distinct groups, animals with copper concentrations between 400 and 600mg/kg dwl were not included in the analysis. Age related changes in copper concentrations have been described previously.[6] We assumed that with our distinction criteria between the two groups with respect to copper concentrations, as well as the histological localization of copper as part of the phenotype, we created well defined distinct groups to avoid the inclusion of phenocopies.

All dogs were client-owned patients of the Department of Clinical Sciences of Companion Animals (Utrecht University). There was no evidence of exposure to drugs or possible exaggerated copper intake, and all dogs were fed commercial dog food. Written owner consent was obtained for all dogs participating in this study.

Results

DNA type II marker retrieval, associations, and linkage analysis

Search in the integrated dog map as well as the ENSEMBL system allowed retrieval of microsatellite markers in a DNA sequence of approximately 500 kb up- and downstream of the candidate genes.

Chi-square-testing was performed for association studies, and a model-independent statistical method was chosen to test for linkage. SimWalk2 software and the Mega2[7] program were used for calculation of non-parametric linkage scores[8].

Both groups of dogs showed an equal distribution of gender (p=0.26) and age (p=0.51).

For each candidate gene a set of up to seven polymorphic microsatellite markers was evaluated. Chi-square testing of one marker in the set for assessment of *ATP7A* revealed a difference between groups (ATP7A-CAA1, p= 0.044). Three more markers approached significance (REN185C11 for ATP7A, p=0.08; CCS-CA1, p=0.075; FH2608 for metallothioneine, p=0.06).

NPL scores were between 0.1 and 1.25. Analysis of two microsatellite markers approached significance (MT1-CA3 for metallothioneine, p=0.1, NPL score = 0.95); and CTR1-CA1, p=0.06, NPL-score 1.25).

Further analyses

Analysis of the 3kB promotor-region of *ATP7A*, as well as all coding regions, including intron/exon boundaries of *ATP7A*, MT-1, CCS, and CTR1 did not reveal differences between affected and normal dogs.

Total cellular RNA was isolated from liver tissue of 18 dogs. Six samples were isolated from affected Labradors with high hepatic copper and associated hepatitis, 6 affected Labradors with high hepatic copper but without hepatitis, and 6 normal Labradors. Quantitative PCR was performed for 3 genes (*ATP7A, CCS,* and *MT-1*). *CTR1* expression was not measured because an expected expression profile for copper loaded dog liver was not available for comparison. Reactions were performed as described previously[9].

The expression profile of affected Labradors with copper-associated chronic hepatitis was in agreement with a reaction of the canine liver to copper loading (fig. 2 and table 1).[3]

Discussion

In this study patients were assumed to share a particular allele haplotype, which is in linkage disequilibrium with the causative gene, and phenotype of the trait.
The actual mode of inheritance could not be determined due to a lack of information regarding all relatives of the analyzed dogs. Therefore a model-independent statistical method was chosen to test for linkage. Although it has been shown previously that approximated inheritance parameters might be more powerful than model free calculations, we did not perform model dependent linkage calculations to avoid the introduction of an error of multiple testing.[10,11]
All results were normal, and gene expression was in agreement with the expected profiles during copper loading of the dog liver. Therefore our results suggest that the investigated genes are intact at the level of genomic DNA, as well as at the transcriptional level in Labrador retrievers with copper-associated chronic hepatitis.
Most likely new genes, which are currently unknown to be involved in copper metabolism, will be discovered with a further genetic workup of the disease in the Labrador. We suggest genome wide analysis and quantitative trait statistics to solve the genetic basis of copper-associated chronic hepatitis in the Labrador retriever.

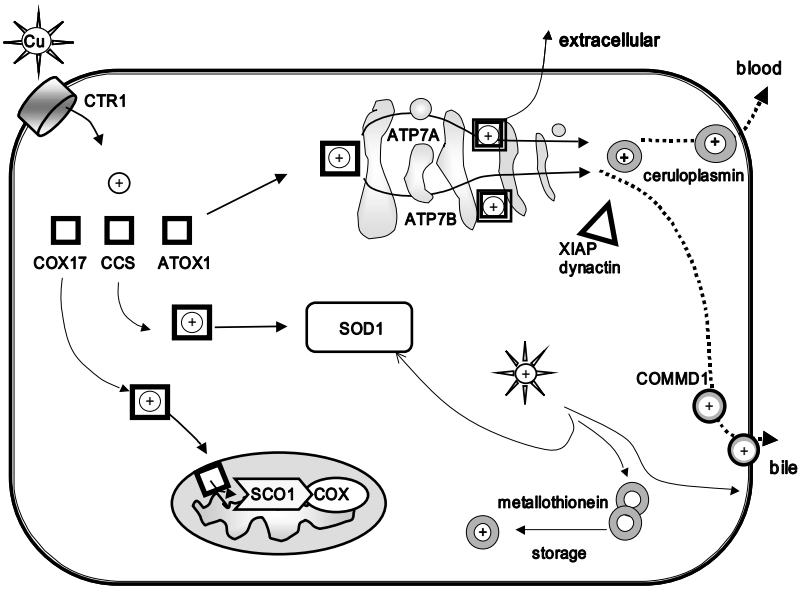

Figure 1: candidate genes involved in copper metabolism
ATP7A and ATP7B: ATPases of the Golgi apparatus, for copper transport across cellular membranes and incorporation into lysosomes and ceruloplasmin. ATP7A: Menkes disease gene, ATP7B: Wilson's disease gene. COMMD1: Copper Toxicosis gene of Bedlington Terriers, CTR1: cellular uptake transporter of copper, COX17, ATOX1 and CCS: transport proteins for intracellular copper trafficking (chaperones), Metallothioneine: non-toxic storage form of copper, ceruloplasmin for blood transport of copper, SOD1: copper containing enzyme superoxide dismutase1, XIAP: X-chromosomal apoptosis inhibitor gene which is reported to interact with COMMD1 as well as ATP7B. Dynactin: suggested interacting partner of ATP7B, COX: mitochondrial cytochrome oxidase.

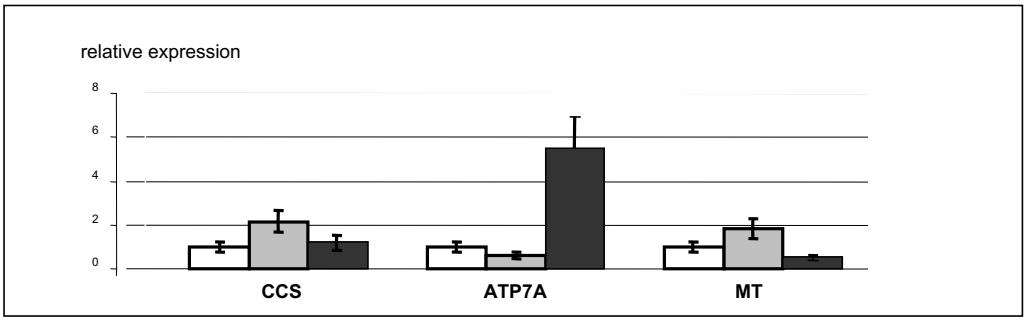

Figure 2: Results from gene expressions measured by qPCR
Y-axis: relative gene expression measured by qPCR. White bar: gene expression in normal Labradors, grey bar: gene expression in affected Labradors with elevated hepatic copper concentrations above 600 ppm but without chronic hepatitis, black bar: gene expression in affected Labradors with copper-assocated chronic hepatitis and elevated hepatic copper concentrations above 600 ppm.

Table 1: Results from genotyping, sequecing and gene expressions measured by qPCR

gene	*genotyping (chi-square test)*			*linkage (NPL score)*	*sequencing*	*gene expression*			
	marker	*location*	*p-value*	*p-value*		*group of dogs*	ratio	SD	p-value
CFA 18 (53.7 MB):	CCS-CA2	53.9	0.6			normal	1	0.24	
CCS	CCS-CA1	53.8	0.26	0.5-0.51	*normal*	high Cu	2.16	0.51	0.08
	REN195J11	55.3	0.075			high Cu and hepatitis	1.19	0.32	0.6
CFA X (63.3 MB):	REN130F03	70.1	0.75						
ATP7A	ATP7A-CA3	63.4	0.26						
	PGK	63.3	0.9	N/A	*normal*	normal	1	0.25	
	ATP7A-CAA1	63.2	0.044*			high Cu	0.62	0.15	0.6
	REN185C11	60.6	0.08			high Cu and hepatitis	5.5	1.47	0.01*
CFA 2 (62.4-5):	FH2608	63.3	0.055						
MT	MT2-CA1	62.5	0.46						
	MT1-CA1	62.3	0.7						
	MT1-CA2	62.4	0.7	0.11-0.5	*normal*				
	MT1-CA5	62.4	0.5			normal	1	0.22	
	MT1-CA3	62.4	0.6			high Cu	1.83	0.47	0.75
	REN154C04	55.7	0.68			high Cu and hepatitis	0.51	0.11	0.12
CFA11 (70.5 MB)	DGN13	71.1	0.8						
CTR1	CTR1-CA1	70.6	0.2	0.06 - 0.5	*normal*	N/A	N/A	N/A	N/A

*: p-value ≤ 0.05, N/A: not assessed, MB: Megabases, MT: metallothionein.

Achnowledgements
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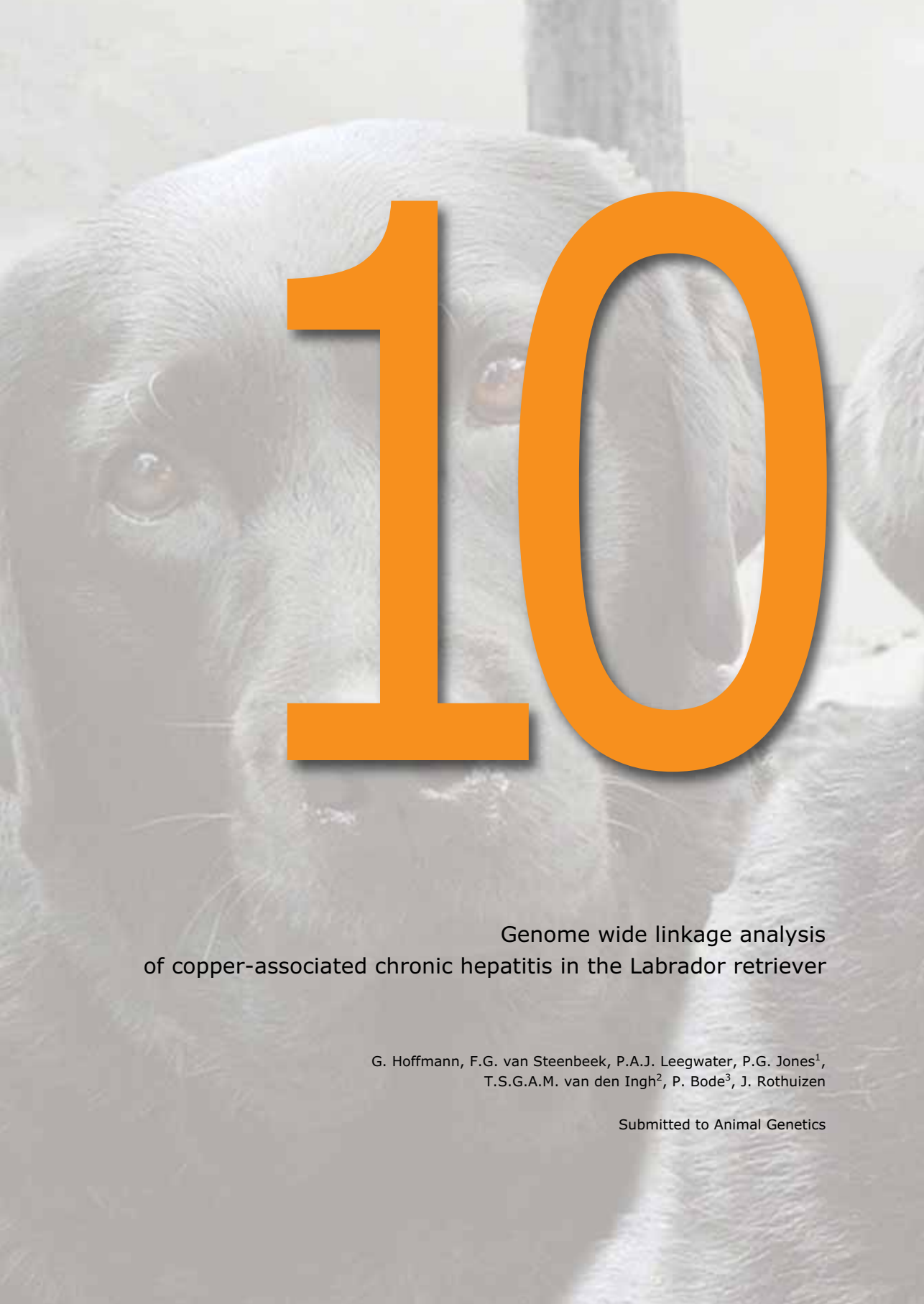

10

Genome wide linkage analysis of copper-associated chronic hepatitis in the Labrador retriever

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Submitted to Animal Genetics

Abstract

Chronic hepatitis from copper accumulation was recently reported in Labrador retrievers from Europe as well as from the United States. The diagnosis of copper-associated chronic hepatitis is based on histologic examination of liver biopsies, and measurement of copper concentrations. Hepatic copper concentrations measured in family members of dogs with copper-associated chronic hepatitis were severely increased compared to hepatic copper concentrations of unrelated control Labradors. The disease is most likely caused by an inherited genetic defect, with an estimated heritability between 0.39 and 0.85 for hepatic copper accumulation.

We investigated genetic linkage of hepatic copper accumulation in a group 31 Labradors by means of a genome wide approach with 289 microsatellite markers. All dogs underwent a physical examination, blood sampling for retrieval of a coagulation profile (aPTT, PT, fibrinogen), platelet count, liver enzyme activities, and isolation of genomic DNA. Liver biopsies for histologic examination, rubeanic acid staining for copper, and quantitative copper measurements were performed in all dogs.

For genetic analysis the phenotype of the disease was defined as an elevated hepatic copper concentration above 600 mg/kg dry weight liver (dw) in addition to elevated staining results for copper with a predominant localization in zone 3 of the liver lobule. The investigated group of 31 dogs contained 13 affected Labradors. Seven dogs were healthy, had liver enzymes in the normal reference range, normal hepatic copper concentrations and normal results from histologic examination of liver tissue. Eleven dogs had copper concentrations in an intermediate zone between 401 and 600 mg/kg dw, or results from quantitative and semiquantitative measurements were not in agreement. These dogs were excluded from the analysis.

Our study revealed 2 regions of possible linkage, with NPL scores of 4 on chromosome 7 and 3.8 on chromosome 28. Fine-mapping in these regions confirmed the high NPL scores and decreased the size of the genomic regions in linkage with hepatic copper accumulation to a length of 1.5-2 Mb.

Introduction

Copper-associated chronic hepatitis of the Labrador retriever was reported recently, to be most likely caused by an inherited genetic defect, and heritability estimates for hepatic copper accumulation ranged between 0.39 and 0.85.[1,2] Clinical signs are nonspecific, mainly consisting in anorexia, vomiting, and weight loss. These signs occur late, and often reflect the end-stage of a fatal cirrhotic liver disease. Female dogs are more commonly affected than male dogs. Diagnosis is dependent upon histologic examination of liver biopsies. Retrieval of liver biopsies is an invasive procedure, which requires specialized veterinary practice, and therefore diagnosis is often missed at an early, treatable stage of the disease, when hepatic pathology is still potentially reversible with appropriate medical care. A relative increase in the hepatocellular enzyme activities above a relative increase in biliary enzyme activities is present in almost all patients with clinical disease.[2]

Results from previous studies showed that at zonal copper concentrations of the magnitude of the affected dogs in this study, a primary metabolic defect of copper homeostasis is always the etiology for copper accumulation, which ultimately leads to chronic inflammation of the liver.[3,4]

Copper accumulation precedes development of hepatitis in Labradors. The disease is comparable to copper toxicosis of other dog breeds, as well as the human copper storage disorder Wilson's disease. Candidate genes involved in these disorders, and genes that play a role in copper metabolism in general were previously excluded as sole etiology for the disease in Labradors (chapter 8). The aim of this investigation was to discover linkage between a genomic region and the phenotype of hepatic copper accumulation in a family of dogs.

Nonparametric linkage analysis (NPL) is a model-free calculation, which is used to assess for allele sharing among affected individuals without assumptions of the inheritance pattern of a disorder. In this analysis genotypes of related individuals with a positive phenotype are expected to exceedingly share the disease-associated haplotype.[5-7]

Materials and methods

Dogs included in the study and DNA isolation

The index patient was a former clinical patient with copper-associated chronic hepatitis at the Teaching Hospital of the Department of Clinical Sciences of Companion Animals (Veterinary Faculty, Utrecht University). The family of this patient was identified by the Dutch Labrador retriever breed club, and invited for participation in this prospective study.

Phenotype

For genetic analysis the phenotype of the disease was defined as elevated hepatic copper concentrations above 600 mg/kg dry weight liver (dw), in addition to elevated staining results for copper with a predominant localization of copper within zone 3 of the liver lobule. Healthy dogs had hepatic copper concentrations below 400 mg/kg dw, together with a normal staining score for copper below 2. The definition of the phenotype "high copper" versus "normal" was based on a previously established breed specific normal range for copper in Labrador retrievers, which was found to be normal below 400 mg/kg dw, and a copper score below 2. In order to clearly confine two distinct groups of dogs, animals with copper concentrations between 400 and 600 mg/kg dw, and dogs for which results from quantitative and semi-quantitative measurements were not in agreement were excluded from the analysis. This decision was made to avoid overlap of groups from a possible influence of age on the copper content of the liver.

All dogs were registered at the Dutch Labrador retriever breed club. There was no evidence of exposure to drugs or possible exaggerated copper intake, and all dogs were fed commercial dog food. From all dogs a medical history was obtained, and all Labradors underwent physical examination, blood sampling, and retrieval of liver biopsies. Blood was sampled for analysis of a coagulation profile (including prothrombin time, activated partial thromboplastin time, and fibrinogen); for analysis of the hepatobiliary enzymes alkaline phosphatase (ALP), and alanine aminotransferase (ALT), as well as measurement of bile acids (BA), and the platelet count. Genomic DNA was isolated from 4 ml EDTA blood using a salt extraction method[8], and frozen at –20ºC until use. Liver biopsies were taken according to the Menghini technique described by Rothuizen.[9] At least two biopsies were fixed in 10 percent buffered formalin for histological examination, and rubeanic acid staining to determine the distribution of copper within liver lobules. One biopsy was frozen in liquid nitrogen for isolation of RNA, and one biopsy was stored in a copper-free container for quantitative copper measurement. A quantitative assay for copper in liver tissue was performed by neutron activation analysis, using the facilities described by Bode, and was measured in lyophilised liver biopsies according to a protocol described by Teske et al.[10,11] Results were reported in mg/kg dry weight liver (dw).

The study was approved by the Utrecht University Institutional Animal Care and Use Committee. All dogs were client-owned, and written owner consent was obtained for all dogs participating in this study.

DNA type II marker retrieval and genotyping

A genome wide linkage analysis was performed with 289 microsatellite markers. The microsatellite markers were selected from an open access 5000cR hybrid radiation map[12]. One of the PCR oligonucleotides to amplify the microsatellite was labeled with either fluorophore 6-FAM, TET, NED, VIC[i], or PET[ii]. The PCR was performed under standard conditions with Amplitaq Gold[b] DNA polymerase. The reaction products were mixed with GS500 size standard and automatically analyzed with a Genetic Analyzer 3100 . The markers are listed in table 1.

Later during this investigation new primers for fine mapping were designed using PRIMER DESIGNER for windows version 2. Forward-primers were ordered with a M13-tail (GTTTTCCCAGTCACGAC). Primer sequences and PCR product lengths are given in table 2.

The forward primers were tailed with M13 sequencing tags, and indirectly labelled with 6-FAM, which allowed analysis on an automated ABI PRISM 3100 Genetic Analyzer. PCR reactions were performed in a PTC-100TM PCR system in a 15 µl volume containing 50 ng template DNA, 50 ng of each oligonucleotide primer, 200 mM dNTP, and 0.5 unit Amplitaq Gold in 1x PCR buffer II with 2.5 mM $MgCl2$[a]. For the M13-tailed primers a mix was used containing 50 ng template DNA, 0.03 µM forward primer, 0.3 µM reverse and labelled-M13 primer, 200 mM dNTP, and 0.5 unit Amplitaq Gold (Perkin Elmer), in 1x PCR buffer II

with 2.5 mM MgCl2. DNA was initially denaturated at 95°C for 5 min and was than subjected to 10 cycles of 95°C for 30 s, annealing for 15 s at 55°C and at 72°C for 30 s, followed by 25 cycles of 92°C for 30 s, annealing for 15 s at annealing temperature according to table1, and at 72°C for 30 s, followed by a final extension step for 10 min at 72°C.
PCR products were run with GS500 size standards on an automated ABI 3130XL DNA Analyzer. Genescan 3.1 (later 4.0) software[a] was used for genotype assessment. All microsatellite markers were evaluated for heterozygosity, and typed in 31 Labrador retrievers.

Linkage analysis
From the pedigree of the dogs it was not possible to clearly identify the mode of inheritance, therefore a model-independent statistical method was chosen to test for linkage. SimWalk2 software was used for calculation of multipoint NPL scores.[13,14] This statistical method calculates values with a minimum of 0.0, and a maximum of 4.0. A value of 0.301 indicates equal evidence for and against linkage. Input files were prepared using the Mega2 program.[15]

Fine mapping with Single Nucleotide Polymorphisms:
For chromosomes with NPL scores above 2.5 further genotyping was performed by analysis of single nucleotide polymorphisms (SNPs) in DNA of 12 dogs of the investigated family. A total of 315 SNPs were selected from a set of 4608 SNPs that were chosen from the public databases where SNPs had been observed in more than one breed. SNP assays were designed optimal to the golden gate assay in the Illumina system and genotyped at Illumina Inc.
Chromosomes 3, 7, 28, and 29 were investigated with genotyping of 108, 112, 39, and 56 SNPs respectively. The average distance between the SNPs was 0.93 Mb (range 0.09-3.6Mb).

Results

DNA of 31 Labrador retrievers was included in the study. The pedigree of the dogs is shown in figure 1. The average age of the dogs was 6.2 years (range 3.8-9.9 years). Fourteen dogs were female (9 spayed, 5 intact) and 17 dogs were male (5 neutered, 12 intact). One affected dog had been diagnosed with chronic hepatitis associated with increased copper concentrations (index patient). The remaining dogs were examined because of their family relation to the former patient. In the affected group 8 dogs were female (7 spayed, 1 intact), and 5 dogs were male (2 neutered, 3 intact). In the normal group there was one intact female, and 6 dogs were male (2 neutered, 4 intact). The normal dogs were clinically healthy, had liver enzymes in the normal reference range, normal hepatic copper concentrations and normal results from histologic examination of liver tissue. Eleven dogs were excluded from the analysis although DNA was available. The phenotype from 2 of these dogs was unknown because owners had declined retrieval of liver biopsies, but agreed to sampling of blood for isolation of DNA. Quantitative copper concentrations of 2 dogs were in the intermediate zone between 401-600 mg/kg copper, and the results from 7 dogs were discordant with respect to quantitative measurements of copper and histologic staining results.
Mean hepatic copper concentrations in the normal group of dogs was 285 mg/kg dw (range 190-330), and the mean hepatic copper concentration in the affected group was 1354 mg/kg dw (range 630-2060). The mean histologic staining results for copper in the affected group of dogs were 2-3+ (range 2+ to 4+). Histologic staining results for copper in the normal group were negative in 4 dogs, and between 0-1+ in 2 dogs.

Genotyping and linkage analysis
All dogs were genotyped with 289 polymorphic microsatellite markers. Ninety markers were homozygous and therefore not informative in the investigated family. These markers were excluded from the analysis. Results from calculation of NPL scores were between 0.0488 and 4. The results are given in Figure 2. Analysis of three regions revealed NPL scores above 3 ($p = 0.0001$, two regions on chromosome 7 and one region on chromosome 28). The according genomic regions were 24.8Mb and 7.4Mb long on chromosome 7 (location 9.04-33.8 Mb and 76.5-83.9 Mb), and 23.6 Mb long on chromosome 28 (location 0-23.6 Mb). In addition the analysis of 2 further regions approached significance. The calculated NPL scores in these additional two regions were 2.97 on chromosome 3 ($p = 0.0006$), and 2.595 on chromosome 29 ($p = 0.0014$).
For fine mapping 315 SNPs were genotyped on DNA of 12 dogs from the investigated family. Results of 64 SNPs were not polymorphic and therefore not informative in the investigated family. Of the remaining informative 251 SNPs 36 SNPs were polymorphic on chromosome 29, 34 SNPs were informative for chromosome 28, 92 SNPs were used for the analysis of chromosome 7, and 89 SNPs were useful to investigate chromosome 3. Calculation of NPL scores of all informative markers for each chromosome revealed NPL scores of 4 on chromosome 28 ($p<0.0001$; 3.4-5.4 Mb), 4 and 3.4 on chromosome 7 ($p<0.0001$ at 17.8-19.3 Mb, and $p=0.0004$ at 80.3-82.2 Mb), 2.29 for chromosome 29 ($p=0.005$ at 5.8-6.3 Mb), and 2.5 on chromosome 3 ($p=0.0032$ at 56.9-58.2 Mb).

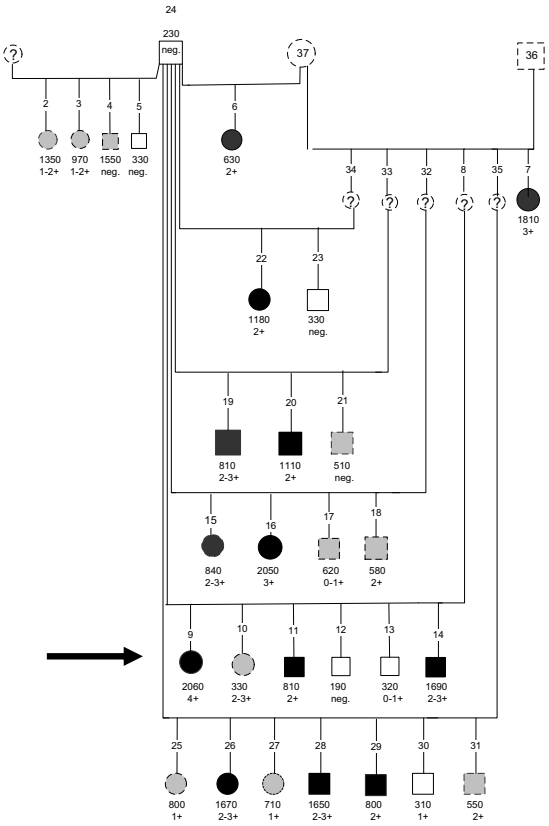

Figure 1: Pedigree of 31 dogs participating in a genome wide genotyping approach to investigate for linkage between hepatic copper accumulation and a microsatellite marker. Square: male dog, circle: female dog, black: affected, white: normal, grey: excluded from genotyping because of unknown phenotype, dotted line: dog did not participate in the study. Numbers underneath the symbols describe hepatic copper concentrations: above = quantitative copper concentration in mg/kg dw, below: semi-quantitative copper concentration from histologic staining with rubeanic acid, arrow: index patient (no.9)

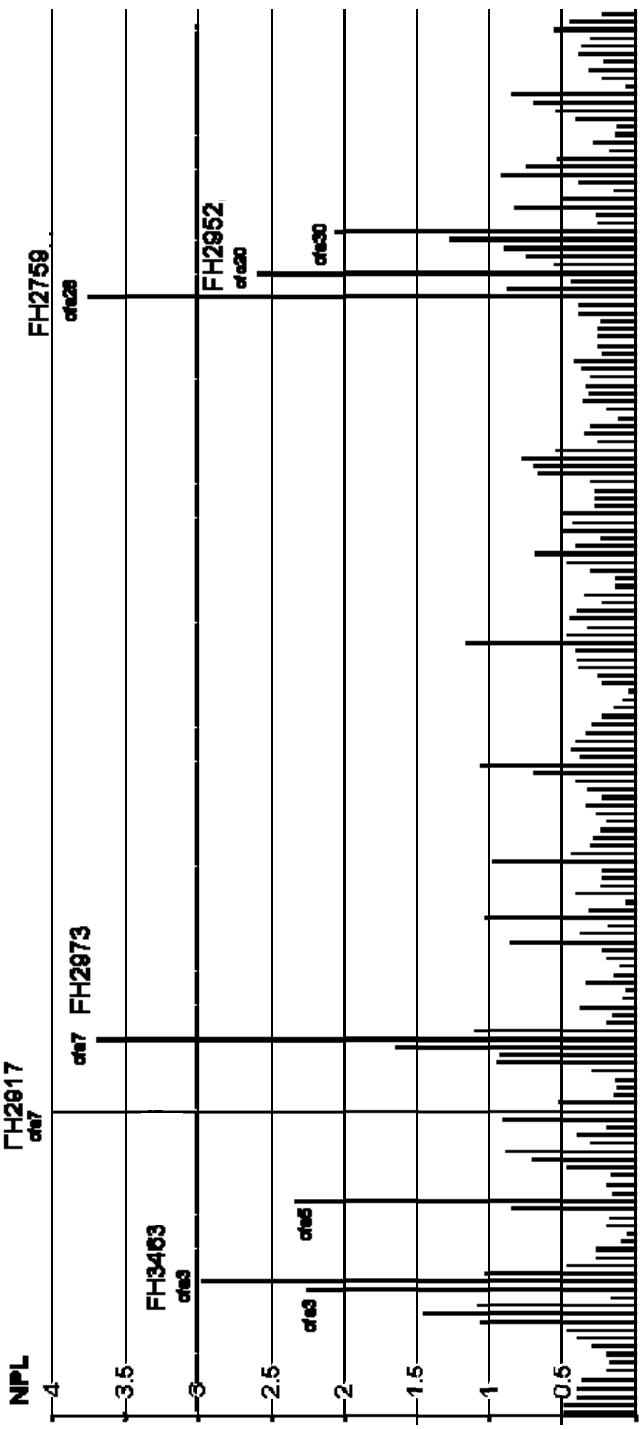

Figure 2: Genome wide genotyping results of 174 microsatellite markers. Calculation of non-parametric linkage revealed 3 regions of significant linkage with hepatic copper accumulation (NPL score above 3: 2 x chromosome 7: FH2917 & FH2973, 1 x chromosome 28: FH2759). In addition two more zones approached significance (FH3463 on chromosome 3 and FH2952 on chromosome 29). All of these intervals were further investigated by genotyping of SNPs.

Table 1: microsatellite markers used in this study ¼

Number	MarkerName	Cfa	Position(Mb)	Forward Sequence	Reverse Sequence	Product	Label	Temp
1	FH2016	1	11	CATTTTTAAGGATGGAGACAGC	AACAGTGTCCCATGGCCTAC	282-322	FAM	55
2	FH2313	1	20	AGTAGAAGAGGCCACGCAAA	CACGAAGAAAGCCATGGTTT	267	VIC	55
3	FH3413	1	25	AGAGTTGAAAGGTTGAAAATGG	TGTGGTCACAAGACTTTAGCC	374	NED	60
4	REN162B09	1	37	CAAACTTGACAGTCTTTTCAGGA	GCATTCAAGATGCACCAATG	180	NED	60
5	REN112I02	1	48	ATAGCCCATGAAATCCA	CCCCAAATACATCCCTACAT	250	VIC	55
6	C01.424	1	59	AGCTTAGCTTACTGCCCTGG	TCCTTTGGTTTTTAGCAGGG	176-195	NED	55
7	C01.251	1	64	TACCACTGTCATTTTTCCATGC	AAGAGGATACCGGTGGCAG	146	FAM	55
8	FH2309	1	85	GACTGAGTTCTTTCAGCACAGTG	GGCAGCCTTATTATTCATGGA	330-430	FAM	50
9	AHT138	1	97	ACTTGGACCAGACAAGGGTG	GGATTTGCCTATGCACTACACA	101-115	FAM	55
10	FH2326	1	108	GAATCCCCAATGTACATGGC	CAGCCATCCAGGAAATCG	251	FAM	55
11	FH2294	1	115	GGGTCCTGGGATCAAGCC	TTAGTAAAGAGACAGCTGATTGCC	222-235	VIC	55
12	UU360Cfa1_124	1	124	GGCTCTGTTCGGTCCTCCTT	GTGTCTTCGTCATCCTTGCACCGACAT	203	VIC	55
13	FH2087U/CPH7	2	11	ACACAACTTTCCATAATACTTCCCA	ATCAATGCTCTCCTCCCCAG	346-355	FAM	55
14	REN280B15	2	25	TTGTTCCCAGACCAACTTCC	AGCATGAAAGGAGGAAAGCA	234	NED	55
15	REN150M24	2	36	CACCAGAATCATTTCCACGA	CTGTGGAGAGCCTGATGTGA	347	NED	55
16	FH2613	2	42	AACAATGAAAAGGAATGCCA	TAATAGCTGCTTTGAAGCCTTC	189	VIC	55
17	FH2237	2	50	AGGCTGACCTTATCTTTCAACTG	CAACACAGATGAAACTGGAAGG	590	NED	50
18	FH2608	2	63	CTACACCCTGCTTGTGCTCT	TCCTCATATAATGTGCTGACCA	246	NED	55
19	C02.894	2	70	TCAGCATCTAGAAAATTAGGT	ACTCATTTTCTCTTATTCTGCAG	141-165	FAM	55
20	AHT111	2	76	CCATACCCAGGATAGTTGAT	CCATCCTGAGGCTAGCTGTG	074-093	FAM	55
21	C02.342	2	85	TCTTTGAAATGAAATGGGCC	GCTCTCATCCCTGTGAAAGC	184-201	VIC	55
22	FH3396	3	4	GGGTGTGGAGCCTGTTTAAG	AGTCCTATGCACTGAGTTCC	435	VIC	55
23	REN161A12	3	13	GCCAAATGTCTCAGATGGGT	TGTCCACAGCTCATGAAAGG	288	NED	55
24	FH2131	3	30	ATGAAGCCTCACGCCAAG	TGATCACACTCATCTCCCCA	361-401	FAM	55
25	FH2320	3	45	AGTATTTTCACAATTGCTCAGTGC	TTTTGGCCTATCTTAAGAGGTCC	237	NED	55
26	REN92B17	3	50	CCATGGCGCACCTACTAT	CAGCCTGCCAAACCACT	211	FAM	55
27	FH3464	3	57	AGCAAAGAATCCAGTCAAGC	AGCTCACAGTCCATTTTATGC	247	FAM	55
28	REN260I04	3	68	CTGTCAGATGCTCTGTCCCA	CCACACAAAGACACACACCC	185	VIC	55
29	FH2316	3	70	AAATGGCCTGACGAATATGC	GTGCCATGGCATATGGTAAA	420	NED	55
30	REN216N05	3	77	GCCACACACATACACCCAGA	CTGCCTCTCTTTCTGTCTCTCA	269	PET	60
31	FH2107	3	84	CATCATTATTGGGCTCCATG	CTGTGTCATCATTAAGCTAAGTCCT	366-389	VIC	55
32	FH4076	3	84	TAAGTTCAAGGCCTGATCCC	ATATCACTATGGGTCAACCTGC	495	FAM	55
33	FH2302	3	92	AGTATTTTCACAATTGCTCAGTGC	TTTTGGCCTATCTTAAGAGGTCC	300-341	FAM	55
34	REN298N18	4	5	AAAACATTTCCCTCCCATCC	ACACCCAAAGCTGCAGAGAT	190	PET	55
35	FH2773	4	9	AAGAGCATCCAGAAATGCAC	ATTCGACCAGTCAGAACCTG	206	FAM	55
36	REN171H02	4	15	TGTCCTCATATGAACGCAGG	GGTCAACACATTGGAGAGGTG	281	PET	55
37	FH3310	4	20	ACTTCTGTGTTTAAATCAGGGC	AGAAAGCACTATTGGGACTGTAG	314	VIC	55
38	FH2412	4	40	GCTCACATGTGTGCATGC	GCACTCCATGAGGCAGGT	159 -191	PET	50
39	REN160J02	4	70	ACCGTGCACAAGTCAGTCAG	AAGCTGAGCCATTCTTTTCC	248	PET	50
40	AHT103	4	75	GGAAACTCAGTTATTTTACA	GCCCTAGAACTTCACACACA	076 -084	PET	50
41	FH2457	4	88	AACCTGGTACTTTGAATTTGCA	TTTGGTCAACATACCTCTCTGG	360	PET	50
42	FH3928	5	9	CATGCTAATCTCTGTGTCATTCC	ATTCTCTTTTTCTGCTCCTTCC	241	NED	55
43	FH2140	5	13	GGGGAAGCCATTTTTAAAGC	TGACCCTCTGGCATCTAGGA	146-171	VIC	55
44	REN42N13	5	16	CACCTGCTTTTCCTCTTTGTA	GCCTTTTAACAGACGGACACA	170	FAM	55
45	ZUBECA6	5	22	GCCATAAGCCCCAAGCCAGCAG	TGCCTCGTCAGCCCCTCTTTCC	302	PET	60
46	FH3702	5	32	AAAAATACCACAGCAGAGAGG	TTCCTTTGGTGATAAAGATTGG	200	VIC	55
47	DTR05.8	5	39	AGTCATTTGGCCTTACTTCA	CAGTGATGTCATGCTGGTAG	398	NED	55
48	REN192M20	5	51	ATTGCAAAGCCCCAGAATGT	TTTTAAGGAGCTTCCCCCAT	185	FAM	55
49	C05.771	5	62	GAGGAAGCCTATGGTAGCCA	CAAGACCTGAATTCCTTGTTCC	500	NED	60
50	FH3450	5	76	ATGAGAAGTGTTCGGCTAGG	AGCGGGTAATCTGCTTGAG	271	PET	55
51	REN122J03	5	82	GTGCGAGTCATCAACAAAT	ACTAAAGCCCATAAATCGTG	197	NED	55
52	CPH14	5	90	GAAAGACAATCCCTGAAATGC	ACCCCATTTATGAGAATCATGT	193	NED	55
53	FH2576	6	10	CCCCAAAATCTTATCATTTGC	CCTGCACTGGGAAAATTAGC	312	FAM	55
54	REN149M14	6	30	AGCATTTTGAATTGGGCACT	CTCCACAAGAGCTGATGGGT	360	FAM	55
55	FH2119	6	47	AGTTGTCAGCATTGTTTTCTCA	TTCAGTCTGGTATGCAATGTCC	175-187	VIC	55
56	C06.636	6	60	GGACCTCATGTCCTACTGTGAG	ACTGTTCCTCTATAGGATGATGCAA	132-171	NED	55
57	FH3303	6	69	TTGTGGTCCATTTTACATTAGG	GCTTACACCACTTGATCATCC	300	FAM	55
58	UU358Cfa6_80	6	80	GAGTGTGCAAGTTCCATCTC	GTGTCTTTCACTGGCAGACACTCTCTA	415	FAM	55
59	AHT109	6	?	ACTTTCATATTACTCTACTG	AACACGTCACTTGCTGTCCA	105	VIC	50
60	FH2226	7	8	GGACTACCCCATTGCATTTG	GAATCGAGTCCCATATCGGG	205	VIC	55
61	FH3972	7	12	ATAAAGCTGGATACAGTTTGGC	AGGCTTTTCTAATGAAAGGGAC	313	FAM	55
62	FH2917	7	18	GTTAAGCATCTGCCTTCAGC	CCAAAAGGAAATTCTTGTCC	490	FAM	55
63	REN162C04	7	29	TTCCCTTTGCTTTAGTAGGTTTTG	TGGCTGTATTCTTTGGCACA	203	FAM	55
64	REN286O18	7	33	CTGGATGTCAATGCAGGTGT	CTGTTGTCACAGGAGGCAGA	306	VIC	55
65	REN200G14	7	50	AGCTTTCCAACCCATTCCTT	TTCTGAAATTCAAGCCAGCA	352	NED	55
66	FH2201	7	62	ATCAACAATGCATGCCACAT	GAGAACAAATAAATGCAAGCCC	154	NED	60
67	FH2581	7	70	ACACTGGGTTGAGTGGTGG	CCAGGGGCTAATATCCCTCT	358	PET	60
68	FH2973	7	81	TCTGGCCCATTTTCTAATTG	CCAAAAACCACTGAAATGTG	244	NED	55
69	FH3241	8	4	AGTTTTAGCCGATCTATTTGG	TCAAGATCCTTGTTTGGTAGG	311	FAM	55
70	FH3425	8	14	TCACTGAATCTTTGATCTCATGG	TGTGACTCATACTGACCATAGG	380	FAM	60
71	C08.410	8	33	GAGGAAAACCAAGTGATTTTGG	ACCTGCAAGTGACCCTCTCT	096-125	FAM	55
72	C08.373	8	45	TATTTAAAAAATCCCAGGCACA	AGCATCAATTAGATGTCAGCG	200	VIC	50
73	C08.618	8	68	CAACCCAGGGTGGAAGC	TAGCAAGAAAATGTGCCCA	188-206	PET	55

Table 1: microsatellite markers used in this study 2/4

Number	MarkerName	Cfa	Position(Mb)	Forward Sequence	Reverse Sequence	Product	Label	Temp
74	C09.173	9	3	ATCCAGGTCTGGAATACCCC	TCCTTTGAATTAGCACTTGGC	110	FAM	55
75	FH2263	9	6	CATGTAGAGTGATTAGTTGGTCTTT	CTGAATATCCTCTGCCCTTC	217	NED	55
76	Pec-1-FAM	9	7.4	GAGGTCAGATGTCAGGAGTA	CTCCTCTCCTTAGACCAGAA	379	FAM	
77	FH2186	9	24	AGAGGGTCTACATGGATTCTGG	TAGAGTCTCAAACAATCCTGTGG	360-500	VIC	60
78	REN278L10	9	37	ACTGTGGGGAGAGGACACAC	CCTCTAGACAAGAGCCACGG	235	FAM	55
79	REN177B24	9	43	GAGGAGCAGGTGAGGCATAG	GCTGCTCTCGATCAACACAC	366	NED	55
80	FH2885	9	50	CTTTTAGGGTGCCTTCAACC	TGGATTATTAAGGGGAATTTAGC	209	FAM	55
81	REN287G01	9	52	CCCATAAGGACCACAGGTTTT	TTCCTAAGGCCCTCCTGATT	187	FAM	55
82	BAC17A4CA-TET	9		GTATTCCTTTTGGTCCTGAGC	GGCAAATACCATGAAGTCAGC		TET	
83	C10.404/FH2537	10	1	AAAAAGTGTAGAGCTTTCTTCAAA	ATTGAGACCCAAGACTGTTAGTG	152-168	FAM	55
84	FH4081	10	12	TAAATTCAATAAGCCATGGAGG	GGTAACAACCAGAGTGTGTGTG	494	FAM	50
85	C10.781	10	16	ACCTCCAAGATGGCTCTTGA	ACGTCGAGCTCCTGGCAT	190	VIC	60
86	RVC8	10	23	GCAAGCCAGCGAGGCTCTTG	CCTGGGTATTCCGTGCCAGG	110	VIC	55
87	FH2293	10	32	GAATGCCCTTCACCTTGAAA	AGGAAAAGGAGAGATGATGCC	222-284	VIC	55
88	REN181G20	10	40	CTTATGGCATTTGGCCTGTC	GGAGTTGATAGTAAATCTTTGCGG	356	FAM	55
89	ZUBECA1	10	49	CTTGAGTGGCTTGAGTGGCTAC	GGGTTTTGGCTGTGAGGATA	284	VIC	55
90	FH2422	10	56	TTGCCCGTCCTATACTCCTG	CCACATGATTTCACTTGTATATGG	238	NED	55
91	FH3381	10	79	CCCAGAAACTCAACTGATGC	AGCTCTTACACGCATTGAGG	283	PET	55
92	FH3203	11	6	GCCCAGAATTAGTAGAAAGAAGG	CTGGCATGTAATTGTTCATAGC	488	VIC	60
93	AHT137	11	10	TACAGAGCTCTTAACTGGGTCC	CCTTGCAAAGTGTCATTGCT	143	VIC	55
94	FH4031	11	12	CTAGCTTATCCTTGGCCTCTC	CCAAGAAATAATGACAACAAGG	321	PET	60
95	REN242K04	11	17	CAAGGGAAGGTCTGATTCCA	CCCTGGGGAATAGGTAGGAA	322	NED	55
96	REN245N06	11	37	TCCTCCCTGGAAAGGAAGAT	GGCAGGAAGGCAGACTGTAG	175	FAM	55
97	FH3393	11	45	GCATTCTCACCACCATGC	CCCACACCTGTCAGAATAGC	318	NED	55
98	REN194N17	11	55	GGTGGAGAAACTTGATGGGA	CCCCATGGAGACCATTCTTA	271	VIC	55
99	C11.873	11	66	CTGGCAGATTACAGGTAGC	GTTCTCCAAAGCACTCAT	133	FAM	55
100	DGN13	11	70	GGAACCCTCAGCTGTTATATGC	GATCTAAGGGCTCCAGAACACC	320	FAM	55
101	FH2200	12	4	CATGATCCTGGAGTCCCG	GAAAGCTGCTTCAGTGGACC	462	FAM	60
102	REN258L11	12	10	TTCTCTGTGTATGTGTACGCTTG	ACACCAAATGTGTGAAGGCA	280	VIC	55
103	FH2152	12	14	ACCCCTCTTCATGCTCTCTG	AGCATATCAGTAGGGAAGGGAG	433	FAM	55
104	REN213F01	12	17	CATTCTGGGCAGGTTTCATT	GGGGACAAATTTCCACTCCT	265	VIC	55
105	FH3711	12	23	CTTGAACCTGTTATCTGATTTCC	GGAGGAGTTAAATCTCAAAACC	238	NED	55
106	REN208M20	12	43	TGCATCATTTGTGGGTGTTT	ATGGAGCATGGGTGAGAAAT	318	VIC	55
107	FH2707	12	50	CACGTGGGCTACCCTAAAG	CAGAAGCAACCAGGTACTGC	208	FAM	55
108	FH2347	12	62	TTAGCTCTTCTGATCTGTCCCC	TCTCTTTCCCACCCCTCC	405	FAM	60
109	C12.852/TBP	12	75	CCTTTCTCTTCTGGAGGAAC	CTGCTGGGATGTCGACTG	98-106	VIC	55
110	UU365Cfa12_75	12	75	TTCCTTCCAGCTCTCCTCTT	GTGTCTTTTCCATGTTGCTGCAGAGTC	347	FAM	55
111	C13.391	13	4	ATTTGAAGTCCGTGGCTCC	GAAACAAAGAGGCAGCGATC	148	PET	60
112	FH3494	13	6	AAGCCTATTTCTCCCTCTGC	CATTCTATTTGCTCCTGTGG	226	NED	55
113	FH3619	13	22	CTGGCAATCACCATTCTACC	GGAAAATCTATTTGTAACGATGG	263	PET	50
114	REN286P03	13	32	GCACATTCACAAAGTGGTGC	GCAATGGAAAGAGGATGGAA	331	PET	55
115	FH2348	13	37	GCATGCAAAGGTGTTAATTGG	ACACAAGGAAGCTTTGGGG	430	VIC	55
116	AHT121	13	52	TATTGCGAATGTCACTGCTT	ATAGATACACTCTCTCTCCG	101-129	PET	55
117	C13.758	13	55	AAGCATCCAGAATCCCTGG	GTTGATTGGGAGATAATCCACA	476	PET	55
118	FH3951	14	9	TTTAAATCAGAAGTGTTCCGAAG	GAGCTTCCTGTGAGGAGACTG	490	FAM	55
119	C14.866	14	15	TGTCATAATAGTTGGAATGAC	TTAGAGCTTACTCATGATATCTG	242	HEX	50
120	FH3725	14	24	GAAAGAACTCACTCAAAACTTCC	AAATGTTACTTCAGAAAAGCTGG	175	VIC	55
121	FH2658	14	36	TCTTAGAAATTGCTGGTGGG	TAAGAAACTGCCAGTCTGTGG	270	FAM	55
122	REN289L09	14	42	GAACCCTGCCAAGGGATAAT	GCTATGGCCCTTTCACAAGT	190	FAM	55
123	FH2763	14	47	CAGGATCATCTCTCCCACTG	CAGAGTCTCCTCTGCCACAC	194	PET	55
124	FH2189	14	49	CCATAAGATACTCAGAAACATGCA	GTGTCTTCACAGTGAGGAGGGGTGG	263	VIC	55
125	PEZ10	14	57	CTTCATTGAAGTATCTATCC	CCTGCCTTTGTAAATGTAAG	282-302	PET	50
126	FH3802	15	4	TTTTCACCAGCTCTGAGATAGC	TTTCTGAGCCACTTTTCCATAG	265	NED	55
127	FH4012	15	14	TCCCTATCATCAAGGAGTTCAC	CATTCCTTCATTTTCACCTCTC	445	NED	
128	REN06C11	15	21	TGCAGGGCAGAGGCTGGAGG	GGGGGTGTCGGTGGAGTTCT	89	FAM	60
129	FH2535	15	31	GTCATTGACAGACTACAAATCTCC	ACAGACTTGCAGTATTTTGTCTG	145	TET	55
130	FH2295	15	43	TCTCGGGGATAGTGTTATAACTCC	GTCAGGAAAAGGACATTTGACC	390	FAM	55
131	REN193M22	15	46	CCATTGGCCATGTATCCTTC	GAGGATGTCATCACTGGCTCT	205	NED	55
132	FH2278	15	58	TCCACTCTGAGCATGGAGC	GCCCAGTTTTCCCAGTAACA	310-330	FAM	55
133	FH3939	15	66	CTTTGTCACGTTTGTCTTGG	CCTTCTCCTCCTATCTCTCAGG	263	NED	55
134	REN214L11	16	9	AAATTGCAAATCTTGGCCC	TCCATGCTAAGACCCCCATA	226	FAM	55
135	FH2670	16	10	GTGGAGTCCTGTGTCTTGATG	CTTCTGTTGAAAAATGCAGTCA	191	NED	55
136	REN85N14	16	21	AAGGCAGGAGGAGGAGCAC	TATGGAGATGGAGGGCACAC	239	VIC	55
137	FH2175	16	34	TTCATTGATTTCTCCATTGGC	AGGACTCTAAAAACTTGCCTCC	251	PET	55
138	REN275L19	16	41	CCTGCATAATCCTGCAATGA	CTATCCTGCTGCACCTAGCC	230	FAM	55
139	FH2155	16	53	TGTAGATGATGGAGACATTGGG	AGGCAAATATGCCAAGGATG	457	HEX	55
140	FH3592	16	57	AGGTGCTGAGCATGTTATCC	GGGTCAAAGTGTTGACATGG	323	NED	55
141	DTRCN1	17	6	AATGCTGACACCAGTAGCTT	TTCTGCCTGTTTATCTGTCA	142	FAM	55
142	FH2321	17	12	CCACTCTGAGTACAGTTGAATTGG	ACAGTTTTAGTTTTGGAGACAGGG	315	FAM	55
143	REN294E18	17	26	CACTGCCATTGCTGTCACTT	CCACCATAATGTCACCCACA	300	FAM	55
144	FH4023	17	40	CAATGGAAAGTAGGCAAATAGC	CTCCACTTCTGATGTCTCCTTC	467	PET	55
145	FH3995	17	45	AAGGAAATGCTTTCTCAAAGAG	GGTCAATATAGTCCAGTGGCAG	436	VIC	55
146	PEZ8	17	63	TATCGACTTTATCACTGTGG	ATGGAGCCTCATGTCTCATC	213-260	FAM	55
147	FH2869	17	67	CATTAACAAAATTGCTTCAGTCC	CACACCAATAACCCAGAACG	204	VIC	55
148	FH4060	18	4	CTAATTTTGGTGGGCTATCAAG	TTGCTTTTGTTGTTTTGTTCAG	428	VIC	55
149	FH3944	18	8	CTGTTGAGGAACAGAGAGAAGC	TGTGTTAGAATGTTGCTTCGAC	462	NED	55
150	REN186N13	18	11	AGGCTCTCCGAGGGTAAGAC	ATTGCTTGGATAAGAGGGGG	369	FAM	55
151	REN54P11	18	21	GGGGGAATTAACAAAGCCTGAG	TGCAAATTCTGAGCCCCACTG	231	PET	
152	GALK	18	26	AATTGAGTTTTTGGGGTGCCTGAG	CAATGAGCTGAGATGGTGTAGTAG	186	FAM	55
153	FH3815	18	41	AGTCGATAACGCTGAGAACC	AGAGCTCAGCAAAAGTCTATGG	338	PET	60
154	FH3824	18	47	AGGAAAAATACCAAACCAGAAA	TTTATCTCTGATTACCTCCTGCC	298	VIC	55
155	REN47J11	18	52	TCTCCTCGCGTGTTTCTG	GGGGACACTCAGAAGGACG	163	FAM	55
156	FH2429	18	61	GATCCACTTTGAATTGATTTTTG	TTCAGCAAATGGTTCTGGAA	173	FAM	55
157	AHT130	18	65	CCTCTCCTGGTAAGTGCTGC	TGGAACACTGGTCCCCAG	108-120	TET	55

Table 1: microsatellite markers used in this study 3/4

Number	MarkerName	Cfa	Position(Mb)	Forward Sequence	Reverse Sequence	Product	Label	Temp
158	FH3969	19	4	TCAGGAATAGACTCAAAATGGG	CACTATCTCTTTCTGGGGACAG	465	PET	50
159	FH2206	19	18	CCTATCTAATTTATTGTTCAGCAAG	TACTGGAAGTAATTCTCCTCTCTCA	540	TET	55
160	FH3313	19	25	TGCACACCCAAAAAGTAAGC	CAATCTGAAGCCAATCTCATC	418	FAM	55
161	FH3491	19	36	CAGGTTCCTTGCTCAGTGG	ACCCAAAGTTGGCACAAGG	312	VIC	55
162	REN213G21	19	46	AAGAAAATCACCAGCCCAAA	CTCCCTTCATCCTTCACCAA	263	VIC	55
163	FH3299	19	54	CCTGGCAGTAGACCAATACC	TCTCCCATGTGACAAGTGC	474	FAM	55
164	PEZ19	20	5	GACTCATGATGTTGTGTATC	TTTGCTCAGTGCTAAGTCTC	186-208	HEX	55
165	REN55P21	20	9	TGGACTCATACAGAACACAG	GTGTCTTCCATACAACAGAATACTACT	258	FAM	60
166	FH2951	20	17	TTACCTCGCAATCCAGTACG	GTGTCTTGGCAGAAATAATCATGTAGGG	415	VIC	50
167	REN100J13	20	25	TGATTGACTCTACTTTACACA	GTGTCTTTATATTAGGCGGTTTTCTTCT	164	FAM	60
168	REN93E07	20	37	TGAGGGCTGCCACTGTAAATA	GTGTCTTGGCCCCCTCACCACTCC	169	VIC	60
169	FH3771	20	47	GGGAAGAATACTGATAAACTGGA	GTGTCTTTCTTTGGTAAAGTGAAAGATTCG	308	VIC	60
170	REN114M19	20	56	AGACCTGTGGTCAGGGAGC	GTGTCTTCCATACAGCCACACCAAGTG	193	FAM	60
171	FH2312	21	3	AAAATAATACTCATCTATATGCTGCC	ACAACATAAGAATGTGTGTCATCA	318	HEX	55
172	FH3624	21	10	GATCAGGATGGAATTTGTGG	GTGTCTTGGATTAAAGTTCTGCTGTCACC	359	FAM	60
173	FH3823	21	20	GATTGAGCCTAGCATCAGACTC	GTGTCTTGCTCACAGCTATTGTCTTTTTG	372	NED	60
174	FH2441	21	33	TAGTTGTGTGCATGATCTCG	GTGTCTTTGGAGAAAGTTCCATGTGCA	129	NED	50
175	FH3803	21	38	TTATATTAGGAAGGAAAGGAGGG	GTGTCTTATGACACATAGGGGAAATTGAG	488	FAM	55
176	FH3880	21	43	GCAGCCTTAGAAGATAAGAAAAGG	GTGTCTTCTCTCACTCTGCGTTCTGC	352	FAM	60
177	REN118B15	21	49	TAAACTGGCGTAACCTCTC	GTGTCTTTCCCAACAGCCAGTCCGTATC	218	VIC	50
178	REN49F22	22	4	GGGGCTCTGTTATTAGGTG	GTGTCTTTCATAAGGCAAAGAAAACC	157	FAM	60
179	FH3355	22	13	TAGAAACAGATTGAGAGGGCAG	GTGTCTTATCTTGAACATCTCTCCTTTGC	479	NED	60
180	REN158P08	22	22	TGGGTGGATAAAGGTGGTGT	GTGTCTTTCAACCTCACTTACTGCCCC	282	VIC	60
181	FH3411	22	37	CAAATGGAAGTGAAAAGAAAGC	GTGTCTTATATGTTCTGGCTGGATCATTC	267	FAM	60
182	REN245C13	22	53	CTGTTCATTGCCCATCCTTT	GTGTCTTGATGTTGGCAGCTTTGTCCT	274	PET	60
183	FH3274	22	55	CCGTAAACACCTATGCAATG	GTGTCTTTCTGAAACAGGACCCATCTG	154	NED	60
184	FH3853	22	64	ATAGCCAAAAGGTAGAAATAATCC	GTGTCTTGTAAGAGGGAGCACAAGTGG	349	NED	60
185	FH2508	23	6	GAACAACTGAGTGTCCACATG	GTGTCTTTTCTTCCATGTTATCTTCCAGG	189	PET	60
186	FH4033	23	11	CCATCAAATAATTGAGACCCTC	GTGTCTTATCACGGACTGAAGACACTTTG	431	FAM	60
187	FH3078	23	12	GCCTTCTTTGGAAAAACACC	GTGTCTTTTCAGGGAATTCTTTCTTGG	199	PET	60
188	FH2626	23	19	TTCTGCCATTCTGGAAAACA	GTGTCTTTCCATGGATCAGCAGCAG	246	NED	50
189	REN113M13	23	31	ATCCCTCAGCACCCACAC	GTGTCTTGTCAAGAGCCAGGAAGGATT	196	VIC	60
190	FH2227	23	38	ATCCCAGAACAGAAGAGAGTCC	GTGTCTTCCTTTGCCTTTGGAATCAAA	565	FAM	60
191	FH2001	23	50	TCCTCCTCTTCTTTCCATTGG	GTGTCTTTGAACAGAGTTAAGGATAGACACG	131	FAM	60
192	REN181K04	23	55	ACAAGCCGACTCTAGCGAAA	GTGTCTTAGATGGGGCCTAACCAAAGT	216	FAM	60
193	FH3750	24	4	GAGCTACCAAGGAATCAAAAGG	GTGTCTTCTCCTCACTTTGACCTACTTCC	193	VIC	60
194	FH3023	24	7	AAGCTCATGGAGTTTCTCACTC	GTGTCTTAGGGAGCAGCTTTGAGTTTC	374	VIC	60
195	FH2159	24	12	GAATCCCACATCGGGCTC	GTGTCTTATTAAGTTTTGAAAGCCAGGTAAG	182	FAM	60
196	REN209L11	24	15	TGATGAAGTCCGATTTATCTCTCTC	GTGTCTTCAAATGAACTTGGGTTTGGG	102	VIC	60
197	FH2495	24	23	ATTTCATATGTGAGGCTGAGATTG	GTGTCTTCAGTGGGAGAAAGATGCCAT	134	PET	60
198	FH3083	24	32	ATTTGCCAGGTACCATTCC	GTGTCTTCAGGTTATTCTGGGCTATGG	216	NED	60
199	FH3287	24	44	AGGAATGCAGCAAAGTGG	GTGTCTTAGTCCATGCACACAGAAGG	482	VIC	60
200	UU361Cfa24_50	24	50	TGTGCGTGTGCGTGTGTCTG	GTGTCTTGTTGTGTGCGGAGCCGAGAT	382	FAM	55
201	FH3977	25	6	TGGGGAGCATACCATTATTTAC	GTGTCTTAACATTGTTGCCAACTGTACTC	472	NED	60
202	FH2318	25	14	CAAGTCTGAGATGAGGCTTGG	TGAGTTCAAATGCCAGCAATC	300	TET	55
203	FH2324	25	17	AGCTCTATGAAAGGTGATTGCC	GTGTCTTAGACAGCCATACAAATGAGAATTG	252	FAM	60
204	FH3979	25	28	TTCCCCTAATGAATAACAATGC	GTGTCTTAGTTCAGTCAGTTAAGCTTCGG	430	VIC	60
205	FH2141	25	35	CAATGCTCCTTAGATTGCAGG	GTGTCTTACCCAAGGAAACTAGAAGGAAG	540	FAM	60
206	FH3627	25	42	CCCACTTAAGCAACACAGC	GTGTCTTGAACCAAGCACCATAGAACC	410	PET	60
207	FH4027	25	49	CGTTCTTTCTTCAAAAGAGTGC	GTGTCTTGCTTGGTCAGTAGAGCATGAG	421	PET	60
208	UU366Cfa25_54	25	54	ACTCCTGCCACCAAGTCTGC	GTGTCTTCGCCTCAGCTTACGGATACC	428	FAM	55
209	UU364Cfa26_4	26	4	ACACTTGTGAGGAGGAGCTA	GTGTCTTCACCTCACCTACACCATGAA	240	FAM	55
210	AHTK211	26	14	TTAGCAGCCGAGAAATACGC	GTGTCTTATTCGCCCGACTTTGGCA	92	FAM	60
211	UU362Cfa26_14	26	14	GATGTTAAGGCCTGAGTAGC	GTGTCTTTAGATAACAGCAGCCTCGAC	235	FAM	55
212	REN299M21	26	20	AAAGTTGCCCACCTGTTGAC	GTGTCTTGACTTGGAGAGGACTGTGCC	242	PET	60
213	DGN10	26	26	TCTGCCTATGTCTCTGCCTCTC	GTGTCTTGGCACCTATTCTGGGAAACTTT	250	VIC	60
214	FH2130	26	35	GCTGTCCTGCACTTTTCCTC	GTTAAGGAATAGTTGGGGGTCC	300	TET	60
215	UU359Cfa27_4	27	4	AAGACTCCTACACACGCATC	GTGTCTTCTGTCCTCATTCAACTGCTG	302	VIC	55
216	FH2289	27	5	CATGGTCTCAGGATCCTAGGA	GTGTCTTCTAAGCATTCTCTCTGATGGTCTT	280	FAM	60
217	UU352Cfa27_9	27	9	AGAACTCTGGCTCCTCTTCC	GTGTCTTCCTTCCTACATCAGCCAACT	421	FAM	55
218	FH4001	27	10	CTATGCAGGATAATACCTTGGC	GTGTCTTTTAAATGTATCACCAAGCTGGC	270	FAM	60
219	REN277O05	27	18	CCTCCTCTCACTTGTCCTGC	GTGTCTTAAATGGTGTCTTCAGCTCCG	334	VIC	60
220	FH2925	27	27	GGTCTATCACCTTGGTTTGG	GTGTCTTTTGGAGCTTTACCTTTCTGC	355	FAM	60
221	REN56C20	27	39	TAAAGTGGGCCTCCTGATA	GTGTCTTGAGTACTCCCTTCCCAACC	249	FAM	60
222	FH4019	27	42	GACCTCCTGTATTAGTAGGGCAC	GTGTCTTCTTTACCTCTGTGATCGTCCTC	458	FAM	60
223	REN181L14	27	46	CGCCAGCATGATAAACAATG	GTGTCTTAGATAACAAGGCTGGGAGCA	177	FAM	60
224	REN72K15	27	48	CCGATGCCTGTCCTTTGA	GTGTCTTACCTGGCCTCTTCTGTTGTCT	250	VIC	60

Table 1: microsatellite markers used in this study 4/4

Number	MarkerName	Cfa	Position(Mb)	Forward Sequence	Reverse Sequence	Product	Label	Temp
225	FH2759	28	4	AGTACTTGAGGCTTGGAGTCAG	GTGTCTTCAAGCTGAGAGCCATGTAGG	197	PET	60
226	FH2208	28	12	ATGTCTGGCTCTGCGCTC	AAACACTCCTGGGAGAGCAA	491	HEX	60
227	FH3033	28	23	CAAATCATGAACACACATAGCC	GTGTCTTGAAGACGTTCCAAATGTGTTG	219	PET	60
228	REN51I12	28	37	ACTTTCTTTGAGCGAGGTG	GTGTCTTCTGGCCTGTGCTGGGTGAG	220	NED	60
229	FH2952	29	6	AAAGGCATGATCCTCAGAGA	GTGTCTTCCAGCTCTTGGTTTCATCAT	371	FAM	60
230	FH3878	29	15	TACCTCAGACAAGCCGAAGG	GTGTCTTTGTCTTGACATTCTGCAAGC	367	NED	60
231	REN165M10	29	21	AACAGCCAAATCATGGAAGC	GTGTCTTAGCACCTCCATCCTTTCCTT	180	VIC	60
232	REN164F23	29	28	GGAGGTGAAAGTGCAATGCT	GTGTCTTGGGAACAGTGGTTACGAGGA	302	FAM	50
233	FH2177	29	40	CCTCAGAAAATGTATGCTGGG	TATAGGGCATCTGGGTGGC	470	TET	60
234	FH3489	30	6	GGCCACGAATGCTATTTACC	GTGTCTTTCCTCATATGTCCTCAACTGC	278	PET	50
235	REN51C16	30	12	CAGTTCATCCTTCCCCCTCTC	GTGTCTTGTGCTAGTCTGGCTGTGCTCA	258	NED	60
236	REN248F14	30	21	CACCTTCGTCTTGTGCAGTT	GTGTCTTACTAGGCAGGGTGAGCTGAA	212	FAM	55
237	FH3632	30	33	AGCTCCCCTACCATTCTCC	GTGTCTTGGCTTAAAATGACAAACATTAGC	238	NED	60
238	FH3053	30	37	GATTAAAAGGGCAAGCAACC	GTGTCTTTCATCTCCAGCTTTTCATGG	193	PET	60
239	LEI-1F11	30	41	TTTCAGAGTTCTGATGCTCC	GTGTCTTGTCAGTTCAATTAGCCAGAG	175	FAM	60
240	UU356Cfa31_4	31	4	ACATGTGCTGTGCTAAGGAG	GTGTCTTGTAGGTTGTCTTGGCTAGGA	178	FAM	55
241	REN43H24	31	10	CAGTGAGCAAAGCAAATGAA	GTGTCTTATGTGAACCCCGCCCAATA	181	PET	55
242	FH2582	31	14	TGGAGTGTGTTCCAAGGTCA	GTGTCTTGTTGTTCCCACAAAAGGCAG	285	FAM	55
243	FH2239	31	28	CCCATTAGCAAATGACTGGG	GAAGTGACTGAGTACCTGAAATCG	580	HEX	50
244	REN110K04	31	30	AAAGAATGGGAAAACTGATAA	GTGTCTTGCCTCTGCCTCTGCCTCTGT	223	FAM	60
245	FH2712	31	37	AAGGTAGTCCCACGATCCTC	GTGTCTTGAGCCCTGTTCTCAGGTTG	186	VIC	60
246	CPH2	32	9	TTCTGTTGTTATCGGCACCA	TTCTTGAGAACAGTGTCCTTCG	100	HEX	55
247	FH2875	32	15	TGATACCCATTAAGTCCATCC	GTGTCTTCAATACCCTGATACCAAAACC	209	VIC	50
248	AHT127	32	26	GGATCAAATCCCACGTCG	GTGTCTTCAAAATTCTACACTCTTCCCCG	171-183	FAM	60
249	FH3294	32	29	CAACATCTCCCTGTTTCTTCC	GTGTCTTTCTCACTTACATGTGGAATGG	358	VIC	60
250	UU357Cfa32_41	32	41	CACAATCTGTAGTGACCAGG	GTGTCTTGATATGGTAGGCTCTCAGTG	267	FAM	55
251	FH2965	33	9	TGACAATTGATATGGGCAGA	GTGTCTTTAGTTCATTAGCACAGTGTCCA	232	PET	60
252	FH3608	33	13	GACCACAAAGAAATCACTTGC	GTGTCTTGACTTGCGGGATGAAAGG	400	FAM	60
253	FH2361	33	19	GCTTGGAAGGTGAGACTGAATG	AGCACTTAGAATGTACCAGGCAC	370	HEX	55
254	REN291M20	33	27	CCTCTAGATCCATCCATATTGTCA	GTGTCTTTGTCCACCCACAGATGAATG	155	FAM	60
255	FH2165	33	32	GGATGGAGTCCCACATCG	GTGTCTTCCTCACCCCAAATTCTTTAGC	326	VIC	55
256	FH3721	34	4	GTTGGCAAATTGAACACCAA	GTGTCTTATTACTTTCAACAACCCACTCC	201	NED	60
257	REN125M11	34	11	TGCTACTAGGGTGGTGATA	GTGTCTTTGTTTGGCTGTAATAGTTT	257	NED	60
258	REN64E19	34	14	TGTATTTTAATGTGGCAGTTT	GTGTCTTGACAAGGACAGGCAATACAGT	146	NED	50
259	REN243O23	34	27	TCTGAGGAAAGCCAATGTGA	GTGTCTTTCATAATGATGCAGCTGGGA	264	VIC	60
260	FH3836	34	35	TAAAATAGGAGTGCTCAGGTGG	GTGTCTTTCCTCTGTCTTCATTTCATGC	457	FAM	55
261	FH4010	34	43	GGTACTCAGGAACTCTTTGTGC	GTGTCTTAACCTGGGCTTCGTAACTG	447	VIC	60
262	REN01G01	35	14	TACATCTCCACATCTACTGA	GTGTCTTGAGACTTAACACAGTATTTG	224	FAM	60
263	REN282I22	35	19	GCCCTGCTGACACTTTCATT	GTGTCTTTATTCTGGAGACTGGGCACC	158	NED	60
264	REN214H22	35	22	TGAAAAGAGCTACTGTTTCTATGC	GTGTCTTCACCTCCTCCATGGGTTTTA	325	FAM	60
265	FH3770	35	26	GGTTTCCTTAGCTGAAAGATGG	GTGTCTTCTCCCTGAAACATATTCAATGC	264	PET	60
266	REN179H15	36	7	AGCCATTTGCCAACCTACAC	GTGTCTTAACACACACCAGTTTTGCCA	314	NED	55
267	REN106I07	36	9	TTCCCCAAGCCACACC	GTGTCTTAACCACTATCCAACTTTAT	211	FAM	60
268	REN252E18	36	16	CAGCATTTCCTCACTTTCCC	GGGGAGATTGTGTATCGGAA	259	FAM	60
269	DTR36.3	36	25	TTCCTAACCAGCTACCAGAG	GTGTCTTTGTTGATATTTGATGTTGCC	211	PET	60
270	UU363Cfa36_33	36	33	CTGGTCTAGGAACAACACAG	GTGTCTTTCAGTGACTTCACCTACTCG	213	VIC	55
271	FH3272	37	5	GGATCCGGGATAGAGCAG	GTGTCTTCTTCCCCAATCTTCCTTGTC	318	VIC	60
272	H10101	37	13	TCAGGCTCATGGGATTGAGACTTC	TGCCATTGCACAGGATATAGGTCCA	305	FAM	50
273	REN67C18	37	22	TCTGTGCGTTTCCGTTTATG	GTGTCTTTTAGTACCTGTTTGTTATCC	137	VIC	50
274	FH2532	37	25	CACGCAGAAAGGCAGAAAG	GTGTCTTTTTCCATAGTGGCTGCATCA	322	NED	60
275	FH2587	37	29	GGCATGAACAAATCAGTGGA	GTGTCTTTTTGCTGTTTAATCCATCTGG	198	NED	60
276	UU355Cfa37_32	37	32	TCATACGTGTACCAGAGTT	GTGTCTTAGTAAATGAGCAAGTAGACA	115	FAM	55
277	UU354Cfa38_4	38	4	ACATCAGAGCGCACAACACA	GTGTCTTTGTGGAATCCTGAGGCTGAG	250	FAM	55
278	UU353Cfa38_13	38	13	TTGTACGGATGCACGTGTGT	GTGTCTTAGAATCTGCACTGCAGGATG	345	FAM	55
279	REN02C20	38	15	AGAAATTGCATCACTCACAT	GTGTCTTGCTGCTCCGAAAACTAACTT	315	FAM	60
280	REN164E17	38	23	GGTCTTCACCCATCACCATT	GTGTCTTTTAGATGGAAAATGTGGCCC	138	VIC	60
281	FH3399	38	26	TCTCTATGCCTGCAGTTTCC	GTGTCTTTTCTGATGCCCTCATAAAGC	256	FAM	60
282	REN230I20	X	12	CACTTAGCAGAGCACCCTCA	GTGTCTTCCAGGTTCACTGCAGCACTA	217	VIC	60
283	REN101G16	X	17	AGACATGTGAACCTGCATG	GTGTCTTTATCATCTCACTCTGGCACA	145	FAM	60
284	FH2584	X	30	GTTAGGTTCACAGTGGGCGT	GTGTCTTACTCAAAGACCTGGAGGGGT	308	VIC	60
285	FH2548	X	30	AAGGGAGGAAACAATGCTGA	GTGTCTTGACATTCAGAGATTTCCGCC	175	VIC	60
286	FH2997	X	35	TCTCTCATCTCTCCCTCTGC	GTGTCTTAAAAACTGACATCAACAAATGC	226	NED	60
287	FH3027	X	40	GTTTCCTCACATGCAAAAGC	GTGTCTTGCTGGAGGTCAAGGATAAGG	212	FAM	60
288	D04614	X	60	ATCAAGTCCCACATCGGGCT	GTGTCTTGTGGTTCTTATCCTTTCTCTTATC	154	NED	55
289	REN144O22	X	125	GAGGCTTGTTTTGTTGGGAA	GTGTCTTGGGCAGAAGTTTTTGACCCA	206	VIC	60

Discussion

A breed predisposition for hepatic copper storage disorders has been reported in the Bedlington terrier[16,17], West Highland White terrier[18,19], Dalmatian[20], Doberman[21-24], and Skye terrier[25,26]. Nevertheless so far all molecular attempts to discover the genetic background of the disease were restricted to research on the disorder in one breed. Copper toxicosis in Bedlington terriers is an autosomal recessive disease causing lysosomal accumulation of copper in the liver of affected dogs, due to a defective COMMD1 gene.[17,27] A deletion of exon 2 of the gene impairs biliary excretion of the metal during cellular copper stress.
The research on copper toxicosis in Bedlington terriers was started with discovery of a microsatellite marker, which was found to be in linkage disequilibrium with the disease phenotype of high copper concentrations in the liver. The same approach was used in the current study. The aim of our investigation was to discover linkage between the haplotype of a chromosomal region and the phenotype of hepatic copper accumulation in a large family of Labrador retrievers, in order to reveal candidate regions for an underlying gene mutation.

Phenotyping
In some examined Labradors of this study isolated hepatic accumulation of copper occurred without histologic signs of chronic inflammation. It appears that dogs may be subject to a prolonged delay period of several years between severe accumulation of copper and development of histologic inflammation, as well as a prolonged second delay period between inflammation revealed during histologic examination and the occurence of clinical signs of the disease. Thus although the metabolic defect is present at young age, clinically obvious copper-associated chronic hepatitis is a disease of late onset in Labradors (average age 7 years, range 2-10 years).[2,28]
In order to define the phenotype of the disease as clear as possible we included in our definition the quantitative copper concentration measured in liver tissue, the semi-quantitative score for copper assessed from staining of liver tissue with rubeanic acid, as well as the centrolobular localization of copper. Our definition of "normal" was based on a previously established breed specific normal range for hepatic copper concentrations (normal below 400 mg/kg dw, normal copper score <2). Of all the dogs that we have examined, we excluded animals with copper concentrations in an intermediate zone between 400 and 600 mg/kg dw from the analysis to account for possible secondary effects on liver copper concentrations (e.g. age). Age related changes in copper concentrations have been described previously.[29] We assumed that with our distinction criteria between the two groups with respect to copper concentrations, as well as the histological localization of copper as part of the phenotype, the inclusion of phenocopies was avoided.
Besides the observation that copper accumulation preceeds development of hepatitis, our definition of the phenotype relies upon the discovery of a study of Spee at al.[3,4] In their study Spee et al compared liver samples of copper toxicosis in Bedlington terriers, with non-copper-associated breeds with chronic extrahepatic cholestasis, or chronic hepatitis, to healthy dogs. The authors were able to find clear distinction criteria to determine whether copper-accumulation is primary or secondary to hepatitis. From their comparison of gene expressions the authors concluded that zonal copper concentrations at the magnitude of the affected dogs in this study indicate a primary copper storage disease.

Genotyping
Linkage Analysis can be used for the assessment of genotyping results within families.
The approach is very succesful for analysis of single and major gene disorders, and especially promising for analysis of diseases with inheritance in mendelian pedigree patterns. Nevertheless this method has also been used for analysis of complex disorders.[5,30,31] Linkage is present if two loci are transmitted from parents to offspring more often than expected by chance. Linkage within a family occurs over larger regions of the genome than linkage disequilibrium within a population. Therefore assessment for linkage within a family requires genotyping of fewer DNA-markers compared to population based studies and the analysis is a good first screening method for identification of probable locations of genes.[5,30]

For disorders of a known mode of inheritance, parametric analysis (LOD-score) is the statistical method of choice to assess for linkage, because this method has more power to detect linkage when compared with nonparametric testing. However, in order to achieve reliable results the allele frequency for the disease allele, as well as the marker alleles have to be estimated accurately, and the model of inheritance should be known. Furthermore LOD scores will vary with the estimated recombination fraction theta (θ).
In this study patients were assumed to share a particular allele haplotype with a non-random association between the disease phenotype of elevated hepatic copper and a particular genetic marker allele (linkage). Several canine genetic diseases have been mapped with the use of microsatellite markers.[32,33]
The actual mode of inheritance could not be determined due to a lack of information regarding all relatives of the analyzed dogs. Therefore a model-independent statistical method was chosen to test for linkage. Although it has been shown previously that LOD score calculations with approximated inheritance parameters might be more powerful than model free calculations, we did not perform model dependent linkage calculations to avoid the introduction of an error of multiple testing.[34-36]
By analysis of microsatellite markers and SNPs in a family of 31 Labradors we have found linkage of hepatic copper accumulation to two regions of 1.5 and 1.9 Mb on chromosome 7, and one region of 2 Mb on chromosome 28. Future analysis will include confirmation and further fine-mapping of the regions by analysis of more markers on DNA of more dogs, as well as the investigation of candidate genes in the indicated regions.
Until the genetic defect underlying copper accumulation in Labradors is identified we will not be able to explain how copper can accumulate without causing hepatitis in this breed. Exhaustion of cellular systems, which protect against oxidative damage by occasional free reactive copper or an additional stress factor, which affects other protective mechanisms of the liver cell, may be necessary for copper to cause hepatitis.
The disease in Labradors is phenotypically comparable to copper toxicosis of other dog breeds, as well as the genetically unresolved human copper storage disorders Indian Childhood cirrhosis and Endemic Infantile Tyrolean Cirrhosis.[16,20,37-40] Results obtained from the study of the canine disease could therefore help to reveal the molecular genetic background of the disease in children.

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11

Gene expression in canine liver cells during copper overload

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Abstract

The objective of this study was to identify genes potentially involved in the metabolism of copper using gene expression microarrays.

Copper toxicity in the liver is mediated by free-radical generation and oxidative stress. To prevent toxic accumulation of copper, liver cells adapt to high copper levels. We used microarray analysis to compare the adaptive responses on gene expression in canine liver cells exposed to high copper levels in vitro. Two canine hepatic cell lines were exposed to elevated copper concentrations. Total RNA isolated at different time points after copper exposure was hybridised to an Affymetrix custom-designed 44K canine array. The arrays were analysed using Genedata Analyst software. Data were corrected for multiple hypothesis testing and tested for significance using the National Institute on Aging array analysis tool. For comparison, data were divided into three separate groups: no added copper, low added copper (5 μM), and toxic concentrations of added copper (50 μM) according to the results from a preceding viability assay. Gene expression of these groups was tested in both cell lines separately at 5 different time points (at the time of exposure = 0 hours, and 2hours, 6 hours, 12 hours, and 24 hours post exposure to copper.)

One hundred and eighty-six genes were differentially expressed at a significance level of 0.05 in the copper treated cell lines compared to controls without copper. Two genes were differentially expressed in both cell lines and at different time points after exposure to copper, and 3 genes were differentially expressed at different time points within one cell line. Quantitative PCR confirmed significant changes of expression in 3 of the genes (*ATP11B, CRIM, HDAC*).

We identified 3 novel genes (*ATP11B, CRIM,* and *HDAC*) that may represent an acute response to copper overload, thus providing insight into the adaptive transcriptional response to copper-induced oxidative stress.

Introduction

All organisms require the uptake of essential amounts of the trace metal copper, which plays a role in many enzymatic reactions. However, in conditions of copper excess the metal may become highly toxic, because free copper leads to the generation of hydroxyl radicals.[1] Hydroxyl radicals can directly damage proteins, lipids, and nucleic acids, and oxidative stress can affect transcription factors, resulting in deregulated gene expressions. Cytokine production in macrophages and other cells can be induced, leading to inflammation, and profibrotic cytokines may favour the production of collagen.[2-5]

Wilson's disease (OMIM 277900) is a human disorder of copper toxicity. Patients accumulate copper in various tissues, particularly the liver and brain. Reduction or absence of the *ATP7B* gene expression reduces the rate of incorporation of copper into ceruloplasmin for excretion into the bloodstream, as well as for biliary excretion. Progressive hepatic copper accumulation results in liver cirrhosis.

In the dog inherited copper toxicosis is a well described disease in the Bedlington terrier, where a deletion of exon 2 in the *COMMD*1 gene (previously called *MURR1*) causes accumulation of copper in hepatocytes, resulting in chronic hepatitis.[6-8]

Other breed-associated hepatic copper storage disorders in dogs occur in the West Highland White terrier, Skye terrier, Doberman Pincher, Dalmatian, and Labrador retriever.[9-15] Mutations of candidate genes involved in the metabolism of copper, like *ATP7B, COMMD1, ATP7A, metallothionein,* copper chaperones and others were excluded in the investigated dog breeds (manuscripts in preparation, chapter 8 and 10). Therefore the genetic background of these disorders of copper toxicity remains unsolved.

Similarities exist between canine copper storage diseases and unresolved disorders of copper metabolism in people.[16-18] For these diseases (Indian childhood cirrhosis and non-indian childhood cirrhosis) a complex ethiology is suggested, with influencing factors from the environment, like high copper intake. The likely influence of environmental factors on the development of the disease suggests that the liver of healthy individuals can compensate to changes in copper exposure by decreasing uptake or increasing excretion of copper, whereas copper metabolism cannot adapt appropriately in predisposed patients.

The aim of our study was to examine the gene expression responses of canine liver cells to toxic copper concentrations, in order to reveal new candidate genes involved in copper metabolism. Messenger RNA expression profiles were investigated in two canine liver cell lines over a time-series of 24 hours to determine the changes in expression profiles of genes involved in the adaptation to high copper levels.

Materials and methods

Cell culture

Canine bile duct epithelial (BDE) cells were acquired from the Amsterdam Medical Centre, Experimental Liver cell bank. Canine hepatocellular carcinoma (cHCC) cells were cultured in house as previously described.[19] Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM[a]) supplemented with 10% fetal calf serum (FCS[a]) and standard antibiotics at 37 ºC with 5% CO_2 and 95% air in a humidified atmosphere as described before.[20]

Cell-counts were performed in both cell lines, and RNA was isolated from a series of dilutions in order to determine the necessary amount of cells for achievement of a minimal total final RNA concentration of 500 ng RNA per isolation.

MTT assay

The mitogenic in vitro activity of copper was measured using the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) proliferation assay.[21] Cells were seeded in each well of a 96-well culture plate at a density of 4500 cells per well in 150 µl DMEM medium containing 1% FCS. Copper chloride dihydrate (Cl2Cu.2H2O[b]) was dissolved in double distilled, deionized water (Milli-Q). Six microliter copper solution were added to 144 µl medium to obtain a concentration of 4 mM copper per 100 µl medium. After an overnight culture starvation period, 150 µl copper containing medium was added in the first row of wells to obtain a concentration of 2 mM copper per 100 µl medium. The medium was serially diluted each step, except for the last column (control cells). After 24 hrs under culture conditions, 0.5 mg/mL MTT[c] were added to each well for 3 hrs. Cell growth was calculated as percentage increase in absorbance of treated cells compared to non-treated cells. The assay was performed in triplicate during two independent experiments.

Copper treatment

BDE cells were seeded in each well of 7 12-well culture plates at a density of 20,000 cells per well in 1 ml DMEM medium containing 1% FCS. Canine HCC cells were seeded in each well of 15 6-well culture plates at a density of 100.000 cells per well in 1 ml of the same medium. Copper chloride dihydrate was dissolved in Milli-Q and added to 2 stocks of DMEM medium to obtain media with copper concentrations of 10 µM (low Cu), and 100 µM (high Cu) respectively. A stock of DMEM medium was left without the addition of copper (no Cu). At four time points (24 hours, 12 hours, 6 hours, and 2 hours prior to RNA isolation) 1 ml of each medium was added to the plated cells in order to obtain copper concentrations of 0 µM, 5 µM, and 50 µM respectively (sixtuplicate for BDE cells, duplicate for cHCC cells).

At time-point 0 hours the medium was extracted from the plates and cells were washed twice with Hank's Balanced Salt Solution (HBSS, Gibco, QQ) and lysed in 500 µl lysis buffer with the addition of 1% beta-mercaptoethanol (from the RNeasy Mini Kit[d]). For BDE cells the content of 2 wells was pooled to obtain triplicates from each treatment.

RNA isolation and quality control

RNA isolation and QPCR were performed as described before.[22] Total cellular RNA was isolated using the RNeasy Mini Kit and treated with DNase-I (Qiagen, Rnase-free DNase kit). Reverse transcriptase-PCR was performed using iScript™ cDNA Synthesis Kit[e]. RNA yields were quantified spectrophotometrically using the Nanodrop® ND-1000 device and set to a 0.1 µg/µl concentration. The Agilent 2100 Bioanalyzer was used as an independent technique to gain information about quantity and quality of the purified RNA. As a control a standard marker supplied by Agilent Technologies for quantity and quality was used. RNA quality was measured in two independent ways: By means of the A260/A280 ratio, and by means of the Bioanalyzer, which displays RNA Integrity Number (RIN-values) indicating the stability of 18S and 28S rRNA.

Amplification of RNA

100 ng total RNA was used to synthesis double stranded cDNA, according to the Affymetrix small sample target labelling protocol VII. The double-stranded cDNA reaction was cleaned up by ethanol precipitation and the precipitated pellet was used in the first cycle, in vitro transcription for cRNA amplification using the MEGAscript T7 kit[f]. The cRNA reaction was cleaned up using RNeasy columns, eluted in a total of 50 µl RNase-free water and quantified on a Nanodrop® ND-100 spectrophotometer.

Affymetrix 44K canine oligonucleotide microarray layout

The array was designed by definition of coding fragments from the 1.2 x poodle genome sequence in collaboration with Celera. An in-house 54,000 protein database and Refseq[g] were used to isolate match islands for dog coding sequences using BLAST[23]. Non redundant sequences matching Refseq (cut-off of e-40) and the protein database (cut-off of e-20) were submitted for oligonucleotide design to Agilent technologies. Three oligonucleotides per sequence were designed and tested against mRNA derived from canine blood, testes and cell culture. Based on maximum performance on the chip and uniqueness in the gene set, one of the three oligonucleotides was selected.

Microarray hybridization, washing, scanning, and image analysis

The cRNA sample underwent a second cycle of cDNA amplification and labelling was carried out according to the Affymetrix small sample target labelling protocol VII. cRNA was synthesised from the double stranded cDNA using a 3'-amplification IVT labelling kit[h] and was subsequently cleaned up using RNeasy columns.

20 µg biotin labelled cRNA were fragmented in 1 x fragmentation buffer (40mM Tris-acetate pH8.1; 100 mM KOAc and 30 mM MgOAc) by heating at 94°C for 35 minutes followed by cooling on ice. 10µg fragmented cRNA per GeneChip were used in each hybridisation cocktail. The hybridisation cocktail containing 0.05mg.ml^{-1} fragmented cRNA, 50 pM control oligonucleotide B2, 1 x eukaryotic hybridisation controls [bioB (1.5pM), bioC (5pM), bioD (25pM), cre (100pM)], 0.1 mg.ml^{-1} herring sperm DNA, 0.5 mg.ml^{-1} acetylated BSA and 1 x hybridisation buffer was heated at 99°C for 5 minutes, followed by 45°C for 5 minutes and then centrifuged at 13,000 rpm for 5 minutes to pellet any insoluble material. The appropriate volume was added into each GeneChip and the arrays hybridised at 45°C, rotating at 60 rpm for 16-17 hours.

The GeneChips were washed and stained using the antibody amplification for eukaryote targets protocol (MIDI-Euk2v3-450) as described in the Affymetrix GeneChip expression analysis technical manual on a GeneChip fluidics station 450. The arrays were scanned on an Affymetrix GeneScanner 3000 using 2 scans per GeneChip.

Statistical analysis of microarray data

Data were imported into Genedata Expressionist Analyst[i] and the Cy3 and Cy5 fluorescence intensities normalised using lowest weighted linear regression (LOWESS).[24] Parametric T-tests carried out on the normalised data identified genes that displayed maximum variation between the groups and minimum variation within the groups, at $p \leq 1x10^{-5}$ and $p \leq 1x10^{-4}$. In addition a pair-wise comparison of gene signal intensity (expression) with fold difference was calculated.

Reverse transcriptase reaction and QPCR

QPCR was performed in duplicate in a spectrofluorometric thermal cycler (iCycler®, BioRad) using iQ™ SYBR® Green SuperMix (BioRad). Gene-expressions were normalized with the average gene-expressions of the endogenous references hypoxanthine phosphoribosyl transferase (HPRT), ribosomal protein S19 (RPS19), and ribosomal protein L8 (RPL8).[22] Primer pairs are indicated in table 1.

Results

MTT assay

Cell viability was stable at a plateau value between 0 and 7.8 µM added copper. Copper dosis response revealed a reduction in cell viability with a slope decline between 31.3 and 62.5 µM added copper. Therefore the addition of 5 µM copper was chosen as low copper environment and the addition of 50 µM copper was chosen as high copper environment for the following experiments.

One hundred and eighty-six genes out of 44,000 were found to be significantly differentially expressed in copper treated canine liver cell lines when compared to the same cell line without the addition of copper (table 2). Eighty two genes were differentially expressed after treatment with a high, toxic dosage of 50 µM copper. *ATP11B* and transcriptionfactor *ELYS* were two genes that showed an increase in expression that increased over time in both cell lines and at different time points when compared to an environment without the addition of copper. The difference occured after 6 hours in cHCC cells, and after 6, 12, and 24 hours in bile duct epithelial cells treated with high and low concentrations of copper. Quantitative PCR of the same cell lines treated with high concentrations of copper confirmed a change of the expression of *ATP11B* over time during copper exposure ($p=0.046$), whereas the difference in expression of transcription factor *ELYS* was not significant ($p=0.12$).

Three genes were only differentially expressed in one cell line. *Ankrd10* was differentially expressed in bile duct epithelial cells after 6, 12, and 24 hours of copper exposure, and cystein rich motor neuron 1 (*CRIM*) and histone deacetylase-like protein (*HDAC*) were differentially expressed after 6 and 24 hours of copper exposure in cHCC cells. The fold-changes in expression are presented in figure 1. Quantitative PCR of the same cell lines treated with high concentrations of copper confirmed a significant change in the expression of *CRIM* ($p=0.05$), and *HDAC* ($p=0.046$), but not *ANKRD10* ($p=0.18$-0.8).

1:

	Gene	oligo sequence	forward primer qPCR	reverse primer qPCR
06	ATP11B	ATAAAACTGGTACACTGACAGAAAATGAGATGCAGTTTCGGGAATGTTCAATTAATGGCA	TGTTGGTGATGGTGCTAATGATGT	TTCTTGCAGCTTGTCTTCCTTCTT
67	ELYS	TTTTGGAGAAACTGGATATGAGCAGAGAAAGTAGCGGAGCTTCAACCAAGTCAGACCAGA	CACCGCCTTTACCATCTCCCTCTC	GTCATCCGCACCTCCAGCAGTCT
66	CRIM	TGCGAAGATGAGAACTGGGATGATGACCAGCTGCTTGGTTTTGAACCCTGCAATGAAAAT	TGAAACAAAGCCAGCCTGTG	TTCGGGCACATAATACCTCTCG
12	ANCRD	TCCATGTCGGATACACTGACAAATGGATGTGTCATCAATGGACATTTGGACTTCCCCTCT	GGTTGATTCAAGCAGGAGCC	CACTGGCATTTCTCAGGTCG
76	HDAC	TGCTGAATGAGAGCTGTGGGGACCATGCTGACACTGACCAGGTCGTGCTTTATGCCGTGA	CCTATACTTGTGCCTGCCTGG	TTCTGTTGAGCGTAGCGGG

ıucleotide sequences and primer pairs and used for identification of the genes ATP11B, ELYS, CRIM, ANCRD, and HDAC during microarray and qPCR
ons.

Table 2 (1/3)

	Gene ID	Gene name	Gene symbol	Function	Copper
1	23200	ATPase, Class VI, type 11B	ATP11B	Transporter	50 µM + 5 µM
2	25909	AT hook containing transcription factor 1, transcription factor ELYS	AHCTF1	Transcription factor	50 µM + 5 µM
3	51232	cystein rich mortor neuron 1	CRIM1	Molecular function unclassified	50 µM + 5 µM
4	55608	ankyrin repeat domain 10	ANKRD10	Molecular function unclassified	50 µM + 5 µM
5	10013	histone deacetylase 6	HDAC6	mRNA transcription regulation	50 µM + 5 µM
6	55737	vacuolar protein sorting 35 homolog (S. cerevisiae)	VPS35	Membrane traffic protein	5 µM
7	2030	solute carrier family 29 (nucleoside transporters), member 1	SLC29A1	Transporter	5 µM
8	11182	solute carrier family 2 (facilitated glucose transporter), member 6	SLC2A6	Carbohydrate transporter	50 µM
9	9123	solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	SLC16A3	Cation transport	5 µM
10	59341	transient receptor potential cation channel, subfamily V, member 4	TRPV4	Cation transport	5 µM
11	84059	G protein-coupled receptor 98	GPR98	Cation transporter	5 µM
12	23400	ATPase type 13A2	ATP13A2	Ion channel	5 µM
13	22796	component of oligomeric golgi complex 2	COG2	Exocytosis	50 µM
14	6782	stress 70 protein chaperone, microsome-associated, 60kDa	STCH	Hsp 70 family chaperone	5 µM
15	539	ATP synthase, mitochondrial F1 complex, O subunit	ATP5O	Hydrogen transporter	5 µM
16	163	adaptor-related protein complex 2, beta 1 subunit	AP2B1	Membrane traffic protein	5 µM
17	10313	reticulon 3	RTN3	Membrane traffic protein	5 µM
18	1201	ceroid-lipofuscinosis, (Batten, Spielmeyer-Vogt disease)	CLN3	Membrane traffic regulatory protein	50 µM
19	9646	Ctr9, Paf1/RNA polymerase II complex component, homolog	CTR9	Molecular function unclassified	50 µM
20	10159	ATPase, H+ transporting, lysosomal accessory protein 2	ATP6AP2	Receptor	5 µM
21	388121	tumor necrosis factor, alpha-induced protein 8-like 3	TNFAIP8L3	Stress response	50 µM
22	58512	discs, large (Drosophila) homolog-associated protein 3	DLGAP3	Transmembrane receptor regulatory/adaptor protein	5 µM
23	60598	potassium channel, subfamily K, member 15	KCNK15	Voltage-gated potassium channel	5 µM
24	64393	zinc finger, matrin type 3	ZMAT3	Zinc finger transcription factor	5 µM
25	23022	palladin, cytoskeletal associated protein	PALLD	Actin binding cytoskeletal protein	5 µM
26	1397	cysteine-rich protein 2	CRIP2	Actin binding cytoskeletal protein	50 µM
27	10486	CAP, adenylate cyclase-associated protein, 2 (yeast)	CAP2	Actin binding cytoskeletal protein	5 µM
28	84033	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	OBSCN	Actin binding cytoskeletal protein	50 µM
29	7139	troponin T type 2 (cardiac)	TNNT2	Actin binding motor protein	50 µM
30	4636	myosin, light chain 5, regulatory	MYL5	actin family cytoskeletal protein	50 µM
31	2038	erythrocyte membrane protein band 4.2	EPB42	Acyltransferase	5 µM
32	64834	elongation of very long chain fatty acids	ELOVL1	Acyltransferase	5 µM
33	1192	chloride intracellular channel 1	CLIC1	Anion channel	5 µM
34	6880	TAF9 RNA polymerase II, (TBP)-associated factor, 32kDa	TAF9	Basal transcription factor	5 µM
35	57491	aryl-hydrocarbon receptor repressor	AHRR	Basic helix-loop-helix transcription factor	5 µM
36	57549	immunoglobulin superfamily, member 9	IGSF9	CAM family adhesion molecule	5 µM
37	2710	glycerol kinase	GK	Carbohydrate kinase	5 µM
38	5214	phosphofructokinase, platelet	PFKP	Carbohydrate kinase	5 µM
39	2645	glucokinase (hexokinase 4, maturity onset diabetes of the young 2)	GCK	Carbohydrate kinase	5 µM
40	90952	endothelial cell adhesion molecule	ESAM	Cell adhesion	5 µM
41	4597	mevalonate (diphospho) decarboxylase	MVD	Cholesterol metabolism	50 µM
42	6389	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	SDHA	Dehydrogenase	5 µM
43	3418	isocitrate dehydrogenase 2 (NADP+), mitochondrial	IDH2	Dehydrogenase	5 µM
44	10901	dehydrogenase/reductase (SDR family) member 4	DHRS4	Dehydrogenase	5 µM
45	3939	lactate dehydrogenase A	LDHA	Dehydrogenase	5 µM
46	10157	aminoadipate-semialdehyde synthase	AASS	Dehydrogenase	5 µM
47	55825	peroxisomal trans-2-enoyl-CoA reductase	PECR	Dehydrogenase	5 µM
48	10051	structural maintenance of chromosomes 4	SMC4	DNA metabolism	5 µM
49	51728	polymerase (RNA) III (DNA directed) polypeptide K, 12.3 kDa	POLR3K	DNA-directed RNA polymerase	5 µM
50	258	ameloblastin (enamel matrix protein)	AMBN	Extracellular matrix	5 µM
51	727897	mucin 5B, oligomeric mucus/gel-forming	MUC5B	Extracellular matrix glycoprotein	5 µM
52	92949	ADAMTS-like 1	ADAMTSL1	Extracellular matrix glycoprotein	5 µM
53	3911	laminin, alpha 5	LAMA5	Extracellular matrix linker protein	5 µM
54	2562	gamma-aminobutyric acid (GABA) A receptor, beta 3	GABRB3	GABA receptor	5 µM
55	4668	N-acetylgalactosaminidase, alpha-	NAGA	Galactosidase	5 µM
56	2589	polypeptide N-acetylgalactosaminyltransferase 1	GALNT1	Glycosyltransferase	50 µM
57	81849	ST6	ST6GALNAC5	Glycosyltransferase	5 µM
58	84750	fucosyltransferase 10 (alpha (1,3) fucosyltransferase)	FUT10	Glycosyltransferase	50 µM
59	2683	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	B4GALT1	Glycosyltransferase	5 µM
60	118491	tetratricopeptide repeat domain 18	TTC18	Glycosyltransferase	5 µM
61	115669	tetratricopeptide repeat domain 6	TTC6	Glycosyltransferase	50 µM
62	50717	WD repeat domain 42A	WDR42A	G-protein	5 µM

Table 2 (2/3)

	Gene ID	Gene name	Gene symbol	Function	Copper
63	5733	prostaglandin E receptor 3 (subtype EP3)	PTGER3	G-protein coupled receptor	50 μM
64	126326	GIPC PDZ domain containing family, member 3	GIPC3	G-protein mediated signaling	5 μM
65	23216	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1	TBC1D1	G-protein modulator	50 μM
66	57231	sorting nexin 14	SNX14	G-protein modulator	50 μM
67	23380	SLIT-ROBO Rho GTPase activating protein 2	SRGAP2	G-protein modulator	5 μM
68	9901	SLIT-ROBO Rho GTPase activating protein 3	SRGAP3	G-protein modulator	5 μM
69	4838	nodal homolog (mouse)	NODAL	Growth factor	50 μM
70	11082	endothelial cell-specific molecule 1	ESM1	Growth factor	5 μM
71	8074	fibroblast growth factor 23	FGF23	Growth factor	5 μM
72	9267	pleckstrin homology, Sec7 and coiled-coil domains 1(cytohesin 1)	PSCD1	Guanyl-nucleotide exchange factor	50 μM
73	55213	regulator of chromosome condensation	RCBTB1	Guanyl-nucleotide exchange factor	5 μM
74	8997	kalirin, RhoGEF kinase	KALRN	Guanyl-nucleotide exchange factor	5 μM
75	3312	heat shock 70kDa protein 8	HSPA8	Hsp 70 family chaperone	5 μM
76	5702	proteasome (prosome, macropain) 26S subunit, ATPase, 3	PSMC3	hydrolase	5 μM
77	26127	FGFR1 oncogene partner 2	FGFR1OP2	immune and defense	5 μM
78	941	CD80 molecule	CD80	Immunoglobulin receptor family member	5 μM
79	5870	RAB6A, member RAS oncogene family	RAB6A	Intracellular signaling cascade	50 μM
80	10178	odz, odd Oz/ten-m homolog 1(Drosophila)	ODZ1	intracellular signaling cascade	5 μM
81	121506	chromosome 12 open reading frame 46	C12orf46	isomerase	5 μM
82	81567	thioredoxin domain containing 5	TXNDC5	isomerase	5 μM
83	3707	inositol 1,4,5-trisphosphate 3-kinase B	ITPKB	Kinase	5 μM
84	7571	zinc finger protein 23 (KOX 16)	ZNF23	KRAB box transcription factor	5 μM
85	55970	guanine nucleotide binding protein (G protein), gamma 12	GNG12	Large G-protein	5 μM
86	10211	flotillin 1	FLOT1	Membrane traffic protein	50 μM
87	10961	endoplasmic reticulum protein 29	ERP29	Membrane traffic protein	5 μM
88	9381	otoferlin	OTOF	membrane traffic protein	5 μM
89	64840	porcupine homolog (Drosophila)	PORCN	Membrane traffic regulatory protein	50 μM
90	23554	tetraspanin 12	TSPAN12	Membrane-bound signaling molecule	5 μM
91	1947	ephrin-B1	EFNB1	Membrane-bound signaling molecule	5 μM
92	24137	kinesin family member 4A	KIF4A	Microtubule binding motor protein	5 μM
93	3835	kinesin family member 22	KIF22	Microtubule binding motor protein	50 μM
94	9576	sperm associated antigen 6	SPAG6	Microtubule family cytoskeletal protein	5 μM
95	6867	transforming, acidic coiled-coil containing protein 1	TACC1	miscellaneous function protein	5 μM
96	118672	chromosome 10 open reading frame 89	C10orf89	Molecular function unclassified	5 μM
97	79668	poly (ADP-ribose) polymerase family, member 8	PARP8	Molecular function unclassified	5 μM
98	220047	coiled-coil domain containing 83	CCDC83	Molecular function unclassified	5 μM
99	4835	NAD(P)H dehydrogenase, quinone 2	NQO2	Molecular function unclassified	5 μM
100	55729	activating transcription factor 7 interacting protein	ATF7IP	Molecular function unclassified	5 μM
101	9627	synuclein, alpha interacting protein (synphilin)	SNCAIP	Molecular function unclassified	5 μM
102	6049	ring finger protein (C3H2C3 type) 6	RNF6	Molecular function unclassified	5 μM
103	152002	chromosome 3 open reading frame 21	C3orf21	Molecular function unclassified	50 μM
104	57636	Rho GTPase activating protein 23	ARHGAP23	Molecular function unclassified	50 μM
105	6100	retinitis pigmentosa 9 (autosomal dominant)	RP9	Molecular function unclassified	50 μM
106	25852	armadillo repeat containing 8	ARMC8	Molecular function unclassified	5 μM
107	158747	motile sperm domain containing 2	MOSPD2	Molecular function unclassified	5 μM
108	152579	sec1 family domain containing 2	SCFD2	Molecular function unclassified	5 μM
109	56951	chromosome 5 open reading frame 15	C5orf15	Molecular function unclassified	5 μM
110	283651	chromosome 15 open reading frame 21	C15orf21	Molecular function unclassified	50 μM
111	63967	claspin homolog (Xenopus laevis)	CLSPN	Molecular function unclassified	5 μM
112	10625	influenza virus NS1A binding protein	IVNS1ABP	Molecular function unclassified	5 μM
113	56244	butyrophilin-like 2 (MHC class II associated)	BTNL2	Molecular function unclassified	5 μM
114	92140	metadherin	MTDH	Molecular function unclassified	5 μM
115	57583	transmembrane protein 181	TMEM181	Molecular function unclassified	5 μM
116	55167	male-specific lethal 2-like 1 (Drosophila)	MSL2L1	Molecular function unclassified	50 μM
117	1535	cytochrome b-245, alpha polypeptide	CYBA	Molecular function unclassified	50 μM
118	83548	component of oligomeric golgi complex 3	COG3	Molecular function unclassified	5 μM
119	9358	integrin, beta-like 1 (with EGF-like repeat domains)	ITGBL1	Molecular function unclassified	5 μM
120	9528	transmembrane protein 59	TMEM59	Molecular function unclassified	50 μM
121	51368	testis expressed sequence 264	TEX264	Molecular function unclassified	5 μM
122	56729	resistin	RETN	Molecular function unclassified	5 μM
123	10362	high-mobility group 20B	HMG20B	Molecular function unclassified	5 μM
124	25814	ataxin 10	ATXN10	Molecular function unclassified	5 μM

Table 2 (3/3)

	Gene ID	Gene name	Gene symbol	Function	Copper
125	10963	stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	STIP1	Molecular function unclassified	5 µM
126	126526	chromosome 19 open reading frame 47	C19orf47	Molecular function unclassified	50 µM
127	146059	congenital dyserythropoietic anemia, type I	CDAN1	Molecular function unclassified	5 µM
128	152137	coiled-coil domain containing 50	CCDC50	Molecular function unclassified	50 µM
129	84223	IQ motif containing G	IQCG	Molecular function unclassified	50 µM
130	23509	protein O-fucosyltransferase 1	POFUT1	Molecular function unclassified	5 µM
131	25998	inhibitor of Bruton agammaglobulinemia tyrosine kinase	IBTK	Molecular function unclassified	5 µM
132	8536	calcium/calmodulin-dependent protein kinase I	CAMK1	Non-receptor serine/threonine protein kinase	5 µM
133	11200	CHK2 checkpoint homolog (S. pombe)	CHEK2	Non-receptor serine/threonine protein kinase	5 µM
134	23264	zinc finger CCCH-type containing 7B	ZC3H7B	Nucleic acid binding	5 µM
135	472	ataxia telangiectasia mutated (groups A, C and D)	ATM	Nucleic acid binding	50 µM
136	349565	nicotinamide nucleotide adenylyltransferase 3	NMNAT3	Nucleotidyltransferase	5 µM
137	6834	surfeit 1	SURF1	Oxidase	5 µM
138	1543	cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	Oxygenase	5 µM
139	11172	insulin-like 6	INSL6	Peptide hormone	5 µM
140	54893	myotubularin related protein 10	MTMR10	phosphatase	5 µM
141	9448	mitogen-activated protein kinase kinase kinase kinase 4	MAP4K4	Protein kinase	5 µM
142	10295	branched chain ketoacid dehydrogenase kinase	BCKDK	Protein kinase	5 µM
143	50488	misshapen-like kinase 1 (zebrafish)	MINK1	Protein kinase	5 µM
144	54961	slingshot homolog 3 (Drosophila)	SSH3	Protein phosphatase	5 µM
145	26469	protein tyrosine phosphatase, non-receptor type 18	PTPN18	Protein phosphatase	5 µM
146	84867	protein tyrosine phosphatase, non-receptor type 5	PTPN5	Protein phosphatase	5 µM
147	8452	cullin 3	CUL3	Proteolysis	50 µM
148	5464	pyrophosphatase (inorganic) 1	PPA1	Pyrophosphatase	5 µM
149	706	translocator protein (18kDa)	TSPO	receptor	50 µM
150	1525	coxsackie virus and adenovirus receptor	CXADR	receptor	50 µM
151	27075	tetraspanin 13	TSPAN13	Receptor	50 µM
152	6741	Sjogren syndrome antigen B (autoantigen La)	SSB	Ribonucleoprotein	5 µM
153	6138	ribosomal protein L15	RPL15	Ribosomal protein	50 µM
154	7818	death associated protein 3	DAP3	Ribosomal protein	5 µM
155	6159	ribosomal protein L29	RPL29	Ribosomal protein	5 µM
156	285855	ribosomal protein L7-like 1	RPL7L1	Ribosomal protein	50 µM
157	10973	activating signal cointegrator 1 complex subunit 3	ASCC3	RNA helicase	5 µM
158	6734	signal recognition particle receptor ('docking protein')	SRPR	RNA-binding protein	5 µM
159	10482	nuclear RNA export factor 1	NXF1	RNA-binding protein	5 µM
160	3735	lysyl-tRNA synthetase	KARS	RNA-binding protein	5 µM
161	5271	serpin peptidase inhibitor, clade B (ovalbumin), member 8	SERPINB8	Serine protease inhibitor	5 µM
162	50846	desert hedgehog homolog (Drosophila)	DHH	signaling molecule	5 µM
163	6440	surfactant, pulmonary-associated protein C	SFTPC	Surfactant	5 µM
164	5743	prostaglandin-endoperoxide synthase 2	PTGS2	Synthase and synthetase	5 µM
165	79746	enoyl Coenzyme A hydratase domain containing 3	ECHDC3	Synthetase	5 µM
166	8544	pirin (iron-binding nuclear protein)	PIR	Transcription cofactor	50 µM
167	9604	ring finger protein 14	RNF14	Transcription cofactor	5 µM
168	8204	nuclear receptor interacting protein 1	NRIP1	Transcription cofactor	5 µM
169	4605	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	MYBL2	transcription factor	5 µM
170	7008	thyrotrophic embryonic factor	TEF	transcription factor	5 µM
171	1869	E2F transcription factor 1	E2F1	transcription factor	5 µM
172	10743	retinoic acid induced 1	RAI1	Transcription factor	5 µM
173	8648	nuclear receptor coactivator 1	NCOA1	Transcription factor	5 µM
174	29082	chromatin modifying protein 4A	CHMP4A	transfer / carrierprotein	5 µM
175	128866	chromatin modifying protein 4B	CHMP4B	transfer / carrierprotein	5 µM
176	29127	Rac GTPase activating protein 1	RACGAP1	Transfer/carrier protein	50 µM
177	10226	mannose-6-phosphate receptor binding protein 1	M6PRBP1	Transfer/carrier protein	50 µM
178	5584	protein kinase C, iota	PRKCI	Transfer/carrier protein	50 µM
179	56994	choline phosphotransferase 1	CHPT1	transferase	5 µM
180	55814	B double prime 1, RNA polymerase III transcr. initiation factor IIIB	BDP1	Translation initiation factor	50 µM
181	10955	serine incorporator 3	SERINC3	Transmembrane receptor regulatory protein	5 µM
182	27020	neuroplastin	NPTN	Transmembrane receptor regulatory/adaptor protein	50 µM
183	64747	major facilitator superfamily domain containing 1	MFSD1	transporter	5 µM
184	2041	EPH receptor A1	EPHA1	Tyrosine protein kinase receptor	5 µM
185	51434	anaphase promoting complex subunit 7	ANAPC7	Ubiquitin-protein ligase	5 µM
186	151112	zinc finger, SWIM-type containing 2	ZSWIM2	Zinc finger transcription factor	5 µM

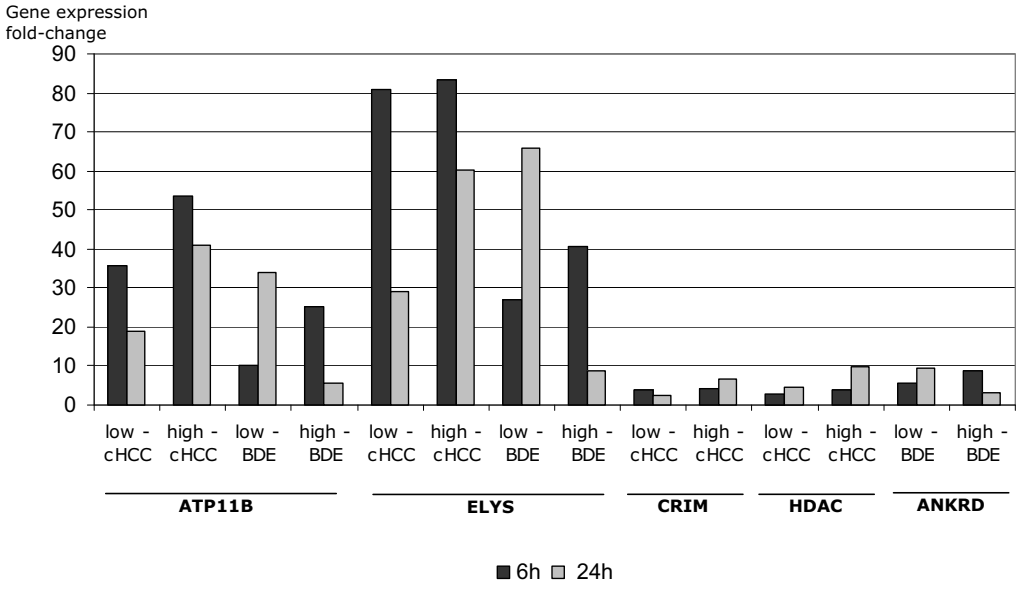

Figure 1: The fold-changes in expression of 5 gene transcripts during micro-array analysis of two canine liver cell lines (BDE: bile duct epithelial cells, and cHCC: canine hepatocellular carcinoma cells) treated with low and high concentrations of copper for 6 hours, and for 24 hours.

Discussion

We examined the time-dependent changes in genome-wide gene expression profiles of canine liver cells during exposure to copper. Our purpose was the identification of novel genes and their associated pathways involved in hepatic copper metabolism.
The overlap of identical transcripts showing changes in gene expression between the two different liver cell lines in this study was minimal. Two genes only showed significant changes in gene expression at different time points in both cell lines (*ATP11B* and *transcription factor ELYS*). Three additional gene transcripts were expressed at different time points within one cell line (*ANCRD10, CRIM*, and *HDAC*). Quantitative PCR confirmed the significance of the results for *ATP11B*, *CRIM*, and *HDAC*, but not for transcription factor *ELYS*, and *ANCRD10*.
Information about *ATP11B* is limited to a few studies. *ATP11B* belongs to the P-type ATPases Class VI. Such ATPases drive uphill transport of ions across membranes. Several subfamilies of P-type ATPases have been identified. One subfamily transports heavy metal ions, such as copper (2+). Other members of this group are *ATP7B* and *ATP7A*, the genes involved in the copper storage disease Wilson's disease, and the copper deficiency syndrome Menkes disease in people. Another subfamily of P-type ATPases Class VI transports non-heavy metal ions, such as H(+), Na(+), K(+), or Ca(+). A third subfamily transports amphipaths, such as phosphatidylserine. (OMIM 605869). The gene is located on dog chromosome 34 at location 18,873,961-18,922,441 (Ensembl).
Cystein rich motor neuron (CRIM) expression was increased 2.3-6.5 times during copper overload. CRIM1 may interact with growth factors implicated in cell differentiation and survival.[25] Sequence analysis predicted that the 1,036-amino acid human protein, which contains a signal peptide, an insulin-like growth factor-binding protein-like domain, 6 highly conserved vWFC cysteine-rich repeat domains, a transmembrane domain, and a 76-residue cytoplasmic tail. (OMIM 606189). The presence of the vWFC region in a number of complex-forming proteins points to the possible involvement of the vWFC domain in complex formation. The domain is named after the von Willebrand factor (vWF) type C repeat which is found in multidomain protein/multifunctional proteins involved in maintaining homeostasis (nature UCSD: http://www.signaling-gateway.org/). The gene is located on dog chromosome 17 at location 31,960,714-32,142,996 (Ensembl).
Interestingly our study revealed a 2.8-9.6 fold increase in expression of Histone deacetylase like protein during copper stress of liver cells. Histone deacetylases (HDACs) are important enzymes for the transcriptional regulation of gene expression in response to cell stress, inhibition of apoptosis, and cell cycle control.[26-28] The primers used in this study were designed for investigation of HDAC 6 on canine chromosome X at location 41,966,283-41,974,521 (Ensembl). Future studies might reveal if the predisposition of female dogs for copper associated liver diseases in Labradors and Doberman pinschers might be brought in context with an upregulated function of this gene or gene products. It is suggested that histone acetylation is important for the control of gene transcription, because binding of genomic DNA with histones prevents transcription interaction with promoter DNA sequences.[29,30] Histone acetylation leads to a loosening of the binding of DNA, allowing for access of transcription factors and initiation of transcription. The balance between histone acetylation and deacetylation is maintained through a balance of the two enzymes histone acetyltransferase and histone deacetylase (HDAC).[31] Other studies found that copper leads to a significant decrease of histone acetylation.[32,33] Due to the finding that cytotoxicity from copper overload is mediated by histone hypoacetylation, HDAC inhibitors were suggested as a new strategy to treat copper-overload diseases.[33]

We chose to look at those genes that were differentially expressed at high significant p values. Although quantitative PCR confirmed the significant changes in expression in many samples, in some cases the identified genes only showed subtle changes in expression levels during quantitative PCR. Technical reasons might account for the difference. The different methods use different normalization tools, and therefore do not allow for a direct comparison between the experiments. The difficulty in combining data from different platforms of gene expression measurement has been highlighted by Jarvinen et al.[34], although, Yauk et al.[35] concluded that when using high quality platforms with the appropriate normalization, the differential gene expression profile is determined primarily by biology rather than the

technology utilized. The expression changes during quantitative PCR were much smaller than expected from the microarray results. Despite this, other authors have shown that small differences in gene expression can be relevant to phenotype.[36] Transcription factors can have a large impact on cellular function despite minimal changes in expression levels and for many metabolic genes, large changes in expression may have no greater predictive value than small changes on the overall pathway.[36-40]

In our study the expression of metallothionein increased only mildly during exposure to a high copper environment. A 1.3 fold and a 1.4 fold increase of the expression of *metallothionein-2* was seen after 6 and 12 hours respectively, and a 1.2, and 1.5 fold increase of the expression of *MT3* was measured after 6 and 24 hours exposure to a high copper environment. *MT1* expression did not change during copper exposure. This is a delay in expression when compared to other studies were the expression of metallothioneins increased during the initial 4 hours of copper treatment. In a study of Muller et al gene expression profiles of HepG2 cells revealed that members of the metallothionein family were upregulated during copper overload. Metallothionein expression reached a maximum after 4 hours of copper treatment, followed by a gradual decrease in expression. It is possible that species differences account for the difference in expression measurements, or that our study missed the peak in gene expression after 4 hours because RNA was isolated at different time points. Both experiments in cell lines are different from a study of Spee et al. who investigated liver tissue of dogs with hepatic copper storage disorders. Spee and his colleagues found the expression of metallothionein to be downregulated in his investigated samples.[41,42] This is in agreement with the finding that the transcriptional response to copper overload in liver in vivo is quite different from the response of cells grown in vitro.[43] The undefined moment of liver biopsy retrieval during the course of a clinical disease, chronicity of the disease, as well as changes from inflammatory mediators and cell death might be responsible for the difference.

The expression of genes known to be involved in copper metabolism did not significantly change as a result of copper overload. This finding is in agreement with the study of Muller et al. Expression changes of the copper influx protein CTR1, the three copper chaperones *CCS1, COX17* and *ATOX1*, and the copper efflux ATPases *ATP7A* and *ATP7B*, as well as *COMMD1, SOD1, glutathione*, and *ceruloplasmin*, were not significant in the previous study, as well as in our results.[43]

Our study examined the gene expression responses of canine liver cells to toxic copper concentrations, and revealed 3 new candidate genes that may be involved in the acute adaptation to high copper levels. Further studies are indicated to assess the function of these genes in vitro, as well as in patients with copper accumulating liver disorders.

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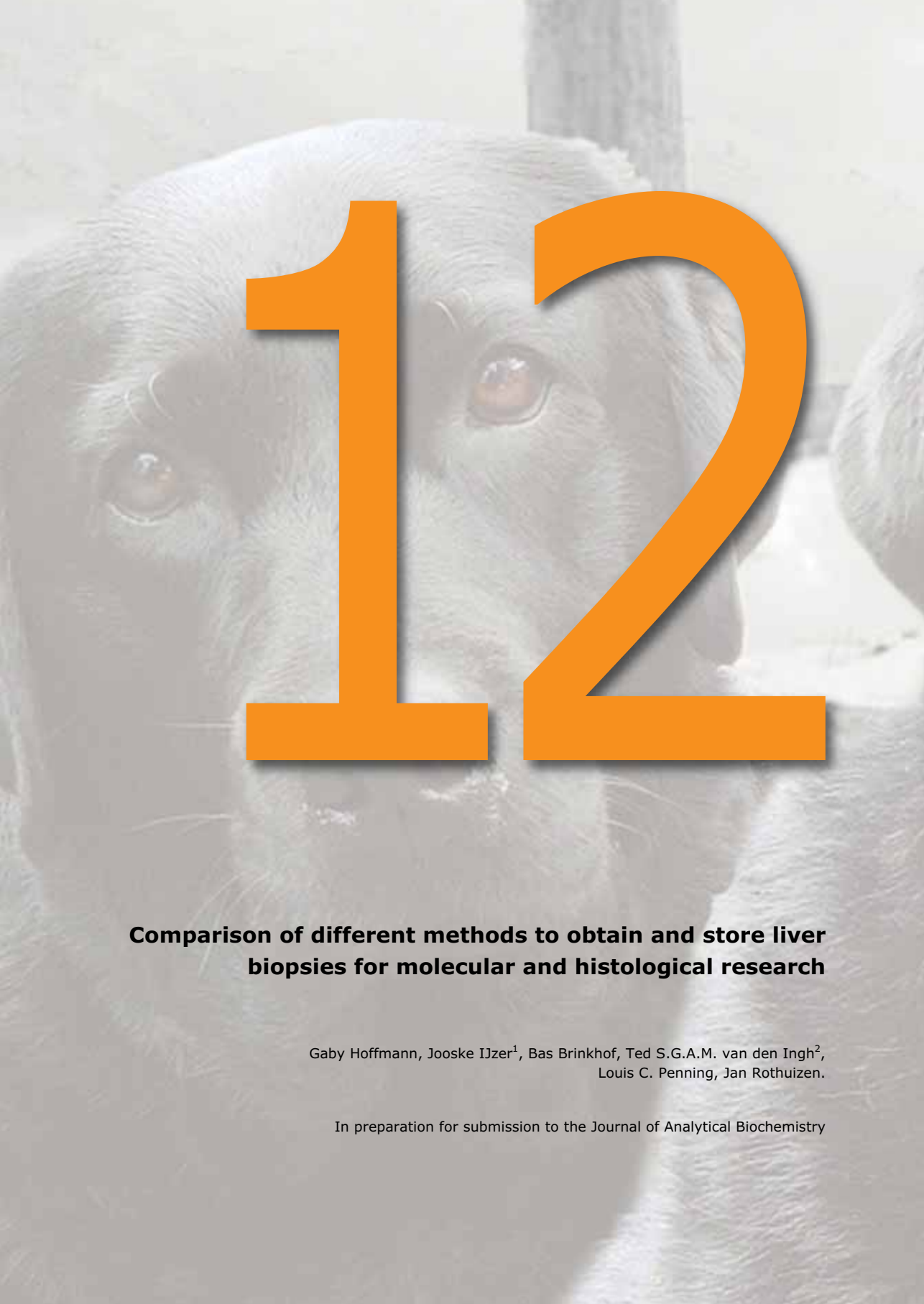

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Comparison of different methods to obtain and store liver biopsies for molecular and histological research

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Abstract

The aim of this study was to assess different sampling techniques, fixation methods, and storage procedures for canine liver tissue. Our objective was to minimize the necessary number of biopsies for molecular and histological research. Three biopsy techniques (wedge biopsy, Menghini, and biopsy gun), four storage media for retrieval of RNA (snap freezing, RNAlater, Boonfix, RLT-buffer), two RNA isolation procedures (Trizol and RNAeasy), and 3 different fixation protocols for histological studies (10%formaldehyde, RNAlater, Boonfix) were compared. Histological evaluation was based on routine Hematoxilin-Eosin (HE) and reticulin (fibrogenesis) staining, as well as the rubeanic acid and rhodanine stains for copper. Immunohistochemical evaluation was performed for cytokeratin-7 (CK-7), multidrug resistance binding protein-2 (MRP-2), canalicular membranes, and Hepar-1 for hepatocytes.
RNA quality was best guaranteed by the combination of a Menghini biopsy with NaCl, followed by RNAlater preservation and RNAeasy mini kit extraction. These results were confirmed by quantitative PCR testing.
Reliable histological assessment of copper by rhodanine or rubeanic acid proved only possible in formalin fixed liver tissue. Reduction of the formalin fixation time to 1-4 hrs improved immunohistochemical reactivity and preservation of good morphology in small liver biopsies.

Introduction

Expression profiling can be used for disease classification, predictions of clinical outcomes or the molecular dissection of affected pathways in hereditary or acquired diseases. Animal models for human diseases facilitate cause-effect studies under controlled conditions and allow comparison with untreated or healthy individuals. Especially the latter can be an ethical or logistical problem in human medicine. More than 300 genetic human disorders are described in dogs (http://www.ncbi.nlm.nih.gov/sites/entrez). Many of these diseases occur in one or just a few of around 400 dog breeds. Single gene diseases are easy to characterize in inbred dog populations, and research of complex diseases profits from the fact that dogs share the human environment. In addition to similarities between dogs and man with respect to physiology, pathobiology, and treatment response, research of breed-related canine behaviour, and phenotypic diversity is promising. Therefore dogs were advocated as a model animal in translational research.[1] Molecular genetic tools available for such comparable research between dogs and men include the in-depth sequencing of the complete dog genome[2,3], a single-nucleotide polymorphism (SNP) data base, containing 2.5 million SNPs[4], and easy access to genetic information of several generations of dogs. In addition, the high degree of inbreeding, which founded the present dog breeds the last few hundreds years, further facilitates the investigations of inheritable gene defects.[5-7] Dog specific micro-arrays are available to perform functional genomic studies. This kind of high-throughput gene expression profiling requires the use of high quality mRNA. Likewise is the quality of mRNA of major impact on the reliability of the results in quantitative RT-PCR (Q-PCR). So far the emphasis in canine molecular biology was put on the use of internal controls for proper Q-PCR measurements and subsequent data analysis.[8-10] However, little information is available that compares different methods of retrieval, isolation and storage of canine tissues for molecular research purposes. Especially liver, but also heart and jejunum, are difficult tissues for retrieval of high quality mRNA.[11]

Liver biopsies, taken for medical and research purposes, are processed for histopathology including immunohistochemistry, as well as RNA and protein isolation. Since these diverse intentions require different fixation and storage methods, clinicians and researchers are often faced with a multitude of different vials, and fluids in order to retain biopsies. In addition, the applications of specific fixation protocols can be necessary, which might require additional training, time, and sophisticated laboratory equipment. Such complexity of tissue handling can challenge the operating personnel, and therefore introduce mistakes, especially in the setup of a multi-centre study, where sampling procedures should be as straightforward as possible. Moreover, in small lesions or advanced diseases, the possibility for retrieval of several biopsies can be limited.

One study described the influence of the size of the biopsy needle in rat liver biopsies on the RNA quality in a subsequent micro-array expression study.[12] The aim of our study was to assess different sampling techniques (with the optimal needle size as described above), fixation methods, and storage procedures for canine liver tissue. Our objective was to optimize the use of a single liver biopsy, in order to minimize the number of necessary biopsies per patient, by evaluation of different methods for RNA isolation and fixation available in our laboratory. Two biopsy techniques were compared. RNA quality was assessed by use of a Bioanalyzer, measuring the stability of 18S and 28S rRNA. Histological evaluation of different fixation methods and fixation times was based on routine hematoxilin-eosin (HE) staining, in addition to the reticulin staining according to Gordon and Sweet, as well as the rubeanic acid, and rhodanine stains for copper. Immunohistochemical evaluation was performed for three different proteins at different (sub)cellular locations i.e. CK-7, MRP-2 and Hepar-1.

Materials and methods

Animals
Projects and procedures were approved by the responsible ethical committees for the use of experimental animals and for use of client-owned animals according to Dutch legislation. Informed owner consent was obtained for all dogs.
For this study, liver tissue was obtained from seven dogs (Table 1). In addition two archival specimens were used as positive controls for staining during histologic examinations. Prior to biopsy retrieval a medical history was obtained from all dogs, and a physical examination was performed. From all client owned dogs, blood samples (Sodium-citrate) were taken for analysis of a coagulation profile, including prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen. EDTA-blood was sampled for measurement of the platelet count.
Surplus animals from orthopedic research revealed, histologically confirmed, healthy livers. These dogs were euthanized immediately prior to extirpation of the liver, using an overdose of pentobarbital via the cephalic vein.

Liver biopsies
Liver biopsies were taken according to the Menghini technique described by Rothuizen[13] and by use of an 16-gauge biopsy needle using an automatic biopsy device[i] (biopsy gun). Liver biopsies retrieved by use of the Menghini technique were kept in physiologic saline solution (0.9% NaCl in sterile water, group Menghini-NaCl) or sterile water (group Menghini water) until transfer into according preservatives. Liver biopsies retrieved with the True-cut gun were kept at room air until transfer into the different storage media.
After the biopsy was taken the retrieved material was either immediately processed further, or kept for 15 minutes, 20 minutes, 25 minutes and 30 minutes before further workup. Further processing included four methods: snap freezing and subsequent storage at minus 70°C, transfer into a sterile 1.5ml vial containing 1ml of RNAlater[ii], Boonfix[iii], or B-RLT[iv]. Biopsies in these vials were kept at 4°C for 2 hrs, and later transferred to minus 20°C and minus 70 °C freezing for long-term storage (2 weeks to 18 months). Additional biopsies retrieved exclusively for histologic examinations were retrieved by the Menghini-NaCl method, and immediately deposited at room temperature (RT) per three in 6 ml containers filled with 10% neutral buffered formaldehyde (formalin). Wedge biopsies (1x1x1cm) were put in a larger container, containing at least 10 cm^3 formalin.

Isolation of RNA and reversed transcriptase.
RNA isolation by means of the RNAeasy kit[v] and Trizol[vi]-mediated RNA isolation were performed according to the manufacturers instructions.
RNA yields were quantified spectrophotometrically using the Nanodrop ND-1000[vii] device and set to a 0.1 µg/µl concentration. The Agilent 2100 Bioanalyzer[viii] was used as an independent technique to gain information about quantity and quality of the purified RNA. As a control a standard marker supplied by Agilent Technologies for quantity and quality was used. One microgram of each total RNA sample was used to synthesize cDNA with an Moloney Murine Leukemia Virus-derived reverse transcriptase according to manufacturer's protocol[ix]. After synthesis the cDNA samples were diluted two times. Details were described previously.[14]
RNA quality was measured in two independent ways: By means of the A260/A280 ratio, which estimates the amount of protein contamination, and by means of the Bioanalyzer, which displays RNA Integrity Number (RIN-values) indicating the stability of 18S and 28S rRNA.

Quantitative PCR.
A SYBR Green based quantitative PCR was performed on a Bio-Rad My-IQ detection system as described previously.[8] PCR conditions and primer sequences can be retrieved from this previous study evaluating canine reference genes for accurate quantification of relative gene expressions.

Histology

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Results

The A260/A280 ratios of all samples in this study were between 1.98 and 2.13, demonstrating that RNA purity was sufficient.

RNA isolation: RNAeasy mini kit versus Trizol
The RNAeasy mini kit isolation was compared to the Trizol mediated isolation protocol in RNAlater fixed Menghini biopsies. RNA-quality of RNA isolated with the RNAeasy mini kit was consistently superior (1 to 1.5 RIN-values higher) to RNA isolated with the Trizol method (Table 2). On the other hand, often the quantity of RNA isolated by the Trizol method was 2-5 fold higher than the quantity obtained with the RNAeasy mini kit (data not shown). Results from assessment of RNA quality prompted us to restrict further comparisons of different RNA fixation protocols to RNA isolated with the RNAeasy mini kit.

Tissue storage for RNA isolation
RNA quality was compared between four methods of biopsy fixation: snap-freezing, Boonfix, B-RLT medium, and RNAlater. Table 3 depicts a comparison for RNA quality after RNA isolation with the commercial RNAeasy mini kit. Three independent results per fixation protocol were measured combined with two different biopsy techniques (biopsy-gun and Menghini biopsy). Snap-freezing, B-RLT, and RNAlater revealed RIN-values consistently within the range required for micro-array (equal to or higher then 8). RIN-values were between 7.9 and 9.3. A slight tendency for higher RIN-values for blind biopsies compared to True-cut biopsies. Since the RNA isolated from liver tissue fixed in Boonfix had RIN-values below 8 (range 7.1-8.1), we excluded Boonfix from further molecular analysis, yet a RIN-value of 7.0 is still considered valuable for Q-PCR testing.

Biopsy technique
RIN-values of biopsy-gun derived RNA were slightly lower then biopsies retrieved by the Menghini technique. The difference in RIN-values was around 1.
The effect of the solution used during the Menghini technique on RNA quality was evaluated in RNAlater preserved/RNAeasy mini kit isolated material. The use of Menghini water was compared to Menghini NaCl. Biopsies for this comparison were retrieved from surplus tissue obtained from one research dog, allowing both measurements of RNA quality and quantity. The RNA yield of Menghini NaCl was more than 5 fold higher than Menghini water. The RNA quality however was comparable (RIN-values above 8). Comparison of RNA quality obtained from biopsies of patients also revealed superior quality of Menghini NaCl biopsies compared to Menghini water sampling (RIN-values up to 8.8 compared to around RIN-values of 6 respectively).

Fixation time
For liver tissue kept in RNAlater additional comparisons were made to reveal a possible influence of the time interval from biopsy retrieval to carry over to the preservative. Time lags of 15, 20, 25, and 30 minutes between biopsy retrieval with the Menghini NaCl method and complete enclosing of the biopsy with RNAlater did not affect RNA quality or quantity.
In addition freezing of liver biopsies kept in RNAlater at minus 20 °C for a period of up to 18 months did not affect RNA quality or quantity.

Gene expression

The optimal number of reference genes for normalization for both Menghini biopsy techniques was determined using the GeNorm program[x]. This analysis was in strong favor for Menghini NaCl above Menghini water. As little as two reference genes (e.g. HPRT, GAPDH or RPS5) gave reliable quantitative data, whereas six or more were needed for water based Menghini biopsies.

Together, the RNA quality was best guaranteed by the combination of a Menghini biopsy in NaCl, followed by RNAlater preservation and RNAeasy mini kit extraction.

Histology

Three different fixation protocols designed for histological studies were compared. Boonfix, RNAlater, and 10% neutral buffered formaldehyde (formalin) fixed samples.

Histological evaluation of 24 hrs formalin fixed wedge biopsies revealed normal liver histology in dogs #1-#4, and control dog #8. Examination of biopsies from dog #5 revealed chronic passive congestion with centrolobular hepatocellular atrophy and a severe non-specific reactive hepatitis. Dogs #6 and #7 had normal hepatic architecture with moderate hepatocellular yellow-brown pigment granulation (copper) in zone III and II and in dispersed Kupffer cells. Hepatitis was not present. Positive copper control dog #9 had severe chronic active hepatitis with a copper score of 5+ (maximal copper accumulation).

HE staining was consistent in all formalin fixed slides regardless of duration of the fixation, which varied from 1 hr to 5 days. There was well preserved tissue architecture, cellular morphology and detail (Fig. 1). Delay of fixation by 30 min. storage in NaCl 0,9% did not show to have any effect. In Boonfix preservative, independent of fixation time, the tissue was well conserved with mild cellular pronounciation, and a mildly enhanced eosinophilic cellular appearance of all cells save erythrocytes which manifested as non-reacting shadows (Fig. 2). Pigmentation in hepatocytes and Kupffer cells was comparable to that seen after formalin fixation. Insufficient tissue preservation occurred centrally in the RNAlater fixed biopsies. Here, cellular borders were ill-defined accompanied by strong eosinophilia and shrinkage of hepatocytes with condensed nuclear chromatin (pycnotic nuclei) and widened sinusoids also containing cells with pycnotic nuclei (Fig. 3). In the well preserved periphery of the biopsy, pigment granules (ceroid/ lipofuscin) in hepatocytes and Kupffer cells appeared similar as in formalin fixation. Storage in minus 20°C did not alter the appearance for Boonfix or RNAlater treated tissue sections.

Reticulin staining accentuated the interstitial reticulin fibres strongly and uniformly in all formalin fixed slides, irrespective of the duration of fixation or delay of fixation by storage for up to 30 min in 0.9% NaCl. Boonfix treated slides stained similarly. In RNAlater, histomorphologic changes in the central core were blemished as described above. In the well preserved periphery of the sections reticulin fibers stained strongly.

Copper staining

Rhodanine stained wedge biopsies of dog #9 copper-associated hepatitis displayed intensely stained red copper granules in the hepatocellular cytoplasm and Kupffer cells. However, in formalin fixed and RNAlater treated Menghini biopsies (dog #6) copper granules stained yellow-brown to faintly red, so no reliable differentiation with lipofuscin pigment was possible. Boonfix treated biopsies exhibited only yellowish copper granules.

In standard rubeanic acid staining many positive black copper granules were present in the hepatocellular cytoplasm and in Kupffer cells of the positive formalin fixed control wedge biopsy (dog #9, Fig. 4). Copper granules in the biopsies of dog #6 stained positive (black) in formalin fixation, but appeared yellowish in both Boonfix (Fig. 5) and RNAlater treated sections, thus differentiation with lipofuscin granules was not possible. Previous washing in formalin did not change the appearance of the positive control or of the specimens. However, previous treatment with HCl rendered all tested sections negative, including the positive control.

CK-7

Formalin fixed sections showed specific brown, granular cytoplasmic staining of cholangiocytes and periportal progenitor cells with negligible background staining, comparable to previous canine studies (Fig. 6).[16,17] Strongest intensity appeared centrally in the 24 hrs fixed wedge biopsy, with a prominent

Hepar1

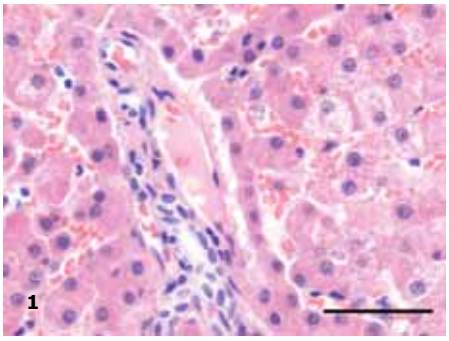
MRP-2

Discussion

In search for an easy-to-use method to acquire, fix and store canine liver biopsies, we used the stability of 18S and 28S rRNA as markers for total RNA and mRNA stability. Histological evaluation was based on HE, reticulin, rhodanine, rubeanic acid staining and three different immunostainings.
RNA quality was best guaranteed by the combination of a Menghini biopsy with NaCl, followed by RNAlater preservation and RNAeasy mini kit extraction. Under optimal biopsy conditions (as was the case for the surplus dog used to compare Menghini-NaCl and Menghini-water in one single dog), no differences in RIN-values between the two techniques were observed. Whether this reflects the fact that exactly the same liver was used, or whether time delay between the biopsy and the actual RNAlater storage as it may occur under clinical situations causes this difference remains unknown. In favor for the first explanation accounts that in the clinical setting the difference was consistent over a large number of biopsies. The evaluation of the optimal number of reference genes needed to obtain reliable data strengthened the observation that the combination of a Menghini-NaCl biopsy followed by RNAlater preservation and an RNAeasy mini kit extraction yield optimal quality canine liver biopsies. The size of the biopsy needle used in this study was based on a previous study on rat liver biopsy techniques, and turned out to be an optimal balance between quantity and quality of the biopsy and the health risks for the animal.[12] This approach of RNA retrieval proved to be a rapid and feasible method for storage for further molecular analysis, and is in agreement with the findings of others for yeast or human tissues.[18]
The quality of the obtained RNA in our approach was feasible for micro-array analysis, which requires the highest possible RNA quality, preferential a RIN value above 8.0. Unfortunately our results show that optimal RNA stabilization was only achieved with media that were unsuitable for histology or immunohistochemistry. HE-staining, and reticulin staining turned out to be insufficiently discriminative in RNAlater. Furthermore for the antibodies tested either the background staining was too high or central staining appeared very poor. The best fixative for immunohistochemistry was fixation of 10% neutral buffered formalin, which was replaced by ethanol after a relatively short fixation period of 2-4 hrs.

The quantity of RNA isolated by use of the Trizol method was 2-5 fold higher than the quantity obtained with the RNAeasy mini kit. However, since the initial amount of liver tissue was not standardized by us, such comparison of yields can be misleading. A more reliable comparison of results from assessment of RNA quality prompted us to restrict further comparisons of different RNA fixation protocols to RNA isolated with the RNAeasy mini kit.

In our experience staining artefacts more frequently occur in small formalin fixed paraffin embedded biopsies. We hypothesized that in the relatively small biopsies overfixation could easily occur. Therefore an effect of the duration of formalin fixation was assessed with subsequent immunohistochemical evaluation of antibodies to proteins at three different (sub)-cellular locations in addition to routine histological staining methods.
Differences of the immunohistochemical reactivity for all three antibodies were found between wedge biopsies and the smaller Menghini tissue samples in this study (Fig. 7). The observation was most pronounced in MRP-2 stained slides where only a very weak signal was evoked in the smaller biopsies. In addition prolonged fixation in formalin caused a signal reduction for CK-7, but did not affect routine HE and reticulin staining (Fig. 1). The difference is most likely due to changes in epitopes required for immunohistochemistry, but less for routine HE and reticulin staining
Indications for possible overfixation by formalin were present in CK-7 and possibly in MRP2 staining. Signal reduction in CK-7 stained biopsies was associated with increased fixation time and was also present in the periphery of wedge biopsies (24 hrs and 5 days fixation). In both situations, prolonged exposure to formalin could explain epitope masking due to protein cross linking of the tissues antigens. Consequently, this antigen masking could result in decreased antigen-antibody reactivity. Occurrence and intensity of this effect will vary per antibody as not all epitopes will be affected similarly [19].
Formalin fixation proved necessary for assessment of copper accumulation in liver tissue. Routine rubeanic acid staining was sufficient in a wedge biopsy (24 hrs) as well as in a Menghini biopsy (8 hrs). Reliable rhodanine staining was limited to one wedge biopsy only. RNAlater or Boonfix treated slides did



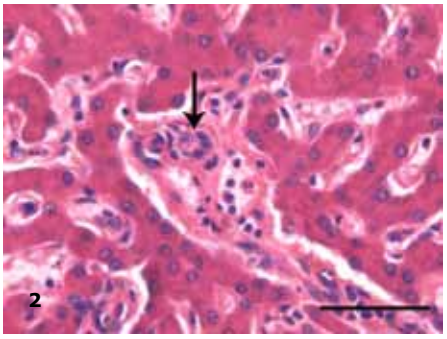
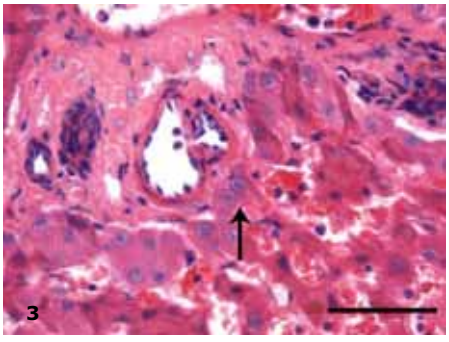


Fig. 1 *Normal liver, dog #1, portal area and periportal parenchyma. The tissue architecture is well preserved, with good contrast and sufficient cellular morphology reflected in distinct cellular and nuclear membranes, and sufficient cytoplasmic details. Needle biopsy, 1 h formalin fixation, HE staining, bar 50 micron.* ***Fig. 2*** *Normal liver, dog #5, portal area with bile duct (arrow) and periportal parenchyma. The tissue is well conserved, and there is mild cellular pronounciation and slightly enhanced eosinophilic appearance of all cells save erythrocytes. Needle biopsy, 8 hrs Boonfix fixation at room temperature, HE staining, bar 50 micron.*



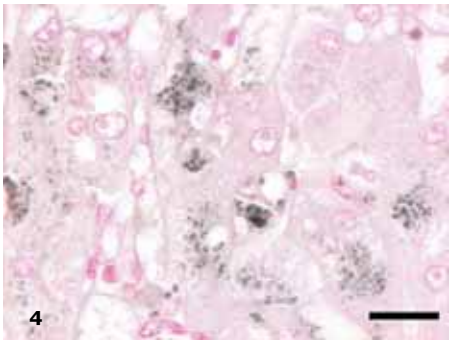
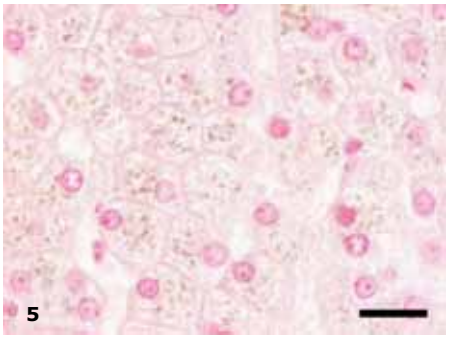


Fig. 3 *Normal liver, dog #5, portal area and periportal parenchyma. Insufficient conservation of tissue architecture in the central part of the biopsy, to the right hand side of the arrow, with ill defined cellular borders, strong eosinophilia and shrinkage of hepatocytes, pycnotic nuclei and artificially widened sinusoids. Needle biopsy, 8 hrs RNAlater fixation at room temperature, HE staining, bar 50 micron.* ***Fig. 4*** *Copper related chronic active hepatitis, dog #9, parenchyma, control tissue. Many, intensely taining copper granules appear in the cytoplasm of hepatocytes and Kupffer cells. Wedge biopsy, 24 hrs formalin fixation, rubeanic acid staining, bar 50 micron.*



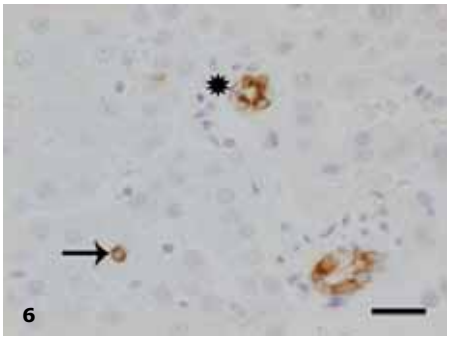
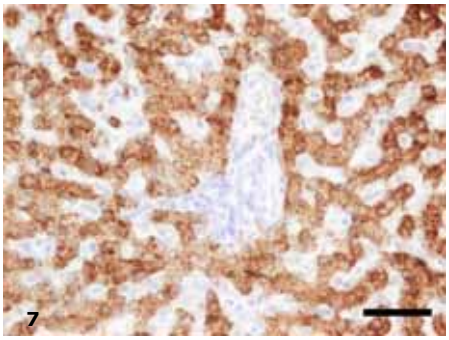


Fig. 5 *Liver with copper storage, dog #6, parenchyma. Intracytoplasmic copper granules stain yellow-brown to faintly red, therefore no reliable differentiation between copper and lipofuscin granules can be made. Needle biopsy, 8 hrs formalin fixation, rhodanine staining, bar 50 micron.* ***Fig. 6*** *Normal liver, dog #2, portal area and periportal parenchyma. Cholangiocytes in the portal tract (asterisk) display a strong signal (brown) in the cytoplasm with negligible aspecific background staining. Also, the parenchyma contains one small, isolated positive periportal cell (arrow), interpreted as a progenitor cell. Needle biopsy, 1 h formalin fixation, CK-7 immunohistochemistry, bar 20 micron.*



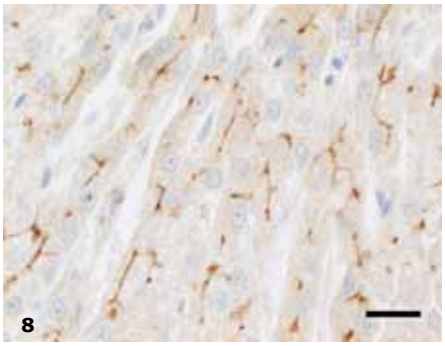


Fig. 7 *Normal liver, dog #5, portal area and periportal parenchyma. All hepatocytes feature strong cytoplasmic reactivity, all other cells are negative. Needle biopsy, 1 h formalin fixation, Hepar1 immunostaining, bar 50 micron.* ***Fig.8*** *Normal liver, dog #8, parenchyma, control tissue. Strong signal (brown) is elicited along the canalicular membranes of all hepatocytes, insignificant background staining. Wedge biopsy, 24 hrs formalin fixation, MRP-2 immunostaining, bar 20 micron.*

Table 1: Animals

animal#	age	sex	breed	remarks
1	1y 8m	f	Labrador retriever	surplus tissue unrelated research
2	1y 8m	m	Labrador retriever	surplus tissue unrelated research
3	1y 8m	m	Labrador retriever	surplus tissue unrelated research
4	5y 10m	f	Labrador retriever	surplus tissue unrelated research
5	3y	m	Beagle	surplus tissue unrelated research
6	6y	f*	Labrador retriever	patient, copper storage no hepatitis
7	2y	f	Mongrel	surplus tissue unrelated research
8	1y	m	Beagle	surplus tissue unrelated research
9	1y 6m	m	Dobermann Pinscher	patient, copper related hepatitis

Table 2. RIN-values after RNA isolation with RNAeasy mini kit or Trizol method

RNAeasy	Trizol
8.1	7.3
8.8	7.4
8.2	6.7

Data of three independent representative isolations. Biopsy was taken with the Menghini technique, RNA was stored in RNAlater. Independent samples were split and divided over the two isolation procedures

Table 3. RIN-values after RNA isolation with RNAeasy kit after different fixation protocols

	minus 70	Boonfix	B-RLT	RNAlater
Biopsy gun	7.9	7.0	8.7	9.2
(dry)	8.7	7.3	8.6	8.5
	8.4	7.2	8.2	8.6
Menghini	8.1	8.1	9.1	9.1
(NaCl)	9.1	7.4	9.3	9.2
	9.0	7.1	9.0	8.5

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[i] Pro-Mag Ultra Automatic Biopsy Instrument, Manan Medical Products, PBN Medicals, Stenløse, Denmark. (22-mm stroke, serial no. 6527)
[ii] RNAlater, Applied Biosystems, Nieuwerkerk a/d lJssel , The Netherlands
[iii] Boonfix, Finetec. CO., Ltd. Tokyo, 183-0044 Japan
[iv] RLT-buffer, QIAGEN, 5911 KJ Venlo, The Netherlands Wodt de afkorting RLT niet toegelicht door de fabrikant?
[v] RNAeasy mini kit, QIAGEN, 5911 KJ Venlo, The Netherlands
[vi] Trizol, Invitrogen BV De Schelp 26 9351 NV Leek, The Netherlands
[vii] Nanodrop, Isogen Life Science, IJsselstein, the Netherlands
[viii] Bioanalyzer, Agilent Technologies Netherlands B.V., Amstelveen, the Netherlands
[ix] iScript cDNA synthesis kit, Bio-rad, Veenendaal, the Netherlands
[x] http://medgen.ugent.be/~jvdesomp/genorm

Discussion and Conclusions

Discussion and Conclusions

Part 1: Chapter 3 – 5: Description of a new disease.

Our approach to define the phenotype of copper-associated chronic hepatitis correctly was threefold.
A. We confirmed the previously described normal reference for hepatic copper concentrations in dogs by establishment of a breed specific normal reference range for Labrador retrievers.
B. We assumed a certain degree of possible phenocopies from age-dependant mild copper accumulation, which has previously been described.[1] Furthermore the histological localization of copper within the centre of the liver lobule was included in the definition of the phenotype. This localization is generally accepted as an indicator for primary copper accumulation[2].
C. We aimed to exclude the influence of environmental factors on the phenotype of the disease by examining affected and normal dogs that were equally distributed throughout the Netherlands, and therefore were considered to receive comparable amounts of copper with their drinking water. In addition did recording of the dietary intake of the dogs reveal comparable amounts of copper in the diets of both groups.
The localization of accumulating copper in the centrolobular region of the liver lobule as it was seen in our study of Labrador retrievers is generally considered an indicator for primary copper accumulation.[2] This consideration was supported by a study of Spee and his colleagues, who were able to find clear distinction criteria to determine whether copper-accumulation is primary or secondary to hepatitis.[3] From their comparison of gene expressions the authors concluded that zonal copper concentrations of the magnitude of the affected Labradors in our study exclusively occur in primary copper storage diseases.
Due to the fact that not all dogs with hepatic copper accumulation had hepatitis, but some developed hepatitis over time, our data suggested that dogs with the disorder may be subject to a prolonged delay period of several years between severe accumulation of copper and development of histologic signs of inflammation. Exhaustion of cellular systems that protect against oxidative damage by occasional free reactive copper, or an additional stress factor that affects other protective mechanisms of the liver cell, may be necessary for copper to cause hepatitis in affected dogs.
Male gender appeared to protect dogs from hepatic copper accumulation. The gender difference was independent from spaying or neutering of the animals. Although further studies seem warranted to confirm our result, because the effect of spaying and neutering approached significance ($p=0.06$). Based on our results at this point it seems unlikely that sex-hormones are involved in the etiopathogenesis of hepatic copper accumulation. It seemed more likely that an explanation for higher copper concentrations in female dogs may be found on the level of DNA, gene expression, or protein function. On the level of DNA higher copper concentrations in female dogs could be explained by a protective gene on the Y chomosome. On the level of gene expression sexual dimorphism could occur, which has recently been shown to develop in liver tissue.[4] Our finding of a difference of copper concentrations with different coat colors was suspected to be an artificial result, because the finding was not true when dogs within one family were examined. The analysis of co-seggregation of the high copper phenotype and coat color within families did not confirm a protective effect of black coat color. Most likely preference of a breeder for certain coat colors in the examined families biased our results. Therefore the investigation of more dogs from more families is advisable. Should a protective effect of the black coat color be confirmed a possible explanation could be co-seggregation of the genes responsible for black coat color with a protective gene against copper accumulation. Likewise a co-seggregation of genes responsible for yellow coat color with the gene responsible for hepatic copper accumulation would be possible. Alternatively one of the copper excretory pathways might be compensating more effectively in black Labradors to prevent copper from accumulating. In mammals, the Menkes protein (ATP7A) has been shown to supply copper to the pigment-forming enzyme tyrosinase within the secretory pathway[5]. Tyrosinase is a copper containing enzyme, which catalyzes the conversion of tyrosine to melanin. Hypothetically high activities of tyrosinase in black colored Labradors might indirectly shuttle more copper away from the liver cell.

Conclusion from part 1:
The findings of our studies suggest that copper-associated chronic hepatitis of Labradors is a liver disease of adult dogs, which is caused by an inherited defect of hepatic copper metabolism. The presenting complaints are non-specific and occur late in the disease process. Male gender appears to protect dogs from hepatic copper accumulation. A protective effect of black coat color awaits further confirmation.

Remaining questions from part 1:
Although 143 dogs were studied prospectively, this remains a relatively small group size and unequal distribution of dogs to different families of certain coat colors might have biased the results. Therefore the investigation of more dogs from more families is advisable for confirmation of the results.
Broader studies are indicated to assess for the incidence of CACH in Labradors of the Netherlands, as well as other countries. We have recently initiated international cooperations and started to investigate liver biopsies from abroad. A pilot study of the first group of dogs from Texas revealed that about 50% of liver biopsies from Labradors with hepatitis had a copper-associated disease (manuscript in preparation).

Part 2 : **Chapter 6 and 7**
Randomized, double-blinded, placebo-controlled treatment studies.

Our decision to treat hepatic copper accumulation prior to the occurrence of hepatic inflammation was based on the observation that copper accumulation precedes the development of hepatitis. A placebo-controlled treatment design was ethically justifiable in this setting, where clinical symptoms of disease were not apparent.
A limitation of our decision to treat hepatic copper accumulation prior to the development of clinical symptoms from liver damage is that our results do not inform about clinical parameters of improvement during treatment.

Penicillamine
The most interesting finding of a treatment trial with penicillamine was that the primary decoppering effect of the drug was supported by our results. Previous reports on penicillamine treatment for advanced copper-accumulating liver diseases raised the question whether the decoppering action of penicillamine is the primary cause of treatment success, or whether the beneficial effect of penicillamine is due to the drug's anti-inflammatory properties[6-12]. In our study a large number of dogs showed minor histologic changes of inflammation at the initiation of penicillamine treatment. Thus inflammation could never be the root cause for copper accumulation in these cases. Hence our study results support that the decoppering effect of penicillamine is the primary cause of an effective treatment with this drug.

Low copper diet and zinc
When dogs with copper accumulation were fed a low-copper diet, copper decreased and stabilized during the assessed time interval within the normal range, with no further decrease in liver copper concentrations between the first and the second control examination which were 8 months apart. A possible explanation for a stabilization of the hepatic copper content might be that the provided diet was not completely devoid of copper, but still contained sufficient maintenance amounts of the metal. Furthermore owner compliance might have decreased after the first control examination which showed that copper concentrations were in the normal range. Owners might have been more tempted to give copper-rich foods as treats to their pets that appeared clinically healthy at this time.
Low-copper diets commercially available for dogs also contain zinc. Zinc has been used for treatment of copper storage disorders in dogs. Plasma zinc concentrations described to suppress copper uptake in dogs are higher than the zinc concentrations achievable with diet alone[13]. Therefore the de-coppering effect of the diet was likely due to the low copper content, which might have worked in concert with a direct protective effect of zinc against oxidative stress in liver cells. Zinc has been shown to induce metallothionein in hepatocytes, thereby binding free copper[14]. Although hepatic copper concentrations

would not directly decrease in this scenario, it is possible that normal cell turnover led to regeneration of liver tissue which did not accumulate copper, because the metal was low in the diet.

Conclusion from part 2:
We expect dogs with elevated hepatic copper concentrations to be at high risk for development of chronic hepatitis and finally cirrhosis, which are potentially irreversible disorders. Our results show that D-penicillamine is an effective first-line treatment to decrease hepatic copper concentrations in Labrador retrievers. Based on our results we recommend dietary management with a low-copper diet against re-accumulation of copper. Adjunctive treatment with zinc does not amplify the de-coppering effect.

Remaining questions from part 2:
The copper-reducing effect demonstrated by our results was most likely due to the low copper content of the diet because previous treatment with penicillamine is unlikely to have a long-lasting action and dogs treated with penicillamine and fed on regular diets with comparable zinc concentrations to the diet used in our study were affected by increasingly elevated hepatic copper levels. However, further studies are necessary to predict if prophylactic dietary management can prevent copper accumulation, and if immunologic adverse effects from penicillamine occur in dogs. Furthermore investigations should address whether copper or iron deficiency might be induced by longterm therapy with penicillamine or diet. Furthermore discussion of the possibility of contamination with other toxins from the diet or the environment might be a point to address in future research.[15,16] Although toxins lead to typical histopathological changes that were not seen in biopsies of Labradors of our study, exposure to toxins might be a theoretical alternative why dietary management with a high quality, low copper diet might have such a prominent de-coppering effect.

Part 3 : Chapter 8 – 13
Molecular genetic studies

Our investigations of CACH in Labradors addressed the recommended questions posed by Burton et al. (introduction of this thesis):

1. What is the phenotype underlying CACH in Labrador retrievers
   Approach: Investigation of the clinical disease, and breed specific normal values (chapter 3 & 4)

2. Is there evidence for aggregation/segregation of the phenotype within families?
   Approach: Family studies (chapter 3 & 4)

3. Is the pattern of aggregation/segregation consistent with the effect of genes?
   Approach: Estimation of heritability (chapter 5)

4. Are haplotypes of known genes involved in the phenotype ?
   Approach: Investigation of candidate genes on the level of DNA-marker analysis & sequencing, as well as mRNA expression measurements (chapter 8 & 9)

5. Where in the genome is a causative gene most likely located ?
   Approach: Linkage studies within extended families
   (chapter 10)

6. Can we be more precise about the location?
   Approach: association studies, haplotype analysis (chapter 10)

7. Are genes in this region affected with respect to their mRNA expression?
   Approach: Gene expression measurements/microarrays (chapter 12)

Phenotyping
Molecular genetic techniques available today, as well as easy access to the compelete dog genome allow for relatively easy and quick assessment of single gene disorders. The remaining difficulties from the view of a researching clinician working on a new disease consists in
A: The correct and precise classification of a disease phenotype as well as the definition of "normal" in order to allow for correct distinction between the two groups.
B: The group size of the study population.
The difficulty of a precise and correct definition of phenotypes for diseases has been reviewed by others.[17,18]
Our results show that the investigated candidate genes are intact at the level of genomic DNA, as well as at the transcriptional level in Labrador retrievers with copper-associated chronic hepatitis. Moreover genome wide linkage analysis did not reveal one single phenotype-associated locus but three. This finding may suggest a multiple gene disorder in the Labrador. Support for this suggestion might be found in the fact that even though we investigated large families of dogs over several generations, our results did not allow us to reveal the inheritance pattern of copper-associated chronic hepatitis in Labradors. The inheritance of the diorder does not appear to follow a simple Mendelian pattern. Further support for the presence of a multiple gene disease or a complex aetiology arises from the similarities between canine copper storage diseases and unresolved disorders of copper metabolism in people. For these diseases (Indian childhood cirrhosis and non-indian childhood cirrhosis) a complex ethiology is suggested, with influencing factors from the environment, like high copper intake.[19-22] Our diet trial revealed that copper concentrations in the diet have an effect on the disease in Labradors.

Microarray

In search for new candidate genes involved in hepatic copper metabolism and protection against oxidative damage from copper we examined the time-dependent changes in genome-wide gene expression profiles of canine liver cells during exposure to copper. The arrays revealed consistent changes in gene expression of the P-type ATPase *ATP11B.* So far literature about this gene is scarce, but our results seem promising, because the gene belongs to the family of *AT*P7B and ATP7A, the genes involved in the copper storage disorders Wilson's disease and Menkes disease. Amongst 2 other genes that showed consistend significant increases in gene expression was the gene for Histone deacetylase 6. Inhibition of the function of this protein has been brought in context with oxidative damage from copper toxicity, and was suggested to be a potential new strategy for the treatment of copper-overload diseases.[23] In the dog this gene is located on the X chromosome. The predisposition of female dogs for copper associated liver diseases (Labradors and Doberman pinschers) might be brought in context with an upregulated function of this gene or gene products in future studies.

Conclusion from part 3:

We identified genetic loci through linkage studies, and genes through gene expression measurement that are new candidate regions for involvement in copper metabolism. Most likely new genes, which are currently unknown to be involved in copper metabolism, will be discovered with a further genetic workup of the disease in the Labrador and other dog breeds.

Outview and remaining questions from part 3:

We will perform quantitative trait statistics to solve the genetic basis of copper-associated chronic hepatitis in the Labrador and the Doberman pinscher. Such analysis requires large groups of healthy and affected dogs and preferably inclusion of all influencing factors in the analysis.

Moreover in our future research we will identify candidate genes located in the regions of linkage for further investigations, and we will begin to address the functionality of the newly found candidate genes in copper homeostasis.

General discussion of the context of this project

Well characterized single gene disorders of copper metabolism include Wilson's disease in people and copper toxicosis in Bedlington terriers.[24,25] In these disorders a single mutated gene leads to severely impaired biliary excretion of copper, resulting in chronic hepatitis (ATP7B in Wilson's disease, and COMMD1 in copper toxicosis of Bedlington terriers). With this work a single gene disorder of a known step in copper metabolism was excluded as aetiology for the copper storage disorder in Labradors.
Other copper accumulating liver diseases like indian childhood cirrhosis and non-indian childhood cirrhosis in people lack the simple inheritance patterns of single gene diseases. The characterization of these complex disorders is more difficult, because the phenotype is assumed to be caused by the interaction of multiple genes that concur concurrently with exposure to environmental risk factors, like high copper intake. Cellular copper metabolism has been described to be highly conserved between different species.[19,20,26] Nevertheless compared to humans dogs have a much higher normal reference range for hepatic copper.[27-31]
Dietary treatment trials at the University of Utrecht revealed an excellent treatment response of dogs with hepatic copper accumulation to feeding of a copper-restricted diet (chapter 7). Therefore we think that it is possible that nutritional contents of copper may have an impact on the higher reference values in dogs, as well as on the development of the disease. In addition to the results of our study a recent investigation of the general dog population in the Netherlands revealed elevated hepatic copper concentrations in liver tissue in a third of all patients with chronic hepatitis (Podervaart/Favier, abstract ECVIM meeting 2007, Hungary, publications in preparation). It may be that normal dogs can compensate for relatively high copper concentrations in their diet, but that affected dogs are genetically more sensitive to copper loading. We think that the results of our study, as well as the study of Poldervaart et al may be relevant for the food industry, because guidelines for minimal and maximum copper concentrations in pet diets, as well as in human nutrition are based on limited research data. Stern and his colleagues from the R. Samuel McLaughlin Center for Population Health Risk assessment, in collaboration with the International Copper association recently analysed the existing data on copper excess and deficiency, and stated that research in this field is needed for reliable modelling of minimum requirements of the trace element, as well as safe upper intake levels for humans and other species.[32] The authors described the difficulty of an exact definition of a safe range of the metal in food and water, that fulfils minimum requirements and avoids toxicity in all groups of normal individuals, as well as individuals which are more susceptible to excess or deficiency, because studies investigating both endpoints are pending. Similar difficulties have been encountered by the European Commissions for Health and Consumer Protection directorate in a recent report, as well as the Committee on Copper in Drinking Water of the National Research Council.[33]
Our results are likely to provide some additional insight into copper metabolism, and we think that the results of our trials will provide a model for additional understanding of hepatobiliary transport of copper during excess and scarcity, which has been assumed to behave in a non-linear fashion, and depend upon the loading of the liver with the metal.[34]
To attain correct guidelines for minimal and maximum copper concentrations in food and water also seems important in view of the possible influence on development of chronic diseases in general. Different studies about oxidative stress from copper accumulation, as well as DNA damage induced by copper which can be a precursors of cancer formation have been reviewed recently.[35,36]
We think that the results of our ongoing genetic work will likely discover new genes of copper metabolism. Thus we will probably soon be able to answer the question "Are there additional copper homeostatic genes ?" that was recently posed by the International Copper association with a clear "Yes, there are !".[32]

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Summary
Zamenvatting
Zusammenfassung
Résumé

Summary

Part 1: Chapter 3 – 5
Description of a new disease.

The objective of part 1 of this thesis was to investigate and describe a new breed related hepatic copper storage disease clinically, as well as histopathologically, and to reveal the heritability of copper accumulation as an inherited trait in the Labrador retriever. These investigations were based on the hypotheses that copper-associated chronic hepatitis in the Labrador is caused by an inherited genetic defect of the hepatic copper metabolism.
Initially the clinical and histopathological presentation of the disease was described in a retrospective case series of Labradors with CACH. The average age of the patients was 7 years, and the most common clinical signs were anorexia, vomiting, and weight loss. Due to a greater relative increase in ALT activity compared to ALP, a primary hepatocellular liver disease was more likely than a cholestatic disease. The clinical and pathological features of the disease were comparable to inherited copper storage diseases of other dog breeds (Bedlington terrier, West Highland White Terrier, Dalmatian, Doberman Pinscher). In all biopsy samples of the investigated dogs, including the prospective examination of family members of affected dogs, hepatocytes that stained positive for copper were always localized in zone 3 (ie, centrolobular). This localization of copper in the centrolobular region of the liver lobule is a strong indicator for primary copper accumulation.
A breed specific normal reference range established from examination results of normal, healthy, unrelated Labrador retrievers (normal below 400mg/kg dry weight) was in agreement with normal reference ranges of other dog breeds. Prospective examination of family members of dogs with CACH identified subclinical hepatic copper accumulation (mean hepatic copper concentration of 1,317 µg/g dwl; range, 402–2,576 µg/g dwl).
The suggestion that copper-associated chronic hepatitis is familial and most likely caused by an inherited genetic defect in the Labrador Retriever was confirmed in a larger study were 143 Labrador retrievers were prospectively assessed for clinical parameters, laboratory results, and liver copper concentrations, as well as histologic signs of inflammation. The results of this larger study revealed that more than two thirds of family members from dogs with CACH have liver copper concentrations above the normal reference range. In addition almost half of the dogs with elevated hepatic copper concentrations have chronic hepatitis. Environmental factors for the accumulation of copper were excluded, because affected and unaffected dogs were equally distributed throughout the Netherlands, and all dogs were fed commercial diets containing comparable concentrations of copper and zinc.
Male gender and black coat color appeared to protect dogs from hepatic copper accumulation, and heritability estimates for copper accumulation ranged between 0.39 and 0.85. The gender difference was independent upon spaying or neutering.

Part 2 : **Chapter 6 and 7**
Randomized, double-blinded, placebo-controlled treatment studies.

Affected Labradors may be subject to a prolonged first delay period of several years between severe accumulation of copper and histological detection of inflammation, as well as a prolonged second delay period between histological detection of inflammation and occurrence of clinical symptoms of the disease.

Penicillamine
The results of this study showed that D-penicillamine is an effective drug to decrease hepatic copper concentrations in affected Labrador retrievers. Based on these results we recommend treatment with penicillamine against hepatic copper accumulation, even before clinical symptoms of disease occur.
Although penicillamine treatment was effective to decrease copper significantly, there are strong individual differences with respect to the decoppering effect of the drug. Therefore at the end of therapy liver copper concentrations were still above the normal reference range of 400mg/kg dry weight in many

dogs. Furthermore due to the fact that CACH is probably caused by an inherited genetic defect lifelong cure cannot be achieved with a single treatment regimen, and re-accumulation of copper is likely.
In order to provide patients with CACH with a more balanced long term control of hepatic copper concentrations the aim of our diet trial was to investigate whether dietary management is effective to influence hepatic copper concentrations in Labradors after treatment with penicillamine, and whether additional treatment with zinc is useful.

Low copper diet and zinc
The most interesting findings of our diet trial were that hepatic copper concentrations decreased significantly into the normal range in all dogs, and that adjunctive treatment with zinc did not amplify the de-coppering effect of the diet. Once within the normal range copper stabilized during the assessed time interval with no further decrease in liver copper concentrations between the first and the second control examination which were 8 months apart.

Part 3 : Chapter 8 – 13
Molecular genetic studies

Our first molecular biologic approach was the investigation of 14 candidate genes in hepatic copper accumulation in 60 Labrador retrievers and 32 Doberman pinschers. Doberman pinschers were included in the study because our investigations started before the dog genome was completely sequenced. At this point in time assessment of candidate genes was more time intensive than it is today (e.g. use of BAC clones). DNA of phenotyped Doberman pinschers was available from previous investigations of Dr. Mandigers and allowed us to investigate for the presence of DNA markers around candidate genes, as well as sequencing analysis, while retrieval of DNA and phenotyping was ongoing in the Labradors.

Phenotyping
The phenotype was defined by elevated hepatic copper concentrations (normal below 400mg/kg, affected above 600mg/kg, exclusion of 400-600mg/kg), as well as the histological localization of copper within the liver lobule.

Genotyping
Microsatellite markers were genotyped for analysis of candidate genes and for genome wide genotyping. In the Labradors these markers were tested for association and linkage. In the Doberman pinschers an association study was performed. Genes for which genotyping of one microsatellite marker approached a difference between groups, were further investigated. These genes were sequenced, and gene expression was measured by quantitative PCR. Our study excluded all investigated candidate genes from involvement in hepatic copper accumulation, under the condition that a single disease gene exists.
For a genome wide genotyping approach 289 microsatellite markers were genotyped, and 174 of these markers were informative and tested for linkage in the family of 31 dogs. This study revealed 3 regions of possible linkage, with NPL scores of 4 and 3.7 on chromosome 7, and 3.8 on chromosome 28. Fine-mapping in these regions confirmed the high NPL scores and decreased the size of the genomic region in linkage with hepatic copper accumulation to a length of about 2 Mb each.

Microarray
Two canine hepatic cell lines were exposed to elevated copper concentrations. Total RNA isolated at different time points after copper exposure was hybridised to an Affymetrix custom-designed 44K canine array. Two genes were differentially expressed in both cell lines and at different time points after exposure to copper, and 3 genes were differentially expressed at different time points within one cell line. We identified 5 novel genes (ATP11B, ELYS, CRIM, HDAC, ANKRD) that may represent an acute response to copper overload.

Discussion

In the discussion the findings of our studies are discussed and an attempt is made to put research on copper metabolism in a general context.

Dietary treatment trials at the University of Utrecht revealed an excellent treatment response of dogs with hepatic copper accumulation to feeding of a copper-restricted zinc-enriched diet (chapter 6). Therefore we think that it is possible that nutritional contents of copper may have an impact on the development of the disease. Stern and his colleagues from the R. Samuel McLaughlin Center for Population Health Risk assessment, in collaboration with the International Copper association recently described the difficulty of an exact definition of a safe range of copper in food and water, because studies investigating both endpoints are pending.[1] Similar difficulties have been encountered by the European Commissions for Health and Consumer Protection directorate in a recent report, as well as the Committee on Copper in Drinking Water of the National Research Council.[2]

Our results are likely to provide some additional insight into copper metabolism. Our ongoing genetic work will likely discover new genes of copper metabolism. Thus we will probably soon be able to answer the question "Are there additional copper homeostatic genes ?" that was recently posed by the International Copper association with a clear "Yes, there are !".[1]

1. Stern BR, M. Solioz, et al. . Copper and human health: biochemistry, genetics, and strategies for modeling dose-response relationships. J Toxicol Environ Health B Crit Rev 2007;10:157-222.
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Samenvatting in het nederlands

Koperstapeling bij de Labrador

Al sinds 1975 wordt onderzoek gedaan naar koperstapelingsziektes bij honden, en de kliniek voor gezelschapsdieren van de Universiteit Utrecht was er af het begin mee bezig. Sinds 2003 wordt nu ook intensief na de oorzaak van koperstapeling bij de Labrador gezocht.
Koperstapeling is een ziekte waarbij koper in de lever opstapelt, omdat het metaal niet op normale manier uit het lichaam kan worden verwijderd. Een teveel aan koper wordt voornamelijk in de lever opgeslagen, en daardoor wordt de lever het sterkste van alle organen aangedaan.
Te veel koper in de lever veroorzaakt oxidatieve schade van de lever. In het begin kan een dergelijk schade nog beperkt worden, omdat er in de lever veiligheidssystemen tegen oxidatieve schade aanwezig zijn. Met toename van koper gedurende jaren is het mogelijk dat deze veiligheidssystemen overbelast worden, en een ontsteking ontstaat.
Leverontsteking (= hepatitis: grieks: hepar = lever, -itis = ontsteking) kan heel verschillend verlopen. Bij mensen is het bekend dat patienten met een leverontsteking gedurende jaren helemaal klachtenvrij kunnen leven. Hetzelfde geldt ook voor de hond.
Het onderzoek over koperstapeling bij de Labrador, dat gedurende de laatste jaren in Utrecht is gedaan wordt in dit boek beschreven. Het onderzoek begon met de klinische waarneming dat vaak Labradors naar de kliniek voor gezelschapsdieren kwamen, waar naast een chronische hepatitis ook een teveel aan koper in de lever was gediagnosticeerd. Met een gebruikelijke behandeling met prednisolon kon bij deze patienten geen verbetering worden verkregen. Daarom was penicillamine gebruikt, een medicijn die de verwijdering van koper uit het lichaam ondersteund. Met dit medicijn kon een genezing van de patienten worden verkregen.

Op grond van de succesvolle behandeling met penicillamine ontstond de **hypothese van dit onderzoek, dat Labradors een raspredispositie voor chronische hepatitis op grond van koperstapeling hebben.**

Hoofdstuk 1 en 2 : Inleiding
In de eerste 2 hoofdstukken van deze thesis wordt de stofwisseling van koper in het lichaam en op cel niveau uitgelegd, en er worden koper-stofwisselingziektes beschreven. Bovendien wordt een overzicht van de verzamelde literatuur over koperstapeling bij honden gegeven.

Hoofdstuk 3 tot 5: Deel 1 – Koperstapeling in de lever, een nieuwe ziekte bij de Labrador retriever
In deze hoofdstukken wordt het klinische beeld van koperstapeling bij Labradors beschreven. Bovendien wordt een prospectief onderzoek van familieleden van Labradors met koperstapeling uitgelegd. De resultaten laten zien dat Labradors met koperstapeling gedurende jaren volledig klachtenvrij kunnen leven, tot het moment dat de honden af en toe iets minder willen eten of iets rustiger blijken dan normaal. Vaak worden deze heel geringe verschijnselen pas achteraf duidelijk, omdat er op het moment van de lichte klachten andere reden mogelijk bleken ("het weer", "de leeftijd", "iets slechts gegeten", "een virus").
Gedurende weken tot maanden kunnen tijden van minder eten of rustiger zijn afwisselen met normaal gedrag. Pas na maanden tot jaren (op een gemiddelde leeftijd van 6-8 jaar) worden de klachten duidelijker, bijvoorbeeld kwijlen en braken. Vanaf dit moment kan het verloop heel ernstig worden: misselijkheid ontstaat doordat de ontgifting van het lichaam door het filterorgaan lever gestoord is. Ook kunnen de huid, de ogen, en de mondslijmvliezen op plekken die normaal rose en wit zijn geel worden (icterus=geelzucht). Op grond van de verzameling van giften in het lichaam kan ook diarree onstaan. In levensgevaarlijke gevallen kan er een dikke buik op grond van een ophoping van vrije vloeijstof onstaan (ascites). In deze gevallen is de lever heel ernstig aangetast en niet meer in staat om belangrijke eiwitten te produceren die het bloed in de bloedvaten houden en daardoor een lekkage in het weefsel en

de buik ontstaan. De honden drinken dan ook meer dan normaal. Bovendien kan een afbraak van bloedcellen worden veroorzaakt door het vrijkomen van grote hoeveelheden koper in het bloed.

Hoofdstuk 6 en 7: Deel 2 – Gerandomiseerde, placebo-gecontrolleerde dubbelblindstudies over de behandeling van koperstapeling bij Labradors

Van meer dan 150 onderzochte honden was bij ongeveer 50 patienten koperstapeling gediagnosticeerd. De patienten worden 3-6 maanden met de medicijn penicillamine behandeld waardoor de uitscheiding van koper uit het lichaam wordt bevorderd. Penicillamine is een medicijn die ook in de humane geneeskunde wordt gebruikt. Gebaseerd op onze studieresultaten is ondertussen bekend dat penicillamine ook bij de Labrador goed werkt. Omdat de opstapeling van koper de oorzaak van een latere hepatitis kan zijn, is een vroegtijdige behandeling met penicillamine een goede preventieve maatregel.
Aansluitend aan de behandeling met penicillamine werden de Labradors met koperstapeling uitgenodigd om aan een voedingstudie deel te nemen. Bij de voedingstudie werdt gedurende 16 maanden een speciaal dieet gegeven dat weinig koper bevat (Waltham Hepatic Support). Dit dieet voer bevat minder koper dan normaal hondenvoer, waardoor de lever minder belast wordt. Bovendien bevat het voer goede hoeveelheden zinc, waardoor de opname van koper in de darmen geremd wordt.

Hoofdstuk 8 bis 13: Deel 3 – Molekulaargenetisch onderzoek:

Om de verantwoordelijke genen te vinden die koperstapeling bij de Labrador veroorzaken, wordt uit een gedeelte van een bloedmonster van iedere hond DNA geisoleerd en voor DNA-analyses gebruikt. Met behulp van een genotypeering met DNA-markers was het mogelijk om alle bekende genen als oorzaak van koperstapeling bij Labradors uit te sluiten. De resultaten van de doorlopende analyses zijn daarom ook interessant voor de humane geneeskunde, omdat wij nu ervan uit kunnen gaan, dat onze resultaten een nieuwe stap in de stofwisseling van koper zullen uitbrengen.

Hoofdstuk 14: Discussie

In de discussie wordt het voorliggende werk in een bredere context beschreven.
Gebaseerd op duidelijke success van een therapie met dieetvoer worden parallelen getrokken met humane koperstapelingsziektes (Indian childhood cirrhosis and non-indian childhood cirrhosis). Deze ziektes worden complex genoemd, omdat de oorzaak gedeeltelijk genetisch verklaarbaar is, en gedeeltelijk milieu-invloeden, zoals een hoge koperconcentratie in de voeding of het drinkwater een rol spelen. Omdat deze ziektes bij mensen zelden voorkomen is het uitermate moeilijk om het meertaal aan genen te herkennen die een verhoogde sensibiliteit tegenover koper veroorzaken. Dit is een wel herkend probleem dat nog explosiever wordt als er naar studies wordt gekeken waarin beschreven staat dat koper oxidatieve schade kan veroorzaken die ook een rol zal kunnen spelen bij het ontstaan van allerlei chronische ziektes. Zo wordt van Europesche en Amerikaanse overheidsinstanties aangegeven dat er nog steds niet voldoende studies bestaan om zekere grenswarden voor koper te bepalen. Een moeielijkheid bestaat erin dat een bepaalde minimale hoeveelheid van levensbelang is, maar een teveel aan koper toxische volgen kan hebben.
Wij denken dat de resultaten van onze vervolgstudies bij Labradors het ons mogelijk zullen maken om een bijdrage te leveren aan de beantwoording van tot nu toe nog onbeantwoorde vragen in het metabolisme van koper bij mens en hond.

Zusammenfassung

Kupferspeicherkrankheit beim Labrador

Bereits seit den 70er Jahren wird an der Kleintierklinik der Universität von Utrecht nach Kupferspeicherkrankheit bei Hunden geforscht und seit 2003 wird nun auch intensiv nach der Ursache dieser Erkrankung beim Labrador gesucht.
Kupferspeicherkrankheit ist eine Erkrankung bei der sich Kupfer im Körper ansammelt, weil es nicht normal ausgeschieden werden kann. Kupfer wird in der Leber angesammelt, weshalb die Leber am stärksten von der Erkrankung betroffen ist.
Zu viel Kupfer in der Leber führt zu oxidativem Schaden der Leber. Am Anfang kann solch ein Schaden noch begrenzt bleiben, weil in der Leber sehr viele Sicherheitssysteme gegen oxidative Schäden eingebaut sind. Bei zunehmenden Kupfermengen in der Leber, über mehrere Jahre hinweg, können diese Sicherheitssyteme jedoch überwältigt werden. Es entwickelt sich eine Entzündung.
Entzündungen der Leber (= Hepatitis: griech: hepar = Leber, -itis = Entzündung) können sehr unterschiedlich verlaufen. Auch beim Mensch ist bekannt, dass Patienten mit lebensbedrohlichen Entzündungen der Leber mehrere Jahre lang völlig beschwerdefrei leben können. Genauso ist es beim Hund.
Die Untersuchungen die in Utrecht während der vergangenen Jahre zum Thema Kupferspeicherkrankheit beim Labrador retriever stattgefunden haben, werden in der vorliegenden Dissertation beschrieben. Die vorliegenden Untersuchungen begannen mit einer klinischen Beobachtung, dass während eines Zeitraums von einigen Jahren häufig Labrador retriever in der Klinik gesehen wurden, bei denen neben einer chronischen Hepatitis auch ein zu hoher Kupfergehalt der Leber diagnostiziert wurde. Nachdem eine konventionelle Behandlung der Patienten mit Prednisolon nicht zur Heilung führte, wurde bei einigen Hunden erfolgreich Penicillamin gebraucht, ein Kupfer bindendes Medikament.

Aufgrund dieser Beobachtungen entstand die **Hypothese der vorliegenden Dissertation, dass beim Labrador retriever eine Rasseprädisposition für chronische Hepatitis aufgrund von Kupferspeicherung besteht.**

Kapitel 1 und 2 : Einleitung
In den ersten zwei Kapiteln dieser Dissertation wird der Stoffwechsel von Kupfer im Körper von Lebewesen, sowie auf zellulärer Ebene beschrieben. Zudem werden speziesübergreifend Kupferspeichererkrankungen beschrieben und erfolgt eine Űbersicht sämtlicher bisher in der veterinärmedizinischen Literatur veröffentlichten Studien der verschiedenen Kupferspeichererkrankungen bei Hunden.

Kapitel 3 bis 5: Teil 1 - Kupferspeicherkrankheit der Leber, eine neue Erkrankung beim Labrador
In diesem ersten Abschnitt der Studie wird das klinische Bild einer neuen Erkrankung beschrieben. Es wird retrospektief anhand der untersuchten Patienten dargestellt wie sich die Erkrankung präsentiert. Zudem wird die prospektieve Untersuchung von Familienangehörigen betroffener Hunde erläutert. Diese Untersuchungen haben ergeben, dass Labradore mit Kupferspeicherkrankheit jahrelang völlig normal erscheinen, bis sie beginnen ab und zu weniger zu essen oder etwas ruhiger zu sein als normal. Häufig werden diese sehr milden Anzeichen erst im Nachhinein deutlich, weil sie unauffällig sind und oft falsch gedeutet werden ("das Wetter", "ein Virus", "irgend etwas gegessen "). Über Wochen bis Monate wechseln sich Perioden von „Unlust am Essen", „Unlust an Bewegung" mit völlig normalem Verhalten ab. Erst nach Monaten bis Jahren (in einem Alter von 6 bis 8 Jahren) werden die Anzeichen deutlicher und es kann zu Speicheln und Erbrechen kommen. Von diesem Zeitpunkt an kann alles sehr schnell verlaufen: Übelkeit entsteht, weil die Entgiftung des Körpers durch das Filterorgan Leber gestört ist. Zusätzlich können gelbe Verfärbungen der Haut (an normalerweise weißen Stellen), der Augen und im Mund

entstehen (Ikterus = Gelbsucht). Aufgrund der Giftansammlungen im Körper kann es auch zu Durchfall kommen. In lebensbedrohlichen Fällen entwickeln die Hunde sogar einen dicken „Trommelbauch" aufgrund von klarer Flüssigkeit im Bauch. In diesen Fällen ist die Leber zu stark geschädigt um wichtige Eiweiße zu produzieren die das Blut zusammen halten, damit es in den Blutgefässen bleibt, anstatt in´s Gewebe zu „sickern". Die Hunde trinken eventuell auch mehr als normal. Außerdem kann es zu einer Zerstörung der Blutzellen kommen, sobald große Mengen an Kupfer aus der Leber ins Blut geschwemmt werden.
Entzündetes Gewebe der Leber kann ab einem bestimmten Stadium nicht mehr vom Körper „repariert" werden. Von diesem Moment an werden die Leberzellen durch eine dem Narbengewebe ähnliche Struktur ersetzt und es entsteht eine Leberzirrhose. Mit zunehmender Zirrhose wird die Leber klein und hart (fest wie ein Radiergummi). Die Erkrankung kommt häufiger bei Hündinnen vor als bei Rüden.

Kapitel 6 und 7: Teil 2 – Randomisierte, Plazebo-kontrollierte Doppelblindstudien zur Behandlung von Kupferansammlungen in der Leber von Labrador retrievern
Von mehr als 150 untersuchten Hunden, wird bei etwa 50 Patienten eine Kupferspeichererkrankung festgestellt. Hunde, bei denen Kupferspeicherkrankheit festgestellt wird, werden 3-6 Monate mit einem Medikament behandelt das Kupfer aus dem Körper ausscheidet. Das Medikament (Penicillamin) kommt aus der Humanmedizin. Aufgrund der Studien in Utrecht ist mittlerweile bewiesen das Penicillamin auch beim Labrador gut hilft. Da die Ansammlung von Kupfer die Ursache für eine spätere Leberentzündung ist, kann mit diesem Medikament eine Leberentzündung vermieden werden. Solch eine frühe und zuvorkommende Behandlung ist wesentlich besser als eine spätere Heilung.
Im Anschluss an die Behandlung mit Penicillamin werden die Labradore mit Kupferspeicherkrankheit eingeladen um an einer Futterstudie teil zu nehmen, bei der über den Zeitraum von 16 Monaten eine spezielle Diät gefüttert wird, die wenig Kupfer enthält (Waltham Hepatic Support). Die Diät enthält weniger Kupfer als normales Hundefutter wodurch der Hund weniger belastet wird. Zusätzlich bindet das Zink im Futter hereinkommendes Kupfer im Darm, sodass Kupfer erst gar nicht aufgenommen werden kann.

Kapitel 8 bis 13: Teil 3 – Molekulargenetische Untersuchungen:
Um die verantwortlichen Gene zu finden, die diese Erkrankung verursachen wird von allen untersuchten Hunden ein Teil des entnommenen Blutes für DNA-Analysen gebraucht. Mit den untersuchten Methoden (Genotypierung mit DNA-Markertechnologie) können alle Gene ausgeschlossen werden, die bisher bekannt sind um beim Stoffwechselgeschehen von Kupfer eine Rolle zu spielen. Darum ist diese Studie interessant für die Humanmedizin, da wir jetzt davon ausgehen können, dass die Resultate der vorliegenden Arbeit einen neuen Schritt im Kupfermetabolismus aufzeigen werden, der bisher unbekannt ist.
Im Anschluss an eine Diskussion mit zusammenfassender Schlussfolgerung finden sich im Anhang ein Dankwort, eine Publikationsliste, sowie ein Lebenslauf der Autorin, und eine Liste der im Text gebrauchten Abkürzungen.

Eindrücke von Teilnehmern:

"Mir wurde versichert, dass ich jeden Moment "Stopp!" sagen kann, falls ich es zu viel finde für meinen Hund und wir dann einfach aufhören …Das gab mir die Freiheit jederzeit selber entscheiden zu können ob ich mitmachen will - oder nicht. Außerdem fand ich es großartig die Chance zu bekommen eine eventuell vorhandene Krankheit die tödlich sein kann, früh zu erkennen und damit auch früh zu behandeln. Ich würde mir ewig Vorwürfe machen, wenn ich meinen Hund nicht untersuchen lasse und dann später -zu spät!- die Erkrankung entdeckt würde…

"Wir mussten eine halbe Stunde auf die Blutresultate warten. Wir haben geklönt und Gaby (die Tierärztin) saß währenddessen gesellig auf dem Fußboden und hat unsere Bliss gekrault. Bliss lag halb bei ihr auf dem Schoß, um mit ihren Pfoten in der Luft so richtig schön vom Kraulen am Bauch zu genießen…"

"Die Zeit verflog und nach einer halben Stunde standen wir zum zweiten Mal im Behandlungszimmer. Eine sehr routinierte Ärztin, die täglich Leberbiopsien nimmt, würde auch jetzt die Biopsie nehmen. Das fand ich sehr beruhigend und zugleich eine absolute Vorraussetzung… an dem Ort wo die Nadel in den Bauch sollte, wurde nach einer örtlichen Betäubung ein sehr kleines Schnittchen gemacht – kleiner als ein Zentimeter. Ich blieb die ganze Zeit beim Kopf von meinem Hund. Freundlich wurde mit meinem Hund gesprochen, mit dem Resultat das der die ganze Zeit ein wenig verlegen mit der Schwanzspitze wedelte. … Alles in allem dauerte die Biopsie-Entnahme nach meinem Gefühl nicht länger als 10 Minuten."

"Resümierend kann ich einzig und alleine sagen dass die Untersuchung mir gut gefallen hat und ich 100% hinter meinem Beschluss stehe mitgemacht zu haben!"

Adaptiert von einer Publikation des Labrador Club Deutschland e. V., Dezember, 2006.

Résumé

Depuis le début des années 70, le Departement des Sciences Cliniques des animaux de compagnie de l´Université d´Utrecht (UKG) procède a des recherches sur la maladie de cuivre contractées par le chien. Depuis 2003, UKG recherche plus précisément les origines et causes de cette maladie chez le Labrador. Elle a pour conséquence une accumulation excessive de cuivre dans le corps et plus particulièrement au sein du foie. Ce phénomène produit des effets lésions oxydantes, qui peuvent être contrôlées au début de la maladie grâce aux défenses anti-oxydantes du foie. Néanmoins l'inflammation qui en résulte se propagent petit a petit au fil des ans. Comme pour l' homme, l'hépatite (du grec hepar = foie et itis qui veut dire inflammation) progresse différemment en fonction de l'individu. Un chien peut survivre sans problèmes majeurs durant plusieurs années. Nous allons donc succinctement présenter dans cette dissertation les résultats des recherches sur cette maladie menées depuis 2003.
L'étude a commencé par observer cliniquement les Labradors atteints de l'Hépatite Chronique et d'une présence trop importante de cuivre dans le foie. Le traitement conventionnel de l'Hépatite Chronique par du Prednisolon étant sans succès, il a été remplacé efficacement par du Penicillamin, un médicament contre les effets du cuivre.
L'hypothèse a donc été faite que le Labrador pouvait être une race ayant des prédispositions pour cette maladie à cause d'une accumulation de cuivre dans le foie.

Chapitres 1 et 2 : Introduction

Dans ces deux premiers chapitres, y seront présenté comment l'organisme du Labrador assimile et traite le cuivre, et les conséquences sur les cellules. Il y sera également présenté un résumé des études sur cette maladie faites sur différents animaux (rats, souris et chiens) et sur l'Homme. Enfin, ce chapitre sera terminé par une vue d'ensemble de la littérature vétérinaire de la toxicité du cuivre sur le chien.

Chapitres 3, 4 et 5 : La toxicité du cuivre, une nouvelle maladie chez le Labrador

Cette partie présentera les données cliniques d'une nouvelle maladie. Tout d'abord les symptômes détectés sur les patients observés, puis le résultat des examens sur la famille des patients. Cela a permis de démontrer que la toxicité du cuivre était bénigne pendant des années, jusqu'à ce que le patient se nourrit moins ou s'il son activité diminue. Fréquemment le propriétaire du patient pensent que les premiers symptômes (relativement faibles) sont dus à un virus ou au changement de temps. Pendant plusieurs semaines les comportements de paresses ou de sous-nutritions sont alternées avec des périodes de comportements normaux. Après plusieurs mois, les symptômes sont plus nettes avec l'apparition de salivation et vomissements. Les chiens boivent plus que normalement. A partir de ce moment, les choses peuvent aller très vite. Le filtre opéré par le foie est défaillant entraînant de fortes nausées et diarrhées, ainsi qu'un jaunissement de la peau sous les yeux et à l'intérieur de la bouche. Dans les cas les plus graves le ventre se gonfle de liquide clair. Dans ce cas. Le foie est trop endommagé pour produire les protéines essentielles qui permettent au sang de rester dans les vaisseaux. De plus les globules rouges peuvent être détruits a cause de la propagation de cuivre dans le sang à partir du foie. Les tissus du foie ne peuvent plus être régénéré entraînant une cirrhose. Enfin le foie devient petit et dur. Il a été également constaté que la maladie apparaissaient plus fréquemment chez les femelles que chez les mâles.

Chapitres 6 et 7 : L'étude comparative de la médicamentation de la toxicité du cuivre sur le foie du Labrador (contrôlée placebo sur deux groupes, patient choisit au hasard)

Sur les 150 chiens participants à l'étude, il a été constaté que approximativement 50 chiens présentaient une intoxication au cuivre diagnostiquées par une biopsie du foie. Ces chiens ont été traités de trois à six mois avec de la Penicillamine, un médicament qui aide à l'élimination du surplus de cuivre. En effet, cette étude à prouvé que la Penicillamine était un remède efficace contre la maladie.

Suivant, les patients sont invités à participer à un suivi nutritionnel où peu de cuivre est présent (Royal Canin/Waltham Hépatique). De plus la présence de zinc dans le traitement nutritionnel permet d'empêcher l'absorption intestinal du cuivre. Et en effet, cette étude à prouvé que Hépatique était un remède efficace contre l' accumulation du cuivre, considéré comme prophylactique.

Chapitres 8, 9, 10, 11, et 12 : L'étude génétique moléculaire

Pour localiser le gêne responsable de la toxicité du cuivre chez le Labrador, il a été prélevé du sang sur chaque patient afin de procéder à une analyse d'ADN. Grâce aux méthodes de recherche, l'étude a pu exclure les gênes ayant un rôle connu dans le métabolisme du cuivre. C'est pourquoi cette étude peut être intéressante pour la médecine humaine, car elle peut conduire à l'identification d'un nouveau gêne qui peut expliquer certaines maladies enfantines causées par l'accumulation de cuivre.

Discussion

Ce chapitre présente les conditions dans lesquelles l'étude a été effectuée. Grâce au succès de notre étude nutritionnelle, des parallèles avec la pathologie humaine ont pu être observées (Indian childhood cirrhosis et non-indian childhood cirrhosis). Ces maladies sont désignées comme complexe car elles ont simultanément une origine génétique et une origine environnementale (présence de cuivre dans la nourriture ou dans la boisson). La rareté de ces maladies chez l'homme rend particulièrement difficile l'identification de gènes qui facilite le développement de la maladie dans un environnement donné. Ce qui rend cette étude encore plus actuelle et passionnante est que les lésions inflammatoires liées au cuivre peuvent avoir une influence sur l'évolution d'autres maladies chroniques. A cause du manque d'études précises, les institutions gouvernementales européennes et américaines ont de la peine à fixer des limites minimales et maximales pour la teneur de cuivre dans l'environnement. En effet le cuivre est un élément essentiel à notre santé, mais une concentration de cuivre trop importante peut entraîner, comme ici, de graves maladies.
Nous pensons que les résultats des études suivantes vont nous permettre d'apporter de bons éléments de réponses à quelques de ces question essentielles.

En appendice, se trouve les remerciements, un curriculum vitae, la liste des publications de l'auteur et la liste des abréviations.

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Traditionally the first expressions of thankfulness always address the promoter of a research project, but in this case there are two first places because this research would not have been possible without the Dutch Labrador retriever Breed Club (**Nederlandse Labrador Vereniging**). I would like to thank all (two- and fourlegged) members of the club: THANK YOU !
This project would not have been possible without your enthusiasm and willingness to track down a possible disease of your favourite breed, and your love for your dogs!
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List of publications

Peer reviewed articles in this thesis

G.Hoffmann, T.van den.Ingh, P. Bode, J.Rothuizen
Copper-associated chronic hepatitis in Labrador retrievers: 15 clinical patients and their family Journal of Veterinary Internal Medicine 2006;20(4):856-61.

G. Hoffmann, T.S.G.A.M. van den Ingh, P.G. Jones, P. Bode, J. Rothuizen Copper-associated chronic hepatitis in Labrador retrievers. Veterinary Journal 2008, submitted

G. Hoffmann, P.G. Jones, C. J. Mesu, T.S.G.A.M.van den Ingh, P. Bode, J. Rothuizen Randomized, double-blind, placebo-controlled treatment with D-penicillamine against hepatic copper accumulation in Labrador retrievers. Journal of Veterinary Internal Medicine 2008, accepted

G. Hoffmann, P.G. Jones, V. Biourge, T.S.G.A.M.van den Ingh, P. Bode, J. Rothuizen Dietary management of hepatic copper accumulation in Labrador retrievers. Journal of Veterinary Internal Medicine 2008, accepted

G. Hoffmann, H.C.M Heuven, P.Leegwater, P.G. Jones, T.S.G.A.M.van den Ingh, P. Bode, J. Rothuizen Heritabilities of copper accumulating traits in Labrador retrievers, Animal Genetics, 2008, In press

G. Hoffmann, P.A.J. Leegwater, F.G.v. Steenbeek, K. Salg, B. Spee, P.G. Jones1,T.S.G.A.M.v. d. Ingh2, P. Bode3, J. Rothuizen
Copper-associated chronic hepatitis in the Labrador retriever
Results from a candidate gene approach, Animal Genetics, 2008, submitted

G. Hoffmann, P.J.J. Mandigers, P.A.J. Leegwater, T.S.G.A.M. van den Ingh, P. Bode , J. Rothuizen Copper-associated hepatitis in the Doberman pinscher - Evaluation of candidate genes, Veterinary Journal, submitted.

G. Hoffmann, J.IJzer1, B. Brinkhof, T.S.G.A.M. van den Ingh2, L.C. Penning, J. Rothuizen Comparison of different methods to obtain and store canine liver biopsies for molecular and histological research
Analytical Biochemistry, 2008, submitted.

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Wirkstoffdatenbank: Nichtsteroidale Antiphlogistika beim Tier
http://www-vetpharm.unizh.ch

Invited lecture's:

2007 Copper–associated chronic hepatitis in Labrador retrievers
ECVIM congress, 2007, Budapest, Hungary
Leitsymptome von Lebererkrankungen
Kleintier Modul Berlin, Continuing education for veterinarians
Primaere Lebererkrankungen
Kleintier Modul Berlin, Cont. education for veterinarians
Akutes Leberversagen
Kleintier Modul Berlin, Cont. education for veterinarians
Vaskulaere Lebererkrankungen
Kleintier Modul Berlin, Cont. education for veterinarians
2006 Copper accumulating liver diseases in dogs and cats
Najaarsdag, Netherlands
Continuing education for practicing veterinarians

Meetings and proceedings

2007
- Copper-associated chronic hepatitis in the Labrador retriever
  Lecture at the ECVIM congress 2007, Budapest, Hungary.

2006
- Copper-associated chronic hepatitis in the Labrador retriever
  Presented at the ACVIM forum 2006, Louisville, KY, USA
- Double-Blind, Placebo-Controlled Treatment with D-Penicillamine
  against Hepatic Copper Accumulation in Labrador Retrievers
  Presented at the ACVIM forum 2006, Louisville, KY, USA
- Evaluation of candidate genes for Copper-associated hepatitis in the Doberman Pinscher, Presented at the Advances in Canine and Feline, Genomics Conference, Davis, CA
- Evaluation of candidate genes for Copper-associated chronic hepatitis in the Labrador retriever, Presented at the Advances in Canine and Feline Genomics Conference, Davis, CA
- Evaluation of candidate genes for Copper-associated hepatitis in the Doberman Pinscher, presented at the ECVIM congress 2006, Amsterdam,NL, 2nd price (2nd best poster presentation)
- Evaluation of candidate genes for Copper-associated chronic hepatitis in the Labrador retriever, presented at the ECVIM congress 2006, Amsterdam,NL

2005
- Copper-associated chronic hepatitis in the Labrador retriever
  Presented at the 16th ECVIM congress, 2005, Glasgow, UK,
  1st price (best oral presentation)

Special achievement's

1.prize: election as best research presentation, 16th ECVIM congress, 2005, Glasgow, topic: Copper-associated chronic hepatitis in the Labrador retriever

2^{nd} prize: election as 2^{nd} best research poster presentation, 17th ECVIM congress, 2006, Amsterdam, topic: Evaluation of candidate genes for Copper-associated hepatitis in the Doberman Pinscher

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Curriculum vitae

Gaby Hoffmann was born September 30, 1970, in Neuss (Germany). After finishing secondary school as third best of her year of 242 students, she studied French at the Institut Catholique in Paris, France, before starting her studies of veterinary medicine at the Faculty of Veterinary Medicine in Hannover (Germany) in 1990. She received her DVM degree in 1996. Subsequent to a six month internship in Japan and Switzerland, she started a research project at the Department of Veterinary Pharmacology & Toxicology, of the Faculty of Veterinary Medicine, at the University of Zurich in Switzerland. In 1998 she received her Doctor of veterinary medicine for her work in Zurich. In the same year she started a residency program for small animal internal medicine at the Small Animal Clinic of the University of Berne in Switzerland, and at the Louisiana State University in the USA. After successful completion of her residency program she came to Utrecht in december 2002, to start her PhD study at the Department of Clinical Sciences of Companion Animals at the Faculty of Veterinary Medicine, of the University of Utrecht, where she is currently employed as a lecturer for small animal gastroenterology (universitaire docent). Gaby Hoffmann is a diplomate of the American College of Veterinary Internal Medicine, as well as the European College of Veterinary Internal Medicine.

Abbreviations

ATOX	AnTiOXidant protein 1
ATP7A	Cu(2+)-transporting ATPase, alpha polypetide
ATP7B	Cu(2+)-transporting ATPase, beta polypetide
BAC	Bacterial Artificial Chromosome
BDE	Bile Duct Epithelial cells
CAH	Copper-associated Hepatitis
CCS	Copper Chaperone for Superoxide dismutase
cDNA	Complement DNA, CopyDNA
CH	Chronic Hepatitis
cHCC	canine Hepatocellular Carcinoma Cells
CACH	Copper-associated Chronic Hepatitis
COMMD1	COpper Metabolism Murr1 Domain-containing protein 1
COX17	Cytochrome c OXidase assembly protein
CP	CeruloPlasmin
CRIM	Cystein rich motor neuron
CT	Copper Toxicosis in Bedlington terriers
CTR1	Copper TRansporter 1
DNA	DesoxiriboNucleic Acid
GAPDH	GlycerAldehyde-3-Phosphate DeHydrogenase
GSH	Glutathione, reduced
GSSG	Glutathione, oxidized
HDAC	Histone deacetylase
HPRT	Hypoxanthine PhosphoRibosyl Transferase
IBD	Identical By Descent
IBS	Identical By State
LD	linkage disequilibrium
LOD	logarithm of odd's
Mb	MegaBase
mRNA	messenger RiboNucleic Acid
MT	Metallothionein
OMIM	Online Mendelian Inheritance of Man
OMIA	Online Mendelian Inheritance of Animals
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR
RNA	RiboNucleic Acid
SNP	Single Nucleotide Polymorphism
SOD1	SuperOxide Dismutase 1
XIAP	X-linked Inhibitor of Apoptosis