Insights in the pathogenesis of Dobermann hepatitis

Inzichten in de pathogenese van Dobermann hepatitis

(met een samenvatting in het Nederlands)

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Contents

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Voor CC, die er gewoon bij had moeten zijn.

Chapter 1

Aim and scope of the study

Introduction

The pathogenesis of chronic hepatitis in Dobermann dogs (also called Dobermann hepatitis) has been under debate for several years. The disease is predominantly seen in female dogs, usually between 4 and 7 years of age, and is in the clinical stage most commonly characterized by anorexia, depression, weight loss, gastrointestinal signs, polydipsia, polyuria and icterus.[4,11,12,33] The histopathological features of Dobermann hepatitis begin centrilobular [15,23,24,31] and in time progress into a micronodular cirrhosis with histological features of fibrosis, piece meal necrosis and progressive lymphocyte and plasma cell infiltration of the portal triads.[4,11,12,33]

The high incidence of chronic hepatitis in Dobermann dogs compared to other breeds suggest hereditary sensitivity.[4,6,11,12,25] Some authors believe the disease to be immune-mediated[26,31] whereas others believe it to be a form of copper toxicosis.[7,15,20,21]

In several dog breeds such as Bedlington terriers,[8,19,27,35] West Highland white terriers,[32,34] Skye terriers,[9] Dalmatians[39] and Anatolian shepherd dogs[3] copper storage has been described as an etiological factor for the development of hepatitis. In 1983 Thornburg et al[33] described 2 Dobermann dogs with a subacute hepatitis and elevated copper concentrations, without any signs of cirrhosis or chronic cholestasis. He concluded that there was evidence that the cause of the liver disease was copper toxicosis in these 2 dogs. A similar observation was done by Speeti et al.[25] They found that in a large group of 626 Dobermann dogs 55 dogs (8.8%) with an elevated ALT, 21 dogs (3.4%) had subclinical hepatitis. They also observed that 19 of 21 dogs with subclinical hepatitis had an elevated copper concentration. These observations are suggestive for a possible copper toxicosis in this breed. However, also an immune-mediated aetiology has been suggested. [4,11,31] The majority of Dobermann dogs with chronic hepatitis are females. In man immune-mediated diseases are more common in females[2] and are especially important in hepatitis.[16,29] Where autoimmune hepatitis (AIH) is strongly associated with inheritance of the HLA A1-B8-DR3 haplotype and particular the DR3 and DR4 haplotypes.[5,14]

Hypothesis 1

In Dobermann dogs exists an autosomal genetic error in metabolism that leads to an abnormal copper metabolism. This results in an increased hepatic copper concentration which may lead to a (subclinical) hepatitis.

Hypothesis 2

Some Dobermann dogs may have a specific DLA (dog leukocyte antigen) configuration that is associated with an abnormal or inadequate response on an (normal) immune-stimulation.[13,37] This may, like in humans,[16,36] introduce autoimmune hepatitis.

In order to investigate the hypotheses several questions were formulated:

1. How often is chronic hepatitis seen in Dobermann dogs and exists a subclinical hepatitis as described by Speeti et al?[25]
2. And if so, is this subclinical hepatitis related with an increase in hepatic copper concentrations?
3. Do Dobermann dogs with subclinical hepatitis and increased hepatic copper concentrations have an impaired copper excretion?
4. Does the increased hepatic copper cause oxidative stress and hence hepatitis?
5. If the increased copper is the result of an genetic error in metabolism which transporter or storage gene is affected?
6. Is it possible to treat Dobermann hepatitis with an immune-modulator or copper chelating drug?
7. Do Dobermann dogs with hepatitis have a specific DLA haplotype like in humans?

A general introduction on the subjects of intracellular transport of copper, the toxicity of copper, copper toxicosis in both humans and dogs, as well as on autoimmunity are outlined in chapter 2. In order to answer question 1, a questionnaire was sent to 340 owners investigating both morbidity and mortality in a Dobermann dog population born between 1993 and 1999 (chapter 3). In addition, a group of 106 clinically healthy Dobermann dogs, all around three years of age, was examined by measuring liver enzymes, and fasting bile acids and by performing a rubeanic stain copper copper on cytological smears of the liver. If a dog would show any abnormality a percutaneous liver biopsy for histopathology and copper measurement would be taken (chapter 4). If possible a follow up would be done regularly to study the presence of copper and the persistence of the subclinical hepatitis (chapter 4).

To answer question 2 five normal Dobermann dogs were compared with five Dobermann dogs with a subclinical hepatitis associated with an increased hepatic copper concentration, by means of an IV administered copper isotope (^{64}Cu).[28,30] Both plasma clearance and bile excretion were studied. Hepatobiliary cholestasis as a possible cause for secondary copper retention was excluded using a ^{99m}Tc- Bis-IDA hepatobiliary scintigraphy[22] (chapter 5).

Question 3 and 4 was addressed by quantitative RT-PCR to determine differentially expressed genes involved in copper metabolism and ROS defences in four groups. One group of normal Dobermann dogs (NDD), a group of Dobermann dogs suffering from a subclinical hepatitis not associated with copper (N-CASH), a group of Dobermann dogs with a subclinical hepatitis and associated with (an abnormally increased) hepatic copper (CASH) and last, Dobermann dogs that already had died from chronic Dobermann hepatitis with increased copper concentrations (DH) (chapter 6).

Question 4 was further addressed by means of candidate gene approach of copper transporter and storage genes (CTR1, Ceruloplasmin, Metallothionein, ATP7A, ATP7B, Murr1, COX17, and ATOX1) (chapter 7).

Questions 5 and 6 are answered by means of three studies. First of all nandrolone laurate, an androgenic anabolic steroid with possible immune-modulating properties[1,10,17,18] was studied in a placebo controlled experiment. Twenty-one Dobermann dogs, all three years of age, with subclinical hepatitis, were scored prior and after four months of treatment with either placebo or the test product (chapter 8). Five Dobermann dogs with a persistent subclinical hepatitis associated with increased hepatic copper concentrations were treated with D-penicillamine[38] for a period of four months. Again the dogs were examined prior and after the treatment (Chapter 9). The last study addresses the variation of DLA alleles in normal Dobermann dogs (NDD), Dobermann dogs with subclinical hepatitis associated with increased hepatic copper concentrations (CASH) and a group of Dobermann dogs that died from chronic Dobermann hepatitis (DH) (chapter 10).

The results of the experiments and the viability of the hypotheses is determined in light of the results in chapter 11.

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Chapter 2

General introduction based on Chronic Hepatitis in Doberman pinschers, a review

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Introduction

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The toxicity of ionic copper and intracellular copper transport

Like zinc (Zn), iron (Fe) and selenium (Se), is copper an essential element for the activity of a number of physiologically important enzymes.[39] Although it acts as a redox co-factor, it is as a free ion highly toxic and able to catalyze the formation of hydroxyl radicals ($OH^{\bullet}$) via the Haber-Weiss reaction.[4,18]

Haber-Weiss reaction:

$$O_2^{\bullet-} + Cu^{2+} \rightarrow O_2 + Cu^{+}$$
$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\bullet}$$
$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{-} + OH^{\bullet}$$

These free radicals can directly damage lipids, proteins and nucleic acids. On average in normal cells there is only one free copper atom per cell.[33]

An adult human normally has an average intake of 2-6 mg copper per day and excretes approximately 1-4 mg/day via the bile.[8] Dietary copper is absorbed across the mucosal membrane of the upper intestine. After entering the bloodstream, it is bound to either albumin, amino acids (histidine, threonine, cysteine) or transcuprein and transported to either the kidney or liver.[13,20,32]

Human cellular copper uptake is mediated by copper transmembrane proteins encoded by CTR1 gene.[74] The copper is preferentially transported as the free monovalent ion, Cu(I).[2] Alternatively bivalent copper, Cu(II), is probably rapidly reduced by intracellular glutathione (GSH). GSH is important for the uptake of copper[14] and has a high affinity for Cu(I). These GSH-Cu(I) complexes may function in intracellular Cu transport.[13] GSH-Cu(I) complexes can donate Cu(I) to Metallothionein (MT) or to the cytoplasmic copper chaperones. These are encoded by the genes COX17,[67] CCS,[67] ATOX1,[31] and distribute the copper to specific cellular compartments. COX17 transports copper to the mitochondrion for incorporation into cytochrome c oxidase.[67] CCS is responsible for the incorporation of copper into Cu/Zn superoxide dismutase (SOD1), one of the most important cytosolic enzymes in the first line against oxidative stress.[20,67] The anti-oxidant protein 1 (ATOX1)[31] transports copper to the copper-transporting ATPases, ATP7A[70] and ATP7B.[5] Both are located in the trans-Golgi network. Subsequently, copper can be incorporated into ceruloplasmin (CP)[8] or bound to MURR1 and transfered outside the cell to respectively blood and bile.[69] MURR1 is the defective gene in copper toxicosis in the Bedlington terriers which lack exon 2, leading to a truncated and inactive protein.[69] The protein is supposed to be associated with the lysosomal vesicle transport

to biliary canalicular membrane for copper excretion. Recently, MURR1 was shown to associate with ATP7B,[59] NFkappaB-IkappaB complex,[17] the anti-apoptotic protein XIAP[6] and an epithelial sodium channel.[1] In hepatocytes, ATP7B incorporates copper in apoceruloplasmin to form ceruloplasmin. Ceruloplasmin, also known as ferroxidase or oxygen oxidoreductase, is a plasma metalloprotein which is involved in peroxidation of Fe(II)transferrin to Fe(III)transferring and forms 90 to 95% of plasma total copper. If there is excess of copper in the hepatocytes, ATB7B is redistributed to vesicles for copper efflux into bile caniculi.[68] In case of excess in other cells, ATP7A is redistributed to small cytoplasmic vesicles and to the plasma membrane where copper is excreted out of the cell.[68]

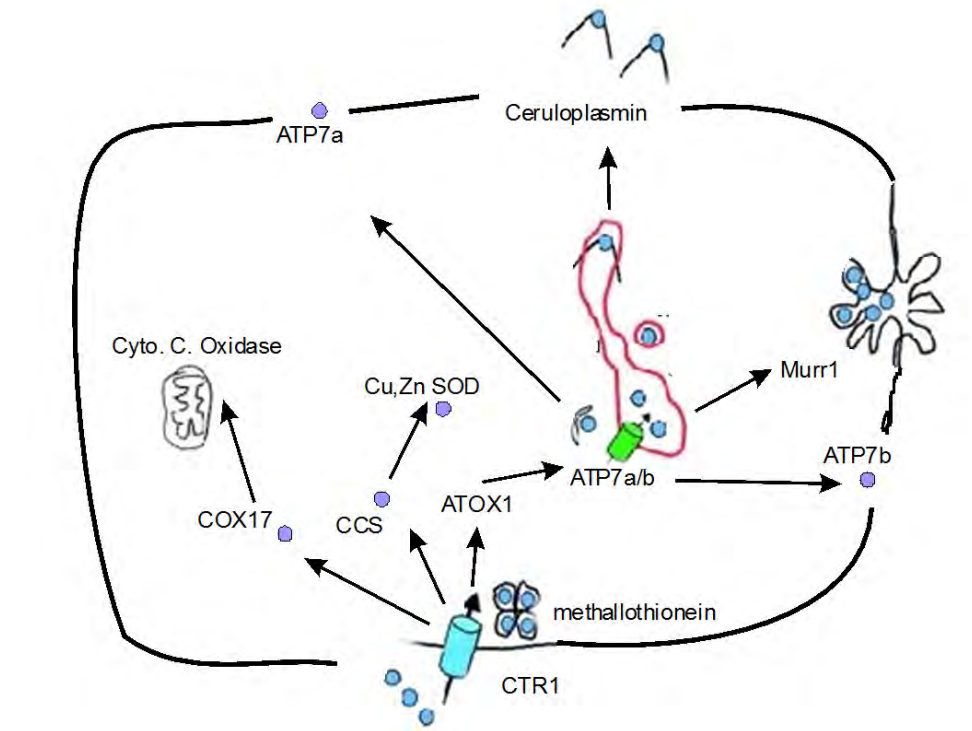

Figure 1: *Model of the proposed pathways and proteins relevant to copper metabolism in the hepatocyte / cell. Based on van der Sluis[68] and Tao et al.[59]*

Finally, free cytoplasmic copper is sequestered by metallothioneins or glutathion (GSH). The small intracellular protein Metallothionein 1A (MT1A) is capable of chelating several metal ions, including copper. Due to the many cysteine residues copper can be strongly bound. Furthermore MT1A is inducible at the transcriptional level by metals and other stress conditions like reactive oxygen species (ROS), UV radiation and hypoxia.[73] Following degradation in the lysosomes, MT1A can donate copper to other proteins.

Copper toxicosis in man

In 1912 Wilson described a progressive lenticular degeneration of the liver.[72] In 1948 it became apparent that copper toxicity was the cause of this disease which became known under the name Wilson's disease.[10] Wilson's disease is characterized by cirrhosis, corneal copper deposits known as Kayser-Fleischer rings, an absence or deficiency of ceruloplasmin in the plasma (below 0.2 g/l), marked elevations of tissue copper concentrations above 50 mg/kg dry matter and increased urinary copper excretion.[53] In Wilson disease the ATPase which transports copper into the trans-Golgi network, is mutated and responsible for this type of autosomal recessive copper toxicosis.[5,7,44] The toxicity of copper has been demonstrated in Long-Evans Cinnamon (LEC) rats which are used as a model for Wilson's disease. In these LEC rats virtually all hepatocytes contain large amounts of copper. Biochemical studies have indicated that a small amount of the cytoplasmic Cu is in the non-metallothionein (MT) fraction which might be responsible for the development of the hepatitis through the generation of free radicals.[13] More recently it was found that chronic copper toxicity in these LEC rats involved the uptake of copper-loaded metallothionein into lysosomes, where it was incompletely degraded and polymerized into an insoluble material, which contained reactive copper. This copper, together with iron, initiated a lysosomal lipid peroxidation, which led to hepatocyte necrosis. Phagocytosis of this reactive copper by Kupffer cells might have amplified the liver damage further.[30]

Other forms of copper toxicosis are the so called non-Wilsonian diseases such as Indian childhood cirrhosis (ICC), endemic Tyrolean childhood cirrhosis (NICC or ETIC) and non-Indian disease known as idiopathic copper toxicosis (ICT), which are in essence all characterized by hepatic copper accumulation due to an impaired copper excretion.[41,42,47,54] In ICT ceruloplasmin levels are normal or elevated and neurological abnormalities are absent.[47] ICC, ETIC and some forms of ICT depend on excessive dietary intake of copper. Both ICT as well as ETIC are believed to be also autosomal recessive disorders.[41,57,58]

Copper toxicosis in dogs

Copper toxicosis has been described in Bedlington terriers,[22,25,43,55,66,68] is most likely present in West Highland White terriers,[62,65] Dalmatians,[71] Skye terriers,[23,38] and is suspected to be present in Labradors[45] and Doberman pinschers.[35,49] The histopathological lesions caused by copper are in some extent specific. At first copper laden hepatocytes are seen centrilobularly associated with necrotic hepatocytes, copper laden macrophages and some lymphocytes, plasma cell and neutrophils.[21,23,25,64,66] In time, the inflammation will become more severe with bridging necrosis, inflammation and fibrosis with ultimately cirrhosis.[19,25,61,64,65,66] It is believed that the copper toxicosis is the result of free intracellular copper causing oxidative stress.[4,18,54] In rats with a chronic copper overload the copper retention and inflammation is first seen in the periportal areas.[16] In dogs with established copper toxicosis, such as the Bedlington terrier the retention starts centrilobularly around the terminal hepatic veins.[21,56,66] This centrilobular retention (zone 3) is therefore believed to be suggestive for a metabolic defect of intracellular copper metabolism.[16]

Diagnosis of elevated copper concentrations

In hepatocytes, copper is stored in lysosomes. Routine haematoxylin-eosin staining may reveal copper laden lysosomes as abundant golden-brown, refractile hepatocellular lysosomal granules[45] but a more reliable estimate can be achieved with a rhodanine and/or rubeanic acid stain of which rubeanic acid is both more sensitive as well as specific[27,49]. Ideally copper is estimated by means of a quantitative copper analysis using instrumental neutron activation analysis (INAA).[3] Compared to this quantitative determination, staining methods are only reliable with values above 1000 mg/kg dry matter.[45] Copper granules become visible with values above 400 mg/kg dry matter.[60,64]

Does copper play a role in Doberman hepatitis?

In most Doberman pinschers, with chronic hepatitis, hepatic copper concentrations are elevated.[9,26,28,49,61,63] Normal hepatic copper concentration in dogs is between 150 to 400 mg/kg dry matter.[19,29,35,45,56] In Doberman pinschers it is reported to be up to 1000 to 2000 mg/kg dry matter.[45,61] Doberman pinschers with chronic hepatitis frequently have cholestasis or cholatestasis, or it is believed to be so.[9,26] In one study it was found that especially in Doberman hepatitis there was a strong correlation between copper concentration and fibrosis.[15] Thus most authors concluded that the elevated copper concentration was secondary to the cholestasis. However there are also reports of Doberman pinschers with elevated copper concentrations that did not have cholestasis.[63] Speeti et al[50] first described a subclinical hepatitis in which again some dogs showed elevated copper concentrations without cholestasis.[48,49] The first studies describing Doberman hepatitis localized the lesions mainly periportal[9,28] which is different from other breeds with copper toxicosis.[23] For that reason it was believed to be of a different nature. However recent studies have proven that the lesions start, like in other breeds, centrilobularly.[35,48,61]

Copper concentrations are still in dispute. Thornburg et al[64] established, in 20 normal Doberman pinschers, by means of a quantitative measurement hepatic copper concentrations. It was reported to be 413 ± 298 mg/kg dry matter and values up to 1500 mg/kg dry matter were found to be present in Doberman pinschers without any sign of inflammation. Values above the reference value of 400 mg/kg dry matter are believed to be abnormal.[19,29,35,45,56] In another study Thornburg[61] described a group Doberman pinschers with chronic hepatitis in a precirrhotic stage of which 30 of 35 had elevated copper concentrations. He concluded that the increased copper was incidental and not the cause of disease. A similar conclusion was drawn by Speeti et al.[49]

Is Doberman hepatitis a suitable model for copper toxicosis in man?

Copper toxicosis in Doberman pinschers is different from Wilson's disease as well as copper toxicosis in other dog breeds. Compared to other breeds, predominantly female Doberman pinschers are affected which is in contrast with other breeds. And treatment with copper chelating drugs such as D-penicillamine has proven to be unsuccessful in the clinical stage of the disease[9] which is again in contrast to results in other breeds.[24,38,45] Compared to Wilson's disease in man there are several differences. First of all ceruloplasmin values are normal or slightly elevated in diseased dogs. Ceruloplasmin values established in 14 normal Doberman pinschers (n=14, median 40 mg/l, range 30 – 90 mg/l) were compared with values of 13 Doberman pinschers with subclinical hepatitis (n=13, median 50 mg/l, range 30 – 110 mg/l) of the same age group (3 to 6 years) and showed no significant statistical difference (p=0.06) (Mandigers, unpublished data). Furthermore the neurological symptoms as well as the eye abnormalities observed in man are absent. Although similarities exists between Doberman hepatitis and the non-Wilsonian diseases (ICC, ETIC and ICT) the disease it still different based on the gender predilection and poor response to treatment.

Is Doberman hepatitis a primary autoimmune hepatitis?

Autoimmune hepatitis in man is an idiopathic disorder affecting the hepatic parenchyma.[12] The characteristic histological picture is that of an interface hepatitis without other changes that are more typical of other liver diseases.[12,36] The disease is predominantly seen in females.[12,37] It was suggested that Doberman hepatitis is most likely to be of an immune mediated nature.[61] The

inflammatory infiltrate of lymphocytes, plasmacytes, the gender difference as well as the unclear role of copper may have led to this hypothesis. There are however several differences between autoimmune hepatitis in humans and Doberman hepatitis. In humans, although not pathognomic, the disease has a periportal/interface or panlobular distribution[12,36] whereas in subclinical or early clinical Doberman pinscher hepatitis, lesions are seen particularly in the centrilobular regions.[35,48,61] One of the criteria to classify the disease in humans as immune-mediated is the excellent response to corticosteroids.[11] An observation in contrast with the treatment results in Doberman pinschers with clinical manifest chronic hepatitis.[9,26,28,46] A possible reason for this lack of efficacy could be the stage of the disease. Based on the pathology Doberman pinschers are in a more advanced stage. Results of treatment in humans is comparably low if the histopathological lesions are too advanced.[12]

Recently Speeti et al[51] described the expression of major histocompatibility complex (MHC) class II antigens in hepatocytes in Doberman hepatitis. The MHC class II expression was seen predominantly centrolobular together with a mononuclear cell infiltration.[51] The expression of MHC class II genes is not normal for hepatocytes[52] and might induce tolerance in autoreactive Th1 cells and may simultaneously favour a Th2 response in uncommitted T cells, and thereby support autoantibody production.[34] It was suggested that the hepatocytes with the MHC class II expression become an antigen-presenting cell for CD4+ T cells, and thus might become a target themselves.[51] Corticosteroid treatment down regulated this MHC class II expression.[51] Based in these results it was concluded that the disease is most likely immune-mediated. However MHC class II expression can also be the result of drugs, viral infections and toxins. All these possibilities, including toxins such as copper, were dismissed by the authors. A conclusion which is not consistent with earlier findings.[35,48,49,61]

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Chapter 3

Morbidity and mortality in a Dutch Dobermann population born between 1993 and 1999

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Abstract

Morbidity and mortality were investigated with questionnaires sent randomly to owners of 928 Dobermann dogs registered over seven different years. The Response rate was 37% (340 owners). In total 81 dogs had died and 259 were still alive. Proportional mortality was high for heart failure (14.8-22.2%), behavioural problems (19.8%), cancer (13.6%), but low for Dobermann hepatitis (3.7%) and cervical spondylomyelopathy (2.5%). A total of 132 of 259 Dobermann dogs were suffering from various disorders. Highest prevalence was observed for skin problems (22.4%) and urinary incontinence (15.8%). Dobermann dogs are, compared to other breeds, at a higher risk of developing urinary incontinence (19.4%) and the risk of developing it after neutering is even higher (37%).

Keywords: *Urinary incontience, Hepatitis, Cervical spondylomyelopathy, Cardiomyopathy*

Introduction

The Dobermann may, like other breeds, develop breed-specific and non breed-specific diseases. Three diseases with a probable hereditary nature have been described. Dobermann dogs have a higher risk of developing and dying from cardiac disease.[2,4,15,16,20,21] There is also a higher incidence of cervical spondylomyelopathy,[3,12,17,23] and chronic hepatitis.[5,9,10,18,19] Other diseases with a breed tendency for Dobermann dogs include behavioural problems[13,16,22] and an increased risk for accidental injury.[2,16] However, current knowledge on morbidity and longevity is either based on a small number of dogs or biased towards a referral hospital population. Although the latter provides reliable data it is not a fair representation of the population seen by practitioners. For instance, chronic hepatitis has a breed-predisposition in Dobermann dogs[5,8,9] and there are two studies estimating the incidence in large groups of normal Dobermann dogs.[10,18] These estimates are based on the incidence of subclinical hepatitis, a disorder which may precede clinical hepatitis, which has an estimated incidence of 5.7% in one study [10], and 3.3 -8.1 % in another[18]. Based on the records of the Department of Clinical Sciences of Companion Animals of Utrecht University the incidence of Dobermann hepatitis in the Netherlands would be 8 of 6538 or 0.12%. There seems to be some discrepancy as recent studies published by Bonnet et al[2] and Proschowsky et al[16] do not mention the disease at all.

This paper describes a questionnaire survey of a large and representative group of Dobermann owners to investigate disease incidence and longevity in the Dutch Dobermann population.

Materials and methods

During December 2002, questionnaires were sent to 928 owners of Dobermann dogs registered by the Dutch Kennel Club. All owners were selected from a total of 6538 registered Dobermann dogs born between January 1993 and December 1999. For each year, seven out of 100 dogs (14.3%) were selected randomly. If no address was known or the owner had moved abroad, the next dog of the same litter was chosen.

The questionnaire, a copy in Dutch is available upon request, consisted of 45 questions and a free post return envelope was included. The first part concentrated on information about neutering, time of neutering and whether the dog was still alive. If the dog had died, the owner was asked to choose from a number of possible diagnoses (Table 1).

Table 1: *Possible answers for the check off list to name the cause of death. Owners were asked to specify the answer.*

Epilepsy	Cervical problems
Back (spinal) problems	Other neurological problems
Joint/skeleton disorders	
Skin	
Mouth/teeth	Nose
Ears	Eyes
Respiratory problems	Coughing
Cardiac problems / heart failure	
Liver problems	Jaundice
(Occasional) vomiting	Gastric problems
(Occasional) diarrhoea	
Renal problems	
Incontinence (passive)	Incontinence (active)
Others	

These questions were presented as a check-off list. Multiple answers were allowed. If an answer was marked, the owner was asked to provide more details and provide, if possible, a statement from the veterinarian consulted. If the dog had died, the owner was asked to return the questionnaire after this section. If the dog was still alive, the questionnaire continued. The second part of the questionnaire aimed at yearly vaccinations, type and brand of food, frequency of feeding, environment of both the owner and dog and exercise given to the dog. The third part

of the questionnaire enquired about the health status of the dog. As before, owners could select from several options. Besides the disorders listed in Table 1, four other disease groups were added (Table 2).

Table 2: *Possible answers for the check off list to name the disorder from which the dog is suffering. Besides these the possible answers from Table 1 were also displayed for check off. Owners were asked to specify the answer.*

Polyuria/polydipsia (specify the amount)
Bladder problems
Anal sack problems
Endocrine problems
- thyroid gland
- diabetic
- pancreatic (other)
- adrenal gland

As in the first part, the owners could select more than one answer and they were requested to elaborate on the checked responses. The last section was a control for the previous question. Owners were asked whether any surgery or diagnostic examination (radiographs, ultrasounds, and blood examination) had ever been performed and if so for what reason. Not all questionnaire responses will be discussed in this article as the second section on food, environment and exercise will be analysed as part of a separate study. No diagnoses were verified by the authors, in other words the accuracy/honesty of the owners and/or veterinarian treating the dogs was relied upon.

Data analysis

The data were analysed using Microsoft Excel (Microsoft software 2002) and the software package Statistix 8.0 for Windows (Analytical Software, P.O. Box 12185, Tallahassee, FL 32317-2185, USA). Descriptive statistics was used for general data such as age, weight, coat colour, etc. For continuous data, t-values were calculated. For categorical data the chi-squared test was used. The level of significance was taken at $P < 0.05$. A survival curve was calculated for nine age categories: 0-1 years of age; 1 up to and including 8 years of age; and 9 years and above. An average yearly mortality was calculated using the true rate formula (approximate denominator) according to Martin et al,[11] as: (number of deaths during the investigated time period for each possible year) / (the average number at risk during that time period for each possible year). The end of 2002 was taken as an endpoint as this was the time when the questionnaires were completed. For all dogs born in 1993 the actual mortality rate for each year could be calculated up to 2002 (in total nine years). For dogs born in 1999 only 3 years could be calculated. This survival curve is compared with the survival estimates published by Egenvall et al.[7] The survival curve was compared to that of two other breeds: one so- called higher risk breed, the Bernese mountain dog, and a lower-risk breed, the Beagle.[7] Proportional mortality, for each cause of death, was calculated as: (number of deaths due to a specific cause) / (number of dogs that died).[2]

Results

From the 928 questionnaires 465 were send to owners of males (50.1%) and 463 to owners of female dogs (49.9%). The response rate was 37% (340 of 928) and all years of birth were equally represented (Table 3). Of the responding owners 153 (45%) had a male and 187 (55%) a female dog.

Table 3: *number of births per year and the number of questionnaires sent out. In total 37% of the owners responded.*

Year	Births	Number of questionnaires send out	Number of questionnaires returned
1993	1021	132	48 (36%)
1994	1129	170	58 (34%)
1995	1255	187	60 (32%)
1996	996	144	65 (45%)
1997	800	114	38 (33%)
1998	737	105	47 (45%)
1999	600	76	24 (32%)
	6538	928 (14.2%)	340 (37%)

Mortality

In total 81 (24%) Dobermann dogs were reported to have died and 259 (76%) were still alive. Approximately 1.4% Dobermann dogs died during their first year (Fig 1) and 50% had died by the time they had reached the age of nine (Fig 1). There was no influence of coat colour (p=0.13) or neutering status (p=0.24) on mortality.

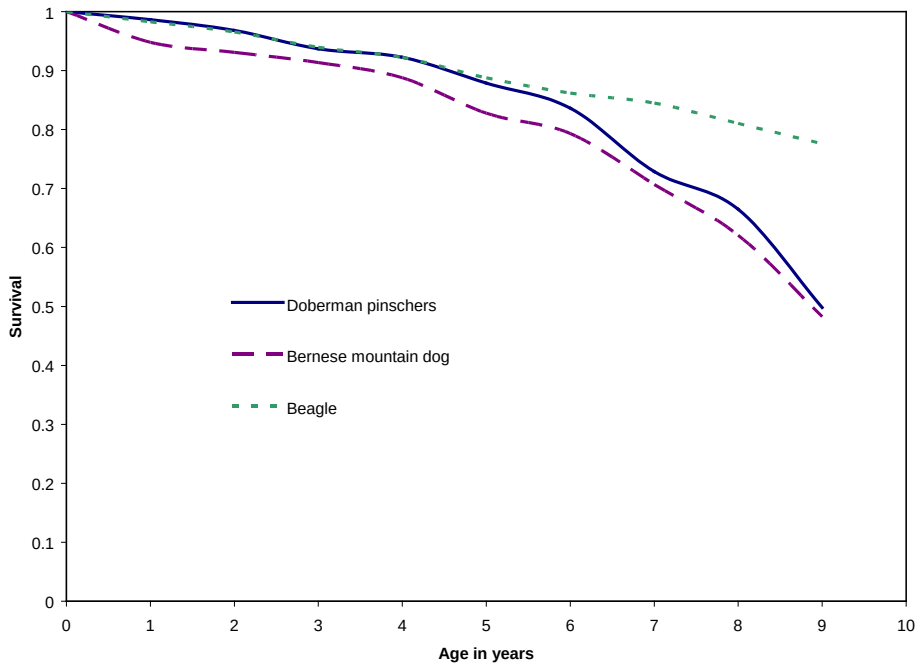

Figure 1: *Survival curve of the investigated group of Dobermann dogs, and for comparison the Bernese mountain dog and Beagle (Reprinted with permission from Egenvall and others 2000b, Age pattern of mortality in eight breeds of insured dogs in Sweden. Preventive Veterinary Medicine, 46:1-14 with permission from Elsevier). The Bernese mountain dog is regarded as a high risk breed, the Beagle as a low risk breed. Based on this curve the Dobermann can be regarded as a high risk breed as well.*

Heart failure was indicated as cause of death in twelve dogs (four females, eight males) and acute heart failure was suspected as the cause of sudden death in a further six dogs (all males), (Table 4).

Table 4: *Proportional mortality, mean age at death, median and range for the causes of death (n=81 dogs).*

Cause of death	No	Proportional mortality	Mean age at death	Median	Range
Heart failure	12	14.8%	5.9	6.5	0.8-8.2
Acute death	6	7.4%	6.5	6.7	4.3-8.8
Epilepsy	3	3.7%	6.3	6.8	4.0-8.0
Cervical instability	2	2.5%	5.5	5.5	5.2-5.7
Gastroenteral	5	6.2%	4.1	3.5	0.5-7.5
Liver cirrhosis	3	3.7%	6.4	6.4	5.1-7.7
Kidney	1	1.2%	4.2		
Tumours	11	13.6%	6.1	5.9	3.9-8.4
Infections	4	4.9%	5.7	5.8	5.1-6.4
Skin	1	1.2%	4.5		
Behaviour	16	19.8%	3.2	2.6	1.0-6.9
Accident	7	8.6%	3.7	2.7	0.9-8.0
Unclear	7	8.6%	3.5	2.8	0.2-6.9
Old age	3	3.7%	7.2	6.8	6.6-8.2

Three dogs had been euthanased as a consequence of epilepsy and two because of cervical spondylomyelopathy. Five dogs were euthanased because of severe gastrointestinal diseases including gastric dilatation and volvulus (two male dogs). Other reasons indicated as a cause of death or reason for euthanasia were liver cirrhosis in three dogs (two females, one male), renal failure in one dog and skin problems in one dog (Table 2). Infections were seen in four dogs including two that died from pneumonia. Seven dogs were reported to have died as a consequence of being hit by a car. Sixteen dogs (11 males, five females) were euthanased because of behavioural problems, 13 because of aggression towards people and three because of sheep-worrying. Tumours were found in 11 dogs: four had a non- specified bone tumour; two had a

liver tumour, and various other tumour types occurred in the remainder. In seven dogs it was not possible to classify the cause of death as the dogs had various clinical signs suggestive of multiple organ failure. Old age was indicated as a reason for euthanasia in only three dogs.

Morbidity

Of the 259 still alive, 201 owners (78%) visited their veterinarian regularly, for example for vaccinations. The other 58 owners reported that they only visited their veterinarian in the event of serious illness. Weight was recorded in 217 dogs. The average weight for male dogs was 39.4 kg (median 40, range 22-60 kg) and for female dogs 32.3 kg (median 32, range 16-48 kg).
The question “Do you consider your animal to be healthy?” was answered unconditionally positive by 213 owners. However, 95 of 213 owners reported several disorders to be present. Only 29 of 259 owners answered that their animal was not healthy.

Various diseases were recorded in 132 dogs and are summarised in Table 5. No significant gender difference was observed for any disorders with the exception of urinary incontinence (37 females, 4 males) and liver disease (2 females). Five female dogs with incontinence were also reported to have polyuria/polydipsia. The owners were asked to quantify water consumption, however none was able to provide a fair estimate. Thirty of the 37 female incontinent dogs were neutered and 22 received medication for it.

Table 5: *The morbidity observed in 132 Dobermann dogs. Multiple answers were possible.*

Organ system	Number	Percentage (of 259 dogs)
Skin	58	22.4%
Eyes	6	2.3%
Ears	4	1.5%
(Occasional) vomiting	7	2.7%
(Occasional) Diarrhoea	4	1.5%
Liver	2	0.8%
Cardiac	2	0.8%
Incontinence	41	15.8%
Polyuria/polydipsia	16	6.2%
Renal problems	1	0.4%
Bladder problems	4	1.5%
Cervical problems	9	3.5%
Backproblems	4	1.5%
Unspecified neurological	1	0.4%
Joint/skeleton problems	18	7%
Epilepsy	2	0.8%
Respiratory	1	0.4%
Tumours	1	0.4%
Thyroid gland	4	1.5%
Diabetes mellitus	1	0.4%

Discussion

The response rate of 37% for this questionnaire is reasonable. The response rate from members of the Danish Kennel club in a study undertaken by Proschowsky et al.[15,16] was 20.5%. The 340 questionnaires returned represent 5% of the total population of Dobermann dogs born in the chosen time period. The disadvantage of this approach of health assessment is that the diagnoses could not be validated by the authors and although owners were asked to provide information from their veterinarian it is possible that some owners might have misclassified their dog.

The survival curve calculated for this time period is comparable to the survival estimate curve of high risk breeds published by Egenvall et al.[7] The Dobermann has a slightly better mortality curve than the Bernese mountain dog which has a survival rate of 33% at 10 years of age.[7] Other "high risk" breeds include Cavalier King Charles spaniel, the Boxer and the German Shepherd dog with 10-year survival rates of 45 to 53%.[7] By comparison, crossbreeds and breeds such as Beagles and Poodles have a survival estimate at 10 years of age of between 74 to 83%.[7]

There are several reasons for this higher risk. The greatest cause of death was behavioural problems (calculated mortality of 19.8%). Studies in the UK and Denmark have a much lower mortality for this category at, respectively, 3.8 %[13] and 9.7%.[16] The difference between the UK population, the Danish population and the Dutch population is remarkable. A possible explanation for this difference could be the difference in genotype from selection for temperament. In the Netherlands Dobermann dogs are commonly used as guard and police training dogs. In the UK they are more typically family pets. It seems reasonable to conclude that continental Dobermann dogs are at a higher risk of euthanasia as a consequence of behavioural problems.

The proportional mortality for heart failure which is perceived by veterinarians as one of the major problems for the breed is 14.8%. This is comparable to 14.4% for a Swedish population[2] and 9.7% in the Danish population.[16] Dilated cardiomyopathy (DCM) is the most common heart disease in the Dobermann [4,20] and is more prevalent in males and between the age of 4 to 10 years.[4] Sudden death, as the only sign of DCM in Dobermann dogs, is estimated at 20% of all DCM cases.[4] In total 7.4% of the deaths (all males) in this study were categorized as sudden death. If these were all due to heart failure cases then the true proportional mortality of heart failure would be 22.2% which is higher than the earlier results published. However, DCM was not a reasonable explanation for all the dogs with sudden death, for example one dog was reported to have died at the age of 10 months. It is, despite the report of the owner, unlikely to be a typical form of DCM as this disease tends to occur in middle-aged dogs.[4] The remainder of the sudden death group were middle-aged male dogs. The average risk of dying from heart failure for all breed is 7%,[2] therefore it is reasonable to conclude that the Dobermann is at higher risk. The proportional mortality of cancer is 13.6%. This is comparable to 12.9% for the Danish study[16] but is in contrast with the UK study which had a calculated figure of 2.67%[13] and the Swedish study which found 22.2%.[2] The number of dogs that had died of cancer is too small to differentiate between the types of tumours. The proportional mortality of cervical spondylomyelopathy was 2.5% which is omparable with the morbidity for this parameter at 3.5%. Although cervical spondylomyelopathy is frequently observed in this breed,the true incidence may be lower and according to this questionnaire it is not an important reason for euthanasia. The proportional mortality for Dobermann hepatitis is 3.7%. Two owners reported that their dog was diagnosed as suffering from chronic hepatitis. Despite therapy efforts, most dogs die within a few months.[5,8,9] Therefore if these two dogs were added to the other three dogs the proportional mortality is 6%. A proportional mortality of 3.7% to 6% is in accordance with the estimates of Speeti et al[18] and Mandigers et al[10]. The estimate of 0.1%, based on our data from the University of Utrecht, is most likely incorrect.

In this study the proportional mortality for old age was 3.7%. Considerably lower than the 20.7% calculated for the UK population.[13] If we add the dogs for which no definite diagnosis could be made the proportional mortality is 12.3% which is comparable with the Swedish study results of 12.4%.[2]
In total 95 of 259 Dobermann dogs had various diseases. The highest incidence was 22.4% for skin problems. This is in accordance with earlier studies[6,15] The second highest disorder was incontinence. Thirty-seven of 154 female dogs (24%) were incontinent. In total 30 (73%) of the dogs suffering from incontinence were neutered. In comparison 53% of the total population was neutered. Okkens et al[14] reported the incidence of incontinence after neutering in all breeds to be 11% and Arnold et al[1] 20%. Therefore it would appear that the Dobermann has a greater risk of developing incontinence after neutering.

In conclusion, the Dobermann is, compared to other breeds, at a higher risk of dying as a consequence of behavioural problems, heart failure and cancer. Cervical spondylomyelopathy and Dobermann hepatitis can be regarded as breed problems but the incidence of the two diseases is low compared to the other disorders. The estimates published on the frequency of Dobermann hepatitis would appear to be accurate. From owners point of view, skin problems, incontinence, heart failure and behavioural problems should be given greater priority.

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Chapter 4

Association between liver copper concentration and subclinical hepatitis in Doberman pinschers

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Abstract

The prevalence of subclinical hepatitis was investigated in a group of 106 randomly selected 3 year old Doberman pinschers. Histopathologic examination of liver samples from 65 dogs (52 dogs with elevated bile acids, alkaline phosphatase activity (AP) and alanine aminotransferase (ALT), or copper granules in hepatocytes in a liver aspirate, and 13 normal dogs) revealed subclinical hepatitis in 22 dogs (19 females and 3 males). Liver copper concentrations measured using instrumental neutron activation analysis (INAA), was significantly higher (419 ± 414 µg/g dry matter; mean ± SD) (p=0.0008) in dogs with hepatitis than those without liver disease (197 ± 113 µg/g; mean ± SD). At 2.6 ± 0.6 years (mean ± SD) hepatitis persisted in 5 of 16 dogs available for examination. One dog with an elevated copper concentration but normal liver subsequently developed subclinical hepatitis after three years. During the follow up period the average copper concentration of the 6 cases with persistent subclinical hepatitis was 939 ± 299 µg/g (mean ± SD) and had continued to rise significantly (p=0.02). The hepatitis in these dogs was associated with apoptotic hepatocytes and copper-laden Kupffer cells in centrolobular regions. The results of this study suggest that there is a relationship between copper storage, hepatocellular damage and hepatitis in Doberman pinschers.

Keywords*: Subclinical Hepatitis; Dogs; Etiology; Copper toxicosis.*

The pathogenesis of chronic hepatitis in Doberman pinschers, also known as Doberman hepatitis, is unknown.[4,9,10,14,19,21,26,28] The disease may be hereditary. The disease is predominantly seen in female dogs and in the clinical stage is most commonly characterized by anorexia, depression, weight loss, gastrointestinal signs, polydipsia, polyuria and icterus. [4,9,10,14,19,21,26,28] Affected Doberman pinschers at this stage have micronodular cirrhosis with histological features comparable to chronic hepatitis in man.[4,9,10,19,21,26,28] Copper concentrations are elevated in the majority of cases, with some authors concluding that the increased copper concentration is the result of concurrent cholestasis.[4,9,10,26] However in 1983 Thornburg et al[28] described 2 Doberman pinschers with a subacute hepatitis without any signs of cirrhosis or chronic cholestasis. He concluded that there was evidence that the cause of the liver disease was copper toxicosis in these 2 dogs. Recently Thornburg[26] described a group Doberman pinschers with chronic hepatitis in a precirrhotic stage of which 30 of 35 had elevated copper concentrations. He concluded that the increased copper was incidental and not the cause of disease based on the fact that the histopathology was comparable in all cases, and that 5 dogs did not have an elevated copper concentration.

Fifty-five dogs from a group of 626 apparently healthy Doberman pinschers of various ages had elevated plasma activity of liver derived enzymes.[19,21] Twenty-one of 23 dogs examined had a subclinical hepatitis and 19 dogs had high liver concentrations. It was concluded that liver copper concentration could be used as a diagnostic criterion.[19] Thus it can be concluded from all these studies that the role of copper in the pathogenesis of Doberman hepatitis is unclear.

There is a consensus that clinical hepatitis in Dobermans is more common in female dogs aged 5 to 7 years. If the clinical disease is indeed the end product of an already existing subclinical form of hepatitis, it should be possible to identify cases at 3 years of age, and follow them over time to analyze the course of the disease. Early recognition of the preclinical cases may also provide better insight in the pathogenetic role of copper, since high liver copper concentrations at that stage may indicate a primary role for copper. In the present study we investigated a random sample of Dutch Dobermans at 3 years of age by blood examination, histological examination of liver biopsies and quantitative liver copper measurements. Follow up studies of all cases with subclinical hepatitis were performed over a period of 2 to 4 years.

Materials and methods

Dogs

To investigate the prevalence of subclinical hepatitis in three-year-old Doberman pinschers a number of dogs were randomly drawn from a group of 967 Dutch Doberman pinschers (150 litters). All dogs were born between August 1, 1995 and July 31, 1996 in the Netherlands. One male and one female dog were selected from each litter born in this period. Their respective owners were then asked to participate in the study. If an animal had died or an owner failed to respond then another animal was selected from the same litter.

Procedure

Owners were requested to withhold feed from the animal 12 hours before the study. A clinical history was recorded and a physical examination performed. A jugular blood sample and 2 to 3 fine needle aspirations from the liver were obtained from all animals. The aspirates were taken in the right 9th or 10th intercostal space at approximately mid thoracic height. The aspirates were smeared on a glass microscope slide, air-dried and then stained with rubeanic acid stain to examine for the presence of hepatic copper granules.[24] Plasma bile acid concentrations, alkaline phosphatase activity (AP) and alanine aminotransferase (ALT) activity were measured from heparinized plasma.

If one or more of the blood variables was increased and/or there were copper granules visible on cytological examination of the liver aspirate then permission was requested from the owner for their dog to have a percutaneous liver biopsy using the Menghini aspiration technique.[12,15] If histologic examination did not reveal abnormalities then the owner was asked if the dog could be used as a control. Four biopsies 2-3 cm in length were taken with a 16-gauge Menghini needle: 2 for histopathological examination and 2 for quantitative copper analysis. The quantitative copper analysis was performed using instrumental neutron activation analysis (INAA) via the determination of ^{66}Cu.[2]

Tissue for histologic examination was fixed in 10% neutral buffered formalin, routinely dehydrated and embedded in paraffin. Microscope slides (4 µm) were stained with haematoxylin-eosin (HE), van Gieson's stain, reticulin stain according to Gordon and Sweet, and with rubeanic acid. Liver samples were semiquantitatively scored in a scale of 0-5 as described,[9] i.e. 0: no copper detectable; 1: solitary liver cells and/or RHS cells containing some copper positive granules; 2: small groups of liver cells and/or RHS cells containing small to moderate numbers of copper positive granules; 3: larger groups or areas of liver cells and/or RHS cells containing moderate numbers of copper positive granules; 4: large area of liver cells and/or RHS cells with many copper positive granules and 5: diffuse presence of liver cells and/or RHS cells with many copper positive granules. The liver samples for the quantitative copper analysis were put in a small copper-free plastic container, freeze-dried and stored until they were analyzed.

At this stage an animal could, based on the histopathological examination, be classified as normal or as having subclinical hepatitis. If an animal could not be classified in either of these 2 groups (undecided) the animal was re-examined after 6 months. All dogs that were found to have subclinical hepatitis and/or to have an elevated copper concentration of more than 400 µg/g dry matter were requested to come back for examination at regular intervals of 6 to 12 months after the initial liver biopsy. The concentration of 400 µg/g dry matter was chosen based on historical data from large numbers of dogs referred to our clinic during the last twenty years. Similar reference values are published elsewhere.[11,17,29] During the follow up period owners were asked not to alter the diet and to record any medication administered. During the first 6 months after recognition all dogs diagnosed with subclinical hepatitis participated in a placebo controlled study investigating the efficacy of nandrolone laureate as therapeutic drug. This drug did not alter the disease process. No other medication was administered to the dogs.

Statistics

Statistical evaluation was implemented using the software package Statistix 8.0 for Windows.[a] Descriptive statistics was used for general data. Copper data were analyzed using a two sample t-test. The level of significance was $P < 0.05$.

Results

Clinical findings

One-hundred and six dogs (47 males and 59 females) met our criteria and were used for evaluation. Physical examination of all 106 dogs was normal. Three females were in heat, 2 showed signs of pseudopregancy, 4 animals were incontinent and one animal was diagnosed earlier having hypothyroidism. Plasma bile acids concentration was elevated in 6 dogs (median value 11 µmol/l; range 11-27 µmol/l; reference value up to 8 µmol/l), ALT in 12 dogs (median value 135 U/L; range 94-226 U/L; reference value 23 to 90 U/L) and AP in 2 dogs (median value 142 U/L; range 135-148 U/L; reference value 25 to 117 U/L). Biopsies were performed on 8 of these 20 dogs.

Copper granules were found on cytological examination in 50 of 106 dogs. Twelve of these dogs had elevated liver enzymes and/or bile acids. Forty four of 50 had biopsies performed for histological evaluation and quantitative copper measurement. Forty eight out of 106 dogs did not have abnormalities on either blood examination or cytology. Of these dogs 13 underwent liver biopsy for histological evaluation and quantitative copper measurement (Table 1).

FNA= fine-needle aspirate

***Table 1**: Results of the 65 dogs from which liver tissue was examined.*

	Normal		Subclinical hepatitis		
	Males	Females	Males	Females	Total
No abnormalities in blood or FNA	6	6	-	1	13
Abnormalities in blood tests only	1	5	-	2	8
Abnormalities in FNA only	9	9	3	11	32
Abnormalities in blood tests and FNA	4	3	-	5	12
Total number of dogs	20	23	3	19	65

Histopathology

Percutaneous Menghini liver biopsies were obtained in 65 of 106 dogs. Nineteen dogs (3 males and 16 females) were diagnosed at first examination as having subclinical hepatitis. In all except one female the results of the screening examinations (blood and cytology) were abnormal. A total of 31 dogs had no histopathological abnormalities. Changes in 15 dogs were borderline (minor non-specific changes at histopathology) and owners were asked to return their dogs after 6 months. After 6 months, 3 additional dogs (all females) were diagnosed with a subclinical hepatitis. The remaining 12 dogs were scored as normal. The histopathological abnormalities were subtle in all cases. The majority demonstrated centrolobular copper-laden hepatocytes and on occasions an apoptotic hepatocyte associated with activated pigmented Kupffer cells, lymphocytes, plasma cells and scattered neutrophils.

Copper analysis

The amount of liver tissue was sufficient for a quantitative copper analysis in 64 of 65 dogs. Copper concentration in 22 dogs with hepatitis (419 ± 414 µg/g dry matter; mean ± SD) was significantly higher (P=0.0008) than in dogs without histological abnormalities (197 ± 113 µg/g dry matter; mean ± SD). However not all dogs with subclinical hepatitis had an elevated copper concentration (Fig. 1). There was no difference between sexes in copper concentration (P=0.2). The females (n=19) had a mean concentration of 449 ± 438 µg/g dry matter (mean ± SD) compared to the males (n=3) with a mean of 229 ± 88 µg/g dry matter (mean ± SD).

Two dogs without histological abnormalities had liver copper concentration above 400 µg/g dry matter. The others were below this concentration. One of these dogs was excluded from this study. It lived on a pig farm and was known to eat pig food which contained high concentrations of copper.

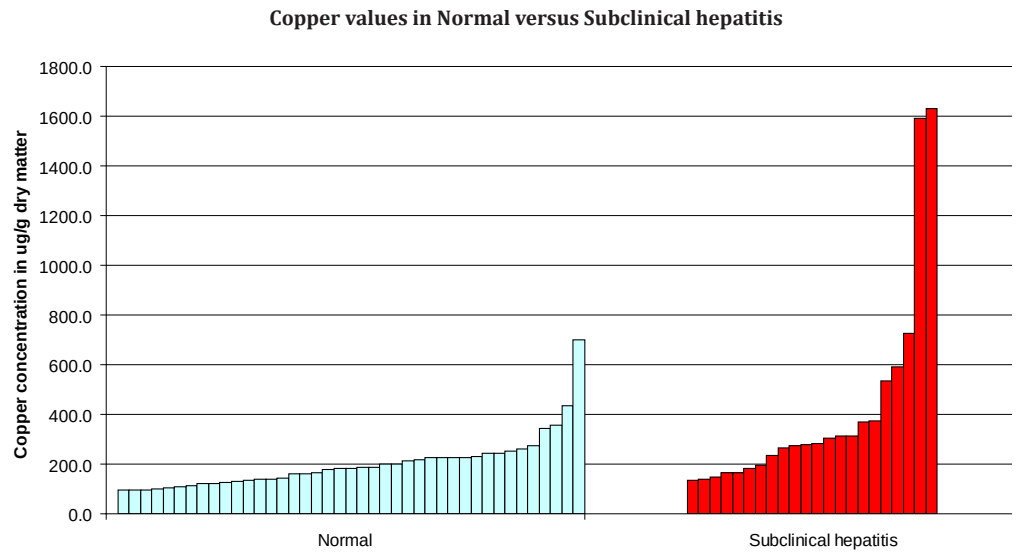

Fig 1: *Liver copper concentrations of dogs; displayed are at the left the dogs without any histological abnormalities and at the right the dogs with subclinical hepatitis. There is significant (p=0.0008) difference between these two groups. Each bar represents value for one dog.*

Follow up after two years

Eighty-two of 106 dogs were without abnormalities after 2 years. All 82 owners were asked to return their animal if any clinical signs of hepatitis developed. After a follow up period of 2 years none of the owners had returned. Owners of the 22 dogs diagnosed with subclinical hepatitis and the owner of the dog with liver copper concentration above 400 µg/g dry matter were requested to present their dog for regular (6 to 12 month) re-examination and transcutaneous liver biopsies. Seven of 23 dogs were lost for follow up before the term of 2 years for reasons not related to their subclinical hepatitis status. Seventeen dogs, 16 with subclinical hepatitis and one without, remained. In some cases we were able to obtain liver biopsies up to 4 years after the initial examination. The average follow up period was 2.6 ± 0.6 years (mean ± SD) after the first examination. Of the 16 dogs diagnosed with subclinical hepatitis, 9 dogs had no histopathological abnormalities at their last 2 examinations and 2 dogs showed a non-specific reactive hepatitis. In 5 of the 16 dogs the subclinical hepatitis persisted at each examination. The dog with a copper concentration above 400 µg/g dry matter but otherwise with no evidence of hepatic disease did develop histological evidence of

subclinical hepatitis after 3 years; the changes have been persistent. All 6 dogs had a histological copper grade of at least 2-3+ and in all dogs apoptotic hepatocytes were found associated with copper-laden Kupffer cells centrolobularly with a slight infiltration of lymphocytes, plasma cells and scattered neutrophils around the hepatic veins.

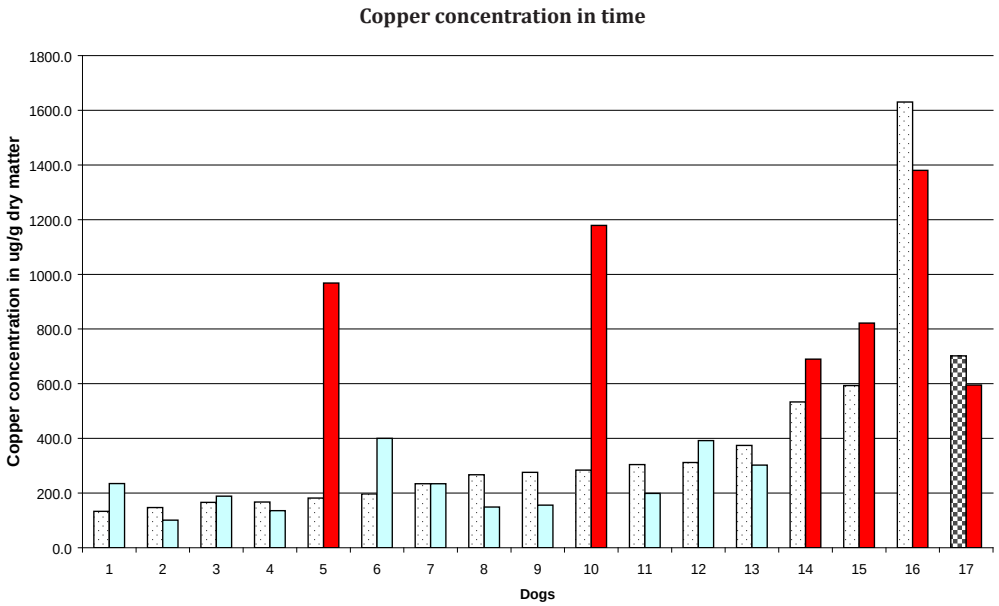

Fig 2: *Copper concentrations after a follow up period of 2.6 ± 0.6 years (mean ± SD). The first bars are the copper concentrations at time of the initial examination. Case number 17 (chessboard filling) showed at that time only an elevated copper concentration without any histopathological abnormality. The second bars are the copper concentrations at the end of the follow up period. Dogs with the dark bars still had subclinical hepatitis.*

The copper concentrations after the follow up period in the groups of dogs with persistent hepatitis (939 ± 299 µg/g dry matter; mean ± SD) and without persistent hepatitis (227 ± 100 µg/g dry matter; mean ± SD) are significantly different (P = 0.002). The dogs in which the hepatitis disappeared during the follow up period all had copper concentrations below 400 µg/g dry matter (Figure 2).

Discussion

In a group of 106 healthy Doberman pinschers at 3 years of age, 22 dogs (21%) appeared to have subclinical hepatitis on the basis of histopathology. Subclinical hepatitis in Doberman pinschers, has been previously described by Speeti et al.[19,21] They found that in a large group of 626 Doberman pinschers 55 dogs (8.8%) had an elevated ALT and 21 dogs (3.4%) had subclinical hepatitis. The percentage of subclinical hepatitis in our study is far higher then the study of Speeti et al.[21] There are several possible explanations. First, unlike the current study, the dogs studied by Speeti et al[21] were not a random selection of a one year cohort. The inclusion criteria used by Speeti et al[21] was that the ALT should be three times higher than the upper reference value. Our criteria were inclusion of any dog with elevation of liver enzymes, bile acids or the presence of copper granules in hepatocytes. For that reason it seems logical to conclude that not all our cases with subclinical hepatitis will later develop Doberman hepatitis. Speeti et al[21] found that 19 of 21 cases with subclinical hepatitis had an elevated copper concentration. By the time their results were published six dogs had died of Doberman hepatitis. In our study we found 21% of dogs had a subclinical hepatitis with both normal and increased copper concentrations. This may indicate that apart from copper also other etiological factors such as infections, deficiencies, toxins, deficient immune status or immune-mediated mechanism may be the cause of the subclinical hepatitis.[5,6,18,31] Although not significant, the majority of dogs with subclinical hepatitis in our study, turned out to be female. This may imply for instance an immune-mediated aetiology as immune-mediated diseases are more common in females[1] and are especially important in hepatitis.[13,23]

The role of copper in the pathogenesis of Doberman hepatitis has been the subject of discussion for many years. In several breeds such as Bedlington terriers,[7,16,22,32] West Highland white terriers,[27,30] Skye terriers,[8] Dalmatians[33] and Anatolian shepherd dogs[3] copper storage has been described as an etiological factor for the development of hepatitis. In the Doberman pinscher its role is unclear. Earlier studies reported normal hepatic copper concentrations up to 500 µg/g dry matter in dogs.[11,17,30,32] However in a study by Thornburg et al[29] copper concentrations in 623 purebred and mixed-breed dogs ranged from less than 100 µg/g dry matter to 6800 µg/g dry matter. In the same study hepatic copper values were measured in 20 healthy Doberman pinschers (without histopathological changes) and were found to range from 150 to 1500 µg/g dry matter (mean ± SD = 413 ± 298 µg/g dry matter). In a more recent study Thornburg[26,25] argued against the role of copper since cases with copper concentrations up to 1500 µg/g dry matter were found without any sign of hepatitis. Also Speeti et al[19,20] argued that an increased copper concentration can only

be used as discriminatory factor for diagnosis of Doberman pinscher hepatitis. In our study the subclinical hepatitis at first examination had a significantly higher copper concentration compared to the normal dogs. However both normal and elevated copper concentrations were seen.

Our data show copper concentrations in normal Doberman pinschers to be less than 400 µg/g dry matter (197 ± 113 µg/g dry matter; mean ± SD). In the follow up study on our dogs with subclinical hepatitis 10 out of 17 cases 'recovered' whereby hepatitis was morphological absent for at least 2 examinations. Furthermore the copper concentration had never been above 400 µg/g dry matter at any stage in the study. The 6 Doberman pinschers (5 females and one male) in which the initial and final copper concentration was above 400 µg/g dry matter or developed pathological copper concentrations during follow up, had persistent hepatitis. They all showed morphologically copper storage centrolobulary with copper-laden Kupffer cells, lymphocytes, plasma cells, scattered neutrophils and on occasion apoptotic hepatocytes. This suggests that there is a relation between copper storage, hepatocellular damage and hepatitis in Doberman pinschers.

In conclusion our study showed normal hepatic copper concentrations in Doberman pinschers less than 400 µg/g dry matter. Subclinical hepatitis was seen in 22 dogs which persisted in only 6 cases with increased copper concentrations suggesting a role for copper in the pathogenesis of Doberman hepatitis.

Footnotes

[a]Analytical Software, P.O. Box 12185, Tallahassee, FL 32317-2185, USA

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Chapter 5

Impaired hepatic ^{64}Cu excretion in Dobermann dogs with subclinical hepatitis

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Abstract

To investigate whether Dobermann dogs have an impaired copper excretion a radioactive copper isotope (^{64}Cu) was used intravenously. Five patients and eight normal dogs (5 normal Dobermann dogs and 3 Beagles) were studied. The patients, five female Dobermann dogs, had a subclinical hepatitis most likely associated with an increased hepatic copper concentration (median 822 mg/kg, range 690 – 1380 mg/kg dry matter). The normal dogs, five Dobermann dogs and three Beagles, had no abnormal liver histopathology and hepatic copper concentrations were considered normal (Dobermann dogs; median 118 mg/kg, range 50 – 242 mg/kg dry matter; Beagles; median 82 mg/kg, range 50 – 88 mg/kg dry matter). Plasma clearance values were in all dogs comparable without significant differences. The excretion of ^{64}Cu into the bile was significantly less for the Dobermann dogs with subclinical hepatitis compared to the normal dogs which suggests an impaired copper excretion. Cholestasis was excluded in all dogs by means of a ^{99m}Tc- Bis-IDA hepatobiliary scintigraphy. The findings suggest that Dobermann dog hepatitis is (partly) caused by a primary copper retention. These findings may have influence on future treatment protocols as well as research on the pathogenesis of the disease.

Keywords: *Aetiology; Ceruloplasmin; Copper toxicosis; Error of metabolism; Hepatic disease*

Introduction

Copper toxicosis with a resulting hepatitis was first described by Wilson in humans[35] and has been described in dogs in Bedlington terriers,[25,32] West Highland White terriers,[31] Sky terriers[8] and Dalmatians.[16] Predominantly female Dobermann dogs are, between 4 and 7 years of age, at a risk of developing chronic hepatitis. The disease is in its clinical stage most commonly characterized by anorexia, depression, weight loss, gastrointestinal signs, polydipsia, polyuria and icterus. Affected Dobermann dogs at this stage have micronodular cirrhosis.[4,9,10]

Although most cases have elevated hepatic copper concentrations the significance of this observations is unclear. Early studies suggest that the rise in hepatic copper might be caused by cholestasis.[4,9,10] Recently Speeti et al[22] described a large group of Dobermann dogs with elevated hepatic copper concentrations. Since cholestasis was only observed in a few dogs they rejected this option as a possible explanation for the rise in hepatic copper.

Recently our group described a group of three year old Dobermann dogs suffering from subclinical hepatitis with increased hepatic copper concentrations.[14] Hepatic copper concentrations in Dobermann dogs without hepatitis were significantly lower from those with hepatitis. Histopathological signs of cholestasis or cholatestasis were not seen in any of these dogs. After a follow-up period of approximately two-and-a-half year hepatitis had persisted in those Dobermann dogs in which copper laden Kupffer cells were found in the vicinity of the inflammatory area's.[14] Findings that are also seen in other breeds with copper toxicosis.[5] Since the hepatic copper concentrations had risen significantly during the follow-up period we concluded that copper may play a more pivotal role in Dobermann dogs developing chronic hepatitis.[14]

The presence of an impaired copper metabolism has been identified in man using radioactive isotopes.[13] The use of radioactive copper isotopes to study copper metabolism in dogs has been described both in Bedlington terriers[3,26] and West Highland White terriers.[3]

In the present study we used ^{64}Cu administered intravenously followed by quantitative collection of the bile and measurement of plasma clearance. To rule out the presence of cholestasis or cholatestasis we performed a quantitative ^{99m}Tc- Bis-IDA hepatobiliary scintigraphy in all dogs.[2,20] Our hypothesis is that Dobermann dogs with subclinical hepatitis associated with increased copper concentrations, have a reduced biliary excretion of copper which supports our hypothesis that this disease is (partly) caused by an abnormal copper metabolism.

Materials and methods

Dogs

Ten Dobermann dogs and three Beagles were examined. The Dobermann dogs were all client-owned and were studied after obtaining an informed consent from the owners. The three Beagles were faculty owned. Permission to carry out this study was obtained from the Ethical Committee on animal experiments (University of Utrecht) as required under Dutch legislation.
The Dobermann dogs came from a randomly selected group of 106 clinically healthy three year old Dobermann dogs, whose owners volunteered to participate in a study investigating the incidence of subclinical hepatitis in this breed.[14]

The 10 Dobermann dogs were divided into two groups: five dogs suffering from a persistent subclinical hepatitis possibly due to copper toxicosis and five normal Dobermann dogs without any histopathological abnormalities. All Dobermann dogs were already examined several times by means of histopathological liver biopsies and quantitative copper analysis.[14] The three Beagles were examined prior to the experiment using the same protocol as for the Dobermann dogs.[14] Both the normal Dobermann dogs as well as the Beagles were used as controls. Prior to the study alkaline phosphatase activity (AP), alanine aminotransferase (ALT), fasting bile acids and ceruloplasmin levels were obtained from a serum sample. Liver biopsies (2-3 cm in length) were taken using the Menghini aspiration technique[12] with a 16-gauge Menghini needle: 2 for histopathological examination and 2 for quantitative copper analysis. The quantitative copper analysis was performed using instrumental neutron activation analysis (INAA) via the determination of ^{66}Cu.[1] Since a copper concentration below 400 mg/kg dry matter is believed to be normal in dogs[6,11,19,26] three groups could be formed based on histopathological results and copper measurements:

1. Dobermann dogs with histologically persistent subclinical hepatitis associated with copper. This group is hereafter called CASH (Copper associated subclinical hepatitis). The mean hepatic copper concentration was 904 mg/kg dry matter (median 822 mg/kg, range 690 – 1380 mg/kg dry matter). All dogs were females with a mean age of 5.8 years (median 5.3 years, range 4.7 – 7.6 years).
2. Dobermann dogs histologically without hepatic abnormalities. This group is hereafter called NDD (normal Dobermann dogs). The mean hepatic copper concentration was 139 mg/kg dry matter (median 118 mg/kg, range 50 – 242 mg/kg dry matter). Three dogs were females and two males with a mean age of 4.9 years (median 4.9 years, range 3.1 – 8.5 years).

3. Three normal Beagles histologically without hepatic abnormalities. This group is hereafter called NB (normal Beagles). The mean hepatic copper concentration was 73 mg/kg dry matter (median 82 mg/kg, range 50 – 88 mg/kg dry matter). All dogs were females with a mean age of 2.5 years (median 2.5 years, range 2.0 – 3.0 years).

Procedure

The copper metabolism was investigated by measuring the ^{64}Cu plasma clearance and excretion into bile. The dogs were fasted 12 hours in advance of the study. Two hours prior to the study the animals were walked after which blood was sampled from the jugular vein, collected in a sterile heparinised tube and centrifuged. Five ml of the sterile plasma was used to administer the ^{64}Cu. The ^{64}Cu isotope was made using metallic copper wire (1.5 mg) that was irradiated for 5 hours in a reactor at a thermal neutron flux rate of $5*10^{16}$ $m^{-2}s^{-1}$, providing an induced activity of approximately 35 $MBq.mg^{-1}$. Upon arrival, approximately four hours after irradiation and one hour prior to the start of the study, the copper wire was dissolved in 50 µl concentrated HNO_3 (10.3 M) after which it was neutralized with 0.15 M NaOH. The normal circulating copper concentration in blood is in the order of 0.3 mg/l[15] which corresponds with approximately 1 mg Cu in the blood pool. The blood pool of dogs for this size is estimated to be approximately 3.5 liter. We took care not to inject more copper than 0.003 mg/kg, which is approximately 10% of the plasma pool. For each experiment, the amount administered was calculated based on these considerations and added to the earlier taken five ml of heparin plasma of the same dog. A small aliquot (0.2 ml) was taken for exact quantification of the injected dose.

For anesthesia the dogs were premedicated 15 minutes prior to induction with atropin[a] at a dose of 0.03 mg/kg intramuscularly. The animals were induced intravenously with 0.5 mg/kg methadone[b] and 0.5 mg/kg midazolam[c] and 0.5 mg/kg of methadone intramuscularly. The methadone was used to constrict the sphincter of Oddi and to prevent the gall-bladder from emptying into the duodenum[34] The dogs were further anesthetized using propofol[d] (based on effect approximately 2 mg/kg) and after intubation maintained on 0.5 liter of oxygen, 1 liter of air and approximately 1% of isofluran[e]. The animals were placed on a heating blanket in right recumbency, put on an infusion with ringer lactate at a rate of 5 tot 10 ml/kg per hour. Urinary catheters were placed. The methadone was readministered three hours after the first injection. Each hour the animals were turned onto the other side. At t=0 a 3 ml blood sample was drawn and the ^{64}Cu was injected intravenously. Blood samples were then taken 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 300 and 420 minutes after injection, and collected in EDTA-coated tubes.

One hour after injection the gall bladder was visualized ultrasonographically[f], punctured with a 22 gauge spinal needle[g] under guidance by ultrasound, and emptied completely by aspiration with a syringe. This procedure was repeated at 120, 180, 300 and 420 minutes after the start of the study. At 420 min. the experiment was terminated. The anesthesia was ended using 10 to 20 mg of Nalaxon[h] (intramuscularly).

The ^{64}Cu activity in plasma, and bile samples was measured with a gamma counter[i] using a window of 450 to 800 keV. Counting time varied from 2 minutes for the first sample to 10 minutes for the last sample. Aliquots of 1000 µl were used except for some of the early bile samples that contained less than 1000 µl. The activities were corrected for decay between administration and measurement.

To examine hepatobiliary dynamics the day after the ^{64}Cu excretion study a ^{99m}Tc-Bis-IDA scintigraphy as described earlier by Rothuizen[20] was performed. A window of 120-160 keV was used to measure the ^{99m}Tc-Bis-IDA activity. The anesthesia protocol used was similar to the day before. Right recumbency was used after placement under the gamma camera[j]. After the administration of 37 to 80 MBq (depending on weight) ^{99m}Tc-Bis-IDA (cephalic vein) the activity in the regions of the liver (excluding the regions of the gall bladder and the aorta and vena cava) were measured at regular time intervals of 5 minutes during 60 minutes, and the time-activity curves of these regions were constructed. From the descending slope of the liver time-activity curve and the ascending flow of activity within the gall bladder, the bile flow was estimated as reported earlier.[20]

Statistics

The data were analyzed using the program Statistix 8.0 for Windows[k]. Descriptive statistics were used for general data such as weight and age. For comparison of the enzyme activity, bile acids, ceruloplasmin, and recovery of ^{64}Cu from the bile between the different groups, a two sample t-test was used. For comparison of the copper concentration a Wilcoxon non-parametric test was used. The level of significance was set at 0.05.

Results

Hepatic copper concentrations for the NDD's and NB's was not significantly different (p=0.09). The difference between CASH's and the NDD's (p=0.008) as well as the NB's (p=0.02) is. The liver derived enzymes AF, ALT as well as bile acids were within reference values for all three groups.

The CASH's had a median ceruloplasmin of 40 mg/l (range 40–75 mg/l), NDD's a median of 40 mg/l (range 30 – 90 mg/l) and the NB's a median of 25 mg/l (range 29 – 50 mg/l). Since reference values of ceruloplasmin in dogs are unknown these data are compared with earlier established ceruloplasmin values evaluated in 14 normal Dobermann dogs (Mandigers, unpublished data). Compared to these data (median 40 mg/l, range 30 – 90 mg/l), ceruloplasmin values are not significant different as well (P=0.32). All dogs exhibited similar uptake and excretion patterns according to the ^{99m}Tc-Bis-IDA scintigraphy. Since all dogs were within normal limits as published earlier by Rothuizen et al (1990), bile flow was concluded to be normal in all dogs.

As can be seen in Fig 1 mean plasma curves of ^{64}Cu for the three groups were similar. ^{64}Cu clearance was 3.4 ± 0.7 ml/min/kg (mean ± SD, n=5) for the NDD's. There was no gender difference. Both the CASH's as well as the NB's fell between the 95% confidence interval of 2.0 and 4.8 ml/min/ kg. Thus there was no statistical significant difference in the clearances for any of these groups.

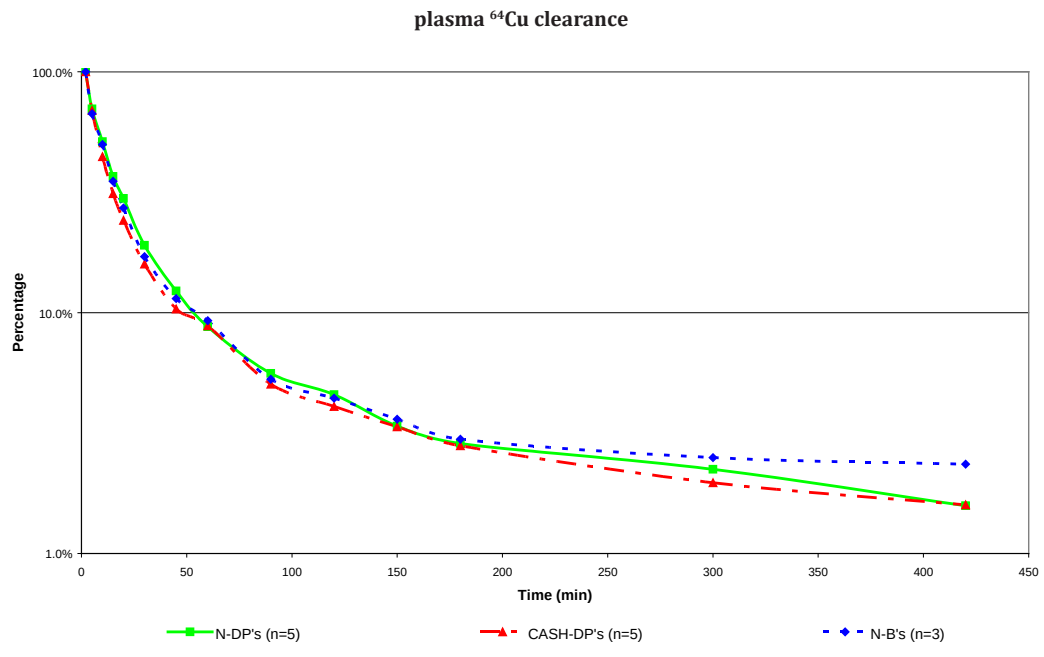

Figure 1: *Plasma 64Cu clearance values for the three groups. The plasma clearance of the CASH group is comparable with the NDD and NB group. This plasma clearance suggests a normal uptake of 64Cu by the hepatocytes in the CASH group.*

In Fig 2 the cumulative 64Cu excretion curves into the bile are shown. At t=60 there was no significant difference between the two groups CASH and NDD (p=0.28) or the combination of NDD and NB versus CASH (p=0.30). However at t=420 the difference between the two groups CASH and NDD (p=0.02) or the combination of NDD and NB versus CASH (p=0.03) was significantly different. At t=420 the median percentage of excretion into the bile was for the CASH group 1.75% (range 1.32 – 1.93%), for the NDD group 2.87% (range 2.3 – 4.3%) and for the NB group 2.72% (range 2.55 – 2.89%).

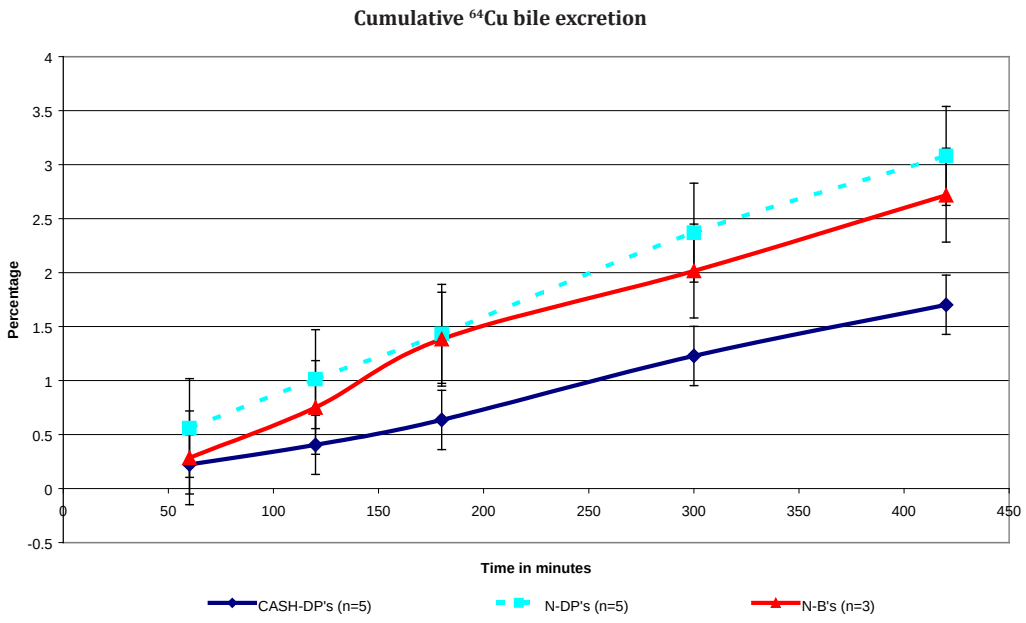

Figure 2: *Cumulative 64Cu excretion into the bile for the three groups. Displayed are mean values + standard error. Note that for the endpoint (t=420 minutes) a significant difference exists between the NB group (p=0.03), the NDD group (p=0.02) and the CASH group. The results suggest that these Dobermann dogs have an impaired 64Cu excretion into the bile.*

The amount of bile collected from the dogs during the experiment varied from 20 to 25 ml and was not significantly different for the CASH versus NDD group (p=0.45) or the combination of the two normal groups versus the CASH group (p=0.47).

Discussion

In previous publications Dobermann hepatitis has been classified as a copper associated hepatitis. There are several reasons that contributed to this classification.

First of all, in dogs with copper toxicosis the lesion of mixed-cell foci and copper retention are located centrilobulary[7,8,30,32] which is suggestive for a metabolic defect of intracellular copper metabolism.[5] In Dobermann dogs it was believed to be mainly periportal.[4,8] However recent studies have shown that the lesions in Dobermann dogs do begin centrilobular as well.[14,21,28]

Secondly it has long been thought that the copper retention was the result of cholestasis. In humans chronic biliary disease or cirrhosis can cause copper accumulation.[17] And although up to now it is still not completely clear whether cholestasis in dogs can cause a copper retention[27] it was believed to be so in Dobermann dogs.[4,9,10]

Hepatobiliary disorders with impaired hepatobiliary function have successfully been studied both in dogs by means of hepatobiliary scintigraphy.[2,20] In the present study all dogs exhibited identical uptake and excretion patterns according to the ^{99m}Tc-Bis-IDA scintigraphy. Based on these findings we can conclude that the increased hepatic copper concentration is not caused by an impaired hepatobiliary function.

Thirdly not all Dobermann dogs with (sub)clinical hepatitis have elevated hepatic copper concentrations. Recently Thornburg[28] described a group Dobermann dogs with chronic hepatitis in a precirrhotic stage of which 30 of 35 had elevated copper concentrations and 5 of 35 did not have an elevated copper concentration. A similar observation was made by Speeti et al[23] However, recently we found that only Dobermann dogs with an elevated hepatic copper concentration continued to have a persistent subclinical hepatitis.[14] An important observation in these dogs with persistent hepatitis is the presence of copper-laden Kupffer cells and macrophages as well as inflammation in the areas with hepatocytic copper storage. Findings suggestive for a primary copper toxicosis.[5]

The results of this study suggest that the Dobermann dogs with a copper-associated subclinical hepatitis have a significant lower ^{64}Cu excretion into the bile compared to normal Dobermann dogs and Beagles. The findings of this study are suggestive for a primary impaired biliary copper excretion. However chronic hepatitis in Dobermann dogs is not simply only a form of copper toxicosis. Compared with the Bedlington terrier, a breed with an established copper toxicosis due to a mutation in the Murr1 gene several differences exist.[33] First of all the clinical course of the disease is different.[4,9,32] Dobermann dogs do not, to our knowledge, develop a haemolytic crisis like

Bedlington terriers. Furthermore the hepatic copper concentrations in Dobermann dogs are rather low compared to Bedlington terriers.[7,18,32] Bedlington terriers reach hepatic concentrations up to 10.000 mg/kg dry matter concentrations, whereas Dobermann dogs with hepatitis have hepatic concentrations between 400 and 1500 mg/kg dry matter.[9,22,29] The fact that the Dobermann dogs develop chronic hepatitis at a lower hepatic copper concentration not only suggests a different error in the copper metabolism but may also suggest that other causes attribute to the disease. Recently Speeti et al[24] found an upregulation of MHC II receptors which suggests an immune mediated origin as well.

The number of dogs included in this study is small. Ideally a larger group of Dobermann dogs was studied. The Dobermann dogs in this study are client-owned and simply increasing the amount of dogs or adding a non copper associated hepatitis group proved to be impossible.

The results of the present study however show that Dobermann dogs with copper associated subclinical hepatitis (CASH) have an impaired biliary excretion of copper. This suggests a primary copper retention.

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Footnotes

[a] Atropin sulfate. Eurovet, Bladel. The Netherlands

[b] Methadone. Pharmacy department, Utrecht University

[c] Midazolam in Dormicum® Roche Netherlands BV, Mijdrecht

[d] Propofol Fresenius Kabi Netherlands BV, Den Bosch

[e] Isofluran Abott Animal Health

[f] High definition Ultrasound system; HDI 3000 ATL (Philips) with a 4-7 MHz broad band Faced-array transducer

[g] Spinal Needle 0.7 * 90 mm

[h] Nalaxon-HCl. Pharmacy department, Utrecht University

[I] Cobra auto-gamma, Packard Bioscience Benelux.

[j] Intergrated Orbiter Gamma Camera System, Siemens Medical Systems, the Netherlands.

[k] Analytical Software, P.O. Box 12185, Tallahassee, FL 32317-2185, USA

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Chapter 6

Differential expression of copper associated and oxidative stress related proteins in a new variant of copper-toxicosis in Doberman pinschers

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Abstract

The role of copper accumulation in the onset of hepatitis is still unclear. We investigated a spontaneous disease model of primary copper-toxicosis in Doberman pinschers to gain insight into the pathophysiology of this new form of copper toxicosis. We used Quantitative Real-Time PCR to determine differentially expressed genes involved in copper metabolism and ROS defences. We investigated different groups ranging from copper associated subclinical hepatitis (CASH) to a clinical chronic hepatitis with high hepatic copper concentrations (Doberman hepatitis, DH). Furthermore, a non-copper associated subclinical hepatitis group (N-CASH) with normal hepatic copper concentrations was added as a control. Most mRNA levels of proteins involved in copper binding, transport, and excretion were around control values in the N-CASH and CASH group. In contrast, many of these (including ATP7A, ATP7B, ceruloplasmin, and metallothionein) were significantly reduced in the DH group. Measurements on defences against oxidative stress showed a decrease in gene-expression of superoxide dismutase 1 (SOD1) and catalase (CAT) in both groups with high copper. Moreover, the anti-oxidative GSH molecule was clearly reduced in the DH group. In conclusion, in the DH group the expression of gene products involved in copper efflux was significantly reduced, which might explain the high hepatic copper levels in this disease. ROS defences were most likely impaired in the CASH and DH group. Overall, this study describes a new inherited copper toxicosis and could provide a molecular base for future treatment in dog and man.

Introduction

Copper (Cu) is an imperative molecule in life and, in contradiction, is highly toxic.[12] Like zinc (Zn), iron (Fe), and selenium (Se), Cu is an essential element for the activity of a number of physiologically important enzymes.[25] Cells have highly specialized and complex systems for maintaining intracellular Cu concentrations.[9] If this balance is disturbed, excess copper can induce oxidative stress which could lead to chronic inflammation.[32,34] Copper induced hepatitis has been described both in humans (Wilson's disease) as in dogs. There are several non-human models of copper toxicosis models, such as the Long-Evans Cinnamon rats and Bedlington terriers. Although the gene underlying Wilson's disease (ATP7B) is deficient in Long-Evans Cinnamon rats,[14,15,27,36] in Bedlington terriers it has been excluded as a candidate for copper toxicosis.[39] The recent discovery of mutations in *MURR1*, responsible for copper toxicosis in the Bedlington terrier, has given rise to the discovery of a new copper pathway.[40] Here we describe a new copper associated chronic hepatitis in Doberman pinschers (also called Doberman hepatitis) characterized by micro-nodular cirrhosis with elevated hepatic copper concentrations.[7,16,29,35] Until recently, the role of copper in the development and progression of hepatitis in the Doberman pinscher had been unclear. Recent studies using intravenous ^{64}Cu clearly show an impaired copper excretion in dogs with hepatitis and elevated copper concentrations (chapter 5). However, ATP7B and MURR1 have been excluded as possible candidates by genotyping (data not shown). Doberman hepatitis can therefore be seen as a new form of copper toxicosis and a possible model for other types of copper toxicosis in humans such a Indian childhood cirrhosis (ICC), non-Indian childhood cirrhosis (NICC), or idiopathic copper toxicosis (ICT).

Intracellular copper is always transiently associated with small copper-binding proteins, denoted copper chaperones, which distribute copper to specific intracellular destinations.[22] One of these copper chaperones is the anti-oxidant protein 1 or ATOX1,[18] which transports copper to the copper-transporting ATPases ATP7A and ATP7B,[41] located in the trans-Golgi network. Copper can then be bound to liver specific ceruloplasmin (CP)[4] or MURR1 and transferred outside the cell to respectively blood and bile.[42] The second chaperone, COX17, is responsible for delivering copper to the mitochondria for incorporation into cytochrome c oxidase.[6] The third chaperone, CCS, is the copper chaperone for superoxide dismutase and is responsible for the incorporation of copper into Cu/Zn superoxide dismutase (SOD1), one of the most important cytosolic enzymes in the defence against oxidative stress.[13,38] CP, also known as ferroxidase or oxygen oxidoreductase, is a plasma metalloprotein which is involved in peroxidation of Fe(II)transferrin to Fe(III)transferrin and forms 90 to 95 % of plasma copper. CP is synthesized in hepatocytes and is secreted into the serum

with copper incorporated during biosynthesis. Metallothionein 1A (MT1A) is a small intracellular protein capable of chelating several metal ions, including copper. It contains many cysteine residues which allow binding and storage of copper. Furthermore MT1A is inducible at the transcriptional level by metals and a variety of stressors such as reactive oxygen species (ROS), hypoxia, and UV radiation.[43] MT1A can donate copper to other proteins, either following degradation in lysosomes or by exchange via glutathione (GSH) complexation.

High hepatic levels of copper induce oxidative stress. There are several important proteins and molecules involved in the defence against oxidative stress. Most of the anti-oxidants can be grouped into either enzymatic defences or non-enzymatic defences.[11] The enzymatic defence against oxidative stress consists of several proteins which have tight regulations such as SOD1 and Catalase (CAT). Non-enzymatic defences against oxidative stress consist of molecules such as alpha-tocopherol, beta-carotene, ascorbate, and a ubiquitous low molecular thiol component, glutathione (GSH).[37] The present study was undertaken to investigate the effect of copper toxicosis on expression of gene-products involved in copper metabolism and oxidative stress in several gradations of copper toxicosis in the liver of the Doberman pinscher.

Experimental procedures

Dogs

Doberman pinschers were kept privately as companion animals. The dogs were presented to the Department of Clinical Sciences of Companion Animals, Utrecht University, either for a survey investigating the prevalence of Doberman (chronic) hepatitis, as described by Mandigers et al[23] or were referred for spontaneously occurring liver disease. All samples were obtained after written consent of the owner. The procedures were approved by the Ethical Committee as required under Dutch legislation.

Groups

Animals are divided in groups based on histopathological examination and quantitative copper analysis. Liver tissue of all Doberman pinschers was obtained using the Menghini aspiration technique.[21,26] Four biopsies 2-3 cm in length were taken with a 14-gauge Menghini needle for histopathological examination and quantitative copper analysis and stored for future quantitative PCR and protein investigations. The quantitative copper analysis was performed using instrumental neutron activation analysis via the determination of ^{66}Cu.[3] Histopathological biopsies were fixed in 10% neutral buffered formalin, routinely dehydrated and embedded in paraffin. Microscope slides (4 µm) were stained with haematoxylin-eosin, van Gieson's stain, reticulin stain according to Gordon and Sweet, and with rubeanic acid. All histological examinations were performed by one experienced board certified veterinary pathologist. All diseased groups, predominantly female, contained at least six animals that were compared with a group of eight age-matched healthy dogs.

Four groups were included in this study (Table 1):

1) Healthy group (n = 8), clinically healthy dogs with normal liver enzymes and bile acids. Histopathology of the liver did not reveal histomorphological lesions. Liver copper concentrations were below 200 mg/kg dry matter.
2) Non-copper associated subclinical hepatitis group (N-CASH, n = 6), dogs with liver enzymes and bile acids within reference values. Although histological examination showed evidence of a slight hepatitis, hepatic copper concentrations were within normal levels i.e. below 300 mg/kg dry matter. The dogs were classified as suffering from subclinical hepatitis, which most likely was the result of a different etiological factor, such as infections, deficiencies, other toxins, deficient immune status or immune-mediated mechanism.[31]
3) Copper associated subclinical hepatitis group (CASH, n = 6), dogs with liver enzymes and bile acids within reference values. At histopathology these dogs showed centrolobular copper-laden hepatocytes, on occasions apoptotic hepatocytes associated with copper-laden Kupffer cells, lymphocytes, plasma cells and scattered neutrophils. These lesions were classified as subclinical copper-associated hepatitis.[10,30] Hepatic copper concentrations were in all dogs above 600 mg/kg dry matter.
4) Doberman hepatitis group (DH, n = 6), dogs with chronic hepatitis and elevated hepatic copper concentrations. All dogs were referred with a clinical presentation of hepatic failure (apathy, anorexia, vomiting, jaundice, and in chronic cases sometimes ascites) and died within 2 months after diagnosis from this disease. Heparinized plasma liver enzymes (alkaline phosphatase and alanine aminotransferase) and fasting bile acids were at least three times elevated above normal reference values. Abdominal ultrasound[1] revealed small irregular shaped echo dense liver. Histopathology showed a micronodular cirrhosis with histological features of fibrosis, hepatocellular apoptosis and necrosis, mononuclear inflammation and bile duct proliferation. These lesions are comparable to chronic hepatitis in man.[31] Hepatic copper concentrations were in all cases above 1500 mg/kg dry matter.

[1] Performed with a high definition Ultrasound system; HDI 3000 ATL (Philips) with a 4-7 MHz broad band Faced-array transducer

Group	n	Hepatic copper	Copper concentrations mg/kg dry matter	Clinical observation
Healthy	8	Normal	100 – 200	No abnormalities
N-CASH	6	Normal	< 300	Sub-clinical hepatitis
CASH	6	Elevated copper levels	> 600	Sub-clinical hepatitis
DH	6	Highly elevated copper levels	> 1500	Chronic hepatitis

RNA isolation and Reverse-transcription polymerase chain reaction

Total cellular RNA was isolated from each frozen Doberman liver tissue in duplicate, using Qiagen RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions. The RNA samples were treated with Dnase-I (Qiagen Rnase-free DNase kit). In total 3 µg of RNA was incubated with poly(dT) primers at 42°C for 45 min, in a 60 µl reaction volume, using the Reverse Transcription System from Promega (Promega Benelux, Leiden, The Netherlands).

Quantitative real-time PCR of oxidative-stress proteins, copper-metabolism and other related signaling molecules

Q- PCR was performed on a total of 17 genes involved in oxidative-stress and copper-metabolism. Real-time PCR was based on the high affinity double-stranded DNA-binding dye SYBR green I (SYBR® green I, BMA, Rockland, ME) and was performed in triplicate in a spectrofluorometric thermal cycler (iCycler®, BioRad, Veenendaal, The Netherlands). For each PCR reaction, 1.67 µl (of the 2x diluted stock) of cDNA was used in a reaction volume of 50 µl containing 1x manufacturer's buffer, 2 mM $MgCl_2$, 0.5 × SYBR® green I, 200 µM dNTP's, 20 pmol of both primers, 1.25 units of AmpliTaq Gold (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands), on 96-well iCycler iQ plates (BioRad). Primer pairs, depicted in Table 2, were designed using PrimerSelect software (DNASTAR Inc., Madison, WI). All PCR protocols included a 5-minute polymerase activation step and continued for 40 cycles at 95°C denaturation for 20 sec, annealing for 30 sec, and elongation at 72°C for 30 sec with a final extension for 5 min at 72°C. Annealing temperatures were optimized at various levels ranging from 54°C till 67°C (Table 2). Melt curves (iCycler, BioRad), agarose gel electrophoresis, and standard sequencing procedures were used to examine each sample for purity and specificity (ABI PRISM 3100 Genetic Analyser, Applied Biosystems). Standard curves constructed by plotting the relative starting amount versus threshold cycles were generated using serial 4-fold dilutions of pooled cDNA fractions from both healthy and diseased liver tissues.

The amplification efficiency, $E(\%) = (10^{(1/-s)}-1)^{*}100$ (s = slope), of each standard curve was determined and appeared to be > 95 %, and < 105 %, over a wide dynamic range. For each experimental sample the amount of the gene of interest, and of the endogenous references glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyl transferase (HPRT) were determined from the appropriate standard curve in autonomous experiments. If relative amounts of GAPDH and HPRT were constant for a sample, data were considered valid and the average amount was included in the study (data not shown). Results were normalized according to the average amount of the endogenous references. The normalized values were divided by the normalized values of the calibrator (healthy group) to generate relative expression levels. Statistical analysis was performed to establish possible significance by using a student T-test.

Table 2: nucleotide Sequences of Dog-Specific Primers for Real-Time Quantitative RT-PCR

Gene	Primer	Sequence (5'-3')	Tm (°C)	Prod size (bp)	Accession number
GAPDH	Forward	TGT CCC CAC CCC CAA TGT ATC	58	100	AB038240
	Reversed	CTC CGA TGC CTG CTT CAC TAC CTT			
HPRT	Forward	AGC TTG CTG GTG AAA AGG AC	56	100	L77488 /
	Reversed	TTA TAG TCA AGG GCA TAT CC			L77489
SOD1	Forward	tgg tgg tcc acg aga aac gag atg	64	99	AF346417
	Reversed	CAA TGA CAC CAC AAG CCA AAC GAC T			
CAT	Forward	TGA GCC CAG CCC TGA CAA AAT G	62	119	AB012918
	Reversed	CTC GAG CCC GGA AAG GAC AGT T			
GSS	Forward	CTG GAG CGG CTG AAG GAC A	62	131	AY572226
	Reversed	AGC TCT GAG ATG CAC TGG ACA			
GPX1	Forward	GCA ACC AGT TCG GGC ATC AG	62	123	AY572225
	Reversed	CGT TCA CCT CGC ACT TCT CAA AA			
CCS	Forward	TGT GGC ATC ATC GCA CGC TCT G	64	96	AY572228
	Reversed	GGG CCG GCC TCG CTC CTC			
p27KIP	Forward	CGG AGG GAC GCC AAA CAG G	60	90	AY455798
	Reversed	GTC CCG GGT CAA CTC TTC GTG			
Bcl-2	Forward	TGG AGA GCG TCA ACC GGG AGA TGT	61	87	AB116145
	Reversed	AGG TGT GCA GAT GCC GGT TCA GGT			
ATOX1	Forward	ACG CGG TCA GTC GGG TGC TC	67	137	AF179715
	Reversed	AAC GGC CTT TCC TGT TTT CTC CAG			
COX17	Forward	ATC ATT GAG AAA GGA GAG GAG CAC	60	127	AY603041
	Reversed	TTC ATT CTT CAA GGA TTA TTC ATT TAC A			
ATP7A	Forward	CTA CTG TCT GAT AAA CGG TCC CTA AA	50	99	AY603040
	Reversed	TGT GGT GTC ATC ATC TTC CCT GTA			
ATP7B	Forward	GGT GGC CAT CGA CGG TGT GC	56	136	AY603039
	Reversed	CGT CTT GCG GTT GTC TCC TGT GAT			
CP	Forward	aat tct ccc ttc tgt ttt tgg tt	62	97	AY572227
	Reversed	TTG TTT ACT TTC TCA GGG TGG TTA			
MT1A	Forward	AGC TGC TGT GCC TGA TGT G	64	130	D84397
	Reversed	TAT ACA AAC GGG AAT GTA GAA AAC			
MURR1	Forward	GAC CAA GCT GCT GTC ATT TCC AA	58	122	AY047597
	Reversed	TTG CCG TCA ACT CTC CAA CTC A			
XIAP	Forward	ACT ATG TAT CAC TTG AGG CTC TGG TTT C	54	80	AY603038
	Reversed	AGT CTG GCT TGA TTC ATC TTG TGT ATG			

Western blot analysis

Pooled liver tissues (n = 6) were homogenized in RIPA buffer containing 1 % Igepal, 0.6 mM Phenylmethylsulfonyl fluoride, 17 µg/ml aprotinine and 1 mM sodium orthovanadate (Sigma chemical Co., Zwijndrecht, The Netherlands). Protein concentrations were obtained using a Lowry-based assay (DC Protein Assay, BioRad). Thirty-five µg of protein of the supernatant was denatured in Leammli-buffer supplemented with Dithiothreitol (Sigma chemical Co.) for 3 min at 95°C and electrophoresed on 10 % Tris-HCl SDS PAGE polyacrylamide gels (BioRad). Proteins were transferred onto Hybond-C Extra Nitrocellulose membranes (Amersham Biosciences Europe, Roosendaal, The Netherlands) using a Mini Trans-Blot® Cell blot-apparatus (BioRad). The procedure for immunodetection was based on an ECL western blot analysis system, performed according to the manufacturer's instructions (Amersham Biosciences Europe). The membranes were incubated with 4 % ECL blocking solution and 0.1 % Tween 20 (Boom B.V., Meppel, The Netherlands) in TBS for 1 hour under gentle shaking. The incubation of the primary antibody was performed at room temperature for one hour, with a 1:2000 dilution of mouse anti-horse metallothionein (DakoCytomation B.V., Heverlee, Belgium). After washing, the membranes were incubated with horseradish peroxidase-conjugated chicken anti-mouse (Westburg B.V., Leusden, The Netherlands) at room temperature for one hour. Exposures were made with Kodak BioMax Light-1 films (Sigma chemical Co.).

Total GSH assay

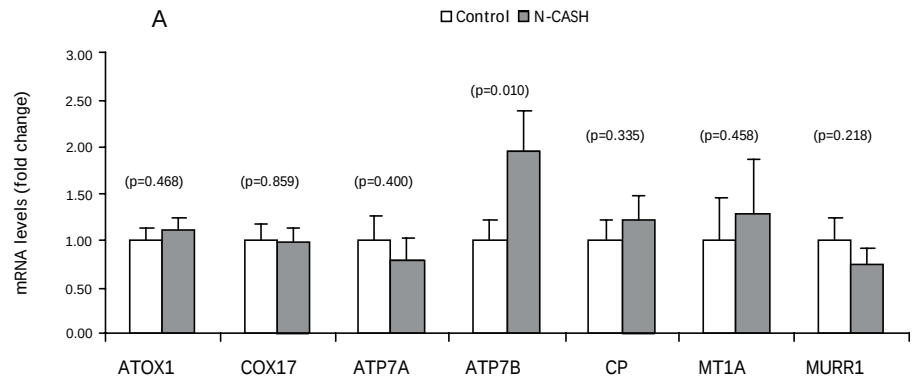
The total amount of GSH was determined by a modified version of a total Gluthathione (tGSH) Determination Colormatric Microplate Assay according to Allen et al.,[2] based on the original Tietze macro assay.[33] Protein samples from Doberman hepatitis (n = 6) and healthy controls (n = 8) were isolated as described in Western blot analysis and subsequently pooled. Total protein concentration was measured using a Lowry-based assay (DC Protein Assay, BioRad). In short 50 µl of the cell-lysate (1 mg/ml) was used in triplicate in a 96-wells plate. The lysates were incubated for 5 minutes with 50 µl of 1.3 mM DTNB, and 50 µl GSH reductase (1.5 U/ml). To start the reaction 50 µl of NADPH (0.7 mM) was added to the wells. Absorbance at 450 nm was measured at start and after 5 minutes. The rate of TNB production (yellow product) was measured in delta absorbance per minute and is directly proportionate with the amount of GSH in the samples. A standard curve was added with known concentrations GSH (0 to 20 µM) in order to determine the GSH concentrations in the samples.

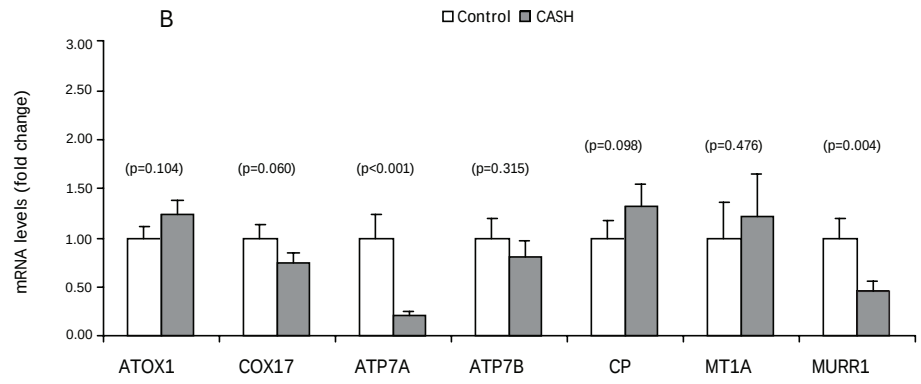
Results

To gain insight into the pathogenesis of copper toxicosis we first measured mRNA levels on several important copper binding gene-products by means of Q-PCR. Because copper toxicity is often associated with oxidative stress we also measured several oxidative stress related gene-products. To determine a possible damaging effect of the oxidative stress we investigated proteins involved in apoptosis and cell-proliferation.

Gene-expression measurements on copper-metabolism related gene-products

In Fig. 1C, several proteins in the Doberman hepatitis group (DH) are reduced compared to healthy controls. In all groups the copper chaperone ATOX1 is not affected, whereas COX17 is decreased three-fold in the DH group and remains unchanged in the non-copper associated subclinical hepatitis group (N-CASH, Fig. 1A) and copper associated subclinical hepatitis group (CASH, Fig. 1B). The mRNA levels of both Trans-Golgi copper transporting proteins ATP7A and ATP7B are decreased, three- and two-fold respectively, in the DH group. Interestingly, mRNA levels of ATP7A are decreased in the CASH group as well (Fig. 1B). In contrast, ATP7B is not affected in the CASH group but is induced two-fold in the N-CASH group. CP mRNA levels are normal except for the DH group where it is decreased two-fold. The same observation was made with measurements on MT1A mRNA, although this protein is decreased four-fold in the DH group. The copper to bile excretion protein MURR1 is unaffected in the N-CASH group but halved in the CASH and DH group.





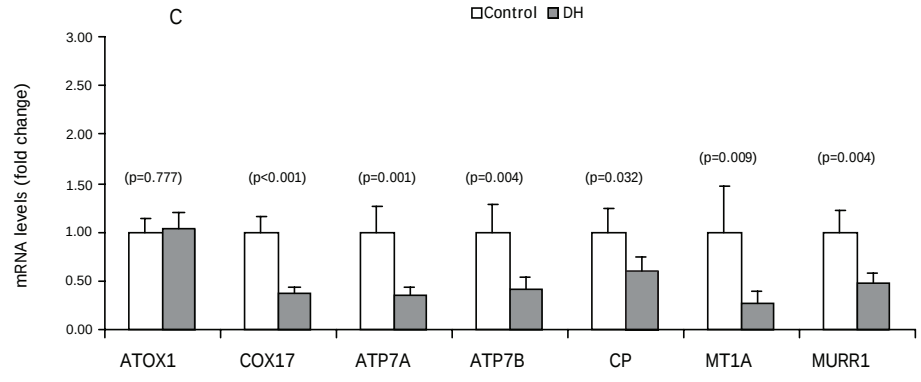


Fig. 1: *Quantitative Real-Time PCR of copper metabolism-related genes. mRNA levels of non-copper associated subclinical hepatitis (n = 6) is shown in (A). mRNA levels of copper associated subclinical hepatitis (n = 6) is shown in (B). mRNA levels of Doberman hepatitis (n = 6) is shown in (C). Data represent mean ± SE.*

Gene-expression measurements on oxidative stress markers

In Fig. 2C, SOD1 and CAT are reduced 7- and 4-fold (respectively) in the DH group compared to healthy controls. This reduction in mRNA levels can be seen in the CASH group (Fig. 2B), where SOD1 and CAT are halved, but are not lowered significantly in the N-CASH group (Fig. 2A). One of the GSH synthesis enzymes GSS is unaffected in the N-CASH group but reduced 2 to 4-fold in the CASH and DH group, respectively. The peroxidase (GPX1) responsible for converting GSSG into its reduced form GSH is induced slightly in mRNA expression in the N-CASH group, and is doubled in the CASH and DH group. The third copper chaperone CCS, responsible for the transport of copper to SOD1, is inhibited 8-fold in the DH group, 2-fold in the CASH group, and remained unchanged in the N-CASH group.

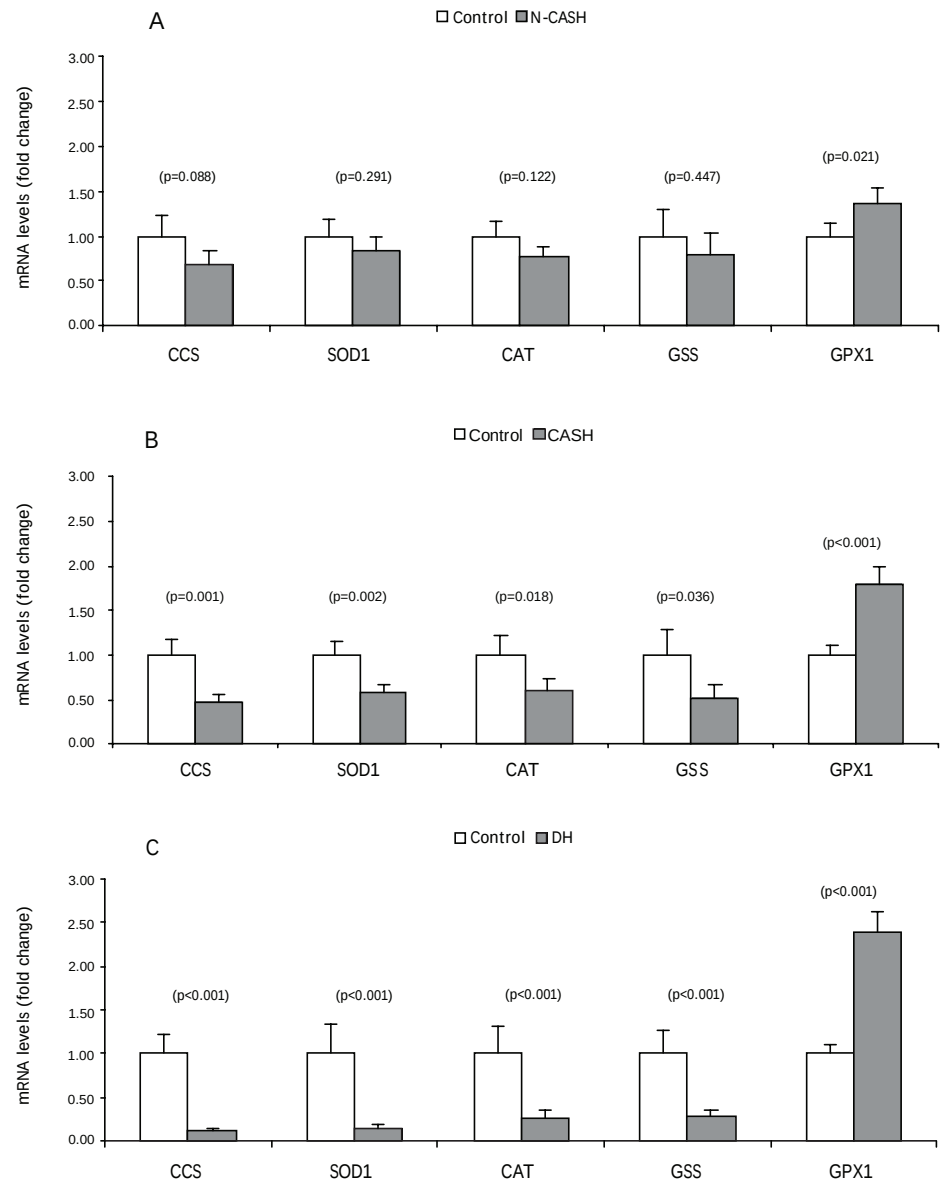


Fig. 2: *Quantitative Real-Time PCR of oxidative stress markers. mRNA levels of non-copper associated subclinical hepatitis (n = 6) is shown in (A). mRNA levels of copper associated subclinical hepatitis (n = 6) is shown in (B). mRNA levels of Doberman hepatitis (n = 6) is shown in (C). Data represent mean ± SE.*

Gene-expression measurements on apoptosis and cell-proliferation

We measured two anti-apoptotic gene-products, viz. Bcl-2, the frequently described anti-apoptotic protein, and XIAP recently associated with MURR1.33 Our apoptosis measurements on Bcl-2 showed no reduction in gene-expression in the N-CASH group (Fig. 3A), but is inhibited 4-fold in the CASH and DH group (Fig. 3B and 3C, respectively). XIAP,5 the inhibitor of apoptosis, is halved in all groups. The most dramatic changes were found in the mRNA levels of the cell-cycle inhibitor p27KIP which is inhibited 24-fold in the DH group, 12-fold in the CASH group, and 3-fold in the N-CASH group.

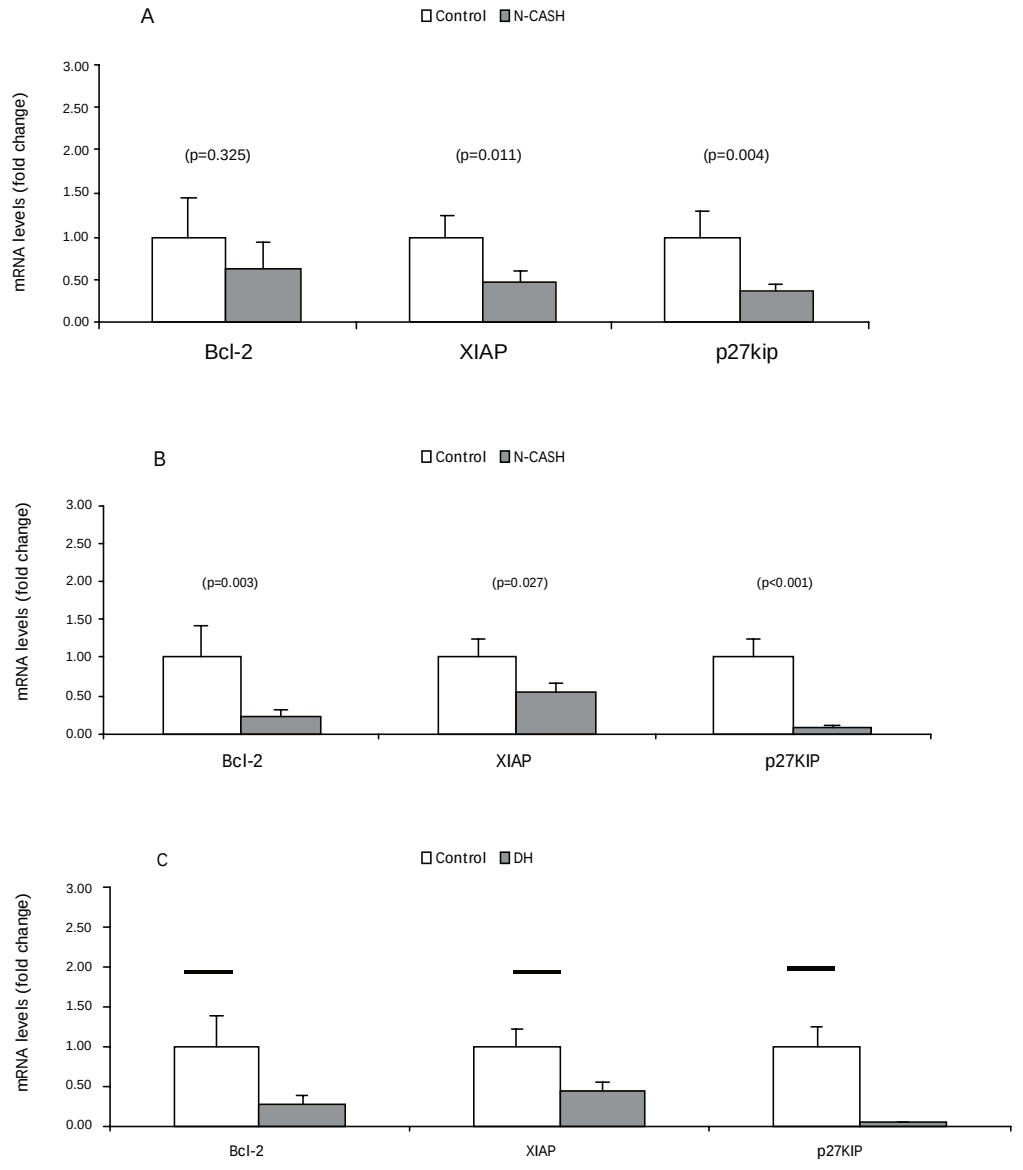


Fig. 3: *Quantitative Real-Time PCR of apoptosis and cell-proliferation related genes. mRNA levels of non-copper associated subclinical hepatitis (n = 6) is shown in (A). mRNA levels of copper associated subclinical hepatitis (n = 6) is shown in (B). mRNA levels of Doberman hepatitis (n = 6) is shown in (C). Data represent mean ± SE.*

Western blots analysis on Metallothionein proteins during copper toxicosis

Measurements on the mRNA levels of MT1A showed a marked decrease in gene expression in the DH group. In order to see if this decrease was also occurring at the protein level, Western blots were performed in order to confirm decreased mRNA levels. Therefore the total amount of metallothionein was determined from Doberman pinschers with chronic hepatitis and high copper (DH-group) levels compared to healthy Dobermans. Metallothionein was detected in both samples, where it was present as a single band of 6 kDa (Fig. 4). Interestingly, the immunoreactive band shows no difference in concentration between the two samples.

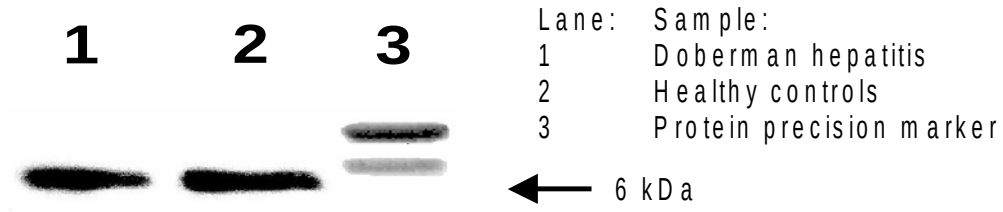


***Fig. 4**: Western blot analysis of the metallothionein proteins. Immunoreactive bands of total metallothionein of pooled fractions of the Doberman hepatitis (DH) group (n = 6) versus healthy controls (n = 8).*

Total Glutathione measurements during copper toxicosis

In order to determine if the decrease in mRNA levels of GSS decreases the GSH levels, we measured the total amount of GSH. Interestingly, in Fig. 5, the total amount of GSH in the high copper group is halved compared to healthy controls.

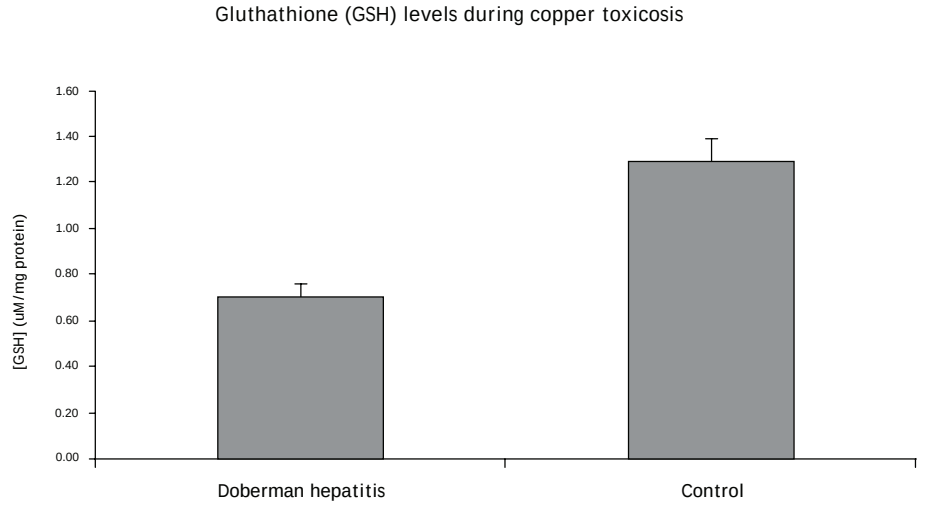


***Fig. 5:** Total glutathione measurements during copper toxicosis in Doberman. Total glutathione levels of pooled protein fractions of the Doberman hepatitis (DH) group (n = 6) versus healthy controls (n = 8).*

Discussion

In the present study the expression of a total of 15 gene products involved in copper metabolism of Doberman pinschers was measured. This provided insight into the molecular pathways of two canine copper-associated hepatic disease models ranging from sub-clinical hepatitis with elevated copper levels (CASH) to severe chronic hepatitis with high hepatic copper levels (DH). Furthermore, these diseases were compared to non-copper associated subclinical hepatitis (N-CASH).

Because of the centrolobular accumulation of copper in the hepatocytes during copper toxicosis in the Doberman a probable defect may be sought in the copper metabolism instead of a secondary effect due to, for instance, cholestasis. Recent findings of Mandigers et al. (chapter 5) indicated that Doberman pinschers with hepatitis and elevated copper concentrations suffer from impaired ^{64}Cu bile excretion which is, together with other studies, conclusive that copper toxicosis exists in the Doberman pinscher.

If copper is sequestered, in time metallothioneins will store the copper in lysosomes as described by Klein et al.[17] They found that chronic copper toxicity in Long-Evans Cinnamon rats involved the uptake of copper-loaded metallothioneins into lysosomes, where it was incompletely degraded and polymerized into an insoluble material, which contained reactive copper. This copper initiated a lysosomal lipid peroxidation, which led to hepatocyte necrosis. Phagocytosis of this reactive copper by Kupffer cells amplified the liver damage. Histological examination of the DH and CASH group samples revealed copper accumulation in lysozomes and copper-laden Kupffer cells similar to that described by Klein et al and therefore can be denoted as benchmarks of chronic exposure to copper.

In our study the gene expression levels of several gene-products involved in copper metabolism seem to be reduced in the DH and CASH group when compared to healthy controls. Short term studies on *in vitro* models all show an induction of MT1A or CP indicative of a higher efflux of copper from the hepatocytes.[8,24] The reductions that are seen in our results could therefore be ascribed to the prolonged or chronic nature of copper accumulation as dogs in the high copper or DH group present clinical signs after 2 years. Our observations are therefore not directly comparable with the short-term induced copper effects *in vitro* but are clinically more relevant, showing the effects of long-term copper accumulation in Doberman hepatitis. However, Western blot experiments on metallothionein, which stores the copper in lysozomes, did not show any reduction at the protein level. This observation could be ascribed to the antibody which binds all metallothioneins including metallothionein 2 (MT2A) which also is present in the liver. If this effect is a compensation for the decrease of MT1A remains to be proven.

In the earlier stages of copper accumulation, comparable to the CASH group, higher amounts of copper can still be excreted. Interestingly, in the N-CASH group ATP7B is indeed induced compared to healthy controls, emphasizing a possible higher efflux of copper. Furthermore, of the two subclinical disease groups the N-CASH group is the only one able to recuperate, whereas the CASH group will eventually turn into clinical hepatitis as seen in the DH group (data not shown). Taken together, our data suggest that in the Doberman pincher copper accumulates in time and finally will have its negative effect on copper metabolism and induce oxidative stress.

Oxidative stress has been ascribed to copper toxicosis as one of the most important negative effects.[28] We can confirm this with four different observations; (i) our measurements showed a decrease in mRNA levels of SOD and CAT, indicative of a reduction in the enzymatic defence against oxidative stress in all groups with copper accumulation; (ii) a reduction of GSS mRNA levels (glutathione synthesis), indicative for a reduced glutathione level in these groups which is one of the most important non-enzymatic molecules against oxidative stress; (iii) the mRNA levels of GPX1 were significantly increased, indicating an increase in GSH oxidation; (iv) the decrease in GSH was confirmed by measuring total glutathione levels in the DH group towards healthy Doberman pinschers. These results indicate a lowered protection against ROS and therefore the dogs with Doberman hepatitis may be more sensitive for exposure to copper. A similar decrease in expression of anti-oxidant enzymes was observed in ApoE-deficient mice in response to chronic inflammation,[1] and inflammatory bowel disease (IBD).[20] This indicates that chronic inflammation (copper toxicosis, atherosclerosis, IBD) is associated with reduced protection against enhanced exposure to ROS.

Other effects of high copper can also be seen in the measurements on apoptosis and cell-cycle. Measurements on Bcl-2 and XIAP indicate a decrease of protection against apoptosis, however the most affected hepatocytes will go into necrosis due to the formation of hydroxyl radicals by the Haber-Weis reaction which is catalyzed by copper.[19] A striking observation was made measuring p27KIP which was shown to be reduced up to 24-fold in the DH group. This could indicate an induction of cell-cycle compared to healthy controls. This could be ascribed to the renewal of affected hepatocytes thus managing the total amount of copper in time, which could contribute to the chronic nature and the late onset of copper toxicosis.

This study is the first to show the effect of prolonged exposure to different copper levels on oxidative stress and copper metabolism in canine livers. Whether differential gene expression is cause-or-consequence of hepatitis is unknown. However, it is conceivable that the reduction

in copper processing gene products might explain copper accumulation and the subsequent oxidative stress. The mechanism behind the differentially expressed genes in the copper related groups is unknown; furthermore we cannot ascribe this as a direct copper effect as it could be an inflammation effect, which remains to be proven. We can conclude that; (i) Doberman Hepatitis is a new variant of inherited copper toxicosis; (ii) there is a clear indication of a reduced copper excretion in the Doberman hepatitis group; (iii) there is a clear correlation between high copper levels and reduced protection against ROS; (iv) this Doberman hepatitis could be a good model to study copper toxicosis and its effects for several human copper storage diseases such as Indian childhood cirrhosis (ICC), non-Indian childhood cirrhosis (NICC), and idiopathic copper toxicosis (ICT), and provide the basis for possible future treatments.

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Chapter 7

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

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Summary

Copper retention in the liver has recently been shown to be most likely a cause of hepatitis in Doberman pinschers. We investigated genes which could be involved in copper associated hepatitis (CASH) by an association analysis of these genes in 30 phenotyped Dobermans. All dogs underwent physical examination, bloodsampling for retrieval of a coagulation profile (APTT, PT, fibrinogen), liver enzyme activities, and isolation of genomic DNA. Liver biopsies for histologic examination, copper staining, and quantitative copper measurements were performed in all dogs. The phenotype of the disease was defined as the association of subclinical or chronic hepatitis with hepatic copper concentrations higher than 600mg/kg dry matter. The group of dogs with hepatitis consisted of 8 dogs which died from DH and 10 dogs with subclinical hepatitis. A group of 12 healthy sex and aged matched Doberman pinschers was used as a control. The candidate genes *CTR1, CP, the metallothionein gene cluster, ATP7A, ATP7B, MURR1, and COX17* were investigated by genotyping of polymorphic markers close to the genes or sequence analysis of genomic DNA.

These genes could be excluded as responsible for copper associated hepatitis in Doberman pinschers.

***Keywords**: Copper toxicosis, Bedlington terrier, gene scan, liver, cirrhosis, Wilson disease*

Introduction

Chronic hepatitis in Doberman pinschers is often characterized by elevated hepatic copper concentrations,[6,13,14,20,21,28,30,31] and copper retention has recently been shown to be most likely a cause of hepatitis in the breed.[18,20] Normal copper concentrations in dog liver range between 150 to 400 mg/kg dry matter.[9,16,20,26,29] 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.[6,13,14] However, increased hepatic copper concentrations were found recently in Dobermans which displayed subclinical hepatitis but were free of cholestasis or cholatestasis. In some of the animals, hepatic copper concentrations continued to rise over a period of 3 years. The copper retention in these dogs was associated with apoptotic hepatocytes and copper-laden Kupffer cells in centrilobular regions,[19,20] suggestive for an intracellular metabolic defect of copper homeostasis.[7] Recently it was observed that Doberman pinschers with CASH, when compared to normal Doberman pinschers, have a decreased bile excretion of IV-administered ^{64}Cu. Cholestasis was excluded by 99mTC-Bis-IDA-scintigraphy in these dogs suggesting a primary copper retention in Doberman pinschers with CASH.[18]

The breed predisposition for copper associated hepatitis in the Doberman pinscher makes a genetic background likely. The aim of our study was to evaluate a possible role of genes known to be involved in copper metabolism and copper associated disorders. Copper associated hepatitis in the Doberman pinscher has phenotypical similarities to known copper storage disease in both men[8,23] as well as dog.[11,24,32,34]

The investigated genes included the Wilson disease gene (*ATB7B*),[3,4,25] Bedlington terrier copper toxicosis gene (*MURR1*),[34] the copper transporter protein gene *CTR1*[36] which is responsible for cellular uptake of copper into mammalian cells, the genes for the intracellular copper transport proteins *COX17*,[33] and the Menkes disease gene *ATP7A*,[35] which encodes a P-type ATPase for copper transport across cellular membranes. Furthermore, the metallothionein gene cluster and the Ceruloplasmin,[5] involved in copper storage and transport respectively, were considered candidates. We tested the hypothesis is that one of these copper transporter or storage genes is mutated in Doberman pinschers with copper associated hepatitis.

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 (DCSCA), Utrecht university either for a survey investigating the prevalence of subclinical hepatitis, as described earlier[20] or were referred for spontaneously occurring liver disease. The procedures were approved by the veterinary ethical committee of the Utrecht University as required under Dutch legislation. The control group consisted of Doberman pinschers (n = 12) which were clinically healthy with normal liver enzymes and bile acids. Histopathology of the liver revealed no histomorphological lesions. Liver copper concentrations, measured by INAA,[2] were below 250 mg/kg dry matter (median 171, range 104 – 245 mg/kg dm).

The second group contained Doberman pinschers (n = 10) with CASH.[20] Liver enzymes and bile acids were within reference values. At histopathology, all dogs showed centrolobular copper-laden hepatocytes, on occasions an apoptotic hepatocyte associated with copper-laden Kupffer cells, lymphocytes, plasma cells and scattered neutrophils. Lesions that classified them as subclinical hepatitis and associated with copper toxicosis.[7,19,20] Hepatic copper concentrations, measured by INAA, were in all dogs above 600 mg/kg dry matter (median 975, range 690 – 1630 mg/kg dm).

The third group (n = 8) consisted of Doberman pinschers with chronic hepatitis and elevated hepatic copper concentrations above 1500 mg/kg dm. All dogs were referred with a clinical presentation of hepatic failure (apathy, anorexia, vomiting, jaundice, and in chronic cases sometimes ascites) and died within 2 months after diagnosis from this disease. Heparinised plasma liver enzymes (alkaline phosphatase (AP) and alanine aminotransferase (ALT) and fasting bile acids were at least three times elevated above normal reference values. Histopathology showed micronodular cirrhosis with histological features of fibrosis, hepatocellular apoptosis and necrosis, mononuclear inflammation and bile duct proliferation. Hepatic copper concentrations, evaluated by means of the histopathological grading system described earlier[13] were in all cases above 1500 mg/kg dry matter.

From all animals whole blood samples were collected in EDTA. In all dogs coagulation profile from citrate plasma (APTT, PT, and fibrinogen) was established. Liver biopsies were taken according to the Menghini technique given by Lettow[17] and Rothuizen.[27] Two biopsies were fixed in 10 percent 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 chronic hepatitis with elevated hepatic copper concentrations above 600 mg/kg dry matter. The pedigrees of affected and unaffected Doberman pinschers included in this study were collected and the familial relationships between these dogs were established. Genomic DNA was isolated using a salt extraction method,[22] and frozen at –20ºC until use.

DNA type II marker retrieval and microsatellite marker analysis

Microsatellite markers residing in the vicinity of 6 candidate genes were retrieved from the literature. Primer sequences and PCR product lengths will be published elsewhere.[12]

The forward primers were labeled with HEX, or FAM fluorescent dyes,[a] which allowed analysis on an automated ABI PRISM 3100 Genetic Analyser.[b] PCR reactions were performed in a PTC-100™ PCR system[c] in a 15 µl volume, as described elsewhere.[12]

PCR products were run with GS500-TAMRA size standard (Applied Biosystems, Foster city, CA) on an automated ABI Prism 3100 Genetic Analyzer.[d] Gene scan 3.1 software was used for genotype assessment. All microsatellite markers were evaluated for heterozygosity and polymorphism, and typed in all Doberman pinschers. To evaluate whether a gene is involved in copper associated (subclinical) hepatitis of the Doberman, we compared allele frequencies between groups. For the genes *ATP7B, MURR1* and *COX17*, with two analyzed microsatellite markers haplotypes of the marker alleles were deduced. The haplotypes of dogs that are homozygous for one or both of the two markers can be assigned unequivocally. The haplotypes of the dogs that were heterozygous in both markers were based on the frequencies of the haplotypes in the unequivocal group. The association of the haplotypes with the phenotype of the disease in Dobermans was assessed.[1,15]

Canine DNA sequencing

DNA type II markers for the genes *ATP7A* and *CTR1* were not or insufficiently polymorphic on Doberman DNA. Therefore all exons of these two genes, 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 literature. One primer of each pair was tailed with M13 forward sequencing primer and the other with M13 reverse sequencing primer, described elsewhere.[12] Obtained sequences were aligned using Seqman of the Lasergene package.[f]

Results

Dogs of a single breed that are affected by a hereditary disease can be expected to share the causative gene and alleles of markers in close vicinity. This will lead to an uneven allele distribution between groups of affected and unaffected dogs. The microsatellite markers close to the genes coding for metallothionein proteins, ceruloplasmin, *ATP7B, MURR1*, and *COX 17* were polymorphic in the study population, and were found to be sufficiently informative. The results from the genotyping of the microsatellite markers as well as of *CTR1* are given in Tables 1 and 2. For each patient both alleles of each microsatellite marker are depicted. None of the deduced haplotypes showed association with the phenotype under autosomal dominant or recessive mode of inheritance.

The DNA marker for the gene *ATP7A* was not polymorphic in the group of Doberman pinschers. Furthermore the genotyping results for *CTR1* were considered insufficiently informative, because of the limited variation in the corresponding microsatellite marker.

Therefore, the coding exons of the candidate genes *ATP7A* and *CTR1*, were analyzed by genomic DNA sequencing. There was no variation in any coding DNA sequence of the examined dogs.

Table 1: *Allele frequencies by patient – gene scan analysis*

		CTR1[a]		ceruloplasmine[a]						MT2[a]	
	Length (bp)[b]	152[b]	154	395	407	409	411	413	430	284	294
patients[e]	1	●[c]	●				●●			●	●
	2		●●[d]			●●				●	●
	3		●●				●●				●●
	4		●●		●●						●●
	5		●●			●	●			●	●
	6	●	●			●	●				●●
	7	●●					●●			●	●
	8		●●			●	●			●	●
subclinical[f]	9			●●							
	10		●●			●	●				●●
	11		●●		●		●				●●
	12		●●			●	●				●
	13									●	●
	14		●●				●	●			●●
	15		●●				●	●			●●
	16		●●			●	●			●	●
	17		●●			●	●				●●
	18		●●				●●				
normal[g]	19		●●				●●				●●
	20		●●		●	●					
	21		●●				●●				●●
	22		●●		●		●				●●
	23									●	●
	24		●●				●●				●●
	25	●	●		●		●			●●	
	26		●●		●		●				●●
	27		●●				●●				●●
	30		●●			●	●			●	●
	31		●●						●●		●●
	32					●●				●	●

For each candidate gene (a) microsatellite markers were investigated. For every microsatellite marker the length in base pairs (b) of both alleles is depicted by two circles of different sizes (c=heterozygote), or of same size (d=homozygote) for each dog. Dogs are grouped into Dobermans with clinical copper associated hepatitis (e: no 1-8), subclinical copper associated hepatitis (f: no 9-18), and normal dogs (g: 19-32). Alleles of equal size are depicted in the same colour for each microsatellite marker.

Table 2: Allele frequencies by patient – results from gene scan analysis

		ATP7B[b]						MURR1					COX17				
		REN68D20[b]		REN128H16				C04107			CF10B19		REN147E03		REN98D17		
Length (bp)[c]		229	235	232	234	236	238	162	164	166	284	294	136	138	208	210	218
patients[d]	1	●●	●	●		●		●●				●●		●●		●●	
	2	●	●	●		●				●●	●		●	●		●●	
	3	●	●				●●	●●				●●		●●		●●	
	4	●	●	●	●			●●				●●	●	●		●●	
	5	●	●					●		●		●●	●	●	●	●	
	6		●●	●	●			●		●	●	●		●●			●
	7	●●				●	●		●	●	●	●		●●		●●	
	8	●	●		●	●								●	●		●
subclinical	9	●	●														
	10		●●	●●						●		●●	●	●	●	●	
	11	●	●		●	●				●●	●●			●●		●	●
	12	●	●	●		●					●	●		●●		●	●
	13	●	●	●		●				●●	●●		●	●		●●	
	14	●	●	●			●		●	●		●●		●●		●●	
	15		●●					●		●		●●	●	●		●	●
	16	●●		●●					●	●	●	●	●	●		●●	
	17		●●							●●		●●	●●		●	●	
	18	●	●					●		●		●●	●	●	●	●	
normal	19	●	●	●			●		●	●	●	●	●●				
	20	●	●		●●			●●				●●	●	●	●	●	
	21	●●				●	●	●		●		●●		●●		●●	
	22		●●	●●						●●		●●	●	●	●	●	
	23	●	●	●		●		●●				●●		●●		●●	
	24	●●				●	●	●		●				●●		●●	
	25	●	●				●●	●		●		●●	●	●		●	●
	26	●	●		●		●	●		●	●	●		●●		●●	
	27	●●				●	●	●●				●●		●●		●●	
	30	●●				●	●	●		●	●	●		●●		●●	
	31		●●	●●				●●				●●	●	●			
	32	●●				●	●	●●				●●					

a: For each candidate gene (ATP7B, MURR1, and COX17) 2 polymorphic microsatellite markers (b) were investigated. c: For each microsatellite marker the length in base pairs of both alleles is depicted by two circles at different sizes (heterozygote), or by two circles of same size (homozygote) for each dog. d: Dogs are grouped into Dobermans with clinical copper associated hepatitis (no 1-8), subclinical copper associated hepatitis (no 9-18), and normal dogs (19-32).

Discussion

We have evaluated 7 candidate genes for copper associated hepatitis in Doberman pinschers. Comparison of the phenotype of copper associated hepatitis in the Doberman with Wilson disease[3,4,25] and copper toxicosis in Bedlington terriers[34] showed similarities in terms of hepatic copper concentrations and histopathological presentation. The genes which cause these disorders

were therefore included as candidate genes. Furthermore genes encoding proteins known to be involved in cellular copper uptake (*CTR1*),[36] intracellular copper transport (*COX17, ATP7B*),[3,10,33] copper storage (*MT1A and MT1B cluster*) and transport in blood (*CP*), were included.[5] Microsatellite markers in close vicinity of the candidate genes were investigated in 30 Dobermans, in order to identify chromosomal segments which 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 consist in the possibility of a polygenic etiology of the disorder. In such a scenario, a molecular genetic analysis would require larger groups of healthy and affected dogs and preferrably inclusion of all factors in the analysis. 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 and that this is not present in healthy dogs. For this reason, dogs were extensively phenotyped and strict criteria were kept to classify the dogs 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 a fully penetrant disease gene exists.

The microsatellite marker for the gene *ATP7A* was not informative in Doberman pinscher DNA. Furthermore, the genotyping results for *CTR1* were considered to be of restricted informative value because the level of heterozygosity of the corresponding microsatellite marker was limited. Strictly, the gene could be excluded as a candidate gene for copper associated hepatitis in our patient group because one patient (nr.7, see Table 1) was homozygous for allele 152 and did not share an allele with the remaining homozygous dogs (nr.2-5) of the patient group. However, it seemed a possibility the disease gene was localized in this region, and the outlier dog nr 7, or one of its ancestors who transmitted the disease gene, was subject to mutation of allele 154 to 152. Therefore, coding exons of the *CTR1* gene were included in the DNA sequence analysis.

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 30 Doberman pinschers by means of a candidate gene approach and we excluded many genes as candidates for mutations in an assumed monogenic etiology.

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Footnotes:

a Life sciences, Bethesda, USA

b Perkin Elmer, Foster City, Ca, USA

c MJ Research Inc, Watertown, Ma, USA

d Applied Biosystems, Foster City, CA

f DNA Star Software

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Chapter 8

A double blinded, placebo controlled study to assess the efficacy of an anabolic steroid in Dobermann dogs with subclinical hepatitis

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Summary

Chronic hepatitis is observed frequently in Dobermann dogs. It is a possible immune-mediated disorder although it has also been labelled a copper-associated hepatitis. In human medicine, androgens are used for treatment of several types of immune-mediated disorders. To evaluate the potential usefulness of nandrolone laurate, an androgenic anabolic steroid, 21 Dobermann dogs, all three years of age, with subclinical hepatitis, were treated in a double blind trial. The dogs were scored prior and after four months of treatment with either placebo or the test product. Prior to and after treatment all dogs were evaluated by clinical biochemistry and liver biopsies. No significant difference was observed for any of the clinical biochemistry values. After four months of treatment eight out of 21 dogs had no histological evidence of hepatitis and five other dogs improved. No significant difference between the two groups was observed. It is concluded that nandrolone laurate at the dosage used is not effective for treatment of subclinical hepatitis.

Keywords: *dog, immune-mediated, hepatitis, treatment*

Introduction

Chronic hepatitis is a disease observed frequently in Dobermann dogs.[5,11,12,27] It is a chronic and rapidly progressive disease characterised by fibrosis, liver cell necrosis and progressive lymphocyte and plasma cell infiltration.[11] Affected dogs usually show clinical signs between 4 and 7 years of age. The aetiology is unknown but it is suspected that the disease is hereditary.[5,11,12,24,27] Many of the dogs studied have elevated hepatic copper concentrations. Some authors have concluded that the increased copper level is a consequence of concurrent cholestasis.[5,11,12] Thornburg (1998) described a group of 35 Dobermann dogs with chronic hepatitis in a precirrhotic stage. He concluded that the increased copper was incidental and not the cause of the disease based on the fact that histopathology was comparable in all dogs and that 5 dogs did not have an elevated copper level. In contrast with this observation, Speeti et al[24] described a group of 40 dogs with subclinical hepatitis and chronic hepatitis with elevated copper concentrations associated with an inflammatory reaction. As only a few dogs showed histopathological signs of cholestasis they suggested a relation between the inflammation and copper accumulation. Similar findings have been described by Mandigers et al.[16] However the centrolobular distribution of inflammation and hepatocyte loss also suggests possible immune-mediated damage.[27] More recently Speeti et al[25] described the expression of major histocompatibility complex (MHC) class II antigens in hepatocytes in Dobermann hepatitis which is also suggestive of an immune-mediated aetiology as well.

Speeti et al[24] described a group of clinically healthy Dobermann dogs where asymptomatic, subclinical hepatitis was diagnosed at a younger age (2 to 4 years of age). The dogs had elevated serum liver enzyme activity and the final diagnosis was made by histological examination of a liver biopsy. In Dobermann dogs both the clinical[5,11,12,27] and subclinical form are predominantly seen in females.[16,24] In humans, immune-mediated disease is also more common in females[2,3] in particular auto-immune hepatitis.[17,28]

Until now the management of Dobermann hepatitis with drugs such as prednisolone, azathioprine, colchicine, ursodeoxycholic acid and D-penicillamine has proven to be unrewarding.[5,11,12,22] Anabolic steroids such as androgens have various indications[20] of which the most well known are inducing erythropoiesis and treatment of metastasised mammary carcinomas in females.[8] However, there are also studies describing the use in immune-mediated disorders. In humans, Ahn et al[1] found that nandrolone decanoate, a synthetic androgen, increased the haematocrit in 12 out of 15 human patients with immune-mediated haemolytic anaemia. The cell membrane bound fraction of

C3 on the erythrocyte decreased significantly suggesting a role in decreasing auto-immunity. In another study nandrolone decanoate appeared to be effective in human patients with immune-mediated thrombocytopenia[18] with comparable results described by Schreiber et al[23] and Pignon et al.[19] However the exact mechanism of action in immune-mediated disorders is unknown.
It is unclear if androgens are effective in canine immune-mediated disease. Bloom et al[4] used it successfully in immune-mediated thrombocytopenia in one dog. Holloway et al[9] studied the successful effect in a dog with immune-mediated anaemia and thrombocytopenia. However, to our knowledge, there are no published controlled studies in a larger series of dogs. The objective of the present study was to investigate in a double blinded study whether nandrolone-laurate may either improve or cure the subclinical hepatitis as seen in Dobermann dogs.

Materials and Methods

Dogs

Twenty-four Dobermann dogs with, based on a liver biopsy, subclinical hepatitis.

Sampling

Dogs (n=106) were selected at random from a group of 946 purebred Dobermann dogs born in the Netherlands between August 1995 and July 1996. All of these dogs were approximately 3 years of age, were client-owned and, according to their owners, clinically healthy. Dogs were included in the study only after fully informed written consent from their owners. Permission for the study was obtained from the Departmental ethical committee (Utrecht University) and the Agency for Registration of Veterinary Medicinal Products (Ministry of Agriculture, Nature and Food quality).
A clinical history was recorded from all dogs and they underwent a physical examination. A blood sample was collected from the jugular vein for liver enzyme activities, bile acid and fibrinogen. The amount of fibrinogen was measured to assess coagulation. Two to three fine needle aspirates were collected from the liver of each dog. The latter were taken from the right lateral side at approximately mid thoracic height of the 9th or 10th intercostal space. The fine needle aspirates were smeared onto a clean slide, air-dried and then stained with rubeanic acid stain to identify copper granules.[26] All of the smears for cytological examination were examined within a 4 week period by an experienced clinical pathologist (Teske). The reference values used for fasting bile acids were less than 9 µmol/L, for alkaline phosphatase (AP) 25 to 117 U/L, for alanine aminotransferase (ALT) 23 to 90 U/L.
All measurements were done in the same laboratory. If one or more of the three liver parameters were increased, and/or increased copper granules were found in the smears, the dogs were further examined by histological examination of large bore needle biopsies. Four liver biopsies were obtained under local anaesthesia with a Menghini needle (16 gauge) as previously described by Lettow.[14] Liver tissue was fixed in 10% neutral buffered formalin, routinely dehydrated and embedded in paraffin, slides (4 µm) were stained with haematoxylin-eosin (HE), van Gieson's stain, reticulin stain according to Gordon and Sweet, and with rubeanic acid. All histological evaluations were performed by one experienced pathologist (van den Ingh) without prior knowledge on the animal's status.

If the histological diagnosis was hepatitis and the dog was clinically healthy the owner was asked to participate in the present study. The liver biopsy was then further classified as: (1) slight hepatitis, (2) minor hepatitis, (3) moderate hepatitis and (4) marked hepatitis.
The criteria for these histological classifications were for (1) slight hepatitis- minimal inflammatory infiltrate in the hepatic parenchyma; (2) minor hepatitis- moderate to marked inflammatory infiltrate in the hepatic parenchyma / slight inflammatory infiltrate in the hepatic parenchyma and a solitary necrotic hepatocyte; (3) moderate hepatitis- slight to moderate inflammatory infiltrate in the parenchyma with some necrotic hepatocytes; and (4) marked hepatitis- moderate to marked inflammatory infiltrate in the parenchyma with several necrotic hepatocytes.
It should be noted that the histopathological abnormalities found in our dogs were subtle. The majority of the dogs showed centrolobular copper-laden hepatocytes and on occasions an apoptotic hepatocyte associated with activated pigmented Kupffer cells, lymphocytes, plasma cells and scattered neutrophils. In none of the dogs there was any histopathological sign of cholestasis. Dogs were excluded from the study if they had received any steroid treatment within 60 days prior to admission, showed overt clinical signs of another disease that might influence the normal response to treatment of liver disease (CH) or were pregnant. Topical or systemic treatment with other androgens, progestagens and/or corticosteroids or the feeding of specially prepared diets was not permitted during the study period.

Dogs were allocated to one of the two treatment groups using a randomization table. The study was set up as a prospective double-blind study: veterinarians were unaware whether they were administering placebo or treatment and the liver biopsies were examined without knowledge of the treatment background. After admission into the study the dogs were treated during four months with a total of four treatments (one injection each month) and re-examined one month after the last injection. After 12 hours fast, a blood sample for measurement of total bile acids, AP and ALT, and a large bore Menghini liver biopsy were taken. Staining and evaluation procedures were as used for

the initial studies. The liver biopsies were classified as described above with one addition: (0) no abnormalities: normal liver. The effect of treatment was assessed using score = classification (after treatment) – classification (prior to treatment)

A dog was classified as having improved if its score was -1 to -4. The classification was "no response" if the score was zero, and a dog was classified as "worsened" if the score was +1 or more. The study was unblinded only after the final score had been calculated.
Only dogs with a complete treatment and examination were evaluated statistically. If, during the study, the clinical condition of the dog deteriorated treatment was stopped. These dogs were considered as treatment failures.

Treatment and dose

The placebo was prepared in identical vials with identical labelling but only contained the arachis oil solution with 10% benzyl-alcohol. The test product was nandrolone laurate (25mg/mL) in a mixture of arachis oil solution with 10 % benzylalcohol (Laurabolin®, Intervet International bv, Boxmeer, The Netherlands). The dose rate was 5 mg nandrolone laurate per kilogram bodyweight (equivalent to 0.2 mL solution per kilogram bodyweight). The treatment was administered by subcutaneous injection by either the investigator or the referring veterinarian. The vials were stored protected from light at 15-25° C.

Statistics

The individual dog was considered the statistical unit and the level of significance (α) was set at 0.05. The statistical evaluation was carried out using the software package Statistix 8.0 for Windows (Analytical Software, P.O. Box 12185, Tallahassee, FL 32317-2185, USA). Descriptive statistics was used for the general data (sex, colour, weight, etc). A median test was used to compare the two groups for weight and age. The possible effect of treatment on the blood chemistry values was evaluated using a paired t-test. The histology scores and the response to treatment were evaluated using a Chi-square test.

Results

Dogs

In total 24 purebred Dobermann dogs (median weight 30 kg, range 25 – 46 kg; median age 3.4 years, range 2.7-7.0 years; three sires and 21 bitches), from different locations from the Netherlands were included in the study. Pedigree analysis showed that there was no over-representation of certain families.

Post admission withdrawals and dogs lost to follow-up evaluation

Twenty-one dogs completed the study. Two females, both treated with the placebo, were lost for evaluation because of owner non-compliance. One female, treated with nandrolone, was euthanized for reasons unrelated to the hepatitis shortly after entering the study. All three dogs were excluded from the study.

Treatment groups

Ten dogs received the nandrolone treatment, eleven dogs the placebo treatment. The median age of the placebo treated dogs was 3.5 years of age (range 3.0-7.0 years) and of the nandrolone treated dogs 3.3 years of age (range 2.7-4.4 years). There was no statistical age difference between the two groups (p=0.18). The median weight of the placebo treated dogs was 30 kg (range 25-36 kg) and of the nandrolone treated dogs 30.5 kg (range 27.5-46 kg). There was no statistical weight difference between the two groups (p=0.85).

Blood examination

Fasting bile acids, AP, ALT and fibrinogen were measured before and after and compared between the two groups. The measurements at the end of the study were not significantly different for any of the values for the two treatment groups (Table 1).

Table 1: Liver enzymes, bile acid and fibrinogen prior to treatment and after four months of treatment. Each dog received each month, during a period of four months, either nandrolone or the placebo. The p values in the 4th and 7th column demonstrated that none of the parameters showed a significant difference when using a paired t-test.

Parameter	Placebo group mean ± SD		p	Nandrolone group mean ± SD		p
	before treatment	after treatment		before treatment	after treatment	
AP (U/L)	58.3 ±21.6	65.6 ± 35.5	0.34	53.7 ± 25.3	45.4 ± 10.5	0.14
ALAT (U/L)	76.9 ± 58.1	56.8 ± 52.9	0.23	41.8 ± 26.5	38.4 ± 13.9	0.73
Bile Acids (umol/l)	3.9 ± 2.2	4 ± 2.5	0.88	2 ± 1.2	5.4 ± 4.6	0.07
Fibrinogen (g/L)	1.4 ± 0.3	1.7 ± 0.4	0.18	1.4 ± 0.5	1.3 ± 0.3	0.53

Cytology

At the start of the study the fine needle aspiration smears showed copper positive granules in the hepatocytes in 19 dogs. On average 6.5 ± 5.6 % of all liver cells contained copper granules. There was no statistical difference between the two groups (p=0.60). Histopathology

Prior to the study all dogs had histological evidence of hepatitis. Eleven dogs showed slight hepatitis (Class 1), seven showed minor hepatitis (Class 2) and three dogs showed a moderate hepatitis (Class 3). None of the dogs had signs of cholestasis. The dogs were assigned randomly to one of the two treatment groups without prior knowledge of their histopathology classification (Table 2). There was no significant difference in histological grading between the two groups prior to the treatment (p=0.43). After completing the treatment eight dogs had no histological evidence of hepatitis (Class 0). Seven dogs had slight hepatitis (Class 1), five minor hepatitis (Class 2) and one dog a moderate hepatitis (Class 3) (Table 2). Again none of the dogs had signs of cholestasis after the treatment period.

Table 2: Classification of the dogs prior to treatment and after four months of treatment. Each dog received each month, during a period of four months, either nandrolone or the placebo. The lesions are classified into five groups. The two groups prior to and after the treatment were not signficantly different.

Histopathology classification	Placebo group number		Nandrolone group Number	
	before treatment	after treatment	before treatment	after treatment
No abnormalities	0	4	0	4
slight hepatitis	6	4	5	3
minor hepatitis	2	2	5	3
moderate hepatitis	2	0	1	1
marked hepatitis	0	0	0	0
Total	10	10	11	11

Table 3: Histopathological classification of twenty-one dogs with subclinical hepatitis prior to treatment and four months after nandrolone or placebo treatment. The third and sixth columns display the score obtained comparing the classification prior and after the treatment. Dogs with a score between –1 and –3 did improve, those with a score of 0 showed no response and those with a score above 1 worsened.

Placebo treated dogs Histopathology classification			Nandrolone treated dogs Histopathology classification		
before treatment	after treatment	score	before treatment	after treatment	Score
2	0	-2	2	0	-2
2	0	-2	3	1	-2
1	0	-1	3	1	-2
2	1	-1	1	0	-1
1	0	-1	1	0	-1
1	1	0	2	1	-1
2	2	0	1	0	-1
2	2	0	1	1	0
3	3	0	1	2	1
1	1	0	1	2	1
1	2	1			

Score after treatment

Of the 21 dogs that completed the study, eleven had been treated with the placebo and ten with nandrolone. Five dogs had a score of –2 (improved), two had been treated with placebos and three were treated with nandrolone (Table 3). Six dogs had a score of –1 (improved); three from the placebo group and three from the nandrolone group. Six dogs did not respond to treatment (no change), of which five were placebo treated and one was nandrolone treated. Three dogs deteriorated of which one was placebo treated and two nandrolone treated. The difference between the two groups was not significant (p=0.19) (Table 4).

Table 4: Results of the two treatment groups after four months of treatment. Although 12 dogs did improve the difference was not significant ($\chi 2$: p=0.19).

	Nandrolone treatment	Placebo treatment
Improved	7	5
No change	1	5
Worsened	2	1
Total	10	11

Suspected adverse reactions to treatment

Of the eleven placebo-treated dogs 10 dogs showed no signs and one was slightly more aggressive. Of the ten nandrolone-treated dogs: six had no side effects; one dog gained two kg in weight; one had some injection site swelling after the first injection; one dog was slightly more aggressive; and one was slightly more timid.

Discussion

Subclinical hepatitis in Dobermann dogs is a histological diagnosis with an unknown aetiology. The expression of the MHC class II antigens[25] and certain histopathological features[27] suggests that the aetiology may be in part immune-mediated. That the disease is predominantly seen in females supports this hypothesis.[28] It was postulated that Nandrolone could be useful in treating subclinical hepatitis because of the effectiveness of anabolic steroids in the treatment of other immune-mediated disorders.[10] Corticosteroids are useful for treatment of autoimmune disease because they inhibit the production of (among others) Interleukin-1 (IL-1), tumour necrosis factor-alpha (TNFα) and other cytokines. Both IL-1 and TNFα are important modulators of the neuro-endocrine-immune communications.[21] In so doing, inhibit T-cell activation, especially T-helper1 and T-helper2 cells of the CD4 subpopulation.[21] T-helper1 cells of the CD4 subpopulation, and the formation of auto-antibodies, play an important role in auto-immunity.[6,21] Corticosteroids have also been shown to induce production of transforming growth factor-beta (TGFβ), which in turn may inhibit the immune response.[21] Nandrolone decanoate, is thought to reduce antibody formation.[10] And although it induces the production of the cytokines IL-1 and TNFα,[10] IL-1 is a potent stimulator of the adrenal corticosteroid production through its influence on corticotrophin-releasing hormone (CRH).[21] Nandrolone decanoate also inhibits the production of interferon-gamma (IFNγ). IFNγ is important for the communication between cells and the immune system. It is for these reasons that nandrolone decanoate is believed to have significant effects on the immune system.[10] Nandrolone laurate is from the same group as nandrolone decanoate.[20]

When treating a liver disease, anabolic steroids should be used with cautions some in particular the oral 17-α-alkyl anabolic steroids, which may possibly induce several types of liver diseases in humans such as liver adenomas, cholestasis, peliosis hepatis, and heptocellular carcinoma.[7,8,13] There is considerably less risk for the injectable, 17-β esters such as nandrolone laurate and decanoate.[13] These possible adverse effects, described in humans are, to our knowledge, not yet described in dogs. To induce a cholestatic hepatitis a hundred fold dose of a 17-β ester would be required.[20] To minimise possible risks, the authors elected to treat the dogs for a brief period of four months with an interval of one application each month. During the treatment period there was no significant difference between the side effects experienced by the two groups. There was also no influence on liver enzyme activities or bile acid.

After four months of treatment eight out of 21 dogs had no histological evidence of hepatitis and five others had improved. However, the responding dogs were equally distributed between the two treatment groups and no significant difference was found. It seems safe to conclude that the drug did not deteriorate the liver status nor did it induce cholestasis. However it did not prove to be effective in treating the subclinical hepatitis.

Several reasons for this lack of efficacy of nandrolone can be suggested. The number of dogs included in the present study is small and the study results would suggest that in some cases subclinical hepatitis will resolve spontaneously. Another reason may be that the dose and dose rate, both advised by the manufacturer, are too low for this particular indication. For example, in people nandrolone decanoate is administered orally at daily intervals.[1] This dose and interval was, among others, based on the current registration status. It is also possible that this type of anabolic steroid is not effective in this type of disease in dogs. Although Dobermann hepatitis is believed to be, among others, immune-mediated, its exact pathogenesis is still unknown.
Of interest is the observation that in eight out of 21 of our dogs with subclinical hepatitis resolved and five more dogs improved after 5 months. This is in contrast with the results of the study of Speeti et al[24] who found that most of their subclinical dogs developed clinical hepatitis. However, there are some differences between their dogs and ours. They selected only dogs in which liver enzymes in plasma were at least three times than the upper reference value. Our dogs had only slightly elevated liver enzyme activities. Speeti's dogs might therefore have had more advanced disease than the dogs in this study. Moreover our group was selected from a three-year-old cohort. Speeti's group consisted of variable ages up to 7 years of age. In addition the prevalence of hepatitis was 9.6% compared to approximately 21% in the present study. A follow-up study on our dogs with subclinical hepatitis suggested that a high copper concentration is important in the development of hepatitis.[15] In this study this factor has not been addressed nor is nandrolone an appropriate choice of treatment for a copper storage hepatopathy.

In conclusion in this small double blind controlled study, no efficacy of nandrolone laurate for treatment of subclinical hepatitis in Dobermann dogs could be demonstrated.

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Chapter 9

Improvement in liver pathology after 4 months of D-penicillamine in 5 Doberman pinschers with subclinical hepatitis

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Abstract

Five female Doberman pinschers with increased hepatic copper concentrations and persistent (3 to 4 years) subclinical hepatitis were treated with D-penicillamine for 4 months. Before and after treatment the dogs underwent clinical, hematological (RBC, WBC, differential and thrombocyte counts), and clinical chemistry (creatinine, ALP, ALT, and total bile acid concentrations) examinations and liver biopsies were examined histologically and their copper content measured quantitatively. No adverse effects were observed during treatment. There was no change in hematology or serum chemistry test results. The mean liver copper concentration was 1036 mg/kg dry matter before treatment and decreased to 407 mg/kg after treatment (P=0.03). The copper concentrations had decreased (by between 134 and 1135 mg/kg dry matter) in all of the dogs. The histopathologic appearance had improved or returned to normal in all 5 dogs. We conclude that D-penicillamine effectively reduced copper retention in these dogs and improved the histopathologic appearance of the lesions. However because D-penicillamine has both copper-chelating and anti-inflammatory properties it is not possible to draw conclusions on the etiology of this disease.

Keywords: *Dogs; Etiology; Copper toxicosis*

Introduction

The pathogenesis of chronic hepatitis in Doberman pinschers has been debated for several years. The disease, first described by Meyer et al[19], has been the subject of several studies and most affected dogs have chronic hepatitis and high liver copper concentrations.[5,13,15,18,26,27,28,31,33] The etiological role of the high copper concentration is unclear. In early studies, it was proposed that copper was an crucial factor in the pathogenesis[5,33] and D-penicillamine was suggested as a potentially effective treatment. However, later studies suggested that increased hepatic copper concentrations were only helpful to confirm the diagnosis[26] but had no etiologic role.[13,27,31] Mandigers et al[18] described a prospective study of a random selection of 3 year-old Doberman pinschers in the Netherlands. This study included a group of dogs with high liver copper concentrations associated with histopathologic evidence of subclinical hepatitis. Follow up over several years showed that persistent hepatitis was only present in dogs with high liver copper concentrations, and that the copper concentration slowly increased over time. The authors concluded that copper might, among other factors, play an important etiologic role in subclinical hepatitis in Doberman pinschers. In an IV-^{64}Cu bile excretion study, it was shown that Doberman pinschers with increased hepatic copper concentrations (> 600 mg/kg dry matter) and subclinical hepatitis excreted ^{64}Cu into the bile significantly more slowly than did normal Doberman pinschers and Beagles (chapter 5). ^{64}Cu excretion could not be attributed to a decreased bile flow or cholestasis[10,13] because hepatobiliary scintigraphy was normal in all of these dogs, and it was concluded that these dogs had impaired copper excretion. The hepatic copper concentrations are low in Doberman pinschers compared with other breeds such as Bedlington and West Highland White terriers, and normally neither Bedlington nor West Highland White terriers have hepatocellular damage or inflammation at copper concentrations measured in Doberman pinschers (between 600 and 1500 mg/kg dry matter).[23,32,34,35] Based on these recent findings, a re-evaluation of the use of D-penicillamine (a chelating agent used for copper-storage hepatopathies[36] that acts by reductive chelation and increased urinary excretion of copper[3]) in the treatment of hepatitis in Doberman pinschers was considered. Furthermore, because clinical hepatitis in Doberman pinschers responds poorly to treatment,[5,13,15,18,26,27,28,31,33] it is of interest to evaluate treatment in an early stage.

In the present study, we selected Doberman pinschers with persistent subclinical hepatitis and increased hepatic copper concentrations from our previous study group.[18] In these Doberman pinschers, subclinical hepatitis may be related to the observed copper retention because, besides other inflammatory cells, copper-laden Kupffer cells were seen. All dogs had hepatic copper

concentrations > 600 mg/kg dry matter and were treated with D-penicillamine for 4 months before re-evaluation. Our hypothesis was that D-penicillamine would decrease hepatic copper content and the extent of subclinical hepatitis.

Materials and methods

Dogs

Five Doberman pinschers with subclinical hepatitis were selected from a group studied earlier.[18] The dogs had been examined every 6 months for 3 to 4 years, and subclinical hepatitis had been present continuously during that period. Copper concentrations had been measured quantitatively every year and had increased gradually over time.[18] Subclinical hepatitis in these dogs was associated with primary copper retention. The inflammation in all dogs, had a centrolobular distribution and was characterized by a mild infiltration of lymphocytes, plasma cells, scattered neutrophils and on occasion apoptotic hepatocytes. Compared to other Doberman pinschers with subclinical hepatitis from the same group,[18] these dogs also had centrolobular accumulations of copper-laden hepatocytes and Kupffer cells. All affected dogs were client-owned and were included in the study after obtaining the informed consent of their owners. Permission to carry out this study was obtained from the Ethical Committee on Animal Experiments (University of Utrecht).

Procedure

One to 2 weeks before the start of the trial, the dogs were fasted overnight and examined the next day. Blood samples were collected into heparinized tubes to allow measurement of enzyme activity (alkaline phosphatase [ALP], alanine aminotransferase [ALT]) and concentrations of total bile acids [BA] and creatinine. Hematology (red blood cell [RBC], white blood cell [WBC], differential WBC and thrombocyte counts) was performed on blood samples collected in EDTA tubes.

Four liver biopsies were taken from each dog using the Menghini aspiration technique with a 16G needle.[17,21] Two of the biopsy samples were submitted for histopathologic examination and 2 for quantitative copper content analysis. Quantitative copper analysis was performed using instrumental neutron activation analysis (INAA) via determination of ^{66}Cu.[2] Samples for quantitative copper analysis were put in a small copper-free plastic container, freeze-dried and stored until analysis. Samples for histopathology were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Slices (4 µm) mounted on slides were stained with hematoxylin-eosin (HE), van Gieson's stain, reticulin stain according to the techniques of Gordon and Sweet, and with rubeanic acid.

Treatment

Treatment then was started with 200 mg D-penicillamine[a] PO q12h for 4 months. The drug was administered 30 minutes before feeding. Owners were asked to report any adverse effects, such as nausea, vomiting, lethargy, fever or skin problems.[22] Owners were instructed to come back to the clinic within 2 weeks after the end of treatment. After the 4-month treatment period, the dogs all underwent the same examinations as before treatment.

One board-certified pathologist (van den Ingh), blinded to the treatment, performed all of the histopathology, and samples from all of the dogs were examined at the same time.

Statistics

The data were entered in a spreadsheet (Microsoft Excel, Microsoft software 2002) and analyzed (Statistix 8.0 for Windows[b]). Descriptive statistics were used for general data. Enzyme activity, hematology and clinical chemistry results were evaluated using a paired t-test. Copper concentrations were evaluated using a Wilcoxon non parametric test. The level of significance (α) was set at 0.05.

Results

All 5 Doberman pinschers were of a similar age (median age, 7.3 years; range, 6.4-8.2 years) and weight (median, 30 kg; range, 29-36 kg) and were the same sex (female). No abnormalities were detected in any of the dogs on physical examination. ALP activity was high (212 and 346 U/L; reference range, 25 to117 U/L) in two dogs and ALT was increased in another dog (502 U/L; reference range, 23 to 90 U/L). All of the other enzyme activities of these and the other dogs were within the reference ranges. Before treatment, hepatic copper concentrations were > 600 mg/kg dry matter in all dogs (median, 1179 mg/kg; range, 630-1330 mg/kg dry matter). Histopathology disclosed subclinical hepatitis in all dogs. In the centrolobular area, apoptotic hepatocytes were found associated with copper-laden hepatocytes and Kupffer cells and there was mild infiltration of lymphocytes, plasma cells and neutrophils scattered around the hepatic veins, as described earlier.[18]

After 4 months of D-penicillamine treatment, no significant difference was observed in the liver enzyme activity, bile acid and creatinine concentrations, hematology (RBC, WBC, differential WBC and thrombocyte count), or body weight. None of the owners observed any adverse effects. After treatment, hepatic copper concentrations (median, 300 mg/kg; range 195 to 815 mg/kg dry matter) were decreased significantly compared with the pre-treatment concentrations (P=0.02). The copper concentration had decreased (by 134 to 1135 mg/kg dry matter) in all of the dogs (see Figure 1).

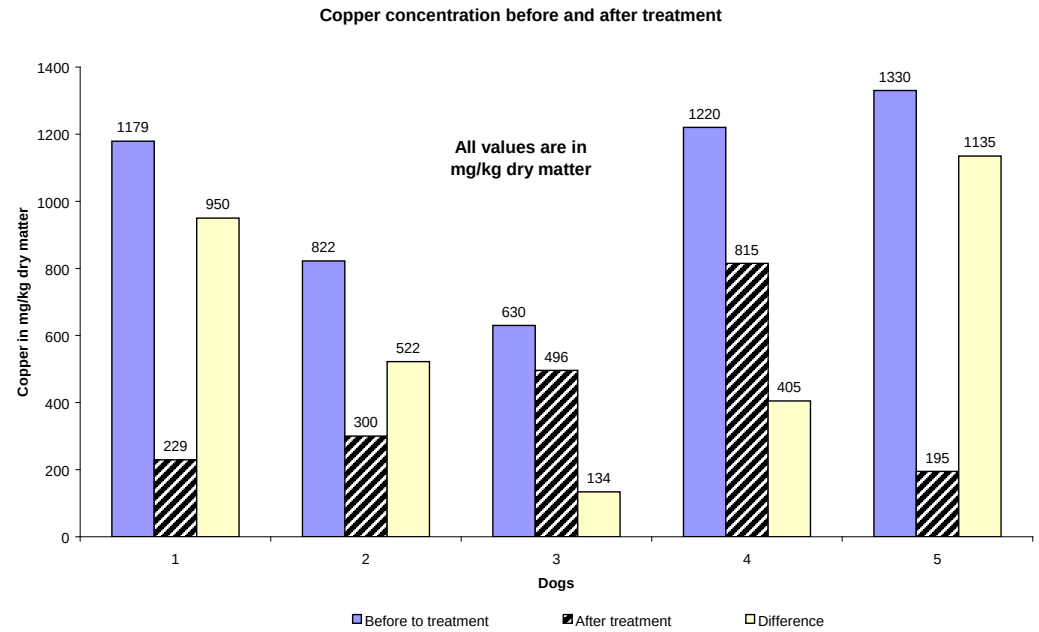

Figure 1: *Hepatic copper concentrations (mg/kg dry matter) before and after treatment with D-penicillamine. Note that there was a reduction in the hepatic copper concentration in all dogs after treatment.*

The histopathologic appearance of the liver had improved, as evidenced by absence of apoptotic cells and copper-laden Kupffer cells in all 5 dogs; in 3 dogs these cells had disappeared completely. There was histologic evidence of a steroid-induced hepatopathy in 3 of the 5 dogs.

Discussion

In this study, 5 Doberman pinschers with subclinical hepatitis were treated with D-penicillamine for 4 months. The exact etiology of subclinical hepatitis still is unclear, and we selected 5 Doberman pinschers in which the histopathology was suggestive for a possible role of copper.

Currently, 4 drugs are used as anticopper agents: D-penicillamine, tetrathiomolybdate, trientine and zinc.[3] D-penicillamine has been available the longest.[36] It prevents the formation or promotes the solubilization of copper-rich particles that occur in the lysosomes of hepatocytes and Kupffer cells.[16] Urinary copper excretion increases during treatment, suggesting active removal of copper from the liver.[7,9,16] Trientine, like penicillamine, is a copper chelator that increases urinary copper excretion.[37] It is comparable to D-penicillamine in many ways.[3] Tetrathiomolybdate, which forms a complex with protein and copper[3] and probably extracts the copper from the blood, was not available to us. Zinc is, compared with the other drugs, non-toxic and easy to obtain. It blocks intestinal absorption of copper by inducing intestinal cell metallothionein[4] but is slow acting and may take several months to have an effect.

There are several reports on the use of D-penicillamine in dogs with copper retention,[11,22,23,24] but, to our knowledge, no controlled dosage study has been performed. Published dosage rates in dogs are between 10 and 15 mg/kg q12h.[23] To avoid possible adverse effects,[3,22] a dose of 200 mg q12h, (approximately 6 mg/kg q12h) was chosen. Compared with the other drugs, D-penicillamine is an aggressive anti-copper agent that produces a large initial negative copper balance.[3] Initial treatment may take 2 to 4 months,[3] which was considered ideal for the small group of Doberman pinschers in the present study. After 4 months of treatment, we observed a significant difference in hepatic copper concentrations. Hepatic copper concentrations in all of these dogs had been increasing for several years, and it seems logical to conclude that D-penicillamine was responsible for decreasing the hepatic copper concentrations in these dogs. Both the treatment period and the dosage chosen were effective in lowering the hepatic copper concentration.

After 4 months of treatment with D-penicillamine, subclinical hepatitis was decreased in all 5 dogs; in 3 dogs it had disappeared completely. It is unlikely that subclinical hepatitis was self-limiting in these dogs,[18] particularly because the 5 dogs described in the present study had subclinical hepatitis for a period of 3 to 4 years. All of the dogs had been examined at regular intervals, and subclinical hepatitis had been persistent during that time. It therefore seems logical to conclude that this drug also was able to reduce the hepatic inflammation in these dogs.

It still is not possible to conclude that subclinical hepatitis was caused by the increased copper concentrations, and that improvement of the hepatitis after the use of D-penicillamine was caused by the decrease in hepatic copper. In humans, D-penicillamine also is used for the treatment of rheumatoid arthritis[1,14,20,25] and is believed to have both anti-inflammatory and immunosuppressive actions.[8,12,14,30] These proposed anti-inflammatory and immunosuppressive effects of D-penicillamine may, have produced the histopathologic improvement observed in the dogs of the present study. Furthermore, in 3 of 5 dogs, we observed steroid-induced hepatopathy. Similar observations were seen earlier in Bedlington terriers treated with D-penicillamine (van den Ingh, unpublished data). Because steroid-induced hepatopathy was not present before treatment, either the drug has steroid-like properties or is able to induce an endogenous steroid response. Earlier, Thornburg[31] described a group of 35 Doberman pinschers and concluded based on morphology that the disease may be immune-mediated. Speeti et al[29] found major histocompatibility complex (MHC) class II antigen expression in hepatocytes of Doberman pinschers with hepatitis, located mainly, in the centrolobular regions. They concluded that this finding was suggestive of Doberman pinscher hepatitis having an immune-mediated etiology. The results of our small trial could support this hypothesis.

In contrast with the immune-mediated hypothesis are the minimal beneficial effects of both D-penicillamine and corticosteroids in clinical Doberman hepatitis.[5,13,15,23] In humans, one of the criteria to classify hepatitis as immune-mediated is an excellent response to corticosteroids.[6] If the disease were solely copper-induced, D-penicillamine should have a better clinical effect in Doberman hepatitis as well.

Based on the findings of this small study, it is not possible to draw conclusions about the etiology of this disease. The results however are of interest. Treatment with D-penicillamine improved hepatic histopathology in these 5 Doberman pinschers with subclinical hepatitis. Early treatment seems reasonable. Together with our group›s recent finding, that impaired IV-^{64}Cu bile excretion occurs in Doberman pinschers (Mandigers et al, unpublished data), several new studies can be initiated. Larger groups of Doberman pinschers with subclinical hepatitis and copper-laden Kupffer cells should be studied. Study groups should include dogs treated with corticosteroids, other immune-modulators and zinc.

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Footnotes

[a] Penicillamine, Cuprimine® MSD, Haarlem, The Netherlands

[b] Analytical Software, P.O. Box 12185, Tallahassee, FL 32317-2185, USA

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Chapter 10

DLA Class II alleles in subclinical and chronic hepatitis in Dobermann dogs

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Abstract

With the recent findings suggestive for copper toxicosis a part of the aetiology of chronic hepatitis in Dobermann dogs has been unclosed. However it is still unclear whether auto-immunity plays a role in the pathogenesis. To investigate the possibility that a specific DLA haplotype is associated with this disease we have genotyped DLA Class II loci in Dobermann dogs. A group of Dobermann dogs that died of chronic hepatitis with high copper concentrations (n=10) and a group of Dobermann dogs with copper associated subclinical hepatitis (n=9) were compared with a group of normal Dobermann dogs (n=21). For each DLA-DRB1, DLA-DQA1 and DLA-DQB1 gene four different alleles were found which combined in five different haplotypes. There was no prevalent DLA allele or haplotype in either of the disease groups. The variation in DLA alleles was extremely limited. The majority of dogs was homozygous for one haplotype that had a frequency of 88.75% in the entire group of Dobermans. Therefore it is not possible to conclude that Dobermann dogs with chronic hepatitis have a specific immunogenic make-up that made them more susceptible compared to other Dobermann dogs.

Keywords: *Copper toxicosis; Chronic Hepatitis; Dog, DLA-DRB1; DLA-DQA1; DLA-DQB1; Immune-mediated; MHC;*

Introduction

Chronic hepatitis is a disease observed frequently in Dobermann dogs.[2,7,8,18,25,29] It is a chronic and rapidly progressive disease characterised by fibrosis, liver cell necrosis and progressive lymphocyte and plasma cell infiltration.[7] The aetiology is unknown but it is suspected that the disease is hereditary. Many of the dogs studied have elevated hepatic copper concentrations and recent studies have shown this to be essential in the aetiology.[17,18] However, there are indications that the disease might be immune-mediated as well.[26,29] In humans, auto-immune hepatitis is most often seen in females[19,31] and strongly associated with inheritance of the HLA A1-B8-DR3 haplotype or the DR4 gene.[3,16] Dobermann hepatitis is also observed more often in female dogs.[2,7,18,26] Speeti et al[26] described the expression of major histocompatibility complex (MHC) class II antigens in hepatocytes in Dobermann hepatitis which contributed to the autoimmune hypothesis as well. MHC molecules determine the level of the immune response generated through the presentation of self and nonself antigens to the immune system[12] and are associated with several autoimmune diseases.[32] In humans there are several diseases with an underlying immune component which are linked to HLA types.[30] With the recently gained knowledge on DLA alleles and haplotypes[10,11,12,32,35] it became opportune to evaluate the autoimmune hypothesis in Dobermann hepatitis by DLA allele typing. We investigated the variation in DLA alleles in normal Dobermann dogs and in Dobermann dogs with chronic hepatitis and in Dobermann dogs with copper associated subclinical hepatitis.[18]

Materials and Methods

Dogs

All samples were obtained from Dobermann dogs privately kept as companion animals after written consent of the owner. The dogs were presented to the Department of Clinical Sciences of Companion Animals, Utrecht University either for a survey investigating the prevalence of Dobermann (chronic) hepatitis, as described earlier[18] or were referred for spontaneously occurring liver disease. The procedures were approved by the ethical committee, Utrecht University as required under Dutch legislation.

1) Normal Dobermann dogs (n = 21, hereafter called N-DP) were clinically healthy and had normal liver enzymes and bile acids in plasma. Histopathology of the liver revealed no histomorphological lesions. Four biopsies 2-3 cm in length were taken as described earlier by Lettow[14] with a 16-gauge Menghini needle: 2 for histopathological examination and 2 for quantitative copper analysis. Tissue for histologic examination was fixed in 10% neutral buffered formalin, routinely dehydrated and embedded in paraffin. Microscope slides (4 µm) were stained with haematoxylin-eosin (HE),

van Gieson's stain, reticulin stain according to Gordon and Sweet, and with rubeanic acid. The quantitative copper analysis was performed using instrumental neutron activation analysis (INAA) via the determination of $^{66}Cu^{1}$ and were all within normal limitis (median 177 mg/kg dry matter; range 101 to 231 mg/kg dry matter). Normal hepatic copper concentration in dogs is between 150 to 400 mg/kg dry matter.[6,9,18,22,28]

2) Dobermann dogs (n = 9, hereafter called CASH) with a subclinical hepatitis as published earlier.[18] Liver tissue was obtained as in the normal dogs. Plasma liver enzymes and bile acids were within reference values. At histopathology all dogs showed centrolobular copper-laden hepatocytes, on occasions an apoptotic hepatocyte associated with copper-laden Kupffer cells, lymphocytes, plasma cells and scattered neutrophils. These lesions classified them as subclinical hepatitis[18] and associated with copper toxicosis.[4] Median hepatic copper concentration, measured by INAA, was 981 mg/kg dry matter; range 690 to 1630 mg/kg dry matter.

3) Dobermann dogs (n = 10, hereafter called DH) with chronic hepatitis and elevated hepatic copper concentrations. All dogs were referred with a clinical presentation of hepatic failure (apathy, anorexia, vomiting, jaundice, and in chronic cases sometimes ascites) and died within 2 months from this disease after diagnosis. Plasma liver enzymes (alkaline phosphatase (AP) and alanine aminotransferase (ALT)) and fasting bile acids were at least three times the upper reference values. Histopathology showed a micronodular cirrhosis with histological features of fibrosis, hepatocellular apoptosis and necrosis, mononuclear inflammation and bile duct proliferation. Hepatic copper concentrations, evaluated by means of the histopathological grading system described earlier[7] were in all cases above 1500 mg/kg dry matter.

From all animals whole blood samples were collected in EDTA. Genomic DNA was isolated using a salt extraction method, and frozen at –20ºC until use.[20]

DNA amplification of DLA region

The locus specific primers were intronic and are shown in Table 1. The primers were M13 tailed and are underlined in Table 1. Primers were obtained from earlier studies: DRB1[34], DQA1 forward primer[33] and the reverse from Kennedy et al.[13] The DQB1 forward primer was designed by Wagner (personal communication) and the reverse by Kennedy (previously unpublished).

PCR reactions were performed with 25 ng genomic DNA in a 15-µl reaction containing 1 x Gibco-BRL buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.33 µM primer and 0.625 U Platinum Taq polymerase (Invitrogen). The following program was used: 10 min initial denaturation step at 94°C, followed by 35 cycles of 30 sec 94°C, 30 sec T_A and 30 sec 72°C, followed by a final extension step of 10 min at 72°C. The following annealing temperatures were used 62°C, 53°C and 68°C for DLA-DRB1, DQA1 and DQB1, respectively.

DNA sequencing

Sequence reactions were performed in a 10-µl reaction containing 2 µl of 5 times diluted PCR product, 1 x sequence buffer (80 mM Tris, 2 mM $MgCl_2$, pH 9.0) 3.2 µM M13 sequencing primer and 1 µl Big Dye terminator Ready Reaction mix (Applied Biosystems). The following program was used: 25 cycles of 30 sec 96°C, 15 sec 55°C and 2 min 60°C. Sequence reactions were purified using multiscreen 96-well filtration plates (Millipore) and were diluted three times before sequencing on the ABI 3100 Genetic Analyzer (Applied Biosystems).

Table 1: *Oligonucleotides used in DNA genotyping*

Primer	DNA sequences[a]
DRB1 forward:	5'-gtt ttc cca gtc acg acc cgt ccc cac agc aca ttt c-3'
DRB1 reverse:	5'-cag gaa aca gct atg act gtg tca cac acc tca gca cca -3'
DQA1 forward:	5'-gtt ttc cca gtc acg acc tca gct gac cat gtt gc-3'
DQA1 reverse:	5'-cag gaa aca gct atg acg gac aga ttc agt gaa gag a-3'
DQB1 forward:	5'-gtt ttc cca gtc acg acc tca ctg gcc cgg ctg tct c-3'
DQB1 reverse:	5'-cag gaa aca gct atg acc acc tcg ccg ctg caa cgt g-3'
M13 Sequencing primers:	
M13 forward:	5'-gtt ttc cca gtc acg ac-3'
M13 reverse:	5'-cag gaa aca gct atg ac-3'

[a]*underlined are tails that were added for DNA sequence analysis with M13 specific primers*

Data analysis

Analysis of the sequence data was performed with Seqman (DNASTAR) software. In this programme the reverse sequence was complemented and aligned with the forward sequence of all Dobermann samples for each DLA product. Each sample was inspected and each site of potential polymorphism was viewed. The different alleles that were found were BLASTed against the NCBI database and

compared with the alleles given on the MHC Sequence Database (www.ebi.ac.uk/ipd/mhc/dla/index.html). Three loci, DLA-DRB1 / DQA1 / DQB1, haplotypes were identified by selecting first the homozygous Dobermans at all three loci. The heterozygous Dobermans were examined and haplotypes were assigned to each of these dogs.

Results

Thirty-five of 40 dogs were homozygous for DLA-DRB1-00601 (Table 2). Five of 40 dogs were heterozygous. Since parental DNA was not available we had to deduce based on the predominant DLA-DRB1-00601 type, the other alleles in the five heterozygous dogs. That meant that in the five other dogs, besides DLA-DRB1-00601, three other DLA-DRB1 alleles were present: DRB1-01501, DRB1-00101 and one allele which has not been published earlier. The predominant DLA-DQA1 allele was 00401. Thirty-two of 40 were homozygous for DLA-DQA1-00401 (Table 2). The other eight dogs had, based on the same deduction as for DRB1 that besides DQA1-00401 three others were present: DQA1-05011, DQA1-00901 and DQA1-00301. For DLA-DQB1 again 32 of 40 were homozygous for DLA-DQB1-01303. Again we deduced for the eight heterozygous Dobermans that besides DQB1-01303 three other alleles were present: DQB1-00701, DQB1-00101 and DQB1-00401.

There was no specific DLA allele that was only seen in either the CASH or the DH group (Table 2). Two dogs, who both died of DH, had DLA-DRB1 alleles that were not present in the group of normal DP. However it was not seen in the CASH group (Table 2).

In total, five different haplotypes were present (Table 3). The majority of dogs were homozygous for the same haplotype. Again there was no specific haplotype that was specific for both the CASH and DH groups except for the two dogs that died of DH (Table 2 and 3).

Table 2:

DLA allele		Number of Dobermann dogs Normal (n=21)	CASH (n=9)	Hepatitis (n=10)	
DRB1	00601	19	9	7
	00601+01501	2		1
	00601+00101			1
	00601+?????			1
DQA1	00401	16	8	8
	00401+05011	3	1	
	00401+00901	2		1
	00401+00301			1
DQB1	01303	16	8	8
	01303+00701	3	1	
	01303+00101	2		1
	01303+00401			1

Table 3: *Haplotypes found and frequencies*

RDB1	DQA1	DQB1	Percentage
00601	00401	01303	88.75%
00601	00511	00701	5%
01501	00901	00101	3.75%
00101	00301	00401	1.25%
????	00401	01303	1.25%

Discussion

In this study we show that there is extremely little variation in the DLA gene repertoire in the Dobermann dog breed. Earlier, Kennedy et al.[10] described DLA-DRB1 and DLA-DQA1 allele frequencies for the Dobermann dog and our results are in agreement with their data. We found three DRB1 alleles that have been found earlier in Dobermans, DRB1-00601, DRB1-01501 and DRB1-00101[11] and one previously unpublished allele. The allele frequency of DRB1-00601 was 93.75%. The distribution of DLA-DQA1 alleles in Dobermann dogs is also limited.[10] One dog was heterozygous for the allele DQA1-00301 (1.25%) and other frequencies were comparable with earlier results of Kennedy and co-workers. No allele frequencies have been published before for DLA-DQB1 in Dobermans. Noteworthy, the variation in this gene was also very limited. The frequent haplotype prevailed in the three groups of Dobermans investigated. Therefore, as a consequence, we found no haplotype with a high frequency in both the CASH and DH groups that would suggest an association with the phenotype.

The Dobermann dog is a newly bred breed and most likely the founding population was small compared to older breeds such as German Shepherd dogs and Labrador retrievers.[10] Furthermore, throughout the years selection has been performed and the obvious consequence could be a highly inbred population or with little variation in DLA molecules. Based on our results it is difficult to conclude if there is a specific genetic make-up that makes the breed more susceptible for (autoimmune) diseases. Dobermann dogs are not necessarily prone to autoimmune diseases. They do have a higher susceptibility for Demodex[36] and hypothyroidism.[21] Both my be immune-mediated diseases. But in another study it was found that they seldom suffered from auto-immune thrombocytopenia.[5] Recently Speeti et al[26] described the expression of major histocompatibility complex (MHC) class II antigens in hepatocytes in Dobermann hepatitis. The MHC class II expression was seen predominantly in centrilobular hepatocytes together with a mononuclear cell infiltration.[26] The expression of MHC class II genes is not normal for hepatocytes[27] and might induce tolerance in autoreactive Th1 cells. Furthermore it may simultaneously favour a Th2 response in uncommitted T cells, and thereby support autoantibody production.[15] It was suggested that the hepatocytes with MHC class II expression become antigen-presenting cells for CD4+ T cells, and thus might become a target to autoimmune destruction.[26] Because corticosteroid treatment down regulated this MHC class II expression[26] it was concluded that the disease is most likely immune-mediated. However MHC class II expression can be the result of drugs, viral infections and toxins. Such causes, including toxins such as copper, were dismissed by the authors, despite the centrilobular coincidence of copper retention reported by them and others.[18,23,24,29]

Conclusion

An association of Dobermann hepatitis with a DLA class II allele would have supported the autoimmune hypothesis. Suprinsingly, this breed virtually displays no DLA variation, which prevents to draw conclusions supporting or denying an autoimmune pathogenesis.

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Chapter 11

General discussion and viability of the hypotheses

The viability of the two hypotheses as stated in the aim and scope of the study (Chapter 1) are being determined in light of the results of this thesis.

First hypothesis

In Dobermann dogs exists an autosomal genetic error in metabolism that leads to an abnormal copper metabolism. This results in an increased hepatic copper concentration which may lead to a (subclinical) hepatitis.

The first question addressed the frequency or incidence of Dobermann hepatitis. Based on earlier studies there is a breed-disposition for chronic hepatitis in Dobermann dogs.[7,13,14] Based on the results of Speeti et al[30] the incidence, in Finland, is around 5.7%. Based on the records of the DCSCA the incidence of Dobermann hepatitis in the Netherlands would be 0.12%. This is based on the number of clinical cases that were admitted between January 1993 and December 1999 to the DCSCA. During that time eight Dutch pedigree Dobermann dogs suffering from DH were referred to the DCSCA. Between January 1993 and December 1999 a total of 6538 Dobermann dogs entered in the pedigree register of the Dutch Kennel Club. Since it is unlikely that all clinical cases are seen at or referred to the DCSCA, questionnaires were sent to 928 owners of Dobermann dogs (Chapter 3). All dogs were registered by the Dutch Kennel Club and were selected from the 6538 registered Dobermann dogs born in the same period as above: January 1993 and December 1999. Three-hundred and forty owners replied. Eighty-one of 340 dogs had died of which, according to the owners, three died of cirrhosis (two females and one male). Diagnosis had been confirmed by histopathology in two dogs and was suspected based on clinical presentation and laboratory work in one.
Based on these results the proportional mortality of cirrhosis, within this group, would be 3.7%. However another two owners that participated in the study reported that their dogs (both females) had been diagnosed by histopathology chronic hepatitis. If we add these two to the other three, proportional mortality would be 6%. This proportional mortality of 3.7% to 6% is in accordance with the estimates of Speeti et al.[30]

To investigate the prevalence of subclinical hepatitis, and the influence of copper, we randomly draw, from a group of 967 three-year-old Dobermann dogs 106 dogs (Chapter 4). All dogs were born between August 1, 1995 and July 31, 1996 in the Netherlands. All dogs were, according to their owners, clinically healthy. Twenty two of these dogs (21%; 3 males and 19 females) showed at histopathology a subclinical hepatitis. However it is unlikely that all of these dogs will develop DH.

To investigate the role of copper, hepatic copper concentration (HCC) was measured in these 22 dogs. It was significantly higher (419 ± 414 µg/g dry matter; mean ± SD) than those without hepatitis (197 ± 113 µg/g; mean ± SD) (p=0.008) (Chapter 4, fig. 1). But if we take into account that a HCC below 400 mg/kg dry matter is considered normal[11,15,25,33] it is unlikely that copper toxicosis is in all of these dogs an aetiologic factor. HCC was only in five dogs (4.7%) above 400 mg/kg dry matter.
If Dobermann hepatitis is caused by copper toxicosis it is unlikely that these cases will recover by themselves. Sixteen of these 22 dogs were available for regular follow up during 2.6 ± 0.6 years (mean ± SD). After the follow up period the subclinical hepatitis persisted only in 5 dogs. After 3 years one dog was added to this group. It originally was without histopathologic abnormalities although it had an elevated HCC (Chapter 4, fig 3). Histopathology in all six dogs showed apoptotic hepatocytes and were associated with copper laden Kupffer cells centrolobulary. Around the hepatic veins there was mild infiltration of lymphocytes, plasma cells and scattered neutrophils. Findings that are regarded typical for copper toxicosis.[10] Furthermore after analysis of the HCC it appeared that the HCC had continued to rise. The 6 dogs with persistent hepatitis, compared with the 'recovered' cases had a significantly higher HCC (p=0.002) (Chapter 4, fig. 2).

A first conclusion could be drawn: the prevalence of Dobermann hepatitis is based on these results, six out of 106 (5.6%). This is in accordance with earlier findings (Chapter 3) and Speeti et al.[30]

It was now also possible to answer the second question: 'is subclinical hepatitis related with an increase in HCC?' Based on the study described in Chapter 4 we can conclude that in Dobermann dog a persistent subclinical hepatitis exists which seems to be associated with copper.

Question three, 'do Dobermann dogs have an impaired copper excretion', was addressed by means of an IV administered copper isotope (^{64}Cu) (Chapter 5). Five Dobermann dogs with a persistent subclinical hepatitis associated with an elevated HCC (hereafter called CASH) were compared with three normal Beagles and five normal Dobermann dogs. The excretion of ^{64}Cu into the bile was significantly different for the CASH group compared to the normal dogs (p=0.02) (Chapter 5, fig. 2). And since plasma clearance curves were comparable in all dogs (Chapter 5, fig. 1) we may conclude that there is evidence for an impaired ^{64}Cu excretion into the bile.

Until recently it was unknown whether cholestasis in dogs can actually cause, like in humans, an increased HCC.[34] Despite the lack of evidence several authors attributed the increase in HCC to the

cholestasis or cholatestasis which is present in Dobermann dogs with chronic hepatitis.[7,13,14] Proof for this hypothesis came from the study by Fuentealba et al,[9] who observed a linear correlation with cholestasis, fibrosis and an elevated HCC in Dobermann dogs with DH.[9] However in contrast with these observations are the reports of Dobermann dogs with elevated copper concentrations that did not have cholestasis.[28,29,30,36]

Since cholestasis or cholatestasis may be difficult to recognize in routine histopathology we performed in our five dogs with CASH and all normal dogs a ^{99m}Tc- Bis-IDA hepatobiliary scintigraphy.[2,26,39] All dogs exhibited similar uptake and excretion patterns and for this reason it seems unlikely that the impaired ^{64}Cu excretion into the bile is caused by cholestasis or cholatestasis.

Based on these results a third conclusion is possible: Dobermann dogs with CASH have a normal bile flow but an impaired ^{64}Cu excretion into the bile. Together with the earlier findings (Chapter 4) it seems reasonable to conclude that Dobermann dogs have a form of copper toxicosis.

Question four, is the increased hepatic copper associated with an abnormal copper metabolism which causes oxidative stress and hence hepatitis, is addressed by means of Quantitative Real-Time PCR. Herewith differentially expressed genes were determined involved in copper metabolism and ROS defences (Chapter 6). mRNA levels of proteins involved in copper metabolism (ATOX1, COX17, ATP7A, ATP7B, CP, MT1A, MURR1), oxidative stress (CCS, SOD1, CAT, GSS, GPX1, GSH) and proteins involved in apoptosis and cell-proliferation (Bcl-2, XIAP, p27kip) were investigated.
Four groups were created to investigate this hypothesis (Chapter 6, table 1):

1) Healthy or normal Dobermann dogs,
2) Dobermann dogs that have a subclinical hepatitis but not associated with copper (N-CASH) (Chapter 4),
3) Dobermann dogs that have CASH and
4) Dobermann dogs that died from Dobermann hepatitis (DH) with elevated HCC.

Interestingly in the DH group the copper metabolism related genes were, except for ATOX1, depressed. In the N-CASH group none were except for ATP7B which was expressed. The CASH group showed a significant decrease in mRNA levels of ATP7A and MURR1 (Chapter 6, fig 1). The fact that there is a clear difference in mRNA levels between N-CASH and CASH/DH seems to suggest that these groups are of a different aetiology. The fact that in the CASH group only ATP7A and MURR1 are depressed compared to all expect ATOX1, in DH seems to suggest that the CASH group is an early form of DH. Similar results were seen for the oxidative stress proteins. The N-CASH group was not significantly different from normal Dobermann dogs whereas both the DH as well as CASH groups showed a significant reduction in CSS, SOD1, CAT and GSS.
Bcl-2, XIAP and p27kip markers for apoptosis and cell-proliferation showed a significant decrease in all groups except for Bcl-2 in the N-CASH group (Chapter 6, fig 3).
Expected for the markers for apoptosis an increase in gene expression was expected in stead of a reduction in mRNA levels. A possible explanation could be that the prolonged period of disease has 'exhausted' the cellular copper metabolism in CASH and DH.

Taken all results into consideration the findings demonstrate a difference in aetiology between N-CASH and CASH/DH. For that reason it may even strengthen our hypothesis that DH is indeed a form of copper toxicosis.

Question five, which copper transport or storage gene is affected, is addressed by means of candidate gene approach (Chapter 7). Genes studied were the gene for Wilson's disease (ATB7B),[4,5,23] Bedlington terrier copper toxicosis (MURR1),[38] the copper transporter protein gene CTR1[44] which is responsible for cellular uptake of copper into mammalian cells. The intracellular copper transport proteins COX17,[37] ATOX1,[17] and the Menkes disease gene ATP7A,[41] which encodes a P-type ATPase for copper transport across cellular membranes. Furthermore metallothionein and ceruloplasmin,[6] involved in copper storage and transport respectively were considered candidates. We evaluated a group of normal Dobermann dogs, CASH and Dobermann dogs that died of DH with increased HCC. A genetic background for copper accumulation and the associated hepatitis in the Dobermann dog is highly likely because of the predisposition of the breed for the disease. By means of a candidate gene approach the three groups of DP were studied. All genes studied were as a genetic cause for DH, provided that there is no polygenic etiology for any of the above mentioned genes.

Hypothesis 2

Some Dobermann dogs may have a specific DLA (dog leukocyte antigen) configuration that leads to an abnormal or inadequate response on an (normal) immune-stimulation.[16,42] This may, like in humans,[21,40] introduce autoimmune hepatitis.

Two questions (Question 6 and 7, Chapter 1) were raised in light of this hypothesis.

Question number six, is it possible to treat Dobermann hepatitis with an immune-modulator or copper chelating drug, is addressed in chapters eight and nine. The first drug that was evaluated was nandrolone laurate (Chapter 8). In human medicine, androgens are used for treatment of several types of immune-mediated disorders.[1,12,22,24,27] For this reason nandrolone was evaluated in 21 Dobermann dogs. The majority that entered came from the study described in chapter 4. All dogs were equally divided into a placebo group and treatment group regardless of their HCC. After four months of treatment eight out of 21 dogs had no histological evidence of hepatitis, five other dogs improved. Although we may not conclude that there is no evidence for an immune-mediated aetiology, we could conclude that nandrolone laurate at the dosage used was not effective for treatment of subclinical hepatitis. Furthermore we observed, most importantly, that some Dobermann dogs with subclinical hepatitis did recover by themselves. In chapter 4 this phenomenon is discussed.

The next drug we evaluated was penicillamine[43] (chapter 9). Penicillamine has both copper chelating as well as anti-inflammatory properties. Regardless of the aetiology the question: 'can you treat CASH?', could be answered with the results of this study. In all five dogs that were treated for four months with D-penicillamine, histopathologic appearance had improved or returned to normal. All dogs had CASH for years. Therefore it is unlikely that they recovered by themselves. Hence CASH can be treated.
The mean HCC in these five dogs was 1036 mg/kg dry matter before treatment and decreased to 407 mg/kg after treatment (P=0.03). Thus penicillamine was able to significantly reduce HCC.
In light of the results discussed in chapter 4, 5 and 6 one can speculate whether this drop in HCC itself introduced the recovery or whether it is the combination of the decrease in HCC and the inflammatory properties of penicillamine. For those reasons different trials are advised with for instance zinc that prevents copper resorbtion from the intestine.[3]

Question number 7, do Dobermann dogs have a specific DLA configuration, is discussed in chapter 10. In humans autoimmune hepatitis is strongly associated with inheritance of the HLA A1-B8-DR3 haplotype and particular the DR3 and DR4 haplotypes. [8,19] Speeti et al[31] described the expression of major histocompatibility complex (MHC) class II antigens in hepatocytes in Dobermann hepatitis. The MHC class II expression was seen predominantly in centrilobular hepatocytes together with a mononuclear cell infiltration.[31] The expression of MHC class II genes is not normal for hepatocytes[32] and might induce tolerance in autoreactive Th1 cells. Furthermore it may simultaneously favour a Th2 response in uncommitted T cells, and thereby support autoantibody production.[18] It was suggested that the hepatocytes with MHC class II expression become antigen-presenting cells for CD4+ T cells, and thus might become a target to autoimmune destruction.[31] Because corticosteroid treatment down regulated this MHC class II expression[31] it was concluded that the disease is most likely immune-mediated. It should be questioned whether this conclusion actually supports the auto-immune hypothesis. MHC class II expression can be the result of drugs, viral infections and toxins. Such causes, including toxins such as copper, were dismissed by the authors, despite the centrilobular coincidence of copper retention reported by them and others.[20,28,29,35] For this reason we investigated the DLA alleles and haplotypes in three groups of Dobermann dogs: normal dogs (N-DP), dogs with CASH and dogs that had died of DH.

For each of the DLA-DRB1, DQA1, DQB1 alleles four alleles were found of which one had not been published earlier.[16] In total five different haplotypes were typed (Chapter 10, Table 2). There was no specific DLA allele or haplotype that was specific for both the CASH and DH groups. Two dogs, both died of DH, had a different DLA alleles compared to the normal DP. However it was not seen in the CASH group (Chapter 10, Table 1).
Although a possible explanation for this lack in difference could be the lack in variation we can exclude this option based on the fact that all three groups showed a comparable polymorphism. However it remains possible that a difference exists on another area of the DLA gene.
An association of Dobermann hepatitis with a DLA class II allele would have supported the autoimmune hypothesis. Our findings however prevent to draw conclusions supporting or denying this autoimmune pathogenesis.

Viability of the hypotheses

In light of the results discussed the following could be concluded:

1. In Dobermann dogs an inborn error of metabolism occurs that results in a decreased biliary-copper excretion (Chapter 5). This copper retention can cause a copper associated (sub) clinical hepatitis (Chapter 4 & 6). The first hypothesis is valid.
2. Dobermann dogs with CASH or DH do not show a variation in DLA haplotypes if compared with normal Dobermann dogs. We have not found proof to support the second hypothesis. The findings discussed in chapters 9 & 10, in light of the results discussed in chapter 4, 5 & 6, do not contradict the first hypothesis. The findings discussed in chapter 8, 9 & 10 do not contradict the second hypothesis.

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Chapter 12

Summary

(met een samenvatting in het Nederlands)

In **chapter 1** chronic hepatitis in Dobermann dogs (Dobermann hepatitis) is introduced as a disease. The high incidence of chronic hepatitis in Dobermann dogs, compared to other breeds, suggests that it is hereditary. However, despite of the many studies the pathogenesis of the disease was still unclear. Two hypotheses were formulated that were addressed in chapters 3 to 10.

Hypothesis 1

In Dobermann dogs exists an autosomal genetic error in metabolism that leads to an abnormal copper metabolism. This results in an increased hepatic copper concentration which may lead to a (subclinical) hepatitis.

Hypothesis 2

Some Dobermann dogs may have a specific DLA (dog leukocyte antigen) configuration that is associated with an abnormal or inadequate response on an (normal) immune-stimulation. This may, like in humans, introduce autoimmune hepatitis.

Chapter 2 is a general introduction on the toxicity of copper and the intracellular copper transport. Both copper toxicosis in man, dogs and its possible role in Dobermann hepatitis are discussed. Based on a review of the literature Dobermann hepatitis has strong resemblances with other types of copper toxicosis in dogs. The last part of this chapter discusses whether Dobermann hepatitis also has immune-mediated components. Compared to auto-immune hepatitis in human, the disease seems to be of a different aetiology. Although there are some studies that looked into the possibility of an immune-mediated aetiology, the results are not clear.

In **chapter 3** the morbidity and mortality were investigated with questionnaires sent randomly to owners of 928 Dobermann dogs registered over seven different years. Response rate was 37% (340 owners). In total 81 dogs had died and 259 were still alive. Proportional mortality was high for heart failure (14.8-22.2%), behavioural problems (19.8%), cancer (13.6%), but low for Dobermann hepatitis (3.7%) and cervical spondylomyelopathy (2.5%). A total of 132 of 259 Dobermann dogs were suffering from various disorders. Highest prevalence was observed for skin problems (22.4%) and urinary incontinence (15.8%). Dobermann hepatitis had a prevalence of 0.8% (two females). If these two dogs would be added to the group of proportional mortality it would be 6%.

Dobermann dogs are, compared to other breeds, at a higher risk of developing urinary incontinence (19.4%) and the risk of developing it after neutering is even higher (37%).

In **Chapter 4** the prevalence of subclinical hepatitis was investigated in a group of 106 randomly selected 3 year old Dobermann dogs. Histopathologic examination of liver samples from 65 dogs (52 dogs with elevated bile acids, alkaline phosphatase activity (AP) and alanine aminotransferase (ALT), or copper granules in hepatocytes in a liver aspirate, and 13 normal dogs) revealed subclinical hepatitis in 22 dogs (19 females and 3 males). Liver copper concentrations measured using instrumental neutron activation analysis (INAA), was significantly higher (419 ± 414 mg/kg dry matter; mean ± SD) (p=0.0008) in dogs with hepatitis than those without liver disease (197 ± 113 µg/g; mean ± SD). At 2.6 ± 0.6 years (mean ± SD) hepatitis persisted in 5 of 16 dogs available for examination. One dog with an elevated copper concentration but normal liver subsequently developed subclinical hepatitis after three years. During the follow up period the average copper concentration of the 6 cases with persistent subclinical hepatitis was 939 ± 299 µg/g (mean ± SD) and had continued to rise significantly (p=0.02). The hepatitis in these dogs was associated with apoptotic hepatocytes and copper-laden Kupffer cells in centrolobular regions. The results of this study suggest that there is a relationship between copper storage, hepatocellular damage and hepatitis in Dobermann dogs.

In **chapter 5** the hepatobiliary copper excretion is examined by means of an intravenously administered radioactive copper isotope (^{64}Cu). Five patients and eight normal dogs (5 normal Dobermann dogs and 3 Beagles) were studied. The patients, five female Dobermann dogs, had a subclinical hepatitis most likely associated with an increased hepatic copper concentration (median 822 mg/kg, range 690 – 1380 mg/kg dry matter). The normal dogs, five Dobermann dogs and three Beagles, had no abnormal liver histopathology and hepatic copper concentrations were considered normal (Dobermann dogs; median 118 mg/kg, range 50 – 242 mg/kg dry matter; Beagles; median 82 mg/kg, range 50 – 88 mg/kg dry matter). Plasma clearance values were in all dogs comparable without significant differences. The excretion of ^{64}Cu into the bile was significantly less for the Dobermann dogs with subclinical hepatitis compared to the normal dogs which suggests an impaired copper excretion. Cholestasis was excluded in all dogs by means of a ^{99m}Tc- Bis-IDA hepatobiliary scintigraphy. The findings suggest that Dobermann dog hepatitis is (partly) caused by a primary copper retention.

In **chapter 6** gene expression of copper related proteins and ROS defences during copper-toxicosis is discussed. A quantitative Real-Time PCR was used to determine differentially expressed genes involved in copper metabolism and ROS defences. Four different groups were investigated ranging normal Dobermann dogs, Dobermann dogs with a subclinical hepatitis not associated with copper (N-CASH), copper associated subclinical hepatitis (CASH) and last clinical chronic hepatitis (DH). Most mRNA levels of proteins involved in copper binding, transport, and excretion were around control values in the N-CASH and CASH group. In contrast, many of these proteins (including ATP7A, ATP7B, ceruloplasmin, and metallothionein) are significantly reduced in the DH group. Measurements on defences against ROS showed reductions in gene-expression of superoxide dismutase 1 (SOD1) and catalase (CAT) in all copper associated groups. In conclusion, reductions as seen in the DH group showed a decreased efflux of copper. Measurements of ROS defences are most likely to be impaired in the CASH and DH group.

In **chapter 7** the results of a candidate gene scan is discussed. Most copper transporter and storage genes (CTR1, Ceruloplasmin, Metallothionein, ATP7A, ATP7B, Murr1, COX17, ATOX1) are evaluated in three groups of Dobermann dogs. First a normal groups of Dobermann dogs (n=12), a group of CASH (n=10) and a group of Dobermann dogs that had died of DH (n=8). For genetic analysis the phenotype of the disease was defined as the association of subclinical or chronic hepatitis with elevated hepatic copper concentrations (above 600mg/kg dry matter). All evaluated genes were excluded as a possible monogenetic cause for the CASH and DH.

In **chapter 8** a double blinded placebo controlled study is described to assess the efficacy of nandrolone laurate in subclinical Dobermann hepatitis. This since Dobermann hepatitis might also be (partly) immune-mediated. In human medicine, androgens are used for treatment of several types of immune-mediated disorders. Twenty-one Dobermann dogs, all three years of age, with subclinical hepatitis, were treated in a double blind trial. The dogs were scored prior and after four months of treatment with either placebo or the test product. Prior to and after treatment all dogs were evaluated by clinical biochemistry and liver biopsies. No significant difference was observed for any of the clinical biochemistry values. After four months of treatment eight out of 21 dogs had no histological evidence of hepatitis and five other dogs improved. No significant difference between the two groups was observed. It is concluded that nandrolone laurate at the dosage used is not effective for treatment of subclinical hepatitis in Dobermann dogs.

In chapter 9 five female Dobermann dogs with increased hepatic copper concentrations and persistent (3 to 4 years) subclinical hepatitis were treated with D-penicillamine for 4 months. Before and after treatment the dogs underwent clinical, hematological (RBC, WBC, differential and thrombocyte counts), and clinical chemistry (creatinine, ALP, ALT, and total bile acid concentrations) examinations and liver biopsies were examined histologically and their copper content measured quantitatively.
No adverse effects were observed during treatment. There was no change in hematology or serum chemistry test results. The mean liver copper concentration was 1036 mg/kg dry matter before treatment and decreased to 407 mg/kg after treatment (P=0.03). The copper concentrations had decreased (by between 134 and 1135 mg/kg dry matter) in all of the dogs. The histopathologic appearance had improved or returned to normal in all 5 dogs. We conclude that D-penicillamine effectively reduced copper retention in these dogs and improved the histopathologic appearance of the lesions. However because D-penicillamine has both copper-chelating and anti-inflammatory properties it is not possible to draw conclusions on the etiology of this disease.

In **chapter 10** DLA Class II allele variations is described. DLA Class II loci were genotyped in a group of Dobermann dogs that died of DH with high copper concentrations (n=10), a group of Dobermann dogs with CASH (n=9) were compared with a group of normal Dobermann dogs (n=21). For each DLA-DRB1, DLA-DQA1 and DLA-DQB1 gene four different alleles were found which combined in five different haplotypes. There was no prevalent DLA allele or haplotype in either of the disease groups. The variation in DLA alleles was extremely limited. The majority of dogs was homozygous for one haplotype that had a frequency of 88.75% in the entire group of Dobermans. Therefore it is not possible to conclude that Dobermann dogs with chronic hepatitis have a specific immunogenic make-up that made them more susceptible compared to other Dobermann dogs.

In **chapter 11** the viability of the hypotheses is discussed in light of the results described in chapters 3 to 10. We concluded that Dobermann dogs have an inborn error of metabolism that results in a decreased biliary copper excretion. This copper retention can cause a copper associated (sub) clinical hepatitis Therefore our first hypothesis was valid.
Since Dobermann dogs with CASH or DH do not show a variation in DLA haplotypes if compared with normal Dobermann dogs we did not found proof to support the second hypothesis. However the findings discussed in chapters 9 & 10, in light of the results discussed in chapter 4, 5 & 6, did not contradict the first hypothesis. The findings discussed in chapter 8, 9 & 10 did not contradict the second hypothesis as well.

Hoofdstuk 1 introduceert chronische hepatitis als ziekte van de Dobermann . De hoge incidentie, in vergelijking met andere rassen, suggereert erfelijkheid. Hoewel diverse studies beschreven zijn is de pathogenese van de ziekte nog steeds onduidelijk. Twee hypothesen werden geformuleerd en deze worden geadresseerd in de hoofdstukken 3 tot en met 10.

De eerste hypothese

In Dobermann honden komt een autosomaal genetisch defect voor dat leidt tot een abnormaal koper metabolisme. Dit heeft tot gevolg dat de koper concentratie in de lever stijgt. Dit kan een hepatitis tot gevolg hebben.

De tweede hypothese

Sommige Dobermann honden hebben een specifieke DLA (hond leukocyten antigeen) configuratie die tot gevolg heeft dat er een abnormale dan wel inadequate respons op een (normale) immuun stimulans plaats vindt. Bij de mens is dit voor auto-immune hepatitis bekend.

Hoofdstuk 2 is een algemene introductie over de toxiciteit van koper en het intracellulaire koper transport. Zowel koper toxicose van de mens, hond en haar mogelijke rol in Dobermann hepatitis worden bediscussieerd. Gebaseerd op een literatuur overzicht heeft Dobermann hepatitis sterke overeenkomsten met andere vormen van koper toxicose zoals beschreven bij de hond.
Het laatste deel van dit hoofdstuk gaat in op het stuk van auto-immuniteit en haar mogelijke rol in deze ziekte. Vergeleken met auto-immuun hepatitis van de mens lijkt Dobermann hepatitis een andere etiologie te hebben. Er zijn enkele studies die in gaan op auto-immuniteit van de Dobermann maar de resultaten zijn niet zo concreet dat het daarmee onomstotelijk bewezen is.

In hoofdstuk 3 worden de morbiditeit en mortaliteit onderzocht aan de hand van enquête resultaten. In totaal werd er aan 928 Dobermann eigenaren, zonder willekeur, een enquête verzonden. Alle Dobermann honden waren in Nederland geregistreerd en waren in de onderzoeksperiode 1993-1999 geboren (7 jaren). Het retour percentage was 37% (340 eigenaren). In totaal bleken er 81 honden overleden te zijn en waren er nog 259 in leven. De proportionele moraliteit was hoog voor hart falen (14.8-22.2%), gedragsproblemen (19.8%), tumoren (13.6%), maar laag voor Dobermann hepatitis (3.7%) en cervicale spondylomyelopathie (de zogenaamde 'wobbler') (2.5%). In totaal 132 van 259 nog levende Dobermann honden bleek te leiden aan diverse aandoeningen. Huidproblemen komen het meeste voor (22.4%) gevolgd door urine incontinentie (15.8%). Dobermann hepatitis

kwam slechts bij twee teven (0.8%) voor. Echter als deze twee honden bij de andere honden gevoegd worden die al overleden zijn is de proportionele mortaliteit 6%.
Dobermann honden hebben, in vergelijking met andere rassen, een hoog risico op het ontwikkelen van urine incontinentie (19.4%). Het risico is na sterilisatie zelfs groter: 37%.

Hoofdstuk 4 beschrijft het voorkomen van subklinische hepatitis. Deze is onderzocht bij een groep van 106, zonder willekeur gekozen, drie jaar oude Dobermann honden. Histologisch onderzoek van de lever werd uitgevoerd bij 65 honden. Deze groep bestond uit 52 honden met verhoogde galzuren, alkalische fosfatase, alanine aminotransferase, of waarbij koper granula in de levercellen zichtbaar waren. Dit laatste was eerder met behulp van een dunne naald aspiratie biopt afgenomen. Tevens werden dertien normale Dobermann honden onderzocht. Bij 22 honden (19 teven en 3 reuen) werd een subklinische hepatitis gevonden. De koperconcentratie van de lever werd gemeten met behulp van een instrumentale neutron activatie analyse (INAA). Deze was significant hoger (419 ± 414 mg/kg droge stof; gemiddelde ± SD) ($p=0.0008$) bij honden met hepatitis dan bij de honden die geen lever ziekte hadden (197 ± 113 mg/kg; gemiddelde ± SD). Na 2.6 ± 0.6 jaren (gemiddelde ± SD) bleek dat de hepatitis persisteerde bij 5 (uit een groep van 16 honden welke voor vervolg onderzoek beschikbaar waren). Een hond die oorspronkelijk alleen een hogere lever koper concentratie had zonder leverafwijkingen kreeg na drie jaar alsnog een subklinische hepatitis. Gedurende de vervolg onderzoek periode bleef de gemiddelde koper concentratie bij deze zes honden met persisterende hepatitis stijgen (939 ± 299 mg/kg droge stof; gemiddelde ± SD). Deze stijging bleek significant te zijn ($p=0.02$). De hepatitis die deze honden hadden bestond uit apoptotische hepatocyten geassocieerd met Kupffer cellen met daarin koper granula. De laesies werden vooral in de centrolobulaire regio gezien. De resultaten van deze studie suggereren dat er een relatie bestaat tussen koper stapeling, levercel schade en de hepatitis van deze Dobermann honden.

In hoofdstuk 5 wordt een experiment beschreven waarbij de hepatobiliaire koper excretie is onderzocht. Dit werd gedaan met behulp van een intraveneus toegediend radioactief koper isotoop (^{64}Cu). Vijf patiënten en 8 normale honden (5 normale Dobermann honden en 3 Beagles) werden bestudeerd. De patiënten, vijf Dobermann teven, hadden een subklinische hepatitis welke hoogstwaarschijnlijk was geassocieerd met koper. De koper concentratie van deze dieren was abnormaal verhoogd (mediaan 822 mg/kg, range 690 – 1380 mg/kg droge stof). De normale Dobermann honden (5) en de Beagles (3) hadden geen histologische leverafwijkingen en een normale lever koper concentratie (Dobermann honden; mediaan 118 mg/kg, range 50 – 242 mg/

kg droge stof; Beagles; mediaan 82 mg/kg, range 50 – 88 mg/kg droge stof). De plasma klaring was voor alle honden vergelijkbaar. Er was geen significant verschil. De excretie van ^{64}Cu naar de gal bleek bij de patiënten duidelijk minder te zijn dan voor de normale honden. Deze resultaten zijn suggestief voor een abnormale koper uitscheiding naar de gal. Een eventuele hepatobiliaire stoornis in de vorm van cholestase werd uitgesloten bij alle honden met behulp van een ^{99m}Tc- Bis-IDA hepatobiliaire scintigrafie. De resultaten van deze studie suggereren dat Dobermann hepatitis (gedeeltelijk) wordt veroorzaakt door een primaire koper retentie.

Hoofdstuk 6 beschrijft de gen expressie van koper gerelateerde eiwitten gedurende een koper toxicose en de verdeding tegen oxidatieve stress (ROS). Middels een kwantitatieve Real-Time PCR werd deze gen expressie gemeten aan de hand van mRNA niveaus voor de eiwitten betrokken bij het koper metabolisme en de verdediging tegen oxidatieve stress (ROS). Vier verschillende groepen werden onderzocht: normale Dobermann honden (N-DP), Dobermann honden met een subklinische hepatitis niet geassocieerd met koper (N-CASH), geassocieerd met koper (CASH) en Dobermann honden die overleden zijn aan Dobermann hepatitis (DH). De meeste mRNA eiwit niveaus welke betrokken zijn bij de koper binding, het transport en de excretie waren vergelijkbaar met de N-DP voor zowel de N-CASH en CASH groep. In contrast hiermee waren de meeste eiwitten (inclusief ATP7A, ATP7B, ceruloplasmine, and metallothionein) die allen significant verlaagd waren in de DH groep. De metingen betreffende de enzymen betrokken bij de verdediging tegen ROS liet een reductie zien in gen expressie voor superoxide dismutase 1 (SOD1) en katalase (CAT) in alle koper geassocieerde groepen. In conclusie, de reductie zoals gezien in de DH groep kan betekenen dat koper vertraagd wordt uitgescheden. In de CASH en DH groep is de verdediging tegen ROS mogelijk verminderd.

In hoofdstuk 7 worden de resultaten van een kandidaat gen scan besproken. De meeste genen betrokken bij koper transport en opslag werden gemeten (CTR1, Ceruloplasmin, Metallothionein, ATP7A, ATP7B, Murr1, COX17, ATOX1) en vergeleken voor drie verschillende groepen. Allereerst een groep van normale Dobermann honden (n=12), een groep van CASH (n=10) en een groep van Dobermann honden die overleden waren aan DH met tegelijkertijd een hoog koper gehalte in hun lever (n=8). Voor de genetische analyse werd het fenotype 'ziek' gedefinieerd als CASH of DH waarbij de lever koper concentratie abnormaal verhoogd is (boven 600mg/kg droge stof). Alle genen die zijn bestudeerd moesten als mogelijke, enkelvoudig verervende, oorzaak uitgesloten worden voor CASH en DH.

In hoofdstuk 8 wordt een dubbel blinde placebo gecontroleerde studie beschreven. Het doel was de effectiviteit van nandrolone laurate te onderzoeken bij subklinische Dobermann hepatitis. Dit werd gedaan omdat de ziekte mogelijk (deels) immuun gemedieerd is. Androgenen worden, in de humane geneeskunde, bij tal van immuun gemedieerde ziekten voor dit doel gebruikt. Een en twintig Dobermann honden, allemaal drie jaar oud en leidend aan een subklinische hepatitis werden in deze dubbel blinde studie onderzocht. De honden werden gescoord voor en vier maanden nadat de maandelijkse behandeling met ofwel het placebo dan wel het test product was gestart. Voor en na de behandeling werden alle honden vergelijken met behulp van bloedonderzoek en lever biopten. Er werd geen significant verschil aangetroffen voor de onderzochte bloedparameters. Na vier maanden behandeling bleek dat 8 van de 21 honden geen hepatitis meer had en vijf andere honden bleken verbeterd. Er werd echter geen significant verschil tussen de twee groepen waargenomen. Er werd geconcludeerd dat nandrolone laurate, met het gebruikte dosis interval en in deze dosering, niet effectief was bij het genezen van de subklinische hepatitis.

Hoofdstuk 9 beschrijft de behandeling van vijf Dobermann teven die al 3 a 4 jaar een persisterende CASH hadden. Lever koper concentraties waren bij alle vijf abnormaal verhoogd. Alle honden werden behandeld gedurende vier maanden met D-penicillamine. Voor en na de behandeling werden de honden klinisch onderzocht en bloed afgenomen. Een aantal hematologische parameters (RBC, leukocyten & differentiatie, thrombocyten aantal), klinische chemie (creatinine, ALP, ALT, en totaal galzuren) evenals leverbiopten werden onderzocht. De leverbiopten werden zowel histologisch bekeken en middels INAA werd de koper concentratie in de lever bepaald.
Er werden geen bijwerkingen waargenomen noch was er verschil voor zowel de hematologische als klinische chemie parameters. De gemiddelde koper concentratie van de lever was 1036 mg/kg droge stof voor de behandeling en nam af tot gemiddeld 407 mg/kg na behandeling (P=0.03). De koperconcentratie was bij alle honden afgenomen (134 and 1135 mg/kg droge stof). De histologische afwijkingen waren bij alle honden normaal of verbeterd. De conclusie is dat D-penicillamine effectief was in het verlagen van de koper concentratie en dat de histologische afwijkingen verbeterd zijn onder invloed van deze stof. Echter D-penicillamine verlaagt niet alleen de koper concentratie maar heeft ook een ontstekingsremmende activiteit. Daarom kan op basis van deze studie niet aangegeven worden wat nu exact de pathogenese is.

In hoofdstuk 10 wordt de DLA klasse II allel variatie beschreven voor drie groepen Dobermann honden: normale Dobermann honden (n=21); CASH (n=9) en Dobermann honden die overleden

zijn aan DH met een hoog koper gehalte in hun lever (n=10). Een specifiek DLA haplotype kan het fenomeen van auto-immuniteit facilliteren. Bij elk van de drie DLA allelen DLA-DRB1, DLA-DQA1 en DLA-DQB1 werden vier verschillende allelen gevonden. In totaal waren er vijf verschillende haplotypes. De variatie in DLA allelen was erg klein. De meerderheid van de honden was homozygoot voor een haplotype welk een frequentie had van 88.75%. Daarom was het niet mogelijk te concluderen dat Dobermann honden een specifieke immunogenetische opmaak hebben die hen gevoeliger maakt voor CASH of DH.

In **hoofdstuk 11** wordt de uitvoerbaarheid van de hypothesen bediscussieerd in het licht van de resultaten van de hoofdstukken 3 tot en met 10. Op basis van de gegevens concluderen we dat Dobermann honden een aangeboren metabool defect hebben waardoor ze een verminderde koperexcretie naar de gal hebben. Deze koper retentie kan een koper geassocieerde subklinische hepatitis veroorzaken. De eerste hypothese is dan ook valide. De resultaten bediscussieerd in de hoofdstukken 9 & 10, bezien in het licht van de resultaten vermeld in de hoofdstukken 4, 5 & 6, spreken de eerste hypothese evenmin tegen.
Dobermann honden met CASH of DH hebben, wanneer ze vergeleken worden met normale Dobermann honden, geen specifiek DLA haplotype. Er is daarom geen bewijs gevonden om de tweede hypothese te onderbouwen. De resultaten besproken in de hoofdstukken 8, 9 & 10 spreken de tweede hypothese niet tegen. De tweede hypothese kon dus niet verworpen of bekrachtigd worden.

List of abbrevations

AIH	Auto-immune hepatitis
ALT	Alkaline aminotransferase
AP	Alkaline phosphatase
ATP	Adenosine 5'-triphosphate
ATOX1	Anti-oxidant protein 1
CASH	Copper associated subclinical hepatitis
CAT	Catalase
CCS	Copper chaperone for SOD
CH	Chronic hepatitis
CO	Cytochroom-oxidase
COX17	Cytochroom c oxidase assembly protein
CP	Ceruloplasmin
CT	Copper toxicosis
CTR1	Copper transport
DCSCA	Department of Clinical Sciences of Companion Animals
DH	Dobermann hepatitis
DNA	Deoxyribonucleic acid
DLA	Dog leukocyte antigen
DTNB	5,5'dithiobis-2-nitrobenzoic acid
ETIC	Endemic Tyrolean Infantile Cirrhosis
ICC	Indian Childhood cirrhosis
ICT	Idiopathic Copper Toxicosis
INAA	instrumental neutron activation analysis
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GPX1	Gluthathione peroxidase 1
GSS	Gluthathione synthetase
GSH	Gamma-glutamylcysteinylglycine
HCC	Hepatic copper concentration
HLA	Human leukocyte antigen
HPRT	Hypoxanthine phosphoribosyl transferase
IV	Intraveneous
MHC	Major histocompatibility complex
m-RNA	Messenger-RNA
MTA1	Methallothionein 1A
NDD	Normal Dobermann dog (FCI style)
NDP	Normal Doberman pinscher (American style)
Q-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR reaction	Real time polymerase chain
SOD1	Cu/Zn Superoxide dismutase
SW	Slechte, niet drinkbare wijn
TNB	2-nitro-5-thiobenzoic acid
UW	Uitstekende Wijn
WD	Wilson disease
XIAP	Anti-apoptotic protein, x-linked inhibitor of apoptosis

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Stellingen
behorend bij het Proefschrift

Insights in the pathogenesis of Dobermann hepatitis

Paulus Justinus Johannes Mandigers

(2005)

1. Een abnormaal kopermetabolisme speelt een rol in de ontwikkeling van Dobermann hepatitis. Het is dan ook onterecht de ziekte nog langer in te delen als koper geassocieerd (dit proefschrift).

2. Dobermann hepatitis hoeft geen dodelijk verlopende ziekte meer te zijn. Het vroegtijdig herkennen en behandelen kan het klinisch worden van de ziekte mogelijk voorkomen (dit proefschrift).

3. De stelling dat Dobermann hepatitis een immuun gemedieerde ziekte is kent een magere wetenschappelijke onderbouwing (dit proefschrift).

4. Hoewel de Dobermann weinig variatie heeft voor het DLA molecuul is de fenotypische variatie, in het uiterlijk, groot. Dit gebrek aan variatie op het DLA molecuul zegt dus weinig over de mate van inteelt.

5. De eigenaar van een Dobermann teef moet meer dan welke eigenaar dan ook een goede afweging maken of sterilisatie wel geïndiceerd is. Dierenartsen moeten op hun beurt eigenaren goed voorlichten over het risico op incontinentie na sterilisatie (dit proefschrift).

6. Het gegeven dat de Amerikanen steevast de oude naam Doberman pinscher als naam gebruiken in de wetenschappelijke pers, in tegenstelling tot de officiële FCI naam Dobermann, is een fraai staaltje van Amerikaans handelen.

7. Een dag is veel te kort om het onderzoek wat je wilt doen uit te voeren.

8. Het is onmogelijk alles te lezen wat er geschreven wordt. Het is vergelijkbaar onmogelijk alles te weten. Er is dus geen basis voor het dogmatische denken.

9. Er is veel goeds te zeggen over het Boeddhisme. Reïncarnatie is slechts een aspect, een ander aspect is het feit dat het nooit fout is om goed te doen.

10. Alleen in de Bourgogne kunnen ze van de pinot noir druif een sublieme wijn maken die qua prijs behoorlijk over het paard is getild.

11. Het schrijven van een proefschrift is vergelijkbaar met het maken van wijn: het is een tijdrovend en ingewikkeld proces dat veel aandacht vergt en waarvan de uitkomst lange tijd onzeker is. De vreugde van een uiteindelijk goed product is echter onovertroffen (stelling uit het proefschrift van dr. Nicola Jägers).

12. Er zijn veel meer soorten wijnen dan momenten op een dag. Wordt het nu niet tijd om echt wat te veranderen aan de duur van een dag?

13. Je kiest eerst de wijn en daarna pas het eten.

14. Het is me dan toch erg lang gelukt succesvol te liegen over mijn leeftijd (dit proefschrift).