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Dietary Management of Labrador Retrievers with Subclinical Hepatic Copper Accumulation

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Background: Genetic and environmental factors, including dietary copper intake, contribute to the pathogenesis of copper-associated hepatitis in Labrador retrievers. Clinical disease is preceded by a subclinical phase in which copper accumulates in the liver.

Objective: To investigate the effect of a low-copper, high-zinc diet on hepatic copper concentration in Labrador retrievers with increased hepatic copper concentrations.

Animals: Twenty-eight clinically healthy, client-owned Labrador retrievers with a mean hepatic copper concentration of 919 ± 477 mg/kg dry weight liver (dwl) that were related to dogs previously diagnosed with clinical copper-associated hepatitis.

Methods: Clinical trial in which dogs were fed a diet containing 1.3 ± 0.3 mg copper/Mcal and 64.3 ± 5.9 mg zinc/Mcal. Hepatic copper concentrations were determined in liver biopsy samples approximately every 6 months. Logistic regression was performed to investigate effects of sex, age, initial hepatic copper concentration and pedigree on the ability to normalize hepatic copper concentrations.

Results: In responders (15/28 dogs), hepatic copper concentrations decreased from a mean of 710 ± 216 mg/kg dwl copper to 343 ± 70 mg/kg dwl hepatic copper after a median of 7.1 months (range, 5.5–21.4 months). Dogs from a severely affected pedigree were at increased risk for inability to have their hepatic copper concentrations normalized with dietary treatment.

Conclusions and Clinical Importance: Feeding a low-copper, high-zinc diet resulted in a decrease in hepatic copper concentrations in a subset of clinically normal Labrador retrievers with previous hepatic copper accumulation. A positive response to diet may be influenced by genetic background. Determination of clinical benefit requires further study.

Key words: Copper-associated hepatitis; Dog; Petfood; Zinc.

Hereditary copper-associated hepatitis in the Labrador retriever is recognized in European[1] and American[2,3] Labrador retriever populations. Unlike autosomal recessive copper-associated hepatitis in the Bedlington terrier,[4] the molecular background of the disease in Labrador retrievers is not completely understood. Genetic[5] as well as environmental factors, including diet,[6] contribute to the disease pathogenesis. The disease is characterized by a subclinical phase during which hepatic copper accumulates without overt clinical signs. Without treatment, continued copper accumulation causes hepatitis and eventually liver cirrhosis. Dogs often are presented in an advanced phase of the disease, which may make treatment less effective. Treatment consists of creating negative copper balance by use of copper chelators such as D-penicillamine,[7] adjusting diet[8,9] or decreasing intestinal copper uptake by zinc supplementation.[8,10]

Abbreviations:

AIC	Akaike's Information Criterion
ALT	alanine aminotransferase
BA	bile acids
CI	confidence interval
dwl	dry weight liver
Kcal	kilocalorie
Mcal	megacalorie

Lifelong, continuous chelation therapy in Labrador retrievers with copper-associated hepatitis may result in copper or zinc deficiency.[7] Therefore, low-copper, high-zinc diets previously were evaluated for their usefulness in the long-term management of Labrador retrievers with copper-associated hepatitis that underwent previous chelation therapy.[8,9] These studies showed that feeding a low-copper, high-zinc diet led to a decrease in hepatic copper concentrations in Labrador retrievers. We therefore hypothesized that feeding a similar diet to Labrador retrievers with increased hepatic copper concentrations may prevent further copper accumulation. Intervention early in the disease process ideally would be started in the subclinical phase in which clinical disease possibly could be prevented or postponed. Therefore, the objective of this study was to investigate the role of a low-copper, high-zinc diet on hepatic copper concentration in Labrador retrievers with subclinical hepatic copper accumulation that had not previously undergone chelation therapy.

Client-owned Labrador retrievers with increased hepatic copper concentrations related to dogs with clinical copper-associated hepatitis were fed a low-copper, high-zinc diet.[a] Effects of diet on hepatic copper

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concentration were evaluated by repeated liver biopsies. The results of this study provide important data for dietary management of Labrador retrievers with increased hepatic copper concentrations.

Materials and Methods

Study Population

Recruitment of Labrador retrievers was performed at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, the Netherlands between 2007 and 2010. An invitation for participation was sent via the Dutch Labrador retriever breed club to owners of Labrador retrievers that were related to 1 of 8 Labrador retrievers previously diagnosed with clinical copper-associated hepatitis. Thirteen dogs were derived from 1 pedigree with a high incidence of severely clinically affected dogs (including 2 deaths). The other dogs (n = 15) were derived from the 7 other pedigrees.

Hepatic biopsy samples were obtained to screen for increased hepatic copper concentrations. Owners of Labrador retrievers with hepatic copper concentrations >400 mg/kg dry weight liver (dwl) were invited to participate in the study. A medical history was obtained from the owners, and a physical examination was performed on all Labrador retrievers by one examiner (HF). Pedigree information was acquired from the registers of the Dutch Labrador retriever breed club.

Clinico-Pathological Examination and Investigation of Liver Biopsy Samples

Blood was collected in sodium citrate for a coagulation profile, including prothrombin time, activated partial thromboplastin time, and fibrinogen concentration. Platelets were counted in EDTA-preserved samples. Alanine aminotransferase (ALT) activity and bile acids (BA), total protein and albumin concentrations were determined using heparinized plasma. At each occasion, at least 3 liver biopsy samples were collected from the left lateral liver lobe using a 14G needle and an automated spring-triggered device[b] under ultrasound guidance. Two biopsy specimens were fixed in 4% neutral buffered formalin and embedded in paraffin. Paraffin sections of liver biopsy samples were stained with hematoxylin and eosin, rubeanic acid[11] and reticulin.[12] Histology was evaluated by a single board-certified veterinary pathologist (TSGAMvdI), who was blinded to all clinical data as well as quantitative copper concentrations in the liver biopsy samples. Grading and staging of biopsy samples was performed according to the system of Ishak et al[13,14] Grading was defined on a scale of 0–5 for necro-inflammatory activity: absent (0), slight (1), mild (2), moderate (3), marked (4), very marked (5). Staging was defined on a scale of 0–4 for the degree of fibrosis: absent (0), mild (1), moderate (2), marked (3), very marked (4). Distribution of copper (centro-lobular or periportal) was evaluated in rubeanic acid-stained sections. Biopsies were only performed when coagulation parameters were within the reference range, which was the case for all biopsy samples obtained in this study. A separate biopsy specimen of at least 5 mg was collected in a metal-free container and freeze dried before quantitative metal determination by instrumental neutron activation analysis.[15] Hepatic metal concentrations were reported in mg/kg dwl.

Diet

All dogs had unrestricted access to tap water and were fed according to their individual caloric needs. Owners were instructed to feed their dog only the low-copper, high-zinc diet[a]. Seven batches of diet were used during the study period. Each production batch was analyzed for moisture, crude protein, crude fat, and crude fibre content by a commercial laboratory[c] according to the procedures described by Association Française De NORmalisation.[16] Copper and zinc concentrations in the diet were analyzed by flame spectrometry.

Possible batch effect on hepatic copper concentrations was analyzed by stepwise backwards linear regression analysis in which batch, copper concentration of the batch, zinc concentration of the batch, and copper/zinc ratio of the batch were studied for association with the difference in hepatic copper concentrations between 2 liver biopsy samples (Δ copper). The model of best fit was determined based on Akaike's information criterion (AIC).

Study Protocol

Dogs were re-evaluated by clinical examination, blood tests (bile acids, ALT, total protein, and albumin) and liver biopsies every 6 months. Owners of dogs with hepatic copper concentrations >800 mg/kg dwl were offered an additional biopsy at 3 months, for minimizing the risk of disease progression with dietary treatment only. The endpoint of the study was defined when dogs reached hepatic copper concentration <400 mg/kg dwl, which was considered normal for hepatic copper concentration in dogs.[17] To assure the health of the dogs and prevent possible progression of disease, dogs were removed from the study when hepatic copper concentrations increased or when the grade of inflammatory activity, the stage of fibrosis or both increased compared to the previous biopsy results. In these cases, dogs were treated medically under the guidance of our hepatology department.

Statistical Analysis

Statistical analysis was performed in R version 3.1.0.[d] Binary logistic regression was used to analyze the data. The dependent variable was defined as "reaching hepatic copper <400 mg/kg dwl during the study period" (yes, no). Independent factors that were analyzed in this model included: sex (male, female), age on entering the study (years), pedigree (being part of the high-risk pedigree, yes/no), and hepatic copper concentration at start of the study (in 100 mg/kg dwl). The model of best fit was determined based on AIC. For the final model, both multivariable and univariable estimates were reported.

Normality of age, hepatic copper concentration, grade, stage, ALT and albumin was assessed by inspecting histograms. Normally distributed data was presented as mean and standard deviation and data that was not normally distributed was presented as median and range.

Ethics

The study was approved by the Utrecht University Institutional Animal Care and Use Committee as well as the Royal Canin ethics committee. Informed consent was obtained from owners of dogs that participated in the study.

Results

Study Population

Twenty-eight Labrador retrievers were enrolled (23 females, 5 males). The mean age of the dogs was 4.9 ± 2.3 years. Results from clinico-pathologic testing and liver biopsies were grouped for the dogs from the high-risk pedigree and for dogs in the other pedigrees (Table 1).

Table 1. Time course, hepatic copper concentration, histological-, and blood parameters for Labrador retrievers treated with low-copper, high-zinc diet.

	Time-points	T0	T1	T2	T3	T4	T5	T6	T7
High-risk pedigree	Number of dogs	13	5	10	5	4	2	1	1
4 M, 9 F	Time (months)	0	2.9 ± 0.6	7.1 ± 1.2	13.1 ± 1.4	20.1 ± 2.3	29.7; 25.3	36.7	43
Age 4.8 ± 2.3 years	Hepatic copper concentration (mg/kg dwl)	1072 ± 627	784 ± 220	848 ± 460	510 ± 201	567 ± 83	690; 573	518	475
	Grade	1 (0–2)	1.5 (1–2)	0 (0–1)	0 (0–1)	0 (0–1)	1; 1	1	1
	Stage	0 (0–0)	0 (0–1)	0 (0–0)	0 (0–0)	0 (0–0)	0; 0	0	0
	ALT (U/L)	45 ± 21	63 ± 16	54 ± 34	50 ± 17	44 ± 9	75; 36	53	39
	Albumin (g/L)	29.6 ± 2.5	27.4 ± 2.1	27.8 ± 2.0	28 ± 1.5	27.8 ± 2.2	28; 28	27	26
Other pedigrees	Number of dogs	15	3	15	7	2			
1 M, 14 F	Time (months)	0	3.1 ± 0.5	6.5 ± 1.2	13.2 ± 1.8	18.2; 21.4			
Age 4.9 ± 2.3 years	Hepatic copper concentration (mg/kg dwl)	786 ± 247	828 ± 300	528 ± 249	454 ± 168	330; 398			
	Grade	0 (0–1)	0 (0–1)	0 (0–2)	0.5 (0–1)	0; 0			
	Stage	0 (0–1)	0 (0–0)	0 (0–1)	0 (0–1)	0; 0			
	ALT (U/L)	40 ± 11	33 ± 5	47 ± 19	56 ± 51	29; 29			
	Albumin (g/L)	29.5 ± 2.3	25.7 ± 1.1	27.7 ± 2.3	25.9 ± 3.1	27; 28			

Data are grouped for dogs being part of the high-risk pedigree (n = 13) and dogs that were part of any of the other pedigrees (n = 15). T0–T7: time-points at which control biopsy samples were obtained. Time in months summarizes the actual time that passed until the dogs returned for their follow-up visits. The number of dogs that were present at each follow-up visit is indicated. Values are indicated as mean ± standard deviation, or as median (range). Laboratory references for ALT were <70 U/L and for albumin 26–37 g/L. When data for ≤2 dogs was present, values for the individual dogs were included in the table.

On entering the study, all dogs appeared clinically healthy based on physical examination and none had a history of clinical signs of liver disease. At the 1st visit, BA, total protein and albumin concentrations were within the normal reference ranges in all dogs. In 1 dog, ALT activity was mildly increased (111 U/L; reference range, <70 U/L). This dog had normal liver histology (grade 0, stage 0) and copper concentration was 570 mg/kg dwl. Mean hepatic copper concentration in the total group was 919 ± 477 mg/kg dwl. Median grade of hepatitis was 0.25 (range, 0–2) and median stage was 0 (range, 0–1). Copper was detected in all but 1 liver biopsy. Distribution of copper throughout the liver lobules was compatible with primary copper accumulation (ie, centro-lobular localization of copper). Eight of 15 dogs (53.6%; 95% binomial CI, 33.9–72.5%) with hepatic copper concentrations >800 mg/kg dwl at the start of the study were admitted for an additional biopsy after 3 months of dietary treatment (T1 in Table 1). Bile acid concentrations were within reference range at all time-points in all dogs.

Diet

Seven commercial batches of the diet were prepared for this study. Means and standard deviations from the diet for each dietary component were as follows: moisture 8.1 ± 0.6%, protein 17.8 ± 0.3%, fat 16.4 ± 1.3%, minerals 4.5 ± 0.1%, nitrogen free extract 50.7 ± 1.2%. The mean metabolizable energy content, calculated using modified Atwater factors,[18] was 3790 ± 77 Kcal/kg. Copper and zinc content, respectively, were 1.3 ± 0.3 mg copper/Mcal and 64.3 ± 5.9 mg zinc/Mcal. The mean zinc-to-copper ratio was 53.5 ± 13.1. In total, 53 measurements for Δ copper were available. There was no association between batch, copper concentration of the batch, zinc concentration of the batch and copper/zinc ratio of the batch and Δ copper.

Follow-up

Fifteen of the 28 dogs reached hepatic copper concentration <400 mg/kg dwl during the study period in a median time of 7.1 months (range, 5.5–21.4 months; Fig 1A,B). Three dogs accumulated hepatic copper despite the diet, and for these dogs the study was terminated at 6.1, 7.6 and 9.1 months after the start of dietary treatment. Three dogs were removed from the study at 5.6, 6.6 and 16 months respectively because of re-accumulation of copper after an initial decrease in copper. Three dogs were removed at the time of the additional biopsy after approximately 3 months because of worsening of hepatitis, despite a decrease in hepatic copper concentration. Four dogs were lost to follow-up because of loss of owner compliance after a median follow-up time of 23 months (range, 12.7–43 months). Three of these dogs were part of the high-risk pedigree with starting hepatic copper concentrations of 690, 900, 927 mg/kg dwl and follow-up times respectively of 43, 25.3 and 20.7 months. The dog from the other group had a hepatic copper concentration of 960 mg/kg dwl and was followed for 12.7 months. None of these dogs reached a hepatic copper concentration <400 mg/kg dwl. Logistic regression analysis was performed using the total data set as well as without the 4 dogs that

were lost to follow-up (Table 2). For both datasets, the logistic regression model with the lowest AIC comprised pedigree and hepatic copper concentration at start of the study. Dogs that were part of the high-risk pedigree and dogs with higher hepatic copper concentrations at the start of the study had a significantly higher risk for not reaching a hepatic copper concentration <400 mg/kg dwl (Table 2).

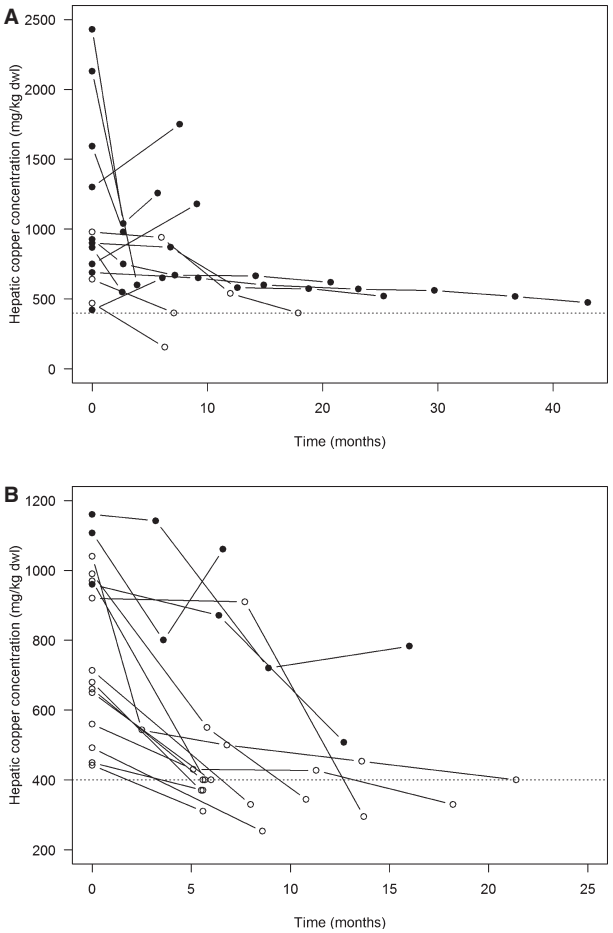

Fig 1. Hepatic copper concentration over time in Labrador retrievers treated with a low-copper, high-zinc diet from a high-risk pedigree (A) and from any of the other pedigrees (B). Hepatic copper concentrations in liver biopsy samples are depicted. At time 0 copper concentrations before the start of dietary treatment is indicated. The filled symbols depict data from dogs that were censored. The open symbols depict data from dogs that reached hepatic copper concentrations <400 mg/kg dwl during the study period. Dotted line: 400 mg copper/kg dwl.

Discussion

Clinical copper-associated hepatitis in Labrador retrievers is preceded by a subclinical phase in which copper accumulates in the liver. Development of clinical disease likely is influenced by environmental factors, including dietary intake of copper and zinc.[6,8,9] The objective of the current study was to investigate the influence of a low-copper, high-zinc diet on hepatic copper accumulation in Labrador retrievers with increased hepatic copper concentrations. The copper concentration in the diet used here (1.3 ± 0.3 mg copper/Mcal) was approximately 3 times lower than copper concentrations that are present in maintenance diets, which were reported to have a median copper concentration of 4.4 mg/Mcal (range, 2.3–9.0 mg/Mcal)[19] or a mean copper concentration of 4.2 ± 1.4 mg/Mcal.[6] The diet contained approximately 1.2 times more zinc (64.3 ± 5.9 mg zinc/Mcal) than maintenance diets, that may contain a median zinc of 53.0 mg/Mcal (range, 33.0–82.2 mg/Mcal)[19] or a mean zinc concentration of 52.4 ± 17.8 mg/Mcal).[6] There were no data available about the copper and zinc concentrations of diets that were fed to the dogs before entering this study. Hepatic copper concentrations in this study were measured in biopsy samples obtained using 14G tru cut needles[b]. Previous studies that compared the reliability of hepatic needle biopsy samples to wedge biopsy samples concluded that results from needle biopsy samples must be interpreted with caution.[20,21] In those studies, 18 G needle biopsy samples were used, which result in a much smaller biopsy volume compared to the 14G biopsy needles that were used in this study. In advanced stages of copper-associated hepatitis, when liver cirrhosis is present, results from hepatic copper determination in needle biopsy samples becomes less reliable, because of the presence of fibrotic tissue and regenerative nodules that contain less copper.

In our study population, there were no dogs with liver cirrhosis and the highest stage of fibrosis identified was stage 1 (mild degree of fibrosis; Table 1) in 8 biopsy samples (of 83 samples). In these 8 samples,

Table 2. Estimates for logistic regression of outcome.

	Multivariable Model			Univariable Model		
	OR	95% CI	*P*-Value	OR	95% CI	*P*-Value
28 dogs						
Pedigree	21.9	2.5–580	.018	13.3	2.5–98	.0050
Copper	1.7	1.1–3.4	.050	1.4	1.1–2.2	.037
24 dogs						
Pedigree	17.2	1.48–696	.050	14	2.2–137	.010
Copper	1.7	1.1–3.7	.076	1.51	1.1–2.4	.035

OR, Odd's ratio.
Copper: OR for hepatic copper at the start of the study (estimates for every 100 mg/kg dwl copper increase).
Pedigree: Being part of the high-risk pedigree (reference category, being part of any of the other pedigrees).

there may have been an increased chance for sampling error, influencing hepatic copper concentration. However, because there was only mild fibrosis present, this effect likely was minimal.

In 15/28 dogs in the study, hepatic copper concentrations decreased to normal upon dietary treatment. However, individual variation in response was present, which was illustrated by the fact that 6/28 dogs actually accumulated hepatic copper despite being fed the diet. The results from this study indicate that a low-copper, high-zinc diet can decrease hepatic copper concentration in the majority of Labrador retrievers at risk for development of clinical copper-associated hepatitis. An individual variation in response to diet may be attributed in part to the genetic background of dogs.

Thirteen of 28 dogs that were recruited for this study were part of a pedigree that was severely affected with copper-associated hepatitis. The index dog of this pedigree died of copper-associated liver cirrhosis a few weeks after giving birth to her fourth litter. Over 4 generations, 20 dogs from this pedigree were affected. In the other 7 pedigrees, the disease frequency was lower and was not known to be extended over several generations.

Although dogs with the highest hepatic copper concentrations were part of the high-risk pedigree, there was no significant difference between hepatic copper concentrations at the start of the study between dogs from the high-risk pedigree and dogs from the other pedigrees. The pedigree effect in the logistic regression analysis was significant in the multivariable model including hepatic copper concentration at the start of the study, indicating that the variation in response to diet may be in part caused by the genetic background of an individual dog. In a complex hereditary disease, such as copper-associated hepatitis in the Labrador retriever, multiple mutations, each with a small effect, are expected to contribute to the phenotype. In a severely affected pedigree, the frequency of disease alleles likely is increased, possibly leading to failure to respond to diet in individual dogs.

After discovery of the genes that are responsible for copper-associated hepatitis in Labrador retrievers and a DNA test becomes available, it may become possible to predict the risk for an individual dog to develop copper-associated hepatitis more precisely compared to prediction based on family history. Ideally, a longitudinal diet trial in dogs at risk in a controlled environment should be performed, aiming to optimize dietary copper and zinc concentrations that possibly can prevent clinical disease in high-risk dogs. The median time to normalization of hepatic copper concentration was 7.1 months (range, 5.5–21.4 months). Dogs that responded to the diet and experienced normalization of hepatic copper concentration had a mean copper concentration of 710 $\pm$ 216 mg/kg dwl at the start of the study and 343 $\pm$ 70 mg/kg dwl at the end of the study. The average decrease rate of approximately 50 mg/kg dwl/month compares to a decrease rate expected with D-penicillamine at a dosage of 20 mg/kg/day in Labrador retrievers.[7] The estimated needed D-penicillamine treatment period is approximately 6 months to obtain normalization of hepatic copper concentration in dogs with a hepatic copper concentration of 710 mg/kg dwl. However, whereas the response to D-penicillamine is predictable, a wide variation was noted in response to diet in the individual dogs. Three dogs accumulated copper despite the diet and 3 other dogs initially showed a decrease, which was followed by an increase in their hepatic copper concentration. Currently, we cannot accurately predict the response to diet, and biopsies are required to monitor response to treatment.

Four dogs in this study did not reach hepatic copper concentrations <400 mg/kg, although they were followed for 12.7–43 months. Their hepatic copper concentrations were between 475 and 620 mg/kg dwl without histological abnormalities when their owners decided to discontinue follow-up liver biopsies. These dogs may represent a subset of dogs that can tolerate slightly increased hepatic copper concentrations without overt progression of histopathological hepatic changes, when kept on a low-copper, high-zinc diet and in this way potentially avoid the need for chelation therapy.

Although the Labrador retrievers in this study had increased hepatic copper concentrations at the start of the study, only 1 dog had increased ALT activity, all other measured blood variables were within normal limits. Also, during dietary treatment, ALT and plasma albumin concentration were not useful in follow-up evaluation. Normal ALT activity, currently considered the most sensitive parameter for parenchymal liver disease,[22] does not rule out hepatic copper accumulation. In the current absence of sensitive and specific biomarkers for copper status and parenchymal liver disease, screening, diagnosis and follow-up of therapy depends on evaluation of liver biopsy samples.

This study was performed using client-owned dogs that were housed in a family environment and we could not control for all other factors possibly influencing copper intake, including copper intake via drinking water and ingestion of other foodstuffs. Copper concentration in drinking water in the Netherlands is under tight regulation. Tap water sampling studies show that the median copper concentration is 50 μg/L and that geographical variation is minimal.[23] With this low concentration of copper in tap water, a critical effect of copper in drinking water on hepatic copper concentrations in this study may be discounted. Owners were strictly and repeatedly instructed to feed their dogs nothing but the provided diet. Although, there is a chance that dogs may have obtained small amounts of other foodstuffs, the large majority of the food intake consisted of the Royal Canin hepatic diet[a], and therefore we consider it unlikely that this variable biased our results.

When we compare our results to a previous study in Labrador retrievers, in which dogs first underwent chelation therapy until hepatic copper concentrations were normalized and were subsequently fed Royal Canin hepatic diet,[9] some differences can be noted. In that study, 3 groups of responders were identified: Dogs that were stable on the diet and did not re-accumulate copper >400 mg/kg dwl for a period of approximately 2 years, dogs that re-accumulated copper between 400 and 800 mg/kg dwl and were stable at that concentration

during the study period of approximately 1.5 years and dogs that re-accumulated copper >800 mg/kg dwl after approximately 1.5 years and needed an additional course of D-penicillamine. The majority of dogs in the present study experienced a decrease in hepatic copper concentration upon feeding the Royal Canin hepatic diet. We can speculate about possible reasons for this difference in response. First, the dogs in the current study had a much higher hepatic copper concentration at the start of the study (919 ± 477 mg/kg dwl) compared to dogs from the previous study (266 ± 125 mg/kg dwl). It may be that because of D-penicillamine therapy, the dogs adjusted to a relatively copper-depleted state and mechanisms for maintaining body copper homeostasis by increasing uptake were up-regulated, resulting in initial copper accumulation in the previous study.

In summary, a low-copper, high-zinc diet[a] alone may be used to decrease hepatic copper to normal concentrations in Labrador retrievers with increased hepatic copper concentration. In some dogs (related to a clinically affected individual), hepatic copper accumulation occurred despite dietary treatment, indicating that response to diet is influenced by genetic background. Currently, it is not possible to predict the response to diet in an individual dog nor are reliable blood biomarkers available. Therefore, the effect of diet on hepatic copper concentration should be evaluated by follow-up liver biopsy samples.

Footnotes

[a] composition of Royal Canin Hepatic dry diet. Ingredients: rice, maize, animal fats, soya protein isolate, hydrolyzed animal proteins, beet pulp, minerals, soya oil, vegetable fibres, fish oil, fructo-oligo-saccharides, marigold extract. Guaranteed analysis: proteins: 16%, fat: 16%, nitrogen free extract: 51.9%, dietary fibre: 7.2%, crude fibre: 2%, minerals: 4.60%, Omega 6: 3.88%, Omega 3: 0.6%, EPA+DHA: 0.21%, calcium: 0.73%, phosphorus: 0.52%, sodium: 0.20%, metabolizable energy : 3737 Kcal/kg (calculated by modified Atwater factors) Vitamin E: 600 mg/kg, Vitamin C: 200 mg/kg, taurine: 2200 mg/kg, lutein: 5 mg/kg.

[b] Pro-Mag Ultra automatic biopsy instrument, Angiotech.

[c] Royal Canin Research Center, Aimargues, France.

[d] http://www.R-project.org

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Conflict of Interest Declaration: Vincent C. Biourge and Adrian L. Watson are employed by Royal Canin. Royal Canin financially supported this study.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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