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ATP7A, *ATP7B*, and *RETN* genotypes in Labrador Retrievers with and without copper-associated hepatopathy

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OBJECTIVE
To determine the frequency of previously reported coding variants in the *ATP7A*, *ATP7B*, and *RETN* genes in a US population of Labrador Retrievers and to explore potential associations of these genotypes with pathologic hepatic copper accumulation.

SAMPLE
Archived hepatic specimens from 90 Labrador Retrievers collected between 2013 and 2021.

PROCEDURES
The Michigan State University Veterinary Diagnostic Laboratory database was searched to identify archived tissues from Labrador Retrievers that had undergone hepatic histopathologic assessment. Cases were classified into control, copper-associated hepatopathy (CAH), and intermediate populations on the basis of histopathologic features and hepatic copper accumulation. The DNA was extracted from archived tissues and genotyped for reported variants in the 3 genes. Allele frequencies were determined, and associations of genotypes with CAH status were evaluated.

RESULTS
29 control dogs, 45 CAH dogs, and 16 intermediate dogs were included in the study. The overall *ATP7A* and *RETN* variant allele frequencies were 30% and 13%, respectively, and were not significantly different among control, CAH, and intermediate populations. The *ATP7B* variant allele frequency was significantly higher in the CAH population (30%) as compared to the control population (13%). However, 21 of 45 (47%) CAH dogs did not have an *ATP7B* variant allele whereas 7 of 28 (25%) control dogs did have an *ATP7B* variant allele.

CLINICAL RELEVANCE
Study results supported a contributory role for the *ATP7B* variant in CAH pathogenesis in Labrador Retrievers. However, the application of genetic testing in a clinical setting is complicated by genotypic variability within healthy and diseased dogs.

Copper is an important functional component of many metalloproteins that are involved in myriad enzymatic processes throughout the body.[1,2] Although copper is an essential micronutrient, excessive tissue copper can overwhelm normal chaperone proteins and result in oxidative stress and inflammation.[3,4] The liver is commonly affected in cases of copper overload given its central roles in copper storage and metabolism.[2,5] Pathologic hepatic copper accumulation, termed copper-associated hepatitis or copper-associated hepatopathy (CAH), is the most common known cause of chronic hepatitis in dogs.[6] Clinical features are varied and can range from asymptomatic dogs with minimal increases in liver enzyme activities to dogs with chronic illness and biochemical evidence of end-stage liver failure.[5–7] Some dogs will even present with acute exacerbations of disease that can mimic hepatotoxicity or infectious hepatopathies.[5,6] Any breed of dog can be affected by CAH, but the Bedlington Terrier, West Highland White Terrier, Dalmatian, Doberman Pinscher, and Labrador Retriever are considered to be predisposed breeds.[8–12]

An autosomal recessive mutation in the *COMMD1* gene resulting in impaired biliary copper excretion and progressive hepatic copper accumulation is responsible for CAH in Bedlington Terriers, but this mutation has not been documented in other CAH-affected breeds.[8,13,14] A complex and incompletely characterized interplay of environmental copper exposures and genetic susceptibilities are thought to be responsible for CAH in most breeds, including the Labrador Retriever.[6] Changes in the Associations of American Feed Control Offi-

cials guidelines for copper supplementation in commercial dog food in the 1990s resulted in the use of more bioavailable forms of copper additives despite there being no evidence of diet-induced copper deficiency at the time.[15,16] These changes likely caused a notable increase in CAH prevalence.[6,17–19] Hepatic copper concentrations increased in dogs regardless of breed after these regulatory guidelines went into effect, and the magnitude of increase was greatest in predisposed breeds such as the Labrador Retriever.[17] These well-established breed predispositions provide support for a contributory genetic etiology of CAH.[5,8–12] Coding variants in the *ATP7A* and *ATP7B* genes, which are where the primary mutations in the copper metabolism disorders of Menkes disease and Wilson disease occur in humans, respectively, have been associated with CAH in a Dutch population of Labrador Retrievers.[20] The *ATP7A* variant was associated with decreased CAH risk, whereas the *ATP7B* variant was associated with increased CAH risk.[20] Abnormalities in these copper transport proteins might be involved in CAH in other breeds as well.[21] The X-linked *ATP7A* protein is involved in the transport of copper from enterocytes into the portal circulation, whereas the *ATP7B* protein is involved in the biliary excretion of copper from hepatocytes.[22] Recently, a variant in the *RETN* gene was associated with lower hepatic copper concentrations in Labrador Retrievers, although the mechanisms for this are unknown.[23]

Genetic testing for the *ATP7A* and *ATP7B* variants in Labrador Retrievers has become widely available in the US in recent years.[24–26] Genetic testing for CAH could decrease disease prevalence and aid clinicians in early disease detection. Indeed, the prevalence of CAH in Bedlington Terriers was reduced from over 46% to < 11% because of genetic testing and selective breeding strategies.[27] However, the *ATP7A* and *ATP7B* variants account for only a small proportion of the variation in hepatic copper concentrations in Labrador Retrievers.[20] This suggests that these variants are genetic modifiers of CAH rather than primary causative mutations. Furthermore, the prevalence and significance of these variants have not been studied in detail in Labrador Retrievers from the US.[28] The inappropriate testing and application of results could have unintended consequences for clinical management of individual patients as well as for the genetic diversity of the population. Additional studies of these variants and their associations with pathologic copper accumulation are needed before routine clinical testing can be recommended. Therefore, our objectives were to investigate *ATP7A*, *ATP7B*, and *RETN* genotypes in well-defined populations of American Labrador Retrievers and to determine the associations of these genotypes with CAH.

Materials and Methods

Case selection

The histopathology database at the Michigan State University Veterinary Diagnostic Laboratory was searched from March 2013 to March 2021 to identify Labrador Retrievers that had undergone histopathologic assessment of hepatic tissue. All cases for which archived formalin-fixed paraffin-embedded tissues were available were considered for inclusion unless the available history indicated the dog had been previously treated for CAH. There were no requirements related to reason for tissue sampling or method of sampling (eg, necropsy evaluation, surgical biopsy, or needle biopsy). Hematoxylin and eosin–stained slides were assessed according to the World Small Animal Veterinary Association guidelines for classification of parenchymal disorders.[29] Rhodanine-stained slides were scored for copper accumulation by use of a previously described system in which scores can range from 0 (no detected copper granules) to 5 (panlobular presence of large numbers of copper granules in hepatocytes, often in association with copper-containing macrophages).[30] Slides were reviewed by a single board-certified pathologist with expertise in hepatic histopathology, and a minimum of 15 portal triads were available for histologic evaluation in all cases.

Quantitative hepatic copper concentrations (reference interval, 137 to 400 µg/g dry matter basis), which were determined by inductively coupled plasma mass spectrometry in the same laboratory,[17,31] were available for some cases in which this analysis was requested as part of the normal submission. Available copper concentration data were used to aid in case characterization. The quantitative analyses were performed on fresh or formalin-fixed paraffin-embedded tissues on the basis of the type of samples submitted to the laboratory and the timing in which quantitative analyses were requested by the submitting veterinarian. The results of quantitative copper determinations in fixed and nonfixed tissues are generally in agreement and would not be expected to alter clinical classification in most cases, although values might be higher in fixed tissues.[32]

Cases were classified into 1 of 3 study populations including control dogs, CAH dogs, and dogs with an intermediate phenotype (IM). Cases were excluded if they did not meet the criteria for inclusion in one of these study populations, the rhodanine-stained slides revealed a predominantly periportal distribution of copper, or the H&E-stained slides contained only neoplastic tissue without any nonneoplastic tissue for assessment. A predominantly periportal distribution of copper would be unusual for CAH, and copper concentrations within neoplastic hepatic tissue do not reflect the copper status of the unaffected, nonneoplastic liver.[6,31]

Control population—Dogs that were ≥ 7 years of age at the time of sample acquisition and had rhodanine scores of ≤ 1 were included in the control population. Dogs with advanced fibrosis or lobular collapse were excluded to ensure that these features did not preclude accurate assessments of copper accumulation.[5,29,30] Copper accumulation is a gradual and chronic process, and the median age at CAH di-

agnosis is approximately 5 to 6 years of age.[5] The inclusion of older dogs with normal hepatic copper stores minimized the possibility that dogs with early or subclinical forms of CAH were erroneously classified as controls.

CAH population—Dogs were classified into the CAH population if they had centrilobular to panlobular copper accumulation and a rhodanine score of ≥ 3. The increased hepatic copper stores in this population were frequently accompanied by histologic evidence of chronic hepatitis, but the histologic features of chronic hepatitis were not a requirement for inclusion. Variability in histopathologic features of dogs with CAH are well recognized, and these criteria are consistent with general guidelines for CAH diagnosis in which most affected dogs have substantial increases in hepatic copper levels that are often accompanied by chronic hepatitis.[4–6,9]

IM population—Hepatic copper concentrations ≥ 1,000 µg/g are generally considered to be toxic, but copper concentrations capable of inducing hepatic damage are not clearly established and likely influenced by multiple factors.[6,33] Some dogs have mild to moderate increases in hepatic copper stores that may or may not be associated with hepatic pathology. It is well recognized that a "gray zone" of copper levels exists in dogs.[6] These dogs pose a diagnostic challenge because it is unknown whether or not this is a mild form of CAH or if the copper accumulation will progress.[6] This important clinical entity warranted study inclusion. Dogs were classified into this phenotypically ambiguous population, termed IM, if they had a rhodanine score of 2, which correlates with mild to moderate increases in hepatic copper accumulation that are often < 1,000 µg/g.[5,18,30]

DNA isolation and genotyping

Tissue sections of approximately 5 X 5 µm were obtained from the archived specimens and used for DNA extraction. The DNA was extracted and genotyped by use of standard methodologies. Samples were genotyped for the previously described missense mutations *ATP7A*:c.980C>T, *ATP7B*:c.4358G>A, and *RETN*:c.19C>T, resulting in amino acid substitutions ATP7A:p.Thr327Ile, ATP7B:p.Arg1453Gln, and RETN:p.Leu7Phe.[20,23] Briefly, custom-made single nucleotide polymorphism genotyping assays specific for the missense mutations *ATP7A*:c.980C>T and *RETN*:c.19C>T were used for the *ATP7A* and *RETN* genotyping. Samples were genotyped for the missense mutation *ATP7B*:c.4358G>A by PCR amplification and restriction enzyme digestion. Samples from all genotypes were further analyzed by direct Sanger sequencing of PCR products at Michigan State University's Genomics Core to validate results. More detailed description of the genotyping is available **(Supplementary Appendix S1)**.

Data and statistical analysis

Data were assessed for normality with Shapiro-Wilk testing and inspection of box plots. Ordinal data (rhodanine scores) and data that were not normally distributed (hepatic copper concentrations) were reported as median and ranges. Demographics, copper scores, and copper concentrations were summarized with descriptive statistics. Demographics and other characteristics of the 3 study populations were compared by use of the Fisher exact test or Kruskal-Wallis test as appropriate. A Fisher exact test also was used to investigate proportionate difference in variant allele frequencies among study populations. Rhodanine scores were compared among selected genotypes by use of Kruskal-Wallis testing or Mann-Whitney *U* testing. Odds ratios and 95% CIs were reported for genotypes that were significantly associated with hepatic copper status. Statistical analyses were performed with commercially available software (Prism version 9.0; GraphPad Software), and for all analyses, $P \leq 0.05$ was considered significant.

Results

Dogs

Ninety Labrador Retrievers met criteria for classification into one of the study populations and had sufficient tissue available for DNA extraction. The median age of the 90 dogs included in the study was 10 years (interquartile (25th to 75th percentile) range, 7 to 11 years), and there were 54 females (51 spayed, 2 intact, and 1 unknown) and 36 males (33 castrated and 3 unknown). Review of rhodanine-stained slides was performed in all 90 study dogs; quantitative hepatic copper concentration data were available for 53 of the 90 dogs. Characteristics of the control (n = 29), CAH (45), and IM (16) populations were summarized **(Table 1)**. There

Table 1—The demographics and hepatic copper data for the control (n = 29), copper-associated hepatopathy (CAH; 45), and intermediate (IM; 16) populations of Labrador Retrievers.

Population	No. of dogs (M/F)	Age (y)	Rhodanine scores	Copper quant (µg/g)
Control	29 (12/17)	11 (9–14)	1 (0–1)	278 (175–291)
CAH	45 (17/28)	7 (2–17)	3.5 (3–5)	1,486 (829–6,475)
IM	16 (7/9)	9 (3–13)	2 (2–2)	526 (412–862)

Male (M) and female (F) are presented as absolute numbers, and age, rhodanine scores, and quantitative hepatic copper concentrations (copper quant) are presented as median and range. Note that quantitative copper values were not available for all dogs. Rhodanine scores ≤ 1 are considered normal, and the reference interval for hepatic copper concentrations is 137 to 400 µg/g.

were more females than males within each population, and the proportion of females in the control (17/29 [59%]), CAH (28/45 [62%]), and IM (9/16 [56%]) populations were not significantly different (P = 0.769).

Rhodanine scores were ≤ 1 in all 29 control dogs, and quantitative hepatic copper concentrations were < 400 µg/g in the 5 control dogs for which these data were available. The primary hepatic histologic diagnoses included normal liver or normal liver with occasional areas of nodular hyperplasia (n = 12), vacuolar degeneration (9), chronic hepatitis (3), acute hepatitis (2), cholestasis (1), cholangiohepatitis (1), and pyogranulomatous hepatitis (1).

Rhodanine scores were ≥ 3 in all 45 CAH dogs. Quantitative hepatic copper concentrations were > 800 µg/g in 35 of 36 CAH dogs for which these data were available, including all 20 CAH dogs that had a rhodanine score of 3. One CAH dog with a rhodanine score of 4.5 (some areas were scored a 4, and some areas were scored a 5) had a hepatic copper concentration of 205 µg/g. This quantitative value was not reflective of the liver copper burden because of the presence of large amounts of fibrosis, regenerative nodule formation, and lobular collapse. The dog remained in the CAH population although the quantitative value was excluded from descriptive analysis. Thirty-eight of 45 CAH dogs had histologic evidence of chronic hepatitis; 4 dogs had mixed, predominantly lymphoplasmacytic, centrilobular inflammatory infiltrates that were not accompanied by any fibrosis; 1 dog had low-level scattered inflammatory infiltrates; and 1 dog had vacuolar degeneration. The remaining CAH dog had a rhodanine score of 4 and an otherwise histologically normal liver except for a hemangiosarcoma in a separate liver lobe.

Rhodanine scores were 2 in all 16 IM dogs; quantitative hepatic copper concentrations ranged from 412 to 862 µg/g in the 12 IM dogs for which these data were available. Primary histologic diagnoses included chronic hepatitis (n = 8), normal liver (3), vacuolar degeneration (2), granulomatous hepatitis (1), low-level scattered to periportal inflammatory infiltrates most consistent with a nonspecific reactive hepatopathy (1), and moderate fibrosis without any accompanying acute or chronic hepatitis (1).

Allele frequencies

The DNA derived from formalin-fixed, paraffin-embedded tissue was successfully genotyped for the wild-type and variant *ATP7A*, *ATP7B*, and *RETN* alleles in 81 dogs (90%), 89 dogs (99%), and 75 dogs (83%), respectively. Overall, the variant allele frequency was 30% (40/135 alleles) for *ATP7A*, 22% (39/178 alleles) for *ATP7B*, and 13% (20/150 alleles) for *RETN*. The *ATP7B* variant allele frequency in the CAH dogs (30% [27/90 alleles]) was significantly higher than that of the control dogs (13% [7/56 alleles]; **Figure 1**). The *ATP7B* variant allele frequency (16% [5/32 alleles]) in IM dogs was not significantly different from controls (P = 0.751) or CAH dogs (P = 0.160). Neither the *ATP7A* variant allele frequencies nor the *RETN* variant allele frequencies were significantly different among the control, CAH, and IM populations.

Genotype associations with copper

Rhodanine scores in dogs heterozygous for the *ATP7B* variant allele were significantly higher than in dogs homozygous for the wild-type allele **(Figure 2)**. Dogs possessing at least 1 *ATP7B* variant allele were 3.1 times (95% CI, 1.3 to 7.6) more likely to be in the CAH population than in the combined control and IM population (P = 0.017). However, 21 of 45 (47%) CAH dogs were homozygous wild-type for the *ATP7B* gene,

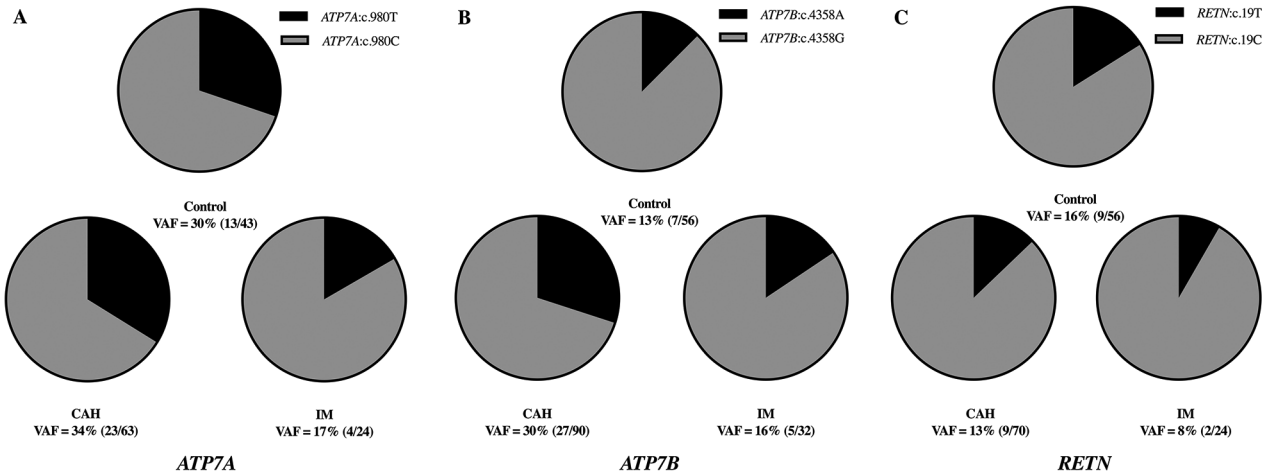

Figure 1—Pie charts depicting variant allele frequencies for the *ATP7A* (A), *ATP7B* (B), and *RETN* (C) genes in the 3 study populations, which included control dogs, dogs with copper-associated hepatopathy (CAH), and dogs that had an intermediate (IM) phenotype. The variant allele frequency (VAF) is depicted below each pie. The *ATP7B* variant allele frequency in CAH dogs was significantly higher than in control dogs (P = 0.016). No other differences in allele frequencies were found among study populations (P > 0.05 for all comparisons).

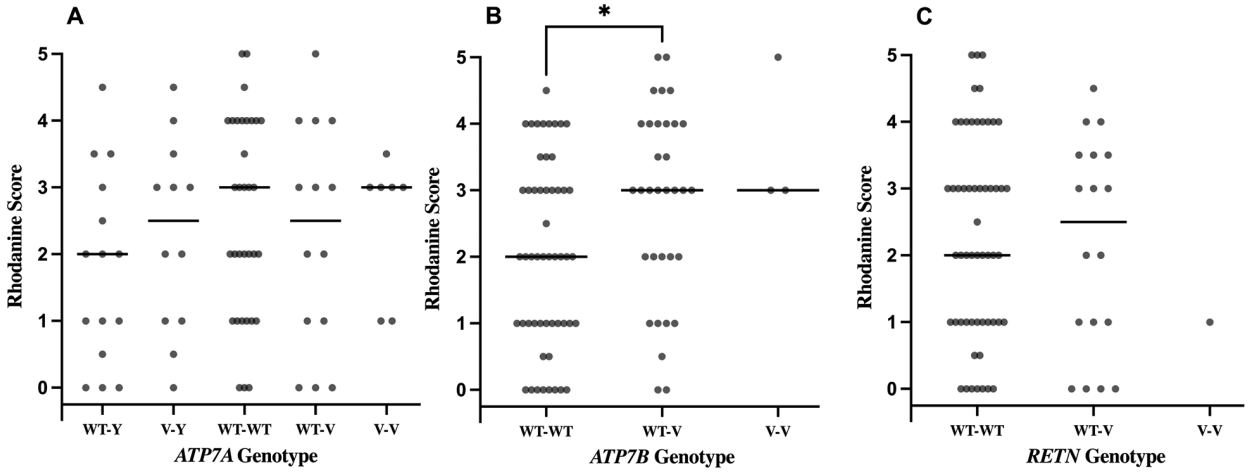

Figure 2—Scatterplots of rhodanine scores in Labrador Retrievers when classified by their *ATP7A* (A), *ATP7B* (B), and *RETN* (C) genotypes. The horizontal lines within each scatter represent the median. Only the *ATP7B* variant was significantly associated with rhodanine scores. Note, the genotypes for *ATP7A* are further divided by sex since this is a sex-linked gene. Also, dogs that were homozygous for variant alleles (V-V) were not included in statistical comparisons because of the low number of dogs within these groupings. V-Y = Hemizygous variant. WT-V = Heterozygous wild-type/variant. WT-WT = Homozygous wild-type. WT-Y = Hemizygous wild-type. *P = 0.018.

whereas 7 of 28 (25%) control dogs and 5 of 16 (31%) IM dogs possessed an *ATP7B* variant allele. Rhodanine scores were not found to vary with the *ATP7A* and *RETN* genotypes (Figure 2).

A total of 14 dogs had at least 1 variant allele for both the *ATP7A* and *ATP7B* genes, and the median rhodanine score in these dogs was 3 (range, 0 to 5). Three of these 14 dogs were controls, 9 dogs were in the CAH population, and 2 dogs were in the IM population. A total of 4 dogs possessed at least 1 variant allele for all 3 genes; 1 dog was in the control population, and the other 3 dogs were in the CAH population.

Only 3 dogs were homozygous for the *ATP7B* variant allele, and all 3 dogs were in the CAH population. One of these 3 dogs was a male that was hemizygous for the *ATP7A* variant and heterozygous for the *RETN* variant. The other 2 dogs were female; one was homozygous variant and the other was heterozygous variant for *ATP7A*, and both were homozygous wild-type for *RETN*. Five of the 7 female dogs that were homozygous for the *ATP7A* variant were in the CAH population; 4 of these 5 dogs were heterozygous (n = 3) or homozygous (1) for the *ATP7B* variant. Six of the 12 male dogs that were hemizygous for the *ATP7A* variant were in the CAH population, and 3 of these 6 dogs were heterozygous (n = 2) or homozygous (1) for the *ATP7B* variant.

Discussion

Our study determined the frequency of *ATP7A*:c.980C>T, *ATP7B*:c.4358G>A, and *RETN*:c.19C>T variants and their association with CAH in an American population of Labrador Retrievers that were well characterized regarding their hepatic histopathologic features. The *ATP7B* protein is a widely distributed copper-transporting P-type ATPase, which has principal effects in the liver, where it is important in the excretion of copper into the bile.[22] Mutations in this protein are responsible for Wilson disease, which is a well-characterized autosomal recessive disorder of copper overload in humans.[22,34] The *ATP7B* variant was twice as common in Labrador Retrievers with CAH as compared to controls, and dogs possessing at least 1 copy of the *ATP7B* variant allele had significantly higher rhodanine scores than dogs homozygous for the wild-type allele. Our findings are in agreement with a large study[20] of over 200 Labrador Retrievers in the Netherlands in which associations of the *ATP7B* variant and pathologic copper accumulation were first described. A previous study[28] of *ATP7A* and *ATP7B* variants in Labrador Retrievers in the US also suggested a role for the *ATP7B* variant in CAH development, but the small number of dogs precluded meaningful assessments of dogs that possessed a single ATP7B variant allele. Coding variants in *ATP7B* are also likely to contribute to copper accumulation in the Doberman Pinscher, although the effect in this breed might be limited to those dogs that are homozygous for the variant allele.[21]

While our findings supported a contributory role for the *ATP7B* variant in the pathogenesis of CAH in Labrador Retrievers in the US, we did not detect any association of the *ATP7A* variant or the *RETN* variant with CAH. The X-linked *ATP7A* also is a widely distributed trans-Golgi network P-type ATPase copper-transporting protein.[22] However, *ATP7A* is highly expressed in the basolateral membranes of enterocytes, where it is involved in transporting dietary copper into the portal circulation.[22] The biological functions of resistin, the protein encoded by the *RETN* gene, are not fully characterized.[35] Resistin has been associated with various metabolic, inflammatory, and autoimmune conditions including liver disease.[36–39] Both *ATP7A* and *RETN* variants were associated with decreased hepatic copper and partly protective against CAH in Dutch populations of Labrador Retrievers.[20,23]

Reasons why these variants were not associated with copper accumulation in our study are unknown. The *ATP7A* and *RETN* variant allele frequencies are similar between US and Dutch populations.[20,23,28] Perhaps other genetic modifiers, differing dietary copper exposures, or a combination of factors contributed to discordant results. It is also possible that a larger sample size would be needed to detect statistically significant effects because the allele frequencies are low and potential clinical consequences are small.

Over 50% of Labrador Retrievers undergoing histopathologic assessment of hepatic tissue at 1 institution had abnormal copper accumulation, and approximately 30% had copper concentrations exceeding 1,000 µg/g.[17] The diagnosis of CAH requires a liver biopsy, and treatment of advanced disease is expensive and associated with variable outcomes.[6,12] Strategies to reduce disease prevalence or aid in the early and minimally invasive diagnosis of CAH would be of immediate impact. Multiple diagnostic laboratories in the US now offer testing for the *ATP7A* and *ATP7B* variants, and diagnostic and breeding recommendations based on genotyping results are available on some of the laboratory websites.[24–26] Our results raise concerns about using *ATP7A* and *ATP7B* variant testing to guide clinical decision-making in the pet population. Even if the *ATP7A* variant protects against copper accumulation, the effect is of questionable clinical relevance. Variant *ATP7A* allele frequencies were nearly identical between CAH and control dogs, and rhodanine scores were similar among all possible *ATP7A* genotypes. Of note, 5 of 7 females homozygous for the *ATP7A* variant and 6 of 12 males hemizygous for the *ATP7A* variant had rhodanine scores ≥ 3 and were in the CAH population.

Although the *ATP7B* variant is associated with heightened risk for CAH, the significance of identifying this variant in Labrador Retrievers in the general pet population is complex. In this study, nearly half of CAH dogs did not possess an *ATP7B* variant allele. If decisions to perform annual screening of liver enzyme activities or diagnostic liver biopsy were based on only genotyping results, many dogs with CAH would be missed with such an approach. Conversely, if an aggressive diagnostic evaluation were pursued in all dogs heterozygous for the *ATP7B* variant allele, non-CAH dogs might be subjected to unnecessary procedures such as liver biopsy. In our study, 25% of older Labrador Retrievers without evidence of copper accumulation (control population) had a variant allele. Further complicating matters is the fact that idiopathic chronic hepatitis also is common in Labrador Retrievers, limiting the value of integrating liver enzyme activities with genotyping results.[18,40,41] The identification of dogs that are homozygous for the *ATP7B* variant would seemingly be of greater importance as all 3 homozygous variant dogs in our study had CAH. A previous study of a Labrador Retriever breeding program also documented that 5 littermates homozygous for the *ATP7B* variant had pathologic copper accumulation at a young age, and hepatic copper concentrations in these 5 dogs were significantly higher than those of the other littermates, which were either heterozygous or homozygous for the wild-type allele.[28] Although the homozygous variant genotype appears to be clinically relevant, it is uncommon in the pet population. Only 3% of dogs in our study were homozygous for the *ATP7B* variant, which is similar to the 2% prevalence reported in over 1,000 dogs undergoing testing in a commercial genetics laboratory.[28]

Testing for the *ATP7B* variant could have more value in breeding programs than in clinical practice settings. The intentional breeding of 2 dogs homozygous for the *ATP7B* variant would be ill-advised considering the heightened risk for CAH in their offspring.[20,28] However, additional breeding decisions based on genotyping results must be made with caution. Even in the Dutch population of Labrador Retrievers, only 12.5% of the heritability of copper accumulation was attributed to the *ATP7A* and *ATP7B* variants.[20] Approximately 25% of the Labrador Retriever population in the US has an *ATP7B* variant allele.[28] Attempts to eliminate this variant could decrease genetic diversity and result in unanticipated consequences. Equally important, nongenetic factors such as the amount and bioavailability of dietary copper play a substantial role in CAH development.[16–19,42] This underscores the need for detailed studies that consider the collective influence of both environmental and genetic factors in disease development and progression. For example, the *ATP7B* variant might be of minimal concern in the absence of excess dietary copper, or dietary copper might minimally alter hepatic copper in the absence of genetic modifiers.

The inclusion and investigation of an IM population was a unique aspect of our study as compared to other studies of CAH.[20,21,23] We thought it was important for this population to be studied and to see if genotyping would offer another tool to further characterize these dogs in the clinical setting. The inclusion of this population could be considered a weakness because clinical outcomes were unknown. Some dogs may have developed progressive copper accumulation over time, whereas others may have remained static. Differing clinicians might have classified some of the IM dogs as CAH, whereas others might have classified some as unaffected. These ambiguities demonstrated that the diagnosis of CAH is not straightforward. Specific criteria for an IM population are not clearly defined, but it is generally agreed upon that such a population exists and is commonly encountered in clinical practice.[6] The IM population also emphasized the importance of integrating history, clinical features, hepatic histopathology, and assessments of hepatic copper stores to guide decision-making in a clinical setting.[6] The *ATP7A* variant did not appear to be responsible for a protective effect against more severe copper accumulation in these dogs as the *ATP7A* variant allele frequency was only 17%. The other variant alleles were also not overrepresented in the IM population, which was most similar to the control population in terms of genotypic diversity. It is possible that environmental factors or unknown genetic factors were the principal reasons for the low-level copper accumulation in some of these dogs.

Copper-associated hepatopathy in most breeds of dogs appears to be a complex disorder, likely involving multiple genes and gene-environment interactions.[17–21] The present study further supported CAH in Labrador Retrievers to be a complex disease with variants in genes such as *ATP7B* having measurable contributory effects across both Dutch and American populations, but also highlighted that many control and CAH dogs lacked variant alleles for any of the 3 studied genes. A notable exception to the genetic complexity of CAH is the Bedlington Terrier. The identification of a linked marker for copper toxicosis in the Bedlington Terrier utilizing a whole genome scanning linkage approach for the first time in the dog led to the discovery of the primary defect in the *COMMD1* gene.[8,13] The Bedlington Terrier highlights the importance of breed-specific investigations because the *COMMD1* gene had not been associated with copper handling in any species prior to this discovery and thus has contributed greatly to current understanding of copper physiology and pathology.[43] Additional studies that can detect other contributing genetic loci are needed in Labrador Retrievers and other CAH-affected breeds. It is possible that studies of each breed may help identify different genes with differing effects on the phenotype of CAH. Such studies have the potential to not only contribute to the understanding and management of CAH in dogs but also to elucidate important steps in copper metabolism that may contribute to understanding other copper-associated diseases across species.[44,45]

A strength of our study was the inclusion of 90 dogs that were phenotypically well-defined in terms of hepatic histopathology. One limitation was that an even larger sample size would be needed to better evaluate the contribution of certain genotypes such as dogs that are homozygous for the *ATP7A*, *ATP7B*, or *RETN* variants. Several hundred cases were initially identified in our database search, but factors such as lack of sufficient archived tissues excluded many cases. Still, the small number of dogs that were homozygous for the *ATP7B* variant in our CAH group pointed to the relatively small role of this genotype in CAH etiology in the US population, which is likely to be true in non-American populations as well.[20,46] We also relied predominantly on scoring of rhodanine-stained slides for disease classification. In clinical settings, the integration of both histochemical staining methods and quantitative copper determinations is recommended.[6] However, our approach was well aligned with recent genetics studies of CAH in which histochemical staining methods were used for disease classification.[14,20,21,23] Furthermore, our criteria of classifying only those with rhodanine scores ≥ 3 as CAH instilled confidence that all CAH dogs were accurately classified, and the inclusion of only older dogs in the control group ensured that early CAH cases were not erroneously classified as controls.

In summary, our study did not identify associations of the *ATP7A*:c.980C>T and *RETN*:c.19C>T variants with CAH in Labrador Retrievers. This does not preclude a role for these variants in hepatic copper homeostasis as variant allele frequencies were low and few dogs were homozygous for variant alleles, but these results do suggest that any possible protective effect against CAH is small. The *ATP7B*:c.4358G>A variant was significantly higher in CAH dogs than in controls. Dogs that were heterozygous for the *ATP7B* variant had higher hepatic copper than did dogs that were homozygous for the *ATP7B* wild-type allele. However, variant alleles were absent in some CAH dogs and present in some control dogs. Dogs homozygous for the *ATP7B*:c.4358G>A variant might be at even greater risk for CAH, but this genotype is found in only 2% to 3% of the general Labrador Retriever population.[28] Practitioners must be mindful of these results when considering the clinical utility of testing Labrador Retrievers for the *ATP7A* and *ATP7B* variants.

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Supplementary Materials

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