Demographic and histopathologic features of dogs with abnormally high concentrations of hepatic copper

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Abstract

Background: Copper associated hepatopathy (CAH) has become an important and prevalent disease since the 1990’s, coincidental with changes in copper (Cu) content in commercial dog foods. Knowing the demographic and histopathologic features related to hepatic Cu concentrations might aid in diagnosing CAH in dogs.

Hypothesis/Objectives: The primary aim was to identify demographic and histopathologic features associated with abnormally high hepatic Cu concentrations.

Animals: Dogs that underwent liver histopathology and Cu quantification at a veterinary diagnostic laboratory between July 2010 and February 2020.

Methods: Data was retrospectively collected from an electronic database. A Gaussian multiple regression model on the log scale was used to evaluate associations between hepatic Cu and a set of demographic and histologic features selected with machine learning methods.

Results: Of 4559 cases meeting criteria, 50% had hepatic Cu > 400 and 19% had Cu > 1000 ppm (parts per million) dry weight (reference range 120-400). Median hepatic Cu was 391 ppm, range 4.5 to 31500. Age was negatively associated (P < .02), but specific breeds (Doberman pinscher, Labrador retriever, and West Highland white terrier) were positively associated with abnormally high hepatic Cu (P < .001). Severity of inflammation (mild, moderate, and severe) and necrosis/apoptosis were associated with abnormally high hepatic Cu (P < .01).

Conclusion and Clinical Importance: Abnormally high hepatic Cu is prevalent in hepatic biopsies from dogs. Machine learning modeling showed that necroinflammation, not cholestasis or cirrhosis, on hepatic histopathology, is predictive of higher hepatic Cu and might be a reliable histologic predictor of CAH.

KEYWORDS

canine, chronic hepatitis, copper associated hepatopathy, liver disease
1 | INTRODUCTION

Copper associated hepatopathy (CAH), is a common and important hepatic disorder in dogs and the prevalence of CAH has increased over time.1,7 Median copper (Cu) has increased 3-fold from the 1980’s to 2000’s.2,8 One study reported that one-third of dogs with chronic hepatitis have copper associated disease.3

A genetic etiology of CAH is strongly suspected given breed predispositions in the Labrador retriever,4,5,8 Bedlington terrier,9-11 Doberman pinscher,12 West Highland white terrier,13,14 and Dalmatian.15 Mutations in Cu transport genes, such as Cu metabolism domain containing 1 (COMMD1) and ABCA12 genes of Bedlington Terriers16-19 or the ATP7B gene of Labrador retrievers and Doberman pinchers, have been associated with CAH.20,21 Environmental factors such as dietary Cu have also been highly implicated.22 Dramatic increases in hepatic Cu were observed after the National Research Council and Association of American Feed Control Officials (AAFCO) regulations mandated more bioavailable Cu compounds in commercial dog food.2,4,22,23 While Cu accumulation has been observed secondary to cholestatic or inflammatory disease in cats and humans,24-31 the impact of these factors has not been thoroughly evaluated and might be minimal in dogs.32

Dogs with CAH are typically young to middle-age and present with asymptomatic elevations in liver enzyme activity that later progress to liver failure.3,6,7 Excessive hepatic Cu causes oxidative stress and hepatocyte injury causing cell death (necrosis or apoptosis) and chronic hepatitis.33-35 Treatment with copper chelation,36-40 dietary Cu restriction,41-43 or a combination is essential to prevent progression. If untreated, some dogs can develop renal Cu accumulation causing kidney injury and Fanconi’s syndrome.44,45

Diagnosis of CAH requires quantification of Cu concentrations and should optimally be accompanied by Cu staining to document excess hepatic Cu stores. Staining typically shows Cu accumulation in a centrilobular location initially, but the distribution can progress periporal to widespread throughout the liver with time.1 However, variation in Cu distribution within the liver,46,47 necroinflammation,6 parenchymal remodeling,47 sampling technique and specimen size,48-50 tissue fixation,49 and an individual dog’s threshold for Cu accumulation4 can affect accurate estimates of Cu and obfuscate the diagnosis of CAH. Thus, knowing the demographic and histologic features most predictive of abnormally high hepatic Cu helps proactively detect, accurately diagnose, and treat CAH to prevent end-stage disease. It is also important to know whether factors such as cirrhosis and cholestasis impact hepatic Cu concentration and the diagnosis of CAH.

Studies reporting the demographic and histologic features of CAH evaluated small cohorts of select predisposed breeds5-16 and most studies reported descriptive features. Furthermore, the prevalence of CAH has grown in both predisposed and nonpredisposed breeds. Thus, reassessing previously noted breed associations and identifying new at-risk breeds is warranted. Further research is also needed to clarify contradictory findings published in previous studies.6 Hepatic Cu was significantly correlated with necroinflammatory activity in 1 study,4 but not in 2 others.51,52 Hepatic Cu was associated with cholestasis in Skye terriers,53 but not in dogs with experimentally induced acute cholestasis or naturally occurring chronic extrahepatic cholestasis.32,54 Thus, the present study aimed to analyze a large dataset using machine learning methods to identify demographic and histopathological features, other than histologic staining with rhodanine, that correlated with abnormally high hepatic Cu in dogs. We hypothesized that middle-aged dogs and breeds such as Labrador retrievers,5,8,12 and Doberman pinchers would have higher hepatic Cu.12 We also expected necroinflammation to be positively correlated with hepatic Cu.

2 | METHODS

2.1 | Datasets

2.1.1 | Retrospective dataset (4559 cases)

The Colorado State University Diagnostic Veterinary Laboratory database was searched to include dogs that had liver tissue submitted for histopathologic analysis and hepatic Cu quantification (performed by flame atomic absorption spectrometry) and reported in ppm (parts per million) dry weight between July 2010 and February 2020. Date of biopsy submission, age, sex, and the presence or absence of histopathologic features in the liver biopsy report were collected and required for inclusion of each case. Histopathologic features of interest included presence and severity of inflammation within the liver, necrosis or apoptosis, cirrhosis, cholangitis or cholangiohepatitis, intrahepatic bile cholestasis, interface hepatitis, and nodular regeneration. If a biopsy report did not contain information regarding severity of inflammation, the case was excluded. Breed information was not required for inclusion, but demographic features including age, sex, intact status, and breed were recorded for all cases where available. Exclusion criteria were cases with duplicate submissions in which case the duplicate was removed, severe tissue artifact or autolysis when mentioned in the histopathologic report, and insufficient sample quantity to perform histopathologic analysis or Cu quantification. A sample quantity of <10 mg was considered inadequate for Cu quantification. Information regarding biopsy technique and specimen quality was not consistently available in the submission or histopathologic report and therefore was not an exclusion criterion. Of 5587 cases, 740 were excluded due to unavailable age or sex information, 177 due to incomplete information regarding histopathologic features, 64 due to duplicate reports, 34 due to severe tissue autolysis or artifact, and 13 due to insufficient sample quantity, which left 4559 cases that met the screening criteria.

Histopathologic reports for each case were reviewed using a coding and scoring scheme developed from WSAVA criteria and definitions agreed upon by a board-certified veterinary histopathologist (Tawfik Aboellail) and 2 board-certified internists (Tarini Vedantham Ullal and David C. Twedt), all with an academic and clinical interest in hepatology. The histopathologist and both internists reviewed 20 randomly selected cases independently and then jointly to reach a consensus on the coding and scoring scheme to be applied. One of the board-certified internists (TVU) independently coded and scored all
Nodular regeneration was marked as present if the ridge or random forest feature importance criteria (randomForest R package v4.6.14), elastic net regression included ridge, lasso, and elastic net methods. Penalized regression coefficients were optimized using 10-fold cross-validation as implemented in the cv.glmnet function (glmnet R package v4.0.2). Penalized regression included ridge, lasso, and elastic net methods. The ridge-lasso tradeoff parameter for elastic net was optimized by minimizing the mean squared error using grid search at evenly spaced intervals from [0, 1] with a step size of 0.1. Penalized regression coefficients were optimized using 10-fold cross-validation as implemented in the “cv.glmnet” function (glmnet R package v4.0.2). Features were deprioritized from further analysis if they were selected against by stepwise selection, showed low feature importance scores by random forest, or had small or nonexistent weights by penalized regression.

2.2.3 | Multiple regression generalized linear modeling and machine learning

Once a set of optimal features was selected, a Gaussian generalized linear model on the log-scale was fit to the retrospective (1.1) and breed datasets (1.2). Selected features and their interactions were included in the models. Candidate models were then compared using the Akaike Information Criterion (AIC) with the selected model having the lowest and optimal AIC. Final regression model residuals were visually checked for compliance with assumptions of homoscedasticity and potential outliers were assessed using quantile-quantile and residuals vs leverage plots.
3 | RESULTS

3.1 | Copper distribution

Complete data except for breed information were available for 4559 cases. Due to missing demographic data, only cases from 2012 to 2020 had complete information. Copper concentrations ranged from 4.5 to 31500 ppm with a median of 391 (Q1-Q3: 225-763), mean 704 ± SD (SD) 1058. Copper distribution showed a right-skewed distribution with a high degree of overdispersion and a heavy right-sided tail. However, on the log-scale, the distribution was roughly Normal with a small amount of right skew. Of 4559 cases, 50% (2233/4559) had hepatic Cu > 400 ppm, 19% (861/4559) had hepatic Cu > 1000 ppm, 33% (1505/4559) had normal Cu (120-400 ppm), and 13% (595/4559) had copper below the normal reference range.

3.2 | Demographic and histologic features

The number of days between the first and last case submitted during the examined study period was 2715. Mean age in the study cohort was 8.1 ± SD 3.5 years. Sex composition consisted of 55% female (2499/4559), 45% were male (2060/4559) and 89% (4037/4559) were neutered or spayed. Breed information was available for 1490 cases of which there were 118 unique breeds. The 3 most abundant breeds were the Labrador retriever (206/1490, 14%), Chihuahua (68/1490, 4.6%), and Shih Tzu (52/1490, 3.5%). Of the dogs with Cu > 1000 ppm, most common breeds were Labrador retriever (65/861, 7.5%), Doberman pinscher (25/861, 2.9%), and West Highland white terrier (13/861, 1.5%). Specimen quality and size were not routinely reported, but all 4559 cases described portal areas, suggesting that there were adequate numbers of portal triads in all samples. Additionally, 3566/4599 reports evaluated sections from 2 or more lobes. Of 4559 cases, 63% (2854/4559) had hepatic inflammation scores characterized as mild in 45% (1292/2854), moderate in 36% (1039/2854), and severe in 18% (523/2854). Other common histopathologic features included necrosis or apoptosis observed in 30% (1378/4559), intrahepatic bile cholestasis in 20% (934/4559), and cholangitis or cholangiohepatitis in 16% (719/4559). Less commonly observed features were cirrhosis in 9% (402/4559), nodular regeneration in 2% (94/4559), and interface hepatitis in 1% (54/4559).

3.3 | Univariate analysis

In the univariate analysis, numerous features were significantly associated with hepatic Cu but to varying strengths (Table 1). Strength of

| Feature | Coefficient estimate | SE | P-value | % Deviance of data |
|---------|----------------------|----|---------|-------------------|
| Day     | 6.1                  | 0.01| .21     | 0.03              |
| Age (years) | -0.04                | 0.004| <.001* | 1.6               |
| Male/female | -0.004               | 0.03| .87     | 0                 |
| Spayed/neutered or intact | -0.10               | 0.04| .02*    | 0.1               |
| Breed | 0.66                  | 0.20| <.001* | 18                |
| Doberman | 0.54                  | 0.03| <.001* | 4.6               |
| Presence of inflammation | 0.94                  | 0.05| <.001* | 13                |
| Severity of inflammation | 0.31                  | 0.13| <.001* | 0.3               |
| Score 0.5 | 0.89                  | 0.04| <.001* | 1.0               |
| Score 1 | 0.89                  | 0.13| <.001* | 0.6               |
| Score 1.5 | 0.94                  | 0.05| <.001* | 2.5               |
| Score 2.0 | 0.94                  | 0.05| <.001* | 0.1               |
| Score 2.5 | 0.94                  | 0.05| <.001* | 0.1               |
| Score 3.0 | 0.94                  | 0.05| <.001* | 0.1               |
| Necrosis or Apoptosis | 0.57                  | 0.03| <.001* | 7                 |
| Intrahepatic Bile Cholestasis | 0.01                  | 0.03| .65     | 0.004             |
| Cholangitis or Cholangiohepatitis | 0.20                  | 0.04| <.001* | 6                 |
| Cirrhosis | 0.57                  | 0.05| <.001* | 2.5               |
| Nodular regeneration | 0.31                  | 0.08| <.001* | 0.3               |
| Interface hepatitis | 0.30                  | 0.13| <.02*  | 0.1               |

Note: This table shows the results of the univariate analyses evaluating the association of each demographic or histologic feature with hepatic copper (Cu) concentrations. For each demographic or histologic feature, coefficient estimate, SE of estimate, and percent deviance are listed. Coefficient estimates and SEs are given on the natural log scale. Significant P values <.05 are marked with an asterisk.
The association was assessed not only with significance of the $P$-value (<.05), but also percent deviance. Date of biopsy submission ($P = .21$), sex ($P = .87$) and intact status ($P = .02$) had minimally weak to no associations with hepatic Cu level, accounting for ≤0.1% of the deviance. Age in years was significantly negatively associated with hepatic Cu ($P < .001$, 1.6% of deviance). Breed accounted for 18% of the deviance and the Doberman pinscher was the breed most significantly correlated with abnormally high Cu ($P < .001$).

Presence of inflammation (4.6% of deviance), each increasing level of severity of inflammation (13% of deviance), necrosis or...

### Table 2: Retrospective Dataset 1.1 (4559 cases) Gaussian Generalized Linear Modeling (GLM) Results of Final Model

| Predictor                        | Coefficient estimate | SE  | $P$-value |
|----------------------------------|----------------------|-----|-----------|
| **Main Effects**                 |                      |     |           |
| Intercept                        | 5.77                 | 0.05| <.001*    |
| Years of age                     | −0.01                | 0.006| .02*      |
| Necrosis/apoptosis               | 0.34                 | 0.11| .003*     |
| Inflammation Severity 1 (mild)   | 0.40                 | 0.09| <.001*    |
| Inflammation Severity 2 (moderate)| 0.93               | 0.10| <.001*    |
| Inflammation Severity 3 (severe) | 1.00                 | 0.15| <.001*    |
| **Interaction Effects**          |                      |     |           |
| Years of age × Necrosis/apoptosis| −0.03                | 0.01| <.001*    |
| Years of age × Inflammation Severity 1 (mild) | −0.01          | 0.009| .19       |
| Years of age × Inflammation Severity 2 (moderate) | −0.04         | 0.01| .002*     |
| Years of age × Inflammation Severity 3 (severe) | −0.05         | 0.01| <.001*    |
| Necrosis/apoptosis × Inflammation Severity 1 (mild) | 0.13            | 0.10| .20       |
| Necrosis/apoptosis × Inflammation Severity 2 (moderate) | 0.28           | 0.10| .004*     |
| Necrosis/apoptosis × Inflammation Severity 3 (severe) | 0.24            | 0.12| .05       |

Note: Coefficient estimates, SEs, and $P$-values of the Generalized Linear Modeling on large dataset 1.1 for the best fitted model by AIC (Akaike Information Criterion) are shown. Coefficient estimates and standard errors are given on the natural log scale. Significant $P$-values <.05 are marked with an asterisk.

### Table 3: Breed Retrospective Dataset 1.2 (1490 cases) Gaussian Generalized Linear Modeling (GLM) Results of Final Model

| Predictor                      | Coefficient estimate | SE  | $P$-value |
|--------------------------------|----------------------|-----|-----------|
| Intercept                      | 5.57                 | 0.08| <.001*    |
| Years of age                   | 0.004                | 0.009| .66       |
| Necrosis/apoptosis             | 0.36                 | 0.16| .03*      |
| Inflammation Severity 1        | 0.46                 | 0.15| .002*     |
| Inflammation Severity 2        | 1.03                 | 0.18| <.001*    |
| Inflammation Severity 3        | 1.29                 | 0.24| <.001*    |
| Breed-Corgi                    | 0.52                 | 0.23| .03*      |
| Breed-Doberman                 | 0.69                 | 0.13| <.001*    |
| Breed-Dalmatian                | 0.70                 | 0.21| .001*     |
| Breed-Labrador retriever       | 0.28                 | 0.07| <.001*    |
| Breed-West Highland White terrier| 0.79              | 0.16| <.001*    |
| Breed-Cavalier King Charles spaniel | 0.75         | 0.29| .01*      |
| Breed-Bloodhound               | −3.63                | 0.86| <.001*    |

Note: Coefficient estimates, standard errors, and $P$-values of the Generalized Linear Modeling on the breed dataset 1.2 for the best fitted model by AIC (Akaike Information Criterion) are shown. Coefficient estimates and standard errors are given on the natural log scale. Significant $P$-values <.05 are marked with an asterisk.
apoptosis (7% of deviance), and cirrhosis (2.5% of deviance) were all significantly and strongly associated with abnormally high hepatic Cu level ($P < .001$; Figure 1A-C and Table 1). Other histopathologic features such as cholangitis or cholangiohepatitis ($P < .001$), nodular regeneration ($P < .001$), and interface hepatitis ($P < .02$), had significant, but weaker associations with Cu level (Table 1) accounting for only 0.1% to 0.6% of the deviance. Intrahepatic bile cholestasis showed no association with Cu level ($P = .65$; Table 1).

3.4 Feature selection

Feature selection revealed that severity of inflammation was the predominant predictor of hepatic Cu concentrations. Of the 1490 cases where breed information was available, a select subset of breeds (Doberman pinscher, corgi, Dalmatian, Labrador retriever, West Highland white terrier, Cavalier King Charles spaniel, and Great Dane) were the most predictive of abnormally high hepatic Cu. Following feature selection, the optimal predictors of hepatic Cu included age,
the subset of breeds previously mentioned, severity of inflammation, and necrosis or apoptosis. The least important features by penalized regression and random forest were sex (male/female), intact status, cholangitis or cholangiohepatitis, cirrhosis, nodular regeneration, intrahepatic bile cholestasis, and interface hepatitis.

3.5 Multiple linear regression model or Gaussian Generalized Linear Modeling (GLM)

Results of Gaussian Generalized Linear Modeling (GLM) showing associations with the optimal features identified by feature selection and hepatic Cu are in Table 2. Severity of inflammation was the most strongly predictive of Cu level with each level of severity (1 = mild, 2 = moderate, 3 = severe) increasingly associated with abnormally high Cu level (mild P < .001, moderate P < .001, and severe, P < .001). Necrosis or apoptosis was significantly associated with hepatic Cu (P = .003), particularly when inflammation was moderate (.004) or severe (P = .05). Years of age was significantly associated with decreased hepatic Cu level (P = .02), particularly when necrosis and apoptosis was present (P < .001) and when inflammation was moderate (P = .002) or severe (P < .001).

Amongst the breed retrospective dataset (1490 cases), severity of inflammation (mild: P = .002, moderate: P < .001, or severe: P < .001) and necrosis or apoptosis (P = .03) were also associated with abnormally high hepatic Cu (Table 3). Additionally, the following breeds had significant correlations with abnormally high hepatic Cu level: Doberman Pinscher (P < .001), Labrador retriever (P < .001), West Highland White Terrier (P < .001), Dalmatian (P = .001), Corgi (P = .03), and Cavalier King Charles spaniel (P = .01; Table 3). Bloodhounds had a strong correlation with decreased hepatic Cu level (P < .001).

4 DISCUSSION

This retrospective study examined hepatic Cu concentrations in a large dataset of dogs over the span of nearly 10 years and used machine learning approaches with regression analyses to evaluate associations with several demographic and histopathologic features. Abnormally high hepatic Cu concentrations were commonly found in this group of dogs. Fifty percent of dogs had hepatic Cu above 400 ppm and 20% above 1000 ppm. Age was negatively associated with hepatic Cu, but select breeds (Doberman pinscher, Labrador retriever, West Highland white terrier, Dalmatian, Corgi, Cavalier King Charles spaniel), presence and severity of inflammation in the liver, and necrosis or apoptosis were associated with abnormally high hepatic Cu. Results highlighted the abundance of abnormally high hepatic Cu in this cohort, identified 2 new breed associations, and supported that necroinflammation, not cholestasis or cirrhosis, is a strong histologic marker of abnormally high hepatic Cu and potentially CAH.

A relatively high proportion of dogs had hepatic Cu above the reference range (> 400 ppm) and >1000 ppm in this study cohort, consistent with reported findings. Frequency of hepatic Cu concentrations above 1000 ppm was reported in this study because necroinflammation is typically observed at levels >1000, although histologic changes can be observed between 600 and 1000 ppm and histologic changes might not be present at levels above 1000. Copper concentrations and the prevalence of CAH increased after 1930 and dramatically rose in the 1990's after regulations increased the bioavailability of copper content of commercial dog foods. However, Cu concentrations might have been inaccurately underestimated due to variable biopsy technique, specimen quality, and hepatic parenchymal remodeling. Examining multiple sections of the liver using digital image analysis of rhodanine-stained liver biopsy sections might have improved the accuracy of Cu quantification. Underestimates of hepatic Cu do not detract from the significant positive associations with abnormally high hepatic Cu identified in this study though they could have resulted in underestimations of the proportions of dogs with abnormally high hepatic Cu (> 400 or >1000 ppm). These proportions likely do not reflect a true incidence or prevalence of CAH because dogs were not definitively diagnosed with CAH and the study cohort consisted of dogs already suspected of hepatic disease. Dogs with abnormally high hepatic Cu can be subclinical and biochemically normal and such dogs are less likely to undergo liver biopsy procedures. Cases in this study cohort were also submitted to a single Diagnostic Veterinary Laboratory and therefore findings might not translate to other geographic regions or the larger global population. However, this study and several other studies demonstrate that abnormally high hepatic Cu is a common, pervasive problem in dogs. Therefore, high Cu in commercial dog foods and the connection to abnormally high hepatic Cu warrants reconsideration of the current regulations for dietary Cu.

In addition to dietary Cu, based on previously reported breed associations (Labrador retriever, Doberman pinscher, Bedlington terrier, West Highland white terrier, Dalmatian) and genetic mutations, genetic factors appear involved. Abnormally high hepatic Cu is much more common in predisposed breeds compared with nonpredisposed breeds and CAH can be heritable. The present study corroborated previously reported breed associations including the Labrador retriever, Doberman pinscher, West Highland white terrier, and Dalmatian. New associations were also found in the Corgi and Cavalier King Charles spaniel in this study. Two case reports of CAH have been published in a Pembroke Welsh Corgi and Cardigan Welsh Corgi but none in Cavaliers. Bedlington terriers were not represented in this dataset likely because of the rarity of this breed and genetic selection that bred out the COMMD1 mutation. A significant negative association with hepatic Cu was found in the Bloodhound breed. It should be considered that this breed might have genetic mutations and protective mechanisms that decrease hepatic Cu load or increase the possibility of Cu deficiency and have been implicated in Labrador retrievers and human patients with Wilson’s and Menkes disease.
Another key demographic finding in this study was that age was negatively associated with Cu, indicating that younger dogs had higher hepatic Cu. Since the mean age of the study cohort was 8 years, this finding was consistent with typical diagnoses of CAH, which occur between 4 and 8 years.² ³ ⁵ ⁸ ¹¹ ¹² A previous study found differing results in that Cu concentrations were significantly higher in older dogs, but in comparison, the dataset of this previous study was much smaller and the analysis was performed by grouping and comparing dogs ≥9 and < 9 years of age.⁶ Although hepatic Cu can progressively accumulate in dogs as they age,⁹ ⁴² ⁷⁴ in a subset of dogs and possibly in certain breeds such as the West Highland white terrier and Bedlington Terrier, Cu levels can plateau or reduce over time⁹ ⁶⁰ ⁷⁰ perhaps because they eventually adapt to high levels of Cu⁷⁰ which has been observed in rodent models.⁷⁵ ⁷⁶ Adaptive mechanisms to reduce hepatic Cu by increasing renal excretion and decreasing intestinal absorption of Cu have also been identified in human patients with Wilson’s disease.⁷² ⁷³ Alternatively, older dogs might develop fibrosis, cirrhosis or nodular areas in which Cu does not accumulate.⁴⁷ ⁴⁹ The negative relationship with age in this study was even more significant in the presence of necrosis, apoptosis, and moderate to severe hepatic inflammation, which further supported that hepatic necroinflammation was associated with abnormally high Cu.

It is well documented in humans²⁴ ⁷⁷ ⁷⁸ and animal models⁶⁶ ⁷⁹ ⁸¹ that intracellular hepatic Cu in its cupric form causes oxidative stress, mitochondrial damage, cell death, and subsequent inflammation. Similarly, dogs with excess hepatic Cu accumulation show signs of greater oxidative stress,³² ⁸² ⁸³ and varying degrees of necrosis or apoptosis and hepatitis.⁴¹ ¹¹ ⁷⁴ Additionally, dogs with hepatitis have significantly higher hepatic Cu.² ⁴ Accordingly, our study found that histologic evidence of hepatocyte cell death (necrosis or apoptosis) and hepatic inflammation of increasing severity were significantly associated with abnormally high hepatic Cu. However, positive associations do not imply causation or explain pathogenesis. Excess Cu might incite a necroinflammatory response (primary Cu accumulation) or conversely, necroinflammation disrupts Cu homeostasis and causes secondary Cu accumulation, defined as copper accumulation secondary to cholestatic and necroinflammatory disorders.¹² ²⁴-²⁷ The distribution of Cu and inflammation in the liver can help differentiate primary from secondary Cu accumulation because primary Cu tends to develop centrilobular initially while secondary Cu accumulates perportal around areas of inflammation.¹ A limitation of this study was that rhodanine staining was not performed on all histological samples but only samples requested by the submitting clinician. This compromised the ability to determine distribution of necroinflammation and correlation of copper location and staining intensity. The rhodanine staining data were considered inadequate to include in the machine learning model. Additionally, the clinical context for cases was lacking to diagnose CAH. Rhodanine stain results, inflammatory distribution, and clinical case details would have been useful to include in a model to predict CAH.

In humans with Wilson’s disease, primary hepatic Cu accumulation is attributed to mutations in the ATP7b copper transporter gene and epigenetic factors, but hepatic Cu can also be mildly increased in cases of viral hepatitis,⁸⁴ ⁸⁵ auto-immune hepatitis,⁸⁶ or alcoholic steatohepatitis,⁸⁷ and further elevated in auto-immune biliary diseases such as primary sclerosing and biliary cholangitis.²⁸ ²⁹ ³⁶ ³⁸ ³⁹ Cu excretion is disrupted in these biliary disorders particularly when there is bile duct injury, hepatic fibrosis, and lobular disarray.⁷⁷ ³⁰ ³¹ ³⁸ ³⁹ However, in dogs, it is unclear whether analogous disease processes lead to higher Cu burden. Dogs diagnosed with idiopathic chronic hepatitis,⁴⁸ ⁵⁰ or biliary diseases often have normal hepatic Cu.³² ⁴⁶ Although 1 study found decreased biliary excretion of Cu in Dobermans with copper associated hepatitis,⁵⁰ a separate study found that chronic extrahepatic bile duct obstruction did not increase hepatic Cu until dogs were administered supplemental Cu intravenously.⁵⁴ Parenchymal remodeling and cirrhosis can reportedly lower Cu measurements because Cu does not typically accumulate within regenerative nodules.⁴⁷ ⁴⁹ However, in a study of 161 dogs with histologically confirmed cirrhosis, 80% had hepatic Cu > 400 ppm and 40% had Cu measurements >1000 ppm, suggesting that cirrhosis does not dramatically lower Cu levels.²² Results in the present study showed that histologic features of interface hepatitis (a feature of chronic hepatitis),³⁵ cholangitis with or without hepatitis, intrahepatic bile cholestasis, nodular regeneration, and cirrhosis were not reliable predictors of hepatic Cu, which supports that these inflammatory, cholestatic, and fibrotic processes do not significantly increase Cu concentrations and that CAH can still be diagnosed in the presence of cirrhosis and nodular regeneration.

There were inherent limitations of this retrospective study such as lack of standardization in biopsy evaluations, unknown biopsy collection methodologies, and incomplete data. Although samples were submitted to a single laboratory, biopsies were read by 21 different histopathologists over the course of the study period. Each histopathologist had preferred descriptors for features, which introduced variability in the reports. Each report had to be reviewed manually to code each parameter, which was time-consuming, labor-intensive, and could have introduced data entry errors. All reports were reviewed by 3 authors (Tarini Vedantham Ullal, Nick Sbardellati and Brooke Gallagher) 2 to 3 times with a standardized coding and scoring system, but in the future, artificial intelligence tools might help analyze such large datasets more efficiently. Given the numerous histopathologists involved in biopsy interpretation, differences in histologic descriptions could have also introduced variability in the results. Although WSAVA guidelines for liver histology interpretation are published, it is unknown whether these criteria were referenced during biopsy evaluations. Additionally, guidelines were published in 2007 and were updated in 2021, after the conclusion of the study. Furthermore, the consensus on canine chronic hepatitis was published in 2019 and was not available for the majority of cases in this study. Due to the small number of cases diagnosed with cirrhosis or interface hepatitis in this study, we suspect some features were underreported. Ideally, a standardized approach and scoring system such as those used in humans⁹¹-⁹³ would have been used to assess and grade histologic features.⁹⁴ However, even when a scoring scheme is applied, evaluation of fibrosis, necrosis or apoptosis, and presence and severity of inflammation is subjective and variable between pathologists⁹⁵ and therefore, there is currently no universally accepted method to score necroinflammation in veterinary medicine. Features
such as cirrhosis and interface hepatitis can be underestimated in humans as well, which can affect the diagnosis of chronic hepatitis. However, this study evaluated associations of demographic and histopathologic features with hepatic Cu and did not require histopathologists and clinicians to accurately diagnose idiopathic chronic hepatitis or CAH. Thus, re-cutting slides for all 4559 cases to obtain standardized, blinded histopathologic evaluations and evaluate interobserver agreement between 1 and 2 histopathologists was not performed. Some bias could have been introduced because histopathologists were not blinded to clinical history, Cu quantification, or rhodamine staining results during biopsy review although this information was variably available. Treatment history was also inconsistently available for cases and therefore, dogs already being treated for CAH or other causes of chronic hepatitis could have been included in the dataset. For example, CAH dogs being treated with anti-inflammatory or immunomodulatory medications would have reduced hepatic inflammation without impacting Cu. However, inclusion of such cases would have biased the data towards not finding an association between hepatic Cu and necroinflammation.

Method of biopsy collection was not standardized in this study. Liver biopsy procedures were performed by many different veterinarians likely using different approaches (laparotomy, laparoscopy, needle biopsy, or necropsy). This variability could have impacted sample quality and consequently, Cu and histologic analyses. Optimally, wedge biopsies would have been collected to maximize diagnostic quality and specimen size. Furthermore, at least 2 lobes should have been sampled to account for interlobular variability and ideally, 3 lobes to diagnose chronic hepatitis. Although biopsy method, specimen size, and quality were not routinely mentioned, most reports were able to evaluate portal areas and described findings in at least 2 or more liver lobes, which assured some level of diagnostic quality. However, because the specific liver lobes biopsied in each case were not reported, the authors could not guarantee that copper quantification was performed on the same lobes evaluated and identified to have necroinflammation histopathologically.

Due to the retrospective nature of the study, 1028 of 5587 cases were excluded due to incomplete age, sex, or histologic data. Ultimately, only cases from the years 2012 to 2020 could be included, which reduced the dataset to 4559. Additionally, only cases from October 2017 onwards had available breed data because the electronic database was not capturing this information in earlier years. This reduced the breed dataset to 1490 cases. Furthermore, breed information was only available for pure-bred dogs. This prevented analyzing whether mixed breed dogs such as Labradoodles were at risk for abnormally high hepatic Cu. Even with these reductions in sample size, this is 1 of the largest retrospective studies investigating hepatic Cu levels in dogs to date, which enabled performing machine learning analyses and strengthened the statistical power of the results. P-values were reported on the log-scale, which made statistically significant results even more significant with respect to absolute values.

In human medicine, machine learning methods similar to those used in this study have been used to develop diagnostic algorithms for many hepatic diseases including nonalcoholic fatty liver disease, hepatocellular carcinoma, and even Wilson’s disease. Most studies have prioritized using noninvasive data (clinical, imaging, and/or multiomics data) instead of liver histology to develop these models. However, future prospective studies in dogs could utilize these machine learning methods on both clinical and histopathologic datasets to develop and validate artificial neural network algorithms similar to those developed for humans. Such algorithms could dramatically improve our ability to screen, predict, diagnose, phenotype, and treat CAH and other hepatic disorders.

5 | CONCLUSION

In conclusion, younger dogs and predisposed breeds such as the Labrador retriever, Doberman pinscher, and West Highland white terriers had abnormally high hepatic Cu. New breed associations were documented in the Corgi and Cavalier King Charles spaniel. Hepatic inflammation of increasing severity and necrosis or apoptosis were the strongest predictors of abnormally high hepatic Cu. Cholestasis, nodular regeneration, or cirrhosis were not significantly associated with hepatic Cu levels, suggesting these processes do not increase hepatic Cu and therefore might not affect the diagnosis of CAH.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

ETHICS STATEMENT

Authors declare human ethics approval was not needed for this study.

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REFERENCES

1. Webster CRL, Center SA, Cullen JM, et al. Acvim consensus statement on the diagnosis and treatment of chronic hepatitis in dogs. J Vet Intern Med. 2019;33:1173-1200.
2. Strickland JM, Buchweitz JP, Smedley RC, et al. Hepatic copper concentrations in 546 dogs (1982-2015). J Vet Intern Med. 2018;32:1943-1950.

3. Poldervaart JH, Favier RP, Penning LC, van den Ingh TSGAM, Rothuizen J. Primary hepatitis in dogs: a retrospective review (2002-2006). J Vet Intern Med. 2009;23:72-80.

4. Johnston AN, Center SA, McDonough SP, et al. Hepatic copper concentrations in labrador retrievers with and without chronic hepatitis: 72 cases (1980-2010). J Am Vet Med Assoc. 2013;242:372-380.

5. Hoffmann G, van den Ingh TS, Bode P, Rothuizen J. Copper-associated chronic hepatitis in labrador retrievers. J Vet Intern Med. 2006;20:856-861.

6. Hoffmann G. Copper-associated liver diseases. Vet Clin North Am Small Anim Pract. 2009;39:489-511.

7. Dirksen K, Fieten H. Canine copper-associated hepatitis. Vet Clin North Am Small Anim Pract. 2017;47:631-644.

8. Smedley R, Mullaney T, Rumbleia W. Copper-associated hepatitis in labrador retrievers. Vet Pathol. 2009;46:484-490.

9. Twedt DC, Stermlieb I, Gilbertson SR. Clinical, morphologic, and chemical studies on copper toxicity of bedlington terriers. J Am Vet Med Assoc. 1979;175:269-275.

10. Robertson HM, Studdert VP, Reuter RE. Inherited copper toxicity in bedlington terriers. Aust Vet J. 1983;60:235-238.

11. Hultgren BD, Stevens JB, Hardy RM. Inherited, chronic, progressive hepatic degeneration in bedlington terriers with increased liver copper concentrations: clinical and pathologic observations and comparison with other copper-associated liver diseases. Am J Vet Res. 1986;47:365-377.

12. Mandigers PJ, van den Ingh TS, Bode P, et al. Association between liver copper concentration and subclinical hepatitis in doberman pinchers. J Vet Intern Med. 2004;18:647-650.

13. Thomburg LP, Shaw D, Dolan M, et al. Hereditary copper toxicity in west highland white terriers. Vet Pathol. 1986;23:148-154.

14. Thomburg LP, Rotthaus G, Dennis G, Crawford S. The relationship between hepatic copper content and morphologic changes in the liver of west highland white terriers. Vet Pathol. 1996;33:656-661.

15. Webb CB, Twedt DC, Meyer DJ. Copper-associated liver disease in dalmatians: a review of 10 dogs (1998-2001). J Vet Intern Med. 2002;16:665-668.

16. van de Sluis B, Rothuizen J, Pearson PL, et al. Identification of a new copper metabolism gene by positional cloning in a purebred dog population. Hum Mol Genet. 2002;11:165-173.

17. Klomp AEM, van de Sluis B, Klomp LWJ, Wijmenga C. The ubiquitously expressed murr1 protein is absent in canine copper toxicity. J Hepatol. 2003;39:703-709.

18. Fedoseienko A, Bartuzi P, van de Sluis B. Functional understanding of the versatile protein copper metabolism murr1 domain 1 (commd1) in copper homeostasis. Ann N Y Acad Sci. 2014;1314:6-14.

19. Haywood S, Boursnell M, Loughran MJ, et al. Copper toxicity in non-commd1 bedlington terriers is associated with metal transport gene abca12. J Trace Elem Med Biol. 2016;35:83-89.

20. Fieten H, Gill Y, Martin AJ, et al. The menkes and Wilson disease genes counteract in copper toxicity in labrador retrievers: a new canine model for copper-metabolism disorders. Dis Model Mech. 2016;9:25-38.

21. Wu X, Mandigers PJ, Watson AL, van den Ingh TSGAM, Leegwater PAJ, Fieten H. Association of the canine atp7a and atp7b with hepatic copper accumulation in doberman dogs. J Vet Intern Med. 2019;33:1646-1652.

22. Center SA, Richter KP, Twedt DC, Wakshlag JJ, Watson PJ, Webster CRL. Is it time to reconsider current guidelines for copper content in commercial dog foods? J Am Vet Med Assoc. 2021;258:357-364.

23. Officials AoAFC. Official Publication. In. Association of American Feed Control Officials: Oxford, IN; 2015.

24. Hurwitz BM, Center SA, Randolph JF, et al. Presumed primary and secondary hepatic copper accumulation in cats. J Am Vet Med Assoc. 2014;244:68-77.

25. Sato C, Koyama H, Satoh H, Hayashi Y, Chiba T, Ohi R. Concentrations of copper and zinc in liver and serum samples in biliary atresia patients at different stages of traditional surgeries. Tohoku J Exp Med. 2005;207:271-277.

26. Takeshima H, Yagi A, Yano M, et al. Hepatic copper accumulation in patients with primary biliary cirrhosis. Nagoya J Med Sci. 1993;55:115-123.

27. Miyamra H, Nakamura Y, Kono N. Survey of copper granules in liver biopsy specimens from various liver abnormalities other than Wilson's disease and biliary diseases. Gastroenterol Jpn. 1988;23:633-638.

28. Elmes ME, Clarkson JP, Mahy NJ, Jasani B. Metallothionein and copper in liver disease with copper retention: a histopathological study. J Pathol. 1989;158:131-137.

29. Evans J, Newman S, Sherlock S. Liver copper levels in intrahepatic cholestasis of childhood. Gastroenterology. 1978;75:875-878.

30. Kowdley KV, Knox TA, Kaplan MM. Hepatic copper content is normal in early primary biliary cirrhosis and primary sclerosing cholangitis. Dig Dis Sci. 1994;39:2416-2420.

31. Gross JB Jr, Ludwig J, Wiensler RH, McCall JT, LaRusso NF. Abnormalities in tests of copper metabolism in primary sclerosing cholangitis. Gastroenterology. 1985;89:272-278.

32. Spee B, Arends B, van den Ingh TS, et al. Copper metabolism and oxidative stress in chronic inflammatory and cholestasis liver diseases in dogs. J Vet Intern Med. 2006;20:1085-1092.

33. Dirksen K, Burgener IA, Rothuizen J, et al. Sensitivity and specificity of plasma alp, alp, and bile acids for hepatitis in labrador retrievers. J Vet Intern Med. 2017;31:1017-1027.

34. Sokol RJ, Twedt D, McKim JM Jr, et al. Oxidant injury to hepatic mitochondria in patients with Wilson's disease and bedlington terriers with copper toxicity. Gastroenterology. 1994;107:1788-1798.

35. van den Ingh TSGAM, Cullen JM, Guy CM, Grinwise HF. Morphological classification of parenchymal disorders of the canine and feline liver. WSSAVA Standards for Clinical and Histological Diagnosis of Canine and Feline Liver Diseases. 2021.

36. Rifkin J, Miller MD. Copper-associated hepatitis in a pembroke welsh corgi. Can Vet J. 2014;55:573-576.

37. Fieten H, Dirksen K, van den Ingh TS, et al. D-penicillamine treatment of copper-associated hepatitis in labrador retrievers. Vet J. 2013;196:522-527.

38. Mandigers PJ, van den Ingh TS, Bode P, Rothuizen J. Improvement in liver pathology after 4 months of d-penicillamine in 5 doberman pinchers with subclinical hepatitis. J Vet Intern Med. 2005;19:40-43.

39. Allen KG, Twedt DC, Hunsaker HA. Tetramine cupruretine agents: a comparison in dogs. Am J Vet Res. 1987;48:28-30.

40. Brewer GJ, Dick RD, Schall W, et al. Use of zinc acetate to treat copper toxicosis in dogs. J Am Vet Med Assoc. 1992;201:564-568.

41. Fieten H, Biourge VC, Watson AL, Leegwater PAJ, van den Ingh TSGAM, Rothuizen J. Nutritional management of inherited copper-associated hepatitis in the labrador retriever. Vet J. 2014;199:429-433.

42. Fieten H, Biourge VC, Watson AL, Leegwater PAJ, van den Ingh TSGAM, Rothuizen J. Dietary management of labrador retrievers with subclinical hepatic copper accumulation. J Vet Intern Med. 2015;29:822-827.

43. Hoffmann G, Jones PG, Biourge V, et al. Dietary management of hepatic copper accumulation in labrador retrievers. J Vet Intern Med. 2009;23:957-963.
1. Appleman EH, Cianciolo R, Mosenco AS, Bounds ME, al-Ghazlat SA. Transient acquired fancer syndrome associated with copper storage hepatothapty in 3 dogs. J Vet Intern Med. 2006;22:1038-1042.
2. Miller AJ, Center SA, Randolph JF, Friesen CH, Miller AD, Warmer KW. Disparities in hepatic copper concentrations determined by atomic absorption spectroscopy, inductively coupled plasma mass spectrometry, and digital image analysis of rhodanine-stained sections in dogs. J Am Vet Med Assoc. 2021;258:395-406.
3. Center SA, McDonough SP, Bogdanovic L. Digital image analysis of rhodanine-stained liver biopsy specimens for calculation of hepatic copper concentrations in dogs. Am J Vet Res. 2013;74:1474-1480.
4. Kemp SD, Zimmerman KL, Panciera DL, Monroe WE, Leib MS, Lanz OI. A comparison of liver sampling techniques in dogs. J Vet Intern Med. 2015;29:51-57.
5. Johnston AN, Center SA, McDonough SP, Warner KW. Influence of biopsy specimen size, tissue fixation, and assay variation on copper, iron, and zinc concentrations in canine livers. Am J Vet Res. 2009;70:1502-1511.
6. Cole TL, Center SA, Flood SN, et al. Diagnostic comparison of needle and wedge biopsy specimens of the liver in dogs and cats. J Am Vet Med Assoc. 2002;220:1483-1490.
7. Schultheiss PC, Bedwell CL, Hamar DW, Fettman MJ. Canine liver iron, copper, and zinc concentrations and association with histologic lesions. J Vet Diagn Invest. 2002;14:396-402.
8. Cedeño Y, López-Alonso M, Miranda M. Hepatic concentrations of copper and other metals in dogs with and without chronic hepatitis. J Small Anim Pract. 2016;57:703-709.
9. Haywood S, Rutgers HC, Christian MK. Hepatitis and copper accumulation in skye terriers. Vet Pathol. 1988;25:408-414.
10. Azumi N. Copper and liver injury--experimental studies on the dogs with biliary obstruction and copper loading. Hokkaido Igaku Zasshi. 1982;57:331-349.
11. Venables WN, Ripley BD. Modern Applied Statistics with S. 4th ed. Springer New York; New York, NY: 2002.
12. Liaw A, Wiener M. Classification and regression by random forest. J Comput Graph Stat. 2002;11:83-106.
13. Venables WN, Ripley BD. Modern Applied Statistics with S. 4th ed. Springer New York: New York, NY; 2002.
14. Cole TL, Center SA, Flood SN, et al. Diagnostic comparison of needle and wedge biopsy specimens of the liver in dogs and cats. J Am Vet Med Assoc. 2002;220:1483-1490.
88. Ritland S, Steinnes E, Skrede S. Hepatic copper content, urinary copper excretion, and serum ceruloplasmin in liver disease. Scand J Gastroenterol. 1977;12:81-88.

89. Ullal T, Ambrosini Y, Rao S, Webster CRL, Twedt D. Retrospective evaluation of cyclosporine in the treatment of presumed idiopathic chronic hepatitis in dogs. J Vet Intern Med. 2019;33:2046-2056.

90. Mandigers PJ, Bode P, van Wees AM, et al. Hepatic (64)Cu excretion in dobermanns with subclinical hepatitis. Res Vet Sci. 2007;83:204-209.

91. Knodell RG, Ishak KG, Black WC, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology. 1981;1:431-435.

92. Ishak K, Baptista A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. J Hepatol. 1995;22:696-699.

93. European Association for Study of L. Easl clinical practice guidelines: Wilson’s disease. J Hepatol. 2012;56:671-685.

94. Vince AR, Hayes MA, Jefferson BJ, Stalker MJ. Hepatic injury correlates with apoptosis, regeneration, and nitric oxide synthase expression in canine chronic liver disease. Vet Pathol. 2014;51:932-945.

95. Lidbury JA, Rodrigues Hoffmann A, Ivanek R, et al. Interobserver agreement using histological scoring of the canine liver. J Vet Intern Med. 2017;31:778-783.

96. Regev A, Berho M, Jeffers LJ, et al. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. Am J Gastroenterol. 2002;97:2614-2618.

97. Balitz D, Shafizadeh N, Peters MG, Ferrell LD, Alshak N, Kakar S. Autoimmune hepatitis: review of histologic features included in the simplified criteria proposed by the international autoimmune hepatitis group and proposal for new histologic criteria. Mod Pathol. 2017;30:773-783.

98. Kemp SD, Zimmerman KL, Panciera DL, Monroe WE, Leib MS. Histopathologic variation between liver lobes in dogs. J Vet Intern Med. 2015;29:58-62.

99. Decharatanachart P, Chaliteerakij R, Tiyarattanachai T, Treerprasertsuk S. Application of artificial intelligence in chronic liver diseases: a systematic review and meta-analysis. BMC Gastroenterol. 2021;21:10.

100. Balsano C, Alisi A, Brunetto MR, et al. The application of artificial intelligence in hepatology: a systematic review. Dig Liver Dis. 2022;54:299-308.

101. Atabaki-Pasdar N, Ohlsson M, Viñuela A, et al. Predicting and elucidating the etiology of fatty liver disease: a machine learning modeling and validation study in the imi direct cohorts. PLoS Med. 2020;17:e1003149.

102. Mao B, Zhang L, Ning P, et al. Preoperative prediction for pathological grade of hepatocellular carcinoma via machine learning-based radiomics. Eur Radiol. 2020;30:6924-6932.

103. Medici V, Czlonkowska A, Litwin T, Giulivi C. Diagnosis of Wilson disease and its phenotypes by using artificial intelligence. Biomolecules. 2021;11:1-11.

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