Lower risk for liver copper accumulation in dogs fed 0.15- 0.24 mg copper/100 kcal vs ≥ 0.31 mg copper/100 kcal

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ABSTRACT

OBJECTIVE: Estimate tolerable dietary-copper (Cu) intake in senior dogs from a primary-care hospital population representing typical pet-dog demographics.

METHODS: Liver samples from 104 dogs euthanized for geriatric-health concerns were collected from 4/07/23-to-4/22/24. Medical records, dietary history, and regional water analyses were acquired. Dietary-Cu (manufacturers typical-analyses or inductively-coupled-plasma-atomic-emission-spectroscopy [ICP-AES, USDA-micronutrient-laboratory]) normalized as mg Cu/100 kcal (energy calculations used modified Atwater factorials). Liver samples underwent histologic evaluation (H&E, rhodanine), rhodanine Cu-scoring, and Cu-quantification (µg/g dry-weight-liver [dwl]). Categorical dietary-Cu intakes were 0.15-0.24 (Cu-restricted) versus 0.31-0.39 and ≥0.40 (Cu-replete) mg Cu/100 kcal. Non-parametric statistics defined significant differences and associations.

RESULTS: 51-male, 53-female, 33-breeds and 29-mixed-breeds had median (95% confidence interval) age, weight, and diet duration of 11.6 (10.6-12.1) years, 23.0 (20.1-25.4) kg, and 8.0 (7.0-8.7) years. No dog fed Cu-restricted diets displayed evidence of Cu-insufficiency. Water Cu concentration was 0.12 (0.08-0.17) ppm. Common histological features (glycogen-type-vacuolation [n=61], reactive-hepatitis [n=20], neoplasia [n=18]) did not associate with liver-Cu concentration. Thirteen Cu-restricted diet dogs had significantly lower liver-Cu than 91-dogs fed Cu-replete-diets. Liver-Cu in Cu-restricted-diet dogs did not exceed 355 µg Cu/g dwl nor stain with rhodanine. Among dogs consuming Cu-replete-diets were 35/91 (38%) with rhodanine positivity, 20/91 (22%) with liver-Cu ≥400µg Cu/g dwl (upper-reference-range) and 14/91 (15%) with liver-Cu ≥600µg Cu/g dwl.

CONCLUSIONS: Findings demonstrate significantly higher liver-Cu concentration with dietary intake ≥0.31 mg Cu/100 kcal vs 0.15-0.24 mg Cu/100 kcal and exonerate water as a critical Cu source.

CLINICAL RELEVANCE: Details inform a tolerable upper Cu-intake of 0.24 mg Cu/100 kcal in studied dogs.

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Copper (Cu) is an essential micronutrient that can adversely affect health if insufficient or excessive.[1-8] Chronic Cu intake exceeding an individual's capacity for neutral Cu balance leads to liver-Cu accumulation with risk for hepatocyte injury. Yet, supranutritional Cu-supplementation is now standard husbandry practice for production animals (e.g., chickens, turkeys, pigs, fish, sheep, cattle, and even deer) to hasten growth and reduce time to market.[2,3,8-12] Unfortunately, this practice has led to food chain and environmental Cu pollution, particularly escalating Cu concentrations in liver and kidney (but not muscle/meat tissue).[2,3,13-15] Similarly, in the commercial pet food industry, direct addition of premix supplements containing Cu to commercial canine diets is the most likely cause of escalating dietary-Cu exposure for dogs.[1]

Finding stainable-Cu in a liver biopsy is abnormal in healthy adult mammals. However, over the last decade, this has become a common finding in canine liver biopsies submitted to veterinary diagnostic centers (e.g., 55-60% of canine liver biopsies submitted to the Cornell University College of Veterinary Medicine Animal Health Diagnostic Center from 2015-2023).[1] Liver-Cu accumulation in dogs has progressively increased since commercialization of dog foods in the 1950s and regulatory recommendations for micronutrient premix supplements containing Cu.[1] Among 1,484 commercial dog foods sampled from 2017-2021 by the American Association of Feed Control Officials (AAFCO), median Cu content approximated 20-30 mg Cu/kg dry matter (DM), equating to ≥0.5-0.8 mg Cu/100 kcal assuming a diet energy density of 3,600-4,000 kcal/kg DM. Such dietary Cu-content reflects derivation from native ingredients as well as premix supplementation.[16] At this level, dietary-Cu intake exceeds minimum adult dog Cu-recommendations declared by AAFCO (0.18 mg Cu/100 kcal)[16,17] and the National Research Council (NRC, 0.15 mg Cu/100 kcal)[18] by ~3-to-5 fold. We suspect that such Cu-intake overwhelms capacity for neutral Cu-balance in a subset of dogs and is causal to copper-associated-hepatopathy (CuAH).[1]

Homeostatic Cu balance is regulated by numerous genes influencing enteric Cu-uptake, Cu-membrane transporters, intracellular and extracellular Cu-chaperones, and hepatocyte biliary canalicular Cu-egress. Complex interactions among these mechanisms influence dietary Cu-tolerance. Unfortunately, because non-invasive biomarkers are not available for diagnosis of CuAH in dogs, liver biopsy requiring general anesthesia is necessitated. The estimated cost of this procedure in veterinary specialty centers approximates $3,000-$8,000 based on a 2021 Veterinary Information Network, Chronic Hepatitis-Copper Hepatopathy Survey. Because of expense and procedural risks, many affected dogs likely remain undiagnosed.

Several factors contribute to the canine dietary Cu conundrum. First, we suspect that initial factorial estimation of minimal Cu-intake for adult non-reproductively active dogs, derived from studies of pregnant / lactating bitches and growing puppies that advised NRC and AAFCO regulatory recommendations, was too generous. Second, there is no declared safe maximum Cu-allowance for dogs in the United States guiding an actionable limit for commercial food. Third, in our experience, quantitative labelling declarations for dietary-Cu are uncommon and when provided describe Cu mg/kg product (as fed or dry matter). This circumstance limits veterinarian and pet-owning consumers from understanding dietary-Cu content and comparing products. Fourth, upon replacement of Cu oxide as the dominant food-grade Cu supplement in 1997 because of its low bioavailability, there was no modification of minimal Cu-allowances despite substitution with more highly bioavailable Cu-supplements (e.g., Cu sulfate, many other forms).[16,19] Last, we are not aware of canine specific studies investigating Cu-premix supplement bioavailability that might guide regulatory recommendations. Nevertheless, concern regarding the causal role of dietary-Cu in canine CuAH remains controversially polarized.[1,16] Understandably, officials of the Food & Drug Administration Center of Veterinary Medicine (FDA-CVM) are wary of modifying dietary-Cu guidelines based only on case-based data derived from veterinary specialists and specialty centers where liver biopsies reflect dogs with suspected

hepatobiliary disease. This population is distinct from demographics of 89.7 million pet dogs in the United States. [1,16,20] It is clear that elucidation of the role of dietary-Cu on liver-Cu accumulation requires further canine specific case-based investigation.[1,16]

The current study targets three objectives: 1) determine Cu-concentration in post-mortem end-of-life liver biopsies collected from humanely euthanized senior dogs (not targeting suspected liver disease) in a large Midwest primary-care veterinary practice, 2) investigate the relationship between dietary Cu-intake and liver-Cu concentration, and 3) estimate a safe tolerable Cu-intake in this population based on absence of rhodanine-stainable liver-Cu and liver-Cu concentration <400 µg Cu/g dry weight liver (dwl).

This study was undertaken to generate case-based evidence that might help advise regulatory actions.

Methods

Animals

Pet dogs (n=104) were euthanized for age-related health concerns except for 3 behaviorally unadoptable senior adult rescues euthanized for severe aggression. Postmortem liver samples were sequentially collected upon guardian consent during a 1-year period (4/07/23-to-4/22/24) at a large primary-care Midwest veterinary hospital (Dickman Road Veterinary Hospital, Battle Creek, MI).

Study Design

Prospective Data Collection

Animal Specific Details: Medical records were reviewed with pertinent information transcribed including breed, age, body weight [BW], and sex.

Dietary Copper: Dietary history derived by owner interview included brand-name(s), specific diet label(s), duration of feeding (owner-specified most consistently fed brands, food type, and flavor[s]) and

any additional foods (e.g., commercial or home-cooked toppers and/or treats) with amounts and frequency of feeding specified. Dietary information was prospectively collected before tissue evaluations (Cu- quantification, Cu-specific staining, histological evaluations). Dietary-Cu content (mg/kg dry matter [DM]) was normalized by expression as Cu mg/100 kcal (diet metabolizable energy determined using modified Atwater factorials). Dietary-Cu in commercial dog foods, dog food toppers, and treats was derived from manufacturer typical analyses. For foods where this information was not provided by manufacturers, C- concentration was determined using validated inductively coupled plasma atomic emission spectroscopy (ICP-AES; Robert W. Holley Center for Agriculture and Health, United States Department of Agriculture, Ithaca, NY). Food samples were homogenized then lyophilized for analysis, as described.[21] Copper content of human foods provided as treats or supplements (e.g., cheese, cottage cheese, vegetables, specified meats), and any home cooked rations were derived using the USDA's comprehensive source of food composition data.[22]

Liver Histopathology & Copper Analyses: Liver biopsies collected from 2-4 liver lobes were immediately fixed in 10% neutral-buffered formalin. Samples from an individual dog were combined for embedding as a single paraffin block to expedite histological evaluations. Sections (7 µM) were stained with hematoxylin and eosin (H&E) and rhodanine (Cu-specific stain). Histological features in all H&E-stained sections were independently assessed by 2 of the authors with expertise in hepatopathology (SAC, ADM). Histological features (spreadsheet enumerated) were scored as present or absent. If present the severity was subjectively qualified as mild, moderate, or severe for: glycogen-type hepatocyte vacuolation, micro- or macro-vesicular lipid vacuolation of hepatocytes, centrilobular hepatocyte or macrophage lipofuscin accumulation, eosinophil focal aggregates, extramedullary hematopoiesis, reactive hepatitis, non-suppurative hepatitis (any zone), suppurative hepatitis (any zone), copper-associated hepatitis, biliary hyperplasia, bile duct distention with mucinous debris, canalicular

cholestasis, nodular hyperplasia, and regenerative nodules. Histological features graded only as present or absent included cirrhosis, portovenous hypoperfusion, neoplasia, and ductal plate malformation. After independent histological interpretations, divergent findings were reconciled into a collaborative opinion.

Rhodanine-stained liver sections were assigned a Cu-score of 0-5, as previously described.[23,24] Digital Cu-analysis was completed for all rhodanine positive samples. Flame atomic absorption spectroscopy (FAAS) measured liver-Cu concentrations in specimens with rhodanine Cu-score ≤1 (Veterinary Diagnostic Laboratory, Fort Collins, CO). Five liver samples were analyzed in triplicate and 4 samples were additionally analyzed by ICP-AES to ensure repeatability of FAAS results (Veterinary Diagnostic Laboratory, Michigan State University, Lansing, MI). Liver-Cu concentrations are expressed as µg/g dry weight liver (dwl). The Cu-content of formalin used for specimen preservation was measured by ICP-AES to assess potential for tissue-Cu contamination.

Regional Water Copper Analyses: Copper analyses of 17 regional water samples collected from geolocations encompassing home vicinities of studied dogs were obtained from county water analyses. Representative samples were collected from free-flowing tap water, public water fountains, well water, regional ground water streams and lakes. Water-Cu concentrations were measured (Alliance Analytical Laboratories, Coopersville, MI) using ICP-AES with 3 replicates. Copper plumbing was denied by dog guardians.

Statistical analysis

Descriptive statistics are reported as median, (mean), 95% Confidence Interval [CI], and (range).

Dietary- Cu ingestion per kg body weight was determined from calculated diet ME using a formula for

senior dogs (1.4[70 x Body Weight(kg)$^{0.75}$]) and consumed diet Cu mg/100 kcal was determined using modified Atwater factorials. Associations between breed, age, BW, duration of diet consumption, dietary Cu concentration (mg/100 kcal), and ingested dietary-Cu mg/kg body weight vs liver-Cu concentrations, and between qualitative rhodanine scores (based on the zonal distribution and number of hepatocytes with stainable-Cu[23,24]) vs liver-Cu concentrations were examined using a Spearman rank correlation with associations ≥ 0.65 designated as strong, 0.45 to 0.64 designated as moderate, and < 0.45 designated as weak, with $P \leq 0.05$ designating significance.

Natural data breaks used as categorical diet groupings (low-risk [0.15-0.24 mg C/100 kcal] vs high-risk [0.31-00.39 and ≥0.40 mg Cu/100 kcal) were justified using R statistics segmented-breakpoint linear regression. Distribution of sex and breed among categorical diet groupings was examined using two-by-two tables and Fisher's exact test. Differences in liver-Cu accumulations between diet groups as mg Cu/100 kcal and as mg Cu/kg BW were examined using a Wilcoxon Rank Sum test; two-sided $P \leq 0.05$ determining significance. Number of dogs with liver-Cu concentration ≥400 µg/g dwl and ≥600 µg/g dwl were enumerated as these cut-points determine actionable clinical recommendations for dietary-Cu restriction or dietary-Cu restriction and d-penicillamine Cu-chelation, respectively.[1] The percentage of dogs consuming diets associated with rhodanine positivity was calculated to represent dogs intolerant to Cu-intake.

Statistical computations (Spearman correlations, Wilcoxon Rank Sum test, two-by-two tables, Fishers exact tests) were completed using Statistix (Version 10, Analytical Software, Tallahassee FL, www.statistix.com). Dot plot graphics were constructed using GraphPad Prism (Version 10.0.0 for Windows, GraphPad Software, Boston, MA, www.graphpad.com). Segmented-linear regression was completed using R Statistics (segmented.package), R Core Team (2024). R: A Language and Environment

for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Results

Animals

Dogs included 51-males, 53-females, and 33-breeds (13-Pit Bull-type dogs, 9-Labrador Retrievers, 5-Chihuahuas, 5-Rottweilers, 4-Golden Retrievers, 4-Shih Tzus, 3-Cocker Spaniels, 3-Miniature Dachshunds, 3-Pugs, 3-Yorkshire Terriers, 2-Pomeranians, and 1-each of Australian Cattle Dog, Bluetick Coonhound, Boxer, Catahoula Leopard Dog, Doberman Pinscher, French Bulldog, Foxhound, German Shepherd, Great Dane, Great Pyrenees, Havanese, Irish Wolfhound, Old English Sheepdog, Pekingese, Pembroke Welsh Corgi, Shar Pei, Siberian Husky, Springer Spaniel, Tibetan Mastiff, Toy Poodle, and Wheaten Terrier) with the remaining 29-dogs of mixed-breeding.

Median (mean) 95% CI (range) for age, BW, and diet duration were 11.6 (11.3) 95% CI 10.6-12.1 (1.6-18.0) years, 23.0 (22.7) 95% CI 20.1-25.4 (1.2-53.6) kg, and 8.0 (7.9) 95% CI 7.0-8.7 (0.7-17.0) years, respectively. There were no significant differences between these values in male versus female dogs. Only 22% (23/104) dogs were < 9 years old.

Liver Histopathology & Rhodanine Scoring

Common hepatic histological features included: glycogen-type vacuolation (n=61), centrilobular macrophage and/or hepatocyte lipofuscin accumulation (n=38), reactive-hepatitis (non-specific inflammatory infiltrates associated with systemic conditions but not consequential hepatic injury, n=20), and neoplasia (n=18); the entirety of classified histologic features and estimated severities are annotated in **Table 1**. Only a single dog, a Doberman Pinscher, had severe CuAH. One other mixed-breed

dog had centrilobular parenchymal collapse associated with Cu-accumulation but only minor inflammatory infiltrates. Ductal plate malformations were suspected in 2 dogs; these displayed characteristic histological features without clinicopathologic abnormalities reflecting a cholangiopathic condition. Rhodanine-staining was positive in 35/104 (34%) dogs, with all 35 dogs consuming ≥ 0.31 mg Cu/100 kcal diets (35/91 [38%]; **Table 2**). Qualitative rhodanine scores had significant strong correlation with measured liver-Cu concentrations (**Figure 1**). Formalin Cu-concentration was <2 µg/mL, discounting Cu- contamination of tissues during fixation.

Liver & Dietary Copper Concentrations & Dietary Copper Ingestion

Among all dogs, median (mean) 95% CI (range) liver-Cu concentration was 248 (318) 267-369 (70-1,795) µg/g dwl with dietary-Cu intake and duration of diet consumption of 0.41 (0.41) 0.39-0.44 (0.15-0.92) mg/100 kcal, 0.19 (0.20) 0.19-0.22 (0.07-0.53) mg Cu/kg BW, and 8 (7.9)7.0-8.7 (0.3-17.0) years, respectively. All but 2 dogs consumed AAFCO compliant commercial dog foods with Cu-concentrations provided by the manufacturer or measured by ICP-AES. Two dogs not eating commercial diets had been routinely fed tortillas, beans, tomatoes and meat scraps [hamburger or pork]) for 7 months. Dietary-Cu intake for 13-dogs consuming 0.15-0.24 mg Cu/100 kcal (hence forth designated as the low-risk Cu diet) was 0.22, (0.21), 0.20-0.23. (0.15-0.24) mg Cu/100 kcal and 0.10, (0.10), 0.09-0.11,(0.07-0.13) mg Cu/kg BW; for 27-dogs consuming 0.31-0.39 mg Cu/100 kcal was 0.34, (0.34), 0.33-0.36, (0.31-0.39) mg Cu/100 kcal and 0.16, (0.17), 0.16-0.19, (0.12-0.26) mg Cu/kg BW, and for 64-dogs consuming ≥ 0.40 mg Cu/100 kcal was 0.44, (0.48) ,0.45-0.51,(0.40-0.92) mg Cu/100 kcal and 0.22, (0.24), 0.22-0.26, (0.15-0.53) mg Cu/kg BW; **Figure 2.** Diets consumed by two dogs delivering 0.15 mg Cu/100 kcal were just within minimum NRC recommendation but below AAFCO guidelines.

Median (mean) 95% CI (range) liver-Cu concentrations for dogs consuming 0.15-0.24 mg Cu/100 kcal were 190, (191),132-250, (79-355) µg/g dwl, for dogs consuming 0.30-0.39 mg Cu/100 kcal were 277, (309),247-371,(129-777) µg/g dwl, and for dogs consuming ≥0.40 mg Cu/100 kcal were 252,(347), 270-424, (70-1,795) µg/g dwl. Six dogs with liver Cu <100 µg/g dwl (5 consuming the low-risk Cu-diet and 1 dog consuming 0.88 mg Cu/100 kcal) had no evidence of Cu-insufficiency (i.e., unexplained non-regenerative anemia, neutropenia, altered coat color or texture, or unexplained neuromuscular abnormalities).[25-27] Commercial diets delivering 0.21-0.24 mg Cu/100 kcal did not include Cu in a premix supplement (Voyager Wholesome Farm Chicken, Voyager Wholesome Ocean Pollack, [Voyager Dog Food Co. Battle Creek, MI]. Diets delivering 0.15 mg Cu/100 kcal were home-cooked. The dog with liver-Cu <100 µg/g dwl that consumed a 0.88 mg Cu/100 kcal diet was a male mixed-breed with severe pemphigus treated for 4-months with high-dose prednisone (2 mg/kg/day) and azathioprine. This dog had a severe diffuse degenerative glycogen-type vacuolar hepatopathy that might have artifactually reduced dwl Cu- concentration secondary to glycogen increased liver weight.[28]

Dogs consuming low-risk Cu-diets had significantly (P = 0.02) lower liver-Cu concentrations than dogs consuming higher Cu-diets (**Figure 2**). No dog fed low-risk Cu-diets had liver-Cu concentration >355 µg/g dwl. Among dogs with higher Cu-intakes (consistent with average Cu-content reported for commercial dog foods[16]) were 20/91 (22%) with liver-Cu concentration ≥400 and 14/91 (15%) with liver-Cu concentration ≥600 µg/g dwl. The Doberman Pinscher with severe CuAH consumed a diet containing 0.42 mg Cu/100 kcal, had rhodanine Cu-score of 5/5, and liver Cu concentration of 1,795 µg/g dwl. Statistical exclusion of this severely affected dog from the liver-Cu vs dietary-Cu comparisons, did not alter analytic significance. Dietary-Cu intake per kg BW was significantly lower (P < 0.0001) in dogs consuming the low-risk (0.15-0.24 mg Cu/100 kcal) diet compared to the other diet categories (**Figure 2**).

Dogs consuming the 0.31-0.39 Cu/100 kcal diet also had significantly lower ($P < 0.0001$) Cu intake per kg body weight compared to dogs consuming the ≥0.40 mg Cu/100 kcal diet.

Ground Water Cu Concentration

Median Cu-concentration for 17 regional water samples was 0.12 (95% CI 0.08-0.17) mg/L, below the EPA upper-tolerance level of 1.3 mg/L for humans.[29]

Discussion

Excessive dietary-Cu provoking liver-Cu accumulation is well-documented in humans, rodents, rabbits, cows, pigs, sheep, and now dogs.[2,3,8-12] Common use of supranutritional Cu-supplements in production animals impacts environmental pollution and food chain Cu-contamination that likely extends to pet food ingredients (especially liver derivatives).[3,11-15] Trepidation regarding liver-Cu accumulation in dogs chronically fed diets exceeding their individual Cu-tolerance is therefore reasonable. Genomic variability among individuals influences a myriad of interactions determining homeostatic Cu-balance and likely explains heterogeneity in dietary-Cu tolerance in dogs observed clinically and corroborated herein. Unfortunately, currently available genetic tests for estimating risk for Cu-associated liver injury have dubious clinical value for most breeds except for Bedlington Terriers (i.e., COMMD1 mutation).[1,30]

Dietary-Cu intake across the studied population was ingested for years. This important variable was embedded in the study design because chronic high Cu-intake can lead to gradual and insidious accrual of hepatocyte-Cu antecedent to clinical signs or histopathological evidence of liver injury.[1,31-33] Among dietary-Cu groups, only the low-risk 0.15-0.24 mg Cu/100 kcal diet can be deemed a safe tolerable Cu-intake for the population studied, based on declared tolerance criteria (i.e., absence of rhodanine-

stainable liver-Cu and liver-Cu concentrations <400 µg/g dwl). The low-risk Cu-diet had dietary-Cu derived from native ingredients without additional Cu-fortified premix supplements (i.e., Voyager Chicken or Pollock recipes, Battle Creek, MI; home prepared foods).

Accumulating case-based evidence (reported herein and a companion study) supports that Cu-intake for adult dogs ranging 0.10-0.25 mg Cu/100 kcal appears protective against liver-Cu accumulation.[35] Additional evidence reported herein incriminates risk for liver-Cu accumulation with consumption of AAFCO reported average/median dietary-Cu content in commercial dog foods (20-30 mg/kg DM (equivalent to ~ 0.55-0.88 mg Cu/100 kcal assuming energy density of 3600-4000 kcal/kg DM).[16,35] Case-based data also predict that an FDA-CVM / AAFCO considered "Cu-limited diet", defined as 15 mg Cu/kg DM and providing no greater than 0.375 mg Cu/100 kcal, also will not prevent liver-Cu accrual in some dogs.[16,35] Likewise, case-based data predicts that the European Union declared maximum Cu-limit of 28 mg/kg DM diet, established because of concerns regarding food animal and environmental Cu-contamination, also will not prevent liver-Cu accrual in some dogs (assuming diets provide 3600-4000 kcal/kg DM, a 28 mg/kg DM limit delivers between 0.70-0.77 mg Cu/100 kcal).[3,35]

Intriguingly, safe-tolerated Cu-intake demonstrated herein and a companion study, aligns with recent toxicologic Cu-limits estimated for humans in the United States and Europe (maximum Cu-intake of 0.04-0.07 mg Cu/kg BW/day), an amount acknowledged to not provoke liver-Cu accrual.[2,4,34] Assuming average energy intake of 2200 kcal/day for a 70 kg person, these Cu-intakes approximate 0.13 to 0.22 mg Cu/100 kcal, complementing our case-based findings.[2,4,35] Extrapolating these limits to dogs, calculated safe Cu-intake for a 10 kg and 30 kg adult dog would approximate 0.4-0.7 mg/day and 1.2-2.1 mg/day, respectively. Estimated resting energy consumption (required kcal) for an average senior dog is $1.4(70 \times \text{kg BW}^{0.75})$, acknowledging that optimal energy requirement varies among dogs, the human

threshold is easily exceeded by diets delivering > 0.24 mg Cu/100 kcal. The average daily metabolizable energy (1.4 [70 x kg $BW^{0.75}$]) for senior dogs weighing 10 kg and 30 kg (estimated metabolic body weights of 5.62 kg and 12.87 kg, respectively) would be 551 and 1261 kcal/day, respectively. Applying the upper safe human Cu-limit predicts ingestion of 0.10-0.23 mg Cu/100 kcal per day, within case-based predicted safe range herein and companion manuscript.[35] Safely tolerated Cu-limits in the current study also align with sufficient-tolerated Cu-intake in puppies (0.15-0.25 mg Cu/100 kcal).[19,25-27] While we caution, that case-based conclusions pertain to studied populations, accumulating coinciding data shows concordant tolerable ranges. Normalizing dietary-Cu intake against energy (mg Cu/100 or1000 kcal basis) as done herein, permits relevant comparisons among products that would assist veterinarians and pet caretakers in identifying a Cu-restricted diet if such were recommended.

It is important to dispel several myths regarding Cu and liver disease in dogs. First, dogs, unlike humans and cats, do not accumulate Cu secondary to cholangiopathic or cirrhotic liver disease.[1,36] Chronic cholangiopathic disorders (e.g., sclerosing cholangitis and chronic destructive lymphocytic cholangiohepatitis in humans and cats, respectively) provoking liver-Cu accumulation are exceedingly rare in dogs. Humans and cats with such disorders are chronically jaundiced and survive for years allowing gradual Cu- accrual secondary to disturbed biliary Cu-egress. Second, liver-Cu accumulation without biochemical or histological evidence of hepatocyte injury is not benign. Liver-Cu accumulation detrimentally increases risk for hepatocyte oxidative stress unbalancing redox homeostasis, leading to mitochondrial injury, and increasing vulnerability to other forms of liver injury.[1,37-39] Indeed, accumulated liver-Cu can augment injury provoked by other conditions (e.g., drug-induced liver disease, hypoxia [i.e., severe anemia, hypovolemia, reperfusion phenomenon], surgical or anesthetic complications, and sepsis).[1,33,38,39] Furthermore, hepatocyte-Cu initiated apoptosis (cuproptosis) is a well verified mechanism of cell death.[38,39]

Discovery of rhodanine-stainable Cu in a liver biopsy is abnormal in adult mammals.[1,40] Because this Cu-specific stain signifies lysosomal and protein-bound entrapped hepatocellular-Cu, positive staining implies an advanced stage of Cu-accumulation.[41-43] Considering that a safe tolerable dietary Cu-intake should not cause positive rhodanine-staining in a liver biopsy, we employed this criterion as evidence of tolerated dietary Cu-intake. The belief that stainable hepatocellular-Cu without concurrent evidence of cellular injury is innocuous implies unawareness of the malicious potential of this transition metal.[1,44-47]

Experimental models of dietary-Cu overload confirm that chronic ingestion of dietary-Cu exceeding an individual's tolerance leads to hepatocyte-Cu accumulation and simultaneous expression of inflammatory and injurious transcriptional elements preceding clinical, biochemical, or histological evidence of liver damage.[45] Inter-individual genomic variation influencing antioxidant defense systems likely also plays a complicating role. Wide variability in onset of hepatic injury in Wilson disease is recognized and thought to reflect epigenetic factors influencing regulation of hepatocyte lipid metabolism, mitochondrial resilience, and release of inflammatory cytokines.[1,46,47]

Liver samples in the present study were not sought, recruited, or pre-selected on clinical suspicion of hepatobiliary disease, in contrast to biopsies submitted through veterinary specialty or pathology centers. Rather, samples studied were sequentially collected from humanely euthanized dogs for terminal conditions or quality of life concerns after guardian approval. Nevertheless, 22% of dogs fed dietary-Cu consistent with average commercial dog foods as reported by AAFCO, had liver-Cu concentrations ≥400 µg/g dwl (a threshold where lifetime dietary Cu restriction is clinically considered), with 15% shown to have liver-Cu concentrations ≥600 µg/g dwl (a threshold where lifetime dietary-Cu restriction and d-penicillamine Cu-chelation are recommended).[1]

Although the population studied herein is epidemiologically small, findings are important considering the lack of longitudinal studies scrutinizing adequacy or toxicity of dietary-Cu intake in non-reproductively active adult dogs. There are four studies of juvenile dogs that explore dietary-Cu insufficiency, sufficiency, or excess.[19,25-27,48] That none of these include mature, non-reproductively active dogs is problematic because juvenile animals require greater Cu-intake compared to adults.[18,25,26,48] Experimentally induced Cu-insufficiency in developing puppies has detailed expected changes: coat color (greying), wiry coat texture, neuromuscular and bone manifestations, and hematologic abnormalities (anemia, neutropenia).[25-27] None of these features were recognized in dogs chronically fed the low-risk Cu-diets. One study in growing puppies demonstrated low bioavailability of Cu oxide compared to Cu sulfate, confirmed Cu-sufficiency at Cu sulfate intake similar to levels designated as low-risk (0.15-0.24 mg Cu/100 kcal) in the present study.[19] Considering that this intake level was sufficient for growing puppies, it is possible that an even lower intake might be sufficient in non-reproductively active adult dogs, consistent with findings in a companion manuscript.[35] Another study involving ~64 dogs (unspecified young age, 4-groups of 6-8 males and 6-8 females) explored tolerance and toxicity of daily Cu-intake at levels of 0.42, 2.1, and 8.4 mg Cu/kg BW fed for 1 year; Cu-gluconate supplemented baseline Cu intake.[48] As extrapolated by AAFCO experts, these values represent dietary Cu-intakes of 0.55-0.61, 2.8-3.1, and 11.2-12.4 mg Cu/100 kcal.[16] Unfortunately, the complete study manuscript is lost with only brief interpretive abridged summaries, without access to biochemical or histological findings. While Cu-accumulation was declared in liver, spleen, and kidney tissue, liver-Cu concentrations are unavailable. We believe the lack of study details strongly disqualifies reference to this study for adjudicating dietary-Cu limits for adult non-reproductively active dogs. A final study involving weaned puppies (2-groups, 5-each) describes the consequences of feeding a Cu-deficient (0.021 mg/100 kcal) and Cu-sufficient (0.25 mg/100 kcal) diet for 5 months.[27] Clinical features of Cu-insufficiency developed within several months with the 0.021 mg/100 kcal diet. Liver-Cu concentrations

(mean ± SD) at 6 months were 19 ± 4 µg/g dwl in Cu-deficient and 246 ± 48 µg/g dwl in Cu-sufficient puppies.[27] Thus, details from historical studies and findings reported herein and a companion manuscript support a safe upper tolerable Cu-intake in non-reproductively active adult dogs of 0.24 mg/100 kcal.[34] However, it is crucial to recognize that the biochemical Cu-formulations and additional dietary components may influence Cu-bioavailability and will impact subsequent nutritional guideline recommendations. Clearly, this topic is broad and complex in need of further case-based investigation.

Currently, veterinary recommendation for dietary-Cu restriction creates an unfortunate conundrum for dog caretakers attempting to research which diets comply with this recommendation (for reasons enumerated in the introduction). Adoption of dietary-Cu content labelling using Cu mg/100 kcal as a universal user-friendly metric for veterinarians and consumers would be an enormous improvement on the status quo, whether or not regulatory experts modify currently endorsed dietary-Cu thresholds.

As reported by AAFCO[16], Cu-content of commercial canine diets designed for all life stages displayed a broad range up to 140 mg Cu/kg DM with average/median concentrations approximating 20-30 mg Cu/kg DM, equivalent to 0.55-0.83 mg Cu/100 kcal assuming 3,600-4,000 kcal/kg diet DM. Considering historic puppy data, findings reported herein, and findings in a companion manuscript, this range of Cu-intake is too high for at-risk dogs.[35] As stated in the introduction, a safe-tolerable upper dietary-Cu limit for dogs should represent the highest daily intake likely to pose no adverse health risk.[49] We assert this to mean there should be no stainable-Cu in a liver biopsy nor hepatic-Cu concentration ≥400 µg/g dwl. Even the current liver-Cu reference limit is likely to be artifactually inflated from over-formulated micronutrient supplements, routine use of Cu-premixes in food production husbandry, and the spill over causing environmental contamination impacting food-chain Cu-exposure.[2,3,50] Indeed, we confirmed that 38% [35 of 91 dogs fed ≥ 0.31 mg Cu/100 kcal] developed stainable hepatic-Cu, thereby qualifying

for dietary-Cu intolerance. Thus, findings reported herein implicate a harmful impact of current common dietary-Cu concentrations in commercial dog foods (for some dogs). Fine tuning of a safe tolerable upper limit applicable to the canine pet population at large, requires further case-based studies, canine-specific investigation of Cu-bioavailability among premix formulations, inter-nutrient interactions (including dietary fats and phytates), and interplay among certain metals (e.g., Cu interactions with cadmium, calcium, molybdate, sulfur, zinc, iron and lead) in dietary formulations.[50] It is relevant also to point out that the daily intake of Cu for studied dogs was estimated from a calculated metabolizable energy requirement using a 1.4 variability quotient applied to resting energy requirement of 70(BW [kg]$^{0.75}$). That quotient is arbitrary and may not be appropriate for all senior dogs; some may have a decline in energy requirement with age due to inactivity and loss of lean body mass.

The focused objective of the present study was to document liver-Cu accumulation consequential to chronic consumption of commercial dog foods with and without premix Cu-supplements. We did not investigate the breadth of factors that might influence liver-Cu accumulation (i.e., multifaceted nutrient interactions and complexity of hepatocyte redox-balance relevant to nutritional components). It is important to bear in mind that the safe upper-tolerance threshold described herein applies to the studied senior pet dog population. Yet, intriguingly, this threshold reconciles with safe dietary Cu-intake derived from juvenile dog studies, findings reported in a companion publication to this manuscript, and thresholds set for human beings. An additional finding in the present study is the unlikely contribution of ground water as a critical source of Cu-intake in the studied population.

The demographics of the studied population more closely reflect the health and nutritional status of senior pet dogs across the United States than samples surveyed from specialty centers or pathology services. Considering that 52% of 59.8 million households with dogs in the United States have at least

one senior or geriatric dog[51], we extrapolate risk for liver Cu ≥400 µg/g dwl in ~6.8 million dogs (that could benefit from lifetime feeding of a Cu-restricted diet) and risk for liver Cu ≥600 ug/g dwl in ~4.7 million dogs (that would benefit from d-penicillamine Cu-chelation with concurrent lifetime dietary Cu-restriction).[1,31,33] Because the current study focused on senior dogs, risk for liver-Cu accumulation in younger dogs remains unexplored. We hope regulatory experts will review the case-based data provided by this study to help advise revision of Cu-limits for adult canine maintenance diets and consider adoption of the user-friendly informative Cu-labelling recommendations we suggest.

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Figure 1. Association between liver copper concentration (µg/g dry weight liver [dwl]) and qualitative copper positive rhodanine scores (1-5) in 35 of 104 dogs euthanatized between 4/07/23-4/22/24 for geriatric health concerns in a primary care veterinary practice; R^2= regression fit, red line depicts linear relationship.

635

636 **Figure 2.** Dot plot of liver copper concentrations (**A**) and estimated dietary copper intake per day (**B**) for
637 104 dogs (described in Figure 1) categorized by dietary groups (0.15-0.24 mg copper/100 kcal, 0.31-0.39
638 mg copper/100 kcal, and ≥ 0.40 mg copper/100 kcal); horizontal line = median, whiskers = 95%
639 confidence intervals.
640 NOTE TO EDITOR: provide jpgs of each did not mount as a single plate as we thought you would have
641 more ability to adjust this with A & B fixed to graphics. The title as loaded jpgs designates which is A
642 and which is B

643

644 Table 1. Enumeration of histological features with estimated severity if relevant, in 104 dogs evaluated

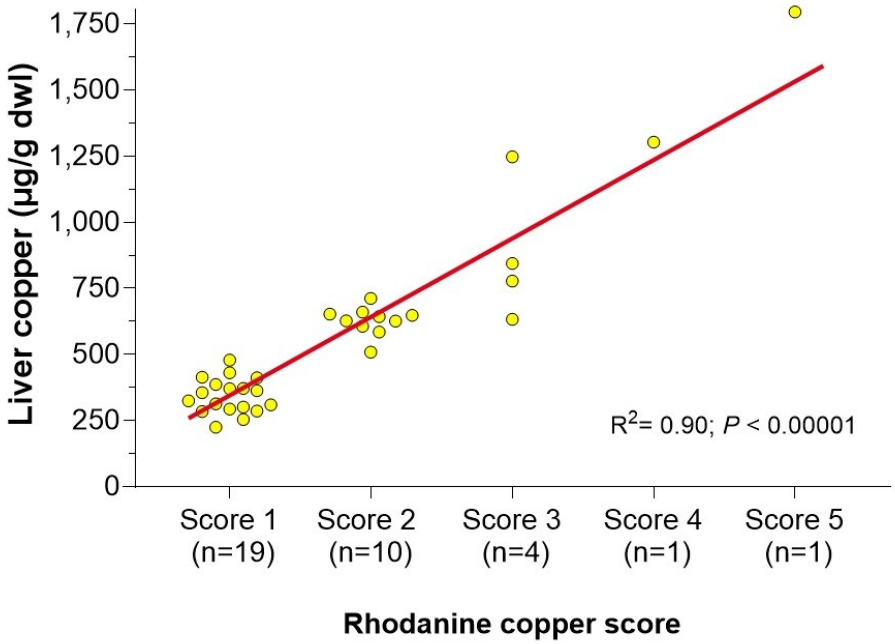
645 for dietary copper intake and liver copper concentration.

Histological Features	Present	Absent	Mild	Moderate	Severe
Glycogen-type vacuolation	61	43	35	20	6
Micro- or macro-vesicular hepatocyte lipid vacuolation	9	95	4	3	2
Centrilobular macrophage & hepatocyte lipofuscin	38	66	15	14	9
Reactive hepatitis	20	84	13	7	0
Eosinophilic focal aggregates	2	102	2	0	0
Extramedullary hematopoiesis	9	95	6	2	1
Non-suppurative hepatitis	7	97	4	2	1
Suppurative hepatitis	3	101	1	1	1
Copper-associated hepatitis	2	102	1	0	1
Biliary hyperplasia	9	95	6	2	1
Bile duct distention	6	98	4	2	0
Canalicular cholestasis	1	103	0	1	0
Nodular hyperplasia	6	98	6	0	0
Regenerative nodules	6	98	1	3	2
Cirrhosis	3	101			
Portovenous hypoperfusion	6	98			
Neoplasia (total of all categories)	18	86			
Lymphosarcoma	7	97			
Hepatocellular carcinoma, adenoma, or dysplastic foci	8	96			
Hemangiosarcoma	2	102			
Metastatic	1	103			
Ductal plate malformation	2	102			

646

Table 2. Median, mean, 95% confidence interval (CI) and range for liver copper concentrations (µg/g dry weight liver [dwl]) in 69 dogs without and 35 dogs with positive rhodanine staining in liver sections. Columns categorize cases based on assigned liver rhodanine scores.

	Liver rhodanine copper score					
	Score = 0	**Score = 1**	**Score = 2**	**Score = 3**	**Score = 4**	**Score = 5**
	Liver copper concentration µg/g dwl					
Dogs: n	69	19	10	4	1	1
Median	201	325	635	811	1,303	1,795
Mean	200	338	627	875	1,303	1,795
95% CI	182-218	305-371	589-665	458-1,294	na	na
Range	70-370	225-479	509-712	633-1,247	na	na



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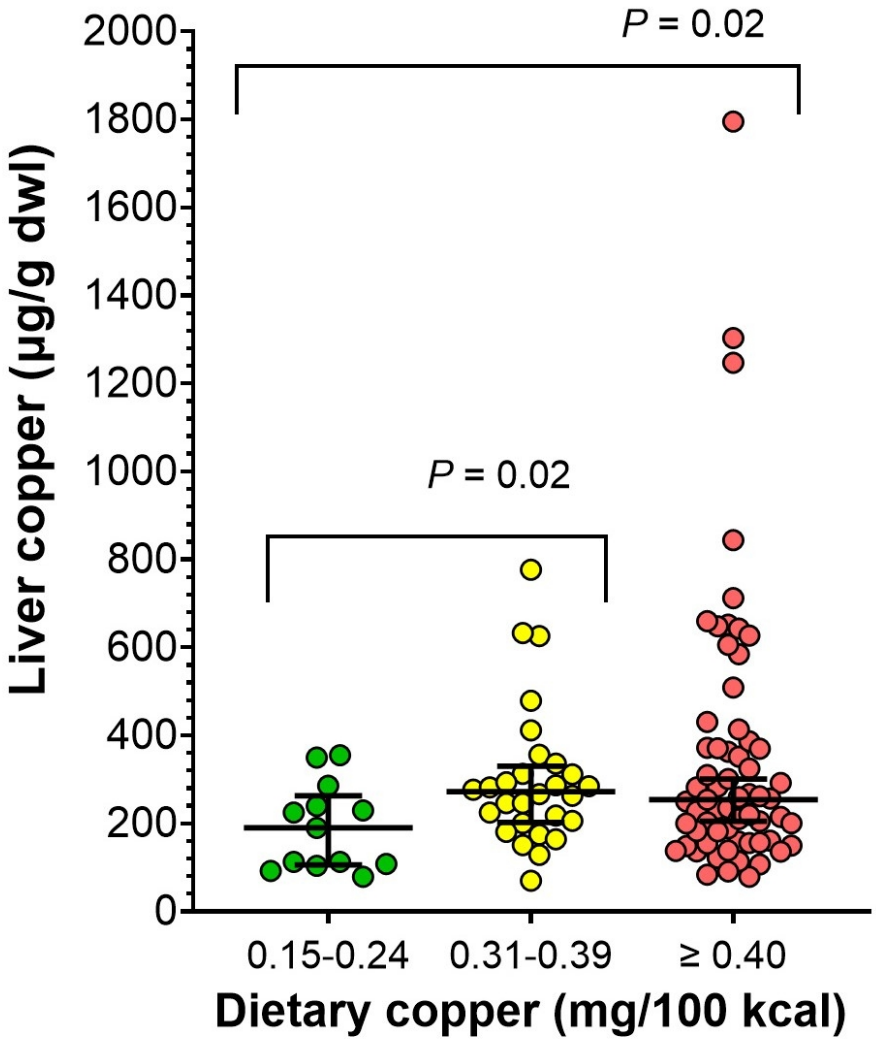

Figure 2. Dot plot of liver copper concentrations (A) and estimated dietary copper intake per day (B) for 104 dogs (described in Figure 1) categorized by dietary groups (0.15-0.24 mg copper/100 kcal, 0.31-0.39 mg copper/100 kcal, and > 0.40 mg copper/100 kcal); horizontal line = median, whiskers = 95% confidence intervals.

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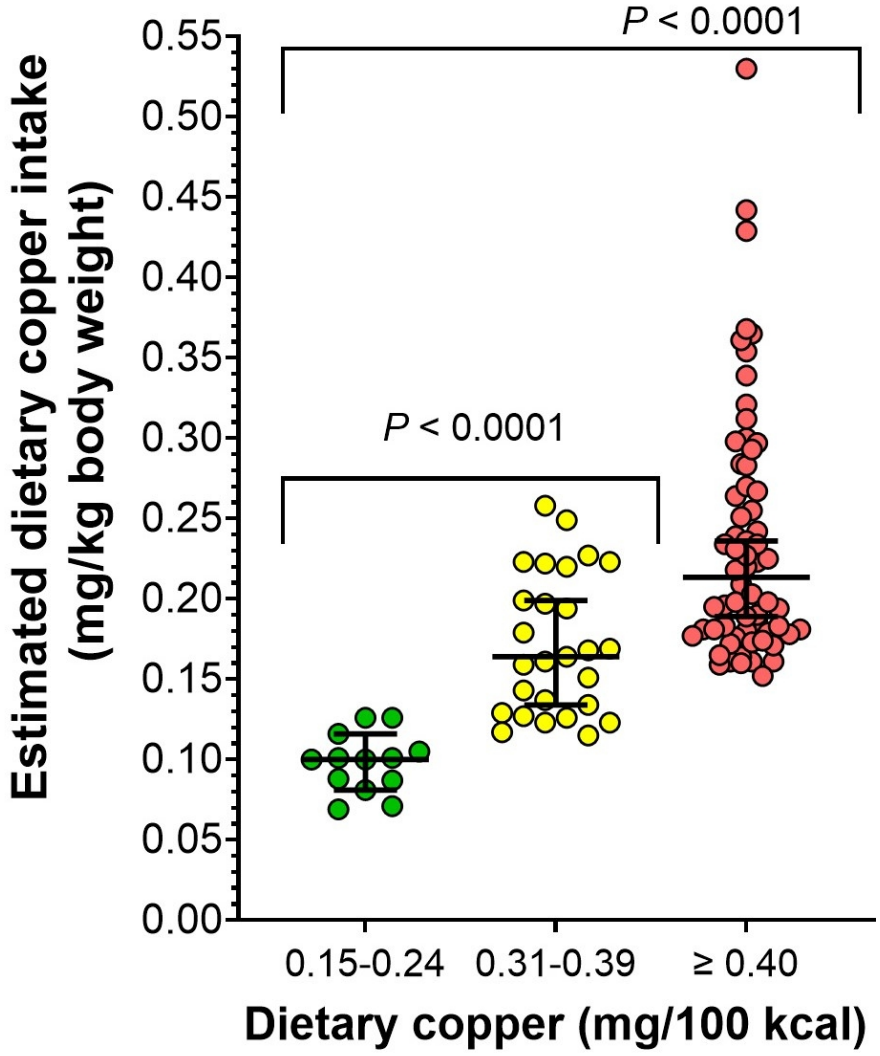

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