ASSESSMENT OF COPPER IN LIVER SPECIMENS FROM CATS

A Dissertation

by

PUNYAMANEE YAMKATE

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Chair of Committee,	Jörg M Steiner
Co-Chair of Committee,	Jonathan A Lidbury
Committee Members,	Jan S Suchodolski
	David C Twedt
	Panagiotis Xenoulis
Head of Department,	Jonathan M Levine

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ABSTRACT

Copper is an essential trace element that can produce reactive oxygen species (ROS). Excess ROS promote oxidative stress, which can lead to hepatic injury. Copper-associated hepatopathy is well-studied in humans and dogs. In contrast, there is limited information describing hepatic copper accumulation in cats. Our studies assessed copper in liver specimens from cats using qualitative (rhodanine staining) and quantitative assessment. We separated liver fractions and evaluated the intracellular distribution of copper in specimens from cats with copper concentrations below and above the upper limit of the reference interval (ULRI: 180 μg/g dry weight liver). To assess oxidative stress, we evaluated the immunohistochemical expression of 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) in the same liver specimens. Finally, to assess apoptosis, we evaluated the immunohistochemical expression of active caspase 3 (Casp-3) in the same specimens.

Hepatic copper concentrations above the ULRI were commonly found in cats regardless of histopathological changes of the liver. Liver specimens from Malaysia had higher copper concentrations compared to those from the United States and Greece. A weak correlation between the hepatic copper score and copper concentrations was found. The intracellular distribution of copper was highest in the cytosol, followed by the nuclear, large granule, and microsomal fractions, respectively in specimens with either copper below or above the ULRI. The immunohistochemical expression of 4-HNE, MDA, and Casp-3 was not significantly different in specimens from cats with liver diseases compared

to those without significant hepatic changes. There was no significant correlation between the hepatic copper score or copper concentrations and these markers.

Our findings suggested that the reference interval for hepatic copper in cats needs to be re-evaluated and should be established based on geographical location. Factors, such as environmental contamination, diet, and intracellular localization of hepatic copper, that may affect copper concentrations and copper staining should be investigated. Our findings of immunohistochemical expression of 4-HNE, MDA, and Casp-3 did not support the utility of these markers in cats with liver diseases. Further studies to assess other potential biomarkers of oxidative stress or apoptosis are warranted. Future studies are needed to better define the importance of hepatic copper in cats.

DEDICATION

I would like to dedicate this doctoral journey to my mother and father, Kunlawadee and Pathom Yamkate, who always have been the source of strength and inspiration, and who endlessly provide the love and support in every way I need.

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NOMENCLATURE

4-HNE	4-hydroxynonenal
ATP7A	ATPase copper transporting Alpha
ATP7B	ATPase copper transporting Beta
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology
CAH	Copper-associated hepatopathy
Casp-3	Caspase 3
CAT	Catalase
CCS	Copper chaperone for superoxide dismutase
COMMD1	Copper metabolism domain containing 1
CuO NPs	Copper oxide nanoparticles
FAAS	Flame atomic absorption spectroscopy
FFPE	Formalin-fixed paraffin-embedded
GSH	Glutathione
GSSG	Glutathione disulfide
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry
MDA	Malondialdehyde
PCH	Primary copper-associated hepatopathy
RI	Reference interval

ROS	Reactive oxygen species
SNVs	Single nucleotide variations
SOD1	Copper-zinc superoxide dismutase
TNF-α	Tumor necrosis factor-alpha
ULRI	Upper limit of the reference interval
WD	Wilson's disease
XIAP	X-link inhibitor of apoptosis

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1. INTRODUCTION

1.1. Copper

Copper is a trace element that acts as a cofactor for several enzymes, such as cytochrome C oxidase (CCO) and copper-zinc superoxide dismutase (SOD1), which are important for growth, development, and maintenance of all living cells.[1, 2] In mammals, dietary copper is mainly absorbed in the proximal small intestine.[3] Copper is taken up by enterocytes and enters the portal circulation. In portal blood, it is carried by proteins, peptides, and amino acids and is transported to the liver.[4] Copper also enters the kidneys, but in smaller amounts.[5, 6]

The liver plays a major role in copper metabolism and homeostasis. Copper is stored in hepatocytes, is delivered to other organs in protein-bound forms, and is eliminated via biliary excretion.[7] Several copper chaperones, enzymes, and proteins are involved in these processes (Figure 1.1).[8] Generally, copper is taken up into hepatocytes via copper transporter 1 (CTR1). Free copper in the cytosol compartment can be sequestered by small copper scavengers, such as metallothionein (MT) or glutathione (GSH). It can also be transported to other designated molecules with the help of copper chaperones. COX17 is a copper chaperone that carries copper to the enzyme cytochrome C oxidase (CCO) in the mitochondria. Copper chaperone for superoxide dismutase (CCS) is another chaperone that delivers copper to SOD1. Antioxidant 1 copper chaperone (ATOX1) carries copper to ATPase copper transporting Alpha and Beta (ATP7A and ATP7B) in the trans-Golgi network where copper is incorporated into

ceruloplasmin (CP) and is excreted into the plasma by ATP7B. ATP7A is ubiquitously expressed in several cell types. The main role of ATP7A is believed to be copper uptake in the intestines. ATP7A helps to transport copper at the basolateral membrane of the enterocytes to the portal circulation. Recently, ATP7A was found to play a role in hepatic copper mobilization in response to peripheral copper deficiency.[9] Under the condition of excess copper, ATP7B facilitates movement of copper into lysosomes and excretes excess copper via the biliary system. The copper metabolism domain containing 1 (COMMD1) is the copper transporter that interacts with ATP7B and facilitates copper excretion. Additionally, copper bound to CCS can also be distributed to X-link inhibitor of apoptosis (XIAP). This protein can inhibit the COMMD1 function by promoting its degradation, which leads to copper accumulation. When XIAP binds to the copper, it can result in self-degradation and a decrease of its ability to inhibit caspase 3, which possibly leads to apoptosis.[10]

Although copper is essential to living organisms, it is also a potentially toxic transition metal, capable of generating reactive oxygen species (ROS). Thus, tight regulation of copper homeostasis is crucial. The disruption of copper metabolism and homeostasis can lead to copper toxicity. Excess ROS produced from copper promote oxidative stress and consequently induce chronic hepatitis, which will eventually progress to hepatic fibrosis and liver failure.[11, 12]

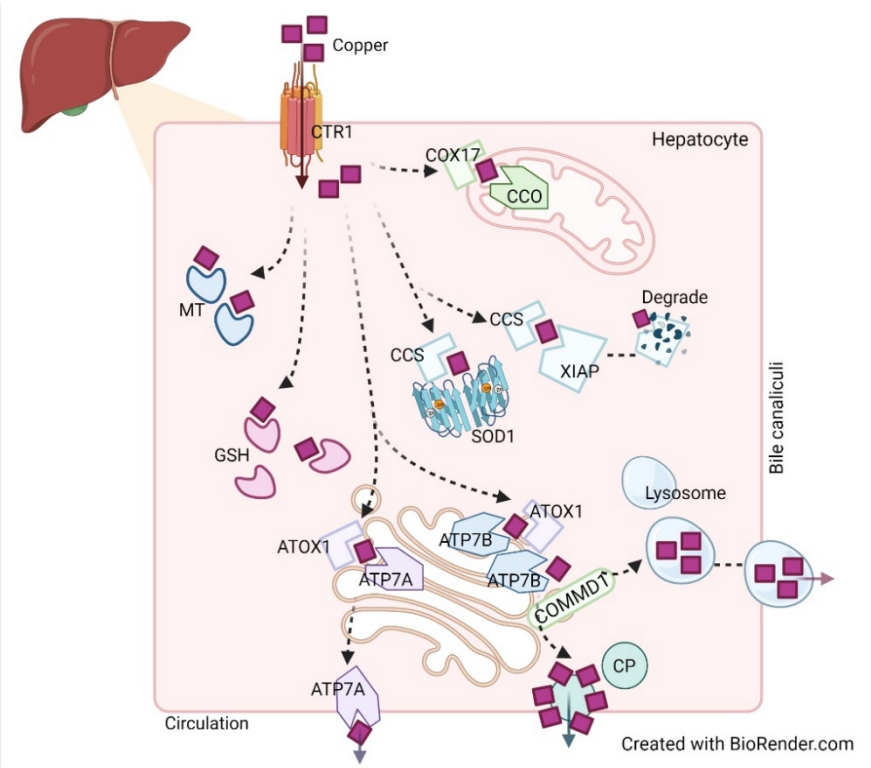

Figure 1.1 Copper metabolism in the liver (created with permission using BioRender)[13]
Copper enters hepatocytes via CTR1. From there, it can be sequestered by MT and/or GSH. Copper chaperones, such as COX17, CCS, ATOX1 help to deliver copper to copper transporters, including CCO, SOD1, ATP7A, and ATP7B. ATP7B delivers copper to CP, which, in turn, is secreted into the circulation. ATP7B also delivers copper to lysosomes and excretes excess copper via biliary excretion. COMMD1 facilitates copper excretion through the biliary system. Copper can be bound to XIAP, which leads to XIAP degradation and decreases its ability of caspase inhibition

1.2. Copper-associated hepatopathy

Liver disease caused by excessive copper accumulation is known as copper-associated hepatopathy (CAH). It is a well-studied and common liver disease in humans and dogs. Copper-associated hepatopathy can be primary, where either a genetic defect of the copper transporter gene leads to a disturbance in the hepatic copper elimination

pathway,[14, 15] or secondary, where the individual is exposed to an increased level of dietary copper. A combination of the two is also possible. In humans and rodent models, hepatic copper accumulation has been reported secondary to cholestasis.[16-19] However, studies in dogs suggest that clinically relevant copper accumulation secondary to cholestasis is not a common finding in this species.[20]

1.3. Copper-associated hepatopathy in humans

One form of CAH in humans is known as Wilson's disease (WD). This disease is an autosomal recessive trait. A gene mutation of the copper transporter *ATP7B* results in a reduction of biliary copper excretion and decreases the rate of copper incorporation into ceruloplasmin.[21] Excessive copper accumulates mainly in the liver and neuronal tissues. Wilson's disease can present as a hepatic or a neurologic form, or a mixture of both.[22] Copper can also accumulate in ocular tissue, leading to a typical circumferential corneal pigmentation, known as Kayser-Fleisher rings.[21, 23] Other copper metabolism disorders in humans include Menkes disease and non-Wilsonian disorders. Menkes disease results from a mutation of the copper transporter gene *ATP7A*. Of note, a mutation of ATP7A results in the opposite circumstance compared to WD in that the mutated copper transporter impairs intestinal copper uptake and leads to copper deficiency.[24] Non-Wilsonian disorders, such as Indian childhood cirrhosis, endemic Tyrolean infantile cirrhosis, and idiopathic copper toxicosis, are characterized by excessive copper accumulation similar to that in patients with WD.[25-27] Clinical signs of the hepatic form of these diseases have been recognized, but neurological forms have not

been reported. The causes of these non-Wilsonian disorders have not yet been fully identified. It has been suggested that genetic susceptibility and environmental factors, including diet, are involved. Furthermore, primary biliary cirrhosis and other cholestatic diseases such as biliary atresia, extrahepatic biliary obstruction, and cholangitis have been reported to lead to copper accumulation in the liver.[6, 16, 19]

1.4. Copper-associated hepatopathy in dogs

Primary CAH has been described in Bedlington Terriers and Labrador Retrievers.[14, 28] A mutation of the *COMMD1* gene has been reported in the Bedlington Terrier. This gene encodes the COMMD1 protein, which interacts with ATP7B protein and mediates biliary copper excretion.[29, 30] The deletion of exon 2 of the *COMMD1* gene shortens the length of COMMD1 protein from 188 amino acids to 94 amino acids.[31] Impairment of COMMD1 function results in excessive hepatic copper accumulation. In Labrador Retrievers, CAH is multifactorial. An inherited predisposition has been reported and is commonly observed in females.[28, 32] Associations between excessive copper accumulation and variants of copper transporting genes, *ATP7B* and *ATP7A,* have been reported in this breed.[33] A mutation of ATP7B causes failure of proper copper excretion and results in increased hepatic copper accumulation. However, a mutation of ATP7A may lead to impaired copper uptake in the intestines. This could attenuate hepatic copper accumulation in the Labrador Retriever. However, these mutations only explain 12% of the heritability of the CAH phenotype.[33] CAH has also been reported in other breeds with a suspected genetic background, including in the West Highland White

Terrier, Skye Terrier, Doberman Pinscher, and Dalmatian.[34-37] The findings in Doberman Pinschers suggest that increased hepatic copper concentrations in this breed may be related to an impaired copper transporter. Decreased expression of messenger ribonucleic acid (mRNA) encoding copper metabolism-related genes, including ATP7A and ATP7B proteins, was found in Doberman hepatitis.[38] Mutations of *ATP7A* and *ATP7B* have been identified in Dutch and North American Dobermans.[39] The researchers suggested that the *ATP7B* mutation was related to increased hepatic copper concentrations. However, the effect of *ATP7A* mutation on hepatic copper could not be determined in that study. Recently, several variants have been identified in genetic sequence of *COMMD1* in Labrador Retrievers and Doberman Pinschers. However, none of the variants were associated with increased hepatic copper concentrations.[40] Additionally, CAH can be found in various non-predisposed purebred dogs and mixed-breed dogs as well.[41, 42]

Diet has been suggested to play a crucial role in copper accumulation in dogs.[12] Guidelines for the copper content in commercial dog foods were made assuming the use of copper oxide. However, more recently, copper sulfate and copper chelates have been used in premixes, which have much higher bioavailability for copper; yet the recommended copper content was not altered. Studies in Labrador Retrievers reported that hepatic copper content is associated with dietary copper concentrations.[43-46]

As mentioned earlier, excessive copper accumulation can promote oxidative stress due to its ability to generate ROS. In turn, oxidative stress can induce hepatic inflammation, necrosis, and fibrosis.[11] Chronic hepatitis induced by copper can

eventually progress to hepatic cirrhosis and liver failure.[12] However, dogs with CAH can be in the subclinical phase for several years. Initially, copper accumulates in the liver without any overt clinical or histologic signs of hepatitis.[32, 47] The clinical signs usually develop when a large enough amount of liver parenchyma is affected. Clinical signs of CAH are nonspecific, including anorexia, lethargy, nausea, vomiting, and weight loss. Clinical signs of hepatic failure, such as ascites, icterus, and/or hepatic encephalopathy, develop when the disease progresses.[6] Routine serum biochemical analysis, such as assessment of liver enzymes is also nonspecific. The gold standard diagnostic is a histopathologic evaluation with copper concentration.[32] Copper accumulation in hepatic tissues can be assessed using copper staining, such as rhodanine or rubeanic acid. Copper accumulation resulting from an inherited defect typically appears in the centrilobular area of the liver.[14] A qualitative copper scoring system was established with a scale of 0 to 5 (Table 1.1). Copper scores of 2 or more are considered abnormal. Also, hepatic copper concentration can be measured by instrumental neutron activation analysis.[48] Copper concentration can also be quantified by spectroscopy methods such as flame atomic absorption spectroscopy or inductively coupled plasma emission spectrometry. Concentrations that fall in the range of 120–400 μg/g dry weight liver are considered normal.[49] Copper concentrations in dogs with primary CAH are usually above 800 μg/g and can reach 10,000 μg/g.[6]

Excessive copper accumulation in dogs can also be a consequence of other hepatobiliary diseases. For instance, cholestatic liver diseases reduce the ability of copper excretion through the biliary system and result in copper accumulation in the

periportal area.[14] Apart from differences in localization of hepatic copper accumulation, the magnitude of copper accumulation secondary to cholestasis is lower when compared with inherited CAH.[32] Dogs with extrahepatic cholestasis were reported to have no or limited copper accumulation based on rubeanic acid staining (copper scores 0–2).[20] Additionally, there were no significant changes in mRNA concentrations of ATP7A and COMMD1 proteins. However, a decrease of mRNA expression of ATP7B was found in this group of dogs and may cause a small increase in copper accumulation. These findings suggest that cholestasis may not be the main cause of copper accumulation in dogs.

Table 1.1 Scoring system for qualitative hepatic copper assessment in canine liver specimens

Score	Accumulation of copper granules in hepatocytes or macrophages
0	None
1	Solitary hepatocytes with some copper granules
2	Small group of hepatocytes with moderate amounts of copper granules
3	Large groups or areas of hepatocytes with moderate amounts of copper granules; macrophages with copper granules may be present
4	Large areas of hepatocytes with many copper granules; macrophages with copper granules usually present
5	Panlobular presence of hepatocytes with many copper granules, usually associated with copper-containing macrophages

1.5. Copper-associated hepatopathy in cats

CAH has not been well characterized in cats to date, with only a handful of studies and case reports describing cats with presumed primary copper-associated hepatopathy (PCH).[50-53] In the two case reports, copper accumulation was localized mainly in the centrilobular area, and these cases had extremely high hepatic copper concentrations (4074 and 4170 µg/g dry weight liver, respectively).[50, 51] The electron-dense aggregates, which are probably lysosomes, were identified in hepatocytes and macrophages in one of these cats during electron microscopic examination. This finding is similar to the one in the Bedlington Terrier with CAH.[51] One study of hepatic copper accumulation in cats found that the copper accumulation varied in localization in the liver. This study did not find an association between copper accumulation and histological diagnosis.[52] Another study suggested that copper accumulation in cats can be primary or secondary to other hepatobiliary diseases.[53] In that study, cats with presumed PCH had copper concentrations (>700 µg/g dry weight liver) higher than those in cats with other hepatobiliary diseases. Additionally, the localization of hepatic copper accumulation appeared to be similar to that in dogs. In PCH cats, copper accumulation was found in the centrilobular area, followed by panlobular deposition. In contrast, cats with cholestatic disorders showed copper accumulation in the periportal and intermediate zone.[53] Recently, a variation of the *ATP7B* gene has been identified in cats with presumed PCH.[54, 55] In a case report, a 9-month-old intact female cat was found to

have higher blood and urine copper concentrations compared to healthy cats. The cytoplasmic copper granules were observed with rhodanine staining. The cat responded to therapy with penicillamine and other symptomatic treatments. Genetic examination was performed with blood samples from the patient and a littermate that had been diagnosed with liver failure. A mutation of the *ATP7B* gene was reported and two single-nucleotide variations (SNVs) of this gene were identified. One of the SNVs was predicted to impair the function of the ATP7B protein.[54] A study of copper-transporting genes revealed these SNVs of *ATP7B* in three cats with suspected PCH. Mutations of the *COMMD1* gene were not identified in these cats. The researchers suggested that *ATP7B* variation is associated with PCH in cats and feline PCH is similar to Wilson's disease in humans. However, the researchers noted that there might be causes other than SNVs in ATP7B that may lead to PCH in some cases.[55] Further investigation is required in a larger number of cats with PCH to provide a better understanding of the pathogenesis of this disease.

1.6. Intracellular distribution of copper in the liver

As described earlier, excess hepatic copper can be toxic because of its ability to produce highly reactive hydroxyl radicals, which induce oxidative stress and can damage liver cells. Therefore, copper homeostasis in hepatocytes is tightly regulated to prevent copper toxicity. Copper is stored in hepatocytes and is distributed to other tissues when there is an excess of copper. Excess copper is also excreted through the biliary system. Copper transporters such as ATP7B and COMMD1 are responsible for the excretion of

excess copper. These copper transporters transfer copper to lysosomal compartments and excess copper is removed to the bile via lysosomal exocytosis.[12]

There are many studies regarding copper homeostasis and the pathophysiological pathways for excessive copper accumulation. Hepatic subcellular fractions can be obtained by the differential centrifugation technique. Differential centrifugation, which is also known as differential velocity centrifugation, is a technique for separation and crude purification of organelles and other subcellular particles based on their sedimentation rate, which depends upon their size.[56] Liver homogenates can be separated into four main compartments; the nuclear fraction, large granule fraction, microsomal fraction, and cytosol fraction.[56] The nuclear fraction contains not only the nuclei of hepatocytes, but also cytoskeleton, plasma membrane, and intact hepatocytes since the differential centrifugation only separates organelles crudely. The large granule fraction contains mitochondria, lysosomes, and peroxisomes. The microsomal fraction contains microsomes and other small vesicles. Lastly, the cytosolic fraction contains intracellular fluid (i.e., cytoplasm), ribosomes, large macromolecules, and other soluble proteins.[57]

Studies in normal control rats found that copper is mainly found in the cytosolic fraction under physiologic conditions.[58] However, in copper-loaded rats, copper concentrations are increased in the large granule fraction.[59] In healthy adult humans, the copper proportion is highest in the cytosol and lesser in the large granule fraction. In contrast, patients with WD have a high copper concentration in the large granule fraction.[60] A study in Bedlington Terriers with CAH found that copper is highly distributed in the fraction containing dense lysosomal granules in these dogs.[61] An

investigation of copper metabolism using electron microscopy reported that copper rapidly accumulated in lysosomes after copper infusion into the intestine of a Bedlington Terrier with CAH. Also, there was a failure of coper excretion through the bile in this dog, compared to a control Beagle.[62] These findings in rats, humans, and dogs suggest that copper, under physiological conditions, is mainly found in the cytosol. After copper loading, copper becomes distributed into the other compartments, especially the large granule fraction. However, no studies to date describe the intracellular distribution of copper in cat livers. An investigation of the intracellular distribution of hepatic copper in cats may help to understand the mechanisms of hepatic copper accumulation in this species.

1.7. Oxidative stress

Free radicals are molecules that have one or more unpaired electrons in their valance orbital. Free radicals are generated through several biological processes from both exogenous and endogenous sources. The liver is responsible for the metabolism, synthesis, storage, and redistribution of several essential nutrients.[63, 64] It is an important organ for free radical production due to its crucial role in metabolic homeostasis. Additionally, several enzymes in the liver can produce free radicals, including diamine oxidase, aldehyde dehydrogenase, tryptophan dual oxidase, liver dehydrogenase, and cytochrome p-450 enzyme system.[63] The most important groups of free radicals in the living system are the ones containing oxygen and nitrogen, known as ROS and reactive nitrogen species (RNS), respectively.[64] The most reactive free radicals are the oxygen-

based hydroxyl radicals and the nitrogen-based peroxynitrite anion.[65] Free radicals are necessary in normal physiological reactions, for example, a defense mechanism against pathogens in granulocytes. Free radicals induce intracellular signal transduction and regulate the expression of the gene, leading to inflammatory reactions against those pathogens.[63, 65] Excessive reactive free radicals can lead to the imbalance between free radicals and antioxidative agents, a situation known as oxidative stress, which can damage host cells and may result in cell death. Excessive free radicals can also react with macromolecules, such as DNA, lipids, or proteins. ROS can induce oxidation of DNA, which may lead to DNA strand break or mutation. In addition, it can react with lipid molecules, particularly polyunsaturated fatty acids. Peroxyl radicals, radicals generated from lipid peroxidation reaction, can alter lipid membrane structure, affecting membrane fluidity and function.[66] Peroxyl radicals can also form reactive aldehyde compounds such as malondialdehyde (MDA) and 4-Hydroxynonenal (4-HNE).[67, 68] These lipid peroxidation products are reactive compounds that are toxic to biological cells. Free radicals can attack several amino acids, such as cysteine, histidine, and methionine, and can cause impaired structure and function. Moreover, ROS can cause mitochondrial dysfunction. They can induce pro-apoptotic factors, alter the oxidative phosphorylation system, and cause mitochondrial DNA damage.[69, 70]

Oxidative stress can be assessed by the measurement of reactive free radicals, oxidative stress products, or antioxidants in biological samples. For example, unpaired electrons in free radicals can be quantified by electron paramagnetic resonance spectroscopy (EPR) with the spin trapping technique.[71] Products of lipid peroxidation,

such as MDA, 4-HNE, are also used as markers for oxidative stress.[72, 73] Glutathione and other enzymatic antioxidants, such as superoxide dismutase, and catalase (CAT), are also used to assess the activity of oxidative stress.[74]

Oxidative stress has been suggested to play a role in the pathogenesis and progression of several diseases, including hepatobiliary diseases in human and veterinary species. Examples of these include copper-associated liver disease, acetaminophen-induced hepatotoxicity, hepatic lipidosis, alcoholic liver disease, and non-alcoholic fatty liver disease.[20, 65, 75-78]

1.8. Oxidative stress and copper accumulation

Free radicals can be generated from free copper atoms. The cupric ion (Cu (II)) can be reduced to cuprous ion (Cu (I)) when superoxide anion radical or biological reductants, such as ascorbic acid or GSH, are present. The cuprous ion can then go on to catalyze hydrogen peroxide and form reactive hydroxyl radicals via the Fenton reaction as described below.[79]

$$Cu\ (II) + O_2^{-\bullet} \rightarrow Cu\ (I) + O_2$$

$$Cu\ (I) + H_2O_2 \rightarrow Cu\ (II) + {}^{\bullet}OH + OH^-$$

Hydroxyl radicals are highly reactive radicals that can damage biological macromolecules, such as DNA and lipids. ROS from excessive copper can break DNA strands and oxidize the bases of DNA. Copper can also promote lipid peroxidation. It is reported to be involved in the oxidation of low-density lipoprotein (LDL) and high-density lipoprotein (HDL).[80, 81] Lipid-derived radicals were identified in copper

challenged rats with vitamin E and selenium deficiency.[2, 82] Rats receiving copper sulfate ($CuSO_4$) were reported to have increased MDA concentrations and decreased GSH concentrations.[83] A study in mice showed that mice exposed to higher copper doses had increased amounts of ROS and protein carbonyls. The activities of glutathione and the mRNA expression levels of superoxide dismutase, CAT, and glutathione peroxidase were decreased.[84] In humans, lipid peroxidation products have been investigated in chronic liver disease. Patients with Wilson's disease had positive immunostaining of both HNE and MDA adducts in the liver.[85] In dogs with copper toxicosis, alterations in the expression of enzymatic and nonenzymatic antioxidants have been reported. Low gene expression of the enzymatic antioxidants, SOD1 and CAT, was reported in Bedlington Terriers with copper toxicosis. Moreover, GSH, a nonenzymatic enzyme, is mainly present in its reduced form and is converted to the oxidized form, glutathione disulfide (GSSG), during oxidative stress. GSH concentrations in dogs with copper toxicosis were decreased, while GSSG concentrations were increased.[20] A study in dogs with chronic hepatitis reported that there was no correlation between immunoreactivity scores of MDA and copper scores.[86] In addition, investigations of copper in ruminants suggest that copper is associated with oxidative stress. Liver tissue from cattle with high hepatic copper concentrations was shown to stain positive for 4-HNE via immunohistochemical analysis. Moreover, a similar pattern on rhodanine stain and 4-HNE immunostaining was identified.[87, 88] To our knowledge, there are a few studies of the immunohistochemical expression of oxidative stress markers in dogs with excessive copper accumulation. Additionally, there are no studies investigating the

immunohistochemical characterization of oxidative stress in cats with hepatic copper accumulation.

1.9. Apoptosis

Apoptosis is naturally-occurring programmed cell death, which is characterized by cytoplasmic shrinkage (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), and plasma membrane blebbing. The intact plasma membrane prevents the cell fragments from leakage and later results in apoptotic bodies, which are cleared by phagocytosis.[89, 90] Apoptosis is involved in physiological functions, such as growth development, immune surveillance, and damaged cell removal. However, it can also be involved in pathological processes, such as the development and progression of neoplasia.[91]

Apoptosis is also known as caspase-dependent cell death. Caspases, which are cysteine proteases, are the key enzymes that cleave proteins at sites adjacent to aspartic acid residues. Usually, they are present in the form of inactive pro-enzymes. Caspases can be divided into initiator caspases, such as caspase 2 and 9, or effector caspases, such as caspase 8 and 10.[92] Apoptosis can be stimulated by two fundamental pathways, extrinsic or intrinsic pathways (Figure 1.2).[90] The extrinsic pathway is initiated by death receptors located at the cell membrane. In the liver, there are three important death receptors, which are the Fas receptor, tumor necrosis factor-alpha (TNF-α) receptor, and TNF-related apoptosis ligand receptor. These three receptors can interact and recruit inactive caspase 8 or 10. The procaspases form homodimers and go through

autoproteolytic cleavage. The activation of caspase 8 or 10 follows the induced proximity model.[93] An active initiator caspase 8 or 10 can directly activate the effector caspase 3 or activate it through the mitochondrial pathway.[90] The direct activation of effector caspases is only found in Type 1 cells, such as thymocytes. Hepatocytes are Type 2 cells, in which caspase 3 is activated through the mitochondrial pathway.[94] The apoptotic mitochondrial pathway involves the activation of the B-cell lymphoma 2 (Bcl-2) protein family. In addition, the mitochondrial pathway can be activated by other factors independent from death receptors, known as the intrinsic pathway. Stimuli of the intrinsic pathway include DNA damages, oxidative stress, endoplasmic reticulum stress, metabolic stress, or hypoxic stress. These stimuli also affect the function of Bcl-2 proteins.[95]

Bcl-2 family proteins consist of pro-apoptotic and anti-apoptotic proteins. These proteins are classified into three groups based on the number of shared Bcl-2 homology (BH) domains. Pro-apoptotic proteins, Bax and Bak, contain three BH domains and are responsible for the initiation of mitochondria outer membrane permeabilization (MOMP).[93] These two proteins are expressed constitutively in the liver.[91] Anti-apoptotic proteins, Bcl-2, Bcl-xL, Bcl-w, A1, Mcl-1, and Boo, contain four BH domains. The last member of the Bcl-2 family is BH3-only proteins, consisting of the activators Bim, Bid, and Puma, and the sensitizers Bad, Bmf, and Noxa.[96] Normally, anti-apoptotic proteins bind to pro-apoptotic proteins to prevent apoptotic activity. When the Bcl-2 family proteins are activated through caspase 8, anti-apoptotic proteins are blocked and the pro-apoptotic proteins later form pores in the mitochondrial membrane. BH3 proteins are

located in the cytoplasm but can be translocated to the mitochondria when activated.[97] BH3 assists apoptosis by binding with the anti-apoptotic proteins and neutralizing their action. Active pro-apoptotic proteins increase mitochondrial permeabilization, which allows cytochrome C release into the cytosol.[91] Cytosolic cytochrome C assembles with the apoptotic protease activating factor 1 (APAF-1) and ATP to form a complex called apoptosome.[98] The apoptosome activates caspase 9 and active caspase 9 activates the effector caspases, such as caspase 3, 6, and 7, which lead to apoptosis.[91]

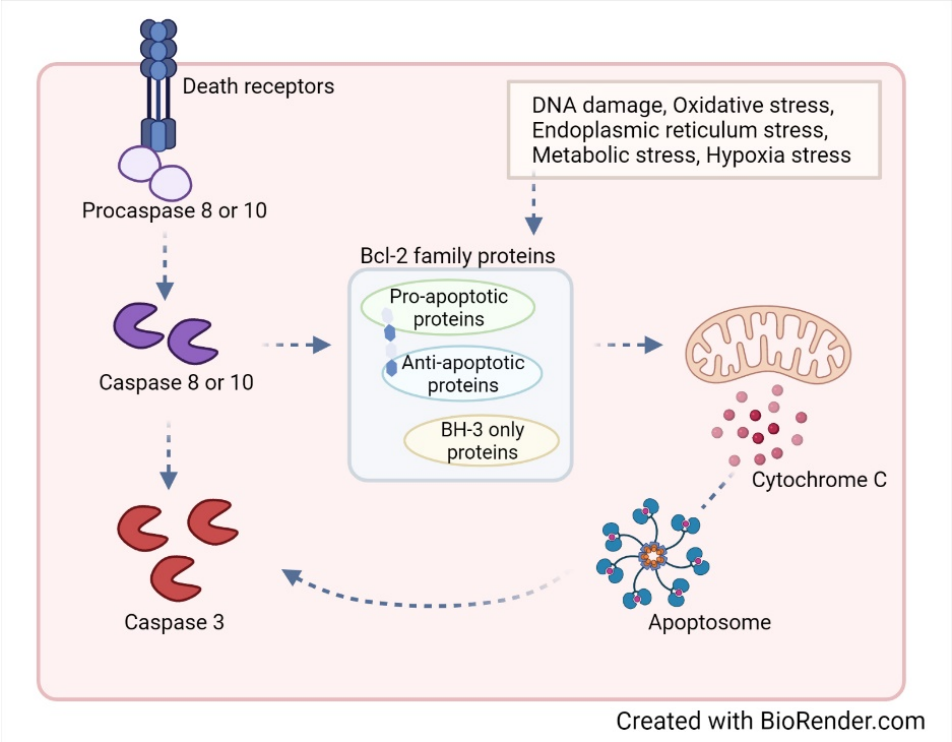

Figure 1.2 Extrinsic and intrinsic pathways of apoptosis (created with permission using BioRender)[13]
The extrinsic pathway or death receptor pathway interacts with procaspase 8 or 10. Procaspases in the homodimer form go through autoproteolytic cleavage and become active. The active caspase 8 or 10 can activate caspase 3 directly or through the mitochondria pathway. Bcl-2 family proteins are involved in this process. These proteins consist of pro-apoptotic, anti-apoptotic, and BH-3 only proteins. Normally, anti-apoptotic proteins bind to pro-apoptotic proteins to prevent apoptotic activity. BH-3 only proteins can bind to anti-apoptotic proteins and assist apoptotic activity. When anti-apoptotic proteins are neutralized, cytochrome C is released from mitochondria. Cytochrome C assembles with APAF-1 and ATP to form a complex called apoptosome. The apoptosome activates caspase 9 that consequently activates caspase 3. Intrinsic factors, including DNA damage, oxidative stress, endoplasmic reticulum stress, metabolic stress, or hypoxic stress activate apoptosis via the mitochondrial pathway

1.10. Apoptosis and copper accumulation

Excessive copper has been suggested to induce apoptosis linked with increased ROS and oxidative stress. In-vitro studies found that copper can induce apoptosis via an intrinsic pathway that involves mitochondrial permeabilization.[99, 100] A study of the mechanism of apoptosis in a rat pheochromocytoma cell line treated with copper identified DNA fragmentation and an increased expression of Bax and Bad proteins, cytochrome C, caspase 9, and caspase 3.[99] The researchers suggested that the intrinsic apoptotic pathway was induced by two mechanisms. Copper accumulation can induce ROS leading to DNA fragmentation. Tumor suppressor gene p53 is activated in response followed by activation of pro-apoptotic Bax protein. This is followed by cytochrome C release and in turn caspase 9 and 3 activation. An increase of Bad, one of the BH3-only proteins, decreases anti-apoptotic Bcl-2 function. It also increases the pro-apoptotic function of Bax, which increases apoptotic activity. However, the role of copper in the

activation of Bad was unknown in this study. A second proposed mechanism is an increase of ROS and Bax, which activates the release of apoptotic-inducing factor (AIF), which later activates caspase-independent apoptosis. A study in hepatocellular carcinoma cells with copper oxide nanoparticles (CuO NPs) also found increased p53 and caspase 3 gene expressions. Also, the ratio of Bax/Bcl-2 was increased, suggesting that the mitochondrial pathway was likely involved.[100] In-vivo studies also support the concept of hepatocellular apoptosis via the intrinsic pathway.[47, 101] Interestingly, the markers involved with the extrinsic apoptotic pathway were also identified.[84] A study in rats receiving CuO NPs and antioxidants showed that rats treated with CuO NPs had elevated liver enzymes and DNA fragmentation. The expression of TNF-α, Bax, and caspase 3 was increased, whereas the expression of Bcl-2 was decreased.[101] A study evaluating the livers from copper-treated mice found increased numbers of apoptotic cells. The investigators found that hepatic ROS levels were higher in mice treated with copper compared to control mice. Also, mRNA levels and protein expression of pro-apoptotic Bak and Bax proteins and cytochrome C were increased. Caspase 9 and caspase 3 expressions were also increased. In contrast, anti-apoptotic Bcl-2 and Bcl-xl expressions were decreased. Moreover, increased mRNA levels and protein expression of death receptors and caspase 8 were also identified, suggesting that the extrinsic pathway was also involved in copper-induced apoptosis.[84] A study in dogs with COMMD-1 deficiency reported an increase in caspase-3 immunoreactivity over time corresponding to copper accumulation as demonstrated histologically.[47] These findings supported the hypothesis that excessive hepatic copper accumulation in dogs can induce

apoptosis, which then results in hepatic injury. However, to date, there are no studies assessing apoptotic activity in the liver of cats.

1.11. Hypotheses and study objectives

The hypotheses of this study are:

1 Qualitative and quantitative copper assessment that have been used as the gold standard in a diagnosis of copper-associated hepatopathy in dogs will allow us to describe the presence of hepatic copper in archived liver specimens from cats and to identify differences in copper content between cats with and without liver disease.

2 Copper quantification of liver fractions will allow us to determine the distribution of copper at a subcellular level and to identify the difference in copper distribution between cats with hepatic copper concentrations below and above the upper limit of the reference interval.

3 Methods for immunostaining for markers of oxidative stress and apoptosis developed in other species can be successfully applied to liver tissue from cats with liver diseases, including those with high hepatic copper concentrations.

The objectives of this study are:

1 To assess hepatic copper content and zonal distribution in cats with various liver diseases and compare them to cats with no significant histopathological changes of the liver.

2 To assess the hepatic copper content in liver specimens from cats from three different geographical regions.

3 To assess the intracellular distribution of hepatic copper in cats with hepatic copper concentrations below and above the reference interval.

4 To assess oxidative stress and apoptosis in cats with various liver diseases through immunohistochemical analysis and compare it to cats with no significant histopathological changes of the liver.

1.12. References

1 Uauy R, Olivares M and Gonzalez M. **Essentiality of copper in humans.** *Am J Clin Nutr* 1998; 67: 952S-959S.

2 Gaetke LM and Chow CK. **Copper toxicity, oxidative stress, and antioxidant nutrients.** *Toxicology* 2003; 189: 147-163.

3 Cousins RJ. **Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin.** *Physiol Rev* 1985; 65: 238-309.

4 Fuentealba IC and Aburto EM. **Animal models of copper-associated liver disease.** *Comp Hepatol* 2003; 2: 1-12.

5 Linder MC and Hazegh-Azam M. **Copper biochemistry and molecular biology.** *Am J Clin Nutr* 1996; 63: 797S-811S.

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

7 Kim B-E, Nevitt T and Thiele DJ. **Mechanisms for copper acquisition, distribution and regulation.** *Nat Chem Biol* 2008; 4: 176-185.

8 Ferenci P, Zollner G and Trauner M. **Hepatic transport systems.** *J Gastroenterol Hepatol* 2002; 17: S105-S112.

9 Kim B-E, Turski ML, Nose Y, et al. **Cardiac copper deficiency activates a systemic signaling mechanism that communicates with the copper acquisition and storage organs.** *Cell Metab* 2010; 11: 353-363.

10 Mufti AR, Burstein E, Csomos RA, et al. **XIAP Is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders.** *Mol Cell* 2006; 21: 775-785.

11 Schultheiss PC, Bedwell CL, Hamar DW, et al. **Canine liver iron, copper, and zinc concentrations and association with histologic lesions.** *J Vet Diagn Invest* 2002; 14: 396-402.

12 Wu X, Leegwater P and Fieten H. **Canine models for copper homeostasis disorders.** *Int J Mol Sci* 2016; 17: 196.

13 BioRender.com. https://app.biorender.com (2021, accessed August 20, 2021).

14 Fieten H, Leegwater PA, Watson AL, et al. **Canine models of copper toxicosis for understanding mammalian copper metabolism.** *Mamm Genome* 2012; 23: 62-75.

15 Amano T, Matsubara T, Nishida T, et al. **Clinically diagnosed late-onset fulminant Wilson's disease without cirrhosis: A case report.** *World J Gastroenterol* 2018; 24: 290.

16 Goldfischer S, Popper H and Sternlieb I. **The significance of variations in the distribution of copper in liver disease.** *Am J Pathol* 1980; 99: 715.

17 Miyamura H, Nakanuma Y and 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.

18 Worwood M and Taylor D. **Subcellular distribution of copper in rat liver after biliary obstruction.** *Biochem Med* 1969; 3: 105-116.

19 Elmes ME, Clarkson JP, Mahy NJ, et al. **Metallothionein and copper in liver disease with copper retention—a histopathological study.** *J Pathol* 1989; 158: 131-137.

20 Spee B, Arends B, Van Den Ingh TS, et al. **Copper metabolism and oxidative stress in chronic inflammatory and cholestatic liver diseases in dogs.** *J Vet Intern Med* 2006; 20: 1085-1092.

21 Sternlieb I. **Perspectives on Wilson's disease.** *Hepatology* 1990; 12: 1234-1239.

22 Huster D. **Wilson disease.** *Best Pract Res Clin Gastroenterol* 2010; 24: 531-539.

23 Ferenci P. **Pathophysiology and clinical features of Wilson disease.** *Metab Brain Dis* 2004; 19: 229-239.

24 Kaler SG. **ATP7A-related copper transport diseases—emerging concepts and future trends.** *Nat Rev Neurol* 2011; 7: 15-29.

25 Tanner MS. **Role of copper in Indian childhood cirrhosis.** *Am J Clin Nutr* 1998; 67: 1074S-1081S.

26 Müller T, Feichtinger H, Berger H, et al. **Endemic Tyrolean infantile cirrhosis: an ecogenetic disorder.** *Lancet* 1996; 347: 877-880.

27 Scheinberg IH and Sternlieb I. **Wilson disease and idiopathic copper toxicosis.** *Am J Clin Nutr* 1996; 63: 842S-845S.

28 Hoffmann G, Van Den Ingh T, Bode P, et al. **Copper-associated chronic hepatitis in Labrador Retrievers.** *J Vet Intern Med* 2006; 20: 856-861.

29 De Bie P, van De Sluis B, Klomp L, et al. **The many faces of the copper metabolism protein MURR1/COMMD1.** *J Hered* 2005; 96: 803-811.

30 Tao TY, Liu F, Klomp L, et al. **The copper toxicosis gene product Murr1 directly interacts with the Wilson disease protein.** *J Biol Chem* 2003; 278: 41593-41596.

31 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.

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

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

34 Thornburg L, Shaw D, Dolan M, et al. **Hereditary copper toxicosis in West Highland white terriers.** *Vet Pathol* 1986; 23: 148-154.

35 Haywood S, Rutgers H and Christian M. **Hepatitis and copper accumulation in Skye terriers.** *Vet Pathol* 1988; 25: 408-414.

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

37 Mandigers P, Van Den Ingh T, Bode P, et al. **Association between liver copper concentration and subclinical hepatitis in Doberman Pinschers.** *J Vet Intern Med* 2004; 18: 647-650.

38 Spee B, Mandigers PJ, Arends B, et al. **Differential expression of copper-associated and oxidative stress related proteins in a new variant of copper toxicosis in Doberman pinschers.** *Comp Hepatol* 2005; 4: 3.

39 Wu X, Mandigers PJ, Watson AL, et al. **Association of the canine ATP7A and ATP7B with hepatic copper accumulation in Dobermann dogs.** *J Vet Intern Med* 2019; 33: 1646-1652.

40 Wu X, Mandigers PJ, Fieten H, et al. **Evaluation of COMMD1 in copper toxicosis in Labrador retrievers and Dobermans.** *Vet J* 2020; 265: 105561.

41 Thornburg L, Rottinghaus G, McGowan M, et al. **Hepatic copper concentrations in purebred and mixed-breed dogs.** *Vet Pathol* 1990; 27: 81-88.

42 Strickland JM, Buchweitz JP, Smedley RC, et al. **Hepatic copper concentrations in 546 dogs (1982-2015).** *J Vet Intern Med* 2018; 32: 1943-1950.

43 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.

44 Fieten H, Hooijer-Nouwens B, Biourge V, et al. **Association of dietary copper and zinc levels with hepatic copper and zinc concentration in Labrador Retrievers.** *J Vet Intern Med* 2012; 26: 1274-1280.

45 Hoffmann G, Jones P, Biourge V, et al. **Dietary management of hepatic copper accumulation in Labrador Retrievers.** *J Vet Intern Med* 2009; 23: 957-963.

46 Fieten H, Biourge VC, Watson AL, et al. **Dietary management of labrador retrievers with subclinical hepatic copper accumulation.** *J Vet Intern Med* 2015; 29: 822-827.

47 Favier RP, Spee B, Schotanus BA, et al. **COMMD1-deficient dogs accumulate copper in hepatocytes and provide a good model for chronic hepatitis and fibrosis.** *PLoS One* 2012; 7: e42158.

48 Bode P. **Instrumental neutron activation analysis in a routine way.** *J Trace Microprobe Teach* 1990; 8: 139-154.

49 Puls R. Mineral Levels in Animal Health: Diagnostic Data. Clearbrook, Canada: Sherpa International, 1988.

50 Haynes JS and Wade PR. **Hepatopathy associated with excessive hepatic copper in a Siamese cat.** *Vet Pathol* 1995; 32: 427-429.

51 Meertens NM, Bokhove CAM and Van Den Ingh TSGAM. **Copper-associated chronic hepatitis and cirrhosis in a European Shorthair cat.** *Vet Pathol* 2005; 42: 97-100.

52 Whittemore JC, Newkirk KM, Reel DM, et al. **Hepatic copper and iron accumulation and histologic findings in 104 feline liver biopsies.** *J Vet Diagn Invest* 2012; 24: 656-661.

53 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.

54 Asada H, Kojima M, Nagahara T, et al. **Hepatic copper accumulation in a young cat with familial variations in the ATP7B gene.** *J Vet Intern Med* 2019; 33: 874-878.

55 Asada H, Chambers JK, Kojima M, et al. **Variations in ATP7B in cats with primary copper-associated hepatopathy.** *J Feline Med Surg* 2020; 22: 753-759.

56 Beaufay H and Amar-Costesec A. **Cell fractionation techniques.** In: Korn ED (ed). Methods in Membrane Biology. Springer, 1976, pp 1-100.

57 López-Alonso M, Prieto F, Miranda M, et al. **Intracellular distribution of copper and zinc in the liver of copper-exposed cattle from northwest Spain.** *Vet J* 2005; 170: 332-338.

58 Smeyers-Verbeke J, May C, Drochmans P, et al. **The determination of Cu, Zn, and Mn in subcellular rat liver fractions.** *Anal Biochem* 1977; 83: 746-753.

59 Gregoriadis G and Sourkes TL. **Intracellular distribution of copper in the liver of the rat.** *Can J Biochem* 1967; 45: 1841-1851.

60 Porter H. **Tissue copper proteins in Wilson's disease: intracellular distribution and chromatographic fractionation.** *Arch Neurol* 1964; 11: 341-349.

61 Johnson GF, Morell AG, Stockert RJ, et al. **Hepatic lysosomal copper protein in dogs with an inherited copper toxicosis.** *Hepatology* 1981; 1: 243-248.

62 Kawamura M, Takahashi I and Kaneko J. **Ultrastructural and kinetic studies of copper metabolism in Bedlington Terrier dogs.** *Vet Pathol* 2002; 39: 747-750.

63 Zhu R, Wang Y, Zhang L, et al. **Oxidative stress and liver disease.** *Hepatol Res* 2012; 42: 741-749.

64 Arauz J, Ramos-Tovar E and Muriel P. **Redox state and methods to evaluate oxidative stress in liver damage: From bench to bedside.** *Ann Hepatol* 2016; 15: 160-173.

65 Cichoż-Lach H and Michalak A. **Oxidative stress as a crucial factor in liver diseases.** *World J Gastroenterol* 2014; 20: 8082.

66 Webb C and Twedt D. **Oxidative stress and liver disease.** *Vet Clin North Am Small Anim Pract* 2008; 38: 125-135.

67 Tuma DJ. **Role of malondialdehyde-acetaldehyde adducts in liver injury.** *Free Radic Biol Med* 2002; 32: 303-308.

68 Poli G, Biasi F and Leonarduzzi G. **4-Hydroxynonenal-protein adducts: A reliable biomarker of lipid oxidation in liver diseases.** *Mol Aspects Med* 2008; 29: 67-71.

69 Sinha K, Das J, Pal PB, et al. **Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis.** *Arch Toxicol* 2013; 87: 1157-1180.

70 Wang C-H, Wu S-B, Wu Y-T, et al. **Oxidative stress response elicited by mitochondrial dysfunction: implication in the pathophysiology of aging.** *Exp Biol Med (Maywood)* 2013; 238: 450-460.

71 McMichael MA. **Oxidative stress, antioxidants, and assessment of oxidative stress in dogs and cats.** *J Am Vet Med Assoc* 2007; 231: 714-720.

72 Yesilova Z, Yaman H, Oktenli C, et al. **Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic fatty liver disease.** *Am J Gastroenterol* 2005; 100: 850-855.

73 Seki S, Kitada T, Yamada T, et al. **In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases.** *J Hepatol* 2002; 37: 56-62.

74 Osman H, Gabr O, Lotfy S, et al. **Serum levels of bcl-2 and cellular oxidative stress in patients with viral hepatitis.** *Indian J Med Microbiol* 2007; 25: 323.

75 Savides M, Oehme F, Nash S, et al. **The toxicity and biotransformation of single doses of acetaminophen in dogs and cats.** *Toxicol Appl Pharmacol* 1984; 74: 26-34.

76 Aronson LR and Drobatz K. **Acetaminophen toxicosis in 17 cats.** *J Vet Emerg Crit Care* 1996; 6: 65-69.

77 Valtolina C and Favier RP. **Feline hepatic lipidosis.** *Vet Clin North Am Small Anim Pract* 2017; 47: 683-702.

78 Verbrugghe A and Bakovic M. **Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis.** *Nutrients* 2013; 5: 2811-2835.

79 Jomova K and Valko M. **Advances in metal-induced oxidative stress and human disease.** *Toxicology* 2011; 283: 65-87.

80 Witting PK, Bowry VW and Stocker R. **Inverse deuterium kinetic isotope effect for peroxidation in human low-density lipoprotein (LDL): a simple test for tocopherol-mediated peroxidation of LDL lipids.** *FEBS Lett* 1995; 375: 45-49.

81 Hillstrom RJ, Yacapin-Ammons AK and Lynch SM. **Vitamin C inhibits lipid oxidation in human HDL.** *J Nutr* 2003; 133: 3047-3051.

82 Kadiiska MB, Hanna PM, Jordan SJ, et al. **Electron spin resonance evidence for free radical generation in copper-treated vitamin E-and selenium-deficient rats: in vivo spin-trapping investigation.** *Mol Pharmacol* 1993; 44: 222-227.

83 Kumar V, Kalita J, Bora HK, et al. **Relationship of antioxidant and oxidative stress markers in different organs following copper toxicity in a rat model.** *Toxicol Appl Pharmacol* 2016; 293: 37-43.

84 Liu H, Guo H, Jian Z, et al. **Copper induces oxidative stress and apoptosis in the mouse liver.** *Oxid Med Cell Longev* 2020; 2020.

85 Paradis V, Kollinger M, Fabre M, et al. **In situ detection of lipid peroxidation by-products in chronic liver diseases.** *Hepatology* 1997; 26: 135-142.

86 Vince A, Hayes M, Jefferson B, et al. **Hepatic injury correlates with apoptosis, regeneration, and nitric oxide synthase expression in canine chronic liver disease.** *Vet Pathol* 2014; 51: 932-945.

87 Lyman D, Clark L and Campbell K. **Copper accumulation in Wisconsin Holsteins with indications of oxidative liver damage.** *J Vet Med Res* 2015; 2: 1021.

88 Strickland JM, Lyman D, Sordillo LM, et al. **Effects of super nutritional hepatic copper accumulation on hepatocyte health and oxidative stress in dairy cows.** *Vet Med Int* 2019; 2019.

89 Kerr JF, Wyllie AH and Currie AR. **Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics.** *Br J Cancer* 1972; 26: 239-257.

90 Reed JC. **Mechanisms of apoptosis.** *Am J Pathol* 2000; 157: 1415-1430.

91 Webster CRL. **Hepatocyte Apoptosis.** In: Arias IM (ed). The Liver: Biology and Pathobiology. 5th ed.: Wiley-Blackwell, 2009, pp 783-802.

92 Lawen A. **Apoptosis—an introduction.** *Bioessays* 2003; 25: 888-896.

93 Malhi H and Gores GJ. **Hepatocyte Apoptosis: Mechanisms and Relevance in Liver Diseases.** In: Arias IM, Alter HJ, Boyer JL, et al. (eds). The Liver: Biology and Pathobiology. 6th ed.: Wiley-Blackwell, 2020, pp 195-205.

94 Özören N and El-Deiry WS. **Defining characteristics of Types I and II apoptotic cells in response to TRAIL.** *Neoplasia (New York, NY)* 2002; 4: 551-557.

95 Zhong H-H, Hu S-J, Yu B, et al. **Apoptosis in the aging liver.** *Oncotarget* 2017; 8: 102640-102652.

96 Baskin-Bey E and Gores GJ. **Death by association: BH3 domain-only proteins and liver injury.** *Am J Physiol Gastrointest Liver Physiol* 2005; 289: G987-G990.

97 Malhi H and Gores GJ. **Cellular and molecular mechanisms of liver injury.** *Gastroenterology* 2008; 134: 1641-1654.

98 Hengartner MO. **The biochemistry of apoptosis.** *Nature* 2000; 407: 770-776.

99 Kawakami M, Inagawa R, Hosokawa T, et al. **Mechanism of apoptosis induced by copper in PC12 cells.** *Food Chem Toxicol* 2008; 46: 2157-2164.

100 Siddiqui MA, Alhadlaq HA, Ahmad J, et al. **Copper oxide nanoparticles induced mitochondria mediated apoptosis in human hepatocarcinoma cells.** *PLoS One* 2013; 8: e69534.

101 Abdelazeim SA, Shehata NI, Aly HF, et al. **Amelioration of oxidative stress-mediated apoptosis in copper oxide nanoparticles-induced liver injury in rats by potent antioxidants.** *Sci Rep* 2020; 10: 1-14.

2. ASSESSMENT OF COPPER ACCUMULATION IN ARCHIVED LIVER SPECIMENS FROM CATS*

Objectives The aim of this study was to assess hepatic copper concentrations and zonal distribution in cat liver specimens.

Methods For this study, 121 archived, formalin-fixed, paraffin-embedded liver specimens from cats were used. Tissue sections were stained for copper with rhodanine and scored from 0 (no copper accumulation) to 5 (panlobular copper accumulation). The tissue specimens were then deparaffinized and hepatic copper concentrations were measured using flame atomic absorption spectroscopy.

Results Tissue samples were categorized into four groups based on histopathologic findings: (1) no significant histopathologic hepatic changes (n = 66); (2) hepatic steatosis (n = 18); (3) inflammatory or infectious disease (n = 24); and (4) neoplasia (n = 13). Of the 121 specimens, 13 (11%) stained positive for copper, with three having a score ⩾3. Thirty-seven specimens (31%) had copper concentrations above the reference interval ([RI] <180 μg/g dry weight liver). Copper concentrations in cats with hepatic inflammatory or infectious disease were significantly higher than cats with hepatic steatosis ($P = 0.03$). Copper-staining score and concentration were positively correlated ($r_s = 0.46$, $P < 0.001$).

Conclusion and relevance Despite the fact that 31% of specimens had copper concentrations above the RI, only 11% showed positive copper staining and only 2.5% had a score ⩾3. Our findings suggest that hepatic copper concentrations greater than the upper

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limit of the RI are relatively common in cats. Further studies to determine the factors that influence hepatic copper staining in cats and to establish contemporary RIs for hepatic copper in healthy cats are warranted.

2.1. Introduction

Copper is an essential trace element that serves as a cofactor for several enzymes that are essential for growth, development and maintenance of living cells.[1, 2] The liver plays a major role in copper metabolism and homeostasis. Copper from the diet is stored in hepatocytes, is exported in a protein-bound form, and is eliminated through biliary excretion.[3] Tight regulation of copper is essential since it is a potentially toxic transition metal that can generate reactive oxygen species (ROS) via the Fenton reaction. The disruption of copper homeostasis can lead to the accumulation of copper and ROS production. Excess ROS promote oxidative stress and consequently induce hepatic necrosis, inflammation and fibrosis.[4, 5] Chronic hepatitis can eventually progress to hepatic cirrhosis and liver failure.[6]

Copper-associated hepatopathy, which results from excessive hepatic copper accumulation, is common in dogs. This disease can be caused by a genetic defect of a copper transporter gene leading to disturbance in the biliary copper elimination,[7, 8] exposure to an increased amount of copper in the diet, or a combination of the two.[7, 9, 10] The mutation of the copper metabolism domain containing 1 (*COMMD1*) gene has been reported in the Bedlington Terrier.[9] In contrast, copper-associated hepatopathy in the Labrador Retriever is multifactorial. An inherited predisposition and an association with the P-type copper-transporting ATPases genes, *ATP7B* and *ATP7A*, have been reported.[10-

[12] Diet has been suggested to play a crucial role in copper accumulation, especially in the Labrador Retriever with a hereditary predisposition.[6, 13] Copper-associated hepatopathy has also been reported in other breeds, including the West Highland White Terrier, Skye Terrier, Doberman Pinscher and Dalmatian.[14-17] Copper accumulation from an inherited defect typically appears in the centrilobular zones of the liver.[7]

In people, hepatic copper accumulation can occur secondary to cholestasis. Cholestatic liver disease decreases the ability of copper excretion through the biliary system and results in copper accumulation in the periportal areas. However, the magnitude of copper accumulation secondary to cholestasis in dogs is low and thus cholestasis may not be an important contributor to copper accumulation in this species.[11, 18, 19]

Copper-associated hepatopathy has not been well characterized in cats, with only a limited number of studies and case reports describing cats with presumed primary copper-associated hepatopathy.[20-23] In two case reports, copper accumulation was mainly localized in the centrilobular area, and both of those cats had extremely high hepatic copper concentrations (4074 and 4170 μg/g dry weight liver [RI] 150–180 μg/g dry weight liver).[22, 23] Electron microscopic examination was performed in one of the cats and showed electron-dense aggregates, which were probably copper laden lysosomes in hepatocytes and macrophages. This finding is similar to the findings in Bedlington Terriers with inherited copper toxicosis.[23]

One study of copper accumulation in cats with various liver diseases revealed that the accumulation was varied in location.[20] Another study suggested that copper accumulation in cats may be primary or secondary to other hepatobiliary diseases.[21] Cats suspected to have primary copper-associated hepatopathy (PCH) had copper

concentrations (>700 µg/g dry weight liver) that were higher than those in cats with other hepatobiliary diseases. Moreover, the areas of copper accumulation appeared to be similar to those seen in dogs. In contrast, cats with cholestatic disorders had copper accumulation in the periportal or intermediate zones.[21] However, no other reliable clinical markers can be used to differentiate between primary and secondary copper associated hepatopathy and possible causes of copper hepatopathy in cats are unknown. Therefore, the role of hepatic copper accumulation in feline liver disease is not fully understood.

The purpose of our study was to assess hepatic copper content and zonal distribution in liver specimens from cats with various liver diseases, comparing them with those with no significant histopathological changes of the liver.

2.2. Materials and methods

2.2.1. Sample collection

In total, 121 archived formalin-fixed paraffin-embedded (FFPE) liver specimens from cats were used. These specimens were collected during non-study related necropsy or surgical biopsy from 2015 to 2016 and were submitted to the Texas A&M Veterinary Medical Diagnostic Laboratory for histopathological analysis.

This work involved the use of non-experimental animals only (including owned or unowned animals and data from prospective or retrospective studies). Established internationally recognized high standards (‘best practice’) of individual veterinary clinical patient care were followed. Ethical approval from a committee was therefore not necessarily required. Informed consent (either verbal or written) was obtained from the owner or legal custodian of all animal(s) described in this work (either experimental or

non-experimental animals) for the procedure(s) undertaken (either prospective or retrospective studies). No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required.

2.2.2. Tissue sample classification

FFPE specimens were cut at 4-µm thickness and tissue sections were stained with hematoxylin and eosin (H&E) and a second set was stained with rhodanine using standard techniques. The H&E-stained slides were used for histopathological analysis using a modified scoring system for inflammation (0 = none; 1 = mild; 2 = mild to moderate; 3 = moderate; 4 = severe)[24] and lipid accumulation (0 = in <80% of hepatocytes; 1 = in ⩾80% of hepatocytes).[21, 25] Tissue samples were classified into four groups based on scores for inflammation, lipid accumulation, and the presence of neoplastic cells (Table 2.1). All tissue sections were evaluated in a blinded fashion by a single board-certified veterinary pathologist (RMG).

Table 2.1 Group classification based on the scores for inflammation, lipid accumulation, and the presence of neoplastic cells

Group	Scoring criteria
No significant histopathological hepatic changes	Inflammation 0–1; lipid accumulation 0
Hepatic steatosis	Inflammation 0–2; lipid accumulation 1

Hepatic inflammatory or infectious disease	Inflammation ⩾2; lipid accumulation 0 Two specimens with an inflammatory score of 1 and a lipid accumulation score of 0 were categorized into this group due to visualization of *Histoplasma capsulatum* organisms
Neoplasia	Presence of neoplastic cells, regardless of inflammatory and lipid accumulation scores

2.2.3. Qualitative copper assessment

The same pathologist also evaluated the hepatic copper accumulation in rhodanine-stained sections. The copper staining was scored using a modified scoring system for copper in dog liver biopsy specimens (Table 2.2).[26]

Table 2.2 Copper scoring system for qualitative copper assessment modified from a scoring system for liver biopsy specimens from dogs

Score	Accumulation of copper granules in hepatocytes or macrophages
0	No copper granule accumulation
1	Variable copper granules
2	Small to moderate numbers of copper granules

3	Moderate to large numbers of copper granules in 50–75% of hepatocytes; copper-containing macrophages may be present
4	Moderate to large numbers of copper granules in >75% of hepatocytes; copper-containing macrophages may be present
5	Panlobular presence of copper granules, usually associated with copper-containing macrophages

2.2.4. Quantitative hepatic copper assessment

FFPE specimens were used for copper quantification since the deparaffinized liver specimens have been shown to have comparable copper concentrations to fresh frozen liver specimens.[27] After tissue sectioning, FFPE specimens were submitted to the Veterinary Diagnostic Laboratories at Colorado State University. They were deparaffinized and hepatic copper concentrations were measured using flame atomic absorption spectroscopy (FAAS). This method of copper quantification has been validated for a sample size of 10 mg dry weight or greater. Thus, specimens that weighed <10 mg dry weight were excluded from the analysis. The RI was 150–180 µg/g dry weight liver and copper concentrations <100 µg/g dry weight liver were considered to be deficient.[28, 29]

2.2.5. Statistical analysis

Data were tested for normality using the Shapiro–Wilk W test and were reported as median (range) values. A comparison of hepatic copper concentrations between groups was performed using the Kruskal–Wallis test with a post-hoc Dunn's multiple comparison

test. The relationship between hepatic copper concentrations and copper-staining scores was evaluated by Spearman's rank correlation. Values of $P < 0.05$ were considered significant. Data were analyzed with commercially available statistical software (JMP Pro version 14, SAS Institute Inc.).

2.3. Results

Initially, 154 liver specimens were considered for use in the study. Twenty-eight specimens were excluded owing to tissue weight <10 mg dry weight. Five specimens were also excluded owing to autolysis on histopathological examination and the remaining 121 specimens were used in this study. The specimens were categorized into one of four groups: no significant histopathological changes, steatosis, inflammatory/infectious disease or neoplasia (found in 13 specimens, comprising four with lymphoma, four with cystadenoma, one each with myelolipoma, hemangiosarcoma, round cell tumor, mast cell tumor and metastatic adenocarcinoma) as shown in Table 2.3.

Table 2.3 Summary of histopathological scoring criteria and median with range of hepatic copper concentrations of 121 cats

Group	Number of liver specimens	Median (minimum–maximum) hepatic copper concentration (µg/g dry weight liver)	Number of liver specimens with copper concentrations above the reference interval (180 µg/g dry weight liver)
No significant histopathological changes	66	124 (17.6–485)	19/66 (29%)
Steatosis	18	76.8* (24.6–421)	3/18 (17%)
Inflammatory/infectious disease	24	194* (27.6–2010)	12/24 (50%)
Neoplasia	13	73.2 (27.8–590)	3/13 (23%)

*There was a significant difference in hepatic copper concentration between specimens with hepatic steatosis and those with inflammatory/infectious disease (P = 0.03)

Distribution of age, sex and breed of cats were analyzed. Information of age and sex was not recorded for six cats and breed information was missing for 11 cats. The median age (n = 115) was 36 months (range 0.5–204 months). Cats with hepatic neoplasia

(median: 138 months, range 24–192 months) were significantly older than those with no significant histopathological changes of the liver (median: 24 months, range 0.5–204; P = 0.002) or those with hepatic inflammatory/infectious disease (median: 36 months, range 0.5–180; P = 0.035). Of the 115 cats, there were 15 intact males (13%), 39 castrated males (34%), 23 intact females (20%) and 38 spayed females (33%). Of the 110 cats, 88 were domestic mixed-breed (80%), four each were Persian or Ragdoll (3.6% each), three each were Bengal or Oriental Shorthair (2.7% each), two were Birman (1.8%), and one each were Bombay, Himalayan, Maine Coon, Norwegian, Siamese, and Tonkinese (0.9% each).

Rhodanine staining was positive in 13/121 specimens (11%). Of these specimens, nine had a score of 1, one had a score of 2, two had a score of 3, and one had a score of 4 (Figure 2.1; see Appendix 1 for individual information of histopathological analysis, copper concentration, copper score and copper distribution). Thirty-seven of 121 specimens (31%) had copper concentrations above the upper limit of the reference interval (Figure 2.2, see Appendix 2). Median hepatic copper concentrations of specimens in each group are shown in Table 2.3. In this study, hepatic copper concentrations were significantly higher in specimens with hepatic inflammatory/infectious disease compared with those with hepatic steatosis (P = 0.03).

Three specimens with copper staining scores $\geqslant$3 had histomorphological diagnoses of no significant histopathological hepatic changes, randomly distributed hepatitis and histoplasmosis and their copper concentrations were 227, 787 and 2010 μg/g, respectively. In the case with no significant histopathological hepatic changes, the copper accumulation was centrilobular. In the other two specimens, copper granules were found in the centrilobular and midzonal areas. The 10 specimens with copper staining scores of 1 to 2

had copper accumulation in various areas of the liver. In total, centrilobular accumulation was noted in four specimens, for one of which the copper was also midzonal; periportal accumulation was reported in three specimens; and midzonal and random accumulations were found in the other three specimens. Copper staining score and copper concentration were positively correlated (r_s = 0.46, P <0.001; Figure 2.3).

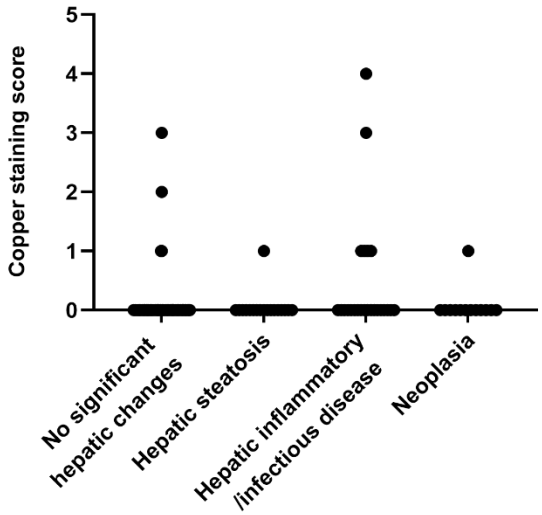

Figure 2.1 Copper staining scores for 121 archived liver specimens from cats
Copper staining intensities were scored from 0 (no copper accumulation) to 5 (panlobular copper accumulation). Positive staining was found in 13 specimens. Only three specimens had copper staining scores ⩾3. Hepatic copper concentrations for these cases were 227, 787 and 2010 µg/g dry weight, respectively

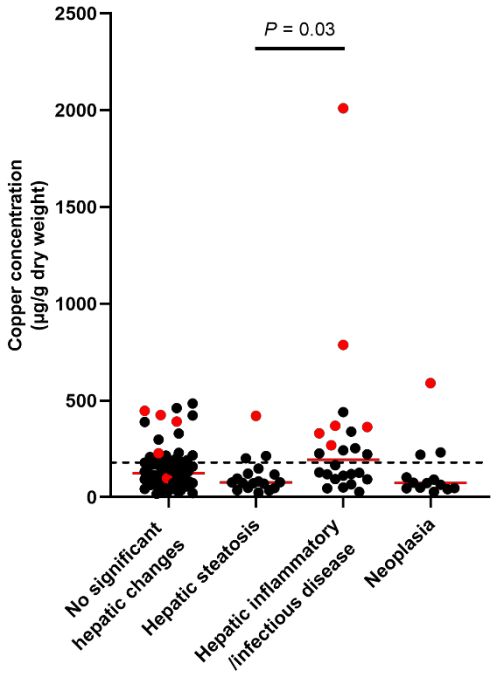

Figure 2.2 Hepatic copper concentrations for 121 archived liver specimens from cats

A total of 37 specimens (31%) had hepatic copper concentrations above the upper limit of the reference interval (<180 µg/g dry weight liver, represented by the dotted line). The red dots represent specimens with positive staining

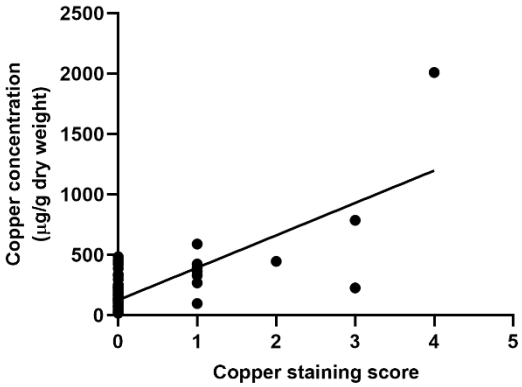

Figure 2.3 Correlation between hepatic copper concentrations and copper (rhodanine) staining scores for 121 archived liver specimens from cats

$r_s = 0.46$, $P < 0.001$

2.4. Discussion

In this study, hepatic copper was assessed in archived cat liver specimens submitted to a veterinary diagnostic laboratory with both qualitative (rhodanine staining) and quantitative approaches. The tissue samples were classified based on histopathological analysis.

Eleven percent of the liver specimens showed positive rhodanine staining. The prevalence in our study resembled that of a previous study, which found positive copper staining in 12 of 104 cats (12%).[20] In that study, the researchers used a different copper scoring scheme with scores ranging from 0 (no staining) to 3 (marked staining). Copper staining in periportal, midzonal or random areas was found in cats with scores of 1 or 2, whereas copper staining in the centrilobular area was found in cats with scores of 2 or 3 with various histopathological diagnoses. In addition, in the same study, copper staining was not present in cats with histologically normal livers.[20] In our study, copper staining in periportal, midzonal or random areas was found in specimens with scores of 1 or 2, whereas copper staining mainly in the centrilobular area was found in specimens with a wide range of scores (1–4) but mainly in those with a score ⩾3. Additionally, 5/66 specimens (7.6%) with no significant histopathological changes had positive copper stains with scores ranging between 1 and 3. The reason for these differences between studies is not known.

In another study of hepatic copper accumulation in cats, the investigators identified cats with presumed PCH had marked centrilobular copper accumulation and copper

concentrations typically greater than 700 µg/g dry liver weight. In contrast, copper accumulation from other causes, especially cholestatic disorders, was found in the periportal areas adjacent to inflammatory infiltrations.[21] As previously discussed, in our study, the pattern of copper accumulation varied; copper staining in specimens with scores ⩾3 was mainly in centrilobular areas, while the copper staining in specimens with scores ⩽2 did not show a specific localization. However, only 3/121 specimens had a copper score ⩾3. Additionally, *H capsulatum* was the likely etiologic agent in one of the three specimens and so concurrent PCH seems unlikely. In the other two specimens, the histological diagnoses were no significant histopathological hepatic changes and randomly distributed hepatitis and the copper staining in these two specimens did not colocalize with inflammation, and so again PCH seems unlikely. Overall, PCH was not convincingly documented in any of the specimens we evaluated.

Measurement of hepatic copper concentrations showed that 37/121 specimens (31%) had copper concentrations above the upper limit of the RI. Nineteen of these specimens showed no significant histopathological changes and 15 of these (78.9%) were negative for rhodanine staining. This suggests that cats can have copper concentrations greater than the upper limit of the RI without any significant hepatic changes and/or copper staining. Considering the previously proposed criteria for copper-associated hepatopathy in cats,[21] in the absence of histological changes and/or copper staining, it is unlikely that hepatic copper concentrations greater than the upper limit of the reference interval are clinically relevant to PCH. However, these samples were evaluated at a single time point and the copper concentrations from these specimens were higher than the reference interval but all still <700 µg/g dry weight liver (the previously speculated threshold for PCH in

cats).[21] Therefore, it cannot be ruled out that the specimens were collected during an early stage of copper-associated hepatopathy. During the initial stage of copper-associated hepatopathy in the Bedlington Terrier, the copper concentration can be increased without histological hepatic alterations and clinical signs.[11] It is also possible that the increased hepatic copper concentrations in these specimens were influenced by other factors, such as diet. The change made to the type of copper premixes used in commercial cat food made in the late 1990s increased the bioavailability of copper. In the Labrador Retriever and other breeds of dogs, increased copper bioavailability in commercial dog food has been suggested to be a risk factor for copper-associated hepatopathy.[13, 30] Furthermore, the RI for hepatic copper content in cats was taken from a textbook published in 1988, and at that time this range (150–180 μg/g dry weight liver) was described as an adequate copper tissue level.[28] There was only one study of dietary copper in cats mentioned in the bibliography of this book.[29] In that study, the copper concentrations in various tissues were obtained from a total of 35, 25-week-old kittens fed with different concentrations of dietary copper. However, it is still unclear exactly how the current RI was derived and it possibly needs to be updated. Establishment of a contemporary RI in a well-characterized group of cats being fed commercial diets would provide useful information for future studies. Additionally, even though copper concentrations measured from FFPE specimens have been shown to be clinically useful in dogs,[27] the effects of formalin on the hepatic copper concentration and the clinical copper assessment in cats have not been investigated. Therefore, fresh liver specimens should be ideally used for the most accurate results.

In the present study, specimens with hepatic inflammatory/infectious disease had significantly higher hepatic copper concentrations than those with hepatic steatosis. Two

specimens in this group had abundant copper accumulation in the centrilobular and midzonal areas. One of these cats had been diagnosed with randomly distributed hepatitis with a copper score of 3 and a hepatic copper concentration of 787 µg/g. The other specimen had *H capsulatum* organisms with copper score 4 and a hepatic copper concentration of 2010 µg/g. These findings are consistent with the ones from a previous study, in which cats with inflammatory or and infectious hepatic diseases also had copper accumulation in the liver.[20] In our study, specimens with hepatic steatosis had a lower median copper concentration than specimens with inflammatory/infectious disease, and 12/18 specimens (67%) had copper concentrations below the deficient level (<100 µg/g dry weight liver). However, these cats were not necessarily copper deficient. This is because copper concentration may be affected by many factors. For instance, a study of hepatic copper concentrations in dairy cattle found a negative correlation between hepatic copper concentrations and hepatic lipidosis.[31] The researchers suggested that increased hepatic fat can have a dilutional copper concentration. Increased fat accumulation displaces other molecules including protein that can bind with copper reducing the measure copper concentration. Additionally, we did not know the dietary history of these cats.

A study in dogs has shown a moderate correlation ($r = 0.66$, $P < 0.001$) between hepatic copper concentration and copper staining score.[30] To our knowledge, no prior study has examined this correlation in cats. In our study, we found a weaker positive correlation ($r_s = 0.46$, $P < 0.001$) between hepatic copper concentration and copper-staining score. The relative weakness of this correlation might be related to the techniques used to measure hepatic copper concentrations and copper accumulation. FAAS, which was used to measure copper concentrations, quantifies chemical elements from the absorption of

radiation by free atoms. FAAS technique uses a flame to evaporate and dissociate the copper elements in tissue samples into atoms.[32] After the atomization, copper atoms absorb the radiation and the absorption is later detected and calculated as the concentration. In contrast, rhodanine, a chelating agent, has a strong affinity for proteinaceous copper deposits.[33, 34] Thus, it has been suggested that it detects protein-bound copper rather than detecting the copper itself.[11, 35, 36] In addition, our ability to precisely define the relationship between hepatic copper staining and concentration from our data is impeded by the low number of specimens with positive copper staining. However, we think it is important to note that in general as copper concentration increases so does copper-staining score. Our findings support that the assessment of hepatic copper accumulation in cats should not rely solely on the rhodanine staining or copper quantification; the two techniques should ideally be performed together.[30]

This study was subject to some limitations. First, we studied in archived liver specimens from a diagnostic lab and so we did not have full clinical records of the cats. We also could not identify the lobe of liver specimens. A study of sample sites in dogs reported no significant difference in copper concentration between liver lobes within dogs.[37] In contrast, uneven distribution and lobar pattern have been reported in rats and humans.[38-40] The specimens in our study may have been collected from various lobes. Thus, the regional variation cannot be ruled out. Moreover, we did not have a healthy control group and instead defined a group without significant histopathological changes of the liver. The group sizes were unequal, and the groups of cats with specific liver diseases were small, which could limit the statistical power and lead to a type II error. Future prospective

studies using a well characterized larger sample size of cats with a variety of hepatobiliary diseases are warranted.

2.5. Conclusion

Hepatic copper concentrations greater than the upper limit of the currently used RI were relatively commonly found in cats regardless of the presence of hepatic histological changes. It is possible that hepatic copper accumulation plays a bigger role in feline liver disease than is currently recognized. Further studies to determine the factors that influence hepatic copper staining in cats and to establish contemporary RIs for hepatic copper in healthy cats are warranted.

2.6. References

1 Uauy R, Olivares M and Gonzalez M. **Essentiality of copper in humans.** *Am J Clin Nutr* 1998; 67: 952S-959S.

2 Gaetke LM and Chow CK. **Copper toxicity, oxidative stress, and antioxidant nutrients.** *Toxicology* 2003; 189: 147-163.

3 Kim B-E, Nevitt T and Thiele DJ. **Mechanisms for copper acquisition, distribution and regulation.** *Nat Chem Biol* 2008; 4: 176-185.

4 Ferenci P, Zollner G and Trauner M. **Hepatic transport systems.** *J Gastroenterol Hepatol* 2002; 17: S105-S112.

5 Schultheiss PC, Bedwell CL, Hamar DW, et al. **Canine liver iron, copper, and zinc concentrations and association with histologic lesions.** *J Vet Diagn Invest* 2002; 14: 396-402.

6 Wu X, Leegwater P and Fieten H. **Canine models for copper homeostasis disorders.** *Int J Mol Sci* 2016; 17: 196.

7 Fieten H, Leegwater PA, Watson AL, et al. **Canine models of copper toxicosis for understanding mammalian copper metabolism.** *Mamm Genome* 2012; 23: 62-75.

8 Amano T, Matsubara T, Nishida T, et al. **Clinically diagnosed late-onset fulminant Wilson's disease without cirrhosis: A case report.** *World J Gastroenterol* 2018; 24: 290.

9 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.

10 Hoffmann G, Van Den Ingh T, Bode P, et al. **Copper-associated chronic hepatitis in Labrador Retrievers.** *J Vet Intern Med* 2006; 20: 856-861.

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

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

13 Fieten H, Hooijer-Nouwens B, Biourge V, et al. **Association of dietary copper and zinc levels with hepatic copper and zinc concentration in Labrador Retrievers.** *J Vet Intern Med* 2012; 26: 1274-1280.

14 Thornburg L, Shaw D, Dolan M, et al. **Hereditary copper toxicosis in West Highland white terriers.** *Vet Pathol* 1986; 23: 148-154.

15 Haywood S, Rutgers H and Christian M. **Hepatitis and copper accumulation in Skye terriers.** *Vet Pathol* 1988; 25: 408-414.

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

17 Mandigers P, Van Den Ingh T, Bode P, et al. **Association between liver copper concentration and subclinical hepatitis in Doberman Pinschers.** *J Vet Intern Med* 2004; 18: 647-650.

18 Thornburg L, Rottinghaus G, McGowan M, et al. **Hepatic copper concentrations in purebred and mixed-breed dogs.** *Vet Pathol* 1990; 27: 81-88.

19 Spee B, Arends B, Van Den Ingh TS, et al. **Copper metabolism and oxidative stress in chronic inflammatory and cholestatic liver diseases in dogs.** *J Vet Intern Med* 2006; 20: 1085-1092.

20 Whittemore JC, Newkirk KM, Reel DM, et al. **Hepatic copper and iron accumulation and histologic findings in 104 feline liver biopsies.** *J Vet Diagn Invest* 2012; 24: 656-661.

21 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.

22 Haynes JS and Wade PR. **Hepatopathy associated with excessive hepatic copper in a Siamese cat.** *Vet Pathol* 1995; 32: 427-429.

23 Meertens NM, Bokhove CAM and Van Den Ingh TSGAM. **Copper-associated chronic hepatitis and cirrhosis in a European Shorthair cat.** *Vet Pathol* 2005; 42: 97-100.

24 Fragkou FC, Adamama-Moraitou KK, Poutahidis T, et al. **Prevalence and clinicopathological features of triaditis in a prospective case series of symptomatic and asymptomatic cats.** *J Vet Intern Med* 2016; 30: 1031-1045.

25 Center SA, Crawford MA, Guida L, et al. **A retrospective study of 77 cats with severe hepatic lipidosis: 1975–1990.** *J Vet Intern Med* 1993; 7: 349-359.

26 Center SA, McDonough SP and 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.

27 Johnston AN, Center SA, McDonough SP, et al. **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.

28 Puls R. Mineral Levels in Animal Health: Diagnostic Data. Clearbrook, Canada: Sherpa International, 1988.

29 Doong G, Keen CL, Rogers Q, et al. **Selected features of copper metabolism in the cat.** *J Nutr* 1983; 113: 1963-1971.

30 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.

31 Strickland JM, Lyman D, Sordillo LM, et al. **Effects of super nutritional hepatic copper accumulation on hepatocyte health and oxidative stress in dairy cows.** *Vet Med Int* 2019; 2019.

32 Brown RJ and Milton MJ. **Analytical techniques for trace element analysis: an overview.** *Trends Analyt Chem* 2005; 24: 266-274.

33 Luxama JD, Carroll MA and Catapane EJ. **Effects of potential therapeutic agents on copper accumulations in gill of Crassostrea virginica.** *In Vivo* 2010; 31: 34.

34 Lindquist R. **Studies on the pathogenesis of hepatolenticular degeneration. II. Cytochemical methods for the localization of copper.** *Arch Pathol* 1969; 87: 370-379.

35 Johnson GF, Morell AG, Stockert RJ, et al. **Hepatic lysosomal copper protein in dogs with an inherited copper toxicosis.** *Hepatology* 1981; 1: 243-248.

36 Johnson G, Gilbertson S, Goldfischer S, et al. **Cytochemical detection of inherited copper toxicosis of Bedlington terriers.** *Vet Pathol* 1984; 21: 57-60.

37 Thornburg L, Beissenherz M, Dolan M, et al. **Histochemical demonstration of copper and copper-associated protein in the canine liver.** *Vet Pathol* 1985; 22: 327-332.

38 Haywood S. **The non-random distribution of copper within the liver of rats.** *Br J Nutr* 1981; 45: 295-300.

39 Faa G, Nurchi V, Demelia L, et al. **Uneven hepatic copper distribution in Wilson's disease.** *J Hepatol* 1995; 22: 303-308.

40 Liggi M, Mais C, Demurtas M, et al. **Uneven distribution of hepatic copper concentration and diagnostic value of double-sample biopsy in Wilson's disease.** *Scand J Gastroenterol* 2013; 48: 1452-1458.

3. ASSESSMENT OF COPPER IN ARCHIVED LIVER SPECIMENS FROM CATS FROM THREE GEOGRAPHICAL REGIONS

Objectives To assess hepatic copper using the qualitative and quantitative approaches in liver specimens from cats from three geographical regions: the United States, Malaysia, and Greece.

Methods In this study, 212 archived liver specimens from cats were identified: 121 from the United States, 58 from Malaysia, and 33 from Greece. Histopathological analysis was performed on hematoxylin and eosin-stained tissue sections. Hepatic copper was evaluated by examining rhodanine-stained tissue sections and quantifying hepatic copper concentrations using flame atomic absorption spectroscopy. Hepatic copper concentrations were then compared between specimens from cats from these three geographical regions.

Results Specimens were categorized into four groups: no significant histopathological hepatic changes, hepatic steatosis, hepatic inflammatory or infectious disease, and neoplasia. More than 30% of specimens in this study had hepatic copper concentrations higher than the upper limit of the reference interval (ULRI: 180 µg/g dry weight liver). More than 50% of the specimens had no significant histological changes. Comparisons of hepatic copper between histopathological groups and cats from geographical regions showed that copper concentrations were higher in specimens with hepatic inflammatory or infectious diseases than those with hepatic steatosis ($P = 0.0148$). Specimens of cats from Malaysia had higher copper concentrations when compared to specimens from cats from the United States and Greece ($P < 0.0001$ and $P = 0.002$, respectively).

Conclusion and relevance Hepatic copper concentrations greater than the ULRI were common in cats even with no significant histopathological changes of the liver. Copper concentrations in specimens of cats from Malaysia were higher than those from the United States and Greece. The reference interval for hepatic copper concentration in cats should therefore be re-evaluated in various groups of cats and should be established based on geographical location.

3.1. Introduction

Copper is one of the trace elements that are essential for cells. Dietary copper is absorbed by enterocytes and the liver plays a major role in copper metabolism and homeostasis.[1] Copper metabolism and its toxicity to the liver, known as copper-associated hepatopathy (CAH), in humans, dogs, and cats are described in Chapter 1. Excessive copper accumulation can be caused by several factors. For humans, major causes are genetic defects of the copper transporter ATPase copper transporting beta (*ATP7B*), which can lead to Wilson's disease.[2] CAH in dogs may also be caused by mutations of copper transporter proteins. A mutation of copper metabolism domain containing 1 (*COMMD1*) has been identified in Bedlington Terriers.[3] In addition, *ATP7B* and *ATP7A* have been suggested to be predisposing causes of CAH in Labrador Retrievers.[4] Decreased expressions of mRNA, encoding copper metabolism-related genes, including ATP7A and ATP7B proteins, were found in Doberman Pinschers.[5] This finding suggested that high hepatic copper in this breed may be related to an impaired copper transporter as well. Later, mutations of *ATP7A* and *ATP7B* were also identified in Dutch and North American Doberman Pinschers.[6] The investigators suggested that the *ATP7B* mutation was related to

increased hepatic copper concentrations. The effect of the *ATP7A* mutation on hepatic copper could not be determined. An evaluation of *COMMD1* in Labrador Retrievers and Doberman Pinschers revealed several variants in the DNA sequence of this gene. However, none of the variants were associated with an increase in hepatic copper concentrations.[7] Recently, studies in cats with presumed primary copper-associated hepatopathy (PCH) found single-nucleotide variations of *ATP7B* and suggested that genetic defects could also cause this disease in cats.[8, 9]

Apart from genetics, several factors have been suggested as potential causes of or contributors to hepatic copper accumulation. Diet has been suggested to be one of the predisposing factors. In humans, copper hepatopathies related to exposure to high dietary copper have been reported, such as Indian childhood cirrhosis, endemic Tyrolean infantile cirrhosis, and idiopathic copper toxicosis.[10-12] Dietary copper is also a potential contributor to increased hepatic copper concentrations in Labrador Retrievers with a susceptible genetic background.[13] Excessive copper accumulation in the liver can also occur secondary to cholestatic disorders, which interfere with biliary copper excretion. Human patients with primary biliary cirrhosis or cats with extrahepatic bile duct obstruction were found to have increased copper accumulation.[14-16] In contrast, the importance of cholestasis induced hepatic copper accumulation in dogs may be lower, since the magnitude of copper accumulation secondary to cholestasis in dogs is lower when compared to dogs with inherited copper-associated hepatopathy.[17-19] Environmental contamination could also be involved, and heavy metal pollution can enter living organisms through several pathways (e.g., ingestion, inhalation).[20-22] Many studies reported that heavy metal pollution can occur via both natural and anthropogenic processes.[22] The natural emissions of heavy

metal or natural weathering can release metals into soil, water, or air. Human activities, such as industry, agriculture, and mining are major causes of heavy metal pollution. Many countries were reported to have various types of metal contamination in soil, water, or even animals.[21-27] The most common heavy metal pollutants are copper, zinc, iron, lead, mercury, and cadmium. The effects of these metal pollutants may not only be limited to humans but also affect other organisms.

The purpose of this study was to assess copper in liver specimens from cats from three geographical regions: the United States, Malaysia, and Greece.

3.2. Materials and methods

3.2.1. Sample population

We identified liver specimens from cats collected from three geographical regions; the United States, Malaysia, and Greece. Samples from the United States were the same 121 archived formalin-fixed paraffin-embedded (FFPE) feline liver specimens from the first study (See chapter 2; section 2.2.1). Specimens from Malaysia were collected from 58 feral cats euthanized for non-study related purposes in the state of Selangor in 2017. Specimens were kept cool (4°C) after collection for 1-2 hours and were transferred to and stored at –80°C until analyzed. Specimens from Greece were collected from 33 cats euthanized for non-study related reasons between 2017 to 2019. Seventeen of these samples were fixed in formalin after collection and the other 16 specimens were kept frozen at –80°C. No live animals were used for the purpose of this study. Therefore, the Institutional Animal Care and Use Committee (IACUC) approval and informed consent were not required.

3.2.2. Tissue sample preparation

For samples from each of the three geographical regions, a different tissue preparation method was used.

Formalin-fixed paraffin-embedded (FFPE) specimens were sectioned at 4-µm thickness and processed with standard histology protocols for histopathological analysis and qualitative copper assessment. The remaining FFPE tissue was submitted for copper quantification.

Each specimen of frozen tissue was divided into two portions. The first was transferred into formalin and embedded in paraffin. The tissue block was cut at 4-µm thickness and processed using standard histology protocols for histopathological analysis and qualitative copper assessment. The second was submitted for quantitative copper assessment.

Formalin-fixed tissues were also cut into two portions. The first portion was embedded in paraffin and processed for histopathological evaluation, including qualitative copper analysis. The other portion was transferred into a 2-mL microcentrifuge tube without any preservatives and submitted for quantitative copper analysis.

3.2.3. Histopathological evaluation

All tissue sections were stained with hematoxylin and eosin (H&E) stain using a standard technique. The tissue slides were evaluated in a blinded fashion by a single board-certified veterinary anatomic pathologist (RGM). Previously published scoring systems for inflammation (0: none; 1: mild; 2: mild to moderate; 3: moderate; 4: severe)[28] and lipid

accumulation (0: in <80% of hepatocytes; 1: in ⩾80% of hepatocytes) were used to assess the sections.[29] Tissue samples were classified into four groups based on the histopathological diagnosis and scores of inflammation, lipid accumulation, and the presence of neoplastic cells (Table 2.1).

Table 2.1 Group classification based on the scores for inflammation, lipid accumulation, and the presence of neoplastic cells

Group	Scoring criteria
No significant histopathological hepatic changes	Inflammation 0–1; lipid accumulation 0
Hepatic steatosis	Inflammation 0–2; lipid accumulation 1
Hepatic inflammatory or infectious disease	Inflammation ⩾2; lipid accumulation 0
Neoplasia	Presence of neoplastic cells, regardless of inflammatory and lipid accumulation scores

3.2.4. Qualitative copper assessment

Tissue sections were stained with rhodanine (5-(4-Dimethylaminobenzylidine) rhodanine; Alfa Aesar, Haverhill, MA) to evaluate for hepatic copper accumulation. The

copper staining was scored from 0 to 5 using a modified scoring system adapted from a scoring system used in dogs (Table 2.2).[30] The copper score was analyzed by the same pathologist in a blinded manner.

Table 2.2 Copper scoring system for qualitative copper assessment modified from a scoring system for liver biopsy specimens from dogs

Score	Accumulation of copper granules in hepatocytes or macrophages
0	No copper granule accumulation
1	Variable copper granules in an occasional hepatocyte
2	Small to moderate numbers of copper granules in <50% of hepatocytes
3	Moderate to large numbers of copper granules in 50–75% of hepatocytes; copper-containing macrophages may be present
4	Moderate to large numbers of copper granules in >75% of hepatocytes; copper-containing macrophages may be present
5	Panlobular presence of copper granules, usually associated with copper-containing macrophages

3.2.5. Quantitative copper assessment

Frozen, formalin-fixed, or FFPE liver specimens were submitted to the Veterinary Diagnostic Laboratories at Colorado State University. The copper concentration was measured by flame atomic absorption spectroscopy (FAAS). The reference interval (RI)

reported by the laboratory is 150–180 µg/g dry weight liver, with copper concentrations <100 µg/g dry weight liver being considered to be deficient.[31]

3.2.6. Statistical analysis

Data were tested for normality using the Shapiro–Wilk W test and were reported as median (range). The relationship between hepatic copper concentrations and hepatic copper staining scores was evaluated by Spearman's rank correlation. Hepatic copper concentrations in each histopathological group and region were compared using the two-way ANOVA model and followed by Tukey's HSD test. We performed the log transformation of copper concentrations for this analysis. In addition, the association between copper status and regions was evaluated using the Chi-Squared test. Values of $P < 0.05$ were considered significant. Data were analyzed using a commercially available statistical software package (JMP Pro, version 15, SAS Institute, Cary, NC).

3.3. Results

3.3.1. Distribution of age, sex, and breed of enrolled cats

For the 121 cats from the United States, age and sex data were not recorded in six cats and breed information was missing for 11 cats. The median age of 115 cats was 36 months (range: 0.5–204 months). Cats with hepatic neoplasia (n = 13; median: 138 months, range: 24–192 months) were significantly older than those with no significant histopathological changes of the liver (n = 66; median: 24 months, range: 0.5–204; $P = 0.002$) or those with hepatic inflammatory/infectious disease (n = 24; median: 36 months, range: 0.5–180; $P = 0.035$). Of 115 cats, there were 15 intact males (13%), 39 castrated

males (34%), 23 intact females (20%), and 38 spayed females (33%). Of 110 cats for which breed data were available, 88 were domestic mixed-breed (80%), four each were Persian or Ragdoll (3.6% each), three each were Bengal or Oriental Shorthair (2.7% each), two were Birman (1.8%), and one each were a Bombay, Himalayan, Maine Coon, Norwegian, Siamese, and Tonkinese (0.9% each).

There was no breed information for the 58 cats from Malaysia. An estimated age was missing for one cat and the estimated age of the remaining 57 cats ranged from 12–180 months. Among 58 cats, there were 31 males (53%) and 27 females (47%).

Also, there was no breed information for the 33 cats from Greece. Estimated age was missing for eight cats and sex was not recorded for three cats. The estimated age ranged from 3–216 months. There were 19 males (63%) and 11 females (37%).

3.3.2. Qualitative copper assessment

Copper staining was positive in 13/121 liver specimens (11%) from the United States. Of these specimens, nine had a score of 1, one had a score of 2, two had a score of 3, and one had a score of 4. Three specimens with copper staining scores $\geqslant 3$ had a histomorphological diagnosis of: 1) no significant histopathological hepatic changes, 2) randomly distributed hepatitis, and 3) histoplasmosis, and their copper concentrations were 227, 787, and 2010 µg/g liver tissue, respectively. In the case with no significant histopathological hepatic changes, the copper accumulation was centrilobular. In the other two specimens, copper granules were found in the centrilobular and midzonal areas. The 10 specimens with copper staining scores of 1 to 2 had copper accumulation in various zones of the liver.

Only 1/58 specimens (1.7%) from Malaysia showed positive copper staining. This specimen was diagnosed as having no significant hepatic changes. The copper was found in centrilobular and midzonal areas with a copper score of 2. The copper concentration of this specimen was 654 µg/g dry weight liver.

None of the specimens from Greece showed positive copper staining.

3.3.3. Quantitative copper assessment

Thirty-seven of 121 specimens (31%) from the United States had copper concentrations above the ULRI (180 µg/g dry weight liver). Among these 37 specimens, 19 of them (51%) had no significant changes of the liver, 12 specimens (32%) had hepatic inflammatory/infectious disease, and three specimens (8%) each had hepatic steatosis, and neoplasia. The correlation between copper concentration and copper staining score was weakly positive ($r_s = 0.46$, $P < 0.001$).

Forty of 58 specimens (69%) from Malaysia, had copper concentrations above the ULRI. Among these 40 specimens, 31 of them (78%) had no significant changes of the liver, five specimens (13%) had hepatic steatosis, and four specimens (10%) had hepatic inflammatory/infectious disease. However, there was no significant correlation between copper staining score and copper concentration ($r_s = 0.21$, $P = 0.114$).

Ten of 33 specimens (30%) from Greece had copper concentrations above the ULRI. Among these 10 specimens, seven specimens (70%) had no significant changes of the liver, 1 specimen each (10%) had hepatic steatosis, hepatic inflammatory/infectious disease, or neoplasia. All these specimens had a copper score of 0. Consequently, it was

not possible to assess the correlation between the copper score and hepatic copper concentration.

3.3.4. Comparison of copper concentrations within and between liver tissue from cats from three geographical regions

Median (range) hepatic copper concentrations in all histopathological groups for cats from each region are reported in Table 3.1 and Figure 3.1.

The copper concentrations were log-transformed for statistical analysis. There was no significant interaction between histopathological groups and geographical regions. However, histopathological groups and geographical regions had a significant effect on the copper concentrations ($P = 0.0157$ and $P < 0.0001$, respectively). Post-hoc testing showed that specimens with hepatic inflammatory/infectious disease had higher copper concentrations than those with hepatic steatosis ($P = 0.0148$; Figure 3.2). Hepatic copper concentrations in specimens from Malaysia were higher than the specimens from the United States and Greece ($P < 0.0001$ and $P = 0.002$, respectively; Figure 3.3).

Additionally, the association between copper status and geographical region was investigated. We classified copper status into two groups: copper concentration below the ULRL and copper concentration above the ULRI. The number of specimens determined by copper status in each geographical region was summarized in Table 3.2. We found that copper status was associated with geographical region ($X^2(\text{df} = 2, \text{N} = 212) = 25.74$, $P < 0.0001$).

Table 3.1 Summary of median (range) hepatic copper concentrations in liver specimens from cats from the United States, Malaysia, and Greece

Histopathological group	Region		
	United States	**Malaysia**	**Greece**
No significant histopathological changes	124.0 (17.6–485, n = 66)	234.5 (43–918, n = 46)	131.0 (41.8–329.1, n = 25)
Steatosis	76.8 (24.6–421, n = 18)	225.0 (91.7–451, n = 7)	134.6 (15.1–254, n = 2)
Inflammatory/infectious disease	194.0 (27.6–2010, n = 24)	250.0 (144–431, n = 5)	146.4 (68.19–289, n = 3)
Neoplasia	73.2 (27.8–590, n = 13)	n/a	80.8 (72.2–733, n = 3)

n/a: not applicable because none of the liver specimens of cats from Malaysia showed neoplastic cells

Table 3.2 Summary of the number of specimens from cats from three different geographical regions classified by the copper status

Region	Copper concentration below the ULRI	Copper concentration above the ULRI

United States	84 (69%)	37 (31%)
Malaysia	18 (31%)	40 (69%)
Greece	23 (70%)	10 (30%)

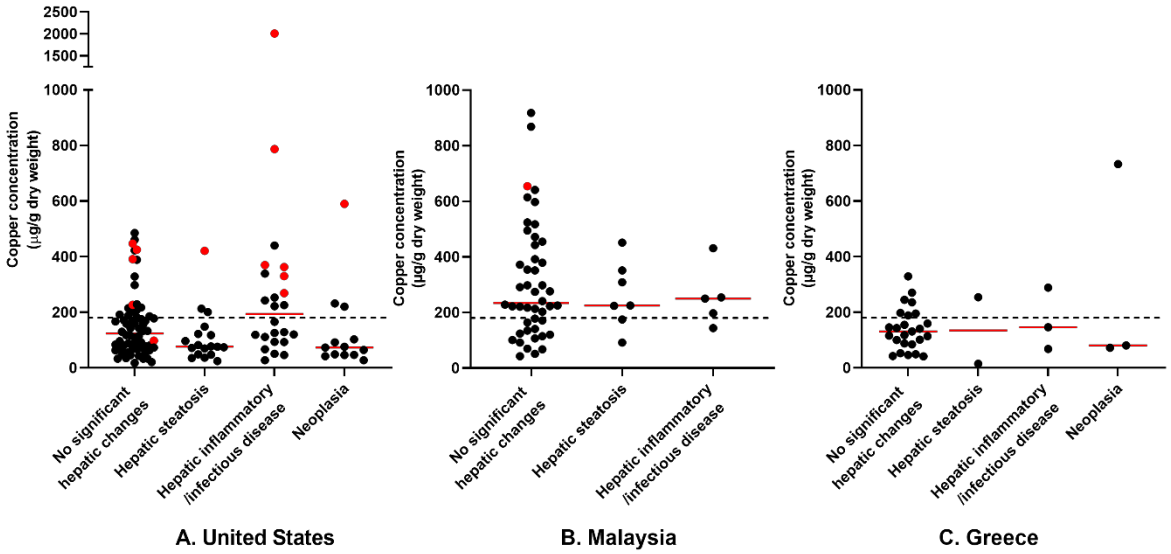

Figure 3.1 Hepatic copper concentrations in 4 histopathological groups of cats from 3 different countries

(A). United States (n = 121); (B). Malaysia (n = 58); (C). Greece (n = 33) Red dot represents specimen with positive copper staining. Red line represents median concentration and the dotted line represents the upper limit of the reference interval (ULRI: 180 µg/g dry weight liver)

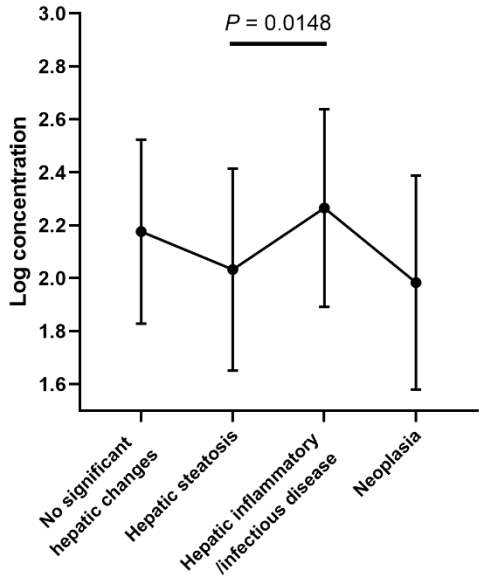

Figure 3.2 Comparison of copper concentrations in liver specimens from cats with different histopathological groups
Mean copper concentrations were higher in specimens with hepatic inflammatory/infectious disease than in those with hepatic steatosis ($P = 0.0148$)

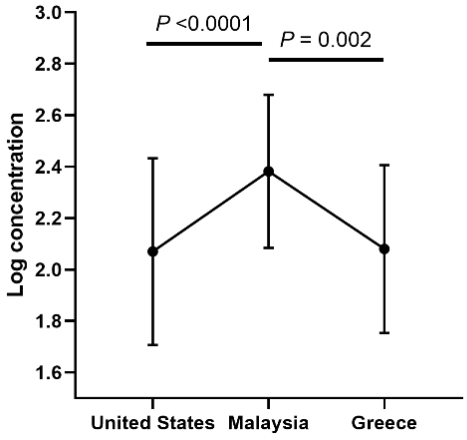

Figure 3.3 Comparison of copper concentrations in liver specimens from cats from three geographical regions
Mean hepatic copper concentrations were higher in liver specimens from cats from Malaysia compared to those from cats from the United States and Greece ($P <0.0001$ and $P = 0.002$, respectively)

3.4. Discussion

A total of 13/121 (11%) of specimens from the United States showed positive staining for copper. Out of these 13 specimens, five of them were diagnosed as having no significant histopathological changes. These specimens had copper scores ranging between 1 and 3. This positive rate of copper staining in our study is similar to that reported by a previous histologic study of copper and zinc in cats.[32] That research study found that 12/104 (12%) specimens had positive copper staining. None of the cats with a histologically normal liver were positive for copper. The reason for the differences between studies is not known. In addition, we found no specific pattern of copper accumulation in specimens with a copper score $\leqslant 2$. The accumulation in the specimens with a copper score $\geqslant 3$ was mainly in the centrilobular area. However, considering the previously proposed criteria,[16] along with our data of copper concentrations and histopathological findings, these specimens were considered unlikely to have PCH. Positive copper staining was found in only one specimen in the specimens from Malaysia and none of the specimens from Greece. None of these specimens were considered likely to have PCH. The positivity rate for copper staining was low even for specimens that had hepatic copper concentrations greater than the ULRI. There are several factors that might potentially affect the quality of copper staining in our study, such as tissue storage time or the handling of tissue before processing. A study of histochemical of copper stains in the liver from dogs reported that liver samples that were fixed in formalin for more than seven months had negative copper staining.[33] However, the researchers could not determine the possible reason for this loss of copper signal. Some of the specimens from Greece were

fixed in 10% neutral formalin for more than one year. Over-fixation may have had an effect on copper content in the liver tissue or may have interfered with the rhodamine stain. In addition, some frozen liver specimens from Greece were found to have freezing artifacts, which compromised the histopathological analysis. Tissue thawing might occur during the handling process, which could have resulted in artifacts that might have interfered with histopathological and qualitative copper analyses.

More than 30% of the specimens had copper concentrations above the ULRI. There were five specimens with copper concentrations >700 μg/g dry weight liver (the speculated threshold concentration of cats with PCH).[16] Among these five specimens, two specimens from cats in the United States were diagnosed as having histoplasmosis or randomly distributed hepatitis. Two specimens from cats in Malaysia had no significant histopathological changes. One specimen from a cat in Greece was found to have metastatic carcinoma. Again, considering the previously published criteria of PCH in cats, none of these specimens were likely to have PCH. It is possible that these specimens were collected during an initial stage of copper-associated hepatopathy. In an early stage of CAH in Bedlington Terriers, elevated hepatic copper concentrations can be found without clinical signs or any histologic changes on a liver biopsy.[17] Moreover, it is also possible that these cats were exposed to other factors that can potentially affect the copper content in the liver, such as dietary copper and environmental heavy metal pollutants. However, these factors may not necessarily have contributed to liver disease.

Interestingly, more than 50% of specimens that had hepatic copper concentrations above the ULRI did not show any significant histopathological hepatic changes. Among these specimens, there were 19/37 (51%) specimens from cats in the United States, 31/40

(78%) specimens from cats in Malaysia, and 7/10 (70%) specimens from cats in Greece. We reviewed hepatic copper concentrations reported in previous studies from 1995–2014.[16, 34-37] Wide ranges of copper concentrations were also found in healthy control cats or cats without hepatobiliary disease similar to the findings in our specimens without significant histopathological changes of the liver (e.g. 9.48–451.64 µg/g dry weight liver in 23 cats,[36] 10.8–394.76 µg/g dry weight liver in 47 cats).[37] It is possible that cats might have a large capacity to store copper in hepatocytes with little or no harmful effects. Considering this, we conclude that a contemporary reference interval established from a large well-characterized group of cats is needed.

We only found a weak correlation between hepatic copper concentrations and copper scores. This contrasted with the finding in dogs that found a moderate correlation ($r = 0.66$, $P < 0.001$).[38] The possible explanation of this discrepancy could be the different forms of copper that the FAAS and rhodanine can detect. The FAAS detects the copper after it has been disassociated from tissue and released as a free atom.[39] In contrast, rhodanine has been suggested to detect the protein that is bound to the copper, not the copper itself.[17, 40-42] A more detailed discussion of the differences between these two modalities to evaluate hepatic copper content can be found in section 2.4.

As mentioned earlier, some of our samples were over-fixated and some showed freezing artifacts. The quality of tissue samples may also interfere with the rhodanine stain as well. Our findings illustrate that clinicians should not solely rely on copper staining or copper quantification and should perform both modalities for a more complete assessment of hepatic copper content.[38] In recent years, researchers have worked to establish a reliable copper measuring tool. The laser ablation inductive-coupled plasma mass spectrometry

(LA-ICP-MS), an elemental bioimaging technique, has been suggested to provide an accurate measurement of hepatic copper in humans and mice with Wilson's disease.[43, 44] This technique can quantify copper in the tissue sample and also visualize the area of accumulation at the same time. Either FFPE or frozen samples can be used with this technique. Further investigations of copper in liver specimens from cats to determine if there are correlations between this technique and rhodanine staining or other quantification methods are needed. If this technique correlates well with other quantification measurements, it could overcome limitations of copper staining.

Specimens with hepatic inflammatory/infectious diseases had higher copper concentrations than specimens with hepatic steatosis ($P = 0.0148$). Our finding agrees with a previous study, which showed that cats with inflammatory and/or infectious hepatic disease have hepatic copper accumulation.[32] These findings indicate that livers affected by inflammation and/or infectious disease possibly have an alteration of copper accumulation. However, the specimens with hepatic steatosis were not necessarily copper deficient due to the fact that many factors could affect copper concentrations, such as a dilutional effects of increased specimen fat content.[45] Furthermore, the model analysis showed that liver specimens from cats from Malaysia had higher copper concentrations, compared with those from the United States or Greece ($P < 0.0001$ and $P = 0.002$, respectively). It is also important to point out that median hepatic copper concentrations for all three disease groups of cats from Malaysia were above the ULRI. The potential reason for these findings could be that these groups of cats might be exposed to copper-contaminated sources in Selangor, the sample collection site. Studies of heavy metal contamination in Malaysia reported that copper and other heavy metals were found in the soil and water due to human

activities, such as chemical industry, agricultural, and mining activities.[46] Studies in Tilapia reported that copper had accumulated highest in the liver of the fish, followed by gills and muscles.[27, 47] However, copper concentration in muscles was below the permissible limit and thus was considered to be safe for human consumption. Nonetheless, the contamination of copper may still enter the food chain cycle of other species and can be harmful to their health. In addition, specimens from Malaysia in our study were collected from feral cats. A study of nutrition in free-roaming cats found that the average copper content of natural diets (e.g., rodents, reptiles, and fish) was two to three times higher than the recommended level for growth and maintenance.[48] The feral cats in our study could also have consumed natural diets that might have contained a high copper content and may also have been exposed to heavy metal contamination in the environment. In the specimens from Greece, 23/33 (70%) specimens were collected from feral cats but 13 of them did not have high copper concentrations. We do not have a definitive explanation for this difference between the three geographical regions. Studies of heavy metal pollution in soil in Greece showed that copper contamination also increased due to industrial and agricultural activities.[23] However, copper contamination was lower than that for other heavy metals and was still below the European Community recommended limits.[25] This could be the possible reason why we did not see copper concentrations above the ULRI in some of these feral cats. It would be interesting to investigate the accumulation of copper and other heavy metals in the liver in a larger group of cats with various liver diseases from other regions. These studies may show that each country or region may require a different reference interval for copper. Furthermore, indoor and outdoor housing may also have an effect on hepatic copper concentrations in cats. Free-roaming cats may have a

higher chance of exposure to environmental contaminants. Further studies on the effect of housing and other environmental factors would provide a better perspective about potential factors of excessive copper accumulation in cats.

There were some limitations of this study. Specimens utilized in this study were collected by different modalities. Some specimens were collected during necropsy, whereas others were collected as biopsies. Furthermore, the specimens were stored under different conditions. Some of these specimens were kept frozen, while others were fixed in a fixative. The difference in tissue condition could have an effect on both copper qualitative and quantitative approaches. Moreover, we did not have full clinical records for the cats enrolled, including a full dietary history. In addition, the sample size of each cat population was unequal and the groups of cats with specific liver diseases were also small. This and the relatively small sample size in some subgroups may have led to a type II error. A larger well-characterized collection of cat liver specimens with a variety of hepatobiliary diseases is required for future studies.

3.5. Conclusion

Our findings suggested that hepatic copper concentrations greater than the ULRI were common in cats, even in those cats with no significant histopathological changes of the liver. Liver specimens from cats from Malaysia had copper concentrations higher than those from the United States or Greece. The reference interval for hepatic copper in cats should therefore be updated and probably established based on geographical locations. Other factors, such as environmental contamination, dietary sources, and housing, may

influence the increased accumulation of hepatic copper. Further investigations regarding those factors in cats are warranted.

3.6. References

1 Fuentealba IC and Aburto EM. **Animal models of copper-associated liver disease.** *Comp Hepatol* 2003; 2: 1-12.

2 Sternlieb I. **Perspectives on Wilson's disease.** *Hepatology* 1990; 12: 1234-1239.

3 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.

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

5 Spee B, Mandigers PJ, Arends B, et al. **Differential expression of copper-associated and oxidative stress related proteins in a new variant of copper toxicosis in Doberman pinschers.** *Comp Hepatol* 2005; 4: 3.

6 Wu X, Mandigers PJ, Watson AL, et al. **Association of the canine ATP7A and ATP7B with hepatic copper accumulation in Dobermann dogs.** *J Vet Intern Med* 2019; 33: 1646-1652.

7 Wu X, Mandigers PJ, Fieten H, et al. **Evaluation of COMMD1 in copper toxicosis in Labrador retrievers and Dobermans.** *Vet J* 2020; 265: 105561.

8 Asada H, Kojima M, Nagahara T, et al. **Hepatic copper accumulation in a young cat with familial variations in the ATP7B gene.** *J Vet Intern Med* 2019; 33: 874-878.

9 Asada H, Chambers JK, Kojima M, et al. **Variations in ATP7B in cats with primary copper-associated hepatopathy.** *J Feline Med Surg* 2020; 22: 753-759.

10 Tanner MS. **Role of copper in Indian childhood cirrhosis.** *Am J Clin Nutr* 1998; 67: 1074S-1081S.

11 Müller T, Feichtinger H, Berger H, et al. **Endemic Tyrolean infantile cirrhosis: an ecogenetic disorder.** *Lancet* 1996; 347: 877-880.

12 Scheinberg IH and Sternlieb I. **Wilson disease and idiopathic copper toxicosis.** *Am J Clin Nutr* 1996; 63: 842S-845S.

13 Fieten H, Hooijer-Nouwens B, Biourge V, et al. **Association of dietary copper and zinc levels with hepatic copper and zinc concentration in Labrador Retrievers.** *J Vet Intern Med* 2012; 26: 1274-1280.

14 Goldfischer S, Popper H and Sternlieb I. **The significance of variations in the distribution of copper in liver disease.** *Am J Pathol* 1980; 99: 715.

15 Elmes ME, Clarkson JP, Mahy NJ, et al. **Metallothionein and copper in liver disease with copper retention—a histopathological study.** *J Pathol* 1989; 158: 131-137.

16 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.

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

18 Thornburg L, Rottinghaus G, McGowan M, et al. **Hepatic copper concentrations in purebred and mixed-breed dogs.** *Vet Pathol* 1990; 27: 81-88.

19 Spee B, Arends B, Van Den Ingh TS, et al. **Copper metabolism and oxidative stress in chronic inflammatory and cholestatic liver diseases in dogs.** *J Vet Intern Med* 2006; 20: 1085-1092.

20 Castro-González M and Méndez-Armenta M. **Heavy metals: Implications associated to fish consumption.** *Environ Toxicol Pharmacol* 2008; 26: 263-271.

21 Hu Y, Zhou J, Du B, et al. **Health risks to local residents from the exposure of heavy metals around the largest copper smelter in China.** *Ecotoxicol Environ Saf* 2019; 171: 329-336.

22 Masindi V and Muedi KL. **Environmental contamination by heavy metals.** In: Saleh HE-DM and Aglan RF (eds). Heavy metals. London, United Kingdom: IntechOpen, 2018, pp 115-132.

23 Golia E, Tsiropoulos G, Füleky G, et al. **Pollution assessment of potentially toxic elements in soils of different taxonomy orders in central Greece.** *Environ Monit Assess* 2019; 191: 106.

24 Harvey P, Handley H and Taylor M. **Widespread copper and lead contamination of household drinking water, New South Wales, Australia.** *Environ Res* 2016; 151: 275-285.

25 Mitsios I, Golia E and Tsadilas C. **Heavy metal concentrations in soils and irrigation waters in Thessaly region, Central Greece.** *Commun Soil Sci Plant Anal* 2005; 36: 487-501.

26 Sany SBT, Salleh A, Rezayi M, et al. **Distribution and contamination of heavy metal in the coastal sediments of Port Klang, Selangor, Malaysia.** *Water Air Soil Pollut* 2013; 224: 1-18.

27 Taweel A, Shuhaimi-Othman M and Ahmad A. **Heavy metals concentration in different organs of tilapia fish (Oreochromis niloticus) from selected areas of Bangi, Selangor, Malaysia.** *Afr J Biotechnol* 2011; 10: 11562-11566.

28 Fragkou FC, Adamama-Moraitou KK, Poutahidis T, et al. **Prevalence and clinicopathological features of triaditis in a prospective case series of symptomatic and asymptomatic cats.** *J Vet Intern Med* 2016; 30: 1031-1045.

29 Center SA. **Feline hepatic lipidosis.** *Vet Clin North Am Small Anim Pract* 2005; 35: 225-269.

30 Center SA, McDonough SP and 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.

31 Puls R. Mineral Levels in Animal Health: Diagnostic Data. Clearbrook, Canada: Sherpa International, 1988.

32 Whittemore JC, Newkirk KM, Reel DM, et al. **Hepatic copper and iron accumulation and histologic findings in 104 feline liver biopsies.** *J Vet Diagn Invest* 2012; 24: 656-661.

33 Thornburg L, Beissenherz M, Dolan M, et al. **Histochemical demonstration of copper and copper-associated protein in the canine liver.** *Vet Pathol* 1985; 22: 327-332.

34 Haynes JS and Wade PR. **Hepatopathy associated with excessive hepatic copper in a Siamese cat.** *Vet Pathol* 1995; 32: 427-429.

35 Meertens NM, Bokhove CAM and Van Den Ingh TSGAM. **Copper-associated chronic hepatitis and cirrhosis in a European Shorthair cat.** *Vet Pathol* 2005; 42: 97-100.

36 Andreani G, Cottignoli S, Perfetti B, et al. **Trace Elements and Metallothionein in Liver and Kidney of Felis catus.** *Biol Trace Elem Res* 2010; 137: 177-189. journal article.

37 Paßlack N, Mainzer B, Lahrssen-Wiederholt M, et al. **Liver and kidney concentrations of strontium, barium, cadmium, copper, zinc, manganese, chromium, antimony, selenium and lead in cats.** *BMC Vet Res* 2014; 10: 163.

38 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.

39 Brown RJ and Milton MJ. **Analytical techniques for trace element analysis: an overview.** *Trends Analyt Chem* 2005; 24: 266-274.

40 Lindquist R. **Studies on the pathogenesis of hepatolenticular degeneration. II. Cytochemical methods for the localization of copper.** *Arch Pathol* 1969; 87: 370-379.

41 Johnson G, Gilbertson S, Goldfischer S, et al. **Cytochemical detection of inherited copper toxicosis of Bedlington terriers.** *Vet Pathol* 1984; 21: 57-60.

42 Johnson GF, Morell AG, Stockert RJ, et al. **Hepatic lysosomal copper protein in dogs with an inherited copper toxicosis.** *Hepatology* 1981; 1: 243-248.

43 Hachmöller O, Aichler M, Schwamborn K, et al. **Element bioimaging of liver needle biopsy specimens from patients with Wilson's disease by laser ablation-inductively coupled plasma-mass spectrometry.** *J Trace Elem Med Biol* 2016; 35: 97-102.

44 Kim P, Zhang CC, Thoröe-Boveleth S, et al. **Accurate Measurement of Copper Overload in an Experimental Model of Wilson Disease by Laser Ablation Inductively Coupled Plasma Mass Spectrometry.** *Biomedicines* 2020; 8: 356.

45 Strickland JM, Lyman D, Sordillo LM, et al. **Effects of super nutritional hepatic copper accumulation on hepatocyte health and oxidative stress in dairy cows.** *Vet Med Int* 2019; 2019.

46 Yunus K, Zuraidah MA and John A. **A review on the accumulation of heavy metals in coastal sediment of Peninsular Malaysia.** *Ecofeminism Clim Change* 2020; 1: 21-35.

47 Taweel A, Shuhaimi-Othman M and Ahmad A. **Evaluation of copper, lead and arsenic level in tilapia fish in Cempaka Lake (Bangi, Malaysia) and human daily/weekly intake.** *Biologia* 2013; 68: 983-991.

48 Plantinga EA, Bosch G and Hendriks WH. **Estimation of the dietary nutrient profile of free-roaming feral cats: possible implications for nutrition of domestic cats.** *Br J Nutr* 2011; 106 S35-S48.

4. ASSESSMENT OF THE INTRACELLULAR DISTRIBUTION OF COPPER IN LIVER SPECIMENS FROM CATS

Objectives This study aimed to assess the intracellular copper distribution in liver specimens from cats with copper concentrations below or above the upper limit of the reference interval (ULRI: 180 μg/g dry weight liver).

Methods Twenty-nine frozen liver specimens from cats were included. Each liver specimen was divided into two pieces for overall copper quantification and tissue fractionation. The copper concentrations in liver specimens and liver fractions were measured by flame atomic absorption spectroscopy.

Results Thirteen liver specimens with copper concentrations below the ULRI showed the highest distribution of copper in the cytosolic fraction followed by the nuclear, large granule, and microsomal fractions. Sixteen liver specimens with copper concentrations above the ULRI showed a similar pattern of distribution. Only one specimen had positive copper staining. No significant difference in the distribution for any fraction between groups was found ($P > 0.05$).

Conclusion and relevance Our findings indicate that similarly to other species, intracellular copper is predominantly found in the cytosolic and nuclear fractions in cats. The distribution of copper between liver specimens from cats with copper concentrations below or above the ULRI was not significantly different. However, such a difference in distribution in cats with primary copper-associated hepatopathy cannot be excluded.

4.1. Introduction

Copper is an essential trace element that serves as a cofactor for several enzymes that are required for growth, development, and maintenance of living cells.[1, 2] The liver plays a major role in copper metabolism and homeostasis. Dietary copper is absorbed from the small intestines and enters hepatocytes from where it is either distributed throughout the body in protein-bound forms, or eliminated through biliary excretion if there is an excess of copper.[3] Copper homeostasis is tightly regulated since excess copper can become a potentially toxic transition metal and generate reactive oxygen species (ROS).[2] Excessive ROS promotes oxidative stress and consequently induces hepatic necrosis and inflammation with possible subsequent fibrosis.[4] Liver injury caused by copper, known as copper-associated hepatopathy, can eventually progress to hepatic cirrhosis and failure.[5] Cats with primary copper-associated hepatopathy (PCH) have been reported to have copper concentrations >700 µg/g (range: 704–7041 µg/g) dry weight liver.[6] The copper accumulation was found to be localized in centrilobular areas associated with most of the hepatocellular histological changes.[6-8] Additionally, excessive copper accumulation is reported to occur in cats secondary to other cholestatic hepatobiliary diseases. Hepatic copper concentrations in these cats were reported to be lower than those with PCH and the accumulation was localized predominately to the periportal regions.[6]

Measurement of copper in subcellular liver fractions may provide an important insight into the pathogenesis of copper-associated hepatopathy. Subcellular organelles, including those found in the liver, can be separated by differential centrifugation, a technique for crude separation and purification of organelles and other particles based on their sedimentation rate which depends upon their size.[9] Using this technique, liver

homogenate can be separated into four main compartments: the nuclear, large granule, microsomal, and cytosol fractions.[9] The nuclear fraction contains the nuclei of hepatocytes. However, since the differential centrifugation only separates organelles crudely, the nuclear fraction also contains cytoskeleton, plasma membrane, and intact hepatocytes. The large granule fraction contains mitochondria, lysosomes, and peroxisomes, whereas the microsomal fraction contains microsomes and other small vesicles. Lastly, the cytosolic fraction contains an intracellular fluid of cytoplasm, ribosomes, large macromolecules, and other soluble proteins.[10]

There are many studies regarding the intracellular distribution of hepatic copper in mammals. In normal control rats, copper was mainly found in the cytosolic fraction.[11, 12] In contrast, in copper-loaded rats, the copper was mainly localized in the large granule fraction.[11] In healthy adult humans, the proportion of copper was highest in the cytosol, whereas, patients with Wilson's disease (WD), a genetic disorder associated with excessive hepatic copper accumulation, had a greater proportion of hepatic copper in the large granule fraction.[13] In Bedlington Terriers with copper-associated hepatopathy, an ultrastructural study using an electron microscope found that copper rapidly accumulated in liver lysosomes after copper infusion into the intestinal lumen. In contrast, there was only a small number of copper granules in bile canaliculi.[14] The cumulative data from the above studies suggested that under physiological conditions, copper is mainly found in the cytosolic compartment of hepatocytes. When rats, humans, and dogs are loaded with copper experimentally or due to spontaneously occurring defects in copper excretion, copper becomes distributed into the other compartments, particularly the large granule

fraction.[11-14] However, to date the intracellular distribution of copper in the liver of cats having normal or increased hepatic copper concentrations has not been reported.

The objective of this study was to describe the intracellular distribution of copper in liver specimens from cats with copper concentrations that were below or above the upper limit of the reference interval (ULRI).

4.2. Materials and methods

4.2.1. Sample collection

Twenty-nine frozen liver tissue specimens from cats were included in this study. The specimens had been collected postmortem from feral cats that were euthanized in Malaysia in 2017 for non-study related reasons. Hepatic wedge biopsy specimens (approximately 2 x 1 x 1 cm in size) were immediately placed on ice for 1-2 hours, and then frozen at –80°C. In 2017, portions of these specimens had been sectioned and were processed using routine histology techniques for histopathological evaluation and qualitative copper assessment. The remainder of these specimens were kept frozen at –80°C until further analysis was performed in 2019. This work did not involve the use of live animals, and therefore, institutional animal care and use committee approval was not required.

4.2.2. Histopathological analysis and hepatic qualitative copper assessment

The formalin-fixed paraffin-embedded tissue blocks were cut at 4-µm thickness. Tissue sections were stained with hematoxylin and eosin (H&E) for histopathological analysis. Tissue specimens were classified based on previously published criteria of

inflammation, lipid accumulation, and the presence of neoplastic cells.[15] The scoring system and classification criteria are described in Appendix 3 and Table 2.1. Tissue sections were also stained with rhodanine for qualitative copper assessment using a modified scoring system adapted from copper staining criteria used in dogs (Table 2.2).[16] All tissue sections were analyzed by a board-certified veterinary anatomic pathologist in a blinded fashion (RMG).

Table 2.2 Copper scoring system for qualitative copper assessment modified from a scoring system for liver biopsy specimens from dogs

Score	Accumulation of copper granules in hepatocytes or macrophages
0	No copper granule accumulation
1	Variable copper granules in an occasional hepatocyte
2	Small to moderate numbers of copper granules in <50% of hepatocytes
3	Moderate to large numbers of copper granules in 50–75% of hepatocytes; copper-containing macrophages may be present
4	Moderate to large numbers of copper granules in >75% of hepatocytes; copper-containing macrophages may be present
5	Panlobular presence of copper granules, usually associated with copper-containing macrophages

4.2.3. Tissue fractionation

Each remaining liver specimen was divided into two pieces. One (at least 50 mg wet weight) was used for overall copper quantification and the second (approximately 1 g wet weight) was processed for fractionation. The samples used for fractionation were individually suspended and homogenized in 0.25 M sucrose, pH 8 in a 1:6 (w:v) ratio at 4°C. The differential centrifugation protocol for tissue fractionation has previously been described.[10] Briefly, the liver homogenate was centrifuged (Centrifuge 5810 R; Eppendorf, Hamburg, Germany) in a fixed-angle rotor (Rotor F-34-6-38; Eppendorf) at 600 × g for 10 minutes to separate the nuclear pellet, and at 8500 × g for 12 minutes to separate the large granule pellet. Then the supernatant was centrifuged (Optima MAX-XP; Beckman Coulter, Brea, CA) with a fixed-angle rotor (MLA-55; Beckman Coulter) at 105,000 × g for 60 minutes to separate the microsomal pellet. The remaining supernatant was considered the cytosolic fraction.

4.2.4. Hepatic copper quantification

All liver specimens and liver fractions were submitted to the Veterinary Diagnostic Laboratories at Colorado State University for copper quantification by flame atomic absorption spectroscopy (FAAS). FAAS is simple to perform with good precision for single-element measurement.[17] In this laboratory, FAAS has been validated for a minimum sample size of 50 mg wet weight. This technique dissociates the copper in tissue samples into atoms using a flame. Copper atoms absorb the light from the lamp and the absorbance is measured.[17] Then, the concentration is calculated in the unit of μg/g wet weight. The wet weight concentration is then multiplied by 4 to be converted to approximate dry weight liver tissue.[18] Based on this laboratory's reference interval, copper concentrations <180

μg/g dry weight liver (i.e., the reported upper limit of the reference interval for cats)[18] were considered normal for the purpose of this study.

4.2.5. Statistical analysis

Statistical analysis was performed using a commercially available statistical software package (JMP Pro version 14; SAS Institute, Cary, NC). The Shapiro–Wilk W test was used to determine the normality of all data sets. Data were reported as median (minimum–maximum) values. The copper distribution in liver fractions was calculated as a percentage of total copper. Then, the distribution in each fraction was compared between liver specimens from cats with copper concentrations below and those above the ULRI, using the Mann–Whitney U test. Statistical significance was set at $P < 0.05$.

4.3. Results

Liver tissues from 29 feral cats, 20 males and nine females were evaluated. The estimated age of the cats ranged from 1 to 7 years. On histopathological evaluation, 25 specimens had no significant histologic hepatic changes, two specimens had hepatic steatosis, and two specimens had hepatic inflammation. One liver specimen stained positive for rhodanine and was given a score of 2 out of 5. The copper accumulated in the centrilobular and midzonal areas. This specimen had no significant histologic hepatic changes and the hepatic copper concentration was 728 μg/g dry weight. The data of individual histopathological analysis and overall hepatic copper concentrations of the specimens in this study are available in Appendices 4 and 5.

The specimens were stratified into two groups based on hepatic copper concentrations. Specimens with copper concentrations below the ULRI (Group 1; n = 13) had a median copper concentration of 113 (range: 37–171) µg/g dry weight. On the other hand, specimens with copper concentrations greater than the ULRI (Group 2; n =16) had a median copper concentration of 317 (range: 204–777) µg/g dry weight. The copper concentration in the fractions of hepatic tissue was reported as a percentage of the total copper concentration (Table 4.1; Figure 4.1).

Table 4.1 Summary of the intracellular distribution of copper in liver specimens from cats with copper concentrations below (Group 1; n = 13) or above (Group 2; n = 16) the upper limit of the reference interval (ULRI)

	Nuclear fraction	**Large granule fraction**	**Microsomal fraction**	**Cytosolic fraction**
Copper concentrations below the ULRI (Group 1)	23.3% (10.6–41.6%)	12.2% (9.1–34.4%)	5.0% (0.8–14.6%)	60.0% (40.0–65.8%)
Copper concentrations above the ULRI (Group 2)	20.8% (8.8–40.3%)	17.2% (2.6–26.2%)	7.0% (1.3–16.5%)	56.8% (36.6–79.2%)

The distribution in each fraction is reported as the median (minimum–maximum) percentage

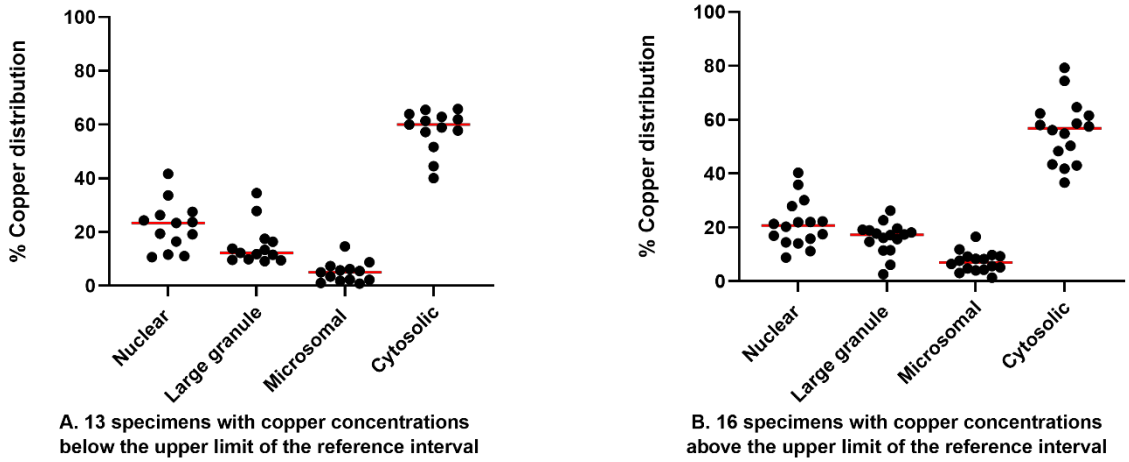

Figure 4.1 The intracellular distribution of copper in liver specimens from cats
(A) 13 specimens with copper concentrations below the upper limit of the reference interval (ULRI: 180 µg/g dry weight), and (B) 16 specimens with copper concentrations above the ULRI. In both groups, copper was found most abundantly in the cytosolic fraction, followed by the nuclear, large granule, and microsomal fractions, respectively. The red lines represent the medians

In group 1, the highest intracellular copper fraction was cytosolic followed by the nuclear, large granule, and microsomal fractions, respectively (Figure 4.1A). Group 2 showed a similar intracellular distribution of copper (Figure 4.1B). There was no significant difference in the intracellular distribution of copper for any of the four liver fractions between the two groups (P >0.05 for each comparison; Figures 4.2A–4.2D).

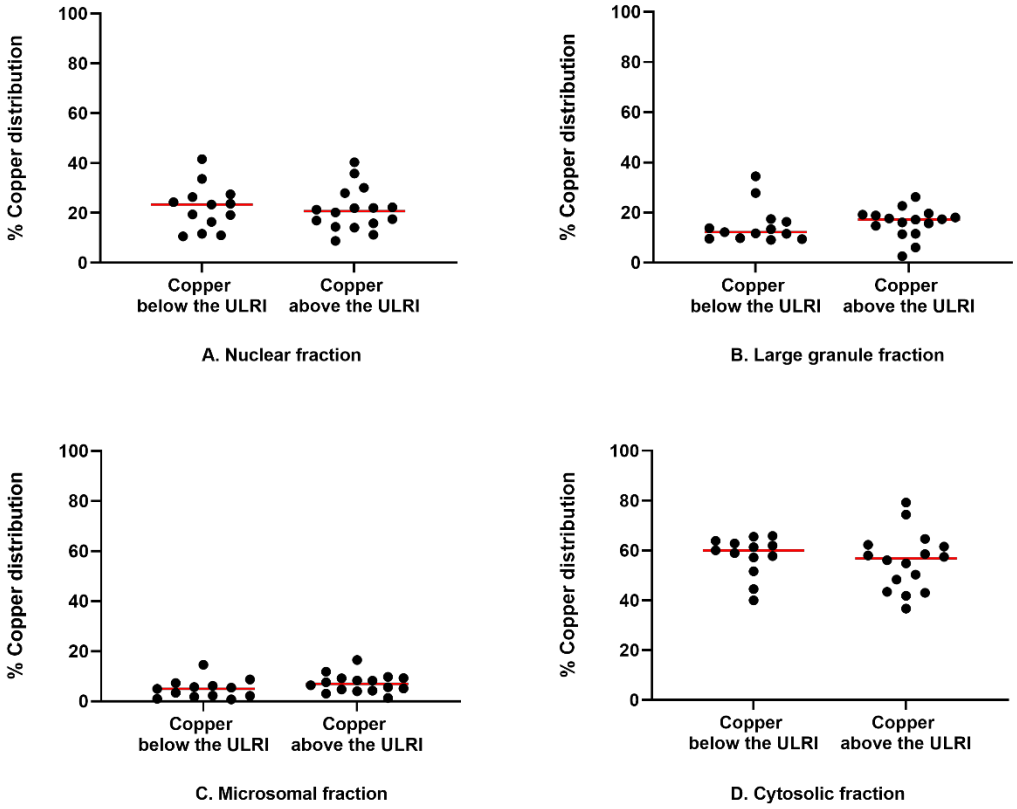

Figure 4.2 Comparison of copper distribution in liver fractions between 13 cat liver specimens with copper concentrations below the upper limit of the reference interval (ULRI) and 16 specimens with concentrations above the ULRI
(A) nuclear fraction ($P = 0.71$), (B) large granule fraction ($P = 0.22$), (C) microsomal fraction ($P = 0.08$), and (D) cytosolic fraction ($P = 0.32$). There was no significant difference in the intracellular distribution of copper between the two groups of liver specimens for any of the fractions. The red lines represent the medians

4.4. Discussion

We determined the intracellular distribution of copper in liver specimens from cats using a differential centrifugation technique to obtain four subcellular fractions. Copper in cat liver specimens with hepatic copper concentrations below the ULRI was found to be most abundant in the cytosolic fraction, followed by the nuclear, large granule, and

microsomal fractions. To our knowledge, this is the first time that the hepatic intracellular copper distribution has been reported in this species. Our findings are similar to those reported for other species, such as rats, dogs, and humans, with normal hepatic copper concentrations.[11-13] Specimens with hepatic copper concentrations above the ULRI had a similar intracellular distribution of copper compared to those with hepatic copper concentrations below this and there was no significant difference in the intracellular distribution of copper between these two groups. Studies in other species in animals with copper-loaded conditions showed significantly higher proportions of copper in the large granule fraction, which contains lysosomes, mitochondria, and peroxisomes,[10] when compared to other fractions.[11, 13] It was also found that copper predominantly accumulated in lysosomes.[14, 19] In contrast to these reports, we did not find a significantly increased proportion of copper in the large granule fraction of our specimens with copper concentrations above the ULRI. However, our sample size was relatively small and only one specimen within the group of elevated copper above the ULRI showed positive rhodanine staining. In addition, only 2/16 in this group of liver specimens had copper concentrations >700 µg/g dry weight liver, the speculated threshold for PCH in cats.[6] Therefore, such a redistribution of copper cannot be ruled out in cats with PCH as this group of cats was not represented in our study. Moreover, 14/16 specimens in this group did not have significant histopathological changes of the liver. The reason why cats without obvious hepatic histological lesions had copper concentrations greater than the ULRI is not known. One possibility is that the reference interval used is not appropriate for feral cats from Malaysia. We also found copper concentrations greater than the ULRI in cat liver tissue submitted to a US-based veterinary laboratory even when hepatic lesions

were not appreciated.[15] Determination of reference intervals for hepatic copper in cats in different regions or countries is warranted.

Our findings are important as the localization of hepatic copper may affect the positivity rate of histological copper staining using rhodanine. A study in humans with Wilson's disease reported that the copper staining rate was low during the early stages of the disease when patients are asymptomatic and that the rate of copper staining was higher in patients with late-stage disease.[20] The authors suggested that diffusely distributed copper in the cytoplasm cannot be observed using copper stains because the concentration is lower than the threshold of detection. In contrast, copper accumulation in lysosomes is dense enough for the stains to show positivity. In our present study, 28/29 specimens were negative for copper staining and all fractionated samples had the highest percentage of copper in the cytosolic fraction. It is possible that the copper in these specimens was freely distributed and therefore below the threshold of detection for the rhodanine stain. Of the 29 specimens, only one showed positive rhodanine staining with a score of 2 and the highest distribution of copper was in the cytosol (48%) following by the nuclear, large granule, and microsomal fractions, (28%, 19%, and 5%) respectively. In this case, we would have expected higher copper concentrations in the large granule fraction representing the hepatic lysosomes. Although the distribution of copper was similar to that of cats with copper concentrations below the ULRI, it is possible that the lysosomal copper concentration was high enough to result in positive staining. The other specimens in group 2 with copper concentrations above the ULRI showed no rhodanine staining. It is conceivable that, while copper was entering lysosomes in these cats, the concentrations were insufficient to reach the detection limit of the stain. In accordance with the findings, we were unable to

determine a threshold of lysosomal copper concentration for rhodanine staining. Further studies into the relationship between the intracellular localization of copper and the rate of copper staining in liver specimens from cats are warranted.

Our study is the first to describe the hepatocellular distribution of copper in cats but is subject to some limitations. The study population consisted of feral cats with no prior medical history. The sample group lacked cats having confirmed PCH, and higher hepatic copper concentrations than seen in most of the cats included in this study are likely required for positive rhodanine staining. Two cats with presumed PCH reported on in two different case reports that had positive copper staining were found to have extremely high hepatic copper concentrations (i.e., 4074 and 4170 µg/g dry weight liver respectively).[7, 8] It is possible that the intracellular distribution of copper would have been different in cat livers with higher hepatic copper concentrations. Secondly, the relatively small and unequal sample size could have led to a type II error, leading to failure to detect a difference in the intracellular distribution of copper between groups in this study. Thus, the evaluation regarding this issue in a greater number of cats, including some with PCH is needed. Although stored frozen tissue samples are reported to provide reproducible subcellular fractionation results,[21] repeating the study using fresh liver samples may have shown a different subcellular copper distribution. Lastly, the crude separation of the cell compartments by differential centrifugation might have resulted in contamination between the fractions. Incorporating enzymatic assays to identify cellular organelles might help to confirm the accuracy of the fractionation process and precise organelle copper localization.

4.5. Conclusion

It has been reported in many species that copper is mainly found in the hepatic cytosol under physiological conditions. Copper is distributed into the large granule fraction in copper-loaded conditions.[11-13] Our study demonstrates that intracellular copper is predominantly found in the cytosolic and nuclear fractions in cats, similarly to other species. We did not find a significant difference in the distribution of copper between liver specimens from cats with copper concentrations below or above the ULRI. However, such a difference in distribution in cats with PCH cannot be ruled out. Further studies in a larger number of cats, including those with PCH, are needed.

4.6. References

1 Uauy R, Olivares M and Gonzalez M. **Essentiality of copper in humans.** *Am J Clin Nutr* 1998; 67: 952S-959S.

2 Gaetke LM and Chow CK. **Copper toxicity, oxidative stress, and antioxidant nutrients.** *Toxicology* 2003; 189: 147-163.

3 Kim B-E, Nevitt T and Thiele DJ. **Mechanisms for copper acquisition, distribution and regulation.** *Nat Chem Biol* 2008; 4: 176-185.

4 Schultheiss PC, Bedwell CL, Hamar DW, et al. **Canine liver iron, copper, and zinc concentrations and association with histologic lesions.** *J Vet Diagn Invest* 2002; 14: 396-402.

5 Wu X, Leegwater P and Fieten H. **Canine models for copper homeostasis disorders.** *Int J Mol Sci* 2016; 17: 196.

6 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.

7 Haynes JS and Wade PR. **Hepatopathy associated with excessive hepatic copper in a Siamese cat.** *Vet Pathol* 1995; 32: 427-429.

8 Meertens NM, Bokhove CAM and Van Den Ingh TSGAM. **Copper-associated chronic hepatitis and cirrhosis in a European Shorthair cat.** *Vet Pathol* 2005; 42: 97-100.

9 Beaufay H and Amar-Costesec A. **Cell fractionation techniques.** In: Korn ED (ed). Methods in Membrane Biology. Springer, 1976, pp 1-100.

10 López-Alonso M, Prieto F, Miranda M, et al. **Intracellular distribution of copper and zinc in the liver of copper-exposed cattle from northwest Spain.** *Vet J* 2005; 170: 332-338.

11 Gregoriadis G and Sourkes TL. **Intracellular distribution of copper in the liver of the rat.** *Can J Biochem* 1967; 45: 1841-1851.

12 Smeyers-Verbeke J, May C, Drochmans P, et al. **The determination of Cu, Zn, and Mn in subcellular rat liver fractions.** *Anal Biochem* 1977; 83: 746-753.

13 Porter H. **Tissue copper proteins in Wilson's disease: intracellular distribution and chromatographic fractionation.** *Arch Neurol* 1964; 11: 341-349.

14 Kawamura M, Takahashi I and Kaneko J. **Ultrastructural and kinetic studies of copper metabolism in Bedlington Terrier dogs.** *Vet Pathol* 2002; 39: 747-750.

15 Yamkate P, Gold RM, Xenoulis PG, et al. **Assessment of copper accumulation in archived liver specimens from cats.** *J Feline Med Surg* 2021; 23: 526-533.

16 Center SA, McDonough SP and 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.

17 Bolann B, Rahil-Khazen R, Henriksen H, et al. **Evaluation of methods for trace-element determination with emphasis on their usability in the clinical routine laboratory.** *Scand J Clin Lab Invest* 2007; 67: 353-366.

18 Puls R. Mineral Levels in Animal Health: Diagnostic Data. Clearbrook, Canada: Sherpa International, 1988.

19 Johnson GF, Morell AG, Stockert RJ, et al. **Hepatic lysosomal copper protein in dogs with an inherited copper toxicosis.** *Hepatology* 1981; 1: 243-248.

20 Goldfischer S and Sternlieb I. **Changes in the distribution of hepatic copper in relation to the progression of Wilson's disease (hepatolenticular degeneration).** *Am J Pathol* 1968; 53: 883-901.

21 Lim J, Menon V, Bitzer M, et al. **Frozen tissue can provide reproducible proteomic results of subcellular fractionation.** *Anal Biochem* 2011; 418: 78-84.

5. ASSESSMENT OF OXIDATIVE STRESS AND APOPTOSIS IN ARCHIVED LIVER SPECIMENS FROM CATS BY AN IMMUNOHISTOCHEMISTRY

Objectives To evaluate markers of oxidative stress (4-hydroxynonenal; 4-HNE, and malondialdehyde; MDA) and apoptosis (active caspase 3; Casp-3) in liver tissue from cats with and without liver diseases by assessing immunohistochemical expression.

Methods We used formalin-fixed paraffin-embedded liver specimens from cats for this study. Specimens were evaluated by routine histology, measurement of copper and iron content, and immunohistochemical analysis. Specimens were classified based on the histopathological analysis and were separated into four groups. Copper was evaluated qualitatively using rhodanine stain and quantitatively by flame atomic absorption spectroscopy. Hepatic iron was qualitatively assessed with Prussian blue stain. Polyclonal antibodies of 4-HNE, MDA, and Casp-3 were used for immunohistochemical analysis.

Results Immunohistochemical expression of 4-HNE (n = 109), MDA (n = 69), and Casp-3 (n = 82) was not significantly different among all groups with liver diseases, nor between specimens with and without significant histopathological hepatic changes. There were no significant correlations between copper scores and the three immunohistochemical markers, neither between copper concentrations and the three markers. A weak positive correlation between iron and MDA scores was found (r_s = 0.382, P = 0.0012).

Conclusion and relevance Our findings indicate that, similar to other species, 4-HNE, MDA, and Casp-3 can be identified in liver specimens from cats by immunohistochemical staining. However, our results did not support the utility of these markers to detect

oxidative stress or apoptosis in cats with liver diseases. Further investigations of other potential biomarkers are warranted.

5.1. Introduction

Reactive oxygen species (ROS) are free radicals or oxidants that play an important role in biological processes in living cells.[1] They are required for physiological responses, such as a defense mechanism of the immune system.[2, 3] Excess ROS can induce the imbalance between free radicals and antioxidative agents, known as oxidative stress. Oxidative stress can damage the structure and function of the cells through pathways, such as oxidation of DNA structure and lipid peroxidation.[4] In turn, these can result in inflammation, cell injury, or even cell death.[5] Oxidative stress has been suggested to be involved in the pathogenesis of several diseases. In human patients and in mouse models, oxidative stress is also involved in the pathogenesis of hepatobiliary diseases, including copper-associated liver disease, acetaminophen-induced hepatotoxicity, hepatic lipidosis, alcoholic liver disease, and non-alcoholic fatty liver disease.[6-9] Apoptosis is one of the characteristics of hepatic injury. It is also known as caspase-dependent cell death and active caspase 3 (Casp-3) is a major enzyme in this process.[10] Apoptosis can be activated either by the extrinsic or intrinsic pathway.[11] The extrinsic pathway involves death receptors and hepatocellular mitochondria.[12] The intrinsic pathway involves mitochondrial signaling.[13] Several factors can activate the intrinsic apoptotic pathway, including DNA damage, oxidative stress, endoplasmic reticulum stress, metabolic stress, or hypoxic stress.[14] These stimuli activate the pro-apoptotic proteins in the B-cell lymphoma 2 (Bcl-2) family of proteins in the mitochondria, which subsequently activate Casp-3.[14]

Excess hepatic copper accumulation in patients with copper-associated hepatopathy can generate highly reactive hydroxyl radicals and induce oxidative stress via the Fenton reaction.[9] These radicals damage macromolecules in hepatocytes, such as DNA, lipid, and proteins. Products of lipid peroxidation, such as 4-hydroxynonenal (4-HNE) or malondialdehyde (MDA), have been used to detect the presence or activity of oxidative stress. Studies in rats that received copper sulfate ($CuSO_4$) found increased MDA concentrations and decreased glutathione concentrations in the liver of these rats.[15] A study in patients with Wilson's disease, an inherited disorder of excessive copper accumulation, reported positive immunostaining for 4-HNE and MDA in liver tissue sections.[16] Rats that received copper oxide nanoparticles (CuO NPs) were found to have elevated liver enzyme activities, hepatic DNA fragments, and expression of serum Casp-3.[17] A study in mice showed that copper-treated mice had increased apoptotic liver cells compared to control mice.[18] Recently, a study in dogs with COMMD-1 deficiency reported an increase of active Casp-3 immunoreactivity in the liver, suggesting that copper accumulation can induce apoptosis over time.[19] However, there is insufficient information on either oxidative stress or apoptotic activity in cats with liver disease, including those related to copper accumulation.

The purpose of this study was to assess the immunohistochemical expression of 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), and active caspase 3 (Casp-3) in liver specimens from cats with various liver diseases and those with no significant histopathological changes. This study also aimed to investigate the relationship between excessive copper accumulation and oxidative stress and apoptosis in liver specimens from cats.

5.2. Materials and methods

5.2.1. Sample collection

We used 109 archived formalin-fixed paraffin-embedded (FFPE) liver specimens from cats. These liver specimens were collected from cats during non-study related necropsy or for surgical biopsy from 2015 to 2016. The specimens were submitted to the Texas A&M Veterinary Medical Diagnostic Laboratory for histopathological analysis. No live animals were used in this study. Thus, the Institutional Animal Care and Use Committee (IACUC) approval was not required. However, due to there being an insufficient amount of tissue, the three immunohistochemical markers were not measured in all specimens (Table 5.1).

Table 5.1 Number of liver specimens used in each immunohistochemistry marker

Group	**4-Hydroxynonenal (4-HNE), n = 109**	**Malondialdehyde (MDA), n = 69**	**Caspase 3 (Casp-3), n = 82**
No significant histopathological hepatic changes	62	42	48
Hepatic steatosis	17	10	12
Hepatic inflammatory/ infectious disease	20	11	13

Neoplasia	10	6	9

5.2.2. Tissue sample classification

The tissue samples were the same population as for our previous study, entitled "Assessment of copper accumulation in archived liver specimens from cats" (chapter 2). We used the same criteria for tissue classification as described in that study. (See chapter 2; section 2.2.2). Tissue samples were classified into four groups based on scores for inflammation (0 = none; 1 = mild; 2 = mild to moderate; 3 = moderate; 4 = severe),[20] lipid accumulation (0 = lipid accumulation in <80% of hepatocytes; 1 = lipid accumulation in ⩾80% of hepatocytes),[21] and the presence of neoplastic cells. Group classification consisted of no significant histopathological hepatic changes, hepatic steatosis, hepatic inflammatory or infectious disease, or neoplasia. Histopathological analysis was performed on Hematoxylin and Eosin(H&E)-stained slides by a board-certified anatomic pathologist (RMG). Hepatic iron was evaluated based on Prussian blue-stained slides. Iron accumulation was scored from 0 to 3 (0: no staining, 1: mild staining, 2: moderate staining, 3: marked staining). The iron scoring system was applied from a system evaluating iron accumulation in liver biopsies from cats.[22]

5.2.3. Hepatic copper assessment

The qualitative copper assessment was evaluated using rhodanine copper staining. The scoring system was adapted from a histological scoring system for copper in liver biopsy specimens from dogs (Table 2.2).[23] The copper score was assigned in a blinded

fashion by the same pathologist (RMG). The quantitative copper assessment was determined using flame atomic absorption spectroscopy at the Veterinary Diagnostic Laboratories, Colorado State University. The laboratory's reference interval for hepatic copper concentrations was 150–180 µg/g dry weight liver.[24]

Table 2.2 Copper scoring system for qualitative copper assessment modified from a scoring system in dog liver biopsy specimens

Score	Accumulation of copper granules in hepatocytes or macrophages
0	No copper granule accumulation
1	Variable copper granules in an occasional hepatocyte
2	Small to moderate numbers of copper granules in <50% of hepatocytes
3	Moderate to large numbers of copper granules in 50–75% of hepatocytes; copper-containing macrophages may be present
4	Moderate to large numbers of copper granules in >75% of hepatocytes; copper-containing macrophages may be present
5	Panlobular presence of copper granules, usually associated with copper-containing macrophages

5.2.4. Immunohistochemistry (IHC) for 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), and active caspase 3 (Casp-3)

All FFPE liver specimens from cats were cut at 4-µm thickness and adhered to charged glass slides. Tissue sections were deparaffinized before IHC was performed. Heat-

induced antigen retrieval in buffer solution was performed using a pressure cooker (121°C) for 10 minutes and followed by blocking of endogenous peroxidases for 10 minutes with 0.3% hydrogen peroxide. After that, blocking of endogenous proteins and other non-specific binding particles was achieved using commercial blocking reagents. Tissue sections were then incubated with polyclonal antibodies in a humidified chamber. Later on, they were washed with 1x of Tris-buffered saline, Tween-20 (TBST) buffer before incubating with anti-rabbit horseradish peroxidase polymer (HRP) (Lab Vision Thermo Fisher Scientific, Fremont, CA) for 15 minutes. The target antigen was detected using a peroxidase substrate kit (Vector Laboratories, Burlingame, CA). The sections were counterstained with modified Mayer's hematoxylin and bluing solution. Finally, they were dehydrated and mounted with a mounting medium. All reagents were used in the amount of 150 µl to cover the whole tissue section. The details of reagents used in antigen retrieval, endogenous protein and other non-specific binding blocking, primary antibody incubation, the chromogen incubation time, and positive control case of the 4-HNE, MDA, and Casp-3 immunostaining are listed in Table 5.2. Negative controls and positive controls were stained similarly, except for the fact that negative controls were incubated with antibody diluent instead of the primary antibody.

Table 5.2 Lists of reagents and positive controls used in the immunohistochemistry of 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), and active caspase 3 (Casp-3) in hepatic tissue sections from cat liver specimens

Primary polyclonal antibody	**Dilution, incubation time, and temperature condition**	**Antigen retrieval buffer**	**Blocking reagent and incubation time**	**Chromogen incubation time**	**Canine positive control case**
4-HNE (Abcam, Cambridge, MA)	1:300, 2 hr, room temperature (RT)	R-Buffer C (Electron Microscopy Sciences, Hatfield, PA)	Protein Block Serum-Free (Dako North America, Carpinteria, CA), 5 min	2.5 min	Subacute hepatitis
MDA (R&D Systems, Minneapolis, MN)	1:500, 1 hr, RT	R-Buffer C (Electron Microscopy Sciences)	UltraVision protein block (Lab Vision Thermo Fisher Scientific), 10 min	3 min	Subacute hepatitis

Casp-3 (Abcam)	1:300, 1 hr, RT	DIVA decloaker (Biocare Medical, Concord, CA)	UltraVision protein block (Lab Vision Thermo Fisher Scientific), 5 min	5 min	Multifocal acute hepatic necrosis

A board-certified veterinary pathologist (PRG) performed the blinded evaluation for positive immunostaining. The number of 4-HNE positive cells were counted in 10 random fields centered on portal triads at 20x magnification using a conventional light microscope. The number of Casp-3 positive hepatocytes were also counted in the same manner with 4-HNE immunoreactivity. The immunoreactivity of MDA was evaluated differently. Positive MDA parenchymal cells were evaluated as the percentage of positive hepatocytes and were scored from 0 to 4 (0: Absent, 1: <10%, 2: 10-30%, 3: 30-60%, 4: >60%). The scoring system was adapted from the criteria used to evaluate MDA staining in liver specimens in dogs with chronic hepatitis.[25]

5.2.5. Statistical analysis

Data were analyzed with a commercially available statistical software package (Prism 8; GraphPad, San Diego, CA). Shapiro–Wilk W tests were used to determine the

normality of data and data were reported as median (range). The data of immunostaining positive cells and scores of each IHC marker were compared between specimen groups using the Kruskal-Wallis test. The correlations between immunoreactivity of three markers and hepatic copper staining scores or copper concentrations were evaluated by Spearman's correlation. The association between the IHC markers and hepatic iron scores was analyzed as well. Lastly, the associations between the histologic distribution (i.e., portal, centrilobular, midzonal, random, or no staining) of MDA and copper staining, and that of MDA and iron staining were analyzed using Chi-square tests. Values of $P < 0.05$ were considered significant.

5.3. Results

Out of 109 cats, the age data was missing for five cats. The median (range) age was four years (0.04–18.8 years). All liver specimens were classified into one of four groups based on the scoring criteria. Data of median (range) of copper scores and copper concentrations are reported in Table 5.3.

Table 5.3 Summary of medians with ranges of hepatic copper scores, copper concentrations, and iron scores in 109 liver specimens from cats

Group	Hepatic copper score median (range) (scores: 0–5)	Hepatic copper concentration median (range) (reference interval: 150–180 µg/g dry weight liver)	Hepatic iron median (range) (scores: 0–3)
No significant histopathological changes	0 (0–3)	123 (17.6–485)	1 (0–2)
Steatosis	0 (0–1)	77.2 (35.5–421)	1 (0–3)
Inflammatory/infectious disease	0 (0–4)	224 (27.6–2010)	1 (0–2)
Neoplasia	0 (0–1)	74.5 (42.3–590)	1 (0–2)

Rhodanine staining was positive in 13 of the 109 liver specimens (12%). Out of these specimens, nine had a score of 1 for copper staining, one had a score of 2, two had a score of 3, and one had a score of 4. Thirty-five of the 109 specimens (32%) had copper concentrations above the upper limit of the reference interval (180 µg/g dry weight liver). Prussian blue staining was positive in 71/109 specimens (65%). Out of the 71 specimens with positive iron staining, 58 had a score of 1, 12 had a score of 2, and one had a score of 3.

Immunostaining cell counts for 4-HNE, MDA, and Casp-3 are reported in Table 5.4. There was no significant difference in positive 4-HNE cells among four histopathological groups (P = 0.18; Figure 5.1). No significant correlation between immunostaining for 4-HNE and copper scores or copper concentrations was identified (r_s = 0.01, P = 0.92 and r_s = -0.12, P = 0.22, respectively). There was no significant difference in MDA scores between groups identified (P = 0.16; Figure 5.2). Finally, a correlation of MDA scores and copper scores or copper concentrations could not be identified (r_s = -0.191, P = 0.11 and r_s = -0.093, P = 0.44, respectively). We also determined the zonal distribution pattern of MDA in these 69 specimens. Twenty-eight specimens were found to have a centrilobular MDA staining. The other 21 specimens had a random distribution of MDA. The association between the histologic distribution of MDA vs. copper staining and the histologic distribution of MDA vs. iron staining were analyzed (Table 5.5). The distribution of MDA was significantly different to that of copper staining (X^2(df = 4, N = 69) = 66.91, P <0.0001). The distribution of MDA was significantly different to that of iron staining (X^2(df = 4, N = 69) = 47.25, P <0.0001). Additionally, no significant difference in active Casp-3 hepatocytes among groups was found (P = 0.05; Figure 5.3). Furthermore, similar to the other two markers, there was no significant correlation between Casp-3 positive hepatocytes and copper scores or copper concentrations identified (r_s = 0.032, P = 0.77 and r_s = -0.031, P = 0.77, respectively).

With reference to the correlations between the three markers and iron scores, there was no correlation between 4-HNE immunoreactivity and iron identified (r_s = 0.075, P = 0.43). In contrast, there was a significant correlation between MDA scores and iron scores (r_s = 0.382, P = 0.0012). Casp3 immunoreactivity was not associated with iron scores (r_s =

0.132, $P = 0.23$). Furthermore, we analyzed the association between age of the cats and IHC markers. We found that age was weakly to moderately correlated with Casp3 and MDA ($r_s = 0.381$, $P = 0.0005$ and $r_s = 0.748$, $P < 0.0001$, respectively)

Table 5.4 Summary of the cells immunostaining positive for 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), or active caspase 3 (Casp-3) in liver specimens from cats

Group	4-hydroxynonenal (4-HNE)	Malondialdehyde (MDA)	Active caspase 3 (Casp-3)
No significant histopathological changes	3 (0–57), n = 62	1 (0–3), n = 42	1 (0–100), n = 48
Steatosis	1 (0–45), n = 17	2 (0–3), n = 10	2 (0–19), n = 12
Inflammatory/infectious disease	2 (0–20), n = 20	2 (0–4), n = 11	4 (1–39), n = 13
Neoplasia	8 (0–32), n = 10	2.5 (2–3), n = 6	5 (0–48), n =9

The data were reported as medians and ranges. There was no significant difference between histopathological groups for any of the three immunohistochemical markers

Table 5.5 Histologic distribution of MDA, copper, and iron staining in 69 liver specimens from cats

Area of accumulation	MDA accumulation N (% of specimens)	Copper accumulation N (% of specimens)	Iron accumulation N (% of specimens)
Zonal portal	0	3 (4%)	4 (6%)
Zonal centrilobular	28 (41%)	3 (4%)	0
Midzonal	0	2 (3%)	9 (13%)
Random	21 (30%)	0	29 (42%)
No positive stain	20 (29%)	61 (88%)	27 (39%)

The data were reported as the number of specimens (N) and the percentage of total specimens. The distribution of MDA was significantly different from that of copper or iron staining (P <0.0001 for each)

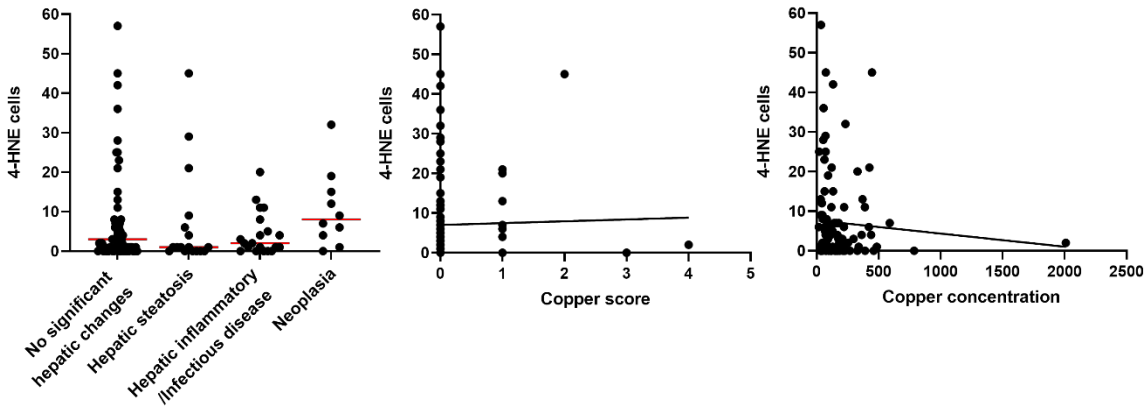

Figure 5.1 4-hydroxynonenal (4-HNE) immunostaining cells in liver specimens from 109 cats

The red lines represent the medians. There was no significant difference in immunostaining between the four groups (P = 0.18). No correlation between 4-HNE immunoreactivity and copper scores or concentrations was identified (r_s = 0.01, P = 0.92 and r_s = -0.12, P = 0.22, respectively)

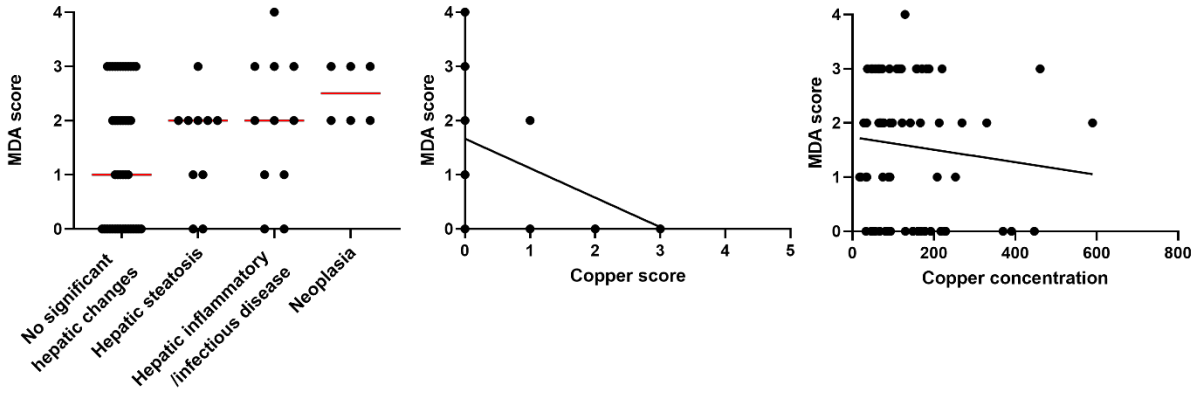

Figure 5.2 Malondialdehyde (MDA) immunostaining in 69 cat liver specimens
The red lines represent the medians. No difference in immunostaining between groups was identified ($P = 0.16$). No correlation between MDA scores or copper scores and concentrations was identified ($r_s = -0.191$, $P = 0.11$ and $r_s = -0.093$, $P = 0.44$, respectively)

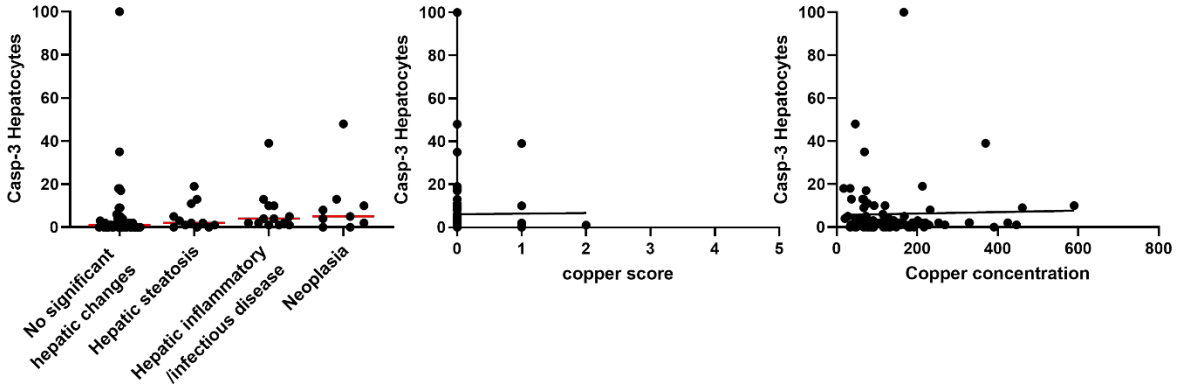

Figure 5.3 Active caspase 3 (Casp-3) immunostaining in 82 cat liver specimens
The red lines represent medians. No significant difference in immunostaining between groups was identified ($P = 0.05$). No correlation between Casp-3 immunoreactivity and copper scores or concentrations was identified ($r_s = 0.032$, $P = 0.77$, and $r_s = -0.031$, $P = 0.77$ respectively)

5.4. Discussion

We used 4-HNE and MDA immunostaining in order to attempt to assess oxidative stress in liver specimens from cats with various liver diseases. Both 4-HNE and MDA are lipid peroxidation products and have previously been suggested to be able to serve as markers of oxidative stress. They can also act as mediators of cell damage because they can interact with other macromolecules and induce damage to those cells.[26] Oxidative stress is detected in several diseases, including liver disease. In our study, we found no significant difference in 4-HNE positive cell counts among specimens in the liver disease groups, nor did we find any difference between those with and without significant histopathological hepatic changes. MDA scores were also not significantly different among these groups. We were unable to identify any significant correlations between these two IHC markers and copper scores or concentrations. Our findings were in contrast to those reported for other species. An immunohistochemical study in rats treated with carbon tetrachloride (CCl_4) reported expression of both 4-HNE and MDA in liver samples.[27] Expressions of 4-HNE and MDA were also found in human patients with chronic liver diseases including Wilson's disease. In contrast, in that study, immunostaining for these markers was not detected in hepatocytes of the normal liver specimens.[16] Also, a study in dogs with chronic hepatitis reported that MDA scores were correlated with hepatic necroinflammatory activity.[25] We studied IHC of 4-HNE and MDA using a similar protocol to this study in dogs with chronic liver diseases (P Yamkate et al., unpublished data). In that study, we included dogs with no significant histopathological hepatic changes and dogs with chronic hepatitis. 4-HNE immunoreactivity was not significantly different between groups and was not significantly correlated with copper scores or concentrations.

In contrast, MDA scores were significantly higher in dogs with chronic hepatitis (*P <0.0001*). We also identified positive correlations between MDA and copper scores or concentrations in these dogs (r_s = 0.530, P = 0.0011, and r_s = 0.684, P <0.0001, respectively). The MDA accumulation was randomly distributed in 57%, portally distributed in 39%, and diffusely distributed in 4% of the total of 28 dogs. Even in dogs with chronic hepatitis where copper was mainly accumulated in the centrilobular area, none of them had a centrilobular distribution of MDA. The findings in cats showed that 49 out of 69 specimens stained positive for MDA and that MDA was centrilobularly distributed in 28/49 (57%) and randomly distributed in 21/49 (43%) of these specimens. The reason for this difference in distribution between species is not known. There was a difference in the histological distribution of MDA vs. copper staining and MDA vs. iron staining (i.e., both copper and iron did not colocalize with this potential marker of oxidative stress as might have been expected). However, the number of samples with very high copper scores in this study was limited and a larger number of specimens with positive copper and iron staining is needed to assess the relationship between the distribution of these redox-active metals and MDA immunostaining.

It should be noted that our sample size for the various disease groups was relatively small, and there were more specimens with no significant histopathological hepatic changes than those with histological abnormalities. Additionally, the number of specimens used to study each IHC marker was unequal. This could have affected the power of statistical analysis and led to a type II error. We found a high number of 4-HNE positive cells and MDA scores in specimens with no significant histopathological hepatic changes. In fact, this group of cats had a wide range of immunostaining scores. It is possible that

hepatic oxidative stress might occur in the cats from which these specimens were collected. However, the consequences of oxidative stress (and products of lipid peroxidation) were not large enough to induce histopathological changes of the liver. Thus, these markers of lipid peroxidation might allow early detection before the lesions can be seen on regular histopathological examination. Unfortunately, we did not have other information about these cats to allow this determination to be made. Medical history, hematology, and serum biochemistry, as well as other diagnostic test results, would be needed to test this hypothesis. Alternatively, this finding could mean that these markers are not good candidates as biomarkers of oxidative stress in the liver in cats.

Copper is known as a toxic heavy metal due to its ability to produce free radicals, which can cause an imbalance between oxidants and antioxidants.[9] Many studies have shown that excess copper accumulation causes increased oxidative stress and apoptosis in the liver.[5, 16, 18, 19, 27-29] Our study in dogs also found positive correlations between hepatic copper accumulation and MDA as well as Casp-3 immunostaining (P Yamkate et al, unpublished data). However, our study in cats did not find any significant correlation between copper accumulation and these IHC markers. It is possible that hepatic copper accumulation in these groups of cats was not severe enough to cause damage to the liver and the lack of convincing primary copper hepatopathy in this cohort of cats supported this hypothesis. Copper concentrations ranged from 17.6–2010 µg/g dry weight in 109 specimens stained for 4-HNE. Thirteen specimens stained positive for copper. Only two specimens had copper concentrations >700 µg/g dry weight liver (the speculated minimum concentration for presumed primary copper-associated hepatopathy in cats)[30] with copper scores of 3 and 4. Copper concentrations of the specimens for MDA and Casp-3 markers

were 17.6–590 µg/g dry weight liver. Of the specimens that stained for these two IHC markers, only eight specimens had positive copper staining. Therefore, this lack of cases with primary copper hepatopathy could be the reason why we did not see the relationship between hepatic copper accumulation and oxidative stress markers. Moreover, if copper in these specimens did not induce ROS and oxidative stress, it is possible that it did not trigger the apoptotic activity. Hence, significant correlation between copper and active Casp-3 could not be identified in this population.

Iron is another essential element for cell function. However, excess iron can also promote free radicals via the Fenton reaction.[9] Therefore, excess iron can induce oxidative stress, inflammation, and hepatic injury similar to that seen due to excess copper accumulation. Many studies in the human medical and veterinary fields have reported the association of iron overload with oxidative stress and hepatic injury.[16, 31, 32] A study in human patients with chronic liver disease found an immunohistochemical expression of 4-HNE and MDA in those with hemochromatosis.[16] Iron-loaded rats also showed increases of hepatic 4-HNE and MDA.[31] Dogs with high hepatic iron concentrations tend to have high copper concentrations and inflammatory lesions in the liver.[32] A study of heavy metals in liver specimens from cats found that hepatic iron accumulation was commonly found.[22] In that study, 86/104 liver specimens (82.7%) had positive Prussian blue staining. Of those 86 specimens, 44% had an iron score of 1, 47% had a score of 2, and 9% had a score of 3. Our study also showed that iron accumulation was commonly found in cats. In total, 71 out of 109 (65%) specimens were positive for iron staining. We used the same iron scoring system as the previous study. We found 58/71 specimens (82%) with a score of 1, 12 (17%) with a score of 2, and one (1%) with a score of 3. However, none of the

specimens were diagnosed as having hemochromatosis. Similar to the finding in the previous study, there was no significant difference in iron scores between groups (P = 0.407).[22] In our study, we found a weak correlation between MDA and iron scores (r_s = 0.382, P = 0.0012). It could be that MDA expression was increased due to oxidative stress caused by hepatic iron accumulation. However, MDA and iron staining did not colocalize as might be expected. In this study, iron scores were not significantly correlated with active Casp-3 immunostaining. Our findings were in accordance with a study in rats loaded with iron for six weeks.[31] The investigators found a remarkable increase of MDA and HNE protein adducts in liver homogenates, compared to the controls. Immunostaining for MDA was also detected in the liver of these rats. In their study, no evidence of hepatic injury or changes of the liver architecture was found. Their findings suggested that iron overload could induce oxidative stress, in subjects without histologically detectable hepatocellular injury. The researchers suggested that noticeable histopathological hepatic changes were not observed in their rat model due to the short duration of iron treatment. It could be possible that the specimens in our study had been exposed to iron for only a short period. Therefore, only a weak correlation between MDA and iron staining was identified, and the iron-induced ROS in these specimens might not be sufficient to induce apoptosis or hepatocellular injury. However, the specimens that we studied were provided by a diagnostic laboratory and we did not have complete clinical records for the cats enrolled. Therefore, to clarify this speculation, further studies of chronic exposure of iron in cats would be needed.

Our study found correlations between the age of cats and immunoreactivity for MDA and active Casp-3 (r_s = 0.381, P = 0.0005 and r_s = 0.748, P <0.0001, respectively).

The process of aging has been suggested to be in part due to oxidative stress and apoptosis.[14] During aging, antioxidant systems decline in function, which can induce oxidative stress. The oxidative stress can trigger apoptosis via the intrinsic pathway through the mitochondria.[13] Moreover, other factors occurring during aging can induce apoptosis, such as instability of DNA, and endoplasmic reticulum stress.[14] The specimens from older cats in our study might reflect these processes. Measurement of other parameters, such as the concentrations of glutathione antioxidant, or 8-hydroxydeoxyguanosine, the products of DNA oxidation, would help to confirm this hypothesis.

The limitations of this study included the small and unequal sample size that may have led to a type II error. The FFPE specimens that we used for this study were provided from a diagnostic laboratory. Tissue processing could alter the immunohistochemical analysis.[33] We expected that the specimens were processed for routine histological analysis within 1–2 days. However, the duration of tissue fixation cannot be determined retrospectively. Over-fixation of tissue specimens may affect the immunohistochemical expression of these markers. Moreover, we measured only two markers for oxidative stress. These compounds might not be the major products of oxidative stress in cats. The Casp-3 marker that we used in this study is an active form of the caspase 3 enzyme. To activate this enzyme, stimuli must be present to trigger either the extrinsic or intrinsic pathways. It is possible that there may have been apoptotic activity that had not yet reached the step of Casp-3 activation. Other markers could be used to detect oxidative stress and apoptotic activity, such as DNA or protein damage, 8-isoprostane, or Bcl-2 family

proteins. Further investigations of these markers in liver specimens from cats are warranted.

5.5. Conclusion

Our study showed that, similar to other species, 4-HNE, MDA, and active Casp-3 are expressed in the liver of cats. However, we were unable to show any significant differences of the immunohistochemical expression of these markers between specimens with or those without liver disease. Moreover, no correlations between hepatic copper and these immunohistochemical markers could be identified. Therefore, our results did not support the utility of these markers to detect oxidative stress or apoptosis in cats with liver disease. Further investigations of these and other potential biomarkers are warranted.

5.6. References

1 Arauz J, Ramos-Tovar E and Muriel P. **Redox state and methods to evaluate oxidative stress in liver damage: From bench to bedside.** *Ann Hepatol* 2016; 15: 160-173.

2 Zhu R, Wang Y, Zhang L, et al. **Oxidative stress and liver disease.** *Hepatol Res* 2012; 42: 741-749.

3 Cichoż-Lach H and Michalak A. **Oxidative stress as a crucial factor in liver diseases.** *World J Gastroenterol* 2014; 20: 8082.

4 Webb C and Twedt D. **Oxidative stress and liver disease.** *Vet Clin North Am Small Anim Pract* 2008; 38: 125-135.

5 Spee B, Arends B, Van Den Ingh TS, et al. **Copper metabolism and oxidative stress in chronic inflammatory and cholestatic liver diseases in dogs.** *J Vet Intern Med* 2006; 20: 1085-1092.

6 Savides M, Oehme F, Nash S, et al. **The toxicity and biotransformation of single doses of acetaminophen in dogs and cats.** *Toxicol Appl Pharmacol* 1984; 74: 26-34.

7 Valtolina C and Favier RP. **Feline hepatic lipidosis.** *Vet Clin North Am Small Anim Pract* 2017; 47: 683-702.

8 Verbrugghe A and Bakovic M. **Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis.** *Nutrients* 2013; 5: 2811-2835.

9 Jomova K and Valko M. **Advances in metal-induced oxidative stress and human disease.** *Toxicology* 2011; 283: 65-87.

10 Lawen A. **Apoptosis—an introduction.** *Bioessays* 2003; 25: 888-896.

11 Reed JC. **Mechanisms of apoptosis.** *Am J Pathol* 2000; 157: 1415-1430.

12 Özören N and El-Deiry WS. **Defining characteristics of Types I and II apoptotic cells in response to TRAIL.** *Neoplasia (New York, NY)* 2002; 4: 551-557.

13 Malhi H and Gores GJ. **Cellular and molecular mechanisms of liver injury.** *Gastroenterology* 2008; 134: 1641-1654.

14 Zhong H-H, Hu S-J, Yu B, et al. **Apoptosis in the aging liver.** *Oncotarget* 2017; 8: 102640-102652.

15 Kumar V, Kalita J, Bora HK, et al. **Relationship of antioxidant and oxidative stress markers in different organs following copper toxicity in a rat model.** *Toxicol Appl Pharmacol* 2016; 293: 37-43.

16 Paradis V, Kollinger M, Fabre M, et al. **In situ detection of lipid peroxidation by-products in chronic liver diseases.** *Hepatology* 1997; 26: 135-142.

17 Abdelazeim SA, Shehata NI, Aly HF, et al. **Amelioration of oxidative stress-mediated apoptosis in copper oxide nanoparticles-induced liver injury in rats by potent antioxidants.** *Sci Rep* 2020; 10: 1-14.

18 Liu H, Guo H, Jian Z, et al. **Copper induces oxidative stress and apoptosis in the mouse liver.** *Oxid Med Cell Longev* 2020; 2020.

19 Favier RP, Spee B, Schotanus BA, et al. **COMMD1-deficient dogs accumulate copper in hepatocytes and provide a good model for chronic hepatitis and fibrosis.** *PLoS One* 2012; 7: e42158.

20 Fragkou FC, Adamama-Moraitou KK, Poutahidis T, et al. **Prevalence and clinicopathological features of triaditis in a prospective case series of symptomatic and asymptomatic cats.** *J Vet Intern Med* 2016; 30: 1031-1045.

21 Center SA. **Feline hepatic lipidosis.** *Vet Clin North Am Small Anim Pract* 2005; 35: 225-269.

22 Whittemore JC, Newkirk KM, Reel DM, et al. **Hepatic copper and iron accumulation and histologic findings in 104 feline liver biopsies.** *J Vet Diagn Invest* 2012; 24: 656-661.

23 Center SA, McDonough SP and 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.

24 Puls R. Mineral Levels in Animal Health: Diagnostic Data. Clearbrook, Canada: Sherpa International, 1988.

25 Vince A, Hayes M, Jefferson B, et al. **Hepatic injury correlates with apoptosis, regeneration, and nitric oxide synthase expression in canine chronic liver disease.** *Vet Pathol* 2014; 51: 932-945.

26 Guéraud F, Atalay M, Bresgen N, et al. **Chemistry and biochemistry of lipid peroxidation products.** *Free Radic Res* 2010; 44: 1098-1124.

27 Hartley DP, Kolaja KL, Reichard J, et al. **4-Hydroxynonenal and malondialdehyde hepatic protein adducts in rats treated with carbon tetrachloride: immunochemical detection and lobular localization.** *Toxicol Appl Pharmacol* 1999; 161: 23-33.

28 Strickland JM, Lyman D, Sordillo LM, et al. **Effects of super nutritional hepatic copper accumulation on hepatocyte health and oxidative stress in dairy cows.** *Vet Med Int* 2019; 2019.

29 Lyman D, Clark L and Campbell K. **Copper accumulation in Wisconsin Holsteins with indications of oxidative liver damage.** *J Vet Med Res* 2015; 2: 1021.

30 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.

31 Khan MF, Wu X, Tipnis UR, et al. **Protein adducts of malondialdehyde and 4-hydroxynonenal in livers of iron loaded rats: quantitation and localization.** *Toxicology* 2002; 173: 193-201.

32 Schultheiss PC, Bedwell CL, Hamar DW, et al. **Canine liver iron, copper, and zinc concentrations and association with histologic lesions.** *J Vet Diagn Invest* 2002; 14: 396-402.

33 Ramos-Vara J and Miller M. **When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry—the red, brown, and blue technique.** *Vet Pathol* 2014; 51: 42-87.

6. CONCLUSIONS

6.1. Summary

To date, there are only few case reports and studies regarding hepatic copper accumulation in cats.[1-4] Cats with presumed primary copper-associated hepatopathy (PCH) were reported to have high hepatic copper concentrations (>700 µg/g dry weight liver). The localization of copper accumulation in the liver of cats was similar to that in the liver of dogs.[4] Recently, a mutation of *ATP7B* has been identified in cats with presumed PCH.[5, 6] Single nucleotide variations (SNVs) were found in this gene and one of the SNVs was predicted to affect the function of ATP7B protein.[6] However, investigation of a larger number of cats with PCH is required to determine the effects of mutations of this copper transporter gene in cats.

The studies reported in this dissertation aimed to provide more information regarding copper accumulation and the consequences of excessive copper accumulation in liver specimens from cats. Among 121 archived specimens, we found 31% of the specimens had copper concentrations above the upper limit of the reference interval (ULRI: 180 µg/g dry weight liver).[7] Out of these specimens, only 11% stained positive for copper and only three specimens had copper scores of ⩾3. A weak correlation between copper staining scores and copper concentrations was identified. Our findings indicate that copper concentrations above the ULRI can be commonly found in cats, regardless of histopathological changes in the liver. None of the specimens we evaluated likely were having PCH based on the histopathological changes observed and hepatic copper concentrations. However, these archived specimens were evaluated at a single time point.

It is possible that these specimens were collected during an initial stage of copper-associated hepatopathy (CAH). Copper concentrations can be increased without any overt histological hepatic changes in this phase.[8] It is also possible that other factors, such as diet, may influence the copper concentration in these specimens. Lacking the full clinical history of these cats was a limitation that stopped us from investigating such other possible factors. Another point that should be taken into consideration for future studies is the RI. This RI was first reported in a textbook published in 1988. There was only one study of dietary copper in 35 cats (25-week-old) mentioned in this book.[9] Thus, it is still unclear how exactly the RI was obtained and a contemporary RI in a well-characterized group of cats is therefore needed.

We also found a discrepancy between the two modalities for hepatic copper assessment (rhodanine staining and quantification). The weak correlation that we found might be related to the methodology of copper assessment techniques.[10-12] FAAS, which was used to quantify copper concentrations, uses a flame to evaporate and disassociate copper elements into atoms. After the atomization, copper atoms absorb the radiation, and the absorption is detected and later calculated into a concentration.[10] In contrast, rhodanine is a chelating agent with a strong affinity for proteinaceous copper deposits.[11] It has been suggested that rhodanine detects proteins bound to copper rather than the copper itself.[8] Further investigation of the relationship between copper staining and copper concentrations in cats is needed to clarify this speculation. Our findings support that the assessment of hepatic copper accumulation should not solely rely only on rhodanine staining or copper quantification. These two techniques should ideally be performed together.[13]

The assessment of hepatic copper in specimens from cats from three geographical locations showed that specimens from cats from Malaysia had higher copper concentrations compared to those from the United States or Greece. Studies of heavy metals in Malaysia reported that copper and other heavy metals were found in the soil and water due to human pollution from chemical industry, agriculture, and mining.[14] Although the level of copper did not exceed the permissible limit for human consumption,[15, 16] this copper contamination might enter the food chain of other species, including cats. Furthermore, the specimens from Malaysia were collected from feral cats that may have consumed natural diets, such as rodents, reptiles, or fish, that were reported to have copper content two to three times higher than the recommended level for growth and maintenance.[17] Therefore, it is possible that environmental contamination and consumption of a natural diet may have played a role in the high hepatic copper concentrations observed in these specimens from Malaysia. Our findings suggest that the RI may not only need to be re-evaluated but that the RI should also be established for different geographical regions.

In our study, many specimens that had copper concentrations above the ULRI were negative for copper staining. These specimens were of FFPE, formalin-fixed, or frozen tissues. Some formalin-fixed specimens were stored in 10% neutral formalin for approximately one year or more. Some of the frozen specimens were found to have freezing artifacts. It is possible that the storage and processing conditions of tissue specimens may have affected the histopathological and qualitative copper analyses. Further studies investigating potential factors that may influence copper staining assessment are needed.

The intracellular distribution of copper has been investigated in humans, rats, and dogs. This investigation can provide an important insight into the pathophysiology of CAH. In healthy individuals, copper is mainly distributed in the cytosol.[18] Under copper-loaded conditions, copper accumulates in these species in the large granule fraction, particularly in lysosomes.[19-21] We assessed the distribution of copper in liver specimens from cats with hepatic copper concentrations below and above the ULRI. Among these 29 specimens, only one specimen stained positive for copper on a rhodanine-stained slide. Regardless of hepatic copper concentration, we found the highest abundance of copper in the cytosolic fraction, followed by nuclear, large granule, and microsomal fractions (decreasing in order of abundance). As it seems unlikely that we had cat specimens with PCH in this experiment, a redistribution of copper in PCH cannot be ruled out. Our findings of the intracellular distribution of copper might explain the failure to detect copper histologically using rhodanine stain. A study in humans with Wilson's disease suggested that localization of hepatic copper may affect the positive rate of histological copper staining.[22] The study reported that the rate of copper staining was low during the early stages of the disease and the rate was higher in human patients with late-stage disease. Human patients in the asymptomatic phase had the highest distribution of copper in the cytosol. The copper was dense in lysosomes in patients with late-stage phase. The researchers presumed that the diffusely distributed copper in the cytoplasm could not be detected with copper stains because the concentration was lower than the threshold of detection. Our study found one cat specimen with positive copper staining. With this proposed speculation, we had expected to see the highest proportion in the large granule fraction that represented hepatic lysosomes. However, the distribution of copper in this

specimen was highest in the cytosol. It is possible that the lysosomal copper concentration was high enough to result in positive staining even though the distribution of copper was similar to that of cats with copper concentrations below the ULRI. A threshold of lysosomal copper concentration for positive rhodanine staining could not be determined from these studies. Further studies investigating the relationship between the intracellular localization of copper and the rate of histological copper staining are needed.

In the last study, involving three immunohistochemical markers, we used the expression of 4-HNE and MDA to assess oxidative stress and Casp-3 to assess apoptosis in liver specimens from cats. In this study, we did not identify a significant difference in the immunohistochemical expression of these markers among groups of specimens with liver diseases, neither between specimens with and without significant hepatic changes. No correlations between these three markers and hepatic copper were found in this species. Furthermore, only a weak correlation between MDA immunostaining and iron staining score was identified. Thus, our work did not support the utility of these three immunohistochemical markers in cats with liver disease. Although 4-HNE and MDA are lipid peroxidation by-products that are commonly used to detect oxidative stress, the products of lipid peroxidation can be generated in other forms, such as propanal and hexanal.[23] It is possible that there may have been oxidative stress induced by copper but 4-HNE and MDA might not be the main forms of lipid peroxidation produced in cats. There may also have been apoptotic activity but it had not yet reached the step of Casp-3 activation or the specimens enrolled may not have had significant apoptotic activity. Further investigations regarding other potential markers that can be used to determine oxidative stress and apoptosis in cats are warranted.

6.2. References

1 Haynes JS and Wade PR. **Hepatopathy associated with excessive hepatic copper in a Siamese cat.** *Vet Pathol* 1995; 32: 427-429.

2 Meertens NM, Bokhove CAM and Van Den Ingh TSGAM. **Copper-associated chronic hepatitis and cirrhosis in a European Shorthair cat.** *Vet Pathol* 2005; 42: 97-100.

3 Whittemore JC, Newkirk KM, Reel DM, et al. **Hepatic copper and iron accumulation and histologic findings in 104 feline liver biopsies.** *J Vet Diagn Invest* 2012; 24: 656-661.

4 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.

5 Asada H, Kojima M, Nagahara T, et al. **Hepatic copper accumulation in a young cat with familial variations in the ATP7B gene.** *J Vet Intern Med* 2019; 33: 874-878.

6 Asada H, Chambers JK, Kojima M, et al. **Variations in ATP7B in cats with primary copper-associated hepatopathy.** *J Feline Med Surg* 2020; 22: 753-759.

7 Puls R. Mineral Levels in Animal Health: Diagnostic Data. Clearbrook, Canada: Sherpa International, 1988.

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

9 Doong G, Keen CL, Rogers Q, et al. **Selected features of copper metabolism in the cat.** *J Nutr* 1983; 113: 1963-1971.

10 Brown RJ and Milton MJ. **Analytical techniques for trace element analysis: an overview.** *Trends Analyt Chem* 2005; 24: 266-274.

11 Luxama JD, Carroll MA and Catapane EJ. **Effects of potential therapeutic agents on copper accumulations in gill of Crassostrea virginica.** *In Vivo* 2010; 31: 34.

12 Lindquist R. **Studies on the pathogenesis of hepatolenticular degeneration. II. Cytochemical methods for the localization of copper.** *Arch Pathol* 1969; 87: 370-379.

13 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.

14 Yunus K, Zuraidah MA and John A. **A review on the accumulation of heavy metals in coastal sediment of Peninsular Malaysia.** *Ecofeminism Clim Change* 2020; 1: 21-35.

15 Taweel A, Shuhaimi-Othman M and Ahmad A. **Heavy metals concentration in different organs of tilapia fish (Oreochromis niloticus) from selected areas of Bangi, Selangor, Malaysia.** *Afr J Biotechnol* 2011; 10: 11562-11566.

16 Taweel A, Shuhaimi-Othman M and Ahmad A. **Evaluation of copper, lead and arsenic level in tilapia fish in Cempaka Lake (Bangi, Malaysia) and human daily/weekly intake.** *Biologia* 2013; 68.

17 Plantinga EA, Bosch G and Hendriks WH. **Estimation of the dietary nutrient profile of free-roaming feral cats: possible implications for nutrition of domestic cats.** *Br J Nutr* 2011; 106 S35-S48.

18 Smeyers-Verbeke J, May C, Drochmans P, et al. **The determination of Cu, Zn, and Mn in subcellular rat liver fractions.** *Anal Biochem* 1977; 83: 746-753.

19 Gregoriadis G and Sourkes TL. **Intracellular distribution of copper in the liver of the rat.** *Can J Biochem* 1967; 45: 1841-1851.

20 Porter H. **Tissue copper proteins in Wilson's disease: intracellular distribution and chromatographic fractionation.** *Arch Neurol* 1964; 11: 341-349.

21 Kawamura M, Takahashi I and Kaneko J. **Ultrastructural and kinetic studies of copper metabolism in Bedlington Terrier dogs.** *Vet Pathol* 2002; 39: 747-750.

22 Goldfischer S and Sternlieb I. **Changes in the distribution of hepatic copper in relation to the progression of Wilson's disease (hepatolenticular degeneration).** *Am J Pathol* 1968; 53: 883-901.

23 Ayala A, Muñoz MF and Argüelles S. **Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal.** *Oxid Med Cell Longev* 2014; 2014.

APPENDIX 1

LIST OF SPECIMENS WITH POSITIVE COPPER STAINING FOR CHAPTER 2

List of specimens with positive copper staining (n = 13) and the information of individual histopathological analysis, hepatic copper concentration, copper score and the area of copper distribution

Liver specimens	**Histopathological analysis of liver specimens**	**Hepatic copper concentration (µg/g dry liver weight)**	**Copper staining score**	**Area of copper accumulation**
1	No significant changes	391	1	Periportal
2	No significant changes	98	1	Centrilobular
3	No significant changes	425	1	Centrilobular and midzonal
4	No significant changes	447	2	Periportal
5	No significant changes	227	3	Centrilobular
6	Hepatic steatosis	421	1	Random
7	Hepatitis with bacterial peritonitis	363	1	Centrilobular
8	Pyogranulomatous hepatitis	370	1	Periportal

9	Histoplasmosis	269	1	Midzonal
10	Histoplasmosis	330	1	Midzonal
11	Randomly distributed hepatitis	787	3	Centrilobular and midzonal
12	Histoplasmosis	2010	4	Centrilobular and midzonal
13	Neoplasia (myelolipoma)	590	1	Centrilobular

n/a: not applicable

APPENDIX 2

LIST OF SPECIMENS WITH COPPER CONCENTRATIONS ABOVE THE UPPER LIMIT OF THE REFERENCE INTERVAL FOR CHAPTER 2

List of specimens with copper concentrations above the upper limit of the RI (ULRI; n = 37) and the corresponding information of histopathological analysis, hepatic copper concentration, copper score and the area of copper distribution

Liver specimens	**Histopathological analysis of liver specimens**	**Hepatic copper concentration (µg/g dry liver weight)**	**Copper staining score**	**Area of copper accumulation**
1	No significant changes	192	0	n/a
2	No significant changes	186	0	n/a
3	No significant changes	389	0	n/a
4	No significant changes	298	0	n/a
5	No significant changes	215	0	n/a
6	No significant changes	391	1	Periportal
7	No significant changes	188	0	n/a
8	No significant changes	461	0	n/a
9	No significant changes	217	0	n/a
10	No significant changes	208	0	n/a

11	No significant changes	182	0	n/a
12	No significant changes	423	0	n/a
13	No significant changes	202	0	n/a
14	No significant changes	227	3	Centrilobular
15	No significant changes	329	0	n/a
16	No significant changes	485	0	n/a
17	No significant changes	425	1	Centrilobular and midzonal
18	No significant changes	447	2	Periportal
19	No significant changes	229	0	n/a
20	Hepatic steatosis	421	1	Random
21	Hepatic steatosis	201	0	n/a
22	Hepatic steatosis	213	0	n/a
23	Histoplasmosis	440	0	n/a
24	Hepatitis with bacterial peritonitis	363	1	Centrilobular
25	Pyogranulomatous hepatitis	370	1	Periportal
26	Randomly distributed hepatitis	787	3	Centrilobular and midzonal
27	Pyogranulomatous hepatitis	339	0	n/a
28	Histoplasmosis	269	1	Midzonal

29	Histoplasmosis	226	0	n/a
30	Hepatitis	222	0	n/a
31	Histoplasmosis	2010	4	Centrilobular and midzonal
32	Histoplasmosis	330	1	Midzonal
33	Hepatitis with fibrinous serositis	243	0	n/a
34	Hepatitis	253	0	n/a
35	Neoplasia (cystadenoma)	232	0	n/a
36	Neoplasia (cystadenoma)	220	0	n/a
37	Neoplasia (myelolipoma)	590	1	Centrilobular

n/a: not applicable

APPENDIX 3

SCORING SYSTEM FOR HISTOPATHOLOGICAL CLASSIFICATION OF LIVER SPECIMENS FOR CHAPTER 4

A modified scoring system for liver specimens based on inflammatory cell accumulation and percentages of hepatocytes showing lipid accumulation

Score	**Accumulation of inflammatory cells**
0	None
1	Mild
2	Mild to moderate
3	Moderate
4	Severe
Score	**Percentage of hepatocytes with lipid accumulation**
0	<80%
1	⩾80%

APPENDIX 4

LIST OF SPECIMENS WITH COPPER CONCENTRATIONS BELOW THE UPPER LIMIT OF THE REFERENCE INTERVAL FOR CHAPTER 4

List of specimens with copper concentrations below the upper limit of the reference interval (ULRI; n = 13). The information of individual histopathological analysis and overall hepatic copper concentration

Liver specimens	**Histopathological analysis of liver specimens**	**Hepatic copper concentration (µg/g dry weight)**
Case 1	No significant changes	135
Case 2	No significant changes	70
Case 3	Hepatic steatosis	92
Case 4	Hepatic inflammation	171
Case 5	No significant changes	102
Case 6	No significant changes	171
Case 7	No significant changes	113
Case 8	No significant changes	94
Case 9	No significant changes	62
Case 10	No significant changes	116
Case 11	No significant changes	143
Case 12	No significant changes	125
Case 13	No significant changes	37

APPENDIX 5

LIST OF SPECIMENS WITH COPPER CONCENTRATIONS ABOVE THE UPPER LIMIT OF THE REFERENCE INTERVAL FOR CHAPTER 4

List of specimens with copper concentrations above the upper limit of the reference interval (ULRI; n = 16). The information of individual histopathological analysis and overall hepatic copper concentration

Liver specimens	**Histopathological analysis of liver specimens**	**Hepatic copper concentration (µg/g dry weight)**
Case 14	Hepatic steatosis	309
Case 15	No significant changes	614
Case 16	No significant changes	289
Case 17	No significant changes	357
Case 18	No significant changes with positive copper staining (score of 2)	728
Case 19	No significant changes	547
Case 20	No significant changes	777
Case 21	No significant changes	204
Case 22	No significant changes	325
Case 23	No significant changes	251
Case 24	Hepatic inflammation	395
Case 25	No significant changes	234

Case 26	No significant changes	282
Case 27	No significant changes	226
Case 28	No significant changes	265
Case 29	No significant changes	480