CANINE CHRONIC HEPATITIS:

DIAGNOSTIC EVALUATION AND COMPLICATING SYNDROMES

A Dissertation

by

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ABSTRACT

Chronic hepatitis is an important disease in dogs that can lead to hepatic fibrosis, portal hypertension, and hepatic encephalopathy. Histological assessment of liver biopsy specimens is currently required to definitively diagnose chronic hepatitis and hepatic fibrosis. To evaluate inter-observer agreement arising from the assessment of canine hepatic fibrosis and necroinflammatory activity, six pathologists assigned scores to histological sections of canine livers. To assess the diagnostic utility of extracellular components as serum markers of hepatic fibrosis, dogs with hepatobiliary disease and healthy dogs were enrolled. For the dogs with hepatobiliary disease hepatic fibrosis was histologically scored. To assess the utility of urine N-methylhistamine as a marker of mast cell mediated inflammation, urine N-methylhistamine to creatinine ratios were measured in dogs with hepatobiliary disease and healthy control dogs. Urine N-methylhistamine to creatinine ratios were compared to hepatic mast cell counts in dogs with hepatobiliary disease. To elucidate the relationship between plasma ammonia concentration and severity of hepatic encephalopathy, and to determine whether factors that precipitate hepatic encephalopathy in humans are associated with the presence of hepatic encephalopathy in dogs previously treated for the disease, the medical records of dogs diagnosed with hepatic encephalopathy were retrospectively reviewed.

There was fair and poor agreement between pathologists assessing hepatic fibrosis and necroinflammation, respectively. This suboptimal agreement needs to be taken into account by clinicians making decisions based on hepatic histopathology

reports. Despite their diagnostic utility for diagnosing hepatic fibrosis in humans the results of this work do not support the utility of the extracellular matrix components studied here to discriminate between dogs with and without hepatic fibrosis. A subset of dogs with hepatobiliary disease had mildly increased urine N-methylhistamine to creatinine ratios, suggesting mast cell mediated inflammation. However, there was no correlation between urine N-methylhistamine to creatinine ratio and hepatic mast cell counts. Severity of hepatic encephalopathy was not significantly correlated with plasma ammonia concentrations and none of the putative precipitating factors for hepatic encephalopathy were associated with the presence of clinical signs of the disease at hospital admission. Further work is needed to better define the pathogenesis of canine hepatic encephalopathy.

DEDICATION

   I dedicate this thesis to my late father Dr. David Patrick Gavin Lidbury whose commitment to lifelong learning was inspirational to me.

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NOMENCLATURE

APSC   Acquired portosystemic collaterals

CH   Chronic hepatitis

CI   Confidence interval

CPSS   Congenital portosystemic shunt

ELISA   Enzyme-linked immunosorbent assay

FP   Fibrosed proportion

HA   Hyaluronic acid

HE   Hepatic encephalopathy

H&E   Hematoxylin & eosin

hpf   High power fields

NMH   N-methylhistamine

OR   Odds ratio

PIIINP   Procollagen type III N-terminal peptide

r_s Spearman's rank correlation coefficient

SIRS   Systemic inflammatory response syndrome

SD   Standard deviation

TIMP   Tissue inhibitor of matrix metalloproteinase

κ Cohen's kappa statistic

κ' Cohen's weighted kappa statistic

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Importance and background

Chronic hepatitis (CH) is a relatively common disease in dogs that can lead to a variety of clinical signs and complicating syndromes.[1] In a study of dogs undergoing necropsy for a variety of reasons, 12% had changes consistent with CH.[2] Left untreated, or in some cases despite treatment, chronic hepatitis is progressive and can ultimately lead to death or euthanasia of the patient. The mean post-biopsy survival times for dogs with idiopathic or copper associated CH were 4.1 months or 8.1 months, respectively.[3] Liver biopsy is needed to definitively diagnose CH but, even after this invasive procedure, for about two thirds of these dogs, no underlying cause can be identified.[3] Much remains to be discovered about the etiopathogenesis of this disease and its complicating syndromes. Additionally, the diagnostic tests currently used by the veterinary profession to diagnose and monitor dogs with CH all have considerable limitations.[4] The development of novel non-invasive tests for the evaluation of patients with this condition and the development of a better understanding of the clinical ramifications of hepatic histological changes are priorities for investigators in the field of canine hepatology.

Etiopathogenesis of chronic hepatitis

Chronic hepatitis is a syndrome that is histologically characterized by hepatocellular necrosis or apoptosis, a mononuclear or mixed inflammatory infiltrate, regeneration, and fibrosis.[5] A variety of factors can lead to hepatic injury and inflammation in dogs, including: drugs (e.g. phenobarbital), toxins (e.g., cycads, aflatoxins, *Amanita phalloides,* and blue-green algae), infectious agents (e.g., *Leptospira* spp., canine adenovirus-1, *Heterobilharzia americana*, and Bartonella sp.), hepatic copper accumulation, and possibly autoimmune disease.[6] However, some of these factors, such as *Amanita phalloides* intoxication, are more likely to cause acute liver injury than CH. In most dogs with CH no underlying cause can be identified and the condition is termed idiopathic CH.[3,7]

Hepatic copper accumulation is identified as the underlying cause in approximately one third of dogs with CH.[3] The liver is the principal recipient of copper that is absorbed from the gastrointestinal tract. Free copper ions can lead to the formation of hydroxyl radicals and subsequently cause oxidative damage to the liver.[8] Copper can accumulate in the liver due to defects in copper metabolism, cholestasis, or possibly increased dietary copper intake.[9,10] The following breeds of dogs are proven or suspected to be predisposed to primary copper accumulation: Bedlington Terrier, West Highland White Terrier, Scottish Terrier, Skye Terrier, Labrador Retriever, Dalmatian, and Doberman Pincher.[8] In the Bedlington Terrier a mutation of the COMMD1 gene that encodes a cellular copper exporter has been identified.[11] It is also possible to diagnose copper associated CH in other dog breeds and in mixed breed dogs. Primary copper

accumulation tends to occur in the centrilobular zones of the liver. In contrast, when copper accumulates secondary to cholestasis it tends be found in the periportal zones of the liver and hepatic copper concentrations are typically lower than those found in dogs with primary hepatic copper accumulation.[8,9]

As previously discussed CH can lead to a number of serious complications. Chronic inflammation of the liver can lead to fibrosis, which can diminish hepatic function as hepatocytes are replaced by collagen and can contribute to the development of portal hypertension.[1] As the liver has a large functional reserve capacity, loss of liver function is detected relatively late in the course of CH. Portal hypertension and decreased hepatic synthesis of albumin contribute to the development of ascites, which is a poor prognostic indicator in these dogs.[12] This is probably because ascites occurs late in the course of CH and usually signifies that irreversible changes to the portal circulation have occurred. Hepatic portal hypertension can also lead to the development of acquired portosystemic collateral blood vessels (APSC).[1] These allow ammonia rich blood from the splanchnic circulation to bypass the liver, which in turn may lead to hepatic encephalopathy (HE). Dogs with hepatic disease also seem to be prone to gastrointestinal ulceration and erosion, possibly because of the effects of portal hypertension.[13]

Hepatic fibrosis

Fibrosis is the deposition of excess fibrous connective tissue in an organ or tissue.[14] Collagen deposition is an essential part of the natural wound healing process,

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but this normal mechanism of tissue repair can become pathological. Although

previously thought to be permanent, extracellular matrix deposition is a dynamic process

and, because of this, fibrosis is a potentially reversible phenomenon.[15] The development

of hepatic fibrosis is an important event in the progression of liver disease and has been

shown to be a negative prognostic factor in humans with chronic hepatitis.[16] A variety of

disease processes can lead to hepatic fibrosis including: reactions to drugs and toxins,

infectious diseases, autoimmune disease, vascular disease, and biliary obstruction. The

commonality amongst these diseases is the development of chronic hepatic

inflammation.[17]

Extracellular matrix is the scaffolding that holds tissues together. In health there

is a balance between matrix deposition and removal. In contrast, in fibrosis there is an

imbalance where the rate of accumulation of matrix exceeds the rate of removal.[14] The

matrix consists of macromolecules such as collagens, non-collagen glycoproteins,

matrix-bound growth factors, glycosaminoglycans, proteoglycans, and matricellular

proteins.[17] In a fibrotic liver there is a marked increase of collagen content, including the

fibril forming collagens (types I, II, and V) and the non-fibril forming collagens (types

IV and VI).[17]

Extracellular matrix is produced by activated myofibroblast cells and activation

of these cells is the key step in the development of fibrosis.[14] In the liver activated

hepatic stellate cells are an important source of extracellular matrix.[17] Quiescent hepatic

stellate cells store vitamin A (retinoid) and are found in the subendothelial space

between hepatocytes and sinusoidal epithelial cells.[14] Portal fibrocytes and bone marrow

derived fibrocytes can also contribute to matrix production in the liver.[18] The relative role that each of these potential sources of myofibroblasts plays in the development of hepatic fibrosis have been difficult to determine, in part because of the limitations of immunohistochemical markers in identifying the origins of myofibroblasts.[18] However, recent studies using genetic markers suggest that hepatic stellate cells are the major contributor.[19] Also, it is now accepted that, although epithelial mesenchymal transition can occur in the liver, it does not lead to the development of true myofibroblasts.[20]

As well as increased production of connective tissue by activated hepatic stellate cells, alterations in the mechanisms responsible for breakdown of extracellular matrix are important in the development of hepatic fibrosis.[21] Early in the course of fibrosis degradation of the normal hepatic matrix occurs.[22] Later in the course of disease, failure to degrade the excess extracellular matrix leads to progression of fibrosis. In patients with progressive fibrosis the activity of matrix-metallopreoteinase-1, which can degrade type I collagen, is diminished because of increased expression of tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2.[22,23]

Diagnosis of hepatic fibrosis

Currently the only way to diagnose hepatic fibrosis in dogs is by histological assessment of a liver biopsy specimen. However, the collection of a liver biopsy is by nature invasive, there is a risk of hemorrhage,[24] and as only a small portion of the organ is evaluated, this technique is susceptible to sampling variation.[25] The expense and risk associated with this procedure means that longitudinal assessment of hepatic fibrosis is

rarely performed in canine patients or in a research setting. Because of these disadvantages serum markers and imaging techniques that allow the assessment of hepatic fibrosis have been developed for use in humans[26], and if they could be utilized for dogs as well, they would be useful, especially for monitoring response to treatment.

In human patients with hepatic fibrosis an increased rate of extracellular matrix turnover results in the release of matrix components into the bloodstream.[26] Hyaluronic acid (HA) is a glycosaminoglycan component of the extracellular matrix that is used as a serum biomarker of hepatic fibrosis in humans.[26] In one study of humans with chronic hepatitis C, the sensitivity and specificity of HA for distinguishing between patients with extensive fibrosis and those with milder fibrosis were 86% and 70%, respectively.[27] Hyaluronic acid has previously been shown to be increased in dogs with hepatic disease including cirrhosis[28] and congenital portosystemic shunts (CPSS).[29] However, the ability of serum HA measurement to differentiate among dogs with different histological stages of hepatic fibrosis has not previously been reported.

Also, in another study of human patients with chronic hepatitis C, measurement of serum procollagen type III N-terminal peptide (PIIINP) concentrations had a sensitivity of 92% and a specificity of 76% for differentiating between those patients with extensive fibrosis and those with milder fibrosis.[27] Serum PIIINP concentrations were increased in growing dogs but not in dogs with chronic bronchopulmonary disease.[30] Serum PIIINP concentrations have not previously been assessed in dogs with hepatobiliary disease.

Tissue inhibitor of matrix metalloproteinase-1 is a protein that inhibits the action of matrix-metalloproteinase-1, thus slowing the degradation of extracellular matrix during fibrosis.[22] In yet another study of human patients with chronic hepatitis C, measurement of serum TIMP-1 concentration had a sensitivity of 75% and a specificity of 70% for differentiating between patients with extensive fibrosis and those with milder fibrosis.[27] To the author's knowledge serum TIMP-1 concentrations have not previously been assessed in dogs with hepatobiliary disease.

Hepatic histological scoring

In human medicine several grading systems for the assessment of hepatic necroinflammatory activity and fibrosis have been developed and are used in clinical patients with chronic hepatitis.[31] For example, the Ishak system is commonly used to score necroinflammatory activity and fibrosis in human liver biopsy specimens.[32] The grade of necroinflammatory activity is derived from separate scores for periportal interface hepatitis, confluent necrosis, focal lytic necrosis, and periportal necrosis. This gives clinicians important information about how active the underlying disease process is. The stage of fibrosis is scored from 0 (no fibrosis) to 6 (cirrhosis/severely fibrosed liver).[32] This gives clinicians important information regarding the chronicity of the disease process. Despite the extensive use of histological scoring systems in human hepatology, there currently is no widely accepted system for use with dogs. Recently, several studies in dogs have used a histological scoring system that was adapted from the Ishak system. [33,34] This modified system was devised by Drs. van den Ingh, Grinwis, and

Rothuizen from the University of Utrecht. Following this protocol necroinflammatory activity is graded between 0 (absent) to 5 (very marked), fibrosis is graded between 0 (absent) to 4 (very marked) and vacuolar change is graded between 0 (absent) to 3 (severe; Table 1).

It has been proposed that histological scoring system should fulfill several criteria including: acceptable inter-observer agreement, repeatability when the same observer performs scoring on different occasions, ease of use, and clinical relevance.[31,32] Poor agreement between pathologists grading the severity of inflammation of histological sections prepared from intestinal biopsies collected from dogs has previously been documented.[35,36] Such disagreement between observers using a scoring system complicates the interpretation of these scores. This lack of agreement limits the clinical utility of examination of intestinal biopsies for the diagnosis of canine enteropathies. To the author's knowledge the inter-observer agreement associated with the histological scoring of fibrosis, necroinflammation, and vacuolar change for canine hepatic biopsy specimens has not yet been reported.

Hepatic encephalopathy

Hepatic encephalopathy is defined as the presence of neuropsychiatric abnormalities in patients with hepatic dysfunction after exclusion of other known brain disease.[37] In humans, HE is divided into three types according to etiology.[37] Type-A HE is due to acute liver failure in the absence of pre-existing liver disease. Type-B HE is associated with portal systemic bypass without intrinsic hepatocellular disease, for

Table 1. Histological grading and staging system for canine chronic hepatitis

Activity (grade)

Grade	Periportal or periseptal interface hepatitis	Focal lytic necrosis	Confluent necrosis
Absent (0)	Absent	Absent	Absent
Minimal (1)	Very mild	1 focus per 10x objective	Absent
Mild (2)	Mild	2-4 foci per 10x objective	Absent
Moderate (3)	Moderate	5-10 foci per 10x objective	Absent
Marked (4)	Marked	>10 foci per 10x objective and/or➔	Confluent or bridging necrosis
Very marked (5)	Marked	>10 foci per 10x objective and/or➔	Bridging or panacinar/multiacinar necrosis

Degree of fibrosis (stage)

Stage	Fibrosis	Bridging fibrosis	Bridging fibrosis with nodule formation
Absent (0)	Absent	Absent	Absent
Mild (1)	Mild fibrous expansion (periportal or central)	Absent	Absent
Moderate (2)	Moderate fibrous expansion	Some bridging fibrosis (PP, CC, or PC)	Absent
Marked (3)	Marked fibrous expansion	Marked bridging fibrosis (PP, CC, or PC)	Absent
Very marked (4)	Marked fibrous expansion	Marked bridging fibrosis (PP, CC, or PC)	Present

This scoring system was adapted from the human Ishak scoring system by Drs. van den Ingh, Grinwis, and Rothuizen, all from the University of Utrecht.

example, congenital portosystemic shunting (CPSS). Type-C HE is associated with cirrhosis and portal hypertension or acquired portal systemic shunting, and is subcategorized according to duration and characteristics. Episodic HE develops over a short period of time and fluctuates in severity. Persistent HE results in cognitive dysfunction and can be classified as being mild, severe, or treatment-dependent.[37] Covert HE is defined as HE occurring in patients with normal mental and neurological status, but abnormal results on specific psychometric tests.[38]

Ammonia is believed to play a central role in the pathogenesis of HE and, in a study of humans with cirrhosis, there was a moderate positive correlation between total venous plasma ammonia concentration and the grade of HE.[39] Ammonia is believed to lead to glutamine accumulation in astrocytes, with subsequent astrocyte swelling and neurological dysfunction.[1,40] Venous plasma ammonia concentrations are commonly increased in dogs with HE, but it is possible for dogs to have HE and have plasma ammonia concentrations that are within the reference interval.[41] The aforementioned study also found a positive correlation between plasma ammonia concentrations and the severity of HE.[41] Measurement of plasma ammonia concentration, where available, often plays a role in the diagnosis of canine HE. In contrast, in human patients measurement of plasma ammonia concentration is not relied upon to diagnose HE.[40]

Several factors are known to precipitate HE in human patients (Table 2) with at least one factor identified in 88 to 90% of those affected.[42,43] Identifying and addressing these precipitating factors plays an important role in patient management, as individuals with one or more of these factors have a worse prognosis than those without.[42] The most

Table 2. Factors believed to precipitate hepatic encephalopathy in humans

Precipitating factor	Proposed mechanism of action
Sepsis	Inflammatory mediators have a synergistic effect with ammonia, increase blood brain barrier permeability, and lead to altered neurotransmission
Gastrointestinal hemorrhage	Increased protein load and ammoniagenesis
Constipation	Dehydration, electrolyte abnormalities, small intestinal dysbiosis, and bacterial translocation
Excess dietary protein	Increased ammoniagenesis
Dehydration	Electrolyte changes, increased renal ammoniagenesis
Drugs	Sedative agents (benzodiazepines, opioids) cause depression of cerebral function Diuretics (cause electrolyte imbalances, alkalosis and dehydration)
Hypokalemia	Leads to movement of intracellular potassium into the extracellular space, extracellular alkalosis, and trapping of ammonium ions within cells
Hyponatremia	Enhanced astrocyte swelling
Alkalosis	Increased access of ammonia to neurons (due to a shift in in the equilibrium from ammonium ions to ammonia, which can pass freely through cell membranes)
Poor compliance with lactulose therapy	Increased ammoniagenesis
Bowel obstruction	Dehydration, electrolyte abnormalities, small intestinal dysbiosis, and bacterial translocation
Uremia	Increased renal ammoniagenesis
Superimposed hepatic injury	Decreased hepatic conversion of ammonia to urea

commonly reported precipitating factors in humans are gastrointestinal bleeding

(18−76% of patients), constipation (3−52%), diarrhea (12−40%), infection (3−52%),

hypokalemia (9−70%), hyponatremia (25−38%), and excess dietary protein (9−52%).[43-45] HE has been described in dogs with CPSS and in dogs with APSC due to portal

hypertension.[46] Several factors have been proposed to precipitate HE in dogs, including:

gastrointestinal hemorrhage, hypokalemia, hyponatremia, high protein diets, and

alkalosis.[47] In a recent retrospective study of dogs with CPSS, hyperammonemia, and

systemic inflammatory response syndrome (SIRS) were found to be associated with HE

but hyponatremia was not.[48] To the author's knowledge, the association of other putative

precipitating factors and HE has not yet been reported in dogs.

Mast cell mediated inflammation

As previously mentioned, canine CH is characterized by mononuclear or mixed

inflammatory cell infiltrates. The role of mast cell mediated inflammation in canine

hepatobiliary disease is poorly understood and mast cells are not commonly noted during

histological evaluation of healthy or diseased canine liver specimens.

Mast cells are derived from myeloid stem cells and can release a variety of

inflammatory mediators including histamine, serotonin, serine proteases, heparin,

thromboxanes, prostaglandins, leukotrienes, and heparin.[49,50] In humans there is some

evidence to support their role in the development of hepatobiliary disease.[51] Firstly, mast

cells have been shown to be present in hepatic tissue from healthy humans, but also in

those from patients with hepatic disease, including chronic liver disease[52], acute

hepatitis[53], primary biliary cirrhosis[54], hepatocellular carcinoma, and cholangiocarcinoma.[55,56] Patients with chronic hepatitis C virus infection with steatosis had higher mast cell densities than those without.[57,58] Another study of patients with chronic hepatitis C found a positive correlation between mast cell density and fibrosis.[58] *In vitro*, mast cell proteases appear to have a profibrotic effect by stimulating fibroblast proliferation.[59] Furthermore, humans with chronic cholestatic hepatic disease frequently complain of pruritus and one study found that patients with cholestasis had higher plasma histamine concentrations than controls.[60] To the author's knowledge there are few studies that have specifically evaluated the role of mast cells in dogs with hepatobiliary disease.

As mast cell degranulation leads to the release of histamine, which is primarily stored in these cells, this substance has been suggested as a potential marker of mast cell degranulation.[49,50] However, histamine is rapidly metabolized and thus is not a practical biomarker.[49,50] N-methylhistamine (NMH) is generated when histamine is metabolized by N-methyltransferase.[61] N-methylhistamine is stable and a method for its measurement in canine urine and fecal samples has been developed.[61,62]

Current challenges in canine hepatology

As discussed above, much remains to be studied about the pathogenesis, diagnosis, and treatment of CH in dogs. However, some of the most germane challenges in canine hepatology are listed below:

i) The pathogenesis of chronic hepatitis

In humans, the discovery of the role of hepatotrophic viruses in the pathogenesis of chronic hepatitis has made it possible to identify the underlying cause of chronic hepatitis in most humans with chronic hepatitis. Unfortunately, this has not been the case for dogs as for most cases an underlying cause cannot be identified. Part of the problem is that chronic hepatitis is often diagnosed late in the course of disease and by that time there is little chance of identifying the causative agent. Histology can provide a histomorphological diagnosis but this does not necessarily lead to an etiological diagnosis.

Possible investigative approach − This challenge is probably the hardest of all to address as canine chronic hepatitis is probably caused by a variety of underlying causes. Powerful molecular biology techniques may provide some answers. Screening for viruses using polymerase chain reaction with degenerate primers and sequencing with high throughput techniques, followed by the appropriate bioinformatics, may lead to the discovery of a previously unrecognized viral or bacterial etiology. Where breed dispositions for this disease occur, genome wide association studies may help identify genotypes that are at increased risk for chronic hepatitis. Gene expression analysis (RNA sequencing, microarray or quantitative real time PCR, proteomics, and metabolomics may also provide clues as to the pathogenesis of this disease. However, the results of these techniques may reflect what is happening at the time of investigation and not reveal what the inciting cause of injury was.

ii) Interpretation of histomorphological changes of the liver

As mentioned previously, histological assessment of a liver biopsy specimen does not always lead to an etiological diagnosis. Additionally, there is often a disconnection between the histological findings reported and what these findings indicate in terms of diagnosis, prognosis, and treatment for the clinician. This is partly because the terminology used to describe these lesions is applied inconsistently, so the same change may be described with several different names. The standardized terminology recommended by the World Small Animal Veterinary Association liver study group should help with this challenge and reduce such inconsistency in the use of terminology[5] but further standardization would beneficial.

Possible investigative approach − a standardized scoring system for lesions seen in biopsy specimens from dogs with chronic hepatitis could be very beneficial. This would make it easier for clinicians to interpret histology reports, and increase inter-observer agreement between pathologists, and be very useful in clinical trials and other research projects. Several such scoring systems have been described for liver specimens from humans. Since the patterns of fibrosis and inflammation are quite similar in human and canine chronic hepatitis it should be possible to adapt one of these systems. The validity of the system would then need to be determined by assessing inter-observer and intra-observer agreement as well as the prognostic value of the system.

iii) Non-invasive assessment of hepatic fibrosis

Currently the only means of assessing canine hepatic fibrosis is by histological evaluation of a liver biopsy specimen. As discussed, this technique has several limitations. Because repeat hepatic biopsy is rarely performed the lack of longitudinal assessment of hepatic fibrosis in canine patients has proven a major hindrance to performing clinical trials for assessing the clinical utility of potentially antifibrotic drugs. Serial evaluation of hepatic biopsy specimens for fibrosis would also be beneficial for monitoring disease progression and response to therapy for individual patients. Non-invasive markers of hepatic fibrosis would therefore be beneficial in the diagnosis and management of canine CH.

Possible investigative approach − in humans the measurement of serum concentrations of extracellular matrix components such as HA, PIIINP, and TIMP-1 has proved reasonably accurate for the diagnosis of hepatic fibrosis.[27] Assessment of the diagnostic utility of these biomarkers should also be performed in dogs. This would involve measuring the concentrations of these substances in the serum of dogs with and without hepatic fibrosis. Hepatic histology could then be used as a gold standard with which to compare their accuracy.

iv) Clinical trials of agents used to treat chronic hepatitis

There is inadequate data to support the efficacy of many of the drugs and nutraceutical agents currently used to treat chronic hepatitis in dogs. Agents that are a

priority to investigate include glucocorticoids, cyclosporine, s-adenosyl-l-methionine, silymarin, and ursodeoxycholic acid.

Possible investigative approach − Multicenter randomized controlled clinical trials assessing the efficacy of above agents should be performed. The enrolled patients should be well characterized and have appropriate follow up, including repeat biopsy. Accurate non-invasive markers for hepatic fibrosis and inflammation could be beneficial, as they would allow more frequent assessment of these parameters.

Hypotheses and objectives

The following hypotheses were formulated for this study:

i. The use of a histological scoring system in dogs will allow assessment of hepatic necroinflammatory activity, fibrosis, and vacuolar change with a high level of inter-observer agreement

ii. Measurement of serum extracellular matrix component concentrations can serve as clinically useful diagnostic markers for canine hepatic fibrosis

iii. N-methylhistamine is a useful biomarker of mast cell induced hepatobiliary inflammation in dogs.

iv. Factors, such as, hyperammonemia, electrolyte abnormalities, and alkalosis that are known to precipitate HE in humans also precipitate HE in dogs

The objectives to prove or disprove the aforementioned hypotheses are:

i. Assessment of inter-observer agreement associated with the use of a histological scoring system for canine chronic liver disease

    a. To assess the inter-observer agreement associated with the use of a scoring system to evaluate hepatic biopsy specimens for necroinflammatory activity, fibrosis, and vacuolar change

    b. To compare fibrosis scores assigned to sections of the same biopsy specimen stained with hematoxylin and eosin (H&E) and picrosirius red

    c. To compare the fibrosis scores assigned to sections of liver by pathologists with data from computer based image analysis

ii. Serum extracellular matrix components as markers of canine hepatic fibrosis

    a. To perform the initial validation of a commercially available enzyme-linked immunosorbent assay (ELISA) for the measurement of HA in humans for use with canine serum

    b. To measure serum concentrations of HA, PIIINP, and TIMP-1 in dogs with various hepatobiliary diseases

    c. To evaluate the correlation between serum HA, PIIINP, and TIMP-1 concentrations and the severity of hepatic fibrosis in dogs

iii.   Urine N-methylhistamine in dogs with hepatobiliary disease

    a. To compare urine NMH concentration between healthy dogs and dogs with various types of hepatobiliary disease

    b. To evaluate the correlation between urine NMH and hepatic mast cell counts, hepatic fibrosis, as well as hepatic necroinflammatory activity

iv.   Putative precipitating factors for canine HE

    a. To determine if there is a relationship between plasma ammonia concentrations and the severity of HE in dogs

    b. To determine what proportion of dogs with HE are affected by factors known to precipitate HE in humans

    c. To determine if there is an association between any of these factors and the presence of HE at the time of admission to a veterinary teaching hospital

CHAPTER II

INTER-OBSERVER AGREEMENT FOR THE HISTOLOGICAL SCORING OF

THE CANINE LIVER

Introduction

In human medicine several grading systems for the assessment of hepatic necroinflammatory activity and fibrosis have been developed and are applied to patients with chronic hepatitis.[31] The Ishak[32] and METAVIR[63] systems are two such systems that are commonly used to score necroinflammatory activity and fibrosis in liver biopsy specimens collected from human patients with chronic hepatitis. Despite the extensive use of histological scoring systems in the field of human hepatology, there is currently no widely accepted system for use in dogs. Recently, several studies in dogs have used a histological scoring system that was adapted from the human Ishak system by Drs. van den Ingh, Grinwis, and Rothuizen from the University of Utrecht.[33,34] According to this scheme necroinflammatory activity is graded between 0 (absent) to 5 (very marked) and fibrosis is graded between 0 (absent) to 4 (very marked; Table 1).

Histological scoring systems should fulfill several criteria including: acceptable agreement between observers, repeatability when the same observer performs scoring on different occasions, ease of use, and clinical relevance.[31,32]. A lack of inter-observer agreement limits the utility of scoring systems. Poor agreement between pathologists evaluating histological sections of canine intestinal biopsy specimens has previously been documented.[35,36] Previous studies have evaluated agreement in the

histomorphological diagnosis made by different observers assessing needle and wedge canine hepatic biopsy specimens[64], intra-observer agreement for a single pathologists assessing various histological features from hepatic biopsy specimens collected using different sampling techniques[65], and the agreement in histomorphological diagnosis for a single pathologist evaluating different liver lobes from the same dog.[66] To the authors' knowledge the inter-observer agreement associated with the histological scoring of fibrosis and necroinflammation from canine hepatic biopsy specimens has not previously been reported.

Cohen's kappa statistic (κ) is frequently used to estimate the agreement of observers for data on nominal scales and represents the level of agreement between users that is beyond the agreement due to chance alone.[67] A κ of 0 represents no agreement beyond that due to chance and a κ of 1 represents complete agreement. For nominal scoring systems, such as the Ishak system, partial agreement between users may be taken into account.[68] A weighted kappa statistic (κ') does this by assigning weights to the different levels of disagreement.[67]

Computerized image analysis has been used to provide quantification of hepatic fibrosis in humans.[69] This technique entails differentially staining collagen fibers with a histological staining solution, for example picrosirius red. The histological section is then digitized and image analysis software is used to calculate the fibrosed proportion (FP). This technique may allow a more objective quantification of hepatic fibrosis than histological scoring by a pathologist. The FP of histological sections of human liver were shown to correlate with the fibrosis score assigned to those sections by a

pathologist.[69] To the author's knowledge the use of computerized image analysis for the assessment canine hepatic fibrosis has not previously been reported.

The primary objective of this study was to assess the inter-observer agreement associated with the use of a scoring system to evaluate canine liver biopsy specimens for fibrosis. The secondary objectives of this study were to assess the inter-observer agreement associated with the use of a scoring system to evaluate liver biopsy specimens for necroinflammatory activity, to compare fibrosis scores assigned to sections of the same biopsy specimen stained with H&E or picrosirius red, and to compare the fibrosis scores assigned to picrosirius red stained sections of liver by pathologists with data from computer based image analysis.

Materials and methods

Forty paraffin embedded specimens of canine liver with varying degrees of fibrosis and necroinflammatory activity were selected from tissue archives at Texas A&M University. The sections were primarily selected to represent the full severity range of hepatic fibrosis. Ten similar specimens were obtained from the Department of Veterinary Pathobiology at North Carolina State University. Thirty-six dogs had chronic hepatopathies, 11 were considered to be free from liver disease, and three had hepatic changes due to congenial portosystemic shunts. No dogs were euthanized, underwent liver biopsy, or any other procedure for the purpose of this study. Seventeen paraffin embedded blocks contained large wedge biopsies collected at necropsy, 17 contained wedge biopsies collected during laparotomy, 11 contained biopsies collected during

laparoscopy with a biopsy forceps (typically 4 to 6), and 5 contained punch biopsies

collected during laparotomy. We opted to use wedge biopsies collected at necropsy,

laparoscopically collected biopsies, and biopsies collected during laparotomy so that

inter-observer agreement could be assessed using only adequately sized specimens. Two

contiguous 5 to 8 μm sections of liver were cut from the paraffin embedded tissue and

mounted onto separate microscope slides. One section was stained with picrosirius red

(PolySciences) and counter stained with Weigert's iron hematoxylin (PolySciences). The

other section was routinely stained with H&E (Polysciences). Whenever possible the

sections were stained in batches to ensure consistency.

A number from 1 to 100 was randomly assigned to each section, of which 50

were stained with H&E and 50 with picrosirius red. The sections were then relabeled

with this number as their only identifier. A digital image of each of section was captured

using a slide-scanning microscope (Nanozoomer 2.0-HT, Hamamatsu Photonics). Six

board-certified veterinary pathologists evaluated the scanned sections or slides. For the

sections stained with picrosirius red the pathologists scored the stage of fibrosis using a

previously published scoring system.[33,34] For the sections of liver stained with H&E the

pathologists evaluated the stage of fibrosis and the grade of necroinflammatory activity

using the same system. The pathologists were blinded to the identity of the sections they

were assessing, the scores assigned by the other pathologists, and the results of image

analysis.

Image analysis software (ImagePro Premier, v9.1, MediaCybernetics) was used

to calculate the FP of each scanned section stained with Picrosirius red at Texas A&M

University ($n = 40$). Briefly, smart segmentation was used to discriminate the red stained collagen fibers. The FP was not calculated for sections from North Carolina State University as a different staining protocol was used so that the same segmentation settings could not be used. For two sections from Texas A&M University it was not possible to discriminate collagen using the same segmentation settings so these sections were removed from this part of the study. For the remaining 38 sections the FP was calculated as the area of the section stained red (collagen fibers) divided by the total stained area of the section.

To evaluate the inter-observer the agreement κ for each pair of observers was calculated. Kappa and κ' for multiple observers was calculated for each scoring category. Kappa and κ' values were interpreted as follows: poor agreement < 0.20, fair agreement 0.21−0.40, moderate agreement 0.41−0.60, good agreement 0.61−0.80, and very good agreement 0.81−1.00.[67] The agreement between observers was also summarized by calculating the frequency of scores assigned by each of the 15 possible pairs of pathologists. The median fibrosis stage assigned to each case for the sections stained with picrosirius red and H&E was compared using the Wilcoxon signed-rank test. The level for statistical significance was set at $\alpha < 0.05$. Spearman's rank correlation coefficient was used to assess the strength of the relationship between the median fibrosis score assigned to each picrosirius red stained section and FP. Statistical analyses were performed using a commercially available software package (Stata v12, StataCorp).

Results

All 100 sections were deemed to be adequate for analysis by the 6 veterinary pathologists.

Agreement between the pairs of pathologists assigning scores for hepatic fibrosis to sections stained with H&E are presented in Figure 1. The median (minimum−maximum) κ for each pair was 0.41 (0.1−0.56). Multiuser κ (95% CI) was 0.35 (0.26−0.44) and multiuser κ' was 0.59 (0.50−0.70). Assignment of fibrosis scores by the 15 possible pairings of pathologists to sections stained with H&E are presented in Figure 2. The pairs of pathologists were in complete agreement 49% of the time, differed by one score level 41% of the time, and differed by more than 1 score level 11% of the time.

Agreement between the pairs of pathologists assigning scores for hepatic fibrosis to sections stained with picrosirius red are presented in Figure 3. The median (minimum−maximum) κ for each pair was 0.40 (0.22−0.56). Multiuser κ (95% CI) was 0.39 (0.30−0.49) and multiuser κ' was 0.64 (0.55−0.73). Assignment of fibrosis scores by the 15 possible pairings of pathologists to sections stained with picrosirius red are presented in Figure 4. The pairs of pathologists were in complete agreement 53% of the time, differed by one score level 42% of the time, and differed by more than one score level 5% of the time.

Agreement between the pairs of pathologists assigning scores for necroinflammatory activity are presented in Figure 5. The median (min−max) κ for each pair was 0.19 (-0.03−0.40). Multiuser κ (95% CI) for the assessment of

necroinflammatory activity was 0.16 (0.10−0.23) and multiuser κ' was 0.43 (0.32−0.55). Assignment of necroinflammatory scores by the 15 possible pairing of pathologists are presented in Figure 6. The percentages represent the frequency at which the 15 possible pathologist pairings assigned scores to the sections. The pairs of pathologists were in complete agreement 34% of the time, differed by one score level 47% of the time, and differed by more than one score level 19% of the time.

There was no significant difference in the median scores assigned by the 6 pathologists for fibrosis between contiguous H&E and picrosirius red stained sections ($P = 0.248$). There was agreement between the scores assigned by an individual observer to contiguous H&E and picrosirius red stained sections from the same dog 58% of the time (Figure 7) and the median (minimum−maximum) κ for the 6 pathologists was 0.46 (0.23−0.61).

The median FP (minimum−maximum) for the sections stained with picrosirius red was 1.8% (0.0−19.6%). There was positive correlation between the median fibrosis score assigned to sections stained with picrosirius red at Texas A&M University and FP (r_s: 0.68; $P < 0.0001$, Figure 8).

Discussion

Using a previously published scoring system there was fair agreement between the 6 board-certified veterinary pathologists when assessing canine hepatic fibrosis and poor agreement when assessing necroinflammatory activity. There was no significant

Figure 1. Pairwise comparison of kappa statistics for the assessment of fibrosis from H&E stained sections

Observer	1	2	3	4	5	6
1		0.47	0.48	0.28	0.41	0.41
2	0.47		0.56	0.17	0.42	0.35
3	0.48	0.56		0.14	0.42	0.48
4	0.28	0.17	0.14		0.18	0.17
5	0.41	0.42	0.42	0.18		0.44
6	0.41	0.35	0.48	0.17	0.44	

The kappa statistic for each of the 15 possible pairings of veterinary pathologists scoring fibrosis for 50 sections of canine liver stained with H&E are presented.

Figure 2. Assignment of fibrosis scores by pathologist pairings for H&E stained sections

Score	Absent	Mild	Moderate	Marked	Very marked
Absent	9.9%	NA	NA	NA	NA
Mild	12.9%	7.2%	NA	NA	NA
Moderate	5.3%	9.7%	14.7%	NA	NA
Marked	0.7%	2.4%	11.9%	3.2%	NA
Very marked	0.0%	0.5%	1.7%	6.0%	13.9%

The percentages represent the frequency at which the 15 possible pathologist pairings assigned hepatic fibrosis scores to the sections. The pairs of pathologists were in complete agreement 49% of the time (dark gray), differed by one score level 41% of the time (light gray), and differed by more than 1 score level 11% of the time (white). NA = not applicable

Figure 3. Pairwise comparison of kappa statistics for the assessment of fibrosis from picrosirius red stained sections

Observer	1	2	3	4	5	6
1		0.50	0.40	0.24	0.29	0.48
2	0.50		0.45	0.29	0.43	0.40
3	0.40	0.45		0.49	0.39	0.56
4	0.24	0.29	0.49		0.22	0.27
5	0.29	0.43	0.39	0.22		0.56
6	0.48	0.40	0.56	0.27	0.56	

The kappa statistic for each of the 15 possible pairings of veterinary pathologists scoring fibrosis for 50 sections of canine liver stained with picrosirius red are presented.

Figure 4. Assignment of fibrosis scores by pathologist pairings for picrosirius red stained sections

Score	Absent	Mild	Moderate	Marked	Very marked
Absent	5.2%	NA	NA	NA	NA
Mild	9.6%	9.7%	NA	NA	NA
Moderate	1.7%	14.0%	18.7%	NA	NA
Marked	0.3%	1.6%	11.2%	3.7%	NA
Very marked	0.0%	0.0%	1.1%	7.5%	15.7%

The percentages represent the frequency at which the 15 possible pathologist pairings assigned fibrosis scores to the sections.

The pairs of pathologists were in complete agreement 53% of the time (dark gray), differed by one score level 42% of the time (light gray), and differed by more than one score level 5% of the time (white). NA= not applicable

Figure 5. Pairwise comparisons of kappa statistics for the assessment of necroinflammatory activity

Observer	1	2	3	4	5	6
1	■	-0.03	0.23	0.14	0.19	0.13
2	-0.03	■	0.06	0.26	0.08	0.06
3	0.23	0.06	■	0.11	0.22	0.26
4	0.14	0.26	0.11	■	0.20	0.37
5	0.19	0.08	0.22	0.20	■	0.40
6	0.13	0.06	0.26	0.37	0.40	■

The kappa statistic for each of the 15 possible pairings of veterinary pathologists scoring necroinflammatory activity for 50 sections of canine liver stained with H&E are presented.

Figure 6. Assignment of necroinflammatory scores by pathologist pairings

Score	Absent	Minimal	Mild	Moderate	Marked	Very marked
Absent	13.2%	NA	NA	NA	NA	NA
Minimal	19.3%	9.1%	NA	NA	NA	NA
Mild	6.1%	11.1%	4.9%	NA	NA	NA
Moderate	2.7%	3.6%	8.7%	3.6%	NA	NA
Marked	0.5%	0.7%	2.3%	4.7%	1.5%	NA
Very marked	0.3%	0.5%	0.7%	1.9%	2.9%	1.9%

The percentages represent the frequency at which the 15 possible pathologist pairings assigned scores to the sections. The pairs of pathologists were in complete agreement 34% of the time (dark gray), differed by one score level 47% of the time (light gray), and differed by more than one score level 19% of the time. NA = not applicable

Figure 7. Summary of agreement for individual pathologists scoring fibrosis from contiguous sections of liver stained with H&E and picrosirius red

Picrosirius red

Score	Absent	Mild	Moderate	Marked	Very marked
Absent	9%	7%	3%	0%	0%
Mild	2%	11%	7%	0%	0%
Moderate	0%	4%	18%	5%	1%
Marked	0%	0%	3%	6%	4%
Very Marked	0%	0%	1%	2%	14%

(H&E, left vertical axis label)

The percentages represent the frequency at which the 6 pathologists assigned that combination of scores for the sections. The pathologists were in complete agreement with their other score 58% of the time (dark gray), differed by one score level 36% of the time (light gray), and differed by more than one score level 6% of the time (white).

Figure 8. Fibrotic proportion and median fibrosis score
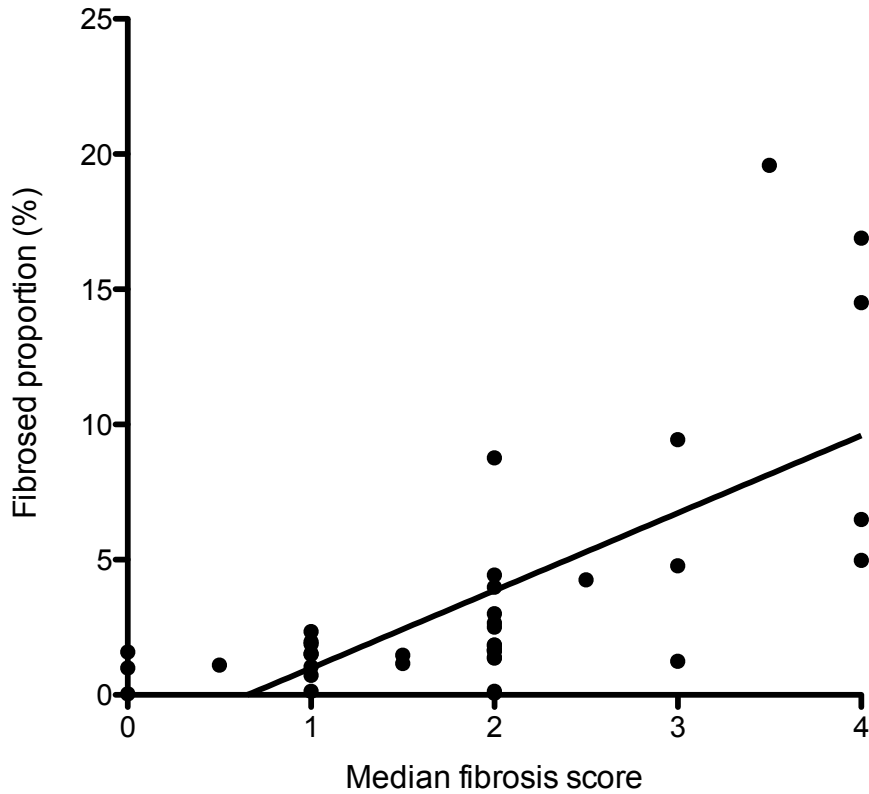
Scatter plot of fibrosed proportion calculated by computer assisted image analysis and the median fibrosis score assigned by 6 pathologists to picrosirius red stained sections of liver from 38 dogs. There was a positive correlation between the median fibrosis scores assigned to each section and the fibrotic proportion of the sections as measured by computer-assisted image analysis ($r_s = 0.677$; $P < 0.0001$).

difference between the median fibrosis score assigned to sections stained with H&E or picrosirius red. There was a positive correlation between the median fibrosis score assigned to sections stained with picrosirius red and the FP of these sections.

In this study there was fair agreement between veterinary pathologists using a previously published system to score canine hepatic fibrosis from H&E and picrosirius red stained sections with a κ of 0.35 and 0.39, respectively. This is comparable to results from a study using the Ishak system to stage hepatic fibrosis in humans where κ was determined to be 0.26 to 0.47, indicating fair to moderate agreement.[68] When the pathologists did not completely agree they often assigned scores that only deviated by one level and they only deviated by more than one level 15% and 5% of the time for H&E and picrosirius red stained sections, respectively. This partial agreement was apparent when κ' was calculated. Weighted-kappa statistics for H&E and picrosirius red stained sections were 0.59 and 0.64, indicating moderate and good agreement, respectively. For clinical decision making purposes it may not be essential to have complete agreement between pathologists as long as there is partial agreement. For example, a clinician may not treat a dog with marked hepatic fibrosis differently from a dog with very marked fibrosis. However, further research is needed to better determine the clinical implications of differences in the severity of canine hepatic fibrosis and necroinflammatory activity. Although the inter-observer agreement associated with using this scoring system to assess canine hepatic fibrosis was suboptimal because of the level of partial agreement, it may be acceptable for clinical use. Agreement as assessed by κ tends to be higher for histological scoring systems with fewer points.[4] Thus, for

clinical use it may be preferable to simplify this scoring system to a four level scale i.e., absent, mild, moderate, and marked. In a research setting, for example to evaluate an anti-fibrotic agent in a clinical trial, it may be preferable to have a scoring system with more levels to detect small differences between study groups, in which case the issue of suboptimal inter-observer agreement should be addressed, potentially by having sections evaluated but more than one observer.

In this study there was poor agreement between pathologists scoring necroinflammation with a multiuser κ of 0.19. This lack of agreement is concerning as it makes it more difficult for clinicians to confidently make treatment recommendation based on histopathological findings. In human medicine it has also proven difficult to develop a grading system for hepatic necroinflammatory activity that has acceptable inter-observer agreement. This may be because of the complexity and subjectivity of the histological grading systems that are necessary to take into consideration all the different features of hepatic necroinflammation, such as interface hepatitis, focal necrosis, confluent necrosis, and portal inflammation. In a study using the Ishak system to assess hepatic necroinflammation in human patients κ for its different components were reported to range from 0.11 to 0.41, indicating poor to moderate agreement.[68] As is the situation in humans[31,68], inter-observer agreement was higher for the assessment of fibrosis than for necroinflammatory activity. This probably reflects the fact that the scoring system for fibrosis is easier to use. For example, bridging fibrosis and nodule formation are relatively simple features to identify histologically. The multiuser κ' for necroinflammatory activity was 0.43, indicating moderate inter-observer agreement.

This reflected the finding that there was often partial agreement between observers. Indeed the scores assigned by pathologists only deviated by more than one level 19% of the time. In the veterinary field, lack of inter-observer agreement is not unique to histological assessment of the liver. A previous study assessing inter-observer agreement when four pathologists assessed sections from 62 dogs and 25 cats undergoing intestinal biopsy using the World Small Animal Veterinary Association gastrointetinal histopatholoigic templates found poor inter-observer agreement with a κ of -0.01 to 0.30.[36] Preliminary data indicate that inter-observer agreement is improved when this scoring system is simplfied.[70] Likewise, before use in a clincal setting, it may be beneficial to simplify the hepatic necroinflmmatory activity scoring system that we used, possibly by collapsing it to four levels like the METAVIR system that is often used to assess chronic hepatitis in humans.[63]

We hypothesized that fibrosis would be easier to detect on picrosirius red stained sections and that these sections would be assigned a higher fibrosis score. However, our finding that there was no significant difference in median fibrosis scores assigned to H&E and picrosirius red stained sections does not support this hypothesis. There was moderate agreement between the fibrosis scores assigned to contiguous H&E and picrosirius red stained sections from the same case (median κ: 0.46). However, the agreement associated with the evaluation of fibrosis from sections stained with picrosirius red, was slightly higher than that from H&E stained sections, κ statistics of 0.35 and 0.39, respectively. Taken together these findings did not demonstrate a clear benefit in staining sections with picrosirius red. However, some pathologists find it

easier to assess fibrosis using this stain than with H&E stained sections, in which case its use may be worthwhile.

There was a positive correlation between the median fibrosis scores assigned to each section and the fibrotic proportion of the sections as measured by computer-assisted image analysis ($r_s = 0.68$; $P < 0.0001$). This is similar to the findings in a study using image analysis to evaluate fibrosis in sections of human livers.[69] This technique has several potential advantages. Firstly, it is more objective than assessment of fibrosis by histological assessment. Additionally, the resulting variable FP is a continuous variable whereas the histological fibrosis score is a nominal variable. This could be useful to detect small changes in hepatic fibrosis in a research setting. It is important to note that the relationship between the median fibrosis score and FP is not linear as the sections with the highest median fibrosis scores had disproportionately high FPs. Furthermore, this technique does not detect key features in the progression of fibrosis, such as the development of bridging fibrosis and therefore it should not be considered to be a direct replacement for the histological assessment of fibrosis.[69]

This study provides useful information about the inter-observer agreement associated with the assessment of canine hepatic fibrosis and necroinflammatory activity. However, it is important to discuss several limitations. Kappa is commonly used to assess the agreement between observers in biomedical research. However, some authors have criticized its use for several reasons. One concern is that κ and κ' are dependent upon the distribution of severity amongst the cases evaluated.[71] The cases enrolled in this study were primarily selected to represent a wide range of the severity

fibrosis and although a wide range of severity of necroinflammatory activity was also represented the distribution among median scores was not uniform. Therefore, κ and κ' for the assessment of inter-observer agreement associated with histological scoring should be interpreted cautiously for this parameter. The weighting systems used to calculate κ' have been criticized as being too subjective.[71] As scoring systems are not routinely used for the assessment of the canine liver most of the observers had not previously used such a scoring system and only one had used this particular system previously. Thus it is possible that if the pathologists had more experience and training using the system evaluated, agreement may have improved. The provision of pictorial templates to the pathologists may have also help to increase agreement. Additionally, our study did not assess intra-observer agreement i.e., the same pathologist assessing the same section on different occasions or the effect of the biopsy technique on inter-observer agreement. This would be worth evaluating in additional studies. Future work may also include the reanalysis of the results of this study using a mixed linear model that should mitigate some of the concerns about the validity of κ and κ'.

In conclusion, there was fair inter-observer agreement when veterinary pathologists assessed canine hepatic fibrosis and poor agreement when they assessed hepatic necroinflammatory activity using this scoring system. This suboptimal agreement, especially for necroinflammation, is concerning, and a simplified version of the scoring system with less possible levels of change may be preferable in a clinical setting as this modification would be expected to improve inter-observer agreement. When investigators design clinical studies evaluating these findings, multiple

pathologists should evaluate specimens or other techniques, such as computerized image analysis, should be used in addition to histological evaluation. This study did not show a difference in the fibrosis scores assigned to sections stained with H&E or picrosirius red, although the level of inter-observer agreement was slightly higher for the latter. Therefore, a clear benefit of the routine staining of hepatic sections with picrosirius red was not demonstrated. Computer-assisted image analysis may offer objective and repeatable assessment of canine hepatic fibrosis but it is unlikely to be a replacement for histological assessment. The utility of this technique for the assessment of hepatic fibrosis in dogs should be further evaluated.

CHAPTER III

SERUM CONCENTRATIONS OF EXTRACELLULAR MATRIX

COMPONENTS IN DOGS WITH HEPATOBILIARY DISEASE

Introduction

The development of hepatic fibrosis is an important event in the progression of liver disease and has been shown to be a negative prognostic factor in humans with chronic hepatitis.[16] Currently, the only way to diagnose hepatic fibrosis in dogs is by histological assessment of a liver biopsy specimen. Liver biopsy is by nature invasive, there is a risk of hemorrhage[24], and as only a small portion of the organ is evaluated, this technique is susceptible to sampling variation.[25] Because of these disadvantages serum markers of hepatic fibrosis have been developed for use in humans.[26]

In human patients with hepatic fibrosis an increased rate of extracellular matrix turnover results in release of matrix components into the bloodstream.[26] Hyaluronic acid is a glycosaminoglycan component of the extracellular matrix that is used as a serum biomarker of hepatic fibrosis in humans.[26] In a study of humans with chronic hepatitis C, the sensitivity and specificity of HE for distinguishing between patients with extensive fibrosis and those with milder fibrosis were 86% and 70%, respectively.[27] Hyaluronic acid has previously been shown to be increased in dogs with hepatic disease including cirrhosis[28] and congenital portosystemic shunts.[29] However, the ability of serum HA measurement to differentiate among dogs with different histological stages of hepatic fibrosis has not previously been reported.

In a study of human patients with chronic hepatitis C, measurement of serum PIIINP concentrations had a sensitivity of 92% and a specificity of 76% for differentiating between those patients with extensive fibrosis and those with milder fibrosis.[27] Serum PIIINP concentrations were increased in growing dogs but not dogs with chronic bronchopulmonary disease.[30] Serum PIIINP concentrations have not previously been assessed in dogs with hepatobiliary disease.

Tissue inhibitor of matrix metalloproteinase-1 is a protein that inhibits the action of matrix metalloproteinase-1, thus slowing the degradation of extracellular matrix during fibrosis.[22] In a study of human patients with chronic hepatitis C, measurement of serum TIMP-1 concentration had a sensitivity of 75% and a specificity of 70% for differentiating between patients with extensive fibrosis and those with milder fibrosis.[27] To the authors' knowledge serum TIMP-1 concentrations have not previously been assessed in dogs with hepatobiliary disease.

The main objective of this study was to assess the clinical utility of measuring HA, PIIINP, and TIMP-1 concentrations for diagnosing canine hepatic fibrosis in dogs. The secondary objective of the study was to perform the initial validation of a commercially available human HA enzyme-linked immunosorbent assay (ELISA) for use with canine serum.

Materials and methods

Dogs over 1 year of age (over 2 years for large and giant breed dogs) with histologically confirmed hepatobiliary disease diagnosed at Gulf Coast Veterinary

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Specialists or Texas A&M University Veterinary Medical Teaching Hospital between 3/1/12 and 3/1/13 were enrolled into this prospective observational cross-sectional study. This age cutoff was used to avoid enrolling growing dogs. The diagnosis of hepatobiliary disease was based on a combination of clinical signs, laboratory testing, diagnostic imaging findings, histological evaluation of a liver biopsy specimen, and in some cases, findings upon surgical exploration of the abdominal cavity. These dogs were divided into four groups: 1) chronic hepatitis; 2) hepatic neoplasia, which could be primary or secondary; 3) CPSS; and 4) other hepatobiliary diseases, including vacuolar hepatopathy, nodular regeneration, and gallbladder mucocele. Where possible an extra liver biopsy specimen was collected from the dogs with hepatobiliary disease for evaluation of the severity of fibrosis.

Healthy staff-owned dogs over 1 year of age (over 2 years of age for large and giant breed dogs) were enrolled at the Texas A&M University Veterinary Medical Teaching Hospital. The health of these dogs was assessed by use of an owner questionnaire, physical examination, complete blood count, serum biochemistry profile, and serum pancreas-specific lipase concentration measurement (Spec cPL, IDEXX Laboratories). Dogs with clinically important abnormalities were excluded from the study.

The study was approved by the Texas A&M University Institutional Animal Use Committee (AUP 2011-215). Informed owner consent was given before enrollment of all the dogs.

At the time of liver biopsy or immediately prior to euthanasia and necropsy, 3−5 mL of blood were collected from the dogs by jugular venipuncture and placed into sterile anticoagulant free tubes. Once a firm blood clot had formed, the blood was centrifuged at 1,300 g for 15 minutes to separate the serum from the red blood cells. The serum was stored at -80 °C until analysis.

Serum HA concentrations were measured with a commercially available human ELISA (Hyaluronan ELISA Kit, Echelon Biosciences). We assessed assay precision by calculating the intra-assay coefficients of variation (%CV) for three samples (low, medium and high concentration) run six times on the same ELISA plate. Reproducibility was assessed by calculating the inter-assay %CV for three samples (low, medium and high concentration) run seven times on different days. We assessed analytical accuracy by calculating observed to expected ratios (%) when equal volumes of two of four canine serum samples were mixed in every possible combination. We assessed assay linearity by calculating observed to expected ratios (%) when five samples were serially diluted, 1:2, 1:4, 1:8, and 1:16, with sample buffer. Serum PIIINP concentrations were measured with a human radioimmunoassay (UniQ PIIINP RIA, Orion Diagnostica) that has previously been validated for use with canine serum.[30] Serum TIMP-1 concentrations were measured using a commercially available canine ELISA (TIMP-1 ELISA, USCN Life Science) that has been validated for use with canine serum by the manufacturer.

Liver biopsies were fixed in neutral buffered formalin, processed for routine histology, and embedded in paraffin. Serial sections of liver were stained with

hematoxylin and eosin and picrosirius red (Picrosirius Red Staining Kit, Polysciences).

Hepatic fibrosis was staged by a board-certified veterinary pathologist (ARH) using a

previously published five-point scoring system (i.e., absent, mild, moderate, marked,

very marked; Table 1)[33,34], which was adapted from the human Ishak system.[32]

Information about the clinical history or serum extracellular matrix component

concentrations was not provided to the pathologist during the scoring process.

The distribution of continuous data was assessed using the Kolmogorov-Smirnov

test and by visual inspection of frequency histograms. Non-parametric data was

expressed as the median (minimum−maximum). Parametric data was expressed as the

mean (±standard deviation). Comparisons of serum concentrations of HA, PIIINP, and

HA amongst the groups of dogs were performed using the Kruskal-Wallis test, followed

by post-testing with Dunn's test as needed. Comparisons of continuous variables

between two groups of dogs were made using Mann-Whitney U tests. Where

appropriate, receiver operating characteristic curve analysis was used to assess

diagnostic accuracy. The relationships between serum HA, PIIINP, and TIMP-1

concentrations and the hepatic fibrosis stage were assessed using Spearman's rank

correlation (r_s). A statistical software package was used for these calculations

(GraphPad, Prism 5, GraphPad Software). Statistical significance was set as $P < 0.05$.

Results

Fifty-nine dogs with hepatobiliary disease were enrolled in the study. The

median age of the dogs was 10 years (minimum−maximum: 1−17). Twenty-four were

neutered male (41%), three were intact male (5%), twenty-eight were spayed female (47%), and four were intact female (7%). The following breeds were commonly represented: five Labrador Retrievers (8%), four Miniature Schnauzers (7%), four Yorkshire Terriers (7%), and 17 mixed breed dogs (29%). Twenty-one dogs (36%) had chronic hepatitis, 19 had hepatobiliary neoplasia (32%), six dogs (10%) had CPSS, and 13 (22%) had other hepatobiliary disease. Forty-five healthy dogs were enrolled into the study, the median age of these dogs was 4 years (1−13). Fifteen were neutered male (33%), one was intact male (2%), 21 were spayed female (47%), and eight were intact female (18%). Commonly represented breeds included: five German Shepherd dogs (18%), three Miniature Schnauzers (7%), three Labrador Retrievers (7%), three Hounds (7%), three Australian Shepherds (7%), and eight Mixed Breed dogs (18%)

Additional liver biopsy specimens for the staging of fibrosis were collected from 48 dogs of the 59 dogs with hepatobiliary disease enrolled in the study (81%). Hepatic fibrosis stage scores were assigned to the dogs as follows: six dogs (13%) had no fibrosis, ten dogs (21%) had mild, 15 dogs (31%) had moderate, eight dogs (17%) had marked, and nine dogs (19%) had very marked fibrosis.

The intra-assay %CV for the HA ELISA was 6.2%, 1.9%, and 12.2% for low, medium, and high concentration samples, respectively. The inter-assay variability %CV for the assay was 15.1%, 13.4%, and 15.3% for low, medium, and high concentration samples, respectively. The mean (±standard deviation; minimum−maximum) observed to expected ratio for spiking recovery experiments was 97.2% (±7.2; 89.5−110.9). The

mean observed to expect ratio for the dilutional parallelism experiments was 96.0% (±16.9; 75.4%−129.3).

Serum HA concentrations were measured in 57 dogs (97%) with hepatobiliary disease and 44 healthy dogs (98%). Healthy dogs, had higher serum HA concentrations (median: 198 ng/mL; 84−1,464) than dogs with neoplasia (median: 123 ng/mL; 82−1,532; $P < 0.01$; Table 3). Otherwise there were no significant differences in serum HA concentrations among the groups of dogs. There was no significant difference in serum HA concentrations between dogs with absent to moderate hepatic fibrosis and those with marked to very marked fibrosis ($P = 0.524$; Table 4). There was no correlation between serum HA concentration and hepatic fibrosis ($r_s = 0.23$; $P=0.120$; Figure 9).

Serum PIIINP concentrations were measured in 50 dogs (85%) with hepatobiliary disease and 44 healthy dogs (98%). There were no significant differences in serum PIIINP concentrations among the groups of dogs ($P = 0.109$; Table 3). There was no significant difference in serum PIIINP concentrations between dogs with absent to moderate hepatic fibrosis and those with marked to very marked fibrosis (Table 4; $P = 0.781$). There was no correlation between serum PIIINP concentration and hepatic fibrosis ($r_s = 0.12$; $P = 0.462$; Figure 10).

Serum TIMP-1 concentrations were measured in 53 dogs (90%) with hepatobiliary disease and 24 healthy dogs (53%). Dogs with hepatic neoplasia had higher serum TIMP-1 concentrations (median: 45 ng/mL; 6−100) than those with chronic hepatitis (median: 14 ng/mL; 6−59; $P < 0.05$; Table 3). There was a trend for the

dogs with neoplasia to have higher serum TIMP-1 concentrations than healthy dogs (median 20 ng/mL; 5−100), but this did not reach statistical significance ($P < 0.1$). Dogswith neoplasia had higher serum TIMP-1 concentrations than dogs with non-neoplastic hepatobiliary disease (median 21 ng/mL; 5−63; $P < 0.01$; Figure 11). The area under the receiver-operator characteristic curve (95% confidence interval) of TIMP-1 for discriminating between dogs with hepatic neoplasia and healthy dogs was 0.75 (0.58−0.91). The area under the receiver-operator characteristic curve of TIMP-1 for discriminating between dogs with hepatic neoplasia and dogs with non-neoplastic hepatic disease was 0.76 (0.60−0.93). Dogs with primary hepatobiliary neoplasia (hepatocellular adenomas, hepatocellular carcinomas, and cholangiocarcinomas) had higher serum TIMP-concentrations than those with tumors those with neoplasia secondarily affecting the liver (lymphoma and hemangiosarcoma), with median concentrations of 46 ng/mL (6−100) and 6 ng/mL (6−12), respectively ($P = 0.022$; Figure 12). There was no significant difference in serum TIMP-1 concentrations between dogs with absent to moderate hepatic fibrosis and those with marked to very marked fibrosis ($P = 0.093$; Table 4). There was a negative correlation between serum TIMP-1 concentration and hepatic fibrosis ($r_s = -0.32$; $P = 0.039$; Figure 13).

Discussion

The main objective of this study was to assess the clinical utility of measuring HA, PIIINP, and TIMP-1 concentrations for diagnosing canine hepatic fibrosis in dogs.

Table 3. Serum concentrations of extracellular matrix components in healthy dogs and dogs with various types of hepatobiliary disease

Analyte	Healthy	Chronic	Hepatobiliary neoplasia	CPSS	Other hepatobiliary disease	P-value
HA	198 ng/mL[*]	155 ng/mL	123 ng/mL[*]	131 ng/mL	156 ng/mL	0.004
median (min−max)	(84−1,464)	(50−3,360)	(82−1,532)	(70−576)	(106−16,223)	
PIIINP	11.3 µg/L	8.9 µg/L	8.1 µg/L	12.5 µg/L	9.9 µg/L	0.109
median (min−max)	(3.8−48.2)	(3.1−38.6)	(3.3−35.3)	(4.1−16.3)	(3.3−50.0)	
TIMP-1	20 ng/mL[***]	14 ng/mL[**]	45 ng/mL[**,***]	26 ng/mL	24 ng/mL	0.011
median (min−max)	(5−100)	(6−59)	(6−100)	(12−34)	(5−63)	

[*]$P < 0.01$ for post-testing between groups, [**]$P < 0.05$ for post testing between groups, [***]$P < 0.1$ for post testing between groups, CPSS = congenital portosystemic shunt, HA = hyaluronic acid, PIIINP = procollagen type III N-terminal peptide, TIMP-1 = tissue inhibitor of matrix metalloproteinase-1

Table 4. Serum concentrations of extracellular matrix components in dogs with different stages of hepatic fibrosis

Analyte	Absent to moderate fibrosis	Marked to very marked fibrosis	P-value
Hyaluronic acid concentration	130 ng/mL	156 ng/mL	0.297
median (min−max)	(50−1,532)	(50−3,360)	
PIIINP concentration	8.6 μg/L	9.8 μg/L	0.524
median (min−max)	(3.3−50.0)	(3.1−38.6)	
TIMP-1 concentration	31 ng/mL	19 ng/mL	0.093
median (min−max)	(5−100)	(6−59)	

HA = hyaluronic acid, PIIINP = procollagen type III N-terminal peptide, TIMP-1 = tissue inhibitor of matrix metalloproteinase-1

Figure 9. Serum hyaluronic acid concentration and hepatic fibrosis stage
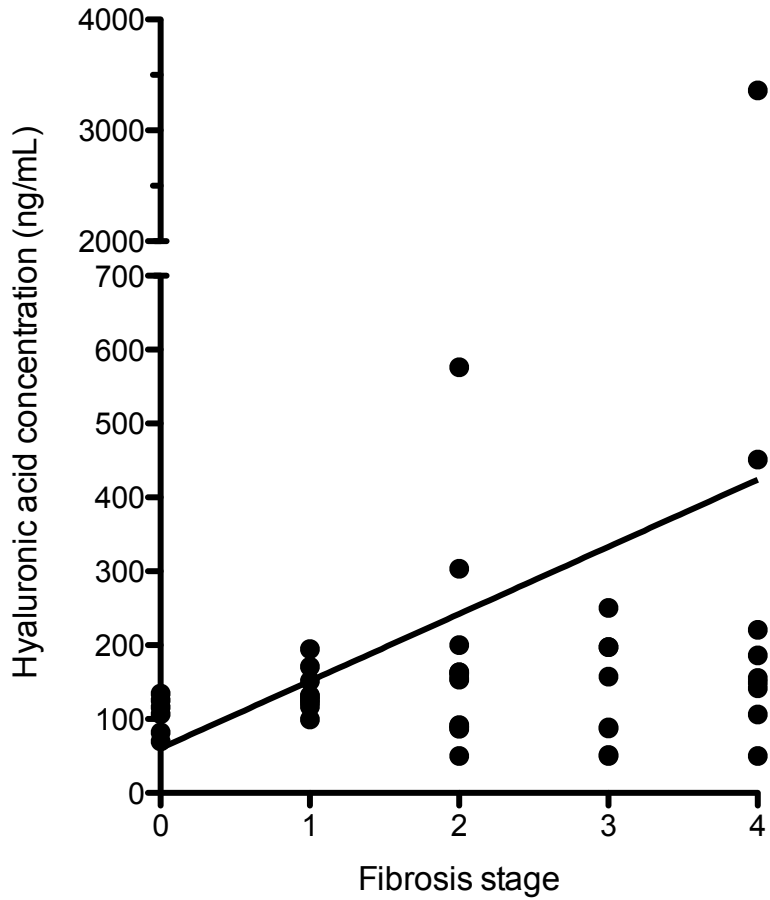
There was no correlation between serum hyaluronic acid concentrations and the stages of hepatic fibrosis (r_s = 0.23; P = 0.120).

Figure 10. Serum procollagen type III N-terminal peptide acid concentration and hepatic
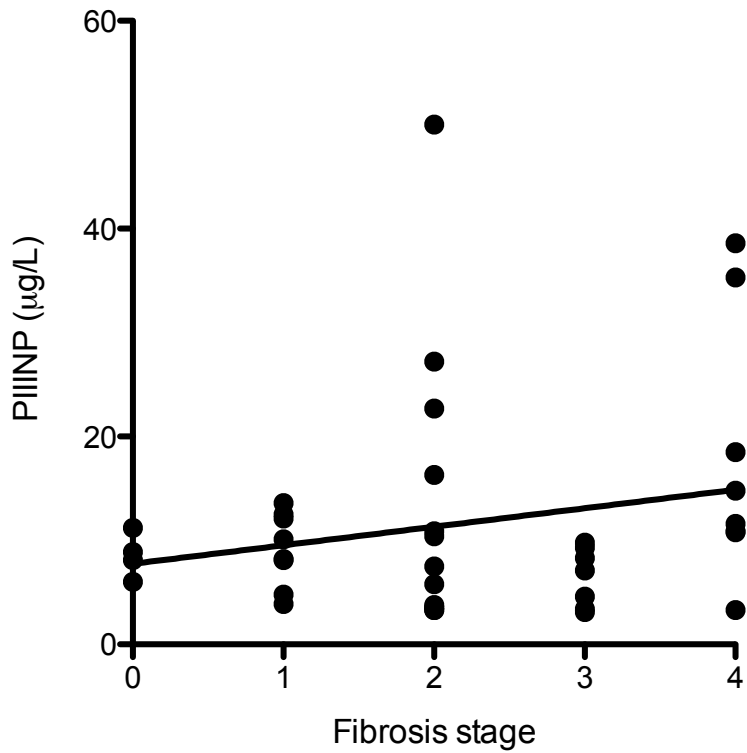
fibrosis stage

There was no correlation between serum procollagen type III N-terminal peptide

(PIIINP) concentrations and the stages of hepatic fibrosis ($r_s = 0.12$; $P = 0.462$).

Figure 11. Serum tissue inhibitor of matrix metalloproteinase-1 concentrations
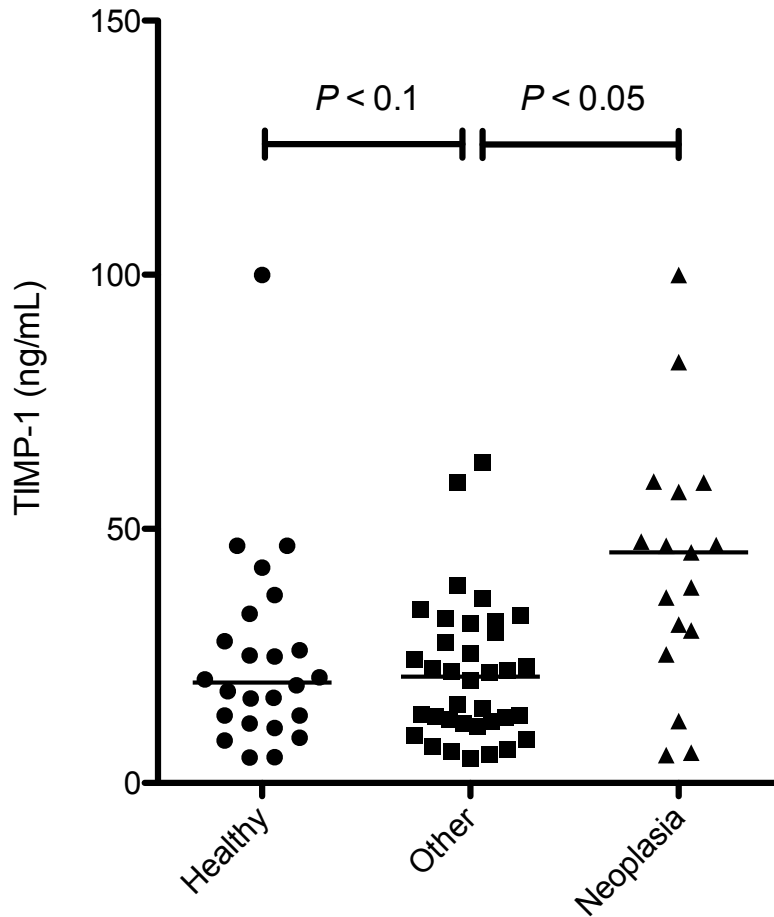
The horizontal bars represent the median serum tissue inhibitor of matrix
metalloproteinase-1 (TIMP-1) concentration for that group. Dogs with hepatic neoplasia
had higher serum TIMP-1 concentrations than those with chronic hepatitis ($P < 0.05$).
There was a trend for the dogs with neoplasia to have higher serum TIMP-1
concentrations than healthy dogs but this did not reach statistical significance ($P < 0.1$).

Figure 12. Serum tissue inhibitor of matrix metalloproteinase-1 concentrations of dogs with primary and metastatic hepatobiliary neoplasia
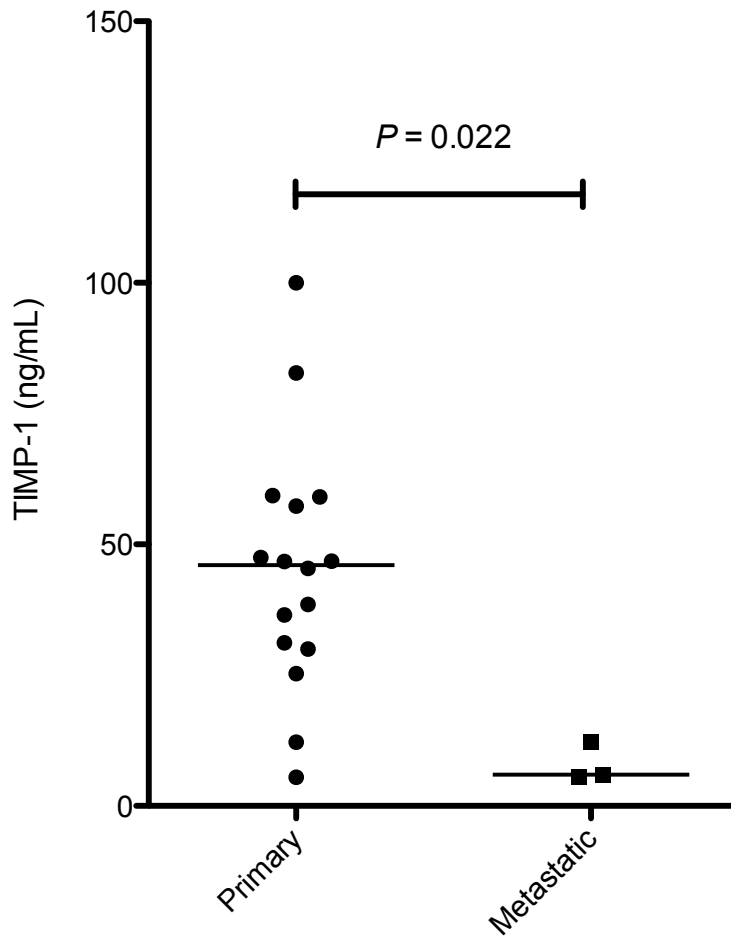
The horizontal bars represent the median serum tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) concentration for that group. Dogs with primary hepatobiliary neoplasia (hepatocellular adenomas, hepatocellular carcinomas, and cholangiocarcinomas) had higher serum TIMP-concentrations than those with tumors those with neoplasia secondarily affecting the liver (lymphoma and hemangiosarcoma).

Figure 13. Serum tissue inhibitor of matrix metalloproteinase-1 concentration and hepatic fibrosis stage
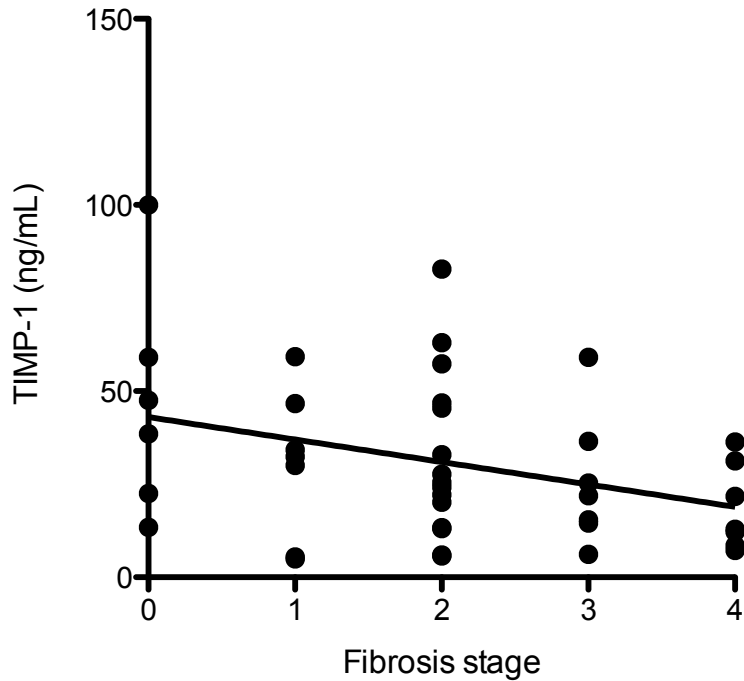
Scatter plot of serum tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and fibrosis stage for 42 dogs with hepatobiliary disease. There was a weak negative correlation between serum tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) concentrations and the stages of hepatic fibrosis (r_s = -0.32; P = 0.039).

The secondary objective of the study was to perform the initial validation of a commercially available human HA ELISA for use with canine serum.

The HA ELISA we used in this study had acceptable precision, accuracy, and linearity for the measurement of canine serum HA concentrations. The inter-assay %CV of 15.1%, 13.4%, and 15.3% were slightly higher than desirable, suggesting suboptimal repeatability. However, we do not think this is likely to have affected the conclusions we reached, as there was not even a trend for serum HA concentrations to be related to the severity of hepatic fibrosis.

The relevance of the finding that dogs with hepatic neoplasia had lower serum HA concentrations than healthy dogs is unknown and neither is the reason why this occurred. However, the differences in the median concentration of HA between the groups was small and therefore may not be clinically important. We are not aware of a similar relationship in humans with hepatic neoplasia. There was no relationship between the severity of hepatic fibrosis and serum HA concentrations in the dogs enrolled in this study. This is in contrast to studies in humans where HA has been shown to be a useful marker of hepatic fibrosis[26,27], and a previous study in dogs where those with hepatic cirrhosis had higher serum HA concentrations than those with non-cirrhotic hepatic disease, those with non-hepatic disease, or healthy dogs.[28] The aforementioned study in dogs used a different assay, an automated latex agglutination assay, than the one we used. However, a previous study using the ELISA that we used in our study found that Chinese Shar Pei dogs with cutaneous mucinosis had higher serum HA concentrations than healthy dogs or Chinese Shar Peis without cutaneous mucinosis[72],

suggesting that this assay is capable of detecting elevated serum HA concentrations in dogs. The median serum HA concentrations of 5 healthy dogs reported in that study was 244 ng/mL (166−302), which is similar to that of the 45 healthy dogs from our study 198 ng/mL (84−1,464). Another possible reason for the discrepancy in results between our study and theirs is that our study contained relatively few dogs with marked or very marked hepatic fibrosis and potentially serum HA concentrations only increase in dogs with very advanced fibrosis.

There was no difference in serum PIIINP concentrations among healthy dogs, dogs with chronic hepatitis, dogs with CPSS, dogs with hepatobiliary neoplasia, or dogs with other hepatobiliary disease. Additionally, no relationship between the severity of hepatic fibrosis and serum PIIINP concentrations was observed. In humans, serum PIIINP concentrations have been shown to be useful in distinguishing between patients with no or mild fibrosis and those with moderate or severe fibrosis.[26,27] The reason why serum PIIINP concentrations were not increased in these dogs with hepatic fibrosis is unknown. One possible explanation is that this protein is not leaked into the bloodstream of dogs with hepatic fibrosis. Type III collagen was increased in the livers of dogs with chronic hepatitis[9], suggesting that this form of collagen is important in canine hepatic fibrosis. It is interesting that a previous study did not find that measurement of serum PIIINP concentrations were increased in dogs with chronic bronchopulmonary disease but concentrations in bronchoalveolar lavage fluid were increased.[30] In humans, PIIINP has also been shown to have a moderately strong positive correlation with the severity grade of hepatic necroinflammatory activity[73], therefore we cannot rule out that this was

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a confounding factor that obscured the presence of a true relationship between PIIINP and the severity of hepatic fibrosis in our study.

Dogs with hepatic neoplasia had higher serum concentrations of TIMP-1 than those with chronic hepatitis and there was a trend for them to have higher serum concentrations than healthy dogs, but this did not reach statistical significance. Additionally, when the dogs with non-neoplastic hepatobiliary disease were combined into one group they had significantly lower serum TIMP-1 concentrations than those dogs with hepatobiliary neoplasia. Serum TIMP-1 had fair diagnostic accuracy for discriminating between dogs with hepatic neoplasia and healthy dogs or dogs with hepatic disease. For example, using a cut-off value of 29 ng/mL, the sensitivity and specificity of serum TIMP-1 measurement for distinguishing between healthy dogs and dogs with hepatobiliary neoplasia were 77% and 75%, respectively. Using the same cut-off, the sensitivity and specificity of TIMP-1 measurement for distinguishing between dogs with non-neoplastic hepatobiliary disease and those with hepatobiliary neoplasia were 77% and 72%, respectively. Additionally, dogs with primary liver neoplasia (hepatocellular adenoma, hepatocellular carcinoma, and cholangiocarcinoma) had higher serum TIMP-1 concentrations than those with lymphoma and hemangiosarcoma. These findings are interesting as humans with a variety of tumors, including, hepatic metastases[74] and pulmonary neoplasms[75] have been shown to have increased serum TIMP-1 concentrations. In one study of humans with hepatic metastases, higher serum TIMP-1 concentrations were shown to be a poor prognostic factor.[74] Dogs with spontaneously occurring mammary tumors have been shown to have a relatively low

tissue TIMP-1 activity when compared to rats with induced mammary tumors[76], but to our knowledge serum TIMP-1 concentrations have not previously been reported in dogs with hepatobiliary neoplasia. Further studies are needed in a larger group of dogs to confirm our findings and to assess serum TIMP-1 concentrations in dogs with other types of neoplasia.

There was a weak negative correlation between serum TIMP-1 concentration and the severity of fibrosis. There was no significant difference in the fibrosis stages assigned to dogs with hepatobiliary neoplasia and those with other hepatobiliary diseases so it is unlikely that the increased serum TIMP-1 concentrations observed in dogs with neoplasia acted as a confounding factor explaining this unexpected negative correlation. There was no difference in serum TIMP-1 concentrations between dogs with absent to moderate fibrosis and those with marked to very marked fibrosis. These findings contrast with those in studies of humans where TIMP-1 is a useful marker of hepatic fibrosis and is positively correlated with its severity.[26,27] The reason for this difference between the two species is not known.

It is important to discuss several limitations of this work. Firstly, there were relatively few dogs (nine out of 48; 19%) that were assigned a score of very marked hepatic fibrosis, so it is possible that this was the reason we failed to find a relationship between any of the extracellular matrix components and the severity of hepatic fibrosis because they are only increased in dogs with very marked fibrosis. However, even if this was the case, the utility of these markers would be limited if they can only distinguish between dogs with mild fibrosis and those with very marked fibrosis. Additionally, we

cannot completely exclude the possibility that some of the dogs that were enrolled in this study had subclinical disease that was causing fibrosis of another organ, therefore increasing their serum extracellular matrix component concentrations, and acting as a confounding factor. However, the group of dogs with hepatobiliary disease that we enrolled in the study would be similar to the population of dogs in which these markers would be used if they were shown to be valuable. Therefore, their lack of apparent diagnostic utility in this population is important to recognize.

The results of this study do not support the utility of measuring serum HA, PIIINP, or TIMP-1 concentrations for the diagnosis of canine hepatic fibrosis. Serum TIMP-1 had fair diagnostic accuracy for discriminating between dogs with hepatic neoplasia and healthy dogs or dogs with hepatobiliary disease. Further studies are needed to confirm this finding.

CHAPTER IV

URINARY N-METHYLHISTAMINE TO CREATININE RATIOS IN DOGS

WITH HEPATOBILIARY DISEASE

Introduction

Mast cells are derived from myeloid stem cells and can release a variety of inflammatory mediators including histamine, serotonin, serine proteases, thromboxanes, prostaglandins, leukotrienes, and heparin.[49,50] In humans there is some evidence to support their role in the development of hepatobiliary disease.[51] Firstly, mast cells have been shown to be present in the livers of healthy humans and those with hepatic disease, including chronic liver disease[52], acute hepatitis[53], primary biliary cirrhosis[54], hepatocellular carcinoma, and cholangiocarcinoma.[55,56] Patients with chronic hepatitis virus infection with steatosis had higher mast cell densities than those without.[57,58] Another study of patients with chronic hepatitis C found a positive correlation between the mast cell density and fibrosis.[58] *In vitro*, mast cell proteases have a profibrotic effect by stimulating fibroblast proliferation.[59] Furthermore, humans with chronic cholestatic disease frequently complain of pruritus and one study found that patients with cholestasis had higher plasma histamine concentrations than healthy controls.[60] The role of mast cell mediated inflammation in canine hepatobiliary disease is poorly defined and mast cells are not commonly noted during histological evaluation of the healthy or diseased canine liver.

As mast cell degranulation leads to the release of histamine, which is primarily stored in this type of cell, histamine has been suggested to be a potential marker of mast cell degranulation.[49,50] However, histamine is rapidly metabolized and, therefore, may not be a practical biomarker.[49,50] N-methylhistamine is generated when histamine is metabolized by the N-methyltransferase enzyme system.[61] N-methylhistamine is stable and a method for its measurement in canine urine and fecal samples has recently been developed and analytically validated.[61,62] In a recent study, seven out of 16 of dogs with chronic gastrointestinal disease were shown to have increased fecal or urinary NMH concentrations, indicating that mast cell mediated inflammation may be important in a subset of these dogs.[77] Urinary NMH to creatinine ratios have also shown to be greatly increased in dogs with mast cell tumors.[62] To the authors' knowledge the utility of NMH as a marker of mast cell induced inflammation in canine hepatobiliary disease has not previously been investigated.

We hypothesized that urinary NMH to creatinine ratio is a useful biomarker of mast cell induced hepatobiliary inflammation in dogs. The main objective of this study was to compare urine NMH concentration between healthy dogs and dogs with various types of hepatobiliary disease. Secondary objectives were to evaluate the correlation between urinary NMH concentrations and hepatic mast cell counts, hepatic fibrosis scores, as well as hepatic necroinflammatory activity in dogs with hepatobiliary disease.

Materials and methods

Dogs with histologically confirmed hepatobiliary disease diagnosed at Gulf Coast Veterinary Specialists or Texas A&M University Veterinary Medical Teaching Hospital between 3/1/12 and 2/28/13 were enrolled into this prospective observational study. The diagnosis of hepatobiliary disease was based on a combination of clinical signs, laboratory testing, diagnostic imaging findings, histological evaluation of a liver biopsy specimen, and, in some cases, findings upon surgical exploration of the abdominal cavity. The dogs were divided into four groups: dogs with chronic hepatitis (CH); dogs with hepatic neoplasia, which could be primary or secondary; dogs with a congenital portosystemic shunt; and dogs with other hepatobiliary diseases, including vacuolar hepatopathy, nodular regeneration, or gallbladder mucocele. Where possible an additional liver biopsy specimen was collected from the dogs for mast cell enumeration, evaluation of fibrosis, and evaluation of necroinflammatory activity.

Healthy staff-owned dogs over 6 months' of age were enrolled at the Texas A&M University Veterinary Medical Teaching Hospital. The health of these dogs was assessed by use of an owner questionnaire, physical examination, complete blood count, serum biochemistry profile, and serum pancreas-specific lipase concentration measurement (Spec cPL, IDEXX Laboratories, Westbrook, ME). Dogs with clinically important abnormalities in any of these parameters were excluded from the study.

The study was approved by the Texas A&M University Institutional Animal Use Committee (AUP 2011-215). Informed owner consent was obtained before enrollment of any of the dogs.

At the time of liver biopsy, 5 mL of urine was collected by cystocentesis and placed into sterile anticoagulant-free tubes. The urine was centrifuged at 20,000 g for 12 minutes to remove cellular material. The supernatant was removed and stored at -80°C until analysis.

Where collected, additional hepatic biopsies were fixed in neutral buffered formalin, processed for routine histopathology, and embedded in paraffin. Sections of liver were stained with hematoxylin and eosin for the evaluation of necroinflammation, picrosirius red for the assessment of fibrosis, and toluidine blue for mast cell enumeration. Hepatic fibrosis (absent, mild, moderate, marked, very marked) and necroinflammatory activity (absent, minimal, mild, moderate, marked, very marked) were scored by a board-certified veterinary pathologist using a previously published scoring system[33,34], which had been adapted from the human Ishak system (Table 1).[32] The total number of mast cells in 10 high power fields (hpf; 400X) for each toluidine blue stained section were counted by a veterinary anatomic pathology resident. Information about the clinical history and urine NMH to creatinine ratio for each case was not provided to the evaluators prior to the scoring/counting process.

Urine N-methylhistamine was measured by stable isotope dilution gas-chromatography/mass spectrometry using a previously analytically validated method.[62] The lower limit of the working range of the assay was 50 pg/μL. Urine creatinine concentrations were used to normalize urine NMH concentrations. Urine samples were diluted 1:20 before measurement of creatinine using an automated chemistry analyzer (Sirrus, Stanbio Laboratory). The urine NMH to creatinine ratio was calculated and

expressed as ng of NMH per mg of creatinine. Samples for which the urine NMH concentration was < 50 pg/µL were removed from the analysis because it was not possible to accurately quantify NMH and therefore it was not possible to calculate or estimate the NMH to creatinine ratio for these samples as the reported ratio could be much higher that it really is for a very dilute urine sample with a low urine creatinine concentration.

Data were tested for normality using the Kolmogorov-Smirnov test and visual inspection of frequency histograms. Non-parametric data are expressed as median (minimum−maximum). Urine NMH to creatinine ratios were compared between healthy dogs and dogs with liver disease using the Mann-Whitney U test. Urine NMH to creatinine ratios were compared among the five groups of dogs using Kruskall-Wallis tests followed by Dunn's post-test as appropriate. A reference interval for urine NMH to creatinine ratios was constructed by calculating the central 95th percentile of the results from healthy dogs. The association between having an elevated urine NMH to creatinine ratio and disease group was assessed using exact methods. The correlations between urinary NMH concentrations and mast cell counts, fibrosis scores, as well as necroinflammatory scores, were assessed using Spearman's rank correlation (r_s). Statistical significance was set as $\alpha < 0.05$. A statistical software package was used for all calculations (GraphPad, Prism 5, GraphPad Software).

Results

Fifty-nine dogs with hepatobiliary disease were enrolled in the study. For 7 of these dogs it was not possible to determine urinary NMH to creatinine ratio because the urinary NMH concentration was < 50 pg/μL. The median age of the remaining 52 dogs was 9.5 years (6 month to 13 years). Sixteen were neutered male (31%), five were intact male (10%), twenty-eight were spayed female (54%), and three were intact female (6%). The following breeds were commonly represented: five Labrador Retrievers (10%), four Miniature Schnauzers (8%), 3 Chihuahuas (6%), and 17 mixed breed dogs (29%). Seventeen dogs (33%) had hepatobiliary neoplasia, 14 (27%) had CH, eight (15%) had hepatic vascular disease (7 of which had CPSS and 1 had microvascular dysplasia), and 13 (25%) had other hepatobiliary disease.

Twenty-five healthy dogs were enrolled into the study. For 3 of these dogs it was not possible to determine urinary NMH to creatinine ratio because the urinary NMH concentration was < 50 pg/μL. The median age of the remaining 22 dogs was 3.5 years (1 to 12 years). Seven were neutered male (27%), none were male intact, 10 were spayed female (38%), and five were intact female (19%). The following breeds were commonly represented: three Labrador Retrievers (12%), four Miniature Schnauzers (15%), two Boston Terriers (8%), and three mixed breed dogs (12%).

Median urine NMH concentrations were 96 pg/μL (13−485) and 104 pg/μL (14−1,114) for healthy dogs and dogs with hepatobiliary disease, respectively ($P = 0.618$). Median urinary NMH to creatinine ratios were 82 pg/μL (<50−291), 143 pg/μL

(<50−286), 199 pg/μL (<50−456), and 142 pg/μL (43−1,114) for dogs with hepatic

neoplasia, CH, vascular, and other hepatobiliary disease, respectively ($P = 0.420$).

Median urinary NMH to creatinine ratios were 71 ng/mg (23−241) and 98 ng/mg

(40−492) for healthy dogs and dogs with hepatobiliary disease, respectively ($P = 0.006$).

Median urinary NMH to creatinine ratios were 90 ng/mg (58−236), 121 ng/mg

(60−423), 128 ng/mg (55−492), and 98 ng/mg (40−326) for dogs with hepatic neoplasia,

CH, vascular, and other hepatobiliary disease, respectively (Figure 14). There was a

trend for healthy dogs to have lower urinary NMH to creatinine ratios than dogs with CH

or dogs with hepatic vascular disease but these differences did not reach statistical

significance ($P < 0.1$ but > 0.05).

The central 95% percentile of the urinary NMH to creatinine ratio for the 22

health dogs was 26−223 ng/mg. Eight of 52 dogs (15%) with hepatobiliary disease had

serum NMH to creatinine ratios > 223 ng/mg. There was no association between disease

group and having a urinary NMH to creatinine ratio > 223 ng/mg ($P = 0.484$; Table 5)

The median hepatic mast cell count for the dogs with hepatobiliary liver disease

was 0 mast cells per 10 hpf (0−24; $n = 46$). Eight of 43 dogs for which fibrosis was

scored (19%) were scored to have no hepatic fibrosis, 10 (23%) to have mild fibrosis, 14

(33%) to have moderate fibrosis, 6 (14%) to have marked fibrosis, and 5 (12%) to have

very marked fibrosis. Ten of 43 dogs (23%) had no necroinflammation, 10 (23%) had

minimal necroinflammation, 11 (26%) had mild necroinflammation, 7 (16%) had

moderate necroinflammation, 5 (12%) had marked necroinflammation, and no dogs had

very marked necroinflammation. There was no significant correlation between urine

NMH concentration and mast cell count (r_s = -0.103; P = 0.496), fibrosis score (r_s = -0.125; P = 0.424) or necroinflammatory score (r_s = -0.091; P = 0.558). There was no significant correlation between urinary NMH to creatinine ratio and mast cell count (r_s = -0.077; P = 0.643), fibrosis score (r_s = 0.256; P = 0.097) or necroinflammatory score (r_s = 0.042; P = 0.788). There was no significant correlation between mast count and fibrosis score (r_s = 0.220; P = 0.184) or necroinflammatory score (r_s = 0.200; P = 0.228).

Discussion

For the current study, dogs with hepatobiliary disease had higher urinary NMH to creatinine ratios than healthy dogs with median ratios of 71 ng/mg and 98 ng/mg, respectively. There was a trend for healthy dogs to have lower urinary NMH to creatinine ratio than dogs with CH or vascular liver disease (CPSS or microvascular dysplasia), but this difference did not reach statistical significance. Equivalent differences between groups were not observed when urine NMH concentrations were compared. This is not unexpected as urine NMH concentrations are affected by differences in urine concentrations, potentially masking differences between groups. This is the reason that we normalized urine NMH concentrations by calculating the urinary NMH to creatinine ratios. In a previous study including 6 healthy dogs the control range for urinary NMH to creatinine ratio was determined to be < 136 ng/mg.[62] However, in the 22 healthy dogs enrolled in our study a preliminary reference range was determined to be 26−223 ng/mg. This difference is probably a reflection that the group of healthy dogs used for the previous study was so small. Eight of 52 dogs (15%) with

Table 5. Urinary N-methylhistamine to creatinine ratio and disease group

Group	Number below RI (%)	Number within RI (%)	Number above RI (%)
Hepatic neoplasia	0 (0%)	16 (94%)	1 (6%)
Chronic hepatitis	0 (0%)	11 (79%)	3 (21%)
Hepatic vascular disease	0 (0%)	6 (75%)	2 (25%)
Other hepatobiliary disease	0 (0%)	11 (85%)	2 (15%)

Urinary N-methylhistamine to creatinine ratios are compared between different disease categories for 52 dogs with hepatobiliary disease and the reference interval of 26−223 ng/mg. There was no significant association between urinary N-methylhistamine to creatinine ratio and disease group ($P = 0.484$). RI = reference interval.

Figure 14. Urinary N-methylhistamine to creatinine ratios for 52 dogs with

hepatobiliary disease
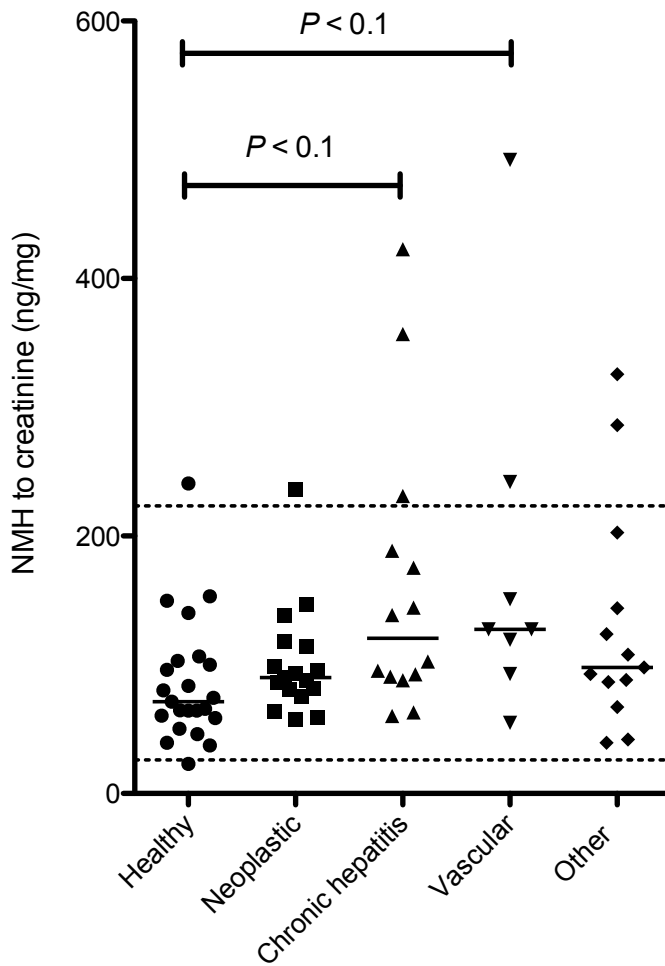
The solid horizontal bars represent the median urinary N-methylhistamine to creatinine

ratio for that group of dogs. The dashed horizontal lines represent the upper and lower

limits of the 95th percentile of urinary N-methylhistamine to creatinine ratios for 22

healthy dogs (26−223 ng/mg). *P*-values are for Dunn's post-test between groups of dogs.

hepatobiliary disease had a urinary NMH to creatinine ratio > 223 ng/mg. There was no association between the disease group and having an increased urinary NMH concentration. The highest recorded urinary NMH to creatinine ratio was 492 ng/mg and this was, unexpectedly, determined in a sample from a dog with a CPSS. This dog did not have any other clinical findings that would account for this increase. Taken together these findings suggest that mast cell mediated inflammation may occur in a subset of dogs with hepatobiliary disease but is not a common finding. This appears to be similar to the situation in dogs with gastrointestinal disease where the median urinary NMH to creatinine ratio in 16 dogs with chronic gastrointestinal disease was 100 ng/mg (63−722) and 4 of 16 (25%) dogs had urinary NMH to creatinine ratios > 223 ng/mg.[77] Another study of 28 dogs with chronic enteropathies found that the median urinary NMH to creatinine ratio was 97 ng/mg (5−474) in that group and that 2 of 28 (7%) had ratios > 223 ng/mg.[78] In comparison 8 dogs with mast cell tumors tended to have much higher urinary NMH to creatinine ratios with a median of 68,760 ng/mg (327−156,600).[62] The lack of comparably high NMH to creatinine ratios in dogs with hepatobiliary disease does not support the hypothesis that mast cell mediated inflammation plays a major role in canine hepatobiliary disease.

We did not find a correlation between urinary NMH to creatinine ratios and hepatic mast cell counts. Similarly, no correlation between urinary NMH concentration and intestinal mast cell counts were found in studies of dogs with gastrointestinal disease.[77,78] There are several possible reasons for this lack of correlation. Firstly, mast cell mediated inflammation may not be a major contributor to canine hepatobiliary

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disease. Therefore, the small differences in NMH to creatinine ratios between groups may not have been due to hepatic mast cell degranulation. Secondly, as we used toluidine blue, which only stains intact mast cell granules, it is possible that the number of mast cells in the sections were underestimated, as degranulated mast cells may not have been identified by this stain. In our study we were unable to find a correlation between mast cell counts and fibrosis or necroinflammation. Once again, this does not support the importance of mast cell mediated inflammation in the development of canine liver disease or hepatic fibrosis. This is in contrast to the situation in humans where, in patients with chronic hepatitis C, a positive correlation between hepatic mast cell density and fibrosis has been reported.[58] We cannot completely exclude the possibility that the limitations in the mast cell staining technique discussed above, or the relatively small number of dogs with CH ($n = 14$), precluded the identification of such an association. It is interesting that humans with cholestatic disease were found to have higher plasma histamine concentrations than healthy people, which suggests that mast cell mediated inflammation may play an important role in these patients who often complain of pruritus.[60] We did not evaluate any dogs with cholestasis. Therefore, we could not determine if this was also the case for dogs.

In our study few mast cells were observed in toluidine blue stained sections of canine liver (median count of 0 mast cells per 10 hpf). Some dogs did have notably higher mast cell counts and the maximum was 24 per 10 hpf, in a dog diagnosed with CH. To our knowledge the mast cell density in the liver of healthy dogs has not been determined. In comparison the median mast cell count from toluidine blue stained

duodenal sections of dogs with chronic gastrointestinal disease was much higher with a median of 44 per 10 hpf (0−170).[77] It is not unexpected that there were fewer mast cells in sections of liver then in sections of the intestine as mast cells are known to be commonly observed when the intestinal tissue is stained with toluidine blue.[79] It would be interesting to also evaluate the location of mast cells within healthy and diseased canine livers to determine whether they are randomly or zonally distributed. Indeed during anaphylaxis the canine intestine is believed to be the main source of histamine release. The liver is also involved in canine anaphylaxis during which portal hypertension and venous congestion develop. However, it is not clear if this occurs secondary to the effects of histamine and other mediators released from gastrointestinal mast cells into the splanchnic circulation[49] or from degranulation of mast cells within the liver itself.

This study is the first to evaluate urinary NMH concentrations in dogs with hepatobiliary disease. However, it is important to point out several limitations. Firstly, the lower limit of the working range of the assay for the measurement of urinary NMH (50 pg/μL) was suboptimal. As a result, 10 dogs had unmeasurable urinary NMH concentrations. Since the results were expressed as urinary NMH to creatinine ratios it was not possible to calculate or even estimate urinary NMH to creatinine ratios from these dogs and therefore they were excluded from the study. Secondly, although 52 dogs with hepatobiliary disease were enrolled into the study, the number of dogs in some of the subgroups was relatively small, which could have resulted in a type II error. For example, it is possible that if more dogs were enrolled, significant differences in NMH

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to creatinine ratios between healthy dogs and dogs with different kinds of hepatic disease, such as CH, may have been observed. Looking at the distribution of NMH to creatinine ratios in dogs with CH there was a lot of overlap with healthy dogs and so, even if more dogs had been enrolled, our interpretation that mast cell mediated inflammation may only be important in a subset of these dogs is unlikely to have changed. Lastly, as previously discussed, we cannot rule out the possibility that the use of metachromatic staining rather than immunohistochemical staining resulted in an underestimation of mast cell counts. Future studies combining immunohistochemical staining for mast cells with computer assisted image analysis should allow for a more accurate assessment of hepatic mast cell density.[57]

In conclusion, although dogs with hepatobiliary disease had higher urinary NMH to creatinine ratios than healthy dogs, there was a lot of overlap between groups. Mast cell counts from toluidine blue stained sections of diseased canine liver were very low. Therefore, mast cell mediated inflammation does not appear to commonly be a major component of hepatobiliary disease in dogs. There was no correlation between urinary NMH to creatinine ratio and hepatic mast cell count, fibrosis score, or necroinflammatory score. Taken together, these findings do not support the utility of urinary NMH concentration or urinary NMH to creatinine ratio as a biomarker in dogs with hepatobiliary disease.

PUTATIVE PRECIPITATING FACTORS FOR HEPATIC

ENCEPHALOPATHY IN DOGS: 118 CASES (1991−2014)[*]

Introduction

Hepatic encephalopathy is a spectrum of neuropsychiatric abnormalities observed in patients with liver dysfunction in which other brain diseases have been ruled out. In human patients hepatic encephalopathy is classified into three types on the basis of etiology.[37] Type A hepatic encephalopathy is caused by acute liver failure in the absence of preexisting liver disease. Type B hepatic encephalopathy is associated with portosystemic bypass without intrinsic hepatocellular disease (e.g., CPSS). Type C hepatic encephalopathy is associated with cirrhosis and portal hypertension or acquired portal systemic shunting, and is subcategorized on the basis of duration and characteristics. Episodic hepatic encephalopathy develops over a short period of time and varies in severity. Persistent hepatic encephalopathy causes cognitive dysfunction and is classified as mild, severe, or treatment-dependent.[37] Subclinical hepatic encephalopathy is associated with normal mental and neurological statuses in conjunction with abnormal results on specific psychometric tests.[38]

Ammonia is believed to have a central role in the pathogenesis of hepatic encephalopathy. In a study[39] of human patients with cirrhosis, there was a moderate

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positive correlation between total venous plasma ammonia concentration and severity of

hepatic encephalopathy. In dogs with hepatic encephalopathy, venous plasma ammonia

concentrations are frequently, but not always, increased from reference limits, and there

is a positive correlation between plasma ammonia concentration and severity of

disease.[41] In veterinary medicine diagnosis of hepatic encephalopathy generally involves

measurement of plasma ammonia concentration when available whereas in human

medicine diagnosis of hepatic encephalopathy is not dependent on measurement of

plasma ammonia concentration.[40]

Several factors are known to precipitate hepatic encephalopathy in human

patients. Identifying and addressing those precipitating factors are important for patient

management because the prognosis for patients with ≥ 1 precipitating factor is worse

than that for patients without precipitating factors.[42] Results of previous studies suggest

that 88% (354/404)[42] to 90% (45/50)[43] of human patients with hepatic encephalopathy

have at least 1 precipitating factor. Precipitating factors most commonly associated with

hepatic encephalopathy in human patients include gastrointestinal bleeding (18%−76%

of patients), constipation (3%−52%), diarrhea (12%−40%), infection (3%−52%),

hypokalemia (9%−70%), hyponatremia (25%−38%), and excess dietary protein intake

(9−52%).[43-45] Hepatic encephalopathy has been described in dogs with CPSS or APSC

secondary to portal hypertension.[46] Factors proposed to precipitate hepatic

encephalopathy in dogs include gastrointestinal hemorrhage, hypokalemia,

hyponatremia, high protein diets, and alkalosis.[47] Results of a study of dogs with CPSS

suggest that hyperammonemia and SIRS, but not hyponatremia, were associated with

hepatic encephalopathy.[48] To our knowledge, studies to elucidate the association between hepatic encephalopathy and other putative precipitating factors in dogs are lacking. The objectives of the study reported here were to elucidate the relationship between plasma ammonia concentration and the severity of hepatic encephalopathy in dogs and to determine whether there is an association between factors that precipitate hepatic encephalopathy in humans and the presence of clinical signs of hepatic encephalopathy at hospital admission in dogs previously treated for the disease.

Materials and methods

The computerized medical record database at the Texas A&M Veterinary Medical Teaching Hospital was searched for records of dogs in which hepatic encephalopathy was diagnosed between October 1, 1991 and September 1, 2014. An investigator (JAL) reviewed all identified records to verify the diagnosis of hepatic encephalopathy and ensure that each dog met the inclusion criteria for the study. Hepatic encephalopathy was diagnosed on the basis of clinical findings, the exclusion of other causes of encephalopathy, evidence of hepatic dysfunction or insufficiency as determined by results of serum biochemical analysis, CBC, urinalysis, diagnostic imaging (typically abdominal ultrasonography or portal scintigraphy), and response to treatment. Plasma ammonia and serum bile acid concentrations were also evaluated when available. A dog was excluded from the study if its medical record was unavailable for review or it did not meet the criteria for hepatic encephalopathy.

Signalment, historical findings (including previous treatments), cause of hepatic encephalopathy, and the results of the physical examination performed at hospital admission, serum biochemical analysis, CBC, diagnostic imaging, and plasma ammonia concentration (when available) were extracted from the record of each dog enrolled in the study. Laboratory test results were evaluated only if the samples were collected within 24 hours after hospital admission. For dogs that were admitted to the teaching hospital on multiple occasions, information was evaluated only from the admission during which hepatic encephalopathy was initially diagnosed. When possible, the cause of hepatic encephalopathy was classified in accordance with a slightly modified version of a classification system[37] used for human patients in which the definition of type C hepatic encephalopathy was broadened to include all types of intrinsic hepatocellular disease rather than just cirrhosis. Specifically, type A hepatic encephalopathy was defined as acute liver failure in the absence of preexisting hepatic disease, type B hepatic encephalopathy was defined as a portosystemic bypass without intrinsic hepatocellular disease (e.g., CPSS), and type C hepatic encephalopathy was defined as intrinsic hepatocellular disease and portal hypertension or acquired portal systemic shunting.

When sufficient information was available for dogs that had been previously treated for hepatic encephalopathy, the severity of hepatic encephalopathy historically and at the time of hospital admission were graded in accordance with a previously described 5-point scale.[80] The historical hepatic encephalopathy grade generally represented the most severe clinical sign recorded in the patient's history. Briefly, grade 0 was assigned to dogs with no clinical signs of hepatic encephalopathy; grade 1 was

assigned to dogs with mildly impaired mobility, apathy, or both; grade 2 was assigned to dogs with severe apathy, mild ataxia, or both; grade 3 was assigned to dogs with hypersalivation, severe ataxia, head pressing, blindness, circling, or any combination of those signs; and grade 4 was assigned to dogs with seizures or that were in a stupor or coma.

The prevalence of factors such as SIRS, gastrointestinal hemorrhage, dietary change or indiscretion, constipation, furosemide treatment, hypokalemia, hyponatremia, alkalosis, and azotemia that are known to precipitate hepatic encephalopathy in human patients[40] was recorded for each dog on the basis of the patient's history and physical examination findings at the time of its first admission to the teaching hospital. Systemic inflammatory response syndrome was diagnosed when at least 2 of the following 4 criteria were met: body temperature $\geq 39.7\ °C$ (103.5 °F) or $\leq 37.8\ °C$ (100.0 °F), heart rate ≥ 160 beats/min, respiratory rate ≥ 40 breaths/min, and WBC count $\geq 12,000$ or $\leq 4,000$ cells/μL or $\geq 10\%$ band neutrophils.[81] Results of serum biochemical analysis for samples obtained only within 24 hours after the first hospital admission were used to determine the prevalence of hypokalemia, hyponatremia, alkalosis, and azotemia. When available, the plasma ammonia concentration measured within 24 hours after the first hospital admission was also recorded.

The distributions of continuous variables were evaluated for normality by visual inspection of frequency histograms and the Kolmogorov-Smirnov test. Results for variables that there not normally distributed were expressed as the median (minimum−maximum), and results for normally distributed variables were expressed as

the mean ± SD. For each historical finding, physical examination result, and precipitating factor, the prevalence within the study population was expressed as the percentage (95% CI). The historic hepatic encephalopathy severity grade was compared with the hepatic encephalopathy severity grade at the time of hospital admission by use of the Wilcoxon signed-rank test. The correlation between plasma ammonia concentration and the hepatic encephalopathy severity grade was assessed with the Spearman rank correlation coefficient. A statistical software program (GraphPad, Prism 5, GraphPad Software) was used for all analyses, and values of $P < 0.05$ were considered significant.

To investigate the relationship between potential precipitating factors for hepatic encephalopathy and the presence of clinical signs of the disease at the time of hospital admission, the study population was divided into 2 groups (i.e., dogs with and without clinical signs of hepatic encephalopathy during the initial physical examination at hospital admission). The respective frequencies of prior treatment for hepatic encephalopathy, SIRS, gastrointestinal hemorrhage, dietary change or indiscretion, constipation, furosemide treatment, hypokalemia, hyponatremia, alkalosis, azotemia, and hyperammonemia were compared between the 2 groups by use of the Fisher exact test. Variables with $P < 0.2$ for the Fisher exact test were included in a multivariable logistic regression model in which the outcome of interest was modeled as the presence of clinical signs of hepatic encephalopathy at hospital admission. The final model was constructed by backward stepwise elimination, and only variables with $P < 0.05$ were retained in the model. The odds ratio (OR) and 95% confidence interval (CI) for each

variable were calculated. These analyses were performed with another statistical software program (PROC LOGISTIC, SAS, version 9.4, SAS Institute).

Results

The database search revealed that 170 dogs were assigned the diagnostic code for hepatic encephalopathy between October 1, 1991 and September 1, 2014. Forty dogs were excluded from the study because their medical records were incomplete or unavailable for review. An additional 12 dogs were excluded from the study because review of their medical records revealed that there was insufficient evidence to diagnose hepatic encephalopathy. Thus, 118 dogs met the criteria for diagnosis of hepatic encephalopathy and were enrolled in the study, of which 46 (39%) were spayed females, 17 (14%) were intact females, 31 (26%) were castrated males, and 24 (20%) were intact males. The median age of dogs at the time of onset of clinical signs was 24 months (minimum−maximum, 1 to 186 months), and the median age of the dogs at the time of admission to the teaching hospital was 32 months (minimum−maximum, 2 to 186 months). The breeds most commonly represented in the study population were Yorkshire Terrier ($n = 17$ [14%]), Miniature Schnauzer (14 [12%]), Chihuahua (7 [6%]), Labrador Retriever (6 [5%]), Poodle (6 [5%]), Pug (4 [3%]), Dachshund (4 [3%]), Cocker Spaniel (3 [3%]), and Pomeranian (3 [3%]).

The cause of hepatic encephalopathy was unknown because of incomplete diagnostic evaluation for 16 (14%) dogs. Type A hepatic encephalopathy was not diagnosed in any of the dogs, whereas types B and C hepatic encephalopathy were

diagnosed for 73 (62%) and 29 (25%) dogs, respectively. Of the 73 dogs with type B hepatic encephalopathy, the disease was attributed to CPSS in 70 (96%), an arteriovenous fistula and APSC in 2 (3%), and microvascular dysplasia in 1 (1%). Of the 29 dogs with type C hepatic encephalopathy, the disease was attributed to intrinsic hepatocellular disease with APSC in 24 (83%) and intrinsic hepatocellular disease without evidence of APSC identified during abdominal ultrasonography in 2 (7%); the remaining 3 (10%) dogs had intrinsic hepatocellular disease but did not undergo diagnostic imaging for evaluation of APSC. Overall, 96 of the 102 (94%) dogs in which the cause of hepatic encephalopathy was identified had some type of macroscopic portosystemic shunting.

The most frequently recorded historical clinical signs were lethargy ($n = 32$ [27%] dogs), altered behavior (31 [26%]), obtundation (29 [25%]), ataxia (28 [24%]), seizures (26 [22%]), head pressing (22 [19%]), ptyalism (22 [19%]), vomiting (21 [18%]), blindness (20 [17%]), circling (15 [13%]), shaking or twitching (14 [12%]), and anorexia or hyporexia (13 [11%]). At the time of hospital admission, abnormal neurological findings were recorded for 56 (47%) dogs, and the most frequently recorded clinical signs were obtundation ($n = 30$ [25%]), ataxia (23 [19%]), paresis (9 [8%]), conscious proprioceptive deficits (8 [7%]), seizures (6 [5%]), stupor or coma (5 [4%]), circling (4 [3%]), abnormally delayed menace response (4 [3%]), tremors (3 [3%]), and blindness, abnormally decreased pupillary light response, head pressing, ptyalism, head tilt, and anisocoria (2 [2%] each).

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The frequency distributions of hepatic encephalopathy severity grades before (historical) and at the time of hospital admission for the study population were summarized (Table 6). A hepatic encephalopathy severity grade at the time of hospital admission could not be assigned to 2 of the 118 dogs because the medical records for those dogs contained insufficient information. The median historical severity grade (3; minimum−maximum, 0 to 4) was significantly ($P < 0.001$) greater than the median severity grade at hospital admission (1; minimum−maximum, 0 to 4). For each of 116 dogs, the medical record maintained by the referring veterinarian prior to the patient's admission to the teaching hospital was available for review, and 50 (43%) dogs were treated for hepatic encephalopathy prior to referral to the teaching hospital.

Plasma ammonia concentration was determined within 24 hours after hospital admission for 83 (70%) of 118 dogs. The median plasma ammonia concentration was 179 μg/mL (minimum−maximum, 15 to 1,350 μg/mL; reference limit, < 50 μg/mL), and 77 (93%) dogs had hyperammonemia. Plasma ammonia concentration was not significantly correlated with either the historical hepatic encephalopathy severity grade ($r_s = 0.16$; $P = 0.156$) or the hepatic encephalopathy grade at the time of hospital admission ($r_s = 0.22$; $P = 0.052$; Figure 15).

Information regarding some precipitating factors for hepatic encephalopathy was unavailable for some dogs; therefore, the denominator used for determining the prevalence varied among the precipitating factors. The putative precipitating factors for hepatic encephalopathy prevalent in dogs at the time of hospital admission were SIRS (prevalence, 14% [16/116]), hyponatremia (7% [7/105]), alkalosis (5% [5/103]),

hypokalemia (5% [5/105]), dietary change or indiscretion (3% [4/118]), furosemide treatment (3% [4/118]), azotemia (3% [3/107]), gastrointestinal hemorrhage (2% [2/118]), and constipation (1% [1/118]). Thirty-six (31%) of the 118 dogs had at least 1 putative precipitating factor for hepatic encephalitis at the time of hospital admission.

Of the 116 dogs for which sufficient information was available to assign a hepatic encephalopathy grade at the time of admission to the hospital, 59 (51%) and 57 (49%) did and did not, respectively, have clinical signs of hepatic encephalopathy recorded during the initial physical examination at the time of admission. Fisher exact test results revealed that prior treatment for hepatic encephalopathy ($P = 0.014$) and hyperammonemia ($P = 0.023$) were significantly associated with whether dogs did or did not have clinical signs of hepatic encephalopathy at the time of hospital admission (Table 7). Factors assessed in the multivariable logistic regression model included prior treatment for hepatic encephalopathy, SIRS, hypokalemia, and hyperammonemia. Hypokalemia, SIRS, and hyperammonemia were sequentially eliminated from the model, and the final model included only prior treatment for hepatic encephalopathy. Dogs with clinical signs of hepatic encephalopathy at the time of hospital admission were less likely to have been previously treated for the disease than were dogs without clinical signs of hepatic encephalopathy at the time of hospital admission (OR, 0.36; CI: 0.17 to 0.78; $P = 0.009$).

Figure 15. Plasma ammonia concentration versus hepatic encephalopathy severity grade

before (A) and at the time of admission (B)
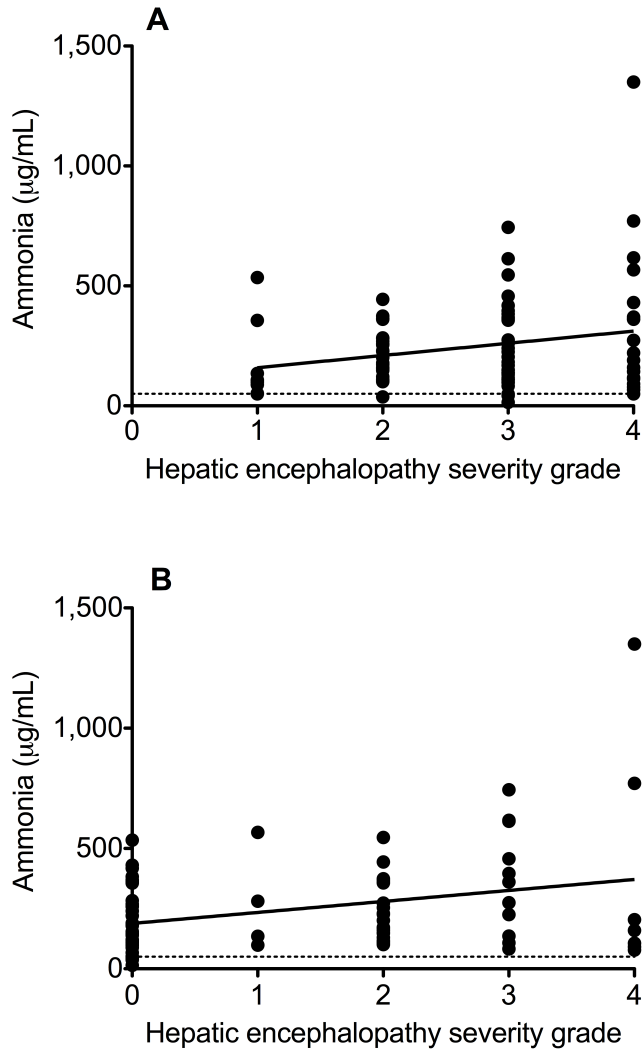
The dashed horizontal line represents the upper reference limit for plasma ammonia

concentration (50 μg/mL). The solid diagonal line represents the line of best fit for the

data.

Table 6. Frequency distributions of hepatic encephalopathy severity grades before
(historical) and at the time of admission to a veterinary teaching hospital

Severity grade	Historical	At hospital admission*
0	1 (1)	57 (49)
1	11 (9)	6 (5)
2	29 (25)	29 (25)
3	54 (46)	14 (12)
4	23 (19)	10 (9)

Values represent the number (percentage) of dogs. Each dog was assigned a hepatic
encephalopathy severity grade on a scale of 0 to 4 as follows: grade 0 = no clinical signs
of hepatic encephalopathy; grade 1 = mildly impaired mobility, apathy, or both; grade 2
= severe apathy, mild ataxia, or both; grade 3 = hypersalivation, severe ataxia, head
pressing, blindness, circling, or any combination of those signs; and grade 4 = seizures,
stupor, or coma.[80] A hepatic encephalopathy severity grade at the time of hospital
admission could not be assigned to 2 of the 118 dogs because the medical records for
those dogs contained insufficient information.

Table 7. Frequency distributions of various putative precipitating factors for hepatic encephalopathy that did and did not have clinical signs of the disease at the time of admission to a veterinary teaching hospital

Factor	Dogs with clinical signs at admission	Dogs without clinical signs at admission	OR (95% CI)*	*P* value**
Prior treatment for HE	18 (32)	32 (56)	0.36 (0.17–0.78)	0.014
SIRS	11 (19)	5 (9)	2.49 (0.80–7.69)	0.176
Gastrointestinal hemorrhage	1 (2)	1 (2)	0.97 (0.06–15.83)	1.000
Dietary change or indiscretion	1 (2)	3 (5)	0.31 (0.03–3.08)	0.360
Constipation	1 (2)	0 (0)	2.95 (0.12–73.95)[***]	1.000
Furosemide treatment	2 (4)	2 (4)	0.96 (0.13–7.10)	1.000
Hypokalemia	4 (7)	0 (0)	8.65 (0.45–165.00)[***]	0.117
Hyponatremia	5 (9)	2 (4)	2.35 (0.44–12.71)	0.443
Alkalosis	2 (4)	3 (6)	0.60 (0.01–3.77)	0.669
Azotemia	3 (5)	0 (0)	6.87 (0.35–136.40)[***]	0.244
Hyperammonemia	45 (98)	33 (83)	9.55 (1.12–81.41)	0.023

Values represent the number (percentage) of dogs unless otherwise indicated. Information regarding some of the precipitating factors was unavailable for some dogs. * Odds ratios were calculated by univariable analysis and the referent was dogs without clinical signs of hepatic encephalopathy at hospital admission. ** For Fisher exact test. *** Calculation was performed by the addition of 0.5 to each group of dogs

Discussion

In the present study, most of the 118 dogs treated for hepatic encephalopathy at a veterinary teaching hospital between October 1, 1991 and September 1, 2014 had type B hepatic encephalopathy, followed by type C hepatic encephalopathy. Although 36 (31%) of those dogs had at least 1 putative precipitating factor for hepatic encephalopathy at the time of hospital admission, none of the precipitating factors evaluated were significantly associated with the presence of clinical signs of hepatic encephalopathy at hospital admission. Dogs that were treated for hepatic encephalopathy prior to hospital admission were less likely to have clinical signs of the disease at hospital admission than were dogs that were not treated for hepatic encephalopathy prior to hospital admission.

In human patients, type C hepatic encephalopathy, which is associated with cirrhosis and portal hypertension or acquired portosystemic shunting, is more common than type B hepatic encephalopathy, which is associated with portosystemic bypass in the absence of intrinsic hepatocellular disease.[40] Conversely, most of the dogs in the present study in which the cause of hepatic encephalopathy was identified ($n = 102$) had type B hepatic encephalopathy (73 [72%]), which was generally attributable to CPSS (70/73 [96%]), whereas the remaining dogs (29 [28%]) had type C hepatic encephalopathy, which was generally attributable to portal hypertension and the development of APSC (24/29 [83%]). Some dogs with intrinsic hepatocellular disease (type C hepatic encephalopathy) were not evaluated for APSC, and in others, APSC was not detected during diagnostic imaging but may have been present (i.e., false negative diagnostic imaging results). Therefore, it is likely the prevalence of APSC was

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underestimated for the dogs of the present study. Macroscopic portosystemic shunting secondary to CPSS or APSC was identified in 96 (94%) of the 102 dogs in which the cause of hepatic encephalopathy was identified; however, it is likely this is also an underestimate of the true prevalence of macroscopic portosystemic shunting in the study population. Regardless, the results of the present study were similar to those of another study in which abdominal ultrasonography was used to identify the cause of hyperammonemia in 90 dogs. In that study,[46] 61 (68%) dogs had CPSS, 17 (19%) dogs had APSC (including arteriovenous fistulae), and 11 (12%) dogs had no macroscopic portosystemic shunting detected. Although type A hepatic encephalopathy associated with acute hepatic failure was not diagnosed in any dogs of the present study, it has been reported in dogs.[41]

The most commonly reported historical and clinical findings (lethargy, altered behavior, obtundation, ataxia, seizures, head pressing or tilt, ptyalism, vomiting, blindness, circling, shaking or twitching, anorexia or hyporexia, abnormally delayed menace and pupillary light responses, anisocoria, conscious proprioceptive deficits, and stupor or coma) for the dogs of the present study were similar are similar to those that have previously been recognized.[47] In human medicine, seizures are rarely associated with hepatic encephalopathy[40]; however, seizure activity was recorded for a substantial proportion (22% [26/118]) of dogs in the present study. The apparent difference in the incidence of seizure activity between humans and dogs with hepatic encephalopathy might be a reflection of the fact that hepatic encephalopathy is generally diagnosed at an earlier stage in human patients than it is in dogs. Human patients with subclinical hepatic

encephalopathy perform poorly on specialized psychometric tests, but do not have an impaired mental status or abnormal neurological examination findings.[38] Unfortunately, there is currently no way to diagnose subclinical hepatic encephalopathy in dogs, and the disease is detected only after clinical signs become apparent. Therefore, it is likely that hepatic encephalopathy is underdiagnosed in dogs.

For the dogs of the present study, the median hepatic encephalopathy severity grade before hospital admission (median grade, 3) was worse than that at hospital admission (median grade, 1). This finding was not surprising because 50 (43%) of the 116 dogs that were assigned a hepatic encephalopathy severity grade both before and at hospital admission were treated for the disease prior to being admitted to the veterinary teaching hospital. Also, dogs that were not treated for hepatic encephalopathy prior to hospital admission were approximately 3 times as likely to have clinical signs of hepatic encephalopathy at the time hospital admission, compared with dogs that were treated for the disease prior to hospital admission.

The protocols used to treat the dogs of the present study for hepatic encephalopathy prior to admission to the veterinary teaching hospital varied, but generally included antimicrobials, lactulose, and some type of dietary intervention. Unfortunately, the protocols varied to such an extent that it was not possible to assess the efficacy of individual interventions. However, the findings of the present study suggested that medical management strategies commonly used to treat dogs with hepatic encephalopathy are effective, at least in the short term.

In the present study, venous plasma ammonia concentration was measured within 24 hours after hospital admission for 83 dogs, of which 78 (94%) had hyperammonemia (ammonia concentration $\geq$ 50 µg/mL). This suggested that most but not all dogs with hepatic encephalopathy have hyperammonemia, a finding that supported the results of another study[41] that involved dogs with hepatic encephalopathy. However, we could not estimate the sensitivity of the presence of ammonemia for detecting dogs with hepatic encephalopathy from our data because the presence of hyperammonemia was used as an inclusion criterion for the study. Consequently, the sensitivity of hyperammonemia for detecting hepatic encephalopathy calculated from the data of the present study would likely be overestimated. Also, at the time of hospital admission, many dogs were receiving lactulose and various antimicrobials, which might have reduced the absorption of ammonia from the intestine at the time the blood sample used to measure plasma ammonia concentration was obtained. Although there was a weak positive correlation (r_s = 0.22) between venous plasma ammonia concentration and the hepatic encephalopathy severity grade at the time of hospital admission, that correlation did not quite reach significance ($P = 0.052$). Results of another study[41] that involved dogs indicate that there is a positive correlation between hepatic encephalopathy severity and both arterial and venous plasma ammonia concentrations. It is possible that the enrollment of additional dogs with hepatic encephalopathy or dogs without a history of hepatic encephalopathy in the present study would have enabled us to detect a significant correlation between plasma ammonia concentration and hepatic encephalopathy severity. Results of studies[39,82] that involved human patients suggest there is a moderate to strong positive

correlation between arterial plasma ammonia concentration or partial pressure and the severity of hepatic encephalopathy. Interestingly, results of another study[83] suggest only a weak correlation between venous plasma ammonia concentration and the severity of hepatic encephalopathy in human patients. Arterial ammonia concentration is generally higher than venous ammonia concentration, and may better reflect the ammonia concentration in the cerebrum.[39] Ammonia in its gaseous form readily enters the brain; therefore, the correlation between the severity of hepatic encephalopathy and the pH-dependent partial pressure of gaseous ammonia is better than the correlation between the severity of hepatic encephalopathy and total arterial ammonia concentration.[82] Because the ranges for plasma ammonia concentration among patients with different hepatic encephalopathy severity grades (including those with a severity grade of 0, or no clinical signs of the disease) overlap, measurement of plasma ammonia concentration is of limited value for detection of individual human or canine patients with hepatic encephalopathy.[39,41] Hence, even though ammonia has a critical role in the pathogenesis of hepatic encephalopathy, other factors such as inflammatory mediators, neurosteroids, and manganese are also important.[40] Dogs with CPSS and clinical signs of hepatic encephalopathy often have serum C-reactive protein concentrations that are increased from reference limits.[84] Additionally, dogs with CPSS[85] or primary hepatitis[86] frequently have blood manganese concentrations that are increased from reference limits.

Thirty-six (31%) of the 118 dogs of the present study had at least 1 putative precipitating factor for hepatic encephalopathy at the time of hospital admission. The precipitating factors for hepatic encephalopathy that were most commonly recorded for

the dogs of the present study were SIRS, hyponatremia, alkalosis, hypokalemia, dietary change or indiscretion, furosemide treatment, azotemia, gastrointestinal hemorrhage, and constipation. The prevalences of those precipitating factors in the dogs of the present study were generally lower than the prevalences of those factors in human patients with hepatic encephalopathy, likely because the most common cause of hepatic encephalopathy in dogs is CPSS, whereas the most common cause of hepatic encephalopathy in humans is cirrhosis, and patients with cirrhosis tend to have more systemic complications.[40]

Results of another study[48] indicate that SIRS is associated with hepatic encephalopathy in dogs with CPSS, and SIRS, with a prevalence of 14% (16/116), was the most commonly recorded precipitating factor for hepatic encephalopathy in the dogs of the present study. The criteria used to diagnose SIRS in the present study were more stringent than those used in another study[87] because we decided it would be preferable to be conservative and reduce the chance for false-positive SIRS diagnoses, which might have contributed to the fairly low prevalence of SIRS in the present study. A variety of mechanisms have been proposed for how inflammation and infection could precipitate hepatic encephalopathy. A synergistic relationship between ammonia and inflammatory cytokines might alter cerebral neurotransmission and increase the permeability of the blood-brain barrier.[88] Also, dogs with CPSS have higher serum C-reactive protein[84] and plasma interleukin-6 concentrations[89] than do dogs without CPSS.

Hyponatremia (7% [7/105]) and hypokalemia (5% [5/105) were the next most common precipitating factors for hepatic encephalopathy recorded for the dogs of the

present study. Hyponatremia is believed to precipitate hepatic encephalopathy by exacerbating the low-grade cerebral edema caused by ammonia dysmetabolism.[90] Hypokalemia causes extracellular alkalosis, which can lead to the trapping of ammonium ions within the cells of the cerebral cortex.[47]

The putative precipitating factors for hepatic encephalopathy evaluated in the present study were chosen on the basis of known precipitating factors for hepatic encephalopathy in humans. For the dogs of the present study, none of those precipitating factors for hepatic encephalopathy were significantly associated with the presence of clinical signs of the disease at the time of hospital admission. However, these findings are specific for the study population and should not be extrapolated to a population that includes dogs with and without a history of hepatic encephalopathy. Thus, the precipitating factors for hepatic encephalopathy evaluated in the present study might instead be comorbid disorders that are not involved in the pathogenesis of hepatic encephalopathy in dogs. The results of the present study differ from those of retrospective study[48] of dogs with CPSS in which SIRS and hyperammonemia, but not hyponatremia, were associated with hepatic encephalopathy. That study[48] differed from the present study in that the dogs with CPSS did not have a history of hepatic encephalopathy, which may account for the conflicting results between the 2 studies. Furthermore, the prevalences of the putative precipitating factors for hepatic encephalopathy in the present study population were fairly low, which could suggest that the study had insufficient power to detect an association between the precipitating factors for hepatic encephalopathy and the presence of clinical signs of the disease at

hospital admission. Conversely, it is possible that there is no association between the putative precipitating factors and the presence of clinical signs of hepatic encephalopathy because those factors are not as critical for the development of hepatic encephalopathy in dogs as they are in humans. Nevertheless, we believe that it is prudent for clinicians to evaluate dogs for the putative precipitating factors for hepatic encephalopathy and manage those factors whenever possible.

The present study had several limitations. As with any retrospective study, our ability to identify dogs that met the criteria for study enrollment and accurately evaluate those dogs was dependent on the correct and complete recording of each subject's history, physical examination findings, and diagnostic test results in its medical record. It is possible that the prevalence of dogs with clinical signs of hepatic encephalopathy at the time of hospital admission was underestimated. Also, the retrospective assignment of hepatic encephalopathy severity grades was difficult because the disease is episodic in nature, and it is possible that clinical signs were not at their worst when the dogs were examined at the veterinary teaching hospital, which could have led to underestimation of severity grade. Underestimation of the hepatic encephalopathy severity grade would have limited our ability to detect a correlation between the plasma ammonia concentration and the severity grade. To minimize the potential effect from underestimation of the hepatic encephalopathy severity grade, we assigned each dog 2 severity grades, 1 of which was based on the patient's history provided by the owner and referring veterinarian and another of which was based on the patient's initial physical examination findings at the time of hospital admission. Additionally, the diagnostic

testing protocol was not standardized. Therefore, the evaluation for portosystemic vascular anomalies varied among dogs, and we were unable to determine the cause of hepatic encephalopathy in some dogs. Ideally, a prospective study should be performed in which each patient undergoes a standardized comprehensive evaluation (e.g. computed tomography angiography for identification of portosystemic vascular anomalies and histological evaluation of a liver biopsy specimen for assessment of intrinsic hepatocellular disease). Although the prevalences of the putative precipitating factors were determined at the time of or within 24 hours after admission to the veterinary teaching hospital to ensure that dogs were assessed for the presence of hepatic encephalopathy as close to hospital admission as possible, it is possible that plasma ammonia or serum electrolyte concentrations changed between the time that the initial physical examination was performed and the time that the blood samples were obtained for analyses. Finally, because of the retrospective nature of the study, evaluation of the putative precipitating factors for hepatic encephalopathy was not standardized. Some factors, such as hypokalemia, are easy to detect, whereas others, such as gastrointestinal hemorrhage, are difficult to diagnose. Consequently, the presence of some factors may have been non-differentially misclassified, which would have shifted the ORs for those factors toward the null and potentially caused a type II error. Further studies are necessary to better elucidate the precipitating factors for hepatic encephalopathy in dogs. Results of the present study indicated that type B hepatic encephalopathy subsequent to CPSS was the most common cause of hepatic encephalopathy in dogs with a history of the disease, followed by type C hepatic encephalopathy subsequent to APSC.

Approximately 31% (36/118) of the dogs had at least 1 putative precipitating factor for hepatic encephalopathy; however, there was no significant association between any of those factors and the presence of clinical signs hepatic encephalopathy at the time of hospital admission. Dogs treated for hepatic encephalopathy prior to hospital admission were less likely to have clinical signs of the disease at the time of hospital admission. Further investigation into the pathogenesis of hepatic encephalopathy in dogs is needed.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Hepatic histological scoring

Histopathological assessment of liver biopsy specimens is currently the only diagnostic method to definitively diagnose CH and to assess the severity of hepatic fibrosis in dogs. However, this technique is expensive, there is a risk of hemorrhage[24], and it is susceptible to sampling variation.[25] Furthermore, there was poor inter-observer agreement when veterinary pathologists evaluated necroinflammatory activity and only fair agreement when they evaluated hepatic fibrosis using a previously published scoring system. Interestingly, the pathologists usually only disagreed by one score level. Thus, the level of agreement observed may be acceptable for fibrosis if partial agreement is taken into account. A retrospective study to determine the prognostic significance of the stage of hepatic fibrosis in dogs with CH is currently being performed. This will allow us to determine the clinical acceptability of this partial agreement. The inter-observer agreement was lower for scoring of necroinflammation than for fibrosis, which is also the case for humans.[31] However, for both the level of agreement was suboptimal. This complicates the interpretation of hepatic histopathology in both a clinical and a research setting. For clinical use where repeatability is important simplification of this scoring system, especially the grading of necroinflammatory activity, may be beneficial, as this would be expected to improve inter-observer agreement. For research studies, where it may be important to detect small changes in fibrosis or necroinflammation, more than

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one pathologist should score sections and/or histological assessment should be augmented by more objective techniques such as computerized image analysis. The work described in this dissertation shown the feasibility of computer assisted image analysis for the assessment of canine hepatic fibrosis. However, further, studies assessing the utility of computerized image analysis to assess canine hepatic fibrosis are needed.

Conclusion

There is suboptimal agreement when veterinary pathologists assess canine hepatic fibrosis and necroinflammatory activity. This is concerning, and a simplified scoring system should be developed for use in a clinical setting. When investigators design studies evaluating these findings, multiple pathologists should examine specimens or other techniques, such as computerized image analysis, should be used in addition to histological assessment.

Diagnosis of hepatic fibrosis

Due to the limitations of the histological assessment of hepatic fibrosis discussed above, the development of non-invasive markers would be beneficial in diagnosing and monitoring dogs with CH. Unfortunately, although HA, PIIINP, and TIMP-1 are promising markers of hepatic fibrosis in humans[27] they did not appear to useful for this purpose in dogs. A previous study did suggest that serum HA has some utility as a marker of canine hepatic fibrosis.[28] Further work to identify and validate other markers

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of fibrosis in dogs is warranted. One appealing approach is to use untargeted proteomics to identify candidate markers from specimens of fibrosed and healthy canine liver. As a next step, these candidate markers would need to be evaluated in a larger group of dogs. Additionally, microRNAs, which are non-coding RNAs, also have the potential to be used as serum markers for hepatic fibrosis.[91]

Another possible approach for the non-invasive assessment of canine hepatic fibrosis would be elastography. In broad terms, this involves creating a shear or strain wave in the tissue of interest. The speed of propagation of this wave is then measured with ultrasound or magnetic resonance imaging.[92] Shear waves pass more quickly through stiff tissue, so the speed of the wave is directly related to the degree of hepatic fibrosis.[92] These techniques have been shown to have good diagnostic accuracy for the detection of hepatic fibrosis in humans with CH[92] but to the author's knowledge have not been used to assess canine hepatic fibrosis.

The finding that serum TIMP-1 concentration has some ability to discriminate between dogs with hepatobiliary disease and healthy dogs or dogs with non-neoplastic hepatic disease is interesting. However, further work is needed to determine if TIMP-1 has any clinical utility for this purpose. This marker is more likely to have potential as a prognostic marker for dogs with hepatocellular carcinoma (and possibly other tumors) rather than as a true diagnostic marker. The reason for this is that hepatocellular tumors are usually readily detected in dogs using abdominal ultrasound and, if after diagnosis they are the surgically excised, a biopsy can be collected for definitive diagnosis.[93]

Conclusion

Measurement of serum PIIINP and TIMP-1 do not appear to be useful biomarkers of canine hepatic fibrosis. The results of the work described in this thesis do not support the utility of serum HA for the assessment of hepatic fibrosis in dogs, however findings from a previous study suggest that this marker may have some utility for this purpose. Further work to identify noninvasive markers of canine hepatic fibrosis is needed.

Mast cell mediated inflammation

A subset of dogs with hepatobiliary disease, including those with CH, had mildly increased urinary NMH to creatinine ratios, suggesting that mast cell mediated inflammation may play a role in these dogs. However, mast cell counts were low in toluidine blue stained sections of liver from dogs with a variety of different hepatobiliary diseases, and there was no correlation between urinary NMH to creatinine ratios and hepatic mast cell counts, fibrosis score, or necroinflammatory score. Taken together these findings do not support the hypothesis that mast cell mediated inflammation plays an important role in canine hepatobiliary disease. They also do not support the utility of urinary NMH to creatinine ratio as a marker of hepatobiliary inflammation in dogs. It is not possible to completely rule out the possibility that mast cell mediated inflammation may be important in a minority of dogs with hepatobiliary disease and studies evaluating hepatic mast cell counts using immunohistochemical staining may allow the more sensitive detection mast cells, especially those that are degranulated.

The development of novel markers of hepatobiliary inflammation would be beneficial for the diagnosis on monitoring of canine liver disease. As CH is characterized by a predominantly lymphoplasmacytic inflammatory infiltrate it may be a better use of resources to develop markers of mononuclear cell inflammation. We also have an ongoing project assessing the efficacy of the acute phase inflammatory marker C-reactive protein and S100-A12, which is a marker of phagocytic cell activation as a biomarkers of hepatic necroinflammatory activity.

Conclusion

Mast cell mediated inflammation does not appear to play an important role in the majority of dogs with hepatobiliary disease and therefore the urine NMH to creatinine ratio does not appear to be a useful biomarker of hepatic inflammation.

Hepatic encephalopathy

Hepatic encephalopathy is an important complication of CH in dogs. The most common cause of HE in dogs is portosystemic shunting, which in turn is most commonly due to a CPSS (type B HE), but can also occur due to APSCs that develop in dogs with hepatocellular disease (type C HE), as is the case in dogs with CH. Hepatic encephalopathy in the absence of macroscopic portosystemic shunting is uncommon in dogs. In dogs with CH, APSCs develop secondary to hepatic portal hypertension. Ammonia plays a central role in the pathogenesis of HE by causing astrocyte swelling and dysfunction through a number of different mechanisms. Therefore, it was not

surprising that there was a weak positive correlation between venous plasma ammonia concentration and the hepatic encephalopathy severity grade at the time of hospital admission, although this correlation did not quite reach significance ($P = 0.052$). Additionally, on univariate analysis there was an association between hyperammonemia and the presence of HE at presentation. In humans other factors that can precipitate HE, such as electrolyte abnormalities, gastrointestinal hemorrhage, and SIRS, have been identified. The roles of such putative precipitating factors in canine HE are not well understood. Although these factors are relatively common in dogs with HE there was no significant association between any of these factors and the presence of clinical signs of hepatic encephalopathy at the time of hospital admission. However, a previous study did find an association between HE and SIRS as well as hyperammonmia.[48] Further investigation into the pathogenesis of hepatic encephalopathy in dogs is indicated. Initially, prospective studies evaluating the association between putative precipitating factors and the development of HE in dogs at risk should be conducted. Techniques, such as magnetic resonance spectroscopy and positron emission tomography, that allow the pathogenesis of HE to be studied *in vivo,* may also help to better define the pathogenesis of canine HE. This in turn may open up the possibility of discovering novel treatments for this syndrome in dogs and humans.

Conclusion

Dogs with HE commonly have at least one putative precipitating factor, such as electrolyte abnormalities. However, it was not possible to demonstrate an association

between any of these factors and the development of HE other than those previously found for hyperammonemia and SIRS. Dogs medically treated for hepatic encephalopathy prior to hospital admission are less likely to have clinical signs of the disease at the time of hospital admission. Further investigation of the pathogenesis of canine HE is needed.

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