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Pathogenesis of Canine Liver Disease

Scott Kilpatrick

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Student Declaration

I declare that the thesis has been composed by myself and that the work has not be submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications has been included. My contribution and those of the other authors to this work have been explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in Chapter 2 was previously published in *The Journal of Small Animal Practice as Whole blood manganese concentrations in dogs with primary hepatitis by* Scott Kilpatrick, Ana Jacinto, Rob D. Foale, Simon W. Tappin, Carolyn Burton, Polly E. Frowde, Clive M Elwood, Roger Powell, Andrew Duncan, Richard J. Mellanby and Adam G. Gow.

Richard Mellanby also acted as supervisor for this Master Thesis. I carried out experimental work, data analysis and writing of the final paper. All authors were involved in experimental work/data collection and approved the material which was also included in the paper.

The work presented in Chapter 3 has been published in *The Veterinary Journal as Plasma cytokine concentrations in dogs with a congenital portosystemic shunt* by Scott Kilpatrick, Adam G. Gow, Rob D. Foale, Simon W. Tappin, Harvey Carruthers, Nicola Reed, Donald A. Yool, Samantha Woods, Ana I. Marques, Rajiv Jalan and Richard J. Mellanby.

Richard Mellanby also acted as supervisor for this Master Thesis. I carried out experimental work, data analysis and writing of the final paper. All authors were involved in experimental work/data collection and approved the material which was also included in the paper.

The work published in Chapter 5 has been published in *PLOS as Presence of systemic inflammatory response syndrome predicts a poor clinical outcome in dogs with a primary hepatopathy by* Scott Kilpatrick, Margaret Dreistadt, Polly Frowde, Roger Powell, Elspeth Milne, Sionagh Smith, Linda Morrison, Adam G. Gow, Ian Handel and Richard J. Mellanby. Richard Mellanby also acted as supervisor for this Master Thesis. I carried out experimental work, data analysis and writing of the final paper. All authors were involved in experimental work/data collection and approved the material which was also included in the paper. Advice for regression analysis was provided by Ian Handel.

Scott Kilpatrick

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CONTENTS

ABSTRACT

Liver disease is a common cause of morbidity and mortality in dogs. Liver diseases can be broadly classified into vascular abnormalities such as congenital portosystemic shunt (cPSS) and parenchymal diseases such as primary hepatitis. cPSS are a well-recognised vascular anomaly in dogs. Despite the importance of liver diseases as a cause of debilitating clinical signs, little is understood about the pathophysiology of the complications of canine liver disease and it is currently difficult to provide accurate prognostic information to owners of dogs with liver disorders.

One of the most common complications of canine liver diseases is the development of neurological signs termed hepatic encephalopathy (HE). The pathogenesis of HE is poorly understood although increases in ammonia, manganese, gastrointestinal-derived endogenous benzodiazepines, cortisol and altered tryptophan metabolism have been linked to the development of neurological signs. Recent studies have also shown an association between inflammation and HE in dogs with cPSS. However, little is known about the role of manganese in the dogs with primary hepatitis and the relationship between inflammation and liver disease has only been crudely assessed to date using tools such as the systemic inflammatory response score.

A small number of studies have examined prognostic markers in dogs with liver disease. However, none have examined the relationship between inflammation and outcome. This is surprising given the accumulating data in human hepatology which indicates that the presence of systemic inflammatory response syndrome (SIRS) is a common and debilitating event in patients with liver diseases. For example, the presence of SIRS has been linked to the

development of complications such as HE and is associated with a poor clinical outcome in humans with liver diseases. In contrast, the relationship between SIRS and clinical outcome in dogs with a primary hepatopathy is unknown.

This thesis aimed to advance understanding of the pathogenesis of hepatic encephalopathy in dogs with liver disease. In addition, the thesis aimed to examine the relationship between inflammation and clinical outcomes in dogs with liver disease. Specifically, the aim of this body of work was to examine manganese and sodium metabolism in dogs with primary hepatitis and to measure cytokines in dogs with a congenital portosystemic shunt. The final aim was to examine the relationship between systemic inflammatory response scores to clinical outcomes in dogs with a primary hepatitis.

This thesis demonstrated that a high SIRS score was associated with a poorer long term survival in dogs with primary hepatopathies. Furthermore, IL-6 concentrations were increased in dogs with a cPSS. Given the well-established role of IL-6 in the pathogenesis of hepatic encephalopathy in humans with acquired liver disease, it is possible that IL-6 may be important in the development of HE in dogs with a cPSS. This thesis also demonstrates that whole blood Mn concentrations are increased in dogs with primary hepatitis and sodium concentrations were rarely outside the reference range in dogs with primary hepatitis. This finding is in contrast with the numerous studies of human patients with liver disease. Overall, these findings highlight the potential role of dogs as a model to examine the pathogenesis of liver disease in human health.

LAY SUMMARY

Liver disease is a common cause of morbidity and mortality in dogs. Liver diseases can be broadly classified into vascular abnormalities such as congenital portosystemic shunt (cPSS) and parenchymal diseases such as primary hepatitis. cPPS are a well-recognised vascular anomaly in dogs. One of the most common clinical signs in dogs with a cPSS is hepatic encephalopathy (HE), which leads to the development of neurological signs. Recent studies have also shown an association between inflammation and HE in dogs with cPSS. This is similar to humans with liver disease.

The underlying aetiology of most cases of canine primary hepatitis is unknown. Consequently, treatments are typically palliative and it is difficult to provide accurate prognostic information to owners. In human hepatology there is accumulating data which indicates that the presence of systemic inflammatory response syndrome (SIRS) is a common and debilitating event in patients with liver diseases. For example, the presence of SIRS has been linked to the development of complications such as HE and is associated with a poor clinical outcome in humans with liver diseases. In contrast, the relationship between SIRS and clinical outcome in dogs with a primary hepatopathy is unknown.

This thesis aimed to better define the role of inflammation in the pathogenesis of canine liver disease. Additional aims were to assess the presence of sodium derangements and whole blood manganese concentrations in dogs with primary hepatitis. The hypothesis of this thesis was that markers of inflammation would be increased in dogs with liver diseases and would be negatively correlated with outcome. As similar findings have been demonstrated in human medicine, the hypothesis was that dogs would be suitable models for human liver disease. We

also hypothesised that whole blood manganese concentrations and derangements in serum sodium concentration would be present in dogs with primary hepatitis.

This thesis demonstrates that a high SIRS score was associated with a poorer long term survival in dogs with primary hepatopathies. Furthermore, IL-6 concentrations were increased in dogs with a cPSS. Given the well-established role of IL-6 in the pathogenesis of hepatic encephalopathy in humans with acquired liver disease, it is possible that IL-6 may be important in the development of HE in dogs with a cPSS. This thesis also demonstrates that whole blood Mn concentrations are increased in dogs with primary hepatitis and sodium concentrations were rarely outside the reference range in dogs with primary hepatitis. This finding is in contrast with the numerous studies of human patients with liver disease. This study, and our earlier work in dogs with a cPSS, indicates that hyponatremia is uncommonly observed in dogs with liver disease. These findings highlight the potential role of dogs as a model to examine the pathogenesis of liver disease in human health.

CHAPTER ONE

Thesis Overview

1.1 Introduction

Liver disease is a common cause of morbidity and mortality in dogs [1]. Liver diseases can be broadly classified into vascular abnormalities such as congenital portosystemic shunt (cPSS) and parenchymal diseases such as primary hepatitis [2]. Primary hepatitis includes all inflammatory disorders of the hepatic parenchyma [2]. In contrast to human medicine, where the type of hepatitis is defined by the inciting cause, few causes of chronic hepatitis have been identified in the dog, and the majority of cases are idiopathic [2]. Primary hepatitis is an important cause of morbidity in humans [3]. In order to further explore the pathogenesis and efficacy of novel therapies, a large number of experimental models have been developed [4]. A widely used model involves the chronic administration of carbon tetrachloride (CCl4) to mice which results in liver fibrosis and cirrhosis [5]. However, it is clear that these models have important differences to human hepatitis both on the histopathological and molecular level [6]. Additionally, the well-controlled experimental set up using these models is very dissimilar to the variable human clinical situation [6]. Taken together, there is an unmet need for additional spontaneous models of hepatitis which better mimic human hepatitis and the human clinical situation for translation of new therapies [7].

1.2 Primary Hepatitis

Primary hepatitis is well recognised in the dog, with one study reporting a clinical prevalence of 0.5% (Poldervaart *et al.*, 2009). Primary hepatitis may be divided into acute and chronic forms, as well as lobular dissecting, granulomatous, and eosinophilic hepatitis as defined by

the WSAVA according to histological criteria (Van den Ingh *et al*., 2006). Of these, chronic hepatitis appears the most common (Poldervaart *et al.*, 2009). Chronic hepatitis, which is used irrespective of the cause and is characterized by the presence of fibrosis, inflammation, hepatocellular apoptosis, and necrosis [8]. Irrespective of the nomenclature used, a morphologic diagnosis should emphasize the type, pattern, and extent of the necrosis and inflammation, as well as the possible cause, and in more prolonged disease, the presence, pattern, and extent of fibrosis and regeneration [9]. Chronic hepatitis is characterized by hepatocellular apoptosis or necrosis, a variable mononuclear or mixed inflammatory infiltrate, regeneration, and fibrosis. The proportion and distribution of these components vary widely, and it is necessary to include in the diagnosis the activity (determined by the quantity of inflammation and extent of hepatocellular apoptosis and necrosis) and the stage of the disease (determined by the extent and pattern of fibrosis and the possible presence of architectural distortion) as well as the possible aetiology [1, 10].

The clinical prevalence of chronic hepatitis may be an underestimate because post-mortem examination of 200 unselected dogs detected the presence of chronic hepatitis in 12% (Watson *et al*., 2010). In contrast to human medicine, where the type of hepatitis is defined by the inciting cause, few causes of chronic hepatitis have been identified in the dog, and the majority of cases are idiopathic (Watson, 2004). As a result, treatment is symptomatic (Watson, 2004) and the prognosis for dogs with chronic hepatitis is poor with a reported median survival time of 18.3 months (Poldervaart *et al.*, 2009).

1.3 Congenital Portosystemic Shunts

Congenital portosytemic shunts (cPSS) are well-recognised vascular anomalies in dogs. Clinical signs are associated with the volume and origin of blood bypassing the liver, resulting in impaired hepatic function, neurological dysfunction, chronic gastrointestinal signs, lower urinary tract signs and delayed growth [11]. Dogs suspected to have a cPSS can be screened with good sensitivity and specificity by measurement of serum bile acids and ammonia [12]. Various imaging modalities can be used to diagnose cPSS in dogs. In most cases the abnormal vessel can be identified using abdominal ultrasonography [13]. In a small proportion of equivocal cases, the vessel can be identified using computed tomographic angiography or direct visualisation or portovenography during coeliotomy [14]. Dogs with a cPSS are initially typically treated with a combination of antibiotics, lactulose and a low protein diet to ameliorate encephalopathy [11]. Once their condition has been stabilised, attenuation of the shunting vessel may be performed after which the prognosis is reasonably good [15].

1.4 Hepatic Encephalopathy

One of the most common presenting signs in dogs with a liver disease is hepatic encephalopathy (HE), which encompasses a range of neurological disturbances that occur in dogs with liver disorders. Clinical signs can include ataxia, unresponsiveness, pacing, circling, blindness, seizures and coma [11]. The pathogenesis of HE in dogs is not fully understood. Hyperammonaemia is considered the most important mediator of HE [16] since ammonia has been demonstrated to be neurotoxic and plasma concentrations are frequently increased in dogs with cPSS. However, the importance of ammonia in driving HE in dogs remains unclear since there is a paucity of studies which have examined whether ammonia

concentrations correlate to the presence and/or severity of HE in dogs and hyperammonaemia alone, which can occur in dogs with urea cycle enzyme deficiencies, does not typically result in HE [17]. Increased plasma concentrations of endogenous benzodiazepines have been demonstrated in dogs with cPSS which have been postulated to be important in causing HE [18]. Other studies have indicated a role for hypercortisolism [19], and altered tryptophan metabolism in the development of HE[20]. Altered manganese metabolism has also been implicated in the pathogenesis of HE as dogs with a cPSS have significantly increased whole blood manganese concentrations [21].

1.5 Inflammation and Liver Disease

Recently, a potential role of inflammation in initiating HE has been demonstrated in dogs with a cPSS. It was found that dogs with a cPSS and HE had higher serum concentrations of c-reactive protein (CRP), a widely used serum biomarker of inflammation, compared to dogs with a cPSS that were asymptomatic and healthy dogs [22]. Dogs with a cPSS that were asymptomatic did not have increased serum CRP concentrations compared to healthy dogs. This observation is similar to several human studies which have shown a strong positive correlation between inflammation and HE [23]. A systemic inflammatory response is frequently present in human patients with liver disease and has been shown to predict mortality and presence of HE [24]. Indeed, the induction of hyperammonaemia with the administration of amino acid solution to cirrhotic patients only resulted in the development of HE in the presence of a systemic inflammatory response [25]. A wide range of studies have explored the role of cytokines, which are a key part of the systemic inflammatory response, in patients with liver disorders with many identifying an increase in plasma concentrations of Interleukin-6 (IL-6) and Tumour Necrosis Factor alpha (TNF-α). For example, serum

concentrations of TNF-α, have been shown to correlate with increasing severity of HE due to chronic liver failure [26, 27]. Other studies have found that IL-6 concentrations are increased in human patients with liver disease [26-28].

There is growing evidence that systemic inflammatory response syndrome (SIRS) is a common and serious disorder among human patients with liver diseases [29-31]. It has been suggested that the presence of SIRS can lead to a further deterioration in liver function resulting in significant morbidity and mortality [32, 33]. The presence of SIRS can compromise the function of various organ systems resulting in Multiple Organ Dysfunction Syndrome (MODS) [34]. In hospitalised human patients, cirrhotics with SIRS have more severe hepatic encephalopathy (HE), are more likely to develop hepatorenal syndrome and have non-reversible renal dysfunction [35]. In acute liver failure, the presence of SIRS, whether or not precipitated by infection, has been implicated in the progression of HE, reducing the chances of transplantation and conferring a poorer prognosis [24]. Furthermore, higher SIRS scores are related to the development of acute liver failure in patients with pre-existing hepatitis [36].

The exact mechanism by which inflammation and hyperammonaemia modulate the neuropsychological function remains to be determined. Astrocytes are the main cells in the brain that metabolise ammonia. Ammonia is considered to initiate HE through the conversion of glutamate and ammonia to glutamine, often resulting in osmotic stress, astrocyte swelling, cerebral oedema and intracranial hypertension [37].

1.6 Manganese and Liver Disease

Many other factors may contribute to the pathogenesis of liver disease in dogs. The impact of primary hepatic disease on the metabolism of other trace elements is less well understood in dogs. In particular, the liver plays a pivotal role in Mn metabolism as the majority of gastro-intestinally absorbed Mn is removed by the liver and excreted into bile such that only approximately 2% of absorbed Mn reaches the systemic circulation (Aschner and Aschner, 2005). One experimental study showed that healthy dogs fed large quantities of Mn daily for 16 months showed no ill-effects and no appreciable increase in blood Mn concentrations. On post-mortem analysis, liver and bile had the highest concentrations of Mn (Reiman and Minot, 1920). Although Mn is an essential mineral, Mn toxicity has been reported in humans and also in experimental animal models (Keen *et al.*, 2000). Studies in humans have shown increased blood Mn concentrations and Mn deposition in the CNS in cases of hepatic insufficiency (Mizoguchi *et al.*, 2001; Rose *et al.*, 1999; Tuschl *et al.*, 2008; Versieck *et al.*, 1974). The deposition of Mn in the CNS is considered to play a role in hepatic encephalopathy and a direct relationship has been demonstrated between blood Mn concentration, MRI hyperintensity consistent with Mn deposition and severity of encephalopathic score in humans (Layrargues *et al.*, 1995; Spahr *et al.*, 1996). In the CNS, Mn is preferentially stored in astrocytes such that Mn concentrations in astrocytes may be almost 200 times the extracellular concentrations (Tholey et al. 1988). Astrocytes are the major cell implicated in the development of hepatic encephalopathy and dysfunction of the astrocyte by Mn is thought to contribute to this (Hazell *et al.*, 2006; Shawcross and Jalan 2005).

1.7 Sodium and Liver Disease

Similar to humans, hyponatremia is often cited to be a complication of canine liver disease and liver disease is often quoted to be an important differential diagnoses of hyponatremia [38-41] . Hyponatremia has also been cited as an important factor in the development of HE in dogs [42, 43]. The severity of the hyponatremia has been linked directly to the severity of the cirrhosis in humans [44, 45]. Portal hypertension, diuretics, large volume paracentesis without administration of albumin and infection have been associated with the development or worsening of hyponatremia [45, 46]. The presence of hyponatremia frequently complicates the management of the cirrhotic patients and those awaiting liver transplants. In addition, hyponatremia is a risk factor for liver transplantation, being associated with a high frequency of complications and reduced short and long term post-transplant survival [44]. Prognostic models incorporating serum sodium concentrations were also better able to predict the urgency and need for transplant [44]. Hyponatremia was a strong predictor of the development of HE in a cohort of cirrhotic patients [47]. Despite the important implication of sodium in human liver disease, few studies have formally examined sodium homeostasis in dogs with primary hepatitis [39, 48-50].

1.8 Thesis Objectives

Liver disease is a common cause of morbidity and mortality in dogs. The pathogenesis of liver disease (particularly primary hepatitis) is poorly understood. It is clear that the role of inflammation is important in the progression and prognosis in human patients with liver disease, but this is poorly defined in dogs. The general objective of this thesis was to better define the role of inflammation in canine liver disease and whether inflammation was linked to prognosis in these cases. Other factors that may contribute to pathogenesis such as sodium

and manganese were also examined. Furthermore, if the findings of this thesis mirrored those seen in human practice, then this thesis aimed to assess if cats and dogs could provide a model system to investigate human disease.

CHAPTER 2

Whole blood manganese concentrations in dogs with primary hepatitis

2.1 Introduction

Primary hepatitis includes all inflammatory disorders of the hepatic parenchyma (consisting of the functional unit of liver lobule (12)), apart from those due to distant endotoxin release (12, 82). Primary hepatitis is well recognised in the dog, with one study reporting a clinical prevalence of 0.5% [1].

The liver has a key role in regulation of many trace elements and various trace element abnormalities have been documented in cases of canine chronic hepatitis (10, 87). Copper metabolism has been investigated most extensively and increased hepatic copper concentrations have been shown to be both a cause and effect of chronic hepatitis. In Bedlington Terriers a primary genetic defect results in copper accumulation within the hepatic parenchyma and subsequent hepatitis due to oxidative damage (69). Conversely, chronic hepatitis can result in reduced biliary excretion of copper and increased hepatic parenchymal concentrations (24).

Similarly, increased hepatic iron concentrations have also been documented in naturally occurring and experimental canine chronic hepatitis (15, 28, 36). Zinc has been shown to be an effective treatment for copper associated hepatitis and hepatic and biliary zinc concentrations have been investigated in canine chronic hepatitis (22, 46, 86).

There have been only a small number of studies which have investigated the role of Mn in canine liver disease. MRI of 13 dogs and 3 cats with a congenital portosystemic shunt (cPSS) detected hyper-intense focal areas in the lentiform nuclei which were considered to be consistent with Mn deposition, although neither blood nor tissue Mn concentrations were assessed in this study (41). In support of this hypothesis, a single case has been reported of a Yorkshire Terrier with a cPSS and a compatible hyper-intense lentiform nucleus which had an increased concentration of Mn in this area on post mortem examination (52). *Gow et al.* have recently demonstrated that whole blood Mn concentrations are increased in cases of canine congenital porto-systemic shunting (90). However, it remains unclear whether dogs with primary hepatitis have altered Mn homeostasis.

The central hypothesis of this study was that dogs with primary hepatitis have altered Mn homeostasis. The aim was to compare whole blood Mn concentrations in dogs with primary hepatitis with those in healthy dogs and in dogs with non-hepatic illness. A second hypothesis was that dogs with primary hepatopathies would not have such profound disturbances of Mn homeostasis as in dogs with a cPSS. This was investigated by comparing whole blood Mn concentrations in dogs with a primary hepatopathy to dogs with a cPSS.

2.2 Materials and Methods

Cases of primary hepatitis, defined as dogs with increased fasting bile acid concentrations, and histopathological confirmation of primary hepatitis from samples collected by either biopsy or post-mortem examination were considered eligible for inclusion into the study. Disease was classified according to WSAVA criteria (22). The type of diet

during the past 7 days was noted for each dog. Consecutive cases of dogs with non-hepatic illness, defined as dogs with illness warranting diagnostic investigations including serum biochemistry which had serum concentrations of albumin, ALT, fasting bile acid concentrations and bilirubin within their respective reference ranges, were enrolled into the study. Dogs with congenital portosystemic shunting confirmed by ultrasonography and/or portovenography were also enrolled. Consecutive cases of healthy dogs, defined as dogs which were considered healthy by their owners and had a normal physical examination when blood sampled for an unrelated primary reason (rabies serology or PCV check before blood donation), were also enrolled into the study.

Whole blood samples were collected from fasted animals. Blood collected via a 21g 5/8" hypodermic needle during clinical jugular sampling was placed into commercial 1.3ml EDTA tubes and retained for Mn measurement. All EDTA samples were frozen within 4 hours of collection and stored at -70°C until they were transported on dry ice to the Scottish Trace Element and Micronutrient Reference Laboratory. Manganese concentrations were determined in whole blood by graphite furnace atomic absorption spectrometry after dilution with Triton X-100 solution. The inter-assay coefficient of variation (CV) was 5.4%. The inter-assay variation was calculated from 10 batches of three internal quality control samples of manganese. The mean Mn concentrations of the three control samples were 760 nmol/L, 490 nmol/L and 320nmol/L; this approach ensured that the inter-assay CV was calculated using a range of Mn concentrations. In summary, both the inter- and intra-assay CVs were very low which would be expected with this analytical technology run from a national centre of excellence for trace element analysis (http://www.trace-elements.co.uk/). Assays CV in this range ensured that are our data, and the conclusions of the study, were highly robust. The study was approved by the University of Edinburgh Ethics Research Committee.

Manganese concentrations, and differences in age, were compared between the dogs with primary hepatitis, hospitalised ill dogs and healthy dogs by a Kruskal-Wallis test with post-test Dunn's multiple comparison test. Manganese concentrations were compared between dogs with primary hepatitis and a cPSS by a Mann Whitney U test. The presence of correlation between Mn concentrations and biochemical values and age was evaluated by Spearman Rank Correlation test. Statistical analysis was performed with a commercial software package (GraphPad Prism version 5.0 for Windows, GraphPad Software) with $p<0.05$ considered significant.

2.3 Results

The group of dogs with non-hepatic illness comprised 31 dogs (19 different breeds, 2 cross-breeds, 4 male entire, 10 male neutered, 13 female neutered, 4 female entire, mean age 59.5 months, range 5 to 132 months). The disease categories were; nasal disease (5), inflammatory disease (4), orthopaedic abnormalities (4), lower urinary disorders (6), poor body condition/ exercise intolerance (5), chronic renal failure (2), focal neoplasia (2) and one each of ovarian remnant syndrome, congenital cardiac disease and rodenticide exposure. Blood samples from healthy dogs were obtained from 16 blood donors and 2 dogs during rabies serology sampling (10 different breeds, one cross-breed, 4 male entire, 11 male neutered, 3 female neutered, mean age 57 months, range 24 to 120 months). 65 dogs with congenital portosystemic shunting (cPSS) (25 breeds, 8 crossbreeds, 21 male entire, 10 male neutered, 26 female entire, 8 female neutered, mean age 19 months, range 3 to 96 months) were also enrolled. Twenty one dogs diagnosed with primary hepatic disease were enrolled (10 different breeds, 6 cross-breeds, 4 male entire, 7 male neutered, 10 female neutered, mean age 84 months, range 6 to 180 months). Eight were confirmed from samples collected post-mortem, and four from a percutaneous spring-loaded biopsy instrument. Nine were collected by surgical biopsy techniques. Eighteen cases were diagnosed with chronic hepatitis, two cases with dissecting lobular hepatitis and one case with cirrhosis. Only one case was being fed a diet formulated for liver disease at the time of sampling. The other twenty dogs were fed a standard proprietary dog food or home cooked diet. The dogs with non-hepatic illnesses and healthy dogs were fed a range of commercial non-clinical diets. There were no significant differences between the age of the dogs in the healthy, non-hepatic illness and primary hepatic disease groups ($p=0.67$).

There was a significant difference in Mn concentrations between dogs with primary hepatic disease, dogs with non-hepatic illness and healthy dogs ($p<0.002$, figure 1). Post test analysis revealed that there was no difference in Mn concentrations between dogs with non-hepatic illness and healthy dogs but a significant difference in Mn concentration between dogs with primary hepatic disease and dogs with non-hepatic illness ($p<0.01$) and dogs with primary hepatic disease and healthy dogs ($p<0.05$). In the primary hepatic disease group, there was no correlation between Mn and fasting bile acid concentrations ($r=0.25$, $p=0.28$), Mn and albumin ($r=-0.19$, $p=0.41$), Mn and ALT ($r=-0.20$, $p=0.39$), Mn and prothrombin time ($r=0.18$, $p=0.48$), Mn and activated thromboplastin time ($r=0.35$, $p=0.16$) or bilirubin and Mn concentrations ($r=0.07$, $p=0.77$).

Dogs with primary hepatitis had significantly lower whole blood Mn concentration compared to dogs with cPSS (median values 1101nmol/L and 2026 nmol/L respectively, $p=0.0005$) (figure 2). There was a significant difference in age between the cPSS and primary hepatitis groups ($p<0.0001$).

2.4 Discussion

The central finding of this study was that whole blood Mn concentrations were significantly higher in dogs with primary hepatic disease compared to both healthy dogs and dogs with non-hepatic illness. Taken together with our earlier study, which reported that dogs with cPSS had increased Mn concentrations, it is clear that dogs with either primary vascular or primary hepatic disease frequently have disturbed Mn homeostasis (21). The first-pass excretion of gastrointestinally absorbed Mn relies on efficient delivery through the portal circulation, functional hepatocytes and functional biliary system. Dysfunction of any

one of these three components will potentially increase delivery of Mn to the systemic circulation (14, 21). Chronic hepatitis and lobular dissecting hepatitis commonly result in dysfunction of all three components; hepatocyte loss by definition in chronic hepatitis, cholestasis due to hepatic architecture disruption of the biliary system and, due to increased portal pressure, recruitment of porto-systemic collateral vessels (31). Consequently, it is technically challenging to establish the relative importance of primary parenchymal diseases, cholestasis and portosystemic shunting on Mn metabolism. This issue has been partially addressed in experimental models of liver disease; a study of CNS Mn deposition in the rat found that rats with induced portocaval shunts had higher concentrations than rats with induced cirrhosis. The shunting percentage was 100% for induced shunts whereas cirrhotic rats shunting percentage ranged from 29-100% (42). One clinical study of humans with cirrhosis demonstrated that all nine patients with hyperintense lesions on MRI had shunting vessels compared to only 2 of 17 patients with no lesions on MRI (59). In the present study, the finding that whole blood Mn concentrations were significantly higher in dogs with cPSS compared to dogs with primary hepatopathy would suggest that shunting vessels have an important role in the pathogenesis of hypermanganesaemia. A further study investigating any correlation between whole blood Mn concentrations with the percentage shunting in dogs with primary hepatitis would further clarify this hypothesis.

Investigation of whole blood Mn concentrations in cases of acute hepatitis may help define the contribution of hepatic dysfunction *per se* to increased Mn concentrations. One study of acute hepatitis in humans demonstrated increased serum concentrations of Mn in acute hepatitis and found correlation with activity of serum aminotransferase in both acute and chronic hepatitis which may suggest that hepatic dysfunction rather than development of

porto-systemic shunting was responsible for the increased systemic concentrations of Mn in these cases (91). Determination of systemic whole blood Mn concentrations in cases of extra-hepatic cholestasis would also be useful to assess the contribution of biliary dysfunction to increased systemic Mn concentrations.

Manganese and iron metabolism are intimately linked. Both metals use divalent metal transporter 1 (DMT1) for absorption from the gastrointestinal tract and also transport into the CNS (84).MT1 expression in both is regulated by iron status via hepcidin (12, 29). Deficiency of iron has been shown to increase CNS Mn uptake whilst iron overload decreases Mn uptake (72).

Hepcidin is mainly produced by hepatocytes and is up-regulated in response to inflammation (43, 72). Because chronic hepatitis (by definition) and to a lesser extent lobular dissecting hepatitis have an inflammatory component, it would be expected that hepcidin may be up-regulated and this, in turn, would reduce gastro-intestinal absorption of Mn (18). In converse, loss of hepatocytes would reduce the liver's ability to produce hepcidin and thus potentially remove negative regulation of DMT1 expression. In support of this, serum hepcidin and prohepcidin concentrations have been found to be lower in humans with chronic hepatitis C compared to healthy controls and correlate with fibrosis (4, 34). Therefore it is likely that the confounding interplay of inflammation, reduced of hepatic function, effects of iron status on hepcidin expression and therefore gastro-intestinal Mn uptake may play a role in the variation in Mn concentrations found in the primary hepatopathy group.

In the primary hepatitis group, 5 dogs had whole blood Mn concentrations which were markedly elevated whilst the remaining 16 had similar concentrations to the non-hepatic illness and healthy groups. This apparent split may be due to a difference in hepatic reserve, or as stated above, due to differing shunt fractions, and larger studies to assess if histological severity and/or shunting fraction correlated with Mn concentrations would be informative.

The main pathological consequence of increased Mn concentrations in hepatic disorders is considered to be neurological dysfunction. The exact mechanism by which Mn causes encephalopathy is unclear although there are both in-vitro and in-vivo studies which suggest that many of the effects are centred on the astrocyte, a key cell in the development of hepatic encephalopathy (35). Astrocytes have been shown to have a high affinity transport mechanism for Mn and cytoplasmic Mn accumulation causes Alzheimer type II changes, which are recognized in post-mortem examination of human hepatic encephalopathy patients (26, 92). Experimental studies have demonstrated a wide variety of potential toxic effects of Mn on the astrocyte, including potentiating the effect of ammonia as well as impairing their glutamate transport (71). Glutamate is the main excitatory neurotransmitter in the brain, and astrocyte uptake is the main method of removal from the synaptic cleft. Glutamate is then converted to glutamine by the astrocyte before being recycled back to the neuron. Inhibition of the astrocyte glutamate transporter GLT-1 by Mn prevents this inactivation and recycling, potentially causing increased concentrations of the neurotransmitter in the synaptic cleft (24). Astrocyte mitochondrial dysfunction and induction of peripheral-type benzodiazepine receptors (PTBR, now termed translocator protein) with increased ligand binding have also been reported with increasing concentrations of Mn (36, 57). Activation of the PTBR causes

neurosteroid production, which are also implicated in astrocyte dysfunction and hepatic encephalopathy (21, 91).

The finding that Mn concentrations are increased in some dogs with primary hepatopathies may be therapeutically relevant since chelation treatment with para-aminobenzoic acid and disodium edetate has been used successfully in human cases of Mn toxicity (33, 75). This has led to the suggestion that it may be of use in controlling Mn and thus hepatic encephalopathy in humans and dogs (22, 36, 48). In support of this, Park *et al.* (2008) used trientine chelation therapy in one human case of chronic hepatic disease with acquired portosystemic shunting and hepatic encephalopathy. Following treatment there was a decrease in whole blood Mn concentrations and a reduction in CNS MRI hyperintensity which was thought to be consistent with a reduction in CNS Mn deposition. These findings correlated with a reported marked improvement in clinical signs.

2.4 Conclusion:

The present study demonstrates that whole blood Mn concentrations are increased in dogs with primary hepatitis. The prognostic significance of increased Mn concentrations, correlation with severity of histopathological changes or acquired shunting fraction and the relationship between Mn and severity of HE in dogs is deserving of further study.

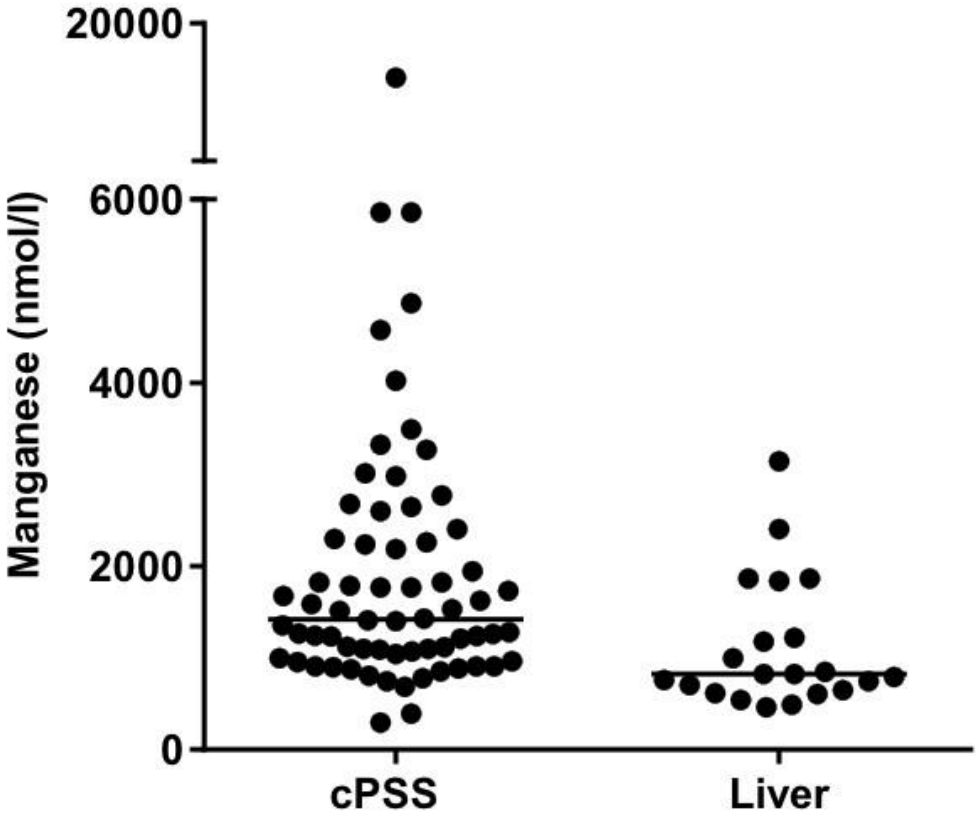

Figure 1: Whole blood manganese concentrations (nmol/l) in dogs with primary hepatitis (liver) and dogs with a cPSS.

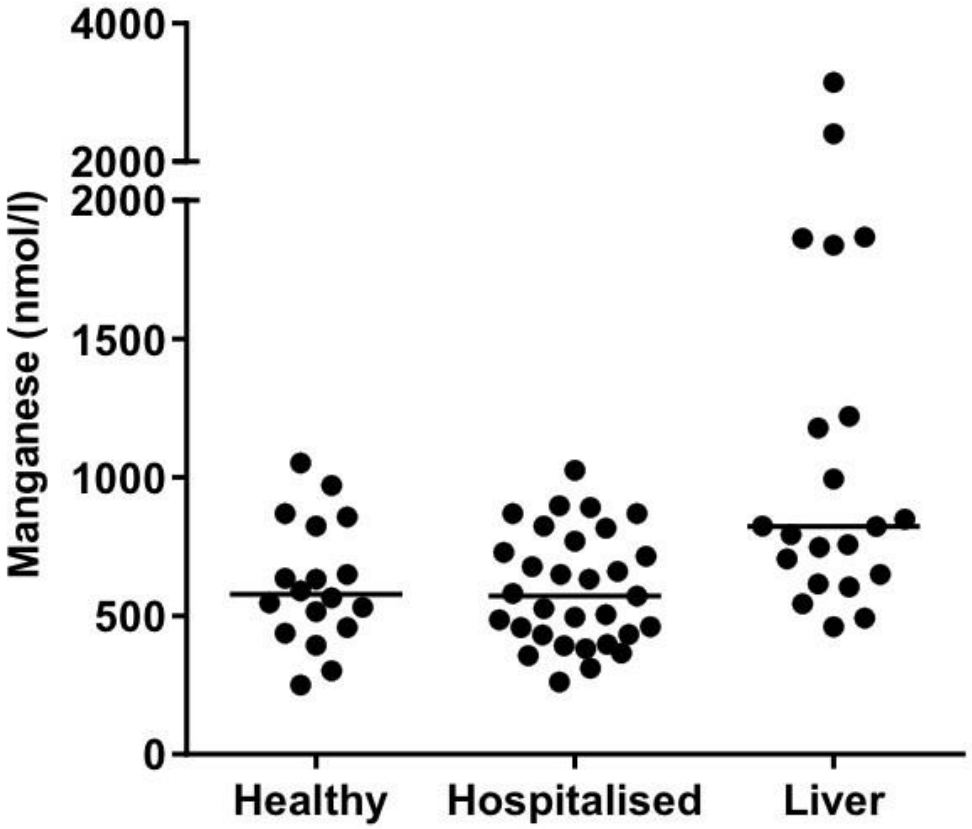

Figure 1: Whole blood manganese concentrations (nmol/l) in dogs with primary hepatitis (liver), dogs with non-hepatic illness (hospitalised) and healthy dogs (healthy).

CHAPTER 3

Plasma IL-6 concentrations are increased in dogs with a congenital portosystemic shunt

3.1 Introduction

Congenital portosytemic shunts (cPSS) are well-recognised vascular anomalies in dogs. Clinical signs are associated with the volume and origin of blood bypassing the liver, resulting in impaired hepatic function, neurological dysfunction, chronic gastrointestinal signs, lower urinary tract signs and delayed growth[11]. Dogs suspected to have a cPSS can be screened with good sensitivity and specificity by measurement of serum bile acids and ammonia [12]. Various imaging modalities can be used to diagnose cPSS in dogs. In most cases the abnormal vessel can be identified using abdominal ultrasonography [13]. In a small proportion of equivocal cases, the vessel can be identified using computed tomographic angiography or direct visualisation or portovenography during coeliotomy[14]. Dogs with a cPSS are initially typically treated with a combination of antibiotics, lactulose and a low protein diet to ameliorate encephalopathy[11]. Once their condition has been stabilised, attenuation of the shunting vessel may be performed after which the prognosis is reasonably good [15].

One of the most common presenting signs in dogs with a cPSS is hepatic encephalopathy (HE), which encompasses a range of neurological disturbances that occur in dogs with liver disorders. Clinical signs can include ataxia, unresponsiveness, pacing, circling, blindness, seizures and coma[11]. The pathogenesis of HE in dogs with a cPSS is not fully understood. Hyperammonaemia is considered the most important mediator of HE[16] since ammonia has been demonstrated to be neurotoxic and plasma concentrations are frequently increased in

dogs with cPSS. However, the importance of ammonia in driving HE in dogs remains unclear since there is a paucity of studies which have examined whether ammonia concentrations correlate to the presence and/or severity of HE in dogs and hyperammonaemia alone, which can occur in dogs with urea cycle enzyme deficiencies, does not typically result in HE[17]. Increased plasma concentrations of endogenous benzodiazepines have been demonstrated in dogs with cPSS which have been postulated to be important in causing HE[18]. Other studies have indicated a role for hypercortisolism[19], and altered tryptophan metabolism in the development of HE[20]. Altered manganese metabolism has also been implicated in the pathogenesis of HE as dogs with a cPSS have significantly increased whole blood manganese concentrations[21].

Recently, we have demonstrated a potential role of inflammation in initiating HE in dogs with a cPSS. We found that dogs with a cPSS and HE had higher serum concentrations of c- reactive protein (CRP), a widely used serum biomarker of inflammation, compared to dogs with a cPSS that were asymptomatic and healthy dogs [22]. Dogs with a cPSS that were asymptomatic did not have increased serum CRP concentrations compared to healthy dogs. This observation is similar to several human studies which have shown a strong positive correlation between inflammation and HE[23]. A systemic inflammatory response is frequently present in human patients with liver disease and has been shown to predict mortality and presence of HE[24]. Indeed, the induction of hyperammonaemia with the administration of amino acid solution to cirrhotic patients only resulted in the development of HE in the presence of a systemic inflammatory response[25]. A wide range of studies have explored the role of cytokines, which are a key part of the systemic inflammatory response, in patients with liver disorders with many identifying an increase in plasma concentrations of Interleukin-6 (IL-6) and Tumour Necrosis Factor alpha (TNF-α). For example, serum

concentrations of TNF-α, have been shown to correlate with increasing severity of HE due to chronic liver failure [26, 27]. Other studies have found that IL-6 concentrations are increased in human patients with liver disease [26-28].

Despite the expanding interest in the role of cytokines in the development of HE in human patients with liver diseases, the concentrations of pro-inflammatory plasma cytokines have not been examined in a cohort of dogs with a confirmed cPSS. Therefore, the aim of the study was to measure plasma IL-2, IL-6, IL-8 and TNF-α concentrations in healthy dogs and dogs diagnosed with a cPSS. We hypothesised that IL-6 and TNF-α concentrations would be increased in dogs with a cPSS compared to healthy dogs.

3.2 Materials and Methods

Consecutive cases of dogs with a cPSS were considered suitable for inclusion into the study. Dogs were diagnosed with a cPSS if the dog had compatible clinical signs and had an abnormal shunting vessel identified via ultrasonography, computerised tomography, intra- operative portovenography, visualised during surgery or by a combination of methods. Dogs which had signs of neurological dysfunction such as altered behaviour, pacing, circling, altered mentation, stupor or seizures within the previous 24 hours were classified as having HE and this was recorded at the time of blood sampling. Consecutive cases of healthy dogs, who were being blood sampled for routine preventive healthcare reasons, were also considered eligible for inclusion into the study. Healthy dogs were defined as dogs which were considered healthy by their owners and that had a normal physical examination. Venous blood samples were collected from both the cPSS and healthy dogs and immediately placed

into lithium heparin blood collection tubes. The samples were then immediately separated, frozen and stored at -80°C until the point of analysis.

Canine pro-inflammatory cytokines (IL-2, IL-6, IL-8 and TNF- α) were measured using Meso Scale Discovery (MSD) multi-spot assay system (A division of Meso Scale Diagnostics, LLC. 9238 Gaither Road, Gaithersburg, MD 20877, USA). This multiplex immunoassay system enables the measurement of biomarkers utilising electrochemiluminescent detection. Each 96-well plate had 4-carbon electrodes in the bottom of each well, each pre-coated with one of the anti-cytokine antibodies of interest. The samples were reconstituted in the assay diluent provided. Assay diluent (25 µl) was added to all wells and the plate sealed and incubated for 30 s at room temperature on an orbital shaker (600 rpm). Samples, standards and controls were added at 25 µl per well. The plate was sealed and incubated for 2 h at room temperature on an orbital shaker (600 rpm). At the end of the incubation the wells were washed three times using 200 µl PBS+0.05%Tween 20, soaking for 30 s and then discarding. Detection antibody was added at 25 µl per well, and the plate sealed and incubated for 1 h at room temperature on an orbital shaker (600 rpm). At the end of the incubation the plate was washed three times as before. 150 µl of the MSD Read Buffer was added to each well and the MSD plates were measured on the MSD Sector Imager 2400 plate reader. The raw data was measured as electrochemiluminescence signal (light) detected by photodetectors and analysed using the Discovery Workbench 3.0 software (MSD). A 4-parameter logistic fit curve was generated for each analyte using the standards and the concentration of each sample calculated.

The plasma concentrations of IL-2, IL-6, IL-8 and TNF- α and ages were compared between the healthy dogs and dogs with a cPSS by a Mann Whitney U test. Significance was set at $p<0.05$.

The study was approved by the University of Edinburgh's Veterinary Ethical Review Committee.

3.3 Results

The cPSS group comprised 36 dogs of 20 different breeds (Table 1). There were 6 neutered males, 15 intact males, 13 intact females and 2 neutered females. The median age of the dogs in the cPSS group was 12 months (range 3-63 months). Hepatic Encephalopathy was present at the time of blood sampling in 29 of the 36 dogs with a cPSS. The healthy control group comprised 25 healthy dogs of 17 different breeds (Table 1). There were 5 neutered males, 8 intact males, 5 intact females and 7 neutered females. The median age of the healthy dogs was 15 months (range 3-72 months). There were no significant differences in the median ages of healthy dogs and dogs with a cPSS.

There were no significant differences in the plasma concentrations of IL-2, IL-8 and TNF- α between the dogs with a cPSS and the healthy dogs (Figure 1, Table 2). There was a significant difference ($p=0.02$) in the median plasma concentration of IL-6 in dogs with a cPSS compared to healthy dogs (Figure 1, Table 2).

Healthy Control	**cPSS**
Stafordshire Bull Terrier	Yorkshire Terrier (5)
Labrador	Labrador (4)
Great Dane	Lakeland Terrier
Bearded Collie (2)	Irish Wolfhound
Yorkshire Terrier (2)	Jack Russell Terrier (3)
Jack Russell Terrier (2)	Dachshund
Crossbreed (6)	West Highland White Terrier (5)
Weimeraner	Pug (4)
Cocker Spaniel	Bichon Frise
British Bulldog	Bassett Hound
Border Terrier	Crossbreed
Bull Mastiff	Fox Terrier
Border Collie	Lhasa Apso
Lhasa Apso	Ibizan Hound
Caviler King Charles Spaniel	Shih Tzu
Golden Retriever	Chihuahua
Springer Spaniel	Border Terrier
	Pyrnean Mountain Dog
	Cocker Spaniel
	Shar Pei

Table 1: Breeds of dogs in cPSS group and healthy group.

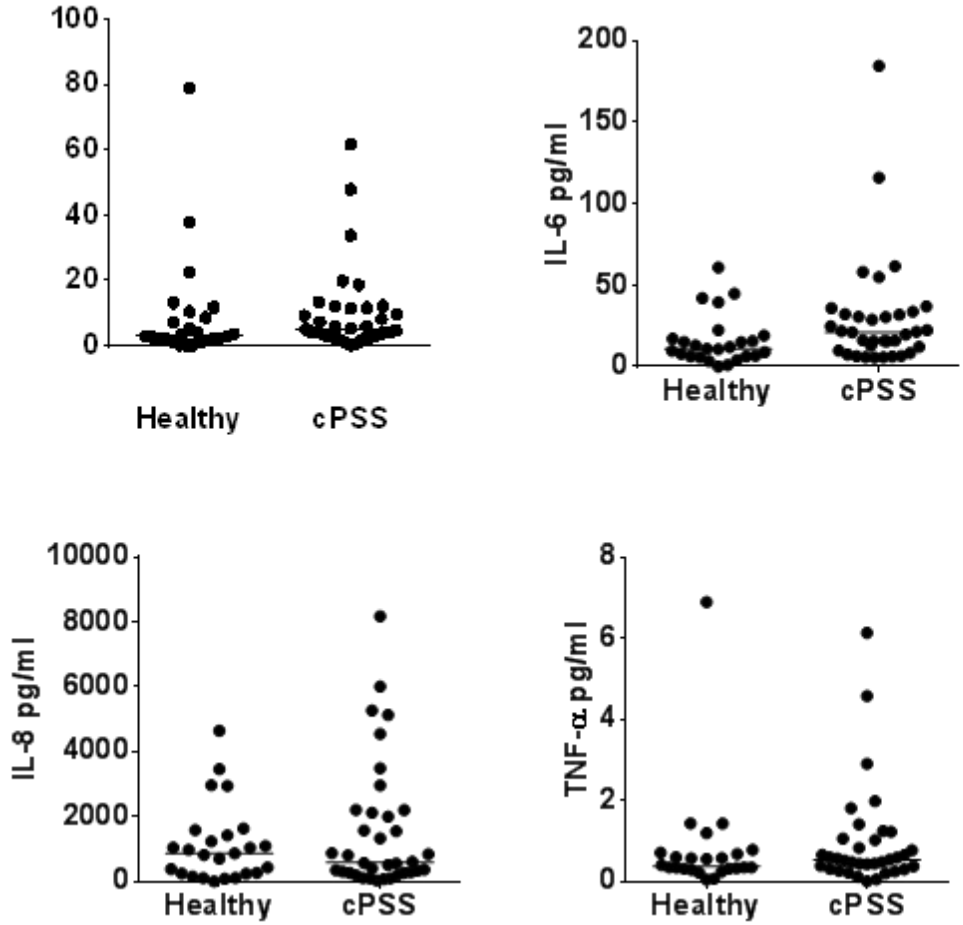

Figure 1: Plasma cytokine concentrations in healthy dogs (control) and dogs with a congenital portosystemic shunt (cPSS).

	Healthy	cPSS	P Value
IL-2 pg/ml	2.90	4.71	0.12
IL-6 pg/ml	10.92	21.11	0.02
IL-8 pg/ml	866.70	589.10	0.79
TNF-α pg/ml	0.38	0.55	0.38

Table 2 : Median concentrations and p values of plasma IL-2, IL-6, IL-8 and TNF-α in healthy dogs and dogs with a congenital portosystemic shunt (cPSS).

3.4 Discussion

This study demonstrated that plasma IL-6 concentrations were increased in dogs with cPSS compared to healthy dogs whereas other pro-inflammatory cytokines such as TNF-α, IL-2 and IL-8 were not significantly different between the two populations of dogs. This finding is consistent with a recent study that found that dogs with intra-hepatic diseases had an increase in serum IL-6 concentrations[51]. It is also consistent with studies in human patients with various liver pathologies. IL-6 concentrations were found to be elevated in patients with acute hepatitis and fulminant hepatic failure [52, 53]. Furthermore, IL-6 concentrations were increased in patients with chronic liver disease and hepatocellular carcinoma [54, 55]. Hill et al. demonstrated that not only were IL-6 concentrations increased in patients with alcoholic hepatitis, but correlated significantly with the severity of disease[56].

Increased IL-6 concentrations in dogs with liver diseases may be important in initiating HE, which was present in the majority of dogs with a cPSS in this study. Interleukin-6 has been shown to be increased in human patients with overt and minimal HE [28, 57]. Additionally, the severity of HE positively correlated with serum IL-6 concentrations. Luo et al. demonstrated that IL-6 and ammonia were independent predictors of HE and that there was a positive additive interaction between IL-6 and ammonia on the presence of HE [28, 57]. Shawcross *et al*. reported that inflammatory mediators, including IL-6 exacerbate neuropsychological alterations induced by hyperammonaemia in cirrhotic patients with HE [58]. Together, these studies suggest that IL-6 may play an important role in the development of HE.

The exact mechanism by which inflammation and hyperammonaemia modulate the neuropsychological function remains to be determined. Astrocytes are the main cells in the brain that metabolise ammonia. Ammonia is considered to initiate HE through the conversion of glutamate and ammonia to glutamine, often resulting in osmotic stress, astrocyte swelling, cerebral oedema and intracranial hypertension [37].

The mechanism by which IL-6 modulates neurological status is unclear although IL-6 can alter signal transduction through some neurotransmitter receptors. Chronic exposure to IL-6 alters metabotrophic glutamate receptor activated calcium signalling in cerebellar neurons [59]. A positive correlation between elevated IL-6 concentrations and increased concentrations of neural glutamine was found in patients with acute liver failure. This suggests that IL-6 might promote cerebral oedema that is the characteristic of HE [60]. Furthermore, there was a negative associated between serum concentrations of IL-6 and memory deficits in patients during low dose endotoxemia and IL-6 has been reported to correlate with cognitive decline in older people [61, 62].

Interleukin-6 may be involved in the mechanism by which ammonia contributes to the pathogenesis of HE. Elevation of serum IL-6 increases fluid phase permeability and ammonia diffusion in the central nervous system derived endothelial cells. This leads to increased ammonia concentration in the brain and subsequent ammonia toxicity to the central nervous system [63]. Experimental models have demonstrated that simultaneous treatment of astrocyte cultures with a mixture of IL-6 and ammonia had a marked additive effect on mitochondrial permeability transition, leading to astrocyte swelling [64].

Additional studies are required to further examine the role of IL-6 in the development of HE in dogs with cPSS. Studies comparing plasma IL-6 concentrations in dogs with a cPSS that have HE with IL-6 concentrations in asymptomatic dogs with a cPSS would be informative. In addition, the serial measurement of plasma IL-6 concentrations in dogs where their HE grade changes or remains static following treatment would also help to clarify the role of IL-6 in initiating neurological dysfunction in dogs with liver diseases.

3.5 Conclusion

The study demonstrates that IL-6 concentrations are increased in dogs with a cPSS. Given the well-established role of IL-6 in the pathogenesis of hepatic encephalopathy in humans with acquired liver disease, it is possible that IL-6 may be important in the development of HE in dogs with a cPSS. Additional studies on the role of IL-6 in the pathogenesis of hepatic encephalopathy in dogs with both acquired and congenital liver disease are warranted. A better understanding of the role of IL-6 and other cytokines in the development of HE may facilitate the development of novel treatment modalities.

3.6 Acknowledgements

We would like to thank all the owners, nurses and veterinarians involved in the care of the dogs included in this study. The study was supported by Petsavers.

CHAPTER 4

Sodium homeostasis in dogs with primary hepatitis

4.1 Introduction

Hyponatremia is a common clinical pathology abnormality in human patients with advanced liver disease, cirrhosis and ascites [47]. Hyponatremia develops due to a disturbance in the renal capacity to eradicate solute-free water [47]. This leads to the retention of water that is out of proportion to the retention of sodium and consequently a hyponatremia with volume excess and increased total body sodium concentrations. This results in a decrease in the plasma sodium concentration and hypo-osmolality. The main pathogenic factor in hyponatraemia is a non-osmotic hypersecretion of arginine vasopressin from the neurophysis due to circulatory impairment [65].

One of the most significant complications of hyponatraemia in humans with liver disease is the development of hepatic encephalopathy (HE). The mechanism(s) by which hyponatremia causes HE in human patients with liver disease is incompletely understood but a shift of water into astrocytes which subsequently causes astrocyte swelling is widely regarded to be important in the development of HE [47]. In experimental models of acute liver failure, the presence of hyponatremia is associated with greater brain swelling compared with normal serum sodium concentrations [66]. Furthermore, in patients with acute liver failure and high grade HE, the administration of hypertonic saline to increase serum sodium concentration reduced the incidence and severity of intracranial hypertension [67].

The hypothesis of this study was that hyponatremia would be a common abnormality in dogs with primary hepatitis. The aim of the study was to document serum sodium concentrations in a cohort of patients with histologically confirmed primary hepatitis.

4.2 Materials and Methods

Medical records from dogs that were presented to the University of Edinburgh Small Animal Hospital were searched for all cases of primary hepatitis. Cases were only included if the dog had histopathological confirmation of parenchymal hepatic disease from samples collected either by biopsy or on post-mortem examination. Information on the dog's signalment and previous medical treatment was also extracted. Dogs which had been previously treated with diuretics or intravenous fluids were excluded from the study. Disease was classified according to WSAVA criteria by a board certified pathologist [2]. The serum sodium concentration and the presence or absence of HE was recorded at initial presentation. The serum was separated and analysed immediately. The presence of HE was evaluated using previously described criteria [43]. The dog was considered to have HE if it had clinical signs of lethargy, inappropriate behavior, disorientation, circling, head pressing or seizures. [68]. Sodium was measured using an ILab650 biochemistry analyser, Diamond Diagnostics, USA.

A Mann Whitney U test was used to compare sodium concentrations in dogs with and without ascites and in dogs with cirrhosis and non-cirrhotic primary hepatopathies.

The study was approved by the University of Edinburgh Veterinary Ethics Research Committee.

4.3 Results

Fifty three cases of histologically confirmed primary hepatitis were identified. Eleven dogs were excluded as they had been treated prior to presentation with diuretics (n= 5) or intra-venous fluid therapy (n=4) or insufficient clinical information was present in their medical records (n=2). The remaining 42 dogs were comprised of 18 different pure breeds and 3 cross-breeds. The gender distribution comprised 8 male entire, 12 male neutered, 11 female neutered and 11 female entire dogs. The median age was 75 months (range 6 to 180 months). The diagnosis of primary hepatitis was confirmed from samples collected at post-mortem examination in 9 cases, and from a percutaneous spring-loaded biopsy instrument in 12 cases. Twenty-one samples were collected by surgical biopsy techniques. Twenty-four cases were diagnosed with chronic hepatitis, 1 case with juvenile hepatic fibrosis, 5 cases with acute hepatitis and 12 cases with cirrhosis.

Thirty nine dogs had sodium concentrations within the reference interval (ref.139-154 mmol/l). Only 3 of the 42 dogs (7.1%) had serum sodium concentrations out with the normal reference interval. Two of these dogs had serum sodium concentrations of less than 139 mmol/l but more than 130 mmol/l. The first case was a ten year old female neutered Labrador. This dog was referred for investigation of acute onset ascites, abdominal pain, inappetance, anorexia and lethargy. Serum sodium concentration was 136 mmol/l at the time of presentation. Histologic diagnosis was of a severe, subacute hepatopathy with marked, multifocal, centrilobular to midzonal and periportal, acute to subacute hepatic necrosis,

hepatocyte drop-out, haemorrhage, histiocytic inflammation and bile duct hyperplasia. The second case was a five year old female Border collie who was referred for investigation of ascites. The dog's serum sodium concentration was 134 mmol/l at the point of presentation. Histological diagnosis was of a chronic active hepatitis. One dog was hypernatremic with a sodium value of 160 mmol/l. This case was a six year old male Doberman Pincher referred for chronically increased hepatic enzymes, inappetance and weight loss. Histologic diagnosis was of chronic hepatitis. Four dogs had clinical signs of HE at the time of measurement of sodium concentration. None of the dogs with serum sodium concentrations outside the reference interval displayed clinical signs of HE.

There was no significant difference between sodium concentrations in dogs with and without ascites (median with ascites 144.0mmol/l, median without 147.0mmol/l, p=0.06). There was no significant difference between sodium concentrations in dogs with cirrhosis compared to dogs with non-cirrhotic primary hepatopathies (median cirrhosis 147.5mmol/l, median non-cirrhotic 147.0mmol/l, p=0.45).

4.4 Discussion

The central finding of this study was that abnormal serum sodium concentrations were not commonly observed in dogs with primary hepatitis. This observation markedly contrasts with the findings from studies of human with liver disease where hyponatremia is frequently observed [46, 47, 69]. For example, in a prospective study of 997 patients with cirrhosis the prevalence of low serum sodium concentration, as defined by a serum sodium ≤135 mmol/l, ≤130 mmol/l, ≤125 mmol/l or ≤120 mmol/l, was 49.4%, 21.6%, 5.7%, and 1.2%, respectively [65]. Hyponatraemia was present in 29.8% of patients admitted for management of cirrhosis,

as defined by a serum sodium concentration of ≤130 mmol/l [70]. Furthermore, the prevalence of hyponatremia was 26% in a population of paediatric patients with end stage liver disease waiting for liver transplantation (as defined by a serum sodium concentration of ≤130 mmol/l) [71].

We recently examined a wide range of clinical and biochemical parameters, including plasma sodium concentrations, in dogs with a congenital portosystemic shunt (cPSS) [68]. The findings in dogs with a cPSS were very similar to the ones in this study with only one of 120 dogs with a cPSS having a plasma sodium concentration below 139mmol/l. Taken together, this study and our earlier work in dogs with a cPSS, demonstrate that abnormalities in sodium concentrations rarely occur in dogs with liver disease.

Previous work in human patients with liver disease and experimental models of liver failure has also linked hyponatremia to the development of HE [46, 47]. In dogs, clinical signs of HE can include lethargy, inappropriate behavior, disorientation, circling, head pressing or seizures [11]. The pathogenesis of HE in dogs is incompletely understood. Hyperammonemia is considered the most important mediator of HE [16] since ammonia has been demonstrated to be neurotoxic and plasma ammonia concentrations are frequently increased in dogs with liver disease [68]. However, ammonia is unlikely to be the sole mediator of HE in dogs with liver disease since it is not absolutely predictive for the presence of neurological disturbances [68]. Furthermore, hyperammonemia alone, which can occur in dogs with urea cycle enzyme deficiencies, does not typically result in HE [17]. Studies have indicated potential roles for hypercortisolism [19], inflammation [22, 68, 72], altered tryptophan metabolism [20] and increased plasma concentrations of endogenous benzodiazepines [18] in the development of

HE. Dogs with liver disease have also been demonstrated to have an increased whole blood manganese concentration, which is neurotoxic and may influence the development of HE [21, 73].

Consequently, the incompletely understood pathophysiology of canine HE has stimulated research into the role of other factors which could potentially initiate HE in dogs. We have previously examined the role of hyponatremia in the development of HE in dogs with a cPSS. We performed a, a multivariable analysis of clinical and biochemical parameters in 120 dogs with a cPSS found no evidence of an association between sodium concentrations and the development of HE in dogs with a cPSS [68]. Although the prevalence of HE in this present study was low, we found no evidence which linked abnormalities in sodium homeostasis with the development of HE in dogs with primary hepatitis. This study, and our earlier study of dogs with a cPSS indicates that hyponatremia is not only rarely observed in dogs with liver disease, but is also unlikely to be an important cause of neurological complications in dogs with liver disease.

It remains unclear why the incidence of hyponatraemia is greater in humans with liver disease compared to dogs with liver disease. The difference in the incidence of hyponatremia between humans and dogs with liver disease may be due to the different reasons for the development of liver disease and the final histopathological findings. Many human patients with liver disease have cirrhosis due to alcoholism which may influence the incidence of electrolyte disturbances [65]. The reason(s) for the species difference in sodium homeostasis in the setting of liver disease is deserving of further study.

4.5 Conclusion

The present study demonstrates that sodium concentrations are rarely outside the reference interval in dogs with primary hepatitis. This finding is in contrast with the numerous studies of human patients with liver disease. This study, and our earlier work in dogs with a cPSS, indicates that hyponatremia is uncommonly observed in dogs with liver disease. In addition, we found no evidence which linked alterations in sodium homeostasis to the development of HE.

4.6 Acknowledgements

We would like to thank the vets, nurses and owners involved in the care of the animals included in this study.

CHAPTER 5

Presence of systemic inflammatory response syndrome predicts a poor clinical outcome in dogs with a primary hepatopathy

5.1 Introduction

Primary hepatopathies are a common cause of morbidity and mortality in dogs with a recent study reporting that over 10 per cent of dogs had evidence of chronic hepatitis at post mortem examination [1, 9, 74]. Primary hepatitis includes all inflammatory disorders of the hepatic parenchyma [2]. In contrast to human medicine, where the type of hepatitis is defined by the inciting cause, few causes of chronic hepatitis have been identified in the dog, and the majority of cases are idiopathic [2]. Although primary hepatitis can be readily diagnosed in dogs through histological examination of a liver biopsy, it remains a challenging and difficult disease to treat [1, 8]. Furthermore, it is difficult to offer clients an accurate prognosis at the time of diagnosis [74].

There is a clear need to clarify the relationship between inflammation and outcome in dogs with a primary hepatopathy. A more detailed understanding of the relationship between inflammation and clinical outcomes may lead to novel therapeutic approaches. The aim of this study was to determine the prevalence of systemic inflammation in a cohort of dogs with primary hepatopathies, and to then examine the relationship between systemic inflammation and patient survival.

5.2 Materials and Methods

Consecutive cases of dogs diagnosed with primary hepatopathies at the Royal (Dick) School of Veterinary Studies or Davies Veterinary Specialists, were considered for inclusion in the study. Cases were only included if the dog had histopathological confirmation of primary parenchymal hepatic disease. Information on the signalment and previous medical treatment was extracted from the clinical records. The histopathological diagnosis was classified according to WSAVA (World Small Animal Veterinary Association) criteria by a board certified pathologist [2]. All blood samples were taken by a veterinary surgeon as part of routine monitoring. The study was approved by The University of Edinburgh Ethics Research Committee and the owners of the dogs gave permission for their animals to be used in this study. The presence of HE was evaluated using previously described criteria [43]. The dog was considered to have HE if it had clinical signs of lethargy, inappropriate behavior, disorientation, circling, head pressing or seizures [68]. If the dog displayed none of these signs, it was considered not to have HE. The presence of ascites was documented if confirmed by ultrasound evaluation. Details of heart rate, respiratory rate, temperature, haematology profile and biochemistry profile were recorded. A SIRS score was calculated for each dog using methodology previously described [75]. The SIRS score could range from 0-4 as each dog was given 1 point when they met each of the following criteria: respiratory rate greater than 20 min-1; heart rate greater than 120 min-1; total white blood cell (WBC) count less than 6 or greater than 16 $x10^9$ L-1 and rectal temperature less than 38.1°C or greater than 39.2°C Therefore, a SIRS score could range from 0 to 4.

Survival of the cohort was initially examined using Kaplan Meier analysis. As some of the dogs were alive at the end of follow up, Cox proportional hazard analysis was used to

estimate the association between survival and selected co-variates. Initially, a uni-variable analysis was performed which explored the relationship between survival and SIRS score. To determine if this relationship persisted in the presence of other possible other confounding variables, a multivariable cox proportional hazard model was constructed which included SIRS score of 0 or 1 (SIRS low) or SIRS score of 2,3 or 4 (SIRS high), sodium, potassium, sex, ALT, ALP, bilirubin, red blood cell count, presence of ascites, sex, age and HE score. A final model was built based on step wise removal of variables from the initial comprehensive model using AIC as a measure of parameter penalised model fit. To confirm the model structure, the impact on AIC of both dropping each of the final variables and adding back in the dropped variables was investigated. Results are presented as a hazard ratio with 95% CI. Statistical analysis was performed in R statistical software package (R Development Core Team (2012)).

5.3 Results

Eighty cases of histologically confirmed primary hepatopathies were identified. Ten dogs were excluded from the study due to incomplete clinical records. The 70 dogs included in this study comprised of 29 different pure breeds and 8 cross-breeds. The sex distribution comprised of 12 entire males, 24 neutered males, 24 neutered females and 10 entire female dogs. The median age was 75 months (range 6 to 180 months). The diagnosis of a hepatopathy was histologically confirmed from samples collected at post-mortem examination in 9 cases, and from a percutaneous spring-loaded biopsy instrument in 12 cases. Forty nine samples were collected by surgical biopsy techniques. Fifty cases were diagnosed with chronic hepatitis (Fig.1), 10 cases with acute hepatitis, 8 cases with cirrhosis (Fig. 2) and 2 cases with copper associated hepatitis (Fig. 3). Fifty-seven dogs were dead at follow up and

13 were alive. Seventeen dogs had a SIRS score of 0, 22 with a score of 1, 25 with a score of 2, 5 with a score of 3 and 1 dog with a score of 4.

The median survival time of the 70 dogs was 38.5 days (95% CI 20-215). To explore the relationship between a systemic inflammatory response and survival, the median survival of dogs with a SIRS score of 0 or 1 (SIRS Low) and dogs with a SIRS score of 2, 3 or 4 (SIRS High) was estimated. The median survival of SIRS Low dogs was 231 days (95% CI 78-not available/ days) and median survival of SIRS High was 7 days (95% CI 3-25/ days) ($p<0.001$) (Fig. 4). To assess whether this relationship persisted in the presence of other co-variates, a Cox proportional hazard model was built. The initial model included SIRS High or low, presence of ascites, HE score, sex sodium, potassium, ALT, ALKP, bilirubin, age, sex and red blood cell count. The final model after variable selection included SIRS, bilirubin, red blood cell concentrations and age (Table 1). The impact of variables dropped from the model was confirmed by calculating the impact on model fit (Δ AIC) when they were individually added back into the model (Table 2).

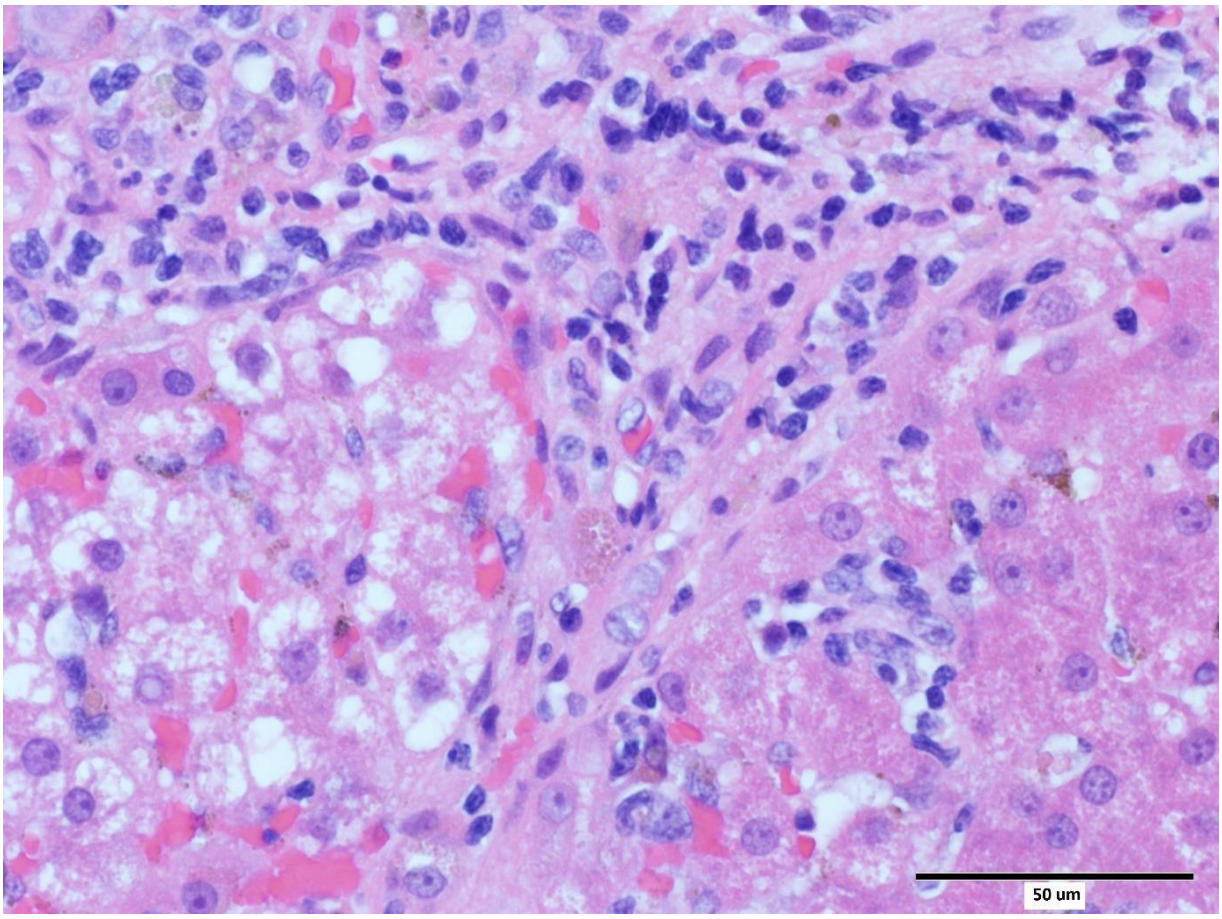

Fig 1: Liver from a case of chronic hepatitis with islands of hepatocytes separated by bands of fibroblasts and collagen, and moderate numbers of macrophages, lymphocytes and plasma cells. Haematoxylin and eosin, scale bar = 50 µ m.

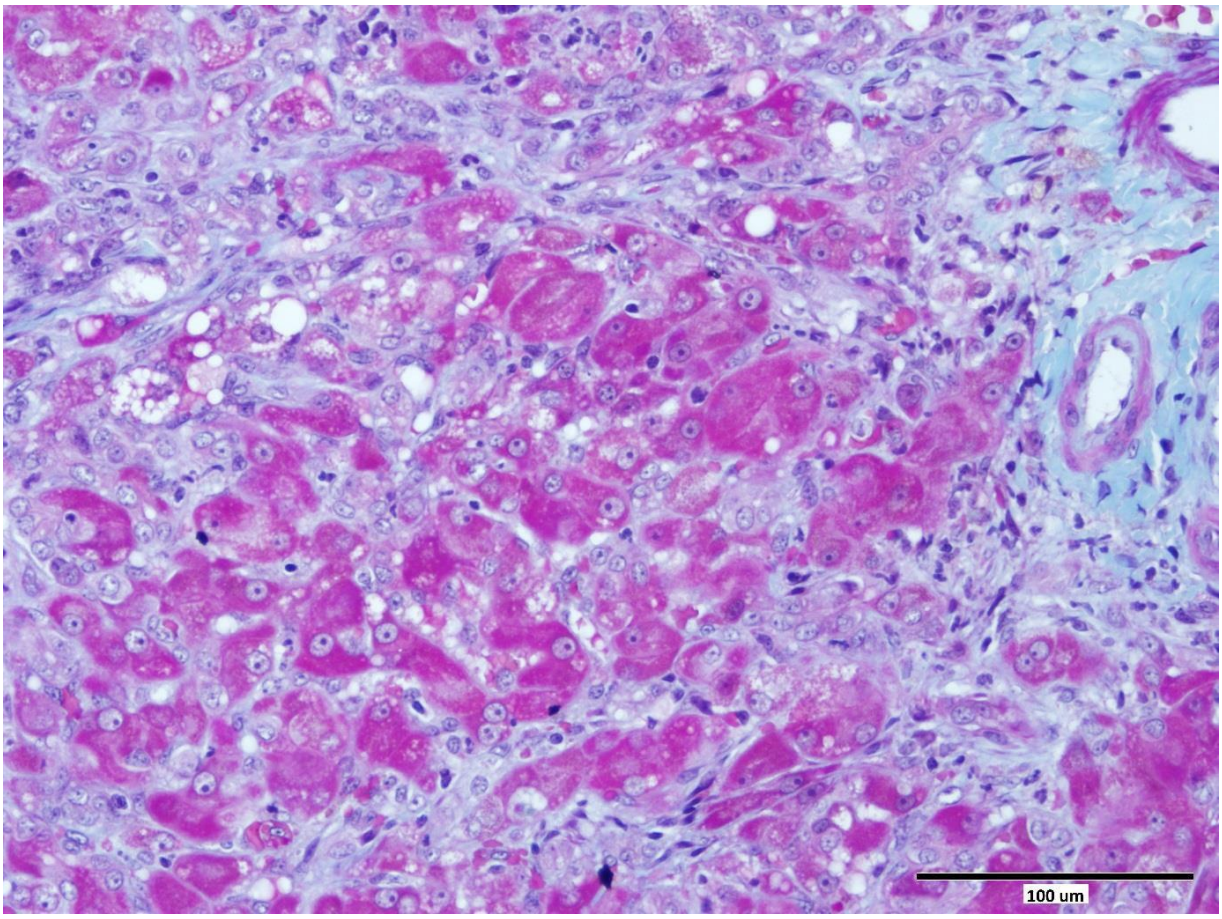

Fig 2: Liver from a case of chronic hepatitis and early cirrhosis showing disruption of the lobular architecture and dissecting fibrosis (green). Masson's trichrome, scale bar = 100 µm.

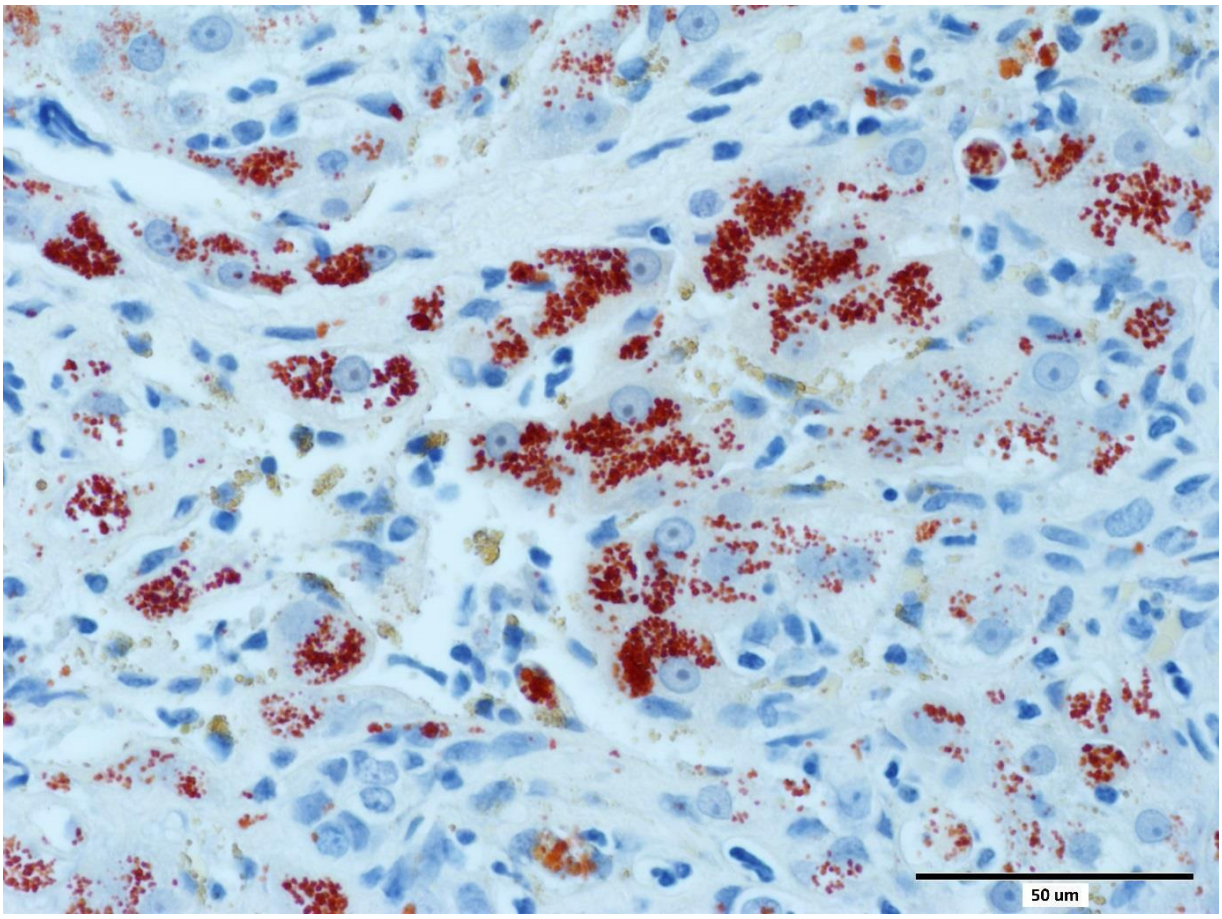

Fig 3: Liver from a case of chronic hepatitis with intracytoplasmic copper accumulation in hepatocytes. Rhodanine red, scale bar = 50 µ m.

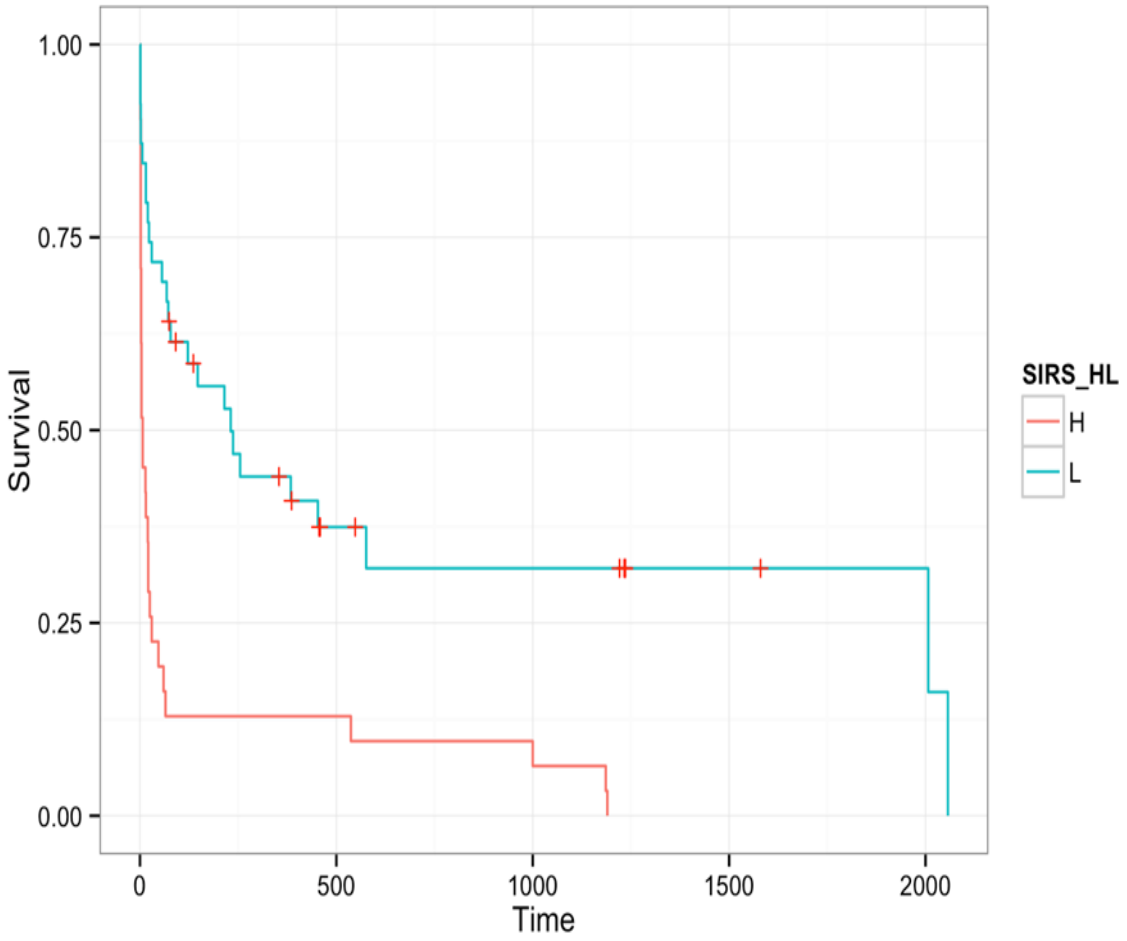

Fig. 4: Kaplan-Meier plot of survival time by SIRS High (red solid line) and SIRS Low (blue dotted line). Tick marks show censored events.

	Hazard Ratio (95 CI)	Δ AIC
SIRS	3.575 (1.857-6.884)	13.3
Bilirubin	1.003 (1.001-1.005)	5.98
Red blood cell	0.768 (0.596-0.990)	2.14
Age	0.909 (0.827-1.000)	1.94

Table 1: Hazard Ratios and 95% confidence interval for terms in final cox proportional hazard model. Δ AIC is the increase in AIC if the term is dropped from model. A positive Δ AIC equates to a poorer model fit.

	Δ AIC
Sodium	0.03
Potassium	1.77
Sex	2.75
Ascites	1.94
HE score	4.77
ALKP	1.98
ALT	0.68

Table 2: Δ AIC for terms not included in final model when added back in final model. A positive Δ AIC equates to a poorer model fit.

5.4 Discussion

In this large two centre study, we have examined, for the first time, the relationship between SIRS, a simple marker of inflammation, and clinical outcome in dogs with primary hepatopathies. The central finding of this study was that the dogs with a primary hepatopathy and a SIRS score of 2, 3 or 4 had a significantly worse clinical outcome than dogs with a SIRS score of 0 or 1. This provides evidence that the presence of systemic inflammation is associated with a poorer clinical outcome in dogs, which is similar to human patients with liver disease. The presence of SIRS was a major predictor of multiple organ failure and strongly correlated with short term mortality in patients with alcoholic hepatitis [30]. In addition, SIRS has recently been associated with acute on chronic liver failure (ACLF) [76].

Inflammation may be associated with a poor outcome in dogs and humans with liver disease for several reasons. Intestinal bacterial overgrowth and altered gut permeability in patients with liver disease is hypothesised to lead to increased translocation of bacteria and endotoxin into the portal circulation [32]. Factors leading to SIRS development in liver disease include the impaired phagocytic function of the reticuloendothelial system which allows endotoxin to reach the systemic circulation in high concentrations[24]. Lipopolysaccharide (LPS) concentrations in the peripheral and portal circulation have been shown to be increased in people with liver cirrhosis [68]. Importantly, LPS concentrations have been shown to predict mortality in patients with alcoholic hepatitis[30]. Endotoxin will activate monocytes and release pro-inflammatory cytokines which have been implicated in the development of hepatic complications such as HE, ascites and variceal bleeding [77]. The disturbances in systemic and hepatic haemodynamics in alcohol-related (ACLF) have also been associated with dysregulated inflammation, multi-organ failure and marked activation of the

sympathetic nervous system [30]. These abnormalities predict high mortality rates in these patients [31].

In our study, the proportion of dogs which had an infection was not accurately defined. In a recent study on human patients with alcoholic hepatitis and SIRS, over 60% of patients had no evidence of infection on clinical or microbiological criteria [31]. Understanding the pathophysiology of sterile inflammation in patients with liver disease with the ambition of developing targeted therapies is one of the major challenges in contemporary hepatology.

Predicting the course and prognosis of disease is an important part of veterinary and human clinical practice. The development of new prognostic markers with documented significance is an important focus of human and veterinary research. Various factors have been demonstrated as negative prognostic factors in dogs with primary hepatitis [78]. These included jaundice, abdominal fluid wave, microhepatica, ascites, enlarged portal lymph nodes, hypoalbuminemia and left shift of neutrophils in the peripheral blood [1]. Consistent with our study, total serum bilirubin was also shown as a negative prognostic factor in idiopathic canine chronic hepatitis [74]. High bilirubin concentrations has also been documented to be associated with a negative outcome in human patients [60, 79]. Furthermore, our prognostic association with low red blood cell count is consistent with the prediction of poor survival with reduced red blood cell counts after surgery in human patients with primary liver cancer [80] and end stage liver disease [81].

Another finding of this study was that abnormal serum sodium concentrations were not commonly observed in dogs with primary hepatitis. This observation contrasts with the

findings from studies of human with liver disease where hyponatremia is frequently observed [46, 47, 69]. For example, in a prospective study of 997 patients with cirrhosis the prevalence of low serum sodium concentration, as defined by a serum sodium ≤135 mmol/l, ≤130 mmol/l, ≤125 mmol/l or ≤120 mmol/l, was 49.4%, 21.6%, 5.7%, and 1.2%, respectively [65]. Borroni et al. reported hyponatraemia in 29.8% of patients admitted for management of cirrhosis, as defined by a serum sodium concentration of ≤130 mmol/l [70]. Furthermore, the prevalence of hyponatremia was 26% in a population of paediatric patients with end stage liver disease waiting for liver transplantation (as defined by a serum sodium concentration of ≤130 mmol/l) [71]. It remains unclear why humans but not dogs develop hyponatraemia with liver disease. We would hypothesise that the differences in the two populations may be due to the different causes of liver disease in dogs compared to humans.

Several studies in human patients with liver disease have examined the role of circulating biomarkers as adjunct markers of systemic inflammation such as LPS, procalcitonin and CRP [82-84]. Cytokines, including interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor (TNF) are key mediators of the inflammatory response [34]. Several studies have measured circulating cytokine concentrations in patients with liver disease. One marker that has been investigated in some detail is IL-6 [54, 61, 72]. Increased serum concentrations of IL-6 have been found in people with different inflammatory diseases and has been correlated with the severity of the disease, prognosis and outcome [85]. Higher circulation levels of IL-6 were significantly associated with a higher risk of developing hepatocellular carcinoma [86]. IL-6 has also been shown to be a useful prognostic marker for canine critical care patients [87].

Our study demonstrates the need to understand more about the development of systemic inflammation in dogs with liver disease and to establish whether reducing systemic inflammation improves outcomes.

5.5 Conclusion

In conclusion, a high SIRS score was found to be associated with a poorer long term survival in our study of dogs with primary hepatopathies. Our study demonstrates the need to understand more about the cause and consequences of inflammation in dogs with liver disorders. Our study demonstrates the need to understand more about the development of systemic inflammation in dogs with liver disease and to establish whether reducing systemic inflammation improves patient outcomes. The impact of ameliorating inflammation in patients with hepatitis is ill defined. Although prednisolone is widely used as first line treatment for hepatitis in both dogs and humans, the therapeutic benefits of prednisolone remain ill-defined in both species [10, 88-90].

5.6 Acknowledgements

We would like to thank the vets, nurses and owners involved in the care of the animals included in this study.

CHAPTER 6

Thesis Summary and Conclusions

In summary the findings of this thesis provide further information about the pathogenesis of canine liver disease. Firstly, whole blood Mn concentrations are increased in dogs with primary hepatitis. Taken together with earlier studies, which reported that dogs with cPSS had increased Mn concentrations, it is clear that dogs with either primary vascular or primary hepatic disease frequently have disturbed Mn homeostasis. The prognostic significance of increased Mn concentrations, correlation with severity of histopathological changes or acquired shunting fraction and the relationship between Mn and severity of HE in dogs is deserving of further study.

The results of this thesis also highlight that sodium concentrations are rarely outside the reference interval in dogs with primary hepatitis. This finding is in contrast with the numerous studies of human patients with liver disease. This study, and our earlier work in dogs with a cPSS, indicates that hyponatremia is uncommonly observed in dogs with liver disease. In addition, we found no evidence which linked alterations in sodium homeostasis to the development of HE.

The results from chapter two have sought to better understand the role of inflammation in the development of HE. IL-6 concentrations are increased in dogs with a cPSS. Given the well-established role of IL-6 in the pathogenesis of hepatic encephalopathy in humans with acquired liver disease, it is possible that IL-6 may be important in the development of HE in dogs with a cPSS. Additional studies on the role of IL-6 in the pathogenesis of hepatic encephalopathy in dogs with both acquired and congenital liver disease are warranted. A

better understanding of the role of IL-6 and other cytokines in the development of HE may facilitate the development of novel treatment modalities.

The final important finding from this work is that a high SIRS score was found to be associated with a poorer long term survival in our study of dogs with primary hepatopathies. Our study demonstrates the need to understand more about the cause and consequences of inflammation in dogs with liver disorders. Our study demonstrates the need to understand more about the development of systemic inflammation in dogs with liver disease and to establish whether reducing systemic inflammation improves patient outcomes.

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APPENDICES

PAPER

Whole blood manganese concentrations in dogs with primary hepatitis

S. Kilpatrick, A. Jacinto, R. D. Foale*, S. W. Tappin*, C. Burton†, P. E. Frowde†, C. M. Elwood†, R. Powell‡, A. Duncan§, R. J. Mellanby and A. G. Gow

Division of Veterinary Clinical Sciences, The Roslin Institute, Royal (Dick) School of Veterinary Studies, Hospital for Small Animals, The University of Edinburgh, Roslin, Midlothian EH25 9RG

*Dick White Referrals, Six Mile Bottom, Cambridgeshire CB8 0UH

†Davies Veterinary Specialists, Higham Gobion, Hitchin, Hertfordshire SG5 3HR

‡Powell Torrance Diagnostic Services, Higham Gobion, Hitchin, Hertfordshire SG5 3HR

§Scottish Trace Element and Micronutrient Reference Laboratory, Department of Clinical Biochemistry, Glasgow Royal Infirmary, Glasgow G4 0SF

OBJECTIVES: Increased whole blood manganese concentrations have been reported in humans with primary liver disease. Due to the neurotoxic effects of manganese, altered manganese homeostasis has been linked to the development of hepatic encephalopathy. Whole blood manganese concentrations are increased in cases of canine congenital portosystemic shunts, but it remains unclear whether dogs with primary hepatopathies also have altered manganese homeostasis.

METHODS: Whole blood manganese concentrations were measured by graphite furnace atomic absorption spectrometry in 21 dogs with primary hepatitis, 65 dogs with a congenital portosystemic shunt, 31 dogs with non-hepatic illnesses and 18 healthy dogs.

RESULTS: The whole blood manganese concentrations were significantly different between dogs with primary hepatitis, dogs with non-hepatic illnesses and healthy dogs (P=0·002). Dogs with primary hepatitis had significantly increased whole blood manganese concentrations compared with healthy dogs (P<0·05) and dogs with non-hepatic illnesses (P<0·01). Dogs with primary hepatitis had significantly lower whole blood manganese concentration compared with dogs with congenital portosystemic shunts (P=0·0005).

CLINICAL SIGNIFICANCE: Dogs with primary hepatopathies have increased concentrations of whole blood manganese although these concentrations are not as high as those in dogs with congenital portosystemic shunts. The role of altered manganese homeostasis in canine hepatic encephalopathy is worthy of further study.

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INTRODUCTION

Primary hepatitis includes all inflammatory disorders of the hepatic parenchyma (consisting of the functional unit of liver lobule, Michalopoulos & DeFrances 1997), apart from those due to distant endotoxin release (Rothuizen & van den Ingh 1998, Van den Ingh *et al.* 2006). Primary hepatitis is well recognised in dogs, with one study reporting a clinical prevalence of 0·5% (Poldervaart *et al.* 2009). Primary hepatitis may be divided into acute and chronic forms, as well as lobular dissecting, granulomatous and eosinophilic hepatitis as defined by the WSAVA according to histological criteria (Van den Ingh *et al.* 2006). Of these, chronic hepatitis appears the most common (Poldervaart *et al.* 2009). The clinical prevalence of chronic hepatitis may be an underestimate because post-mortem examination of 200

unselected dogs detected the presence of chronic hepatitis in 12% of dogs (Watson *et al.* 2010). In contrast to human medicine, where the type of hepatitis is defined by the inciting cause, few causes of chronic hepatitis have been identified in the dog, and the majority of cases are considered idiopathic (Watson2004). As a result, treatment is symptomatic (Watson 2004) and the prognosis for dogs with chronic hepatitis is poor with a reported median survival time of 18·3 months (Poldervaart *et al.* 2009). One of the most serious consequences of primary hepatitis is the development of hepatic encephalopathy which can cause significant morbidity (Rothuizen 2009).

The liver has a key role in regulation of many trace elements and various trace element abnormalities have been documented in cases of canine chronic hepatitis (Schultheiss *et al.* 2002, Pressler *et al.* 2010). Copper metabolism has been investigated most extensively and increased hepatic copper concentrations have been shown to be both a cause and effect of chronic hepatitis. In Bedlington terriers, a primary genetic defect results in copper accumulation within the hepatic parenchyma and subsequent hepatitis due to oxidative damage (Haywood *et al.* 1996). Conversely, chronic hepatitis can result in reduced biliary excretion of copper and increased hepatic parenchymal concentrations (Hoffmann *et al.* 2009). Similarly, increased hepatic iron concentrations have also been documented in naturally occurring and experimental canine chronic hepatitis (Fuentealba *et al.* 1997, Schultheiss *et al.* 2002, Soubasis *et al.* 2006). Zinc has been shown to be an effective treatment for copper-associated hepatitis and hepatic and biliary zinc concentrations have been investigated in canine chronic hepatitis (Schultheiss *et al.* 2002, Hoffmann *et al.* 2009, Pressler *et al.* 2010).

The impact of primary hepatic disease on the metabolism of other trace elements is less well understood in dogs. In particular, the liver plays a pivotal role in manganese (Mn) metabolism as the majority of gastrointestinally absorbed Mn is removed by the liver and excreted into bile such that only approximately 2% of absorbed Mn reaches the systemic circulation (Aschner & Aschner 2005). One experimental study showed that healthy dogs fed large quantities of Mn daily for 16 months showed no ill-effects and no appreciable increase in blood Mn concentrations. On post-mortem examination, liver and bile had the highest concentrations of Mn (Reiman & Minot 1920). Although Mn is an essential mineral, Mn toxicity has been reported in humans and also in experimental animal models (Keen *et al.* 2000). Studies in humans have shown increased blood Mn concentrations and Mn deposition in the central nervous system (CNS) in cases of hepatic insufficiency (Versieck *et al.* 1974, Rose *et al.* 1999, Mizoguchi *et al.* 2001, Tuschl *et al.* 2008). The deposition of Mn in the CNS is considered to play a role in hepatic encephalopathy and a direct relationship has been demonstrated between blood Mn concentration, magnetic resonance imaging (MRI) hyperintensity consistent with Mn deposition and severity of encephalopathic score in humans (Layrargues *et al.* 1995, Spahr *et al.* 1996). In the CNS, Mn is preferentially stored in astrocytes such that their Mn concentrations may be up to approximately 200 times the extracellular concentrations (Tholey *et al.* 1988). Astrocytes are the major cell implicated in the development of hepatic encephalopathy and dysfunction of the astrocyte by Mn is thought to contribute to this (Shawcross & Jalan 2005, Hazell *et al.*2006).

There have been only a small number of studies which have investigated the role of Mn in canine liver disease. MRI of 13 dogs and 3 cats with a congenital portosystemic shunt (cPSS) detected hyperintense focal areas in the lentiform nuclei which were considered to be consistent with Mn deposition, although neither blood nor tissue Mn concentrations were assessed (Torisu *et al.* 2005). In support of this hypothesis, a single case has been reported of a Yorkshire terrier with a cPSS and a compatible hyperintense lentiform nucleus which had an increased concentration of Mn in this area on post-mortem examination (Torisu *et al.* 2008). A recent study demonstrated that whole blood Mn concentrations are increased in cases of canine cPSS (Gow *et al.* 2010). However, it remains unclear whether dogs with primary hepatitis have altered Mn homeostasis.

The central hypothesis of this study was that dogs with primary hepatitis have altered Mn homeostasis. The aim was to compare whole blood Mn concentrations in dogs with primary hepatitis with concentrations in healthy dogs and in dogs with non-hepatic illness. A second hypothesis was that dogs with primary hepatopathies would not have such profound disturbances of Mn homeostasis as in dogs with a cPSS. This was investigated by comparing whole blood Mn concentrations in dogs with a primary hepatopathy and those with a cPSS.

MATERIALS AND METHODS

Study population

Cases of primary hepatitis, defined as dogs with increased fasting bile acid concentrations, and histopathological confirmation of primary hepatitis from samples collected by either biopsy or post-mortem examination were considered eligible for inclusion into the study. Disease was classified according to WSAVA criteria (Van den Ingh *et al.* 2006). The type of diet during the past 7 days was noted for each dog. Consecutive cases of dogs with non-hepatic illness, defined as dogs with illness warranting diagnostic investigations including serum biochemistry which had serum concentrations of albumin, alanine aminotransferase (ALT), fasting bile acid concentrations and bilirubin within their respective reference intervals, were enrolled into the study. Dogs with cPSS confirmed by ultrasonography and/or portovenography were also enrolled. Consecutive cases of healthy dogs, defined as dogs which were considered healthy by their owners and had a normal physical examination when blood sampled for an unrelated primary reason (rabies serology or PCV check before blood donation), were also enrolled into the study.

Sampling methods

Whole blood samples were collected from fasted animals. Surplus blood collected via a 21g 5/8″ hypodermic needle from the jugular vein for clinical diagnostic purposes was placed into commercial 1·3 mL Ethylenediaminetetraacetic acid (EDTA) tubes and retained for Mn measurement. All EDTA samples were

frozen within 4 hours of collection and stored at −70°C until they were transported on dry ice to the Scottish Trace Element and Micronutrient Reference Laboratory. Manganese concentrations were determined in whole blood by graphite furnace atomic absorption spectrometry after dilution with Triton X-100 solution. The inter-assay coefficient of variation (CV) was 3·1% and the intra-assay CV was 5·4%.

The study was approved by the University of Edinburgh Ethics Research Committee and owner consent was obtained.

Statistical analysis

Manganese concentrations, and differences in age, were compared between the dogs with primary hepatitis, hospitalised ill dogs and healthy dogs by a Kruskal-Wallis test with post-test Dunn's multiple comparison test. Manganese concentrations were compared between dogs with primary hepatitis and a cPSS by a Mann-Whitney U test. The presence of correlation between Mn concentrations and biochemical values and age was evaluated by Spearman Rank Correlation test. Statistical analysis was performed with a commercial software package (GraphPad Prism version 5.0 for Windows; GraphPad Software) with $P<0·05$ considered significant. Non-parametric testing was done because of the size of the groups under analysis.

RESULTS

The group of dogs with non-hepatic illness comprised 31 dogs (19 different breeds, 2 crossbreeds, 4 male entire, 10 male neutered, 13 female neutered, 4 female entire, mean age 59·5 months, range 5 to 132 months). The disease categories were nasal disease (n=5), inflammatory disease (n=4), orthopaedic abnormalities (n=4), lower urinary tract disorders (n=6), poor body condition/exercise intolerance (n=5), chronic renal failure (n=2), focal neoplasia (n=2) and one each of ovarian remnant syndrome, congenital cardiac disease and rodenticide exposure. Blood samples from healthy dogs were obtained from 16 blood donors and 2 dogs during rabies serology sampling (10 different breeds, one crossbreed, 4 male entire, 11 male neutered, 3 female neutered, mean age 57 months, range 24 to 120 months). Sixty-five dogs with cPSS (25 breeds, 8 crossbreeds, 21 male entire, 10 male neutered, 26 female entire, 8 female neutered, mean age 19 months, range 3 to 96 months) were also enrolled. Twenty-one dogs diagnosed with primary hepatic disease were enrolled (10 different breeds, 6 crossbreeds, 4 male entire, 7 male neutered, 10 female neutered, mean age 84 months, range 6 to 180 months). Eight were confirmed from samples collected during post-mortem examination, and four by using a percutaneous spring-loaded biopsy instrument. Nine were collected by surgical biopsy techniques. Eighteen cases were diagnosed with chronic hepatitis, two cases with dissecting lobular hepatitis and one case with cirrhosis. Only one case was being fed a diet formulated for liver disease at the time of sampling. The other 20 dogs were fed a standard proprietary dog food or home cooked diet. The dogs with non-hepatic illnesses and healthy dogs were fed a range of commercial non-clinical diets. There were no significant differences between the age of the dogs in the healthy, non-hepatic illness and primary hepatic disease groups (P=0·67).

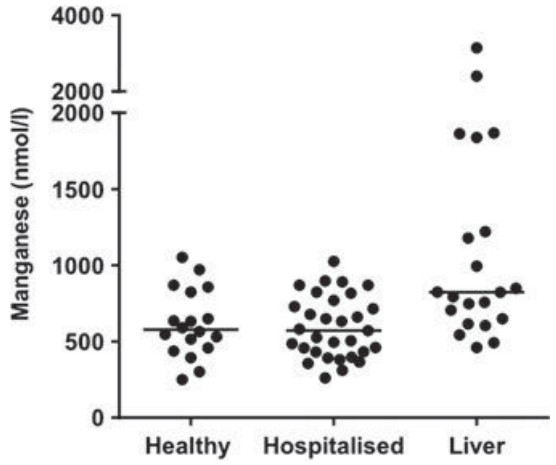

FIG 1. Whole blood manganese concentrations (nmol/L) in dogs with primary hepatitis (liver), dogs with non-hepatic illness (hospitalised) and healthy dogs (healthy)

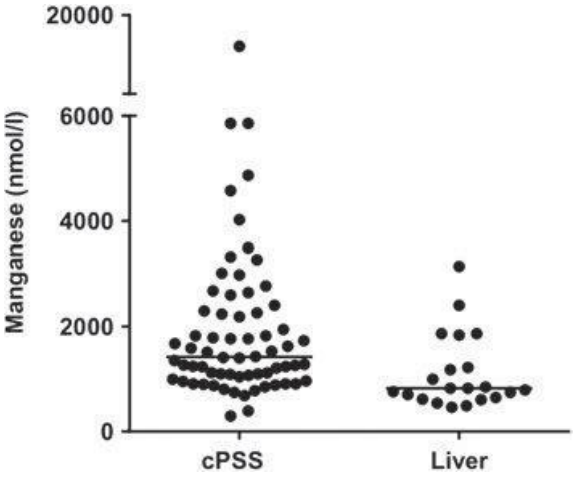

FIG 2. Whole blood manganese concentrations (nmol/L) in dogs with primary hepatitis (liver) and dogs with a congenital portosystemic shunt (cPSS)

There was a significant difference in Mn concentrations between dogs with primary hepatic disease, dogs with non-hepatic illness and healthy dogs ($P<0·002$, Fig 1). Post test analysis revealed that there was no difference in Mn concentrations between dogs with non-hepatic illness and healthy dogs but a significant difference in Mn concentration between dogs with primary hepatic disease and dogs with non-hepatic illness ($P<0·01$) and dogs with primary hepatic disease and healthy dogs ($P<0·05$). In the primary hepatic disease group, there was no correlation between Mn and fasting bile acid concentrations ($r=0·25$, $P=0·28$), Mn and albumin concentration ($r=-0·19$, $P=0·41$), Mn and ALT activity ($r=-0·20$, $P=0·39$), Mn and prothrombin time ($r=0·18$, $P=0·48$), Mn and activated thromboplastin time ($r=0·35$, $P=0·16$) or Mn and bilirubin concentrations ($r=0·07$, $P=0·77$).

Dogs with primary hepatitis had significantly lower whole blood Mn concentration compared to dogs with cPSS (median values 1101 and 2026 nmol/L, respectively, P=0·0005; Fig 2). There was a significant difference in age between the cPSS (median age six months) and primary hepatitis groups (median age 84 months) (P<0·0001).

DISCUSSION

The central finding of this study was that whole blood Mn concentrations were significantly higher in dogs with primary hepatic disease compared to both healthy dogs and dogs with non-hepatic illness. Taken together with an earlier study, which reported that dogs with cPSS had increased Mn concentrations, it is clear that dogs with either primary vascular or primary hepatic disease frequently have disturbed Mn homeostasis (Gow *et al.* 2010). The first-pass excretion of gastrointestinally absorbed Mn relies on efficient delivery through the portal circulation, and both functional hepatocytes and a functional biliary system. Dysfunction of any one of these three components will potentially increase delivery of Mn to the systemic circulation (Krieger *et al.* 1995, Rose *et al.* 1999). Chronic hepatitis and lobular dissecting hepatitis commonly result in dysfunction of all three components: hepatocyte loss by definition in chronic hepatitis, cholestasis due to hepatic architecture disruption of the biliary system and, because of increased portal pressure, recruitment of portosystemic collateral vessels (Rothuizen 2009). Consequently, it is technically challenging to establish the relative importance of primary parenchymal diseases, cholestasis and portosystemic shunting on Mn metabolism. This issue has been partially addressed in experimental models of liver disease, a study of CNS Mn deposition in the rat found that rats with induced portocaval shunts had higher concentrations than rats with induced cirrhosis. The shunting percentage was 100% for induced shunts, whereas cirrhotic rat shunting percentage ranged from 29 to 100% (Rose *et al.* 1999). One clinical study of humans with cirrhosis demonstrated that all nine patients with hyperintense lesions on MRI had shunting vessels compared to only 2 of 17 patients with no lesions on MRI (Inoue *et al.* 1991). In this study, the finding that whole blood Mn concentrations were significantly higher in dogs with cPSS compared to dogs with primary hepatopathy would suggest that shunting vessels have an important role in the pathogenesis of hypermanganesaemia. A further study investigating any correlation between whole blood Mn concentrations with the percentage shunting in dogs with primary hepatitis would further clarify this hypothesis.

Investigation of whole blood Mn concentrations in cases of acute hepatitis may help define the contribution of hepatic dysfunction per se to increased Mn concentrations. One study of acute hepatitis in humans demonstrated increased serum concentrations of Mn in acute hepatitis and found a correlation with activity of serum ALT in both acute and chronic hepatitis which may suggest that hepatic dysfunction rather than development of portosystemic shunting was responsible for the increased systemic concentrations of Mn in these cases (Versieck *et al.* 1974). Determination of systemic whole blood Mn concentrations in cases of extra-hepatic cholestasis would also be useful to assess the contribution of biliary dysfunction to increased systemic Mn concentrations.

Manganese and iron metabolism are intimately linked. Both metals use divalent metal transporter 1 (DMT1) for absorption from the gastrointestinal tract and also transport into the CNS (Garrick *et al.* 2003). DMT1 expression in both is regulated by iron status via hepcidin (Brasse-Lagnel *et al.* 2011, Li *et al.* 2011). Deficiency of iron has been shown to increase CNS Mn uptake whilst iron overload decreases Mn uptake (Aschner & Aschner 1990). Hepcidin is mainly produced by hepatocytes and is up-regulated in response to inflammation (Park *et al.* 2001, Nicolas *et al.* 2002). Because chronic hepatitis (by definition) and to a lesser extent lobular dissecting hepatitis have an inflammatory component, it would be expected that hepcidin may be up-regulated and this, in turn, would reduce gastrointestinal absorption of Mn (Rothuizen 2006). In converse, loss of hepatocytes would reduce the liver's ability to produce hepcidin and thus potentially remove negative regulation of DMT1 expression. In support of this, serum hepcidin and prohepcidin concentrations have been found to be lower in humans with chronic hepatitis C compared to healthy controls and correlate with fibrosis (Jaroszewicz *et al.* 2008, Tsochatzis *et al.* 2010). Therefore, it is likely that the confounding interplay of inflammation, reduced hepatic function, effects of iron status on hepcidin expression and therefore gastrointestinal Mn uptake, may all play a role in the variation in Mn concentrations found in the primary hepatopathy group.

In the primary hepatitis group, 5 dogs had whole blood Mn concentrations which were markedly elevated whilst the remaining 16 had similar concentrations to the non-hepatic illness and healthy groups. This apparent split may be due to a difference in hepatic reserve, or as stated above, due to differing shunt fractions, and larger studies to assess if histological severity and/or shunting fraction correlated with Mn concentrations would be informative.

The main pathological consequence of increased Mn concentrations in hepatic disorders is considered to be neurological dysfunction. The exact mechanism by which Mn causes encephalopathy is unclear although there are both in vitro and in vivo studies which suggest that many of the effects are centred on the astrocyte, a key cell in the development of hepatic encephalopathy (Prakash & Mullen 2010). Astrocytes have been shown to have a high affinity transport mechanism for Mn and cytoplasmic Mn accumulation causes Alzheimer type II changes, which are recognized in post-mortem examination of human hepatic encephalopathy patients (Hazell *et al.* 2006, Rama Rao *et al.* 2007). Experimental studies have demonstrated a wide variety of potential toxic effects of Mn on the astrocyte, including potentiating the effect of ammonia as well as impairing their glutamate transport (Jayakumar *et al.* 2004). Glutamate is the main excitatory neurotransmitter in the brain, and astrocyte uptake is the main method of removal from the synaptic cleft. Glutamate is then converted to glutamine by the astrocyte before being recycled back to the neuron. Inhibition of the astrocyte glutamate transporter GLT-1 by Mn prevents this inactivation

and recycling, potentially causing increased concentrations of the neurotransmitter in the synaptic cleft (Hazell & Butterworth 1999). Astrocyte mitochondrial dysfunction and induction of peripheral-type benzodiazepine receptors (PTBR, now termed translocator protein) with increased ligand binding have also been reported with increasing concentrations of Mn (Hazell *et al*. 1999, Yin *et al*. 2008, Ahboucha 2011). Activation of the PTBR causes neurosteroid production, which is also implicated in astrocyte dysfunction and hepatic encephalopathy (Hazell & Butterworth 1999, Ahboucha 2011).

The finding that Mn concentrations are increased in some dogs with primary hepatopathies may be therapeutically relevant since chelation treatment with para-aminobenzoic acid and disodium edetate has been used successfully in human cases of Mn toxicity (Ky *et al*. 1992, Herrero Hernandez *et al*. 2006). This has led to the suggestion that it may be of use in controlling Mn and thus hepatic encephalopathy in humans and dogs (Riordan & Williams 1997, Torisu *et al*. 2008, Tuschl *et al*. 2008). In support of this, Park *et al*. (2008) used trientine chelation therapy in one human case of chronic hepatic disease with acquired portosystemic shunting and hepatic encephalopathy. Following treatment there was a decrease in whole blood Mn concentrations and a reduction in CNS MRI hyperintensity which was thought to be consistent with a reduction in CNS Mn deposition. These findings correlated with a reported marked improvement in clinical signs.

Conclusion

This study demonstrates that whole blood Mn concentrations are increased in dogs with primary hepatitis. The prognostic significance of increased Mn concentrations, correlation with severity of histopathological changes or acquired shunting fraction and the relationship between Mn and severity of hepatic encephalopathy in dogs is deserving of further study.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Short Communication

Plasma cytokine concentrations in dogs with a congenital portosystemic shunt

Scott Kilpatrick [a], Adam G. Gow [a,b], Rob D. Foale [c], Simon W. Tappin [c], Harvey Carruthers [d], Nicola Reed [a], Donald A. Yool [a], Samantha Woods [a], Ana I. Marques [a], Rajiv Jalan [e], Richard J. Mellanby [a,b,⇑]

[a] Royal (Dick) School of Veterinary Studies, Division of Veterinary Clinical Sciences, Hospital for Small Animals, The University of Edinburgh, Midlothian EH25 9RG, United Kingdom
[b] Roslin Institute, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Midlothian EH25 9RG, United Kingdom
[c] Dick White Referrals, Station Farm, London Road, Six Mile Bottom, Cambridgeshire CB8 0UH, United Kingdom
[d] Tay Valley Veterinary Centre, Perth PH2 0PA, United Kingdom
[e] UCL Institute of Hepatology, Royal Free Hospital, Upper Third Floor, UCL Medical School, Pond Street, London NW3 2QG, United Kingdom

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abstract

Congenital portosystemic shunts (cPSS) are a well-recognised vascular anomaly in dogs. Recent studies have shown an association between inflammation and hepatic encephalopathy (HE), which is a common clinical syndrome in dogs with a cPSS. Pro-inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)-a are frequently increased in the plasma of human patients with liver disease and have been implicated in the development of HE.

In the current study, plasma concentrations of IL-2, IL-6, IL-8 and TNF-a were measured using a multiplex electrochemiluminescence immunoassay in 36 dogs with a cPSS and compared to 25 healthy dogs. There were no significant differences in plasma IL-2, IL-8 and TNF-a concentrations between the two groups; however, plasma concentrations of IL-6 were significantly higher in dogs with a cPSS compared to healthy dogs ($P = 0.02$).

Congenital portosystemic shunts (cPSS) are a well-recognised vascular anomaly in dogs. One of the most common clinical signs in dogs affected with a cPSS is hepatic encephalopathy (HE). Recently, we demonstrated the potential role of an inflammatory response, whereby dogs affected with HE, associated with a cPSS, had higher serum concentrations of C-reactive protein (CRP), compared to healthy dogs or dogs with a cPSS that were asymptomatic (Gow et al., 2012). Similar observations have been made in humans affected with liver disease (Coltart et al., 2013). Several groups of researchers have investigated the role of cytokines, which are key mediators of the systemic inflammatory response, in human patients with liver disorders, with several of such studies identifying elevated plasma concentrations of interleukin (IL)-6 and tumour necrosis factor (TNF)-a (Odeh et al., 2005; Luo et al., 2012a). The aim of the current study was to measure IL-2, IL-6, IL-8 and TNF-a in the plasma of healthy dogs and in those affected with a cPSS. The study was approved by the University of Edinburgh's Veterinary Ethical Review Committee (VERC 110308). Dogs were diagnosed with a cPSS if they had compatible clinical signs and had an abnormal shunting vessel identified via ultrasonography, computerised tomography, intra-operative portovenography, visualised during surgery or by a combination of these methods. Healthy dogs, undergoing blood sampling for routine health monitoring, were included in the study and were defined as those that were considered healthy by their owners and who had a normal physical examination. Venous blood samples were collected from both the cPSS dogs and healthy control animals and immediately placed into lithium heparin anticoagulant blood collection tubes. Samples were spun then plasma separated, frozen and stored at −80 °C until cytokine analysis was performed.

Canine pro-inflammatory cytokines (IL-2, IL-6, IL-8 and TNF-a) were measured using a multiplex electrochemiluminescence immunoassay system[1] (Meso Scale Discovery; MSD). Assay diluent (25 μL) was added to all wells, plates sealed and incubated for 30 min at room temperature on an orbital shaker (600 rpm). Samples and standards, diluted in assay diluent, were added at 25 μL per well. Plates were again sealed and incubated for a further 2 h at room temperature with shaking. At the end of the incubation period, wells were washed three times with 200 μL/well phosphate-buffered saline (PBS), supplemented with 0.05% Tween 20 (Sigma – Aldrich) for 30 s, then discarded. Detection antibody was added at

⇑ Corresponding author at: Royal (Dick) School of Veterinary Studies, Division of Veterinary Clinical Sciences, Hospital for Small Animals, The University of Edinburgh, Midlothian EH25 9RG, United Kingdom. Tel.: +44 1316 507650.
E-mail address: Richard.mellanby@ed.ac.uk (R.J. Mellanby).

[1] See: http:// www. mesoscale.com/CatalogSystemWeb/Documents/Canine_ProInflam_Panel_3.pdf.

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Table 1
Breeds of dogs in cPSS group and healthy group.

Healthy control	cPSS
Staffordshire Bull terrier	Yorkshire terrier (5)
Labrador	Labrador (4)
Great Dane	Lakeland terrier
Bearded Collie (2)	Irish Wolfhound
Yorkshire terrier (2)	Jack Russell terrier (3)
Jack Russell terrier (2)	Dachshund
Crossbreed (6)	West Highland White terrier (5)
Weimaraner	Pug (4)
Cocker Spaniel	Bichon Frise
British Bulldog	Bassett hound
Border terrier	Crossbreed
Bull Mastiff	Fox terrier
Border Collie	Lhasa Apso
Lhasa Apso	Ibizan hound
Cavalier King Charles Spaniel	Shih Tzu
Golden Retriever	Chihuahua
Springer Spaniel	Border terrier
	Pyrenean Mountain dog
	Cocker Spaniel
	Shar Pei

25 μL per well, plates sealed and incubated for a further 1 h at room temperature with shaking. Plates were washed three times and 150 μL of MSD Read Buffer added to each well, then electrochemiluminescence measured using the MSD Sector Imager 2400 plate reader. Plasma cytokine concentrations and ages were compared between the healthy dogs and dogs affected with a cPSS, using the Mann Whitney *U* test with a *P*-value <0.05 considered to be significant.

The cPSS group consisted of 36 dogs of 20 different breeds (Table 1). There were 15 entire males, six neutered males, 13 entire females and two neutered females. The median age of the dogs in the cPSS group was 12 months (range 3 – 63 months). Hepatic encephalopathy was present at the time of blood sampling in 29/36 dogs with a cPSS. The healthy control group comprised 25 healthy dogs of 17 different breeds (Table 1). There were eight entire males, five neutered males, five entire females and seven neutered females. The median age of the healthy dogs was 15 months (range 3 – 72 months). There was no significant difference in the median ages of healthy dogs and dogs with a cPSS.

There were no significant differences between groups in the plasma concentrations of IL-2, IL-8 and TNF-α, but there was a significant difference ($P = 0.02$) in the plasma IL-6 concentrations comparing dogs with a cPSS and healthy dogs (Fig. 1, Table 2). This finding is consistent with a recent study that found dogs with intra-hepatic diseases had increased serum IL-6 concentrations (Neumann et al., 2012). It is also consistent with studies in human patients with various types of liver pathology (Tilg et al., 1992).

Increased circulating IL-6 concentrations in dogs with liver disease might be an important factor in initiating HE, which was present in the majority of dogs with a cPSS in the present study. IL-6 has been shown to be increased in human patients with overt and minimal HE, which correlates with the severity of the disease

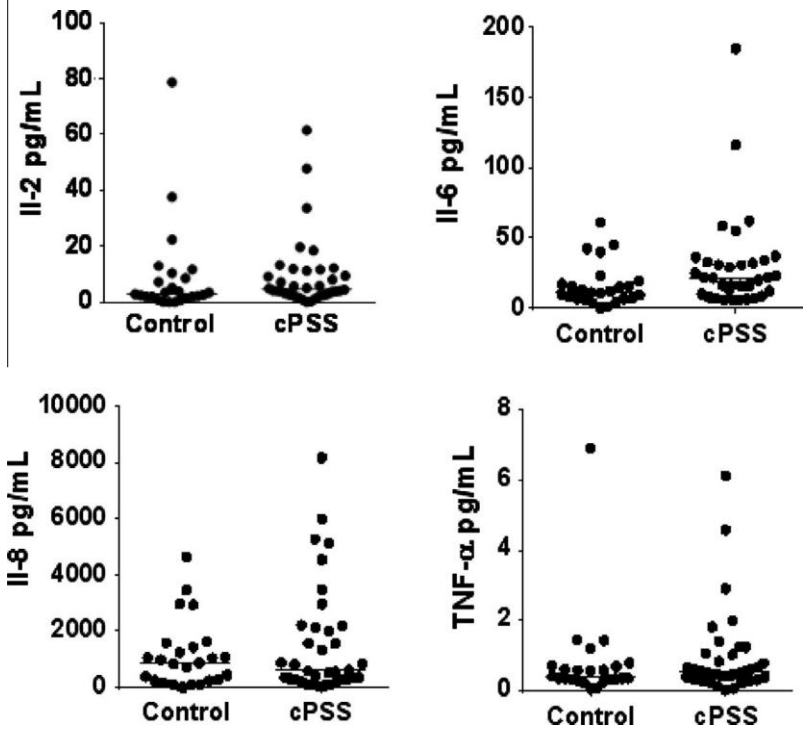

Fig. 1. Plasma IL-2, IL-6, IL-8 and TNF-α concentrations in healthy dogs and dogs with a congenital portosystemic shunt (cPSS), measured by electrochemiluminescence immunoassay. The median cytokine concentration is shown as a horizontal line for each group.

Table 2
Median concentrations and *P*-values of plasma IL-2, IL-6, IL-8 and TNF-α in healthy dogs and dogs with a congenital portosystemic shunt (cPSS).

Cytokine	Healthy (pg/mL)	cPSS (pg/mL)	*P*-value	Lower limit of detection (pg/mL)	Upper limit of detection (pg/mL)
IL-2	2.90	4.71	0.12	1.38	20,000
IL-6	10.92	21.11	0.02	3.01	10,000
IL-8	866.70	589.10	0.79	0.31	10,000
TNF-α	0.38	0.55	0.38	0.20	5000

(Luo et al., 2012a,b). The mechanism by which IL-6 could potentially modulate neurological status is unclear, although chronic exposure to IL-6 alters metabotrophic glutamate receptor-activated calcium signalling in cerebellar neurons, which could promote cerebral oedema (Nelson et al., 2004). Furthermore, elevated IL-6 concentrations have been reported to correlate with cognitive decline in humans (Weaver et al., 2002).

IL-6 might be involved in the mechanism by which ammonia contributes to the pathogenesis of HE. Experimental models have demonstrated that simultaneous treatment of astrocyte cultures with a mixture of IL-6 and ammonia had a marked additive effect on mitochondrial permeability transition, leading to astrocyte swelling (Alvarez et al., 2011). Future studies examining the cause of the increased plasma IL-6 concentration and longitudinally, monitoring IL-6 concentrations following treatment in a large cohort of dogs would be informative.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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RESEARCH ARTICLE

Presence of Systemic Inflammatory Response Syndrome Predicts a Poor Clinical Outcome in Dogs with a Primary Hepatitis

Scott Kilpatrick[1], Margaret Dreistadt[1], Polly Frowde[2], Roger Powell[3], Elspeth Milne[1], Sionagh Smith[1], Linda Morrison[1], Adam G. Gow[1], Ian Handel[1], Richard J. Mellanby[1]*

1 Royal (Dick) School of Veterinary Studies and The Roslin Institute, The University of Edinburgh, Hospital for Small Animals, Easter Bush Veterinary Centre, Roslin, Midlothian, United Kingdom, 2 Davies Veterinary Specialists Limited, Manor Farm Business Park, Higham Gobion, Herts, United Kingdom, 3 PTDS, Unit 2a, Manor Farm Business Park, Higham Gobion, Herts, United Kingdom

* Richard.Mellanby@ed.ac.uk

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Abstract

Primary hepatopathies are a common cause of morbidity and mortality in dogs. The underlying aetiology of most cases of canine hepatitis is unknown. Consequently, treatments are typically palliative and it is difficult to provide accurate prognostic information to owners. In human hepatology there is accumulating data which indicates that the presence of systemic inflammatory response syndrome (SIRS) is a common and debilitating event in patients with liver diseases. For example, the presence of SIRS has been linked to the development of complications such as hepatic encephalopathy (HE) and is associated with a poor clinical outcome in humans with liver diseases. In contrast, the relationship between SIRS and clinical outcome in dogs with a primary hepatitis is unknown. Seventy dogs with histologically confirmed primary hepatitis were enrolled into the study. Additional clinical and clinicopathological information including respiratory rate, heart rate, temperature, white blood cell count, sodium, potassium, sex, presence of ascites, HE score, alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin and red blood cell concentration were available in all cases. The median survival of dogs with a SIRS score of 0 or 1 (SIRS low) was 231 days compared to a median survival of 7 days for dogs with a SIRS score of 2, 3 or 4 (SIRS high) ($p<0.001$). A Cox proportional hazard model, which included all other co-variables, revealed that a SIRS high score was an independent predictor of a poor clinical outcome. The effect of modulating inflammation on treatment outcomes in dogs with a primary hepatitis is deserving of further study.

Introduction

Primary hepatopathies are a common cause of morbidity and mortality in dogs, with a recent study reporting that over 10 per cent of dogs had evidence of chronic hepatitis at post mortem examination [1–3]. Primary hepatitis includes all inflammatory disorders of the hepatic

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parenchyma [4]. In contrast to human medicine, where the type of hepatitis is defined by the inciting cause, few causes of chronic hepatitis have been identified in the dog, and the majority of cases are idiopathic [4]. Although primary hepatitis can be readily diagnosed in dogs through histological examination of a liver biopsy, it remains a challenging and difficult disease to treat [1, 5]. Furthermore, it is difficult to offer clients an accurate prognosis at the time of diagnosis [2].

There is growing evidence that systemic inflammatory response syndrome (SIRS) is a common and serious disorder among human patients with liver diseases [6–8]. It has been suggested that the presence of SIRS can lead to a further deterioration in liver function resulting in significant morbidity and mortality [9, 10]. The presence of SIRS can compromise the function of various organ systems resulting in Multiple Organ Dysfunction Syndrome (MODS) [11]. Hospitalised human patients with cirrhosis and SIRS have more severe hepatic encephalopathy (HE), are more likely to develop hepatorenal syndrome and have non-reversible renal dysfunction [1 2]. In acute liver failure, the presence of SIRS, whether or not precipitated by infection, has been implicated in the progression of HE, reducing the chances of transplantation and conferring a poorer prognosis [13]. Furthermore, higher SIRS scores are related to the development of acute liver failure in patients with pre-existing hepatitis [1 4].

There is a clear need to clarify the relationship between inflammation and outcome in dogs with a primary hepatitis. A more detailed understanding of the relationship between inflammation and clinical outcomes may lead to novel therapeutic approaches. The aim of this study was to determine the prevalence of systemic inflammation in a cohort of dogs with primary hepatitis, and to then examine the relationship between systemic inflammation and patient survival.

Materials and Methods

Consecutive cases of dogs diagnosed with primary hepatopathies at the Royal (Dick) School of Veterinary Studies or Davies Veterinary Specialists, were considered for inclusion in the study. Cases were only included if the dog had histopathological confirmation of primary parenchymal hepatic disease. Information on the signalment and previous medical treatment was extracted from the clinical records. The histopathological diagnosis was classified according to WSAVA (World Small Animal Veterinary Association) criteria by a board certified pathologist [4]. All blood samples were taken by a veterinary surgeon as part of routine clinical management of each patient. The study was approved by The University of Edinburgh Ethics Research Committee and the owners of the dogs gave permission for their animals' data to be used in this study.

The presence of HE was evaluated using previously described criteria [15]. The dog was considered to have HE if it had clinical signs of lethargy, inappropriate behavior, disorientation, circling, head pressing or seizures [16]. If the dog displayed none of these signs, it was considered not to have HE. The presence of ascites was documented if confirmed by ultrasound evaluation. Details of heart rate, respiratory rate, temperature, haematology profile and biochemistry profile were recorded. A SIRS score was calculated for each dog using methodology previously described [17]. The SIRS score could range from 0–4 as each dog was given 1 point when they met each of the following criteria: respiratory rate greater than 20 min heart rate greater than 120 min; total white blood cell (WBC) count less than 6 or greater than 16 x10^9 L and rectal temperature less than 38.1˚C or greater than 39.2˚C Therefore, a SIRS score could range from 0 to 4.

Survival of the cohort was initially examined using Kaplan Meier analysis. As some of the dogs were alive at the end of follow up, Cox proportional hazard analysis was used to estimate the association between survival and selected co-variates. Initially, a uni-variable analysis was

performed which explored the relationship between survival and SIRS score. To determine if this relationship persisted in the presence of other possible other confounding variables, a multivariable Cox proportional hazard model was constructed which included SIRS score of 0 or 1 (SIRS low) or SIRS score of 2, 3 or 4 (SIRS high), sodium, potassium, sex, ALT, ALP, bilirubin, red blood cell count, presence of ascites, age and HE score. A final model was built based on step wise removal of variables from the initial comprehensive model using AIC as a measure of parameter penalised model fit. To confirm the model structure, the impact on AIC of both dropping each of the final variables and adding back in the dropped variables was investigated. Results are presented as a hazard ratio with 95% CI. Statistical analysis was performed in R statistical software package (R Development Core Team (2012)).

Results

Eighty cases of histologically confirmed primary hepatopathies were identified. Ten dogs were excluded from the study due to incomplete clinical records. The 70 dogs included in this study comprised of 29 different pure breeds and eight cross-breeds. The sex distribution comprised of 12 entire males, 24 neutered males, 24 neutered females and 10 entire female dogs. The median age was 75 months (range 6 to 180 months). The diagnosis of hepatitis was histologically confirmed from samples collected at post-mortem examination in 9 cases, and from a percutaneous spring-loaded biopsy instrument in 12 cases. Forty nine samples were collected by surgical biopsy techniques. Fifty cases were diagnosed with chronic hepatitis (Fig 1), 10 cases with acute hepatitis, eight cases with cirrhosis (Fig 2) and two cases with copper associated hepatitis (Fig 3). Fifty-seven dogs were dead at follow up and 13 were alive. Seventeen dogs had a SIRS score of 0, 22 had a score of 1, 25 had a score of 2, five had a score of 3 and one dog had a score of4.

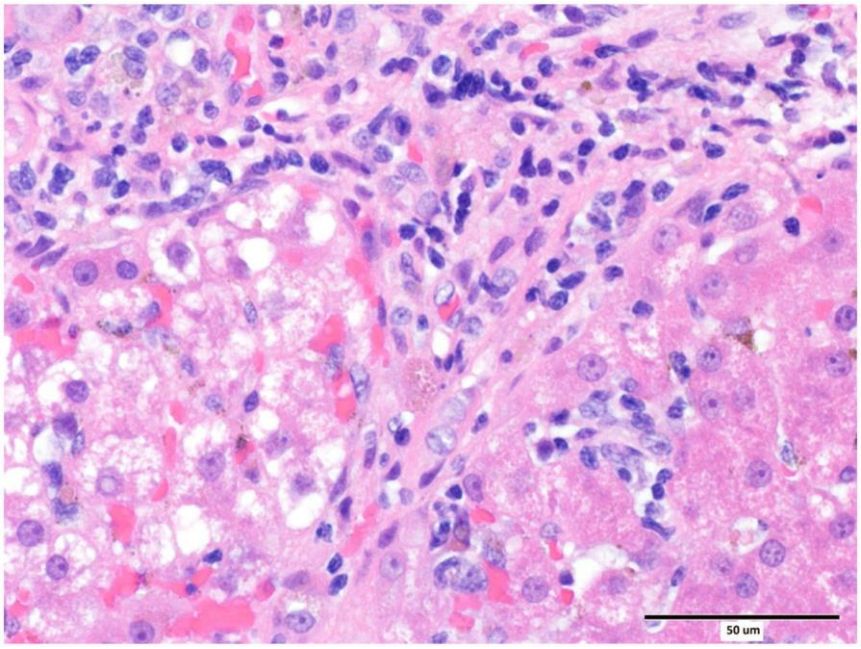

Fig 1. Liver from a case of chronic hepatitis with islands of hepatocytes separated by bands of fibroblasts and collagen, and moderate numbers of macrophages, lymphocytes and plasma cells. Haematoxylin and eosin, scale bar = 50 μm.

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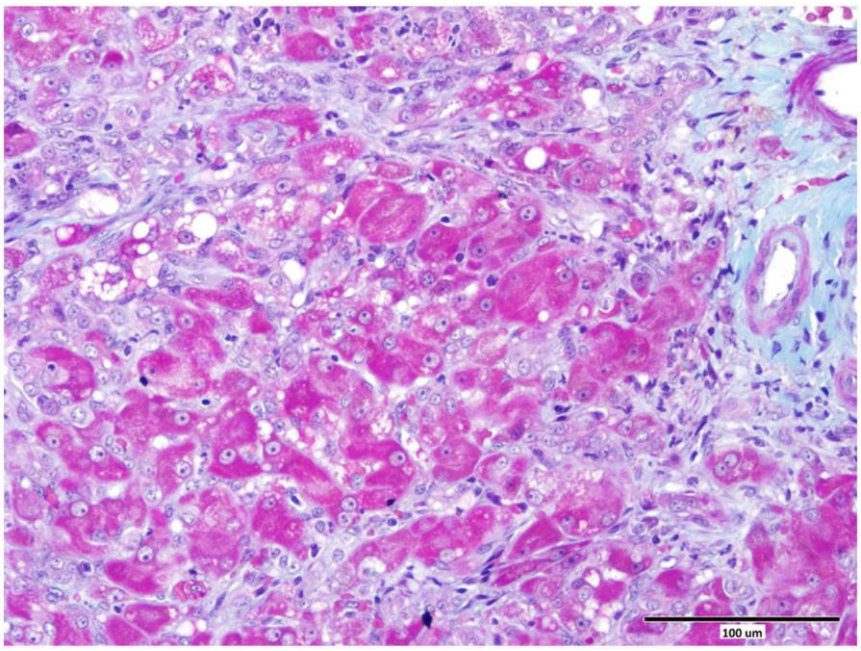

Fig 2. Liver from a case of chronic hepatitis and early cirrhosis showing disruption of the lobular architecture and dissecting fibrosis (green). Masson's trichrome, scale bar = 100 μm.

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The median survival time of the 70 dogs was 38.5 days (95% CI 20–215). To explore the relationship between a systemic inflammatory response and survival, the median survival of dogs with a SIRS low and dogs with a SIRS high was estimated. The median survival of SIRS low dogs was 231 days (95% CI 78-not available/ days) and median survival of SIRS high was 7

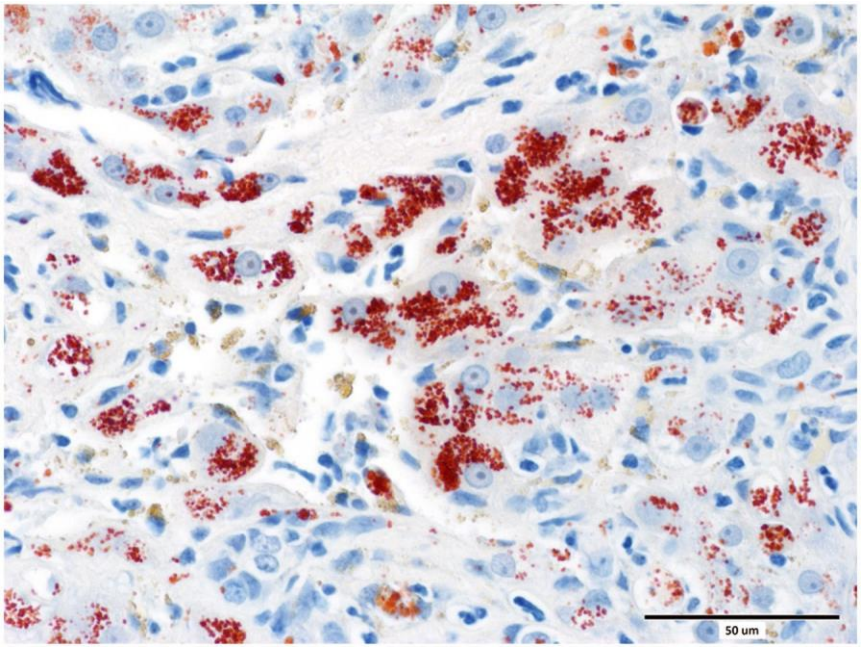

Fig 3. Liver from a case of chronic hepatitis with intracytoplasmic copper accumulation in hepatocytes. Rhodanine red, scale bar = 50 μm.

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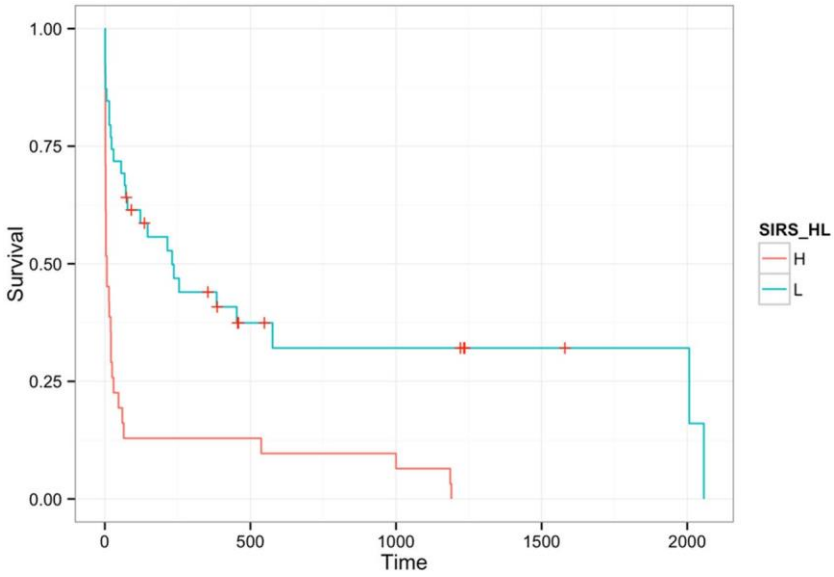

Fig 4. Kaplan-Meier plot of survival time in days by SIRS high (red solid line) and SIRS low (blue dotted line). Tick marks show censored events.

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days (95% CI 3-25/ days) ($p<0.001$) (Fig 4). To assess whether this relationship persisted in the presence of other co-variates, a Cox proportional hazard model was built. The initial model included SIRS high or low, presence of ascites, HE score, sex, sodium, potassium, ALT, ALP, bilirubin, age, sex and red blood cell count (S1 File). The final model after variable selection included SIRS, bilirubin, red blood cell concentrations and age (Table 1). The impact of variables dropped from the model was confirmed by calculating the impact on model fit (Δ AIC) when they were individually added back into the model (Table 2).

Discussion

In this large two centre study, we have examined for the first time, the relationship between SIRS, a simple marker of inflammation, and clinical outcome in dogs with primary hepatitis The central finding of this study was that the dogs with a primary hepatitis and a SIRS score of 2, 3 or 4 had a significantly worse clinical outcome than dogs with a SIRS score of 0 or 1. This provides evidence that the presence of systemic inflammation is associated with a poorer clinical outcome in dogs, which is similar to human patients with liver disease. The presence of SIRS was a major predictor of multiple organ failure and strongly correlated with short term mortality in patients with alcoholic hepatitis [7]. In addition, SIRS has recently been associated with acute on chronic liver failure (ACLF) [18].

Table 1. Hazard Ratios and 95% confidence interval for terms in final Cox proportional hazard model. Δ AIC is the increase in AIC if the term is dropped from the model. A positive Δ AIC equates to a poorer model fit.

	Hazard Ratio (95% CI)	Δ AIC
SIRS	3.575 (1.857–6.884)	13.3
Bilirubin	1.003 (1.001–1.005)	5.98
Red blood cell	0.768 (0.596–0.990)	2.14
Age	0.909 (0.827–1.000)	1.94

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Table 2. △ AIC for terms not included in final model when added back in final model. A positive Δ AIC equates to a poorer model fit.

	△ AIC
Sodium	0.03
Potassium	1.77
Sex	2.75
Ascites	1.94
HE score	4.77
ALP	1.98
ALT	0.68

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Inflammation may be associated with a poor outcome in dogs and humans with liver disease for several reasons. Intestinal bacterial overgrowth and altered gut permeability in patients with liver disease is hypothesised to lead to increased translocation of bacteria and endotoxin into the portal circulation [9]. Factors leading to SIRS development in liver disease include the impaired phagocytic function of the reticuloendothelial system which allows endotoxin to reach the systemic circulation in high concentrations [13]. Lipopolysaccharide (LPS) concentrations in the peripheral and portal circulation have been shown to be increased in people with liver cirrhosis. Importantly, LPS concentrations have been shown to predict mortality in patients with alcoholic hepatitis[7]. Endotoxin can activate monocytes and release pro-inflammatory cytokines which have been implicated in the development of hepatic complications such as HE, ascites and variceal bleeding [19]. The disturbances in systemic and hepatic haemodynamics in alcohol-related liver failure (ACLF) have also been associated with dysregulated inflammation, multi-organ failure and marked activation of the sympathetic nervous system [7]. These abnormalities predict high mortality rates in these patients [8].

In our study, the proportion of dogs which had an infection was not accurately defined. In a recent study of human patients with alcoholic hepatitis and SIRS, over 60% of patients had no evidence of infection, based on clinical or microbiological criteria [8]. Understanding the pathophysiology of sterile inflammation in patients with liver disease, with the ambition of developing targeted therapies, is one of the major challenges in contemporary hepatology.

Predicting the course and prognosis of disease is an important part of veterinary and human clinical practice and the development of new prognostic markers with documented significance is an important focus of human and veterinary research. Various factors have been demonstrated as negative prognostic indictors in dogs with primary hepatitis [20]. These include jaundice, abdominal fluid wave, microhepatica, ascites, enlarged portal lymph nodes, hypoalbuminemia and left shift of neutrophils in the peripheral blood [1]. Consistent with our study, total serum bilirubin was a negative prognostic indicator in idiopathic canine chronic hepatitis [2]. High bilirubin concentrations have also been documented to be associated with a negative outcome in human patients [21, 22]. Furthermore, our prognostic link with low red blood cell count is consistent with the prediction of poor post-surgical survival associated with reduced red blood cell counts in human patients with primary liver cancer [23] and end stage liver disease [24].

Another finding of this study was that abnormal serum sodium concentrations were not commonly observed in dogs with primary hepatitis. This observation contrasts with the findings from studies of humans with liver disease where hyponatremia is frequently observed [25–27]. For example, in a prospective study of 997 patients with cirrhosis the prevalence of low serum sodium concentration, defined as a serum sodium $:::;135$ mmol/l, $:::;130$ mmol/l, $:::;125$ mmol/l or $:::;120$ mmol/l, was 49.4%, 21.6%, 5.7%, and 1.2%, respectively [28]. Borroni et al.

reported hyponatraemia in 29.8% of patients admitted for management of cirrhosis, as defined by a serum sodium concentration of ::;130 mmol/l [29]. Furthermore, the prevalence of hyponatremia was 26% in a population of paediatric patients with end stage liver disease awaiting liver transplantation (as defined by a serum sodium concentration of ::;130 mmol/l) [30]. It remains unclear why humans frequently develop hyponatraemia with liver disease while dogs do not. We hypothesise that the differences may reflect the different causes of liver disease in dogs compared to humans.

Several studies in human patients with liver disease have examined the role of circulating biomarkers as adjunctive markers of systemic inflammation, such as LPS, procalcitonin and C reactive protein [31– 33]. Cytokines, including interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor (TNF), are key mediators of the inflammatory response [1 1]. Several studies have measured circulating cytokine concentrations in patients with liver disease. One marker that has been investigated in some detail is IL-6 [34–36]. Increased serum concentrations of IL-6 have been found in people with different inflammatory diseases and has been correlated with the severity of disease, prognosis and outcome [37]. Higher circulation levels of IL-6 were significantly associated with a higher risk of developing hepatocellular carcinoma [38]. IL-6 has also been shown to be a useful prognostic marker for canine critical care patients [39].

Our study demonstrates the need to understand more about the development of systemic inflammation in dogs with liver disease and to establish whether reducing systemic inflammation improves outcomes.

Conclusion

In conclusion, a high SIRS score was associated with poorer long term survival in our study of dogs with primary hepatitis Our study demonstrates the need to understand more about the causes and consequences of inflammation in dogs with liver disorders. Our study demonstrates the need to understand more about the development of systemic inflammation in dogs with liver disease and to establish whether reducing systemic inflammation improves patient outcomes. The impact of ameliorating inflammation in patients with hepatitis is ill defined. Although prednisolone is widely used as a first line treatment for hepatitis in both dogs and humans, its therapeutic benefits remain ill-defined in both species [40–43].

Supporting Information

S1 File. Clinical and biochemical data from the 70 dogs with histologically confirmed hepatitis reported in this study.
(XLSX)

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Author Contributions

Conceived and designed the experiments: RJM SK. Performed the experiments: SK RJM. Analyzed the data: SK RJM IH. Contributed reagents/materials/analysis tools: SK MD PF RP EM SS AGG IH RJM LM. Wrote the paper: SK MD PF RP EM SS AGG IH LM RJM.

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