NOVEL BIOMARKER DISCOVERY IN DOGS WITH CHRONIC HEPATITIS

A Dissertation

by

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ABSTRACT

Chronic hepatitis can present a diagnostic challenge, with different types of chronic hepatic disease being associated with similar clinical and laboratory findings. Clinical signs of chronic hepatitis are often non-specific; therefore, this disease is frequently diagnosed in an advanced stage that makes successful intervention less likely. Differentiating dogs with chronic hepatitis from those with acquired hepatopathies is often difficult requiring invasive diagnostic procedures such as laparoscopic liver biopsy. There is also a need for biological markers that are able to stage chronic hepatitis non-invasively. Currently, the only way to establish a definitive diagnosis of chronic hepatitis in dogs is through the histopathological assessment of a liver biopsy specimen. The acquisition of a liver biopsy specimen, although considered the gold standard, is invasive and has limitations and risks. The identification of biomarkers that are differentially expressed in dogs with chronic hepatitis could contribute to the development of novel diagnostic tests for this disease and provide insight into its pathogenesis. The objective of these studies was to identify candidate biomarkers that are differentially expressed in the liver, serum, or urine of dogs with chronic hepatitis. The hepatic proteome, serum and urine metabolome, and hepatic microRNA transcriptome were analyzed by 2-dimensional fluorescence difference gel electrophoresis coupled to nanoflow liquid chromatography tandem mass spectrometry, gas chromatography – quadrupole time of flight mass spectrometry, and high-throughput small RNA Illumina sequencing, respectively. Differential hepatic protein expression of cytokeratin 18 and annexin 5 was demonstrated in dogs with chronic hepatitis. Untargeted metabolite profiling documented a decreased

ratio of branched chain amino acids to aromatic amino acids in the serum of dogs with a congenital portosystemic shunt. The assessment of serum trans-4-hydroxyl-proline confirmed a decreased concentration in dogs with chronic hepatitis. Untargeted metabolomic profiling of urine in dogs with chronic liver disease documented alterations in metabolites involved in glutathione, arginine, proline, and nitrogen metabolism in addition to metabolites involved in fatty acid biosynthesis. Differential microRNA expression of transcripts implicated in hepatic fibrosis and apoptosis was demonstrated in dogs with chronic hepatitis. Further targeted assessment of the novel biomarkers identified in these studies is needed to determine their utility.

DEDICATION

A MOTHER'S SŪTRA

My mother always said I am no one. I am no body. She did not recognize the profound truth to which those words point. While I know I am not my body. Death will show me. While I recognize the insignificance of all the things one clings to. Death takes them all away. While I have been told that all men and women of all races are equal. Death makes us all the same. There is no person or experience that can teach you what death has to teach you. How paradoxical that death holds within it one of life's greatest lessons. The sutra is to die before death. Become no thing, no one, no body. So, you can be.

Mother, Laurel C. Lawrence, in memoriam. Through your selflessness, your amazing grace is known. Through your nothingness, your divinity remembered.

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All other work conducted for the dissertation was completed by the student independently.

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NOMENCLATURE

%CV	Coefficient of variation
DQC	Dilution quality control
LC-MS-MS	Liquid chromatography–tandem mass spectrometry
LLOQ	Lower limit of quantification
PVDF	Polyvinylidene difluoride
TTBS	Tween - Tris buffered saline
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
GGT	Gamma-glutamyl transferase
DIGE	Difference gel electrophoresis
MWM	Molecular weight marker
kDA	Kilodalton
NL	Nonlinear
CK18	Cytokeratin 18
ANX5	Annexin 5
AAA	Aromatic amino acids
BCAA	Branched chain amino acids
HMGB1	High mobility group box-1
GSTα	Alpha glutathione-S-transferase
TGF-β1	Transforming growth factor beta-1
TIMP	Tissue inhibitor of metalloproteinase

MMP	Matrix metalloproteinase
2-DE	2-dimensional gel electrophoresis
miRNA	MicroRNA
cDNA	Complementary DNA
PCR	Polymerase chain reaction

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. **Canine chronic hepatitis**

Chronic hepatitis is the most common type of liver disease in dogs with idiopathic chronic hepatitis being the most frequently observed form [1]. Chronic hepatitis is associated with hepatocellular damage resulting in an aberrant repair process that can lead to end-stage liver disease characterized by fibrosis of the liver and the replacement of normal liver architecture into abnormal regenerative liver nodules with portal – central vascular anastomoses [2]. End-stage hepatic disease, determined histologically, carries a poor long term prognosis with a reported 0.4 month survival time [3,4]. In contrast, chronic hepatitis diagnosed prior to the development of ascites and cirrhosis carries a reported survival from diagnosis to death of 18.3 – 36.4 months [3,4]. Cases of chronic hepatitis are often diagnosed in an advanced stage where treatment is less likely to be effective and thus early identification of dogs with chronic hepatitis before clinical signs develop is crucial. The lack of robust biomarkers for canine chronic hepatitis limits the non-invasive evaluation of this disease. The histological assessment of a liver biopsy specimen continues to be the gold standard for the assessment of hepatocellular necrosis, inflammation, and fibrosis, as well as providing a histomorphological diagnosis. Liver biopsies however, are not without risk or limitations and are often indicated for patients with a compromised health status. Risks inherent to liver biopsy include hypotension, hemorrhage, and collateral damage to adjacent organs [41]. Patients with suspected chronic hepatitis can also have ascites, coagulopathies, or thrombocytopenia and at increased risk of complications secondary to liver biopsy. Apart from this, chronic hepatitis can be heterogeneously distributed

rendering evaluation of a relatively small liver biopsy specimen susceptible to sampling bias [42]. Furthermore, repeated biopsies to evaluate disease progression or treatment response are impractical due to the increased risk of complications and financial burden. Novel non-invasive biomarkers are therefore, needed to stage, monitor, and diagnose chronic hepatitis.

A number of non-invasive biomarkers of hepatocellular injury have been identified and validated. The most widely used biomarker of hepatocellular injury is serum alanine aminotransferase (ALT) activity with an increased serum ALT activity being considered the gold standard for detection of hepatocellular injury [5,6]. Alanine aminotransferase is predominantly found in the liver with lower enzyme activities found in skeletal and cardiac muscle. Serum enzyme activity increases are associated with either reversible or irreversible hepatocellular damage [7]. Serum alanine aminotransferase activity has a reported sensitivity for detecting liver disease in symptomatic dogs that varies from 60% – 76% [8]. There is little known about the sensitivity of biomarkers including that of alanine aminotransferase, to detect hepatocellular injury in patients with subclinical disease. A study by Dirksen et al. reported sensitivities of alanine aminotransferase, alkaline phosphatase, and bile acids for identifying subclinical chronic hepatitis of 71%, 35%, and 13% respectively [9]. Alanine aminotransferase, although considered sensitive for the detection of hepatocellular injury does not indicate when the injury occurred in terms of determining drug or toxin exposure. Also, alanine aminotransferase activity is inducible with drug therapy and thus even marked serum elevations may result from increased hepatocellular content independent of hepatocellular injury. Alanine

aminotransferase activity does not always correlate with clinical data, which makes interpreting disease severity difficult.

There are several candidate biomarkers of hepatocellular injury in the human literature that have demonstrated increased sensitivity and specificity when compared to alanine aminotransferase. MicroRNAs are a class of small noncoding RNA that regulates post-transcriptional gene expression. Various studies have demonstrated the ability of microRNAs of hepatic origin to function as stable and sensitive non-invasive biomarkers of hepatocellular injury in animal models and in human patients with normal or increased serum ALT concentrations. Several studies have indicated that hepatocyte-derived microRNAs may have a higher sensitivity than serum ALT for hepatocellular injury [10-12]. A recent study in Labrador Retrievers found microRNA-122 to be highly sensitive and specific for the detection of hepatocellular injury with the ability to identify more patients with hepatocellular injury than ALT activity [13]. MicroRNA-122 was also able to identify patients with high hepatic copper contents but normal ALT activity [13]. MicroRNA-122 is the only biomarker of hepatocellular injury that has been found to be more sensitive than alanine aminotransferase in the veterinary literature.

Cytokeratin 18 (CK18) is an intermediate filament protein expressed in hepatocytes [14]. The cleavage of CK18, mediated by caspase, occurs during the structural cellular rearrangement of apoptosis [14]. Passive release of full-length cytokeratin 18 occurs in cellular necrosis where the release of cytokeratin 18 fragments occurs in apoptosis [15]. Immunoassays have been used to measure either caspase-cleaved cytokeratin 18

(suggestive of apoptosis) and or full-length cytokeratin 18 (suggestive of necrosis). These markers have been evaluated for the monitoring of chemotherapeutic drugs and the assessment of apoptosis in patients with chronic hepatic diseases in humans [16]. Elevations in the serum concentrations of cytokeratin 18 in individuals with acute hepatic disease was associated with a poorer prognosis and CK18 was increased in some patients with serum ALT activity within the reference interval [16].

Glutamate dehydrogenase is an enzyme found in the mitochondria of hepatocytes with small amounts in the kidney and the central nervous system; however, serum activity originates almost exclusively from the liver [5]. Glutamate dehydrogenase is conserved across many species, which makes it a good candidate biomarker. An experiment in rats exposed to methapyrilene, dexamethasone, cyproterone, isoniazid, and lead nitrate showed that glutamate dehydrogenase elevations were of greater magnitude and lasted longer than those of alanine aminotransferase [17]. This study concluded that glutamate dehydrogenase was more specific and less inducible than alanine aminotransferase. An inducible enzyme, active during the second phase of hepatic detoxification, known as glutathione-s-transferase catalyzes the conjugation of glutathione with metabolites that were formed during phase one of detoxification [16]. There are four isozymes of glutathione-s-transferase (i.e.alpha, pi, mu, and theta) expressed in humans and mammalian species [16]. The alpha glutathione-s-transferase (GST) class is of particular interest because it is expressed in hepatocytes. Rats administered hepatotoxins to cause hepatocellular damage demonstrated serum GSTα concentrations that correlated with necro-inflammatory histopathology scores; however, the GSTα concentrations were not

informative regarding prognosis or disease severity [18]. A case report of patients with self-induced acetaminophen toxicity all developed increased serum glutathione-S-transferase alpha activities; however, alanine aminotransferase activities were within the reference interval in affected individuals up to 6 hours after intoxication [5].

A protein released passively by necrotic cells called high mobility group box-1 (HMGB1) is able to activate the immune system by binding Toll-like receptors [16]. This protein has been used to indicate cellular death in studies of drug toxicity when measured in the serum. Serum levels of HMGB1 and the modified form acetyl-HMGB1 have been correlated with histopathologic assessment of hepatocellular inflammation and necrosis [16]. Furthermore, circulating HMGB1 concentrations are associated with circulating alanine aminotransferase concentrations in drug-induced acute hepatocellular damage and elevations of acetyl-HMGB1 were associated with a worse prognosis [16].

Argininosuccinate synthetase is an important enzyme for the synthesis of arginine and is a critical enzyme in several metabolic processes that require arginine. Argininosuccinate synthetase has been shown to increase earlier and decrease faster than alanine aminotransferase in rodent models of liver injury caused by ischemia-reperfusion and by galactosamine-induced sensitization to endotoxin and these results were similar in human acetaminophen overdose patients [19]. Argininosuccinate synthetase levels were increased in serum after treatment with endotoxin alone while aminotransferase activities were normal suggesting that argininosuccinate synthetase levels are more sensitive for

hepatocellular damage [19]. Argininosuccinate synthetase also appears to demonstrate a greater diagnostic range than alanine aminotransferase with greater maximum elevations.

There are few biomarkers of hepatic fibrosis that have been assessed in the veterinary literature but several biomarkers of fibrosis that have been assessed in the human with hepatic disease. The identification of the stellate cell in the liver as the major source of extracellular matrix deposition has led to the identification of several molecules that result in the activation of hepatic stellate cells and fibrogenesis. Serum and urinary biomarkers of hepatic fibrosis can be divided into two groups. There are biomarkers of hepatic fibrosis that indicate altered liver function but are not correlated with the deposition of extracellular matrix (e.g. serum transaminase activities) called indirect biomarkers [20]. These indirect markers of hepatic fibrosis are utilized most often for the diagnosis of end stage liver disease or cirrhosis [20]. Direct markers of hepatic fibrosis reflect extracellular matrix (ECM) turnover and play roles in the non-invasive staging of liver disease [20]. Hyaluronic acid is a nonsulfated glycosaminoglycan distributed in connective tissue and is a chief component of the ECM. Hyaluronic acid has been evaluated as a serologic biomarker for hepatic fibrosis and has a reported sensitivity of 70% to 80% for differentiating mild fibrosis from moderate to severe fibrosis and a specificity of 75% to 100% [21]. Preliminary research indicates the measurement of circulating hyaluronic acid may have a role in the assessment of hepatic fibrosis in dogs. A study of chronic hepatitis in dogs found that those with end-stage hepatic fibrosis had higher circulating hyaluronic acid concentrations healthy control dogs, dogs with extra-hepatic disease, and dogs with non-fibrotic hepatic disease [21].

A cytokine, hypothesized to have a role in fibrosis, is transforming growth factor beta-1 (TGF-β1). Some of the functions of TGF-β1 include inhibition of cell division, inhibition of neoplastic cells, and suppression of the immune system; however, TGF-β1 causes apoptosis and fibrosis in the liver [22]. TGF-β1 has been investigated as a biomarker of liver fibrosis in dogs by measuring circulating TGF-β1 in healthy control dogs and in dogs with liver disease. Circulating TGF-β1 concentrations in the dogs with marked hepatic fibrosis was significantly different ($P < 0.001$) from concentrations in healthy control dogs [23]. This suggests a possible role for this cytokine in the assessment of hepatic fibrosis in dogs.

The FibroVet test, released by Echosens is an index that evaluates various biological markers that are combined to form an index of hepatic fibrosis. A research abstract reported a study of this index to have the ability to differentiate dogs with hepatic fibrosis from those without fibrosis with an accuracy of 90% to 100%.

There are several indirect and direct biomarkers for the assessment of hepatic fibrosis in the human literature. The indirect biomarkers that have been evaluated include serum activities of alanine aminotransferase, the ratio of serum aspartate and alanine aminotransferase activities, platelet count, and prothrombin index [20]. Additional fibrosis indices include the combination of prothrombin time, serum gamma glutamyl transferase activity, and blood apolipoprotein A1 concentration to form the PGA index [20]. The PGA index with the inclusion of serum α2-macroglobulin concentration results in improved performance and is known as the PGAA index [20]. Direct markers of hepatic

fibrosis assessed in humans are characterized by their molecular structure and include collagens, glycoproteins, polysaccharides, collagenases, and cytokines [20]. Examples of collagens assessed in humans include pro-collagen one and three, which are pro-peptides released into the serum during ECM formation and type four collagen released during ECM degradation. Glycoproteins and polysaccharides that have been assessed include hyaluronic acid, tenascin, and laminin that have been identified in regions of matrix deposition. Collagenases and their inhibitors include the metalloproteinases and the tissue inhibitors of metalloproteinase. Cytokines that have been examined include TGF-β, which is involved in liver fibrosis. A study by Oberti et al. evaluated candidate biomarkers of hepatic fibrosis (i.e., PIIINP, hyaluronic acid, laminin, and TGF-β) in 243 human patients with chronic viral hepatitis and found that hyaluronic acid, PIIINP, laminin, and TGF-β achieved diagnostic accuracies of 86%, 74%, 81%, and 67%, respectively [24]. Circulating concentrations of tissue inhibitor of metalloproteinase (TIMP)-1 and matrix metalloproteinase (MMP)-2 have also been evaluated as biomarkers of hepatic fibrosis in chronic liver disease. A study by Boeker et al. found the ability of circulating MMP-2 to detect hepatic fibrosis was unacceptable with a sensitivity and diagnostic accuracy of 7% and 58%, respectively [25]. Circulating MMP-2 was able to predict end-stage liver disease with a sensitivity and specificity of 83% and 100%, respectively [25]. Similarly, circulating TIMP-1 concentrations were able to identify hepatic fibrosis with a sensitivity and specificity of 67% and 88%, respectively [25]. Furthermore, circulating TIMP-1 concentrations were able to identify patients with end-stage liver disease with a sensitivity and specificity of 100% and 56%, respectively and the diagnostic accuracy of circulating

TIMP-1 for hepatic fibrosis was not significantly different than hyaluronic acid but superior to serum transaminase activity and serum albumin [25].

In summary, there is a lack of clinically applicable non- invasive biomarkers for the detection of hepatocellular damage and hepatic fibrosis that drives biomarker discovery research in veterinary medicine in general and in canine chronic hepatitis more specifically.

1.2. **Biomarker discovery**

A biological marker (biomarker) is defined by the Biomarkers Definitions Working Group as a parameter that can be objectively measured as an indicator of a normal biological process, pathogenic process, or a pharmacologic response to a therapeutic intervention [26]. Biomarkers have important roles in disease detection and in the monitoring of overall health status. The discovery of novel biomarkers using classes of -omics data has become an important objective in academic and commercial research. This is driven in part by the potential of biomarkers to serve as a surrogate for specific disease states while permitting a more comprehensive understanding of biology in health and disease. Biomarker research is composed of three phases: discovery (i.e., the quantitative identification of biomarker candidates), verification (i.e., the screening of candidates to identify the most promising candidates), and validation (i.e., the analytical and clinical evaluation of candidate biomarkers in a large diverse patient cohort). The discovery of biomarkers is based on the principle that the comparison of physiological states, phenotypes or changes across control and disease patient groups can identify differences in the abundance of molecules that can serve as candidate biomarkers [27]. A spectrum of pathological injury occurs in canine

chronic hepatitis including inflammation, oxidative stress, apoptosis, and fibrosis. Potential biomarkers for each of these mechanisms are therefore of interest.

1.3. **Proteomic analysis for biomarker discovery**

The recent advances in genomics research and mass spectroscopic technology has contributed to the development of the field of proteomics. The proteome encompasses the entire protein complement expressed by a genome and proteomics is the study of this protein complement. Proteomics is further defined as the global identification, characterization, and quantification of the proteins present in a biological sample or organism in specified conditions and is an effective technique for biomarker discovery [28]. There are several available proteomic platforms; however, the most commonly used platform is 2-dimensional gel electrophoresis (2-DE). The proteome is extracted from a biological sample and then separated according to their isoelectric points and their molecular weights to generate protein profiles in 2-DE. Typically, mass spectrometry is then utilized to produce mass spectra that is then compared to a library for identification of proteins in differentially expressed protein spots. During the development of disease proteins are altered or modified and these altered proteins can be detected in tissue, blood, urine, or other body fluids. Chronic inflammatory hepatobiliary disease has been demonstrated to result in changes to proteins found in hepatic tissues in humans [29]. There is a single proteomic study related to canine liver disease that has been published to date [30]. This study examined soluble proteome of the liver from 3 Bull Terriers (2 diseased, 1 control) with inherited lethal acrodermatitis and identified differentially expressed proteins [30]. The proteomic analysis of hepatic tissue from dogs with chronic hepatitis may identify novel protein biomarkers for liver disease. To the authors

knowledge, prior to the one presented in this dissertation, there are no published studies on the hepatic proteome of dogs with chronic hepatitis.

1.4. **Metabolomic analysis for biomarker discovery**

Metabolomics, the systematic study of small molecular weight molecules in cells, tissues or whole organisms, has been used in biomarker discovery for a range of diseases [31]. The analytical platforms used, includes nuclear magnetic resonance and mass spectrometry, and can characterize thousands of metabolites simultaneously. The power of metabolomics results from its ability to detect small alterations in metabolites that may precede a gross alteration in phenotype. Metabolomic studies can be classified as targeted or untargeted based upon when metabolites are identified. An untargeted metabolomic study is used for global analysis of all detectable analytes in a sample including the annotation of known and unknown analytes through database searches [32]. This approach is suited to biomarker discovery. A targeted metabolomic study employs tandem mass spectrometry or gas chromatography–mass spectrometry selected ion recording to determine precise and accurate relative abundances and concentrations of a known metabolite [32]. This approach is suited to validation and verification of a known metabolite. The application of metabolomics in veterinary medicine to detect global changes of populations of endogenous low molecular weight metabolites in biological samples in dogs with chronic hepatitis may allow the identification of surrogate disease profiles. A couple metabolomic studies have already been performed in veterinary medicine to characterize the changes in the metabolome that occur in hepatobiliary disease. Gookin et al. recently performed an untargeted metabolomic analysis of the serum and the hepatic duct bile of dogs with a gallbladder mucocele and identified a number of

compounds that were able to differentiate between healthy dogs and dogs with a gallbladder mucocele [33]. Another veterinary study employed a metabolomic strategy to evaluate the plasma metabolome of dogs with a congenital portosystemic shunt and dogs with acquired hepatic disease and detected alterations in the plasma bile acid and phospholipid profiles of dogs with a congenital portosystemic shunt [34]. The comprehensive analysis utilized in the metabolomic research platform can offer a more sensitive means of detecting small molecular alterations related to canine hepatobiliary disease.

1.5. **MicroRNA transcriptomics for biomarker discovery**

MicroRNAs(miRNAs) are a class of small noncoding RNAs that are important regulators of posttranscriptional gene expression [35]. The miRNA transcriptome is the complete repertoire of miRNA in a cell or organism. The miRNA transcriptome reflects the miRNA transcripts that are being expressed at a given moment. Comparison of transcriptomes between experimental groups allows the identification of genes that are differentially expressed in distinct sample populations, disease states, or in response to different treatments. Transcriptomics is a high throughput technology enabling the relative quantification of expression of thousands of gene transcripts and with sequencing platforms, it is possible to assess the entire complement of transcripts within a cell or tissue. There are several platforms available to characterize and quantify the miRNA transcriptome, that include hybridization-based or sequence-based approaches. The hybridization-based approach usually involves the incubation of fluorescently labelled complementary DNA (cDNA) with customized microarrays. A sequence-based approach determines the cDNA sequence of the miRNAs. DNA high-throughput deep sequencing

technology provides the platform for mapping and quantification of the miRNA transcriptome. Briefly, a sample of miRNA is reverse transcribed into a cDNA fragment library. Each cDNA fragment is then sequenced to obtain short reads. Following sequencing, the short reads are then aligned to a reference genome to construct a transcription map consisting of the transcript identity and level of expression for each transcript.

MiRNAs are emerging as promising candidate biomarkers for a number of biliary and hepatic diseases in humans [36,37]. In human patients, several hepatocyte-derived miRNAs, such as miR-122, miR-21, and miR-22, have been demonstrated to be sensitive biomarkers for acute or chronic liver damage caused by various forms of liver injury [12,38]. Other studies have also demonstrated different serum concentrations of miRNAs: miR-21, miR-31, miR-122, miR-145, miR-146a in human patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma [39,40]. There are only a few targeted transcriptomic studies in dogs with chronic liver disease. Dirksen et al. have analyzed the serum of dogs with various hepatobiliary diseases including chronic hepatitis for the presence of specific miRNA biomarkers identified in previous human studies [5,6,9,25]. These studies found circulating miR-122 and miR-148a values were elevated in the serum of Labradors with liver injury, and miR-122 values increased more markedly than those in the control group. Other findings included the ability to differentiate between parenchymal, biliary, and neoplastic hepatobiliary diseases with a miRNA panel consisting of miR-21, miR-122, miR-126, miR-200c, and miR-222. Transcriptomics represents a valuable approach to molecular pathway discovery and biomarker

development, and this has been demonstrated in comparative human studies. There are no published studies of untargeted transcriptomic studies of liver tissue in dogs with chronic hepatitis to the authors' knowledge.

The overall hypothesis of the studies presented here is that there is a difference of the hepatic proteome, serum and urine metabolome, and hepatic microRNA transcriptome between healthy dogs and dogs with chronic hepatitis. Experiments will allow identification of potential biomarkers that hold power for the differentiation of dogs with chronic hepatitis from healthy control dogs.

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2. PROTEOMIC ANALYSIS OF LIVER TISSUE FROM DOGS WITH CHRONIC HEPATITIS*

Chronic hepatitis is the most common hepatic disease in dogs. Copper accumulation is an important cause of chronic hepatitis in dogs however; the etiology in most dogs cannot be determined. Clinical signs of chronic hepatitis are often non-specific; therefore, this disease is frequently diagnosed in an advanced stage that makes successful intervention less likely. Early diagnosis of chronic hepatitis in dogs would thus be beneficial. The identification of proteins that are differentially expressed in dogs with chronic hepatitis could contribute to the development of novel diagnostic markers for this disease and provide insight into its pathogenesis. The objective of this study was to identify novel proteins that are differentially expressed in the liver of dogs with chronic hepatitis. Hepatic tissue was collected from 8 healthy dogs during ovariohysterectomy and from 8 dogs with histologically confirmed chronic hepatitis. The proteome of the liver samples was extracted by mechanical disruption and detergent-based cell lysis and differentially labeled prior to analysis by 2-dimensional fluorescence difference gel electrophoresis. Spots with an absolute fold change value > 2.0 were selected for further analysis. Protein identification was achieved by nanoflow liquid chromatography tandem mass spectrometry. Differential expression of select proteins was validated by Western blot.

Five protein spots were differentially expressed between patients with chronic hepatitis and healthy control dogs. From these 5 protein spots 11 proteins were identified. Differential expression of cytokeratin 18 and annexin 5 were confirmed by Western blot analysis. Differential protein expression was shown between dogs with chronic hepatitis and healthy control dogs. Upregulation of cytokeratin 18 in chronic hepatitis may suggest increased hepatocellular apoptosis and necrosis, whereas upregulation of annexin 5A suggests increased hepatocellular apoptosis. Further studies are needed to determine whether either protein has diagnostic utility.

2.1. **Introduction**

Chronic hepatitis is the most common type of liver disease in dogs with chronic hepatitis being the most frequently observed form [1]. Chronic hepatitis is associated with apoptosis/necrosis and an inflammatory infiltrate that is often mononuclear. This results in a repair process that can lead to hepatic fibrosis including end-stage liver disease characterized by fibrosis of the liver and the conversion of normal liver architecture into abnormal liver nodules with portal-central vascular anastomoses [2]. End-stage hepatic disease carries a poor long-term prognosis with a reported median survival time of 0.4 months [3,4]. Chronic hepatitis, in contrast diagnosed at an earlier stage carries a reported median survival from diagnosis to death of 18.3 – 36.4 months [3,4]. Cases of chronic hepatitis are often diagnosed at an advanced stage where treatment is less likely to be effective and thus early identification of dogs with chronic hepatitis and hepatic fibrosis, ideally before clinical signs develop, would be beneficial.

The histological evaluation of a liver biopsy specimen continues to be the gold standard for the assessment of hepatocellular necrosis, inflammation, fibrosis, and for a definitive diagnosis of chronic hepatitis. The current lack of robust biomarkers for canine chronic hepatitis restricts the non-invasive evaluation of this disease. A number of non-invasive biomarkers of hepatocellular injury have been identified and validated in dogs and humans. For example, the most widely used biomarker for hepatocellular injury is serum alanine aminotransferase activity [5]. Alanine aminotransferase is predominantly found in the liver with lower level enzyme activities found in skeletal and cardiac muscle. Serum enzyme activity increases are associated with either reversible or irreversible hepatocellular damage [6]. Serum alanine aminotransferase activity has a reported sensitivity for detecting histopathologic evidence of hepatocellular injury in symptomatic dogs that varies from 60% - 76% [7]. Little is known about the sensitivity of biomarkers including alanine aminotransferase to detect hepatobiliary injury in the subclinical disease phase. A recent study by Dirksen et al. found that serum alanine aminotransferase and alkaline phosphatase activities and serum bile acid concentrations have a sensitivity for detecting histologic evidence of copper-associated chronic hepatitis in the subclinical phase of 71%, 35%, and 13% respectively [8]. Additionally, serum ALT activity can also be increased due to extrahepatic diseases such as pancreatitis, enteritis, and endocrinopathies [1]. Therefore, a more sensitive and specific marker of hepatocellular injury in dogs with chronic hepatitis would be beneficial. There is also an unmet need for novel non-invasive diagnostic tests for hepatic fibrosis, hepatic function, and hepatic neoplasia in dogs.

Proteomics is the large-scale characterization of the protein complement of a cell, tissue, or organism and has emerged as a powerful tool in biomarker discovery. Chronic inflammatory hepatobiliary disease has been demonstrated to result in changes to proteins found in hepatic tissues in humans [9]. There is one proteomic study of the canine liver to the authors knowledge. This study evaluated the soluble liver proteome from bull terriers affected with inherited lethal acrodermatitis [10]. Proteomic analysis of canine hepatic tissue from dogs with chronic hepatitis may lead to the identification of novel candidate biomarkers that could later be evaluated in serum or urine as well as having the potential to provide new insights into the pathogenesis of this disease.

The aim of this study was to identify proteins that are differentially expressed between hepatic tissue from dogs with chronic hepatitis and healthy control dogs. We hypothesized that dogs with chronic hepatitis will exhibit differential hepatic protein expression compared to healthy control dogs.

2.2. **Material and methods**

2.2.1. **Patients and procedures**

Eight canine patients undergoing liver biopsy for routine diagnostic purposes that were histologically confirmed to have chronic hepatitis and 8 healthy control dogs undergoing ovariohysterectomy were enrolled at the Texas A&M University Veterinary Medical Teaching Hospital between September 2014 – August 2017. Informed client consent was obtained for each patient and the study was approved by the Texas A&M University Institutional Animal Care and Use Committee (Animal Use Protocol #2014-320 and 2015-0043). Patients with suspected chronic hepatitis underwent laparoscopic liver biopsy and

5 to 8, 5 mm closed cup forceps liver biopsy specimens were collected. The same 5 mm closed cup forceps were used to collect 3 – 4 liver biopsy specimens from healthy dogs at the time of ovariohysterectomy. One biopsy specimen, approximately 0.5 g of liver tissue from each patient was flash frozen in liquid nitrogen within 30 minutes of acquisition and stored at -80°C. One liver biopsy specimen was collected in a sterile red top tube for copper quantification by atomic adsorption spectroscopy (expressed as μg/g dry weight) and another was collected and stored in a sterile specimen cup for aerobic and anaerobic culture and susceptibility testing. The remaining liver tissue from dogs in each group was fixed in neutral buffered formalin for routine histological processing. Histological sections from formalin-fixed paraffin embedded tissues were stained with hematoxylin and eosin, picrosirius red, and rhodamine. The diagnosis of chronic hepatitis and healthy control were based on clinical signs, routine serum biochemistry panels, and histological assessment of liver specimens by a board-certified veterinary pathologist according to the World Small Animal Veterinary Association Liver Standardization Group Guidelines [11]. The stage of hepatic fibrosis, the grade of necroinflammatory activity, and the semiquantitative assessment of copper content were assessed using a previously published scoring scheme [2,12].

2.2.2. **2-Dimentional Fluorescence Difference Gel Electrophoresis**

2.2.2.1. **Protein processing and labeling**

Each liver biopsy specimen was placed in a 1.5 mL ground glass pestle and tube tissue grinder and homogenized in 10 mM Tris-HCL/1% CHAPS buffer. The overall protein concentration was determined by Bradford protein assay using bovine serum albumin as a standard. The proteome from each liver sample was precipitated with

choloroform/methanol and dissolved in 1 mL of DIGE labeling buffer (30 mM Tris, 7 M urea, 2M Thiourea, 4% CHAPS, pH 8.5 buffer) [13]. Samples were fluorescently labeled by reacting 50 µg of protein with 200 pmol CyDye DIGE Fluors (GE Healthcare). One sample was labeled with Cy3 while the other was labeled with Cy5 to allow for differential labeling. A pooled sample containing equal amounts of each sample was labeled with Cy2. The labeling reactions were quenched with 10 mM lysine.

2.2.2.2. **2-Dimentional difference gel electrophoresis**

Cy2-, Cy3- and Cy5-labeled samples were mixed together, and rehydration buffer was added (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% pharmalytes (GE Healthcare), 40 mM dithiothreitol (DTT), and 0.002% bromophenol blue) to a final volume of 450 µl and used to rehydrate an immobilized pH gradient strip (24 cm; pH 3–10NL; GE Healthcare) by passive diffusion at 20°C for 12h. Isoelectric focusing was performed on an IPG Phor 3 horizontal electrophoresis system (GE Healthcare) with a program of 0.5 kV for 1h, holding at 0.5 kV for 5 h, ramping to 1 kV over 1 h, ramping to 8 kV over 3 h, holding at 8 kV until 60 kV*h, and holding at 0.5 kV for 4 h. Each focused strip was then equilibrated in two steps: 15 minutes in a reducing equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8 with 30% (v/v) glycerol, 2% (w/v) SDS, 0.01 bromophenol, and 10 mg/mL DTT), followed by 15 minutes in an alkylating equilibration buffer where DTT was replaced by 25 mg/mL iodoacetamine. The equilibrated IPG strips were then placed directly on top of polymerized 12% SDS gels and sealed with an agarose sealing solution (25 mM Tris, 192 mM, glycine, 0.1% SDS, 0.5% (w/v) agarose, and 0.02% bromophenol blue). Gels were run in cooled tanks with DIGE buffer on an Ettan Dalt-6 (GE Healthcare) at 1 W per gel until the bromophenol blue dye front reached the bottom of the gel.

2.2.2.3. **Image acquisition and analysis**

Gel images were obtained on a Typhoon FLA 9500 (GE Healthcare) at an excitation wavelength of 473 nm for Cy2, 532 nm for Cy3, and 635 nm for Cy5 labeled samples. The resolution was set at 100 μm. DeCyder 2-D Differential Analysis Software (v 6.5, GE Healthcare) was utilized for data analysis. The software subtracts the background signal using a subtraction algorithm and quantifies spot intensity based on normalized spot volume. The normalization is ratiometric with Cy3 and Cy5 images being expressed as a ratio of the corresponding Cy2 internal standard protein spot. Further, DeCyder was also used to match spots between gels and determine significant changes in protein expression and the absolute fold change ratio. Absolute fold change ratio was derived from the normalized spot volume standardized against the intragel standard providing a measure of the magnitude of expression differences between identified spots. Protein spots present in the gels were matched using Decyder software from which a pick list was generated. Spot detection was verified visually. Statistical significance was determined by a two-sided student's t-test. Post-hoc testing was performed using Bonferroni's multiple comparisons test and significance was set at $q < 0.05$. Proteins with an absolute fold change value > 2.0 between dogs with chronic hepatitis and healthy controls were selected for further analysis. All gels were fixed overnight in 10% methanol and 7.5% acetic acid.

2.2.2.4. **Spot picking and protein processing**

Ettan Spot Handling Workstations (GE Healthcare, Chicago, IL USA) were used to cut out the selected protein spots from a protein gel, perform an in-gel tryptic digestion with recombinant porcine trypsin (Promega, Madison, WI) and extract the peptides from the gel. Extracted trypsin peptides were concentrated by SpeedVac. Protein identification was

achieved by nanoflow liquid chromatography tandem mass spectrometry. Subsequently, peptides from the MS were identified using the MASCOT search engine. The MASCOT program (v2.2) searched the mouse genome using the following parameters for protein identification: 1) one missed cleavage by trypsin; 2) monoisotopic peptide masses; 3) peptide mass tolerance of 1.2 Da; and 4) fragment mass tolerance of 0.8 Da. Further, oxidation of methionine (variable modification) and carbamidomethylation (fixed modification) of cysteine were taken into consideration by MASCOT in the protein identification. Peptides were matched to proteins at a minimum of two peptides. Protein identification was verified by Scaffold (Proteome Software, Portland, OR).

2.2.2.5. **Western blotting**

Select proteins (annexin A5 and cytokeratin 18) that were identified in spots that showed differential expression in hepatic tissue from dogs with chronic hepatitis (based on the 2D-DIGE results) were validated using standard Western blotting techniques. The following antibodies were used: annexin V (1:1000, rabbit polyclonal, Abcam, Cambridge, MA, USA) and cytokeratin 18 (1:20000, mouse monoclonal, Abcam, Cambridge, MA, USA). Liver samples were homogenized in 10 mM Tris-HCL/1% CHAPS buffer. The protein concentration was determined with the Bradford protein assay using a bovine serum albumin standard. Equal protein loading for each lane was confirmed by immunoblotting for anti-Lamin B1 (1:5000, rabbit monoclonal, Abcam, Cambridge, MA, USA). Protein samples were boiled following addition of Laemmli loading dye, separated on precast gels (BIO-RAD 8-16% Mini-PROTEAN TGX), and transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked in 5% milk 0.05% Tween - Tris buffered saline (TTBS) overnight at 4ºC, incubated with primary antibodies diluted in 5%

milk 0.05% TTBS overnight at 4ºC. Membranes were washed in 5% milk 0.05% TTBS and incubated with an HRP-conjugated anti-rabbit or goat IgG 1/20000 dilution in 5% milk 0.05% TTBS. PVDF membranes were washed in 5% milk 0.05% TTBS, incubated in ECL-plus, and the signal was detected using an Amersham Imager 600 (GE Healthcare, Chicago, IL USA). Densitometry was performed using the ImageQuantTM TL (GE Healthcare Life Sciences, Chicago, IL USA) software.

2.2.2.6. **Statistical analysis**

Differences between groups were analyzed for statistical significance by two-sided student's T-test. Post-hoc testing was performed using Bonferroni's multiple comparisons test. Significance was set at $q < 0.05$.

2.3. **Results**

2.3.1. **Dog demographics and clinical characteristics**

The demographics and clinical characteristics for the dogs enrolled into the study are shown in Table 1.

Table 2.1. Demographics and clinical characteristics of dogs enrolled into the study. Reprinted with permission [27].

Patient	Disease	Age[a]	Sex	Breed	FIS	AS	HVS	CuS	Q Liver Cu	ALT	ALP	TBILI	GGT
227448	Chronic Hepatitis	12	SF	Labrador Retriever	4	2	1	1	667	466	557	0.2	16
229439	Chronic Hepatitis	6	CM	Doberman Pincher	3	1	0	2	196	989	283	0.3	12
203392	Chronic Hepatitis	9	M	Labrador Retriever	2	2	2	0	463	577	230	0.2	<10
231640	Chronic Hepatitis	5	M	Doberman Pincher	2	2	2	3	3450	750	334	0.2	18
229216	Chronic Hepatitis	4	SF	German Shorthaired	1	2	1	2	1080	231	98	0.4	<10
220269	Chronic Hepatitis	11	SF	Labradoodle	2	2	2	3	1230	663	179	0.3	22
175660	Chronic Hepatitis	14	CM	Rat Terrier	3	0	3	0	170	476	327	<0.1	12
231108	Chronic Hepatitis	9	F	Rottweiler Dog	4	1	0	0	257	121	122	0.2	8
229461	Healthy Control	0.5	F	Catahoula Hog Dog	0	0	2		181				
211715	Healthy Control	6	F	Greyhound Dog	0	0	0		168				
227893	Healthy Control	1	F	Chihuahua	0	0	0		280				
229383	Healthy Control	5	F	Staffordshire Terrier	0	0	2		153				
229057	Healthy Control	1	F	Labrador Retriever	0	0	1		124				
229382	Healthy Control	3	F	Walker Coonhound	0	0	0		96.3				
231847	Healthy Control	0.5	F	Labrador Retriever	0	0	0		316				
229270	Healthy Control	0.5	F	Australian Shepherd	0	0	0		158				

[a]Age (years), SF – Spayed female, M – Male, CM – Castrated male, F – Intact female, M – Intact male, FIS – Fibrosis score, AS – Activity score, HVS – Hepatocellular vacuolation score, CuS – Copper Score, Q Liver Cu – Quantitative liver copper, ALT – Alanine transaminotransferase, ALP – Alkaline phosphatase, GGT – Gamma-glutamyl transferase

2.3.2. **Liver biopsy specimens**

All liver biopsies were assessed to be of adequate diagnostic quality with > 12 portal triads per biopsy specimen [14]. The liver biopsy specimens of control dogs were free from any remarkable histopathological changes.

2.3.3. **Identification of candidate biomarkers for chronic hepatitis**

Liver tissue specimens from eight dogs with chronic hepatitis and eight healthy control dogs were adequate to show a reproducible liver proteome spot pattern of approximately 2,500 spots. Each spot could contain 1 or multiple proteins. Labeled spots were selected for analysis (Fig.1). Image analysis revealed 5 differentially expressed protein spots ($q < 0.05$ and fold change > 2.0) that were selected for protein identification.

Altogether 5 differentially expressed protein spots contained 11 different proteins (Table 2). Spot 1440 showed a 3.18-fold increase in abundance (for chronic hepatitis dogs compared to healthy control dogs; $q = 0.00095$) and contained annexin 5, regucalin, and haptoglobin (Table 1). For protein disulfide – isomerase A4, from spot 472 a 2.33-fold increase was detected ($q=0.0000325$). Additionally, we found a 2.24-fold increase in abundance of spot 779 that contained formimidoyltransferase-cyclodeaminase isoform X1, annexin A6, and UDP-glucose 6-dehydrogenase ($q=0.00033$) (Table 2).

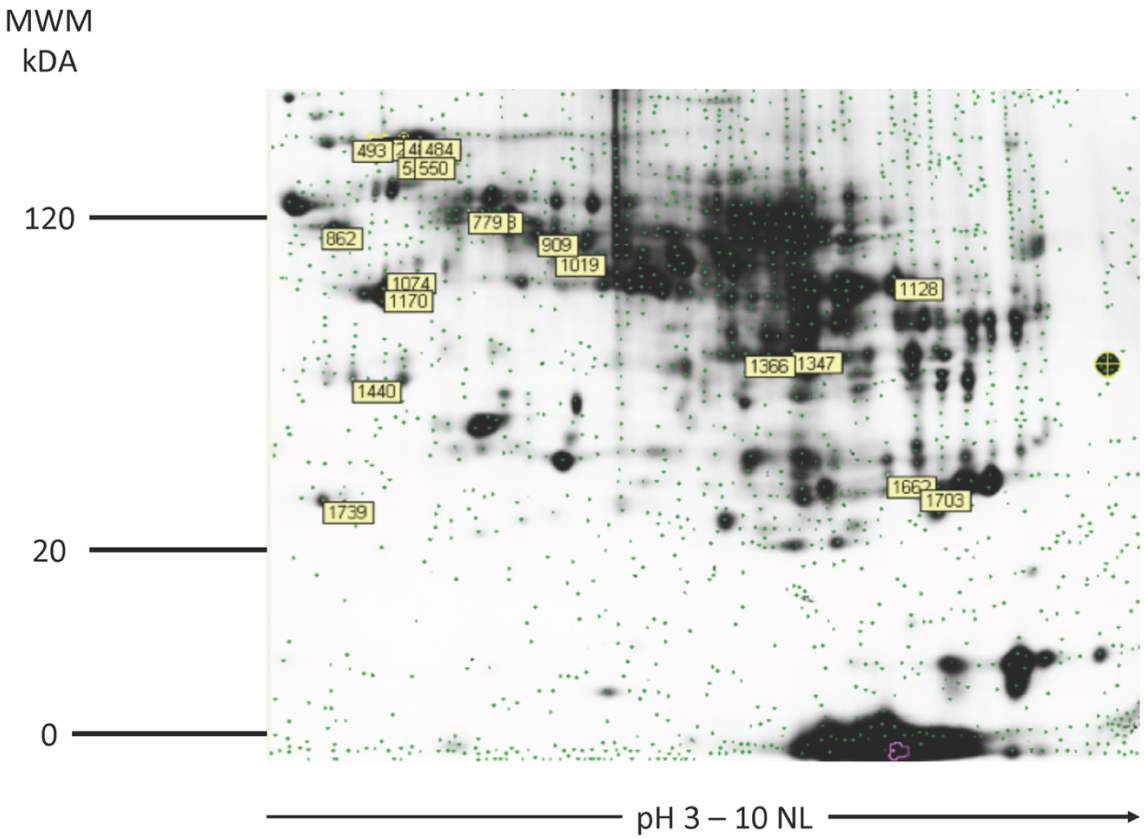

Figure 2.1. 2-D gel image of the master gel with labeled picked proteins spots. Proteome pattern of canine liver tissue by means of 2D – DIGE. For protein analysis, proteins were labeled with Cy2, Cy3, and Cy5, separated in the first dimension using an immobilized pH gradient, and subsequently, in the second dimension, by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein spots were detected using a fluorescence scanner (MWM – molecular weight marker; kDA – kilodalton, NL – nonlinear). Reprinted with permission [27].

Table 2.2. Summary of proteins identified from differentially expressed spots. Reprinted with permission [27].

Spot	Identified Protein	GENE	GO Biological Process	Fold ∇	Peptide No.	q Value	P Value
1047	Succinyl CoA	SUCLG1	cellular metabolic process	2.75	10.00	0.031	0.0062
1047	Cytokeratinl 8	KRT8	hepatocyte apoptotic process	2.75	6.00	0.031	0.0062
1047	Cytokeratin 18	KRT18	hepatocyte apoptotic process	2.75	7.00	0.031	0.0062
1440	Annexin A5	ANXA5	negative regulation of apoptosis	3.18	6.00	0.00095	0.00019
1440	Regucalcin	RGN	L-ascorbic acid biosynthesis	3.18	8.00	0.00095	0.00019
1440	Haptoglobin	HP	acute - phase response/antioxidant	3.18	3.00	0.00095	0.00019
460	Protein Disulfide-Isomerase A4	PDIA4	cell redox homeostasis	2.24	3.00	0.00295	0.00059
460	Gamma-glutamyltransferase 2	TGM3	protein tetramerization	2.24	3.00	0.00295	0.00059
472	Protein Disulfide-Isomerase A4	PDIA4	cell redox homeostasis	2.33	3.00	3.25E-005	6.50E-006
779	Formimidoyltransferase	FTCD	cellular metabolic process	2.24	7.00	3.30E-004	6.60E-005
779	Annexin A6	ANXA6	apoptotic signaling pathway	2.24	6.00	3.30E-004	6.60E-005
779	UDP-glucose 6-dehydrogenase	UDGH	glycosaminoglycan biosynthesis	2.24	4.00	3.30E-004	6.60E-005

2.3.4. **Analysis of cytokeratin 18 and annexin 5 in the liver of dogs with chronic hepatitis using immunoblotting**

Two proteins were selected for Western blot analysis. The proteins, cytokeratin 18 and annexin 5 were confirmed to contribute to the increased relative abundance in the liver proteome of dogs with chronic hepatitis (n = 7, n=4) compared to healthy controls (n = 7, n=4), respectively (Table 2; Table 3; Fig. 2 and Fig. 3). Not all dogs were used to conserve the limited quantity of available protein.

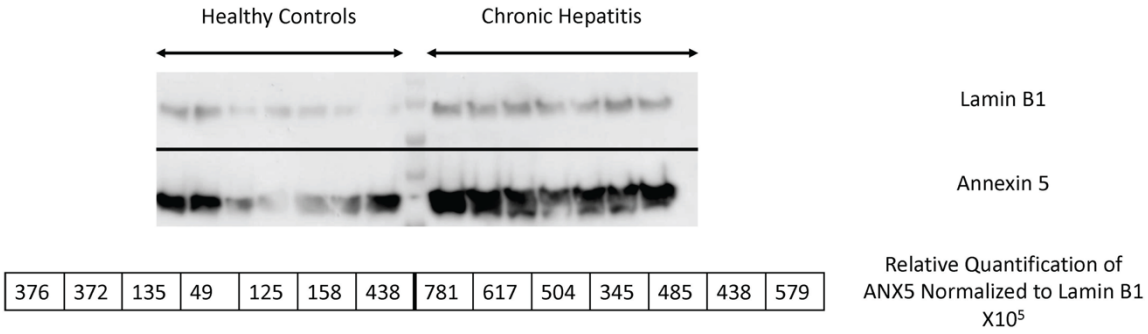

Figure 2.2. Relative abundance of annexin 5 in the hepatic tissue of dogs with chronic hepatitis by Western Blot analysis. Annexin 5 was assessed in the liver proteome of 7 dogs with chronic hepatitis and compared to 7 healthy control dogs. Lamin was used as a loading control.
Reprinted with permission [27].

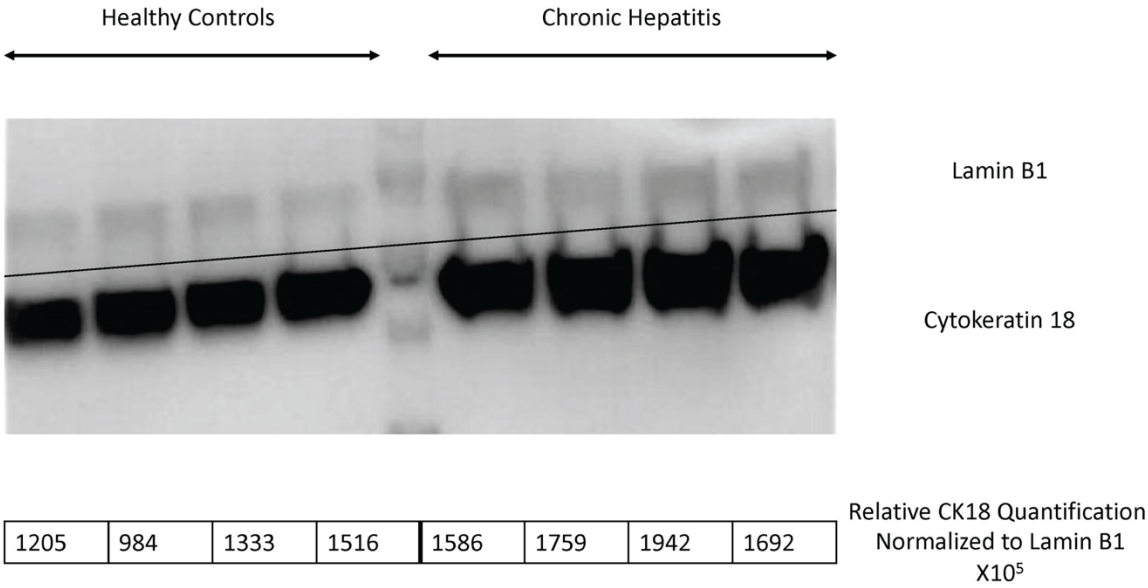

Figure 2.3. Relative abundance of cytokeratin 18 in the hepatic tissue of dogs with chronic hepatitis by Western blot analysis. Cytokeratin 18 was assessed in the liver proteome of 4 dogs with chronic hepatitis and compared to 4 healthy control dogs. Lamin was used as a loading control.
Reprinted with permission [27].

Table 2.3. Patient allocation for Western blot analysis
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Patient ID#	Disease	FIS	Age[a]	Sex	Breed	WB CK18	WB ANX5
227448	Chronic Hepatitis	4	12	SF	Labrador Retriever	X	
229439	Chronic Hepatitis	3	6	CM	Doberman Pincher	X	X
203392	Chronic Hepatitis	2	9	M	Labrador Retriever	X	X
231640	Chronic Hepatitis	2	5	M	Doberman Pincher	X	X
229216	Chronic Hepatitis	1	4	SF	German Shorthaired Pointer		X
220269	Chronic Hepatitis	2	11	SF	Labradoodle		X
175660	Chronic Hepatitis	3	14	CM	Rat Terrier		X
231108	Chronic Hepatitis	4	9	F	Rottweiler Dog		X
229461	Healthy	0	1	F	Catahoula Hog Dog		
211715	Healthy	0	6	F	Greyhound Dog	X	X
227893	Healthy	0	1	F	Chihuahua	X	X
227893	Healthy	0	5	F	Staffordshire Terrier	X	X
229057	Healthy	0	1	F	Labrador Retriever		X
229382	Healthy	0	3	F	Treeing Walker Coonhound	X	X
231847	Healthy	0	1	F	Labrador Retriever		X
229382	Healthy	0	1	F	Australian Shepherd		X

[a]Age (years), SF – Spayed female, M – Male, CM – Castrated male, F – Intact female, M – Intact male, FIS – Fibrosis score, WB – Western blot, CK18 – Cytokeratin 18, ANX5 – Annexin 5

2.4. **Discussion**

Our study utilized a gel-based, quantitative proteomics approach to identify differentially expressed proteins in the liver of dogs with chronic hepatitis compared to healthy control dogs. We detected 2,500 protein spots in the liver biopsy samples from dogs. We identified 5 protein spots that showed differential expression with an absolute fold change of at least 200% (2.00 absolute fold change) representing 11 proteins was significantly different

between groups. These proteins warrant investigation as potential biomarkers of canine chronic hepatitis. Several of the identified proteins such as cytokeratin 18, cytokeratin 8, annexin 5, and annexin 6 are involved with hepatocyte apoptosis. Only cytokeratin 18 and annexin 5 were validated by Western blot in this study. All other identified proteins need to be validated to confirm these differences.

Hepatocellular apoptosis is a key event in the pathophysiology of many chronic liver diseases of dogs and humans, and it is associated with liver fibrogenesis and the development of cirrhosis [15-18]. The effector caspases are activated in the final common pathway of apoptosis. Cytokeratin 18 is the major intermediate filament protein in the liver and the most prominent substrate of caspases during hepatocellular apoptosis [19]. Apoptotic cell death of hepatocytes is associated with release of caspase-cleaved CK18 fragments in the bloodstream and in contrast, the cytosolic pool of uncleaved CK18 is released from hepatocytes during necrosis [20]. Multiple studies have assessed human serum or plasma CK18 concentrations and suggested the potential use of CK18 fragments as a noninvasive biomarker for the diagnosis or staging of chronic liver disease [21]. Furthermore, several studies of nonalcoholic fatty liver disease in humans have demonstrated that caspase activation and liver cell apoptosis were prominent features of this disease. Additionally, the degree of apoptosis correlated with the severity of hepatitis and the stage of fibrosis [22]. Serum CK18 is a marker of hepatocyte apoptosis in humans and correlates with histologic activity more accurately than serum alanine aminotransferase [23]. Annexin 5A (ANXA5) is a negatively charged phospholipid-binding protein that is widely used as a marker of apoptosis. Annexin 5A binds to

negatively charged phospholipids, such as phosphatidylserine that are exposed on the cell surface during apoptosis [24,25]. Increased hepatic annexin 5A expression could therefore indicate ongoing apoptosis. Annexin A5 and CK18 have not been previously evaluated in canine liver disease. The apoptotic pathway may be altered in canine chronic hepatitis and other proteins in this pathway should be further investigated in a targeted study.

This is the first study to evaluate the liver proteome of dogs with chronic hepatitis. However, limitations of our study include the heterogeneity of the dogs in the chronic hepatitis group with one copper-associated chronic hepatitis case and 7 idiopathic chronic hepatitis cases. Examining specific subpopulations of dogs with chronic hepatitis may be advantageous. Another limitation is the fact that we only looked at proteins with an absolute fold change of > 2.0 between groups to avoid false discovery, which eliminated a number of proteins from further analysis. Another limitation is the use of only female dogs as a control group; however, the impact of this limitation is unknown. The healthy control group dogs were significantly younger than the chronic hepatitis dogs due to selection bias. The impact of this limitation is unknown, although, age-related changes in the liver proteome have been described in rats [26]. Finally, this initial study has a limited sample size that could have led to type II error. Further work that includes measurement in blood or urine and validation in a diverse clinical population will be required to establish whether the identified proteins have any diagnostic utility.

Proteomic evaluation of liver tissue from dogs with chronic hepatitis demonstrated differential protein expression compared to healthy controls. The increased expression of

two proteins involved in the apoptotic pathway, cytokeratin 18 and annexin 5 was confirmed with Western blotting in these tissues. Further work will be required to establish whether these proteins have diagnostic utility over currently available biomarkers.

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3. UNTARGETED METABOLOMIC PROFILING OF SERUM FROM DOGS WITH CHRONIC HEPATIC DISEASE*

3.1. **Introduction**

Chronic hepatopathies in dogs represent a spectrum of diseases that include idiopathic chronic hepatitis, copper-associated chronic hepatitis, drug-associated chronic hepatitis, breed-associated metabolic errors, congenital portosystemic vascular anomalies, lobar dissecting hepatitis, and granulomatous hepatitis [1]. Further characterization of metabolic changes that occur in these diseases will increase our understanding of disease pathophysiology, which may improve disease diagnosis, management, or treatment. Although an early accurate diagnosis is important for an improved clinical outcome, achieving a definitive diagnosis can be cost prohibitive and invasive with the examination of a liver biopsy specimen regarded as the gold standard. The identification of non-invasive biomarkers that can reliably characterize chronic hepatopathies is desirable and may have clinical implications.

The liver is a central organ for regulating metabolism, and a variety of metabolic disturbances are seen in patients with chronic liver disease [2,3]. Data from studies in human and animal models have documented alterations in hepatic lipid metabolism, protein metabolism, energy metabolism, cytokine metabolism, and increased generation

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of reactive oxygen species in patients with chronic liver disease [4-6]. Global metabolomic profiling, the untargeted quantification of small molecules in biologic samples, allows for a comprehensive analysis of changes in several metabolic and signaling pathways and their interactions [7-9]. This platform utilizes nuclear magnetic resonance spectroscopy or mass spectrometry to measure low molecular weight metabolites permitting the formation of a metabolite profile. Metabolite profiles can be altered by a variety of physiological and pathological processes, and therefore global changes in such profiles may signal the presence of a particular disease [10,11]. Characteristic metabolite profiles that can discriminate between various types of liver disease have been identified in human studies [12-17]. To the authors' knowledge there are no previous studies that have evaluated serum metabolomics in dogs with chronic liver diseases however, a similar study examined the plasma metabolome in canine chronic liver disease and found significant differences [18].

The present study examined the serum metabolome of dogs with chronic hepatitis, dogs with a congenital portosystemic shunt, and clinically healthy dogs. We hypothesized that there would be a difference in serum metabolites between groups and that the three groups would have significantly different metabolomes.

3.2. **Materials and methods**

3.2.1. **Animals**

Serum was collected from canine patients examined at the Texas A&M University College of Veterinary Medicine Veterinary Medical Teaching Hospital between September 2011 and August 2017 with histologically confirmed chronic hepatitis or an ultrasonographic

diagnosis of a congenital portosystemic shunt. Informed client consent was obtained for each patient and the study was approved by the Texas A&M University Institutional Animal Care and Use Committee (Animal Use Protocols #2014-0142, 2014-0320, and 2015-0043). Patients with chronic hepatitis underwent a laparoscopic liver biopsy and 5 to 8, 5 mm closed cup forceps liver biopsy specimens were collected. One liver biopsy specimen was collected in a sterile red top tube for copper quantification by atomic absorption spectroscopy (expressed as μg/g dry weight) and another was collected and stored in a sterile specimen cup for aerobic and anaerobic culture and susceptibility testing. The remaining liver tissue from dogs with chronic hepatitis was fixed in neutral buffered formalin for routine histological processing. Histological sections from formalin-fixed paraffin embedded tissue were stained with hematoxylin and eosin, picrosirius red, and rhodamine. The diagnosis of chronic hepatitis was based on clinical signs, routine serum biochemistry panels, and histological assessment of liver specimens by a board-certified veterinary pathologist according to the World Small Animal Veterinary Association Liver Standardization Group Guidelines[19] Dogs with a hepatic copper level > 400 μg/g dry weight, centrilobular copper accumulation, and an associated inflammatory infiltrate were characterized as copper-associated chronic hepatitis. The stage of hepatic fibrosis, the grade of necroinflammatory activity, and the semiquantitative assessment of copper content were assessed using a previously published scoring scheme [20,21]. Dogs with suspected congenital portosystemic shunting based on compatible clinical and/or clinicopathologic signs were definitively diagnosed on the basis of two-dimensional, grey-scale ultrasonography as previously described [22]. Dogs with a history of current systemic disease such as hyperadrenocorticism, cancer, or others were excluded from the

study. All patients with chronic hepatitis or a congenital portosystemic shunt had a complete abdominal ultrasound and were accessed for acquired portosystemic shunting sonographically as described previously [38]. Dogs with acquired portosystemic shunting were excluded from this study. Dogs presented for a wellness examination to the Texas A&M University College of Veterinary Medicine Veterinary Teaching Hospital were used as healthy controls subsequent to a normal physical examination. Informed client consent was obtained for each dog and sample collection was approved by the Texas A&M University Institutional Animal Care and Use Committee Animal Use Protocol #2014-0251.

3.2.2. **Serum sample collection**

Serum from all dogs was obtained from non-heparinized whole blood samples that were submitted to the clinical pathology service at the Texas A&M University College of Veterinary Medicine where serum was separated by means of centrifugation at 2150 × g for 10 min at 4°C. Serum biochemical analysis was performed using an Ortho Vitros 4600 analyzer and the leftover serum was stored at 4°C for less than 72 hours before retrieval by study investigators for storage at -80°C. All serum samples were stored for a similar length of time. Food was withheld for a minimum of 12 hours prior to blood sample collection.

3.2.3. **Medical record data collection**

The information recorded from the medical record of dogs with chronic hepatitis and congenital portosystemic shunting included age, breed, sex, diet, medical treatment at the time of enrollment, serum biochemical markers of liver disease, and histologic or sonographic diagnosis, respectively. For control dogs recorded medical record data

included age, breed, sex, diet, medical treatment at the time of enrollment, and serum biochemical markers of liver disease.

3.2.4. **Serum metabolome analysis**

Untargeted metabolomics analysis was performed by the West Coast Metabolomics Center at the University of California (Davis, CA) on a fee-for-service basis. Serum aliquots were extracted by degassed acetonitrile. Internal standards C8-C30 fatty acid methyl ethers were added, and the samples were derivatized by methoxyamine hydrochloride in pyridine and subsequently by N-methyl-N-trimethylsilyltrifluoroacetamide for trimethylsilylation of acidic protons. Analytes were separated using an Agilent 6890 gas chromatograph (Santa Clara, CA) and mass spectrometry was performed on a Leco Pegasus IV time of flight mass spectrometer (St. Joseph, MI) following a published protocol [23]. Unnamed peaks were excluded from statistical analysis.

3.2.5. **Statistical analysis**

3.2.5.1. **Univariate analysis**

Differences in the abundance of serum metabolites between the chronic hepatitis, congenital portosystemic shunt, and healthy control groups were evaluated using a Kruskal-Wallis test. Univariate analysis was performed using JMP Pro 13 (JMP Software, Marlow, England). P-values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate and significance was set at $q < 0.10$.[24] Post hoc testing was performed with Dunn's Test of multiple comparisons using JMP Pro 13 and significance was set at $p < 0.05$.

3.2.5.2. **Multivariate analysis**

Data reported as peak height were log transformed prior to multivariate analysis. Principle component analysis, random forest analysis, and hierarchical cluster analysis was performed using MetaboAnalyst 4.0 (http://www.metaboanalyst.ca)[25].

3.3. **Results**

3.3.1. **Selection criteria of case and control dogs**

There were 12 healthy control dogs enrolled in the study based on a normal physical examination and the absence of any reported clinical signs. Three of the 10 dogs with a portosystemic shunt had an intrahepatic anomaly, while the other 7 had an extrahepatic anomaly. All 10 dogs had blood ammonia concentrations > 50 μ/dL; however compatible clinical signs were only reported in four. Three of the six dogs with chronic hepatitis had copper-associated chronic hepatitis and 3 had idiopathic chronic hepatitis based on histopathologic findings and a quantitative liver copper level. There was no evidence of acquired portosystemic shunting in any of the dogs enrolled in this study.

3.3.2. **Animal population**

The demographics of the healthy control dogs, congenital portosystemic shunt dogs, and chronic hepatitis dogs enrolled into the study are summarized in Table 1. Breeds of dogs included in each study group are summarized as supplementary data (Supplemental Table 1). There was no significant difference in the distribution of sexes between groups. There was a statistically significant difference in age with chronic hepatitis dogs being older than healthy control or congenital portosystemic shunt dogs. There was a statistically significant difference in weight with chronic hepatitis dogs weighing more than healthy control or congenital portosystemic shunt dogs.

Table 3.1 Control (n=12), congenital portosystemic shunt (n=10), and chronic hepatitis (n=6) dog demographics
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Heading	Control (n = 12)	Congenital Portosystemic Shunt (n = 10)	Chronic Hepatitis (n = 6)	p-value
Age in years (mean +/- SD)	3.75 +/- 1.9	3.3 +/- 2.0	8.67 +/- 4.0	0.0006
Sex (M/F)	6/6	4/6	2/4	0.78
Weight kg (median/range)	15.5/3 - 32	6.5/2 - 35	30.3/10 - 48.6	0.02

Table 3.2 Supplemental Table 1. Control (n=12), congenital portosystemic shunt (n=10), and chronic hepatitis (n=6) dog breeds, diet, and medical treatment at time of sample collection. AB – antibiotic therapy, L – lactulose therapy, NC – nutraceutical therapy (denamarin, and/or ursodiol).
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Group	Breed	Diet	Medical Therapy
Chronic Hepatitis	Rat Terrier	Maintenance	NC
Chronic Hepatitis	Labrador Retriever	Maintenance	NC
Chronic Hepatitis	Labradoodle	Hill's l/d	AB
Chronic Hepatitis	German Short Haired Pointer	Hill's l/d	None
Chronic Hepatitis	Doberman Pincher	Hill's l/d	None
Chronic Hepatitis	German Shepherd Dog	Maintenance	None
Control	German Shepherd Dog	Maintenance	None
Control	Australian Shepherd	Maintenance	None
Control	Pomeranian	Maintenance	None
Control	Labrador Retriever	Maintenance	None
Control	Silky Terrier	Maintenance	None
Control	Cavalier King Charles Spaniel	Maintenance	None
Control	Mixed Dog	Maintenance	None
Control	Silky Terrier	Maintenance	None
Control	Labrador Retriever	Maintenance	None
Control	Miniature Pinscher	Maintenance	None
Control	Australian Shepherd Dog	Maintenance	None
Control	Mixed Dog	Maintenance	None
Congenital Portosystemic Shunt	Miniature Schnauzer	Hill's l/d	AB
Congenital Portosystemic Shunt	Rhodesian Ridgeback	Maintenance	None
Congenital Portosystemic Shunt	Shih Tzu	Royal Canin Hepatic	AB, L
Congenital Portosystemic Shunt	Australian Shepherd Dog	Hill's l/d	AB
Congenital Portosystemic Shunt	Australian Heeler	Hill's l/d	AB
Congenital Portosystemic Shunt	Mixed Dog	Maintenance	None
Congenital Portosystemic Shunt	Yorkshire Terrier	Hill's l/d	AB, L
Congenital Portosystemic Shunt	Pug	Royal Canin Hepatic	AB, L
Congenital Portosystemic Shunt	Lhasa Apso	Hill's l/d	None
Congenital Portosystemic Shunt	Dachshund	Hill's l/d	AB

3.3.3. **Laboratory and pathology findings**

Serum biochemistry values relevant to hepatic inflammation, cholestasis, and the blood ammonia concentration from the dogs with congenital portosystemic shunting are summarized in Table 2. Serum biochemistry values relevant to hepatic inflammation, cholestasis, and the liver copper level from the dogs with chronic hepatitis are summarized in Table 3. The histologic diagnosis and the anatomic pathology scores from the dogs with chronic hepatitis are provided as supplementary data (Supplemental Table 2). Serum biochemistry values relevant to hepatic inflammation and cholestasis from control dogs are provided as supplementary data (Supplemental Table 3).

Table 3.3 Serum biochemistry variables pertinent to hepatic inflammation and cholestasis and blood ammonia in dogs with a congenital portosystemic shunt (n=10). Reprinted with permission [39].

Clinical Pathological Variable	Median	Range	Number (%) of dogs with a result outside reference interval	Reference interval
Alkaline phosphatase (IU/L)	126.5	38 – 343	3/10 (30)	24 – 147
Alanine transaminase (IU/L)	181.5	53 – 407	6/10 (60)	10 – 130
Gamma-glutamyl transferase (IU/L)	12	<10 – 15	0/10 (0)	0 – 25
Total Bilirubin (mg/dL)	0.35	<0.1 – 0.5	0/10 (0)	0 – 0.8
Ammonia (µg/dL)	136.5	73 – 696	10/10 (100)	< 50

Table 3.4 Histologic diagnosis and anatomic pathology scores from dogs with chronic hepatitis (n=6).
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Case No.	Histologic Diagnosis	FS	AS	HVS	CS
1	Copper Associated Chronic Hepatitis	2	2	2	2
2	Copper Associated Chronic Hepatitis	1	2	1	2
3	Copper Associated Chronic Hepatitis	3	3	2	3
4	Idiopathic Chronic Hepatitis	3	1	0	2
5	Idiopathic Chronic Hepatitis	1	2	3	3
6	Idiopathic Chronic Hepatitis	3	0	3	0

FS – fibrosis score, AS – activity score, HVS – hepatovacuolization score, CS – copper score

Table 3.5. Serum biochemistry variables pertinent to hepatic inflammation and cholestasis and hepatic copper concentrations in dogs with chronic hepatitis (n=6).
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Clinical Pathological Variable	Median	Range	Number (%) of dogs with value outside reference interval	Reference interval
Alkaline phosphatase (IU/L)	283	98 – 481	4/6 (66.7)	24 – 147
Alanine transaminase (IU/L)	519	231 – 989	6/6 (100)	10 – 130
Gamma-glutamyl transferase (IU/L)	12	<10 – 32	1/6 (16.7)	0 – 25
Total Bilirubin (mg/dL)	0.3	<0.1 – 0.4	0/6 (0)	0 – 0.8
Tissue copper (µg/g dry weight)	494.5	170 – 1080	3/6 (50)	120 - 400

Table 3.6. Serum biochemistry variables pertinent to hepatic inflammation and cholestasis in control dogs (n=10).
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Clinical Pathological Variable	Median	Range	Number (%) of dogs with a result outside reference interval	Reference interval
Alkaline phosphatase (IU/L)	126.5	38 – 343	0/10 (0)	24 – 147
Alanine transaminase (IU/L)	181.5	53 – 407	0/10 (0)	10 – 130
Gamma-glutamyl transferase (IU/L)	12	<10 – 15	0/10 (0)	0 – 25
Total Bilirubin (mg/dL)	0.35	<0.1 – 0.5	0/10 (0)	0 – 0.8

3.3.4. **Effect of congenital portosystemic shunting and chronic hepatitis on serum metabolomics**

A total of 126 named serum metabolites were identified of which 50 differed significantly ($p \leq 0.05$; $q<0.10$) between healthy control dogs, congenital portosystemic shunt dogs, and chronic hepatitis dogs (Table 4). Principle component analysis plots showed a clustering of the variables based on disease classification (Figure 1). The distribution of the most significant metabolites separating the three experimental groups was visualized with a heat map (Figure 2). Every column represents a different sample and each box represents a metabolite in the sample. Increased abundances are shaded red, whereas decreased abundances are shaded blue. The congenital portosystemic shunt group showed greater abundances of the aromatic amino acids tyrosine and phenylalanine and a decreased abundances of the branched chain amino acids leucine, isoleucine, and valine when compared to healthy dogs and dogs with chronic hepatitis. Random forest analysis identified metabolites that had the highest discriminatory power between the three groups (Figure 3). Individual compounds in serum that contributed the most to the accuracy of disease classification are shown in Figure 3. A subset of those highly-discriminatory metabolites were selected, and individual sample results were plotted in Figures 4 and 5.

Table 3.7 Serum metabolites that differed significantly between healthy control dogs (n=10), dogs with a congenital portosystemic shunt (n=12), and dogs with chronic hepatitis (n=6). KW – Kruskal – Wallis Test.
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Compound Name	p.value_KW	q.value_KW
xylitol	4.70E-05	0.0036214
phenylalanine	5.75E-05	0.0036214
valine	0.00018392	0.0062971
tyrosine	0.00023471	0.0062971
phenylethylamine	0.00029986	0.0062971
cysteine	0.00026692	0.0062971
serine	0.00042437	0.0070513
leucine	0.00050367	0.0070513
2-deoxypentitol	0.00047524	0.0070513
methionine	0.00068492	0.0084815
ethanolamine	0.00074045	0.0084815
threonine	0.00090305	0.0088613
3-phenyllactic acid	0.00091426	0.0088613
arachidic acid	0.0010602	0.0095421
lactamide	0.0017013	0.014291
methionine sulfoxide	0.001935	0.014342
hypoxanthine	0.0019338	0.014342
isoleucine	0.0021926	0.015348
trans-4-hydroxyproline	0.0025673	0.017025
succinic acid	0.0032266	0.01848
mannose	0.0032044	0.01848
creatinine	0.0031961	0.01848
uric acid	0.0039235	0.021196
inosine	0.0040372	0.021196
pyruvic acid	0.0046021	0.023195
pseudo uridine	0.0053592	0.025971
stearic acid	0.0061125	0.027506
aspartic acid	0.0059262	0.027506
glycolic acid	0.007269	0.031583
2-deoxytetronic acid	0.0078535	0.032985
myristic acid	0.010268	0.041736
hexuronic acid	0.01128	0.044415

Table 3.7 Continued

Compound Name	p.value_KW	q.value_KW
thymidine	0.01279	0.048833
4-hydroxyphenylacetic acid	0.013265	0.049157
ribonic acid	0.015176	0.054359
proline	0.015531	0.054359
sucrose	0.01748	0.059429
myo-inositol	0.017923	0.059429
urea	0.019469	0.062901
uracil	0.020081	0.063256
ribose	0.022043	0.066128
cytosin	0.02172	0.066128
2-ketoisocaproic acid	0.025132	0.073642
conduritol-beta-epoxide	0.028493	0.081594
citric acid	0.033803	0.090622
allantoic acid	0.033014	0.090622
4-hydroxymandelic acid	0.033103	0.090622
pipecolinic acid	0.035076	0.092074
5-methoxytryptamine	0.038389	0.098715

Figure 3.1 Principal component analysis of the serum metabolome showing clustering of samples based on group.
Reprinted with permission [39].

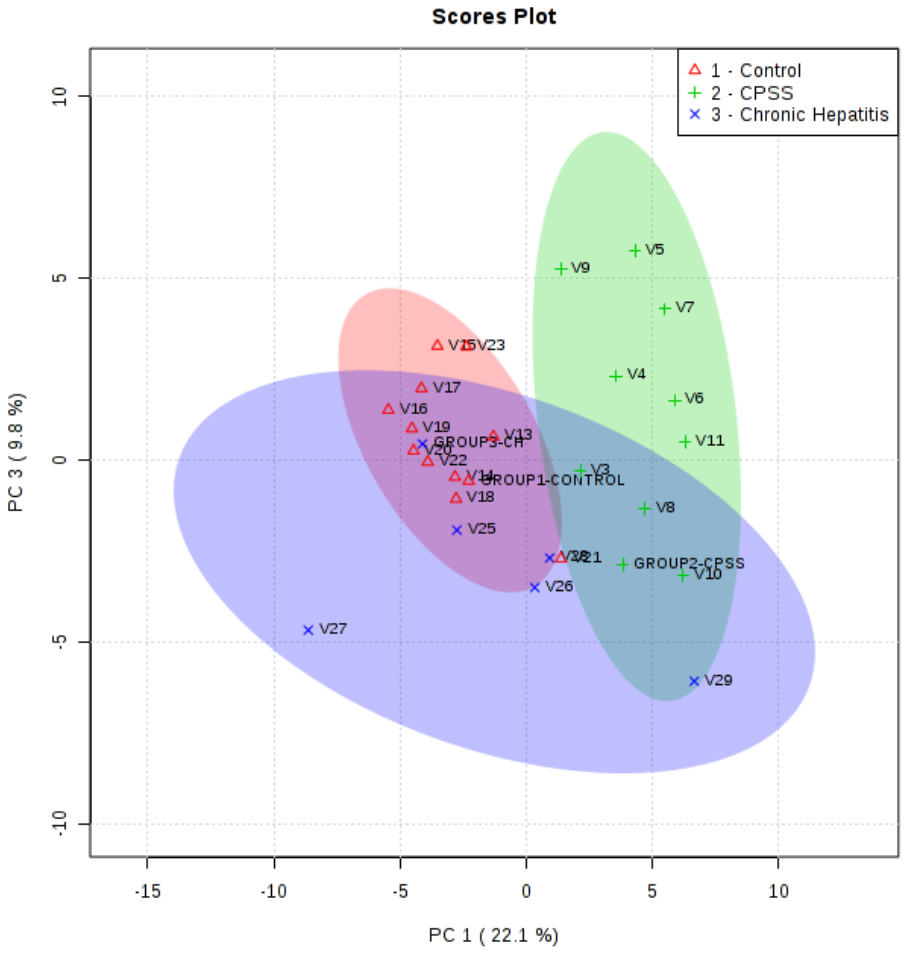


Figure 3.2 . Heatmap of the most abundant metabolites in all groups, as identified by VIP scores in PLS-DA.

Each sample is represented by a single column. The higher the intensity of the red color, the higher the abundance of the metabolite (CPSS – Congenital portosystemic shunt; CH – Chronic hepatitis). Reprinted with permission [39].

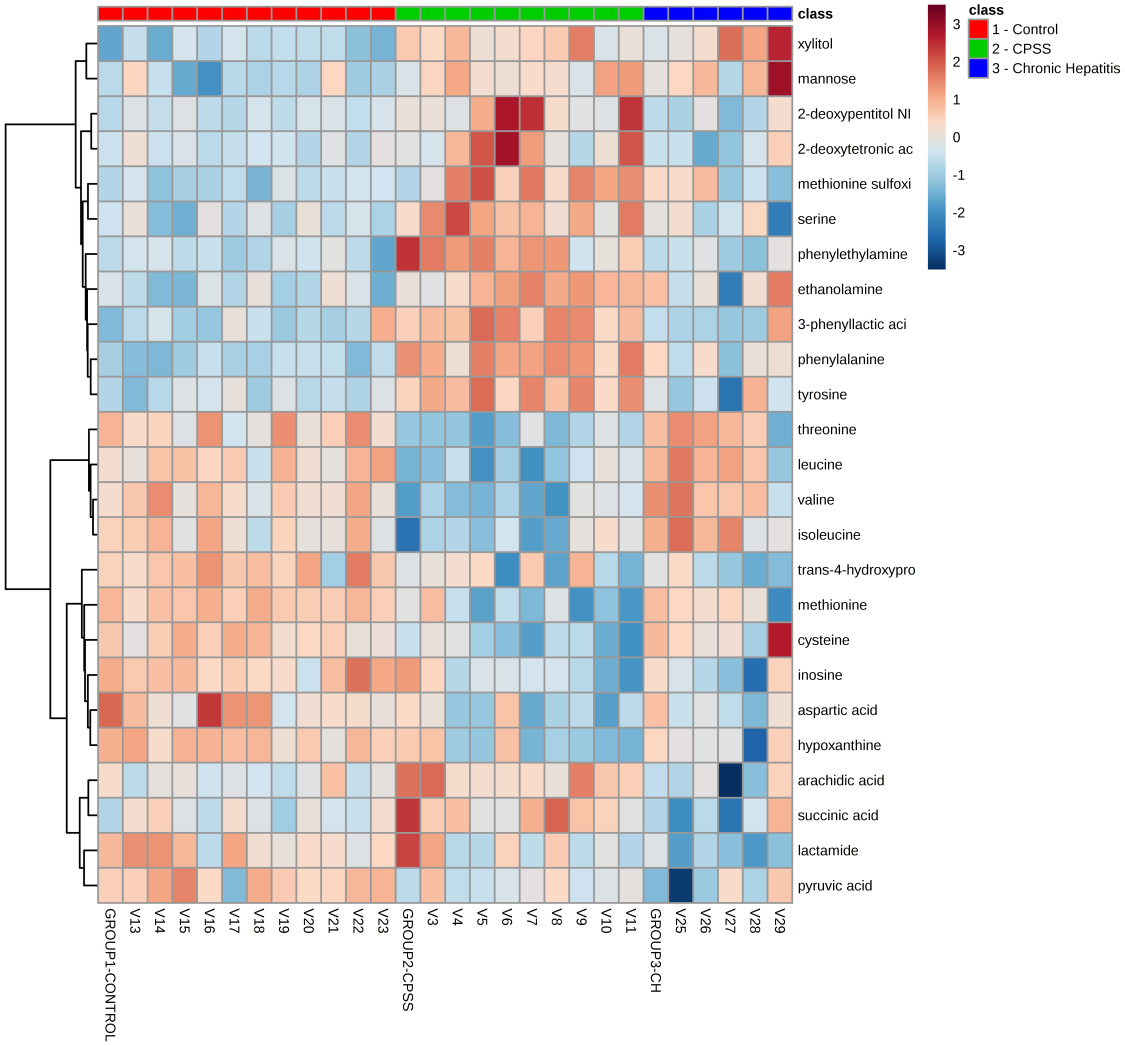


Figure 3.3 Top 15 metabolites in serum (left panel) in rank order of importance for differentiating between control dogs, congenital portosystemic shunt dogs, and chronic hepatitis dogs.

Data were generated by Random Forest analysis. Compounds contributing the most to the accuracy of disease classification were identified as those having the highest mean decrease accuracy (MDA) of the predicted classification when permuted within the dataset. Reprinted with permission [39].

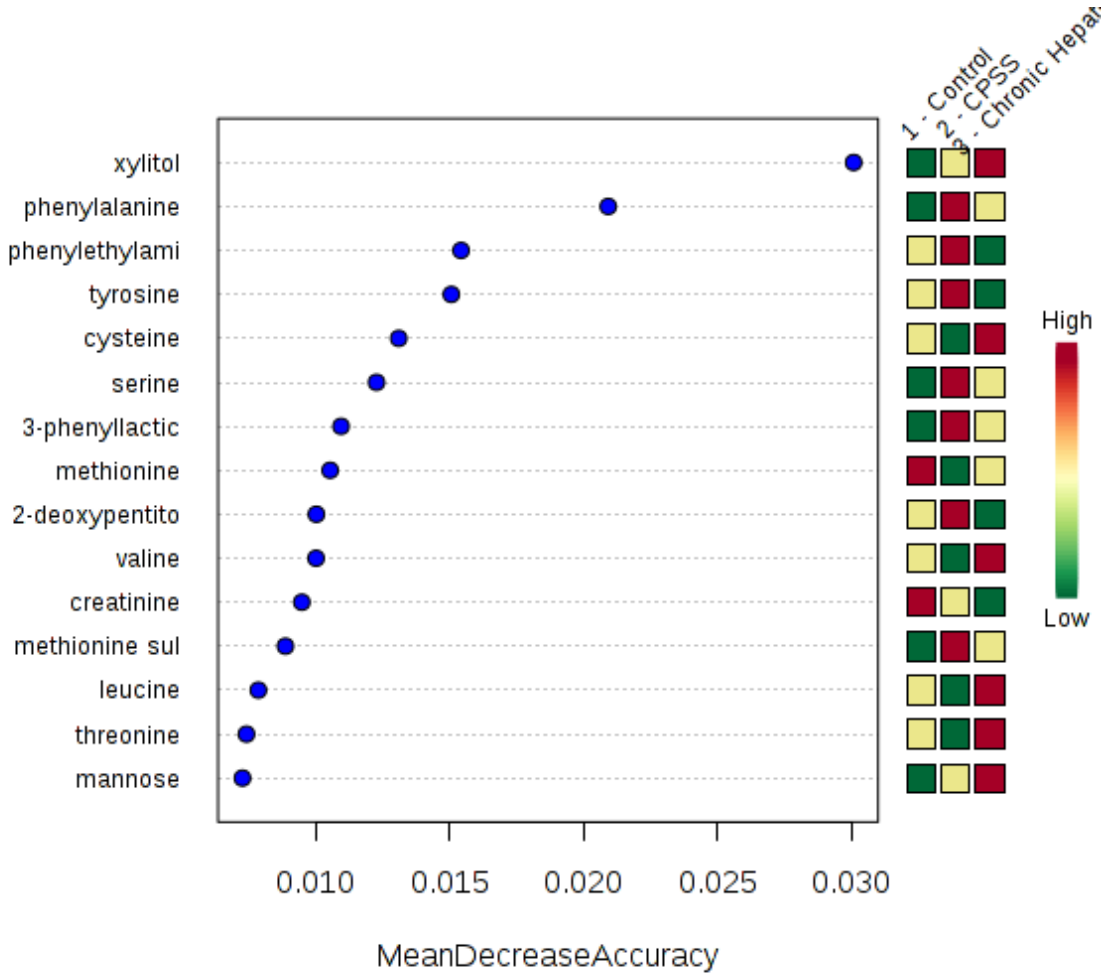


Figure 3.4 Branch chain amino acids and aromatic amino acids identified as potential biomarkers of congenital portosystemic shunting and chronic hepatitis in dogs.

Each metabolite was identified by Random Forest or univariate analysis as important for differentiation of dogs into either control, congenital portosystemic shunt, or chronic hepatitis groups. The abundance of phenylalanine was significantly increased in dogs with a congenital portosystemic shunt compared to control dogs and the abundance of tyrosine was significantly increased in dogs with a congenital portosystemic shunt compared to control dogs and chronic hepatitis dogs. The abundance of leucine, isoleucine, and valine was significantly decreased in dogs with a congenital portosystemic shunt compared to control dogs and chronic hepatitis dogs. Reprinted with permission [39].

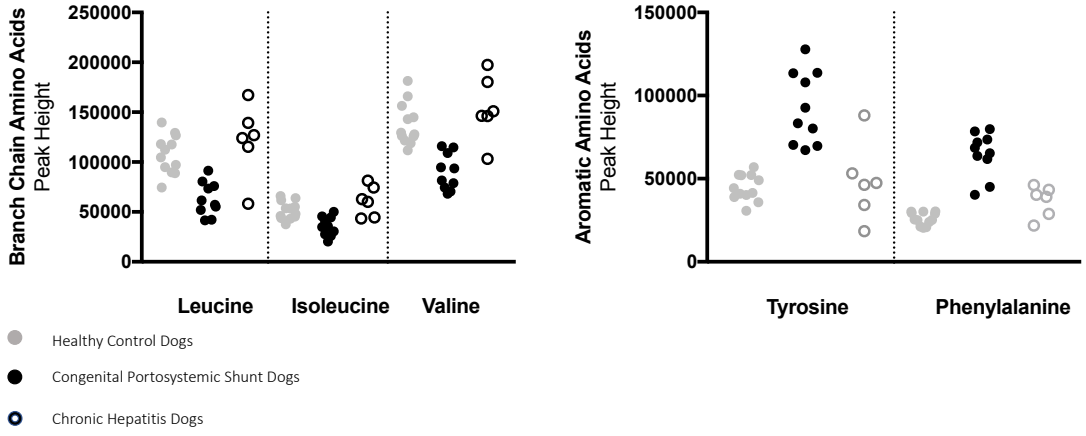
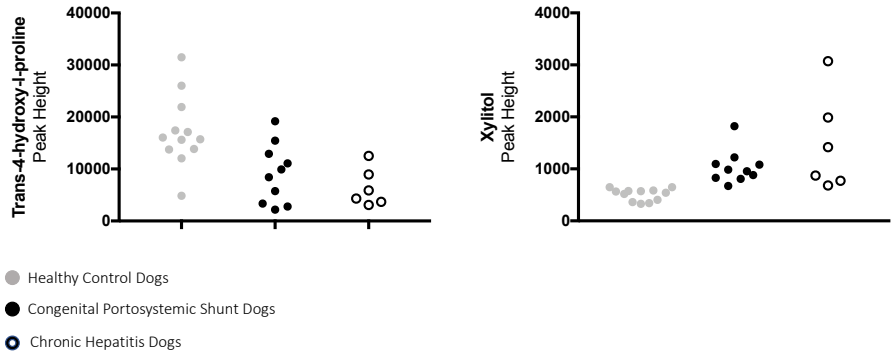


Figure 3.5 Selected serum metabolites with potential value as biomarkers of a congenital portosystemic shunt or chronic hepatitis dogs.

Each metabolite was identified by Random Forest and/or univariate analysis as important for the differentiation of dogs into either control, congenital portosystemic shunt, or chronic hepatitis groups. The serum abundance of trans-4-hydroxy-l-proline was significantly increased in control dogs compared to dogs with a congenital portosystemic shunt or chronic hepatitis. The serum abundance of xylitol was significantly decreased in control dogs compared to dogs with a congenital portosystemic shunt or chronic hepatitis. Reprinted with permission [39].



3.4. **Discussion**

This study identified extensive metabolic abnormalities in the serum of dogs diagnosed with a congenital portosystemic shunt or chronic hepatitis, with significant alterations in 50 of 126 named serum metabolites. These metabolites are known to be involved in a variety of processes, including aminoacyl-tRNA biosynthesis, branch chain amino acid biosynthesis and degradation, proline metabolism, phenylalanine metabolism, citrate cycle, and pantothenate and coenzyme A biosynthesis.

3.4.1. **Abnormal amino acid metabolism**

Significant abnormalities were observed in the state of conjugation and the relative amounts of individual amino acids in dogs with congenital portosystemic shunts and dogs with chronic hepatitis compared to healthy control dogs. Among these findings was a

significant increase in the abundance of the aromatic amino acids (AAAs) tyrosine and phenylalanine, and a significant decrease in the branched chain amino acids (BCAAs), leucine, isoleucine, and valine in dogs with a congenital portosystemic shunt (Figure 4). The decreased serum ratio of BCAAs to AAAs characterized of hepatic cirrhosis in humans and is attributable to several factors, including reduced nutritional intake, hypermetabolism, and ammonia detoxification by skeletal muscle [26]. A low serum BCAA/AAA ratio reduces biosynthesis and secretion of albumin in hepatocytes, and is also associated with a less favorable prognosis in human patients with chronic liver disease [27,28]. BCAAs are a constituent of protein and in addition are a source of glutamate, which detoxifies ammonia by glutamine synthesis in skeletal muscle [26]. The changes in the metabolism of BCAAs and AAAs that occur in liver disease may also have a role in the pathogenesis of end-stage liver disease complications, such as hepatic encephalopathy and hypoalbuminemia [29]. Additionally, the serum concentration of the AAA tyrosine has been found to increase early in the course of chronic liver disease in humans and to positively correlate with histologic fibrosis scores [30]. A low serum BCAA/AAA ratio was only observed in dogs with a congenital portosystemic shunt and may represent decreased hepatic function due to hypoperfusion consistent with previous studies [31,32]. This was not observed in the dogs in this study with chronic hepatitis, however none of these dogs had any evidence of acquired portosystemic shunting. The BCAA/AAA ratio is considered an indicator of hepatic insufficiency that decreases with the severity of hepatic dysfunction or porto-systemic shunting [32]. Further studies evaluating the BCAA/AAA ratio over the course of chronic liver disease in dogs and dogs with acquired portosystemic shunting are warranted. This is the first study to the author' knowledge to

examine the serum metabolome of dogs with chronic liver disease with an untargeted approach. Additional research to characterize the role of these metabolic changes in disease diagnosis, stratification, and treatment is warranted.

3.4.2. **Potential biomarkers for dogs with a congenital portosystemic shunt and dogs with chronic hepatitis**

A number of compounds in serum were individually identified for their value in distinguishing between healthy dogs, dogs with chronic hepatitis, and dogs with congenital portosystemic shunts. Random forest analysis identified 15 metabolites that were able to differentiate dogs with congenital portosystemic shunts (100% accuracy) and dogs with chronic hepatitis (83% accuracy) from apparently healthy dogs (Figure 3). There was a significant decrease in serum proline and hydroxyproline in the dogs with a congenital portosystemic shunt and dogs with chronic hepatitis compared to healthy control dogs (Figure 5). Hydroxyproline is a non-proteinogenic amino acid that is fairly specific to collagen in mammals and its serum concentration reflects collagen metabolism in tissues with a high metabolic turnover of this protein [33]. Additional research is warranted to determine the significance of this finding for collagen metabolism in canine patients with hepatic fibrosis and the role of this molecule as a marker of disease progression. There was also a significant increase in xylitol in the serum of dogs with chronic hepatitis and dogs with a congenital portosystemic shunt compared to healthy control dogs (Figure 5). Xylitol, a five-carbon polyalcohol is widely distributed in nature with trace amounts of xylitol produced by animals [34]. Although xylitol can enter almost all cells of an organism the liver cells are especially permeable [34]. This molecule, which is part of xylose metabolism has been found to be increased in the serum and urine of

humans with liver disease [17,35,36]. The role of this metabolite in liver disease has not been fully characterized.

3.4.3. **Future potential medical strategies**

There are limited evidence-based strategies for the medical management of dogs with chronic hepatitis or a congenital portosystemic shunt. A number of compounds identified in this study have a potential therapeutic role in the management of patient with liver disease. Studies in humans have shown that the intravenous or oral supplementation of BCAAs improves not only nutritional status, but also prognosis and quality of life in patients with chronic liver disease [14]. The presence and impact of a significant deficiency or over-abundance of any of these compounds will require validation in a targeted quantitative analysis.

3.4.4. **Study limitations**

Limitations of this study would include the influence of concurrent drug administration on our findings and the lack of inclusion of a group of dogs with other forms of hepatic disease such as acute hepatitis. Some of our findings may also be observed in dogs with extra-hepatic disease and the effect of diet on circulating metabolites was not considered. Control dogs were not assessed for portosystemic shunting; therefore, the possibility of subclinical portosystemic shunting in this group exists. These findings will need to be validated in a prospective targeted study in healthy control dogs, chronic hepatitis dogs, congenital portosystemic shunt dogs, and dogs with other forms of hepatic or gastrointestinal disease. Another limitation of the study reported here is the discrepancy in the ages of dogs with chronic hepatitis compared to healthy control dogs and dogs with a congenital portosystemic shunt. It is possible that the random forest analysis identified

some compounds simply on the basis of a difference in age between these groups of dogs. Since metabolites in serum are affected by the cumulative effect of complex physiological processes across all tissues in the body, it is not possible to definitively determine the organ, cell type, or intracellular compartment from which identified compounds originated. Furthermore, the impact of renal excretion versus reabsorption, and intestinal microbial metabolism on the types and quantity of compounds detected in the serum is unknown. Metabolomic investigation of the urine of dogs with chronic hepatitis and congenital portosystemic shunting compared to healthy control dogs would provide considerable additional insight into the metabolic abnormalities identified in this study.

3.4.5. **Conclusion**

The untargeted metabolomic profiling of serum from dogs with chronic hepatitis or dogs with a congenital portosystemic shunt demonstrated significant semi-quantitative differences in 50 of 126 named serum metabolites, including branch chain amino acids, aromatic amino acids, xylitol, and hydroxyproline. Further validation of these results in a targeted study and determination of their utility as clinical biomarkers is warranted and under way.

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4. DEVELOPMENT, VALIDATION, AND APPLICATION OF A LC-MS/MS METHOD FOR THE QUANTITATIVE DETERMINATION OF TRANS-4-HYDROXY-L-PROLINE IN THE SERUM OF DOGS WITH CHRONIC HEPATITIS[*]

4.1. **Introduction**

Chronic hepatitis is the most common liver disease in dogs [1]. The cause of chronic hepatitis in dogs is often unknown and called idiopathic chronic hepatitis. Recognized causes of chronic hepatitis in dogs include hepatic accumulation of copper and drug-induced liver injury. Chronic hepatitis is histologically characterized by hepatocellular apoptosis or necrosis, a variable mononuclear or mixed inflammatory infiltrate, regeneration, and fibrosis [1]. The prognosis for dogs with chronic hepatitis is guarded, with variable progression to end-stage liver disease and fibrosis. Fibrosis is defined by the overgrowth, hardening, and scarring of various tissues and is attributed to excess deposition of extracellular matrix components, including collagen [2]. Efforts have been made to replace examination of liver biopsy specimens with measurement of noninvasive markers of liver fibrosis to aid in diagnosis and monitoring response to treatment in dogs with liver disease.

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Complications of cirrhosis or end-stage liver disease are the primary causes of death related to chronic hepatic diseases. Progressive liver fibrosis resulting in the development of end-stage liver disease is characteristic of chronic liver disease; thus, the assessment of liver fibrosis can be a predictive factor for liver-related fatalities. The stage of liver fibrosis has been correlated with mortality rates and prognosis in chronic hepatic diseases of dogs [3,4] and humans [5]. Noninvasive markers of liver fibrosis are desired.

Trans-4-hydroxy-L-proline is a nonproteinogenic, nonessential amino acid found in collagen and other extracellular proteins. Trans-4-hydroxy-L-proline is an important constituent of collagen and is a collagen-specific amino acid, with approximately 1% of the amino acid found in elastin [6]. Serum trans-4-hydroxy-L-proline concentration has been correlated with fibrosis in humans with chronic hepatic disease [7,8].

The purpose of the study reported here was to develop and validate a simple, efficient, and reliable LC-MS-MS method for the quantitative determination of endogenous trans-4-hydroxy-L-proline in canine serum. The clinical applicability of the method was assessed by analyzing the trans-4-hydroxyl-L-proline content in the serum of dogs with chronic hepatitis. We hypothesized that dogs with chronic hepatitis would have higher serum concentrations, compared with concentrations in healthy control dogs.

4.2. **Materials and methods**

4.2.1. **Samples**

Blood samples previously collected from 20 dogs with histologically confirmed chronic hepatitis and from 20 healthy control dogs that had been examined at the Texas A&M

University Veterinary Teaching Hospital from November 2014 through December 2015 were used for the study. Blood samples were immediately centrifuged at 1,800 X g for 10 minutes at 4°C; serum was harvested and stored at −80°C until analysis. For dogs that underwent laparoscopic liver biopsy or exploratory celiotomy with surgical liver biopsy and removal of bile from the gallbladder as part of standard diagnostic testing, a 1-mL sample of bile and 1 additional liver biopsy specimen were collected for an unrelated unpublished study. Four to 6 liver biopsy specimens were acquired during a typical liver biopsy. Of these, 1 biopsy specimen was placed in a sterile red top tube and used for tissue copper quantification, 1 biopsy specimen was placed in a sterile specimen cup and submitted for aerobic and anaerobic bacterial culture and susceptibility testing, and 1 to 3 biopsy specimens were fixed in neutral-buffered formalin, processed for routine histologic examination, and embedded in paraffin. Serial sections of liver tissue were stained with H&E, eosin, or Picrosirius Red[a] stains or a combination of these stains. Hepatic fibrosis, inflammation, and hepatocellular vacuolation were scored by a single board-certified veterinary pathologist who was not aware of the clinical data; scores were assigned by use of an established 5-point scoring system (0 = absent, 1 = mild, 2 = moderate, 3 = marked, and 4 = very marked). Informed client consent was obtained for each dog, and the study was approved by the Texas A&M University Institutional Animal Care and Use Committee (animal use protocols No. 2017-0351CA and 2015-0043).

4.2.2. **LC-MS-MS**

The LC-MS-MS system consisted of a high-performance liquid chromatography system[b] and a mass spectrometer.[c] Sample acquisition and data analysis were performed with commercial software.[d] A C18 column[e] was used to achieve chromatography separation.

The mobile phase consisted of 0.1% formic acid (solvent A) and 0.1% formic acid in methanol (solvent B). The gradient was 0 to 5 minutes, 10% solvent B to 40% solvent B; 5 to 7 minutes, 40% solvent B to 95% solvent B; 7 to 9 minutes, 95% solvent B; 9 to 9.1 minutes, 95% solvent B to 10% solvent B; and 9.1 to 13 minutes, 10% solvent B. Injection volume was 10 μL, total assay time was 13.1 minutes, and flow rate was 0.4 mL/min.

The mass spectrometer was operated with electrospray ionization in the positive ion mode. Instrument parameters and transitions were optimized, with flow infusion set at a rate of 10 μL/min. Electrospray parameters used were static spray voltage of 3,500 V, ion transfer tube temperature of 325°C, and vaporization temperature of 350°C. Sheath gas, auxiliary gas, and sweep gas were 45, 13, and 1 Arb, respectively. Collision gas was at 200 Pa. Injector needles were washed with water and acetonitrile between successive injections. Autosampler carryover was monitored by performing blank injections. Column performance was monitored by measuring retention times.

4.2.3. **Standard solutions, calibrations, and internal control samples**

Trans-4-hydroxy-L-proline[f] (purity ≥ 99%) and trans-4-hydroxy-L-proline-2,5,5-D3g (purity > 98%) were used as internal standards. High-performance liquid chromatography–grade acetonitrile[h] was purchased, and 18 MOhm water was purified with a water purification system.[i]

Stock solutions (concentration, 0.1 mg/mL) were prepared by dissolving 0.1 mg of D3-trans-4-hydroxy-L-proline in 1 mL of water. Standard working solutions were then prepared by diluting stock solutions with water to obtain concentrations of 0.5, 1, 2.5, 5,

10, 25, 50, 100, and 250 ng/mL. Calibration standards were prepared by spiking 50 μL of blank canine serum with a freshly prepared working solution to achieve concentrations of 0.5, 1, 2.5, 5, 10, 25, 50, 100, and 250 ng/mL; solutions were then extracted with 100% acetonitrile to achieve standard solutions with concentrations of 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2.5 ng/mL.

4.2.4. **Sample preparation**

Canine serum samples (50 μL) were thawed at room temperature (21°C) and placed in a 1.5-mL Eppendorf tube; 200 μL of 100% acetonitrile was added, and the mixture was mixed in a vortex device. Samples were then centrifuged at 17,500 X g at 4°C for 5 minutes. An aliquot (200 μL) of supernatant was transferred to a clean 1.5-mL Eppendorf tube. Another 50 μL of supernatant was transferred to a 2-mL glass vial with a glass insert; a sample of 10 μL was removed from this glass vial and injected into the LC-MS-MS system.

4.2.5. **Analytic validation**

The LC-MS-MS method was analytically validated in accordance with US FDA guidelines[10] with respect to linearity, accuracy, precision, and recovery.

4.2.6. **Linearity**

Linearity of the assay was tested on 6 replicates of each of 3 concentrations of DQCs (2X, 5X, and 10X); the DQCs were dilutions of a high concentration (5.0 ng/mL) of a quality control standard solution. Calculated concentration measurements were compared to the nominal concentration for each DQC.

4.2.7. **Selectivity**

Selectivity of the method was assessed by comparing chromatogram responses of 6 lots of naïve canine serum with canine serum spiked with the internal standard.

4.2.8. **Calibration curve and the LLOQ**

Calibration standards were prepared by spiking canine serum samples with 0.5 μL of working standard solutions or the internal standard. A calibration curve was constructed with a linear range of 0.01 to 2.50 ng/mL. Calibration curves were prepared for each analytic run by plotting the back-calculated concentration against the nominal concentration. Linearity was assessed by use of linear regression with appropriate weighting. The LLOQ was determined to be acceptable when 5 replicates of the lowest calibration standard had accuracy and precision deviations of < 20%.

4.2.9. **Accuracy and precision**

Accuracy was evaluated for 4 quality control samples on 5 consecutive days. Accuracy was determined as the percentage difference between the mean of observed concentrations and the theoretical concentration (relative error); this value was required to be within ± 10% for all samples. Intra-assay and inter-assay precision was determined by assaying 3 samples at each of 3 quality control concentrations in 5 assays and on 5 consecutive days, respectively. The CV was used to express precision; CV values were required to be ≤ 10%.

4.2.10. **Recovery**

Recovery was determined by comparing the peak areas of extracted endogenous samples with low, mid, and high concentrations (1.8, 2.8, and 4.8 ng/mL, respectively) with the peak areas for postextraction spiked samples. Recovery percentage of analyte was

calculated by dividing peak areas of the analyte obtained for the extracted exogenous samples with those of postextraction spiked samples.

4.2.11. **Matrix effect**

Matrix effect of canine serum on the analysis of trans-4-hydroxy-L-proline concentrations was determined by comparing peak areas of the analyte in extracted blank serum samples to those obtained for standard solutions at the corresponding concentrations. The matrix effect was evaluated at 3 quality control concentrations (0.01, 0.10, and 0.50 ng/mL).

4.2.12. **Statistical analysis**

Differences between groups were analyzed for statistical significance by the Mann-Whitney U and Kruskal-Wallis test where appropriate using commercially available software.[j] Significance was set at $p < 0.05$.

4.3. **Results**

4.3.1. **Sample**

Serum samples were obtained from 20 dogs with chronic hepatitis and 20 healthy control dogs. The dogs with chronic hepatitis comprised 9 Labrador Retrievers, 4 Doberman Pinchers, 4 crossbreds, 1 Dachshund, 1 Rat Terrier, and 1 Vizla. There were 11 spayed females, 6 castrated males, 2 sexually intact males, and 1 sexually intact female. Fibrosis score was 1 for 3 dogs, 2 for 9 dogs, 3 for 6 dogs, and 4 for 2 dogs. Median age of the dogs was 9 years (range, 3 to 9 years). The healthy dogs comprised 4 Labrador Retrievers, 3 crossbreds, 2 Boston Terriers, 2 Golden Retrievers, and 1 each of Alaskan Malamute, American Bull Terrier, Australian Shepherd, Boxer, Chihuahua, English Cocker Spaniel, Miniature Schnauzer, Schipperke, and Siberian Husky. There were 9 castrated males, 8 spayed females, 2 sexually intact females, and 1 sexually intact male. Median age of the

dogs was 3 years (range, 0.5 to 12 years). The dogs with chronic hepatitis were significantly ($P = 0.007$) older than the control dogs.

4.3.2. **LC-MS-MS**

Fragment ion spectra, including quantification and confirming ions, of trans-4-hydroxy-L-proline and the deuterated internal standard D3-trans-4-hydroxy- L-proline were m/z 132 and m/z 135.1, respectively (Figure 1). Trans-4-hydroxy- L-proline and the internal standard D3-trans-4-hydroxy- L-proline were detected under optimized conditions (Figure 2). There were no substantial interfering peaks for blank canine serum at the retention times of trans-4-hydroxy-L-proline and D3-trans-4-hydroxy-L-proline.

4.3.3. **Calibration and linearity**

The calibration curve for trans-4-hydroxy-L-proline in canine serum was linear over the concentration range of 0.01 to 2.50 ng/mL (r = 0.9997) and had consistent slope values when evaluated by use of weighted (1/2X) linear regression. The equation for the slope of the calibration curve of trans-4-hydroxy-L-proline was y = 9,000,000x – 69,198, and the LLOQ was 0.005 ng/mL.

4.3.4. **Precision and accuracy**

Intra-day and interday precision and interday accuracy were determined for measurement of trans-4-hydroxy-L-proline in canine serum. Intraday precision (CV) for measurement of trans-4-hydroxy-L-proline in canine serum ranged from 2.1% to 3.0% (Table 1). Interday precision (CV) for measurement of trans-4-hydroxy-L-proline in canine serum ranged from 3.2% to 5.3%. Interday accuracy (relative error) for measurement of trans-4-hydroxy-L-proline in canine serum ranged from –0.5% to 7.8% (Table 2).

Table 4.1.—Precision and of LC-MS-MS analysis of the trans-4-hydroxy-l-proline concentration in canine serum.
Reprinted with permission [22].

Sample		Measured mean ± SD (ng/mL)	Precision (%) *
Intraday (n = 5)			
	1	0.81 ± 0.03	3.0
	2	1.75 ± 0.04	2.1
	3	4.26 ± 0.12	2.5
Interday (n = 5)			
	1	0.81 ± 0.05	5.3
	2	1.72 ± 0.09	4.5
	3	4.23 ± 0.15	3.2

*Precision represents the CV.

Table 4.2.—Accuracy of LC-MS-MS analysis of the trans-4-hydroxy-l-proline concentration in canine serum.
Reprinted with permission [22].

Nominal concentration (ng/mL	Measured mean ± SD (ng/mL)	Accuracy (%) *
0.10	0.10 ± 0.001	–0.5
0.25	0.24 ± 0.010	5.5
0.50	0.46 ± 0.010	7.8
1.00	1.02 ± 0.010	–2.3

*Accuracy was evaluated for 4 quality control samples on 5 consecutive days and is reported as the relative error.

4.3.5. **Recovery**

Extraction recovery was calculated by comparing the peak areas of extracted endogenous canine serum samples with low, mid, and high concentrations with those of postextraction spiked samples. Recovery for trans-4-hydroxy-L-proline was 48.9% for 1.8 ng/L, 55.1% for 2.8 ng/mL, and 47.2 % for 4.8 ng/mL (overall mean recovery, 50.4%).

4.3.6. **Matrix effect**

A possible matrix effect was evaluated for 3 concentrations during method development of the assay for measurement of trans-4-hydroxy-L-proline in canine serum (Table 3). Overall mean matrix effect of blank serum was 25.1%.

Table 4.3.—Matrix effect of canine serum on the trans-4-hydroxy-L-proline concentration.
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Observed concentration (ng/mL)	Peak area when dissolved in matrix serum (arb. unit)	Peak area when dissolved in mobile phase (arb. unit)	Matrix effect (%)
0.01	93,223	116,247	19.8
0.10	738,634	1,187,546	37.8
0.50	3,570,732	4,334,020	17.6

4.3.7. **Assessment for clinical application**

Median concentration of trans-4-hydroxy-L-proline in the serum of dogs with chronic hepatitis (0.24 ng/mL) was significantly ($P = 0.007$) lower than the median concentration in the serum of healthy control dogs (0.78 ng/mL; Figure 3). Concentration of trans-4-hydroxy-L-proline for dogs with mild fibrosis (fibrosis score, 1 or 2) was significantly (P = 0.02) lower that of healthy control dogs (0.20 and 0.78 ng/mL, respectively). There was not a significant difference in concentrations between dogs with mild or marked (fibrosis score, 3 or 4) fibrosis and between dogs with marked fibrosis and healthy control dogs (Figure 4).

4.4. **Discussion**

An LC-MS-MS method for the quantification of trans-4-hydroxy-L-proline in canine serum was developed and analytically validated in the study reported here. Linearity of the calibration curve was indicated by a high correlation coefficient (r > 0.9997), which

indicated a strong correlation between the peak area ratio and the concentration of trans-4-hydroxy-L-proline in the linear range. The method described had a linear range of 0.01 to 2.50 ng/mL, and the LLOQ was 0.005 ng/mL. Selectivity for trans-4-hydroxy-L-proline was indicated by identification of the peak without interference. Intraday and interday precision and accuracy were within 15%, as stipulated by FDA guidelines. Mean percentage recovery was 50.4%; however, recoveries for trans-4-hydroxy-L-proline and the deuterated internal standard were consistent and similar, which allowed adjustment of the low recovery and fulfilled FDA validation guidelines. The method had acceptable performance in terms of sensitivity (ie, LLOQ), precision, and accuracy and had a relatively short run time for analysis and relatively simple sample preparation. Trans-4-hydroxy-L-proline concentrations were significantly lower in the serum of dogs with chronic hepatitis, compared with concentrations in serum of healthy control dogs.

Hydroxyproline is fairly specific for collagen in mammals, and serum concentrations of hydroxyproline are a reflection of collagen metabolism in tissues with a high metabolic turnover of this protein [11]. The quantity of hydroxyproline in the liver of rats [12,14], humans [13], and dogs [15] were increased in proportion to the degree of hepatic fibrosis. During conditions of increased fibrogenesis (eg, hepatic fibrosis), serum concentrations of hydroxyproline are increased in humans [7] and rats [21].

Results of the present study that indicated lower serum hydroxyproline concentrations in dogs with chronic hepatitis than in control dogs were unexpected. The healthy control dogs used in the study were screened on the basis of a client questionnaire, evaluation of

serum biochemical values, and results of liver function testing. However, the possibility of occult histologic liver disease cannot be completely excluded without examination of liver biopsy specimens. Although hydroxyproline is relatively specific for collagen, increased tissue concentrations have also been detected in patients with fibrotic pulmonary disease [16], fibrotic cardiac disease [17], and fibrotic renal disease [18]. The effect of fibrosis in nonhepatic organs on serum hydroxyproline concentrations is unknown and was not examined in the present study. The possibility of concurrent or occult nonhepatic fibrosis cannot be excluded, and the effect on serum hydroxyproline concentrations could not be determined in the study reported here.

The serum concentration of hydroxyproline in dogs with mild hepatic fibrosis were lower than in healthy control dogs in the present study; evaluation of a larger group of dogs with a wider range of fibrosis scores may be warranted. Serum hyperprolinemia in humans is a specific feature of alcoholic liver cirrhosis [6,8]. Human patients with nonalcoholic cirrhosis or other forms of chronic liver disease have serum proline and hydroxyproline concentrations indistinguishable from those of healthy control subjects [6,8]. On the other hand, patients with alcoholic hepatitis have serum proline and hydroxyproline concentrations significantly higher than those in patients with other forms of liver disease [6,8]. It has been hypothesized that alcohol inhibits proline metabolism or facilitates its release from hepatocytes [6,8]. The pathophysiology of alcohol-induced serum hyperprolinemia in humans with chronic hepatitis likely differs from that of dogs with chronic hepatitis because dogs do not develop alcohol-induced hepatitis.

Trans-4-hydroxy-L-proline was significantly lower in the serum of dogs with chronic hepatitis than in the serum of healthy control dogs (median concentrations of 0.20 and 0.78 ng/mL, respectively. Age-related changes in the liver have been described in humans and includes a decrease in volume, decrease in blood flow, mitochondrial dysfunction, and an increase in susceptibility to fibrosis [19]. Age-related fibrosis has not been characterized in humans. The groups of dogs were not matched on the basis of age in the study reported here. The serum available for the control group was obtained from dogs owned by veterinary students and faculty and staff of the veterinary teaching hospital, and these dogs typically were young. Therefore, we cannot rule out that the difference in the concentration of hydroxy-L-proline between groups reflected a difference attributable to age. Age-matched healthy control dogs with histologic confirmation of disease status would be needed to more accurately assess the trans-4-hydroxy-L-proline concentrations in the control dogs. Therefore, age may have contributed to the finding of a lower serum trans-4-hydroxy-L-proline concentration in dogs with mild fibrosis, which may have included patients of all ages that may have had occult liver disease, compared with the concentration in healthy control dogs.

Furthermore, total urinary hydroxyproline concentration normalized on the basis of the urine creatinine concentration was found to be a better indicator of collagen metabolism in bone disease than was the serum hydroxyproline concentration [20] To the authors' knowledge, total urinary hydroxyproline concentration has not been evaluated in dogs with liver disease and serum proline or trans-4-hydroxy-L-proline concentrations have not been evaluated in dogs with hepatobiliary disease. A larger study may be warranted to

determine whether lower serum trans-4-hydroxy-L-proline concentrations are a feature of dogs with chronic hepatitis, are correlated with the fibrosis score, and can be used as a noninvasive biomarker of hepatic fibrosis in this species. A noninvasive biological marker of liver fibrosis would not replace histologic examination of a biopsy specimen, but it could be used to assess response to therapeutic interventions and as an objective tool for prognostic assessment. Additional research is also warranted to determine the importance of decreased serum trans-4-hydroxy-L-proline concentrations for collagen metabolism in canine patients with hepatic fibrosis and the role of this marker molecule in disease progression.

4.5. **Footnotes**

a. Picrosirius Red staining kit, Polysciences, Warrington, Pa.

b. UltiMate 3000 HPLC, ThermoFisher Scientific, Waltham, Mass.

c. TSQ Quantiva triple-quadrupole mass spectrometer, ThermoFisher Scientific, Waltham, Mass.

d. TraceFinder software, version 3.3, ThermoFisher Scientific, Waltham, Mass.

e. Synergi 4 μm fusion–RP, 150 mm X 2 mm, Phenomenex, Torrance, Calif.

f. TLC, Sigma-Aldrich, St Louis, Mo.

g. CDN Isotopes, Pointe-Claire, QC, Canada.

h. Millipore, Milford, Mass.

i. EMDMillipore, Burlington, Mass.

j. GraphPad Prism Software, La Jolla, CA

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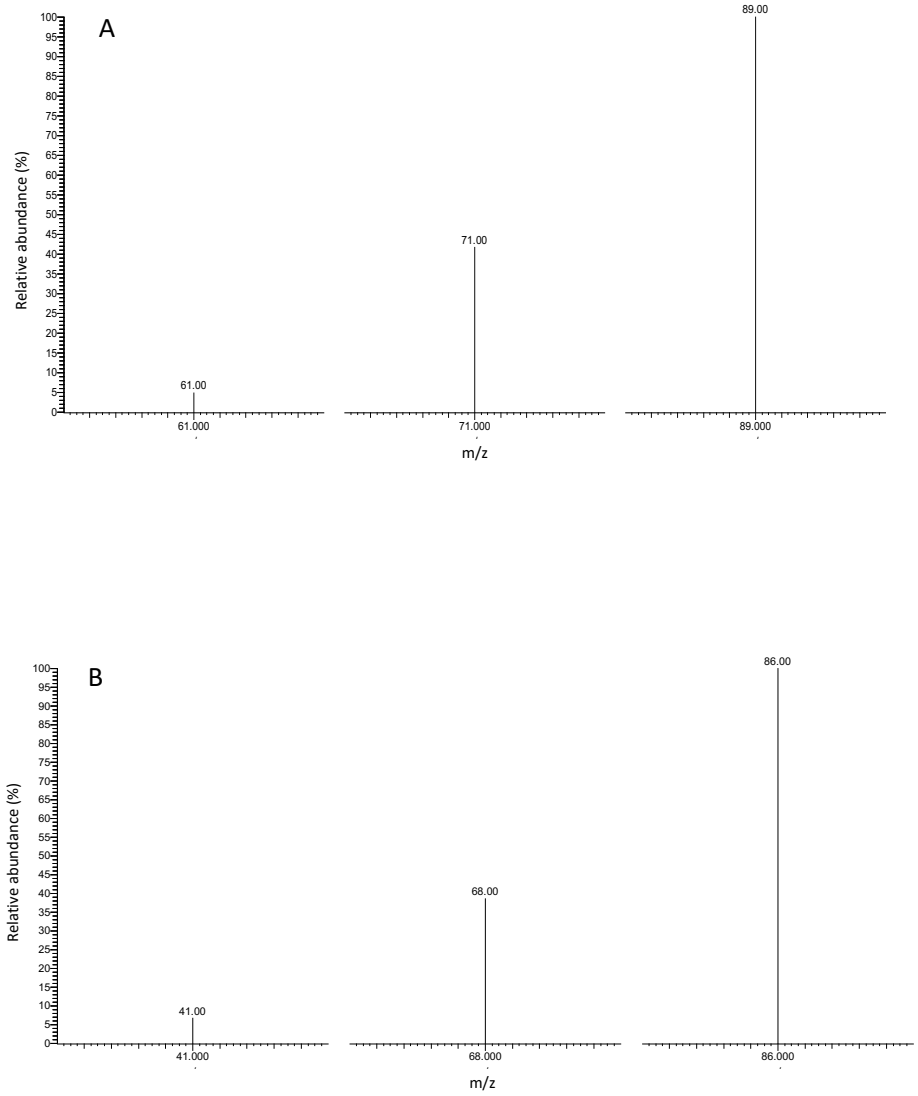


Figure 4.1. Product spectra for LC-MS-MS of trans-4-hydroxy-l-proline (A) and trans-4-hydroxy-l-proline-2,5,5-D3 (B).
Reprinted with permission [22].

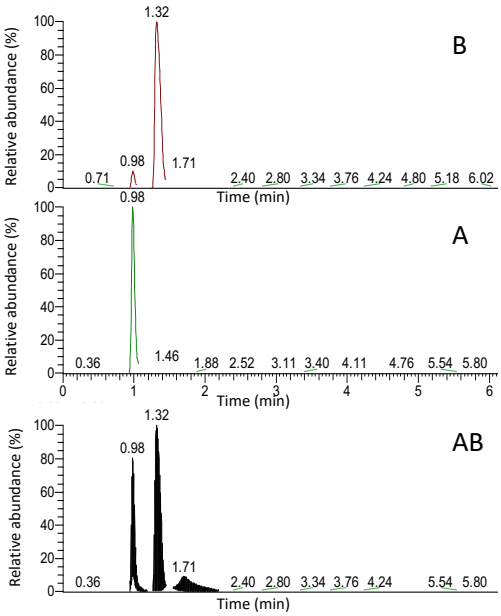


Figure 4.2. The LC-MS-MS chromatogram for canine serum with endogenous trans-4-hydroxy-L-proline (A), canine serum spiked with an internal standard (trans-4-hydroxy-L-proline-2,5,5-D3; B), and the summation of both (C). Reprinted with permission [22].

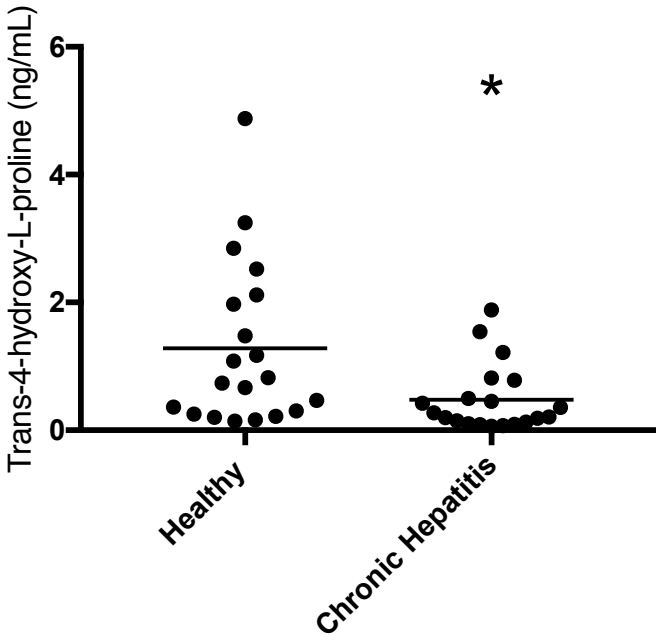


Figure 4.3. Serum trans-4-hydroxy-L-proline concentrations in 20 dogs with chronic hepatitis and 20 heathy control dogs. Each circle represents results for 1 dog, and the horizontal line represents the median for the group. *Median value differs significantly (P = 0.007) from the median value for the healthy dogs.
Reprinted with permission [22].

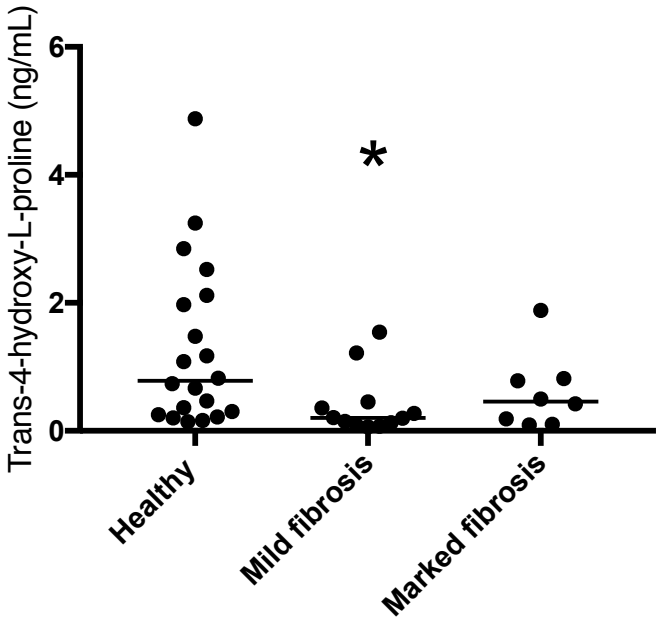


Figure 4.4. Serum trans-4-hydroxy-l-proline concentrations in 12 dogs with chronic hepatitis and mild fibrosis, 8 dogs with chronic hepatitis and marked fibrosis, and 20 healthy control dogs. *Median value differs significantly (P = 0.02) from the median value for the healthy dogs. See Figure 3 for remainder of key.
Reprinted with permission [22].

5. UNTARGETED METABOLOMIC PROFILING OF URINE FROM DOGS WITH CHRONIC HEPATIC DISEASE

5.1. **Introduction**

Chronic hepatic disease includes idiopathic chronic hepatitis, copper-associated chronic hepatitis, drug-associated chronic hepatitis, breed-associated metabolic errors, congenital portosystemic vascular anomalies, hepatocellular carcinoma, lobar dissecting hepatitis, and granulomatous hepatitis [1]. Differentiating these diseases can pose a diagnostic challenge due to the similarities of clinical signs and laboratory findings between different diseases. Although an early accurate diagnosis is important for an improved clinical outcome, achieving a definitive diagnosis can be cost prohibitive and invasive with the histological examination of a liver biopsy specimen regarded as the gold standard. The identification of non-invasive biomarkers that can reliably characterize chronic hepatic disease is desirable and could have clinical implications.

The liver is a central organ for regulation of metabolism, and a number of metabolic disturbances are seen in chronic liver disease patients [2,3]. Data from studies in human and animal models have documented alterations in hepatic lipid metabolism, protein metabolism, energy metabolism, cytokine metabolism, and increased generation of reactive oxygen species in patients with chronic liver disease [4-6]. Global metabolomic profiling, the untargeted quantification of small molecules in biologic samples, allows for a comprehensive analysis of changes in several metabolic and signaling pathways and their interactions [7-9]. This platform utilizes nuclear magnetic resonance spectroscopy or

mass spectrometry to measure low molecular weight metabolites permitting the formation of a metabolite profile. Metabolite profiles can be altered by a variety of physiological and pathological processes, and therefore global changes in such profiles may signal the presence of a particular disease [10,11]. Characteristic metabolite profiles that can discriminate between various types of liver disease have been identified in human studies [12-17]. There are no previous studies that have evaluated the urine metabolome of dogs with chronic hepatic disease to the authors' knowledge.

The aim of the present study was to compare the urine metabolome of healthy dogs, dogs with chronic hepatitis, dogs with hepatocellular carcinoma, and dogs with a congenital portosystemic shunt. We hypothesized that there would be a difference in urine metabolites between groups and that the four groups would have significantly different metabolomes.

5.2. **Materials and methods**

5.2.1. **Patients and procedures**

Adult dogs with definitively diagnosed chronic hepatic diseased diagnosed at Texas A&M University Veterinary Medical Teaching Hospital or Gulf Coast Veterinary Specialists between January 2011 and December 2014 were enrolled in this prospective observational study. The diagnosis of chronic hepatic disease was based on clinical signs, serum biochemical evaluation, diagnostic imaging findings, and the histological assessment of a liver specimen by a board-certified veterinary pathologist. Liver biopsy specimens were collected by diagnostic laparoscopy, where five to eight total specimens were collected from different liver lobes using forceps, or during celiotomy, where one to four wedge or

punch biopsies were collected from one or more liver lobes. The liver biopsy specimens were fixed in neutral buffered formalin for routine histological processing. Histological sections from formalin-fixed paraffin embedded tissue were stained with hematoxylin and eosin. Additional stains were performed at the discretion of the anatomic pathologist and attending clinician. Dogs with a suspected congenital portosystemic shunt were evaluated by ultrasonography as previously described [18]. Dogs presented for a wellness examination to the Small Animal Hospital at Texas A&M University, College Station, Texas were used as healthy controls. The health of these dogs was assessed by performing a physical examination, owner questionnaire, complete blood count, and a serum biochemistry profile. Dogs with clinically important abnormalities were excluded from the study. The study was approved by the Texas A&M University Institutional Animal Care and Use Committee (Animal Use Protocol #2014-0320). Informed client consent was obtained for each dog prior to enrollment in the study.

5.2.2. **Urine sample collection**

Urine from all dogs was obtained by cystocentesis. Urine samples were at ambient temperature for less than 30 minutes before being placed on ice, and then frozen within 1 hour of sampling for storage at -80°C. Samples collected at Gulf Coast Veterinary Specialists were kept at -80°C until shipment on dry ice to Texas A&M University, where they were stored at -80°C. All dogs had free access to water; however, food was withheld for a minimum of 12 hours prior to urine sample collection.

5.2.3. **Urine metabolome analysis**

Untargeted metabolomics analysis was performed by the West Coast Metabolomics Center at the University of California (Davis, CA) on a fee-for-service basis. Urine aliquots were normalized by urine creatinine concentration measured by a SIRRUS Clinical Chemistry Analyzer and extracted by degassed acetonitrile. Internal standards C8-C30 fatty acid methyl ethers were added, and the samples were derivatized by methoxyamine hydrochloride in pyridine and subsequently by N-methyl-N-trimethylsilyltrifluoroacetamide for trimethylsilylation of acidic protons. Analytes were separated using an Agilent 6890 gas chromatograph (Santa Clara, CA) and mass spectrometry was performed on a Leco Pegasus IV time of flight mass spectrometer (St. Joseph, MI) following a published protocol [19]. Unnamed peaks were excluded from statistical analysis.

5.2.4. **Statistical analysis**

5.2.4.1. **Univariate analysis**

Univariate analysis was performed using JMP Pro 13 (JMP Software, SAS Software Inc., Marlow, England). The dataset was tested for normality using the Shapiro-Wilk test and because it did not meet the assumptions of normal distribution, comparisons between healthy dogs, dogs with chronic hepatitis, dogs with hepatocellular carcinoma, and dogs with a congenital portosystemic shunt groups were determined using the non-parametric Kruskal-Wallis test. The resulting p-values were adjusted for multiple comparisons using the Benjamini-Hochberg's False Discovery Rate and significance was set at $q < 0.10$ [20]. Post hoc testing was performed with Dunn's Test of multiple comparisons using JMP Pro 13 and significance was set at $q < 0.05$.

5.2.4.2. **Multivariate analysis**

Data reported as peak height were log transformed prior to multivariate analysis. Principle component analysis, random forest analysis, and hierarchical cluster analysis was performed using MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) [21].

5.3. **Results**

5.3.1. **Selection criteria of case and control dogs**

There were 10 healthy control dogs enrolled in the study based on a normal physical examination, owner questionnaire, complete blood count, serum biochemistry profile. and the absence of any reported clinical signs. There were 10 dogs with chronic hepatitis and 6 dogs with hepatocellular carcinoma enrolled in the study based on histologic examination of a liver biopsy specimen. There were 5 dogs with a congenital portosystemic shunt enrolled in the study.

5.3.2. **Animal population**

The demographics of the healthy control dogs, dogs with chronic hepatitis, dogs with hepatocellular carcinoma, and dogs with a congenital portosystemic shunt enrolled into the study are summarized in Table 1. Breeds of dogs included in each study group are summarized in Table 2. There was a significant difference in the distribution of age and sexes between groups.

Table 5.1. Summary of demographic information of dogs enrolled into the study.

Heading	Control (n = 10)	C. Hepatitis (n = 10)	H. Carcinoma (n = 6)	Congenital PSS (n = 5)	p-value
Age in years (median, range)	4.0, 1-8[A]	10.8, 2-12 [AB]	10.5, 5-13[B]	3.0, 0.5-5 [A]	0.001
Sex (M/F)	5/5[A]	5/5[A]	6/0[B]	5/0[B]	0.033

C. Hepatitis – Chronic hepatitis, H. Carcinoma – Hepatocellular carcinoma, Congenital PSS – Congenital portosystemic shunt, M – intact or castrated male, F – intact or spayed female, groups with same superscript are not significantly different.

Table 5.2. Breed distribution of dogs enrolled into the study.

Group	Breed
Healthy	English Setter
Healthy	Golden Retriever
Healthy	Crossbreed Dog
Healthy	Crossbreed Dog
Healthy	Crossbreed Dog
Healthy	English Pointer
Healthy	Standard Schnauzer
Healthy	Crossbreed Dog
Healthy	Boston Terrier
Healthy	Boxer
Chronic Hepatitis	Crossbreed Dog
Chronic Hepatitis	Bassett Hound
Chronic Hepatitis	Labrador Retriever
Chronic Hepatitis	Cirneco Dell'Etna
Chronic Hepatitis	Miniature Schnauzer
Chronic Hepatitis	West Highland White Terrier
Chronic Hepatitis	Crossbreed Dog
Chronic Hepatitis	Labrador Retriever
Chronic Hepatitis	Yorkshire Terrier

Table 5.2 Continued

Group	Breed
Chronic Hepatitis	Australian Shepherd Dog
Hepatocellular Carcinoma	Crossbreed Dog
Hepatocellular Carcinoma	Chihuahua Dog
Hepatocellular Carcinoma	Crossbreed Dog
Hepatocellular Carcinoma	Siberian Husky Dog
Hepatocellular Carcinoma	Crossbreed Dog
Hepatocellular Carcinoma	Chihuahua Dog
Congenital Portosystemic Shunt	Pug
Congenital Portosystemic Shunt	Maltese Terrier
Congenital Portosystemic Shunt	Rat Terrier
Congenital Portosystemic Shunt	Miniature Schnauzer
Congenital Portosystemic Shunt	Maltese Terrier

5.3.3. **Differences in the urine metabolome between dogs with chronic hepatitis, hepatocellular carcinoma, and a congenital portosystemic shunt**

A total of 220 named urine metabolites were identified of which 37 differed significantly ($p \leq 0.05$; $q<0.10$) between healthy control dogs, dogs with chronic hepatitis, dogs with hepatocellular carcinoma, and dogs with a congenital portosystemic shunt (Table 3). Principle component analysis plots showed a clustering of the enrolled patients based on disease classification (Fig. 1). The distribution of the most significant metabolites separating the four groups was visualized with a heat map (Fig. 2). Random forest analysis identified metabolites that had the highest discriminatory power between the four groups (Fig. 3). Urine metabolites involved in glutathione, nitrogen, arginine, and proline metabolism and fatty acid biosynthesis that differed significantly between groups were plotted in Figs. 4 - 6.

Table 5.3. Urine metabolites that differed significantly between healthy control dogs, dogs with a congenital portosystemic shunt, dogs with hepatocellular carcinoma, and dogs with chronic hepatitis.
KW – Kruskal-Wallis H Test

Compound Name	p-value_KW	q-value_KW
gluconic acid	0.000251	0.021188
succinic acid	0.000331	0.021188
glutamine	0.000367	0.021188
glucose	0.000385	0.021188
mannose	0.000511	0.022470
sorbitol	0.000812	0.028368
glycyl proline	0.000904	0.028368
valine	0.001032	0.028368
2-deoxypentitol	0.001229	0.028483
gluconic acid lactone	0.001321	0.028483
putrescine	0.001457	0.028483
cellobiose	0.001554	0.028483
xylitol	0.002287	0.038710
3-ureidopropionate	0.003685	0.057910
tryptophan	0.003995	0.058586
citric acid	0.005117	0.069541
adenine	0.005374	0.069541
ethanolamine	0.005783	0.070113
threonine	0.006092	0.070113

Table 5.3 Continued

Compound Name	p-value_KW	q-value_KW
3-hydroxybutyric acid	0.006374	0.070113
heptadecanoic acid	0.007225	0.075687
palmitic acid	0.009100	0.079666
glycerol	0.009313	0.079666
tyrosine	0.009734	0.079666
lysine	0.010036	0.079666
2,5-dihydroxypyrazine	0.010055	0.079666
2-picolinic acid	0.010088	0.079666
pyrogallol	0.010139	0.079666
isomaltose	0.010669	0.080940
2,3-dihydroxybutanoic acid	0.011770	0.081349
2-hydroxybutanoic acid	0.012612	0.081349
ornithine	0.012808	0.081349
alloxanoic acid	0.013170	0.081349
2-deoxytetronic acid	0.013342	0.081349
stearic acid	0.013582	0.081349
ascorbic acid	0.013668	0.081349
glucoheptulose	0.013681	0.081349

Figure 5.1. Principal component analysis of the urine metabolome showing clustering of samples based on group. The scores plot showed a clustering of groups. CPSS – Congenital portosystemic shunt; HCC – Hepatocellular carcinoma

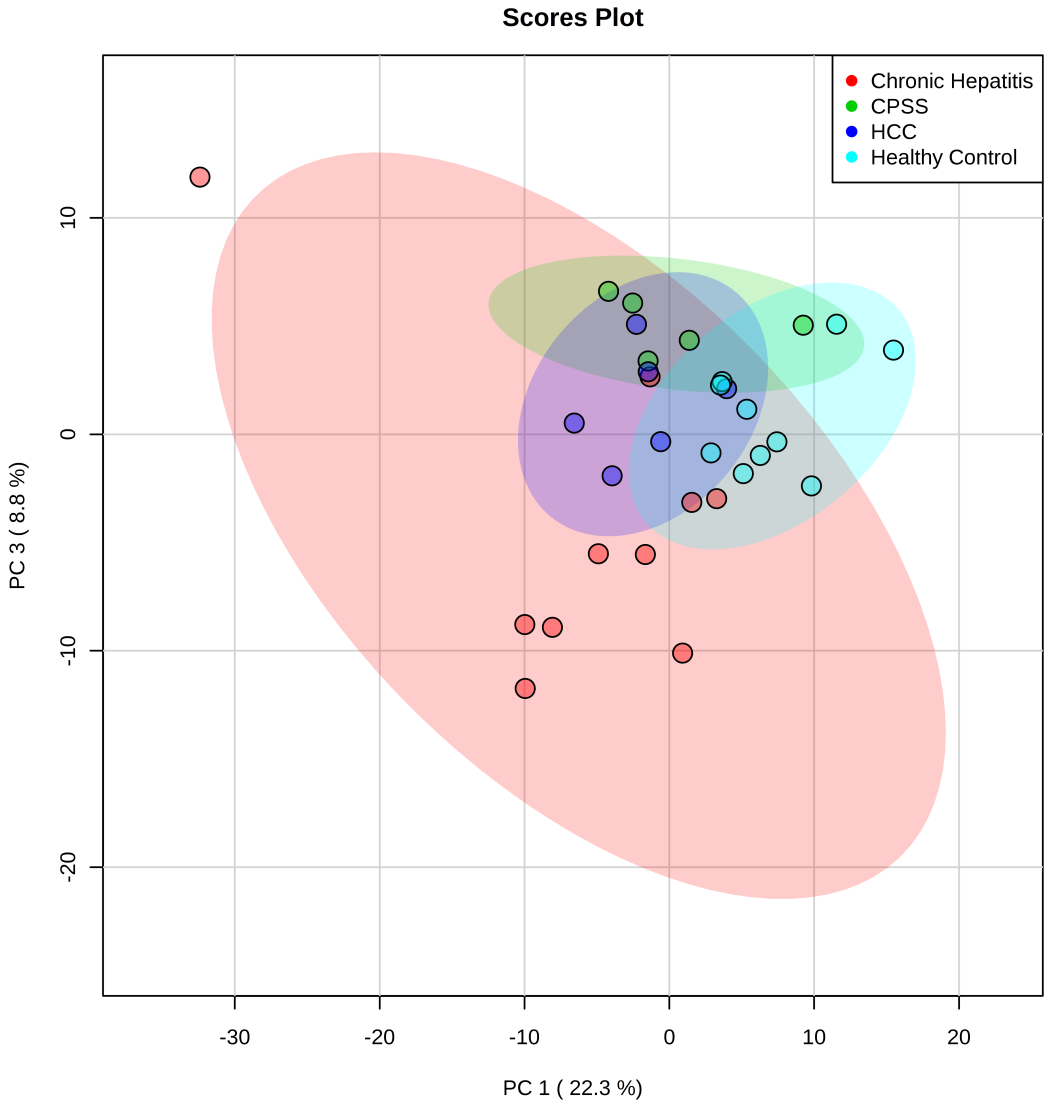


Figure 5.2. Heatmap of the most abundant metabolites in all groups, as identified by VIP scores in PLS-DA. Each sample is represented by a single column. The higher the intensity of the red color, the higher the abundance of the metabolite (CPSS – Congenital portosystemic shunt; HCC – Hepatocellular carcinoma).

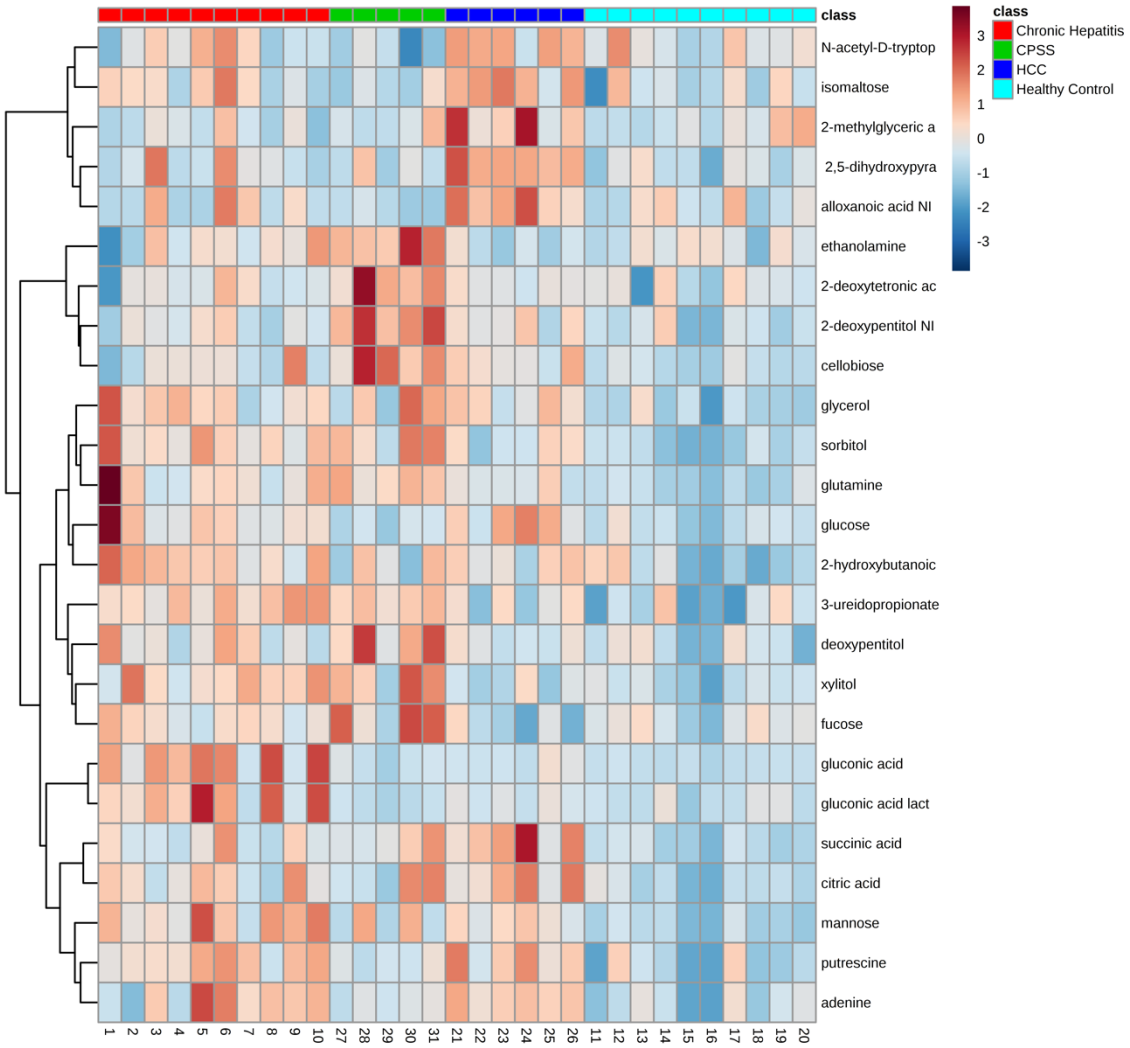


Figure 5.3. Top 15 metabolites in urine (left panel) in rank order of importance for differentiating between dogs with chronic hepatitis, dogs with a congenital portosystemic shunt (CPSS), dogs with hepatocellular carcinoma (HCC), and healthy control dogs.

Data were generated by random forest analysis. Compounds contributing the most to the accuracy of disease classification were identified as those having the highest mean decrease accuracy (MDA) of the predicted classification when permuted within the dataset.

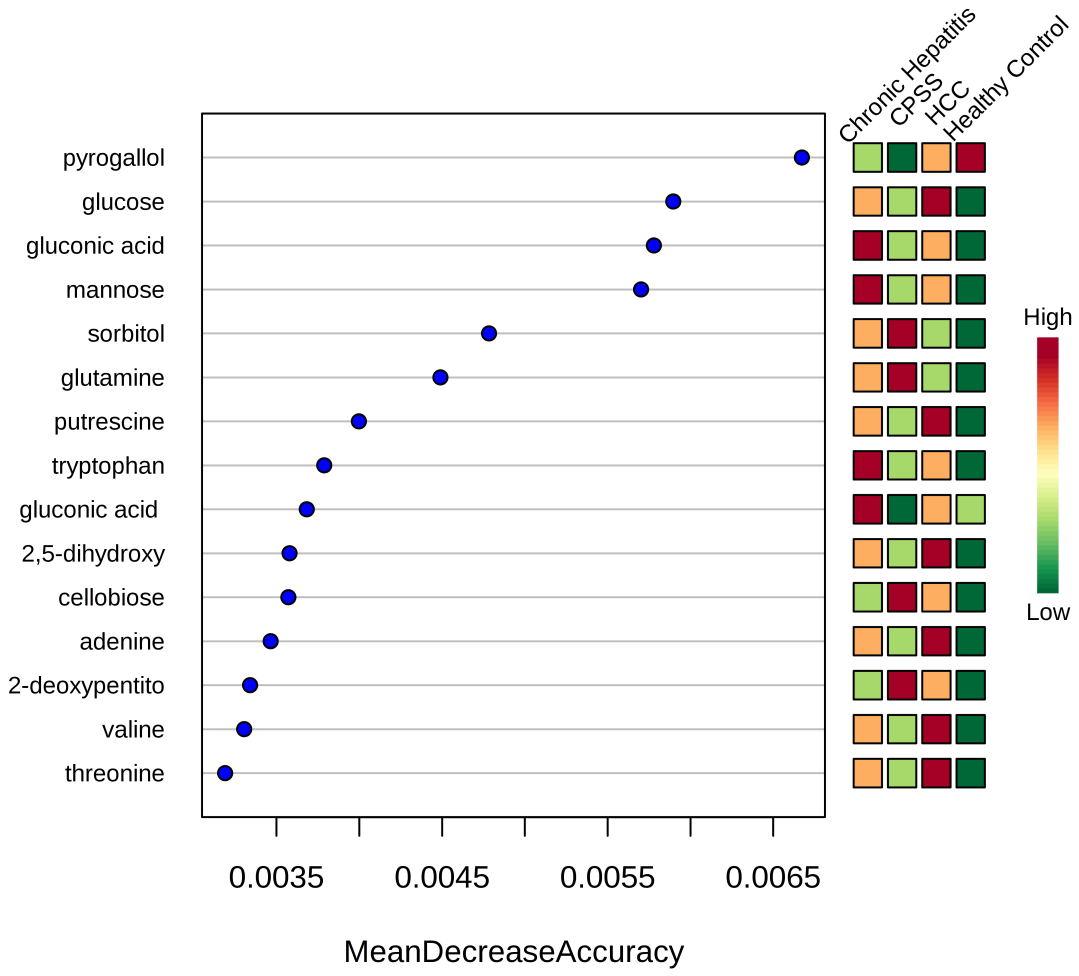


Figure 5.4. Urine metabolites of glutathione metabolism with potential value as a biomarker of hepatocellular carcinoma, chronic hepatitis, or a congenital portosystemic shunt in dogs.

Each metabolite was identified by random forest and/or univariate analysis as important for the differentiation of dogs into either control, congenital portosystemic shunt, hepatocellular carcinoma, or chronic hepatitis groups. Ornithine was significantly different between groups ($p = 0.013$) and increased in the urine of dogs with hepatocellular carcinoma compared to control dogs ($q = 0.04$). Putrescine was significantly different between groups ($p = 0.002$) and increased in the urine of dogs with chronic hepatitis and hepatocellular carcinoma compared to control dogs ($q = 0.006$, $q = 0.01$, respectively). Ascorbic acid was significantly different between groups ($p = 0.14$) and increased in the urine of dogs with hepatocellular carcinoma compared to dogs with a congenital portosystemic shunt ($q = 0.02$).

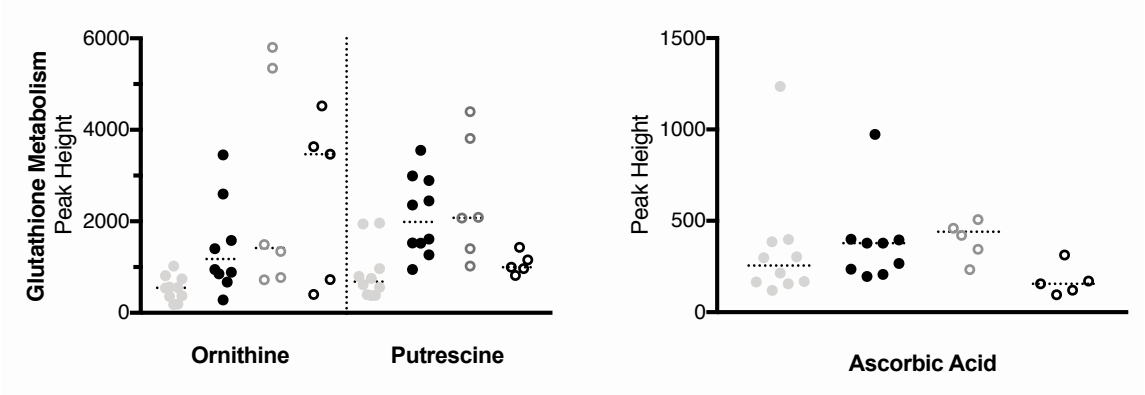


Figure 5.5. Urine metabolites of nitrogen metabolism, arginine, and proline biosynthesis with potential value as a biomarker of hepatocellular carcinoma, chronic hepatitis, or a congenital portosystemic shunt in dogs.

Each metabolite was identified by random forest and/or univariate analysis as important for the differentiation of dogs into either control, congenital portosystemic shunt, hepatocellular carcinoma, or chronic hepatitis groups. Tyrosine was significantly different between groups ($p = 0.01$) and decreased in the urine of dogs with chronic hepatitis compared to control dogs ($q = 0.01$). Glutamine was significantly different between groups ($p = 0.0004$) and increased in the urine of dogs with chronic hepatitis and a congenital portosystemic shunt compared to control dogs ($q = 0.005$, $q = 0.0009$, respectively). Tryptophan was significantly different between groups ($p = 0.004$) and increased in the urine of dogs with chronic hepatitis compared to control dogs ($q = 0.002$).

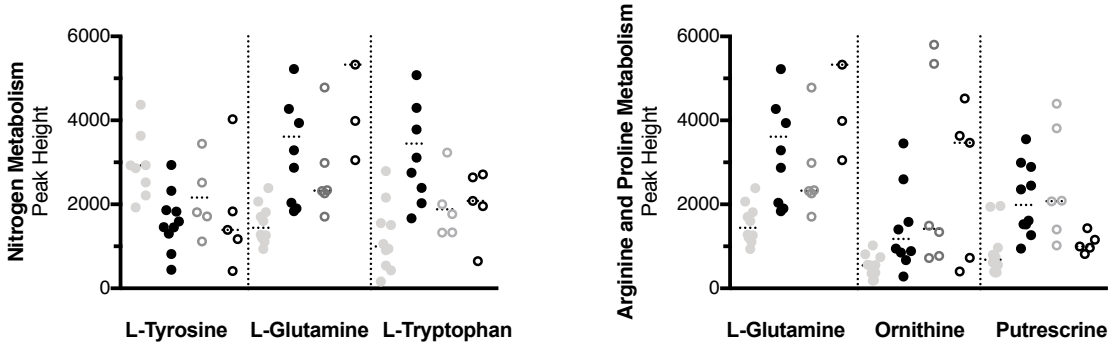
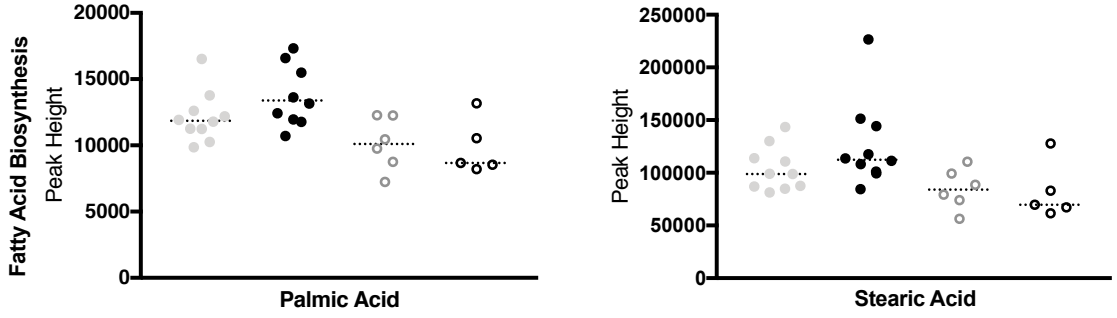


Figure 5.6. Urine metabolites of fatty acid biosynthesis with potential value as a biomarker of hepatocellular carcinoma, chronic hepatitis, or a congenital portosystemic shunt in dogs.

Each metabolite was identified by univariate analysis as important for the differentiation of dogs into either congenital portosystemic shunt, hepatocellular carcinoma, or chronic hepatitis groups. Palmic acid was significantly different between groups ($p = 0.01$) and increased in the urine of dogs with chronic hepatitis compared to dogs with hepatocellular carcinoma or a congenital portosystemic shunt ($q = 0.04$, $q = 0.03$, respectively). Stearic acid was significantly different between groups ($p = 0.01$) and decreased in the urine of dogs with a congenital portosystemic shunt compared to dogs with chronic hepatitis ($q = 0.04$).



5.4. **Discussion**

This study identified metabolic abnormalities in the urine of dogs diagnosed with chronic hepatitis, hepatocellular carcinoma, or a congenital portosystemic shunt with significant alterations in 37 of 220 named metabolites. These metabolites are known to be involved in a variety of processes, including glutathione metabolism, nitrogen metabolism, arginine and proline metabolism, and fatty acid biosynthesis.

5.4.1. **Abnormal amino acid metabolism and fatty acid biosynthesis**

Significant differences were noted in the relative amounts of metabolites in the urine of dogs with chronic hepatitis, hepatocellular carcinoma, or a congenital portosystemic shunt compared to healthy control dogs. Among these findings was a significant difference in three metabolites derived from glutathione metabolism, ornithine, putrescine, and ascorbic acid between groups. Ornithine was significantly increased in the urine of dogs with hepatocellular carcinoma compared to healthy control dogs ($q = 0.0410$). Putrescine was significantly increased in the urine of dogs with chronic hepatitis and hepatocellular carcinoma compared to healthy control dogs ($q = 0.0056$, $q = 0.010$ respectively). Ascorbic acid was significantly increased in the urine of dogs with hepatocellular carcinoma compared to dogs with a congenital portosystemic shunt ($q = 0.02$). Glutathione (GSH, L-γglutamyl-L-cysteinyl- glycine) is a tripeptide thiol synthesized in all mammalian cells [22]. Glutathione detoxifies reactive molecules by either spontaneous conjugation or a GSH-S-transferase catalyzed reaction. The liver, kidney, and lungs are important sources of GSH; however, systemic concentrations of GSH and its substrates are predominantly hepatically derived [23]. Hepatocytes are unique, compared with other cells, because they synthesize large quantities of GSH that can be either used locally or

effluxes into the systemic circulation and bile [23]. The discovery of increased urine concentrations of three metabolites derived from glutathione metabolism in dogs with chronic hepatitis and hepatocellular carcinoma compared to healthy control dogs and dogs with a congenital portosystemic shunt may represented altered glutathione metabolism. Decreased quantities of glutathione have been found in the liver of dogs with various liver diseases including chronic hepatitis [22]. The increased utilization of the glutathione pathway resulting in decreased liver tissue concentrations may result in increased urine levels of products derived from glutathione metabolism. There are no previous studies of the urine metabolome in dogs with hepatic disease; however, similar findings of increased tyrosine, glutamine, ornithine, valine, threonine, and lysine have been reported in the urine of humans with Wilson's disease [24].

Significant differences in amino acids involved in nitrogen metabolism; tyrosine, glutamine, and tryptophan and were detected. There was a significant decrease of tyrosine in the urine of dogs with chronic hepatitis compared to healthy control dogs ($q = 0.01$), a significant increase of glutamine in the urine of dogs with chronic hepatitis or a congenital portosystemic shunt compared to healthy control dogs ($q = 0.0045$ and $q = 0.0009$, respectively), and a significant increase of tryptophan in the urine of dogs with chronic hepatitis compared to healthy control dogs ($q = 0.0016$). Glutamine, ornithine, and putrescine are also reaction intermediates of arginine and proline metabolism. Most amino acids are synthesized and degraded in the liver; thus, injury to the liver can result in abnormalities in the metabolism of amino acids and the release of amino acids from hepatocytes [24,25]. There are no previous studies of the urine metabolome in dogs with

hepatic disease; however, urine amino acid levels of lysine, valine, threonine, and tyrosine are markedly increased in humans with the inflammatory liver disease, nonalcoholic steatohepatitis disease are elevated when compared to healthy controls [26]. These results of this study are similar to the finding of our study. Alterations in amino acid metabolites are thought to represent an adaptive physiological responses to hepatic stress in humans with nonalcoholic steatohepatitis and our results may represent a similar response in dogs [27].

Additionally, significant differences in two metabolites of fatty acid biosynthesis; palmitic and stearic acid were detected. There was a significant decrease of palmitic acid in the urine of dogs with hepatocellular carcinoma or a congenital portosystemic shunt compared to dogs with chronic hepatitis and a significant decrease of stearic acid in the urine of dogs with hepatocellular carcinoma compared to dogs with chronic hepatitis ($q = 0.038$, 0.026, and 0.035 respectively). Long chain free fatty acids (FFAs) can be divided into saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids based on the presence of double bonds. The best characterized FFAs in the context of apoptosis are palmitic acid, a 16 carbon length saturated fatty acid, oleic acid, an 18 carbon length monounsaturated fatty acid, and stearic acid, a 18 carbon length saturated fatty acid [28-30]. Saturated fatty acids can induce apoptosis in many different cell types and in hepatocytes, the saturated fatty acids, palmitic acid, and stearic acid lead to a concentration- and time-dependent lipoapoptosis [31]. Thus, the increase in these fatty acids could contribute to hepatocellular apoptosis. The role of FFAs in canine liver disease

has not been characterized and therefore, the significance of these findings if validated in a targeted prospective study is unknown.

5.4.2. **Potential biomarkers for dogs with chronic hepatitis, hepatocellular carcinoma, or a congenital portosystemic shunt**

A subset of compounds in urine were identified for their value in differentiating healthy control dogs from those diagnosed with chronic hepatitis, hepatocellular carcinoma, or a congenital portosystemic shunt. Random forest analysis identified 15 metabolites that were able to classify healthy control dogs (100% accuracy), dogs with chronic hepatitis (70% accuracy), dogs with hepatocellular carcinoma (33% accuracy), and dogs with a congenital portosystemic shunt (40% accuracy) (Figure 3). Further evaluation of these findings in a prospective targeted study is warranted.

Limitations of this study include the influence of concurrent drug administration or diet on our findings. The results of this study will need to be validated in a prospective targeted study in dogs with chronic hepatitis, hepatocellular carcinoma, or a congenital portosystemic shunt. The metabolites in urine can be affected by the cumulative effect of complex physiological processes across all tissues in the body, it is not possible to definitively determine the organ, cell type, or intracellular compartment from which identified compounds originated. Furthermore, the impact of renal excretion versus reabsorption, and intestinal microbial metabolism on the types and quantity of compounds detected in the urine is unknown. Metabolomic investigation of the serum of dogs with chronic hepatitis, hepatocellular carcinoma, or a congenital portosystemic shunt compared

to healthy control dogs would provide considerable additional insight into the metabolic abnormalities identified in this study and is currently underway.

Untargeted metabolomic profiling of urine from dogs with chronic hepatitis, hepatocellular carcinoma, and a congenital portosystemic shunt demonstrated significant semi-quantitative differences in 37 of 220 named metabolites, including those involved in glutathione, nitrogen, arginine and proline metabolism, and fatty acid biosynthesis. Further validation of these results in a targeted study and determination of their utility as clinical biomarkers is warranted.

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6. ILLUMINA MICRORNA SEQUENCING OF LIVER TISSUE FROM DOGS WITH CHRONIC HEPATITIS

6.1. **Introduction**

Chronic hepatitis is associated with apoptosis, necrosis, and an inflammatory infiltrate that is often predominantly mononuclear. Those processes can initiate a repair process that can progress to end-stage liver disease characterized by fibrosis of the liver and the replacement of normal liver architecture with abnormal liver nodules with portal-central vascular anastomoses [2]. End-stage hepatic disease carries a poor long term prognosis with a reported median survival time of 0.4 months [3,4]. Chronic hepatitis, in contrast diagnosed at an earlier stage carries a reported median survival from diagnosis to death of 18.3 – 36.4 months [3,4]. Dogs with chronic hepatitis are often diagnosed at an advanced stage where treatment is less likely to be effective. Therefore, biological markers are needed that can identify dogs with chronic hepatitis, hepatocellular inflammation, or hepatic fibrosis, before clinical signs develop.

MicroRNAs (miRNAs) are small non-coding naturally occurring RNAs that regulate gene expression by suppressing translation of target messenger RNAs [5,6]. They exert critical functions in many physiological processes and are consequently involved in the pathogeneses of various diseases. Various miRNAs have been shown to have potential as biomarkers for a variety hepatobiliary diseases in humans, including chronic hepatitis C, non-alcoholic fatty liver disease, rejection after liver transplantation, drug- or alcohol-induced liver injury, intrahepatic cholangiocarcinoma, and hepatocellular carcinoma [7-

13]. MicroRNAs are expressed in a number of tissues; however, certain miRNAs are organ specific [14]. Hepatic tissue expresses a number of distinct miRNAs that include miR-122, the single most abundant hepatic miRNA and it is estimated to make up 70% of the total hepatically derived miRNA [15]. Because microRNAs are well conserved across species and highly stable in blood, they are good candidate biomarkers for liver disease in dogs.

The aim of this study was to identify miRNAs that are differentially expressed in hepatic tissue from dogs with chronic hepatitis compared to healthy control dogs using Illumina small-RNA sequencing. We hypothesized that dogs with chronic hepatitis will exhibit differential hepatic miRNA expression compared to healthy control dogs.

6.2. **Materials and methods**

6.2.1. **Animals**

Eight dogs undergoing liver biopsy for routine diagnostic purposes that were subsequently histologically confirmed to have chronic hepatitis and 8 healthy control dogs undergoing ovariohysterectomy were enrolled at the Texas A&M University Veterinary Medical Teaching Hospital between September 2014 and August 2017. Informed client consent was obtained for each patient and the study was approved by the Texas A&M University Institutional Animal Care and Use Committee (Animal Use Protocols #2014-320 and 2015-0043). Patients with suspected chronic hepatitis underwent laparoscopic liver biopsy and 5 to 8, 5 mm closed cup forceps liver biopsy specimens were collected. The same 5 mm closed cup forceps were used to collect 3 – 4 liver biopsy specimens from healthy dogs at the time of ovariohysterectomy. One biopsy specimen, representing approximately

0.5 g of liver tissue from each patient was placed in RNAlater (Invitrogen, Carlsbad, CA) for a minimum of 12 hours, the RNAlater was then decanted, and the specimen was stored at -80°C until further analysis. One liver biopsy specimen was collected in a sterile red top tube for copper quantification by atomic adsorption spectroscopy (expressed as μg copper per g dry weight) and another was collected and stored in a sterile specimen cup for aerobic and anaerobic culture and susceptibility testing. The remaining liver tissue from each dog in each group was fixed in neutral buffered formalin for routine histological processing. Sections from formalin-fixed paraffin embedded tissues were routinely stained with hematoxylin and eosin, picrosirius red, and rhodamine. The diagnosis of chronic hepatitis and healthy control were based on clinical signs, routine serum biochemistry panels, and histological assessment of liver specimens by a board-certified veterinary pathologist according to the World Small Animal Veterinary Association Liver Standardization Group Guidelines [16]. The stage of hepatic fibrosis, the grade of necroinflammatory activity, and the semiquantitative assessment of copper content were assessed using a previously published scoring scheme [2,17].

6.2.2. **Total RNA extraction**

Liver tissue stabilized in RNAlater was homogenized with a tissue homogenizer (PRO Scientific, Oxford, CT). The extraction of total RNA was performed using the RNeasy Plus Mini Kit (QIAGEN, Germantown, MD) according to the manufacturer's instructions and stored at –80°C until analysis. RNA pellets were eluted to sequencing grade water (Amresco, Solon, OH), and the quality of total RNA analyzed by the Bioanalyzer Nano 6000 chip (Agilent Technologies, Santa Clara, CA). Only samples with an RNA Integrity Number (RIN) greater than 7.0 were used for further experiments.

6.2.3. **Small RNA library preparation and Illumina sequencing**

Small RNA library preparation for each sample in each group was based on Illumina's TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, CA) following the manufacturer's protocol. Briefly, RNA 3′ and 5′ adapters were ligated to target microRNAs in two separate steps. Reverse transcription reaction was conducted to the ligation products to create single stranded cDNA. The cDNA was amplified by PCR using a common primer and a primer containing the index sequence. Libraries were sequenced on an Illumina GA Iix (SCS 2.8 software; Illumina, San Diego, CA), with 50 single end sequencing and 2 lanes. Sequence reads were extracted from the image files using the GitHub 2.16microRNA profiling pipeline (GitHub Inc. San Francisco, CA, USA).

6.2.4. **Small RNA sequence and data analysis**

After filtering adaptor sequences and removal of contaminated reads, the clean reads were processed for computational analysis. The obtained reads were aligned against the dog reference genome (*Canis lupus familiaris* Boxer genome, NCBI MegaBLAST). Subsequently, the sequences were analyzed by a BLAST search against the miRNA database, miRBase (version14.0 and 19.0). To prepare for differential miRNA expression analysis, miRNAs in the library of each case in both groups were normalized to obtain the expression of transcripts per million using the following formula: Normalized expression = (Actual miRNA sequencing reads count / Total clean reads count) × 1,000,000. The normalized expression values were input into the R/Bioconductor package *edgeR* [18] for differential expression analysis, and rlog transformation was applied as a preprocessing step. The *edgeR* package implements a state-of-the-art- statistical method specifically designed for detecting differential expression with RNA-Seq data. Briefly, an

overdispersed Poisson probability regression model was fit to each miRNA, and differential expression was assessed via hypothesis testing of the regression model coefficients; for testing, we used the *edgeR* implementation of QL F-tests. To accommodate the extreme multiple testing involved in a differential expression analysis, raw p-values were translated into q-values (analogs to p-values when the error measure used is false discovery rate (FDR) instead of Type I error rate) [19].

6.3. **Results**

6.3.1. **Patient demographics**

The demographics of the dogs enrolled into the study are shown in Table 1.

Table 6.1. Demographics and clinical characteristics of dogs enrolled into the study.

Disease	Age[a]	Sex	Breed	FIS	AS	HVS	CuS	Q Liver Cu	ALT	ALKP	TBILI	GGT
C. Hepatitis	12	SF	Labrador Retriever	4	2	1	1	667	466	557	0.2	16
C. Hepatitis	6	CM	Doberman Pincher	3	1	0	2	196	989	283	0.3	12
C. Hepatitis	9	M	Labrador Retriever	2	2	2	0	463	577	230	0.2	<10
C. Hepatitis	5	M	Doberman Pincher	2	2	2	3	3450	750	334	0.2	18
C. Hepatitis	4	SF	German Shorthaired	1	2	1	2	1080	231	98	0.4	<10
C. Hepatitis	11	SF	Labradoodle	2	2	2	3	1230	663	179	0.3	22
C. Hepatitis	14	CM	Rat Terrier	3	0	3	0	170	476	327	<0.1	12
C. Hepatitis	9	F	Rottweiler Dog	4	1	0	0	257	121	122	0.2	8
Control	0.5	F	Catahoula Hog Dog	0	0	2		181				
Control	6	F	Greyhound Dog	0	0	0		168				
Control	1	F	Chihuahua	0	0	0		280				
Control	5	F	Staffordshire Terrier	0	0	2		153				
Control	1	F	Labrador Retriever	0	0	1		124				
Control	3	F	Walker Coonhound	0	0	0		96.3				
Control	0.5	F	Labrador Retriever	0	0	0		316				
Control	0.5	F	Australian Shepherd	0	0	0		158				

Table 6.1 Continued

[a]Age (years), SF – Spayed female, M – Male, CM – Castrated male, F – Intact female, M – Intact male, FIS – Fibrosis score, AS – Activity score, HVS – Hepatocellular vacuolation score, CuS – Copper Score, Q Liver Cu – Quantitative liver copper, ALT – Alanine transaminotransferase, ALKP – Alkaline phosphatase, GGT – Gamma-glutamyl transferase, C. Hepatitis – Chronic hepatitis

6.3.2. **MicroRNA sequencing analysis**

MicroRNAs were evaluated in canine liver tissue from 8 healthy control dogs and 8 dogs with chronic hepatitis. There were 50,666,671 and 45,949,375 RNA reads on average per sample, respectively that equaled 48,308,023 RNA reads on average per sample. A total of 772,928,372 high-quality reads were subjected to analysis. The differential expression of hepatic miRNA was evaluated using QL F-test**.** There were 176 total miRNAs detected by sequencing and of these hepatically derived miRNAs, 37 were found to be differentially expressed between groups (Table 2) The miRNAs highlighted in gray are liver-specific [44-47]. Among the differentially expressed miRNAs, 29 were up-regulated and 8 were down-regulated in the liver of dogs with chronic hepatitis compared to the liver of healthy control dogs.

Table 6.2. Differentially expressed microRNAs in the liver tissue of dogs with chronic hepatitis

MICRORNA	EXP.	CONTROL (MEAN +/- SD)	C. HEPATITIS (MEAN +/- SD)	LOGCPM	PVALUE	FDR
CFA-LET-7D.MIMAT0034396	Up	40.75+/-2.28	159.87+/-4.73	7.170	0.000	0.011
CFA-LET-7E.MIMAT0006608	Up	460.17+/-16.5	977.53+/-35.65	9.586	0.005	0.056
CFA-MIR-1199.MIMAT0031123	Down	0.5+/-0.08	0.07+/-0.01	0.397	0.001	0.017
CFA-MIR-1296.MIMAT0034413	Up	4.5+/-0.2	9.33+/-0.44	3.167	0.001	0.019
CFA-MIR-148B.MIMAT0006663	Down	983.92+/-44.97	975.33+/-42	9.675	0.010	0.092
CFA-MIR-150.MIMAT0006602	Up	47.83+/-2.09	470+/-32.27	8.068	0.004	0.047
CFA-MIR-15B.MIMAT0006676	Up	59.33+/-3.21	222.33+/-12.66	7.419	0.000	0.003
CFA-MIR-181A.MIMAT0006707	Up	2853.25+/-101.62	3191.8+/-96.07	11.512	0.000	0.012
CFA-MIR-1838.MIMAT0006644	Up	2.83+/-0.2	10.8+/-0.58	3.243	0.002	0.039
CFA-MIR-1840.MIMAT0006679	Up	0.92+/-0.06	5.27+/-0.23	2.323	0.000	0.006
CFA-MIR-193A.MIMAT0006735	Up	132.67+/-4.91	265.93+/-9.59	7.925	0.002	0.030
CFA-MIR-193B.MIMAT0006699	Down	23.08+/-0.82	20.93+/-0.73	4.487	0.008	0.088
CFA-MIR-197.MIMAT0006698	Up	41+/-2.05	125.47+/-5.01	6.763	0.000	0.006
CFA-MIR-30D.MIMAT0006616	Down	14524.17+/-578.09	12996.53+/-390.08	13.532	0.003	0.045
CFA-MIR-326.MIMAT0009894	Up	6.17+/-0.21	10.47+/-0.6	3.204	0.000	0.006
CFA-MIR-328.MIMAT0006688	Up	17.5+/-0.79	39.53+/-1.36	5.180	0.000	0.012
CFA-MIR-331.MIMAT0009895	Up	28.83+/-1.24	45.2+/-2.66	5.327	0.001	0.019
CFA-MIR-339-1.MIMAT0011134	Up	249.33+/-15.74	364.47+/-22	8.215	0.002	0.039
CFA-MIR-350.MIMAT0006704	Up	21.5+/-1.74	28.67+/-2.77	4.392	0.006	0.070
CFA-MIR-365.MIMAT0001540	Up	917.08+/-51.23	1193.87+/-57.81	10.030	0.002	0.039
CFA-MIR-423A.MIMAT0006742	Up	743+/-21.41	1623.27+/-42.22	10.477	0.010	0.092
CFA-MIR-433.MIMAT0006712	Up	0.25+/-0.03	1.13+/-0.11	0.819	0.011	0.100
CFA-MIR-483.MIMAT0009901	Up	9.25+/-0.55	17.47+/-1.03	3.812	0.010	0.092
CFA-MIR-500.MIMAT0006759	Up	154+/-6.34	210.07+/-7.43	7.483	0.005	0.056

*** MARKERS HIGHLIGHTED IN GRAY ARE LIVER-SPECIFIC.**

Table 6.2 Continued

MICRORNA	EXP.	CONTROL (MEAN +/- SD)	C. HEPATITIS (MEAN +/- SD)	LOGCPM	PVALUE	FDR
CFA-MIR-504.MIMAT0009907	Up	15.92+/-0.77	60.47+/-2.56	5.828	0.000	0.001
CFA-MIR-505.MIMAT0009908	Up	12.42+/-0.59	29.6+/-1.13	4.638	0.011	0.100
CFA-MIR-568.MIMAT0009914	Down	0.33+/-0.06	0+/-0	0.386	0.003	0.045
CFA-MIR-574.MIMAT0006673	Up	24.75+/-1.35	114.4+/-5.04	6.458	0.001	0.027
CFA-MIR-8821.MIMAT0034311	Down	0.33+/-0.06	0+/-0	0.298	0.001	0.027
CFA-MIR-8825.MIMAT0034315	Up	0.08+/-0.02	0.27+/-0.04	0.401	0.001	0.027
CFA-MIR-8854.MIMAT0034349	Up	0.25+/-0.03	0.4+/-0.05	0.509	0.010	0.092
CFA-MIR-8859A.MIMAT0034354	Up	293.33+/-10.47	542.8+/-23.64	8.705	0.008	0.088
CFA-MIR-8881.MIMAT0034381	Up	0+/-0	0.27+/-0.06	0.369	0.003	0.045
CFA-MIR-8883.MIMAT0034385	Down	0.08+/-0.02	0.07+/-0.01	0.267	0.005	0.057
CFA-MIR-8887.MIMAT0034391	Down	2.42+/-0.2	1.13+/-0.15	1.441	0.000	0.006
CFA-MIR-8908B.MIMAT0034415	Up	0.42+/-0.08	0.67+/-0.08	0.665	0.000	0.015
CFA-MIR-92A.MIMAT0006653	Up	1611.67+/-57.32	3017+/-120.17	11.245	0.003	0.045

*** MARKERS HIGHLIGHTED IN GRAY ARE LIVER-SPECIFIC.**

6.4. **Discussion**

The microRNA expression profile of liver tissue from dogs with chronic hepatitis was compared to the expression profile of liver tissue from healthy control dogs using Illumina small-RNA sequencing. After correcting for multiple comparisons, 37 miRNAs were found to be significantly differentially expressed: 29 were up-regulated and 8 were down-regulated (Table 2). This is the first report of miRNA expression profiling of liver tissue from dogs with chronic hepatitis to the author's knowledge.

6.4.1. **Potential microRNA biomarkers of canine chronic hepatitis**

There are no previous studies of the microRNA profile of liver tissue from dogs with chronic hepatitis; however, there are limited studies that characterize the utility of specific serum miRNAs in dogs with chronic hepatitis and a study of the microRNA profile of liver tissue from Beagle dogs with drug-induced hepatotoxicity [20-22]. Furthermore, there are many human studies of microRNA profiling of the liver, serum, and plasma from patients with chronic liver disease [23-25].

Fibrosis is the final common outcome of all chronic liver diseases, including chronic hepatitis in dogs [26]. Hepatic stellate cells, the matrix-producing of the liver, once activated transform into myofibroblast-like cells that secrete extracellular matrix leading to liver fibrosis [27]. MiRNAs identified in the study presented here that have been previously identified as playing a role in hepatic stellate cell activation or hepatic fibrosis includes cfa-mir-181a, cfa-mir-150, and cfa-let-7d [28-30]. A study by Brockenhausen et al. found that miR-181a exhibited a direct effect of inducing hepatocyte epithelial–mesenchymal transition, replaced TGF-β-induced effects in vitro, and hepatocyte

expression in vivo was increased in liver cirrhosis, similar to the findings of our study [29]. MiR-150, another important suppressor of hepatic stellate cell activation which is crucial in the development of liver fibrosis, has been found to inhibit type I and IV collagen in activated hepatic stellate cells [31]. Honda et al. demonstrated the anti-fibrotic effect of miR-150 in fibroblasts through the regulation of integrin β3, phosphorylated Smad3 and COL1A1 expression [32]. Previously, Li et al. found that the expression of miR-150 was up-regulated in the serum of human patients with chronic hepatitis B compared with control patients [33]. Recently, it has also been shown that the serum level of miR-150 was able to discriminate mild from severe fibrosis in human patients with schistosomiasis[34]. The expression of cfa-mir-150 was increased in the liver tissue of dogs with chronic hepatitis; however, confirmation of this finding and serum evaluation may be warranted. The let-7 family of miRNAs has been shown to act as a negative regulator of hepatic fibrosis in a mouse model via the down-regulation of TβRI [35]. Matsuura et al. has also showed that the levels of circulating let-7 family members in plasma declined significantly over time during the progression of fibrosis in chronic hepatitis C, with an AUC value of 0.776, 0.734 and 0.790 for let-7a, let-7c and let-7d, respectively [36]. Similar to these observations, a study by Cai et al. found that let-7d (AUC: 0.6270) circulating in serum could discriminate between mild and severe schistosomiasis induced hepatic fibrosis [34]. Similarly, the expression of cfa-let-7d was increase in liver tissue of dogs with chronic hepatitis in the current study; however, confirmation of this finding and serum evaluation is warranted. These findings suggest a potential role of cfa-mir-181a, cfa-mir-150, and cfa-let-7d in hepatic fibrosis.

Aberrant apoptosis is an important feature of chronic liver disease and is associated with worsening fibrosis [37,38]. A miRNA identified in the current study previously identified as playing a role in apoptosis is cfa-mir-15b, which has been proposed as a non-invasive biomarker of chronic liver disease [28,39,40]. Specifically, the administration of miR-15b and miR-16 was shown to inhibit hepatic stellate cell proliferation and induce apoptosis by induction of the mitochondrial-associated anti-apoptosis protein, Bcl-2 in a murine model of acute liver failure [40]. The increased expression of cfa-mir-15b in the liver tissue of dogs with chronic hepatitis in the current may indicate, if validated a role of cfa-mir-15b in canine chronic hepatitis.

There is growing evidence that indicates a role of miRNAs in the modulation of liver inflammation through targeting various signaling molecules. A number of miRNAs has been implicated in inflammatory responses: miR-155, miR-132, miR-125b, miR-146a, miR-150, miR-181, let-7 and miR-21 [41,42] and of these miR-150, miR-181, let-7 were altered in the liver of dogs with chronic hepatitis and if validated could indicate a role in canine inflammatory liver disease.

There are no previous deep miRNA sequencing studies of serum, plasma, or liver in dogs with chronic hepatitis to the authors' knowledge; however, there have been a number of targeted studies of circulating miRNAs (miR-21, miRNA-126, miR-200c, miR-222, miR-122, miR-29a, miR-148a, miR-181b, miR-133a, miR-17-5) that have been performed in dogs with chronic hepatitis [20,21,44]. There were no overlapping transcripts identified in our study of hepatic miRNA deep sequencing with those evaluated in targeted studies

in canine serum. The circulating miRNAs evaluated in dogs were chosen based on their associations with chronic liver disease in humans; however, the serum of dogs with chronic hepatitis has not been profiled and it is unknown if the same changes occur. MiRNA concentrations in the liver have been found to correlate with serum concentration for a number of miRNAs [49,50]. MiRNAs from the liver may enter the serum passively through apoptosis and necrosis or actively through secretion of exosomes and viral particles [51]. Therefore, although miRNA levels in the serum may estimate miRNA activity in the liver it is unknown if the same circulating miRNA profile occurs in human and canine liver diseases.

There are a small number of studies that have identified 55 liver specific miRNAs in humans [45-48] of the 300 different miRNAs found in liver tissue [49] and 8 of these were differentially expressed in our study (cfa-let-7d, cfa-let-7e, cfa-mir-15b, cfa-mir-181a, cfa-mir-193a, cfa-mir-193b, cfa-mir-483, cfa-mir-92a). MiRNA transcripts selected for validation should be considered in light of their suspected function and/or tissue-specificity.

There are several limitations of this study that should be noted. MiRNAs and their targets form complex regulatory networks and a single miRNA can bind to and regulate many different mRNA targets and, conversely, several different miRNAs can bind to and cooperatively control a single mRNA target [43]. The modulation of any target gene may be regulated by the combined efforts of transcription factors, miRNA, protein methylation, ubiquitination and other factors. The results reported here are from liver tissue sequencing

and the acquisition of a liver biopsy is invasive and limit the examination of miRNAs in liver tissue. The evaluation of the identified miRNAs in serum is likely warranted. These initial findings due to the probability of a type 1 error should be validated with quantitative PCR to confirm these initial findings.

In conclusion, the present study showed the differential expression of hepatic miRNAs between dogs with chronic hepatitis and healthy control dogs. The data presented here suggested that small-RNA Illumina Sequencing is a powerful approach to study miRNA expression profiling. Further investigations are needed to validate these findings and determine diagnostic utility.

6.5. **References**

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7. CONCLUSIONS

7.1. **Application of proteomics in biomarker discovery**

The recent advances in both genomic sequencing and mass spectroscopic technology has driven the progress that has been made in the field of proteomics. Proteomics has an important role to play in the discovery of disease biomarkers in veterinary medicine and this is not limited to canine hepatic disease. The pathogenesis of disease, including chronic hepatitis, involves changes in the expression of proteins and this provides the foundation for the discovery of candidate protein biomarkers by characterization of the proteome. Global analysis of the proteins in a biological sample can identify proteins that can improve the diagnosis, staging, prognosis, and treatment monitoring of disease. There are a number of protein biomarkers currently utilized in the evaluation of dogs with suspected chronic hepatitis (i.e., alanine aminotransferase, aspartate aminotransferase); however, deficiencies discovered with the current non-invasive biomarkers include an unacceptable frequency of false positives, false negatives, poor sensitivity, and lack of tissue specificity. all of which can decrease confidence in the utility of the biomarker. The development of more reliable, informative biomarkers or panels of biomarkers for the evaluation of liver disease in dogs is needed. The study presented in section 1 identified 11 candidate protein biomarkers in the liver tissue of dogs with chronic hepatitis of which two related to apoptosis, cytokeratin 18 and annexin 5, were confirmed by western blot analysis. Cytokeratins (CKs) represent a multigene family of cytoskeletal proteins that are present in large quantities in epithelial cells where they form bundles of intermediate-sized filaments [1-4]. There are 21 different CK polypeptides expressed in development- and

cell type-specific patterns that have been identified in humans [5-7]. Two CK subfamilies, type I and type II CKs have been distinguished and at least one member of each subfamily has to be present for intermediate filament assembly [7]. Among CKs, the pair of CK8 (type II) and CK18 (type I) is of special interest in liver disease because these two polypeptides are the only CKs present in hepatocytes [8]. Furthermore, toxic liver damage is drastically enhanced in the absence of CK8 [9].

Reflections and musing: Evaluation of other unconfirmed proteins is important to realize the full potential of this study and this technology. Protein molecules with potential based on their role in hepatocellular apoptosis would include cytokeratin 8 and annexin A6. Furthermore, the analytical validation of existing human diagnostic tests or the development of a diagnostic test for the measurement of cytokeratin 18 and annexin 5 in canine samples is needed to determine the utility of measurement of these proteins in a clinical population. The potential of the proteomic research platform to identify candidate biomarkers for chronic hepatitis in dogs is great; however, increasing the resolution for separating low abundant proteins and increasing the sensitivity for identifying small quantities of proteins would further strengthen this technique. Additionally, the need for a well characterized study population cannot be overstated. This is the first study in veterinary medicine to utilize the proteomic platform for biomarker discovery in canine chronic hepatitis, however, further studies are imperative to determine their diagnostic potential.

7.2. **Application of metabolomics in biomarker discovery**

The interdisciplinary field of metabolomics combines analytical chemistry, mass spectrometry, and sophisticated data analysis to characterize the lipids, sugars, nucleotides, amino acids, organic acids and many other low molecular-weight compounds in a biological specimen. These context-dependent metabolites will vary in concentration based on the physiology of the cell, tissue, organ or organism. The application of this technology to biomarker discovery can assist with the discovery of signals influenced by genetics, the environment or their interaction. The value offered by assessment of the metabolome resides in the identification of early signals of cellular dysregulation that may occur prior to the development of gross disease. Because metabolites represent the downstream expression of genome, transcriptome, and proteome, they more closely reflect the phenotype of an organism at a specific time and the analysis of metabolic differences between unaltered and altered pathways could provide insight into underlying disease pathology. The studies presented in sections 3 – 5 have identified a number of metabolites that are altered in the serum or urine of healthy control dogs when compared to dogs with chronic hepatitis, hepatocellular carcinoma, or a congenital portosystemic shunt.

Specifically, the ratio of branched chain amino acids to aromatic amino acids was decreased in the serum of dogs with a congenital portosystemic shunt. Human patients with advanced liver disease demonstrate decreased serum concentrations of branched chain amino acids and increased serum concentrations of the aromatic amino acids, phenylalanine and tyrosine resulting in a low ratio of branched chain amino acids to aromatic amino acids, a ratio called the Fischer ratio [10]. A low Fischer ratio has been

associated with hepatic encephalopathy and becomes more marked with the progression of liver diseases. The ratio has also been used to determine the prognosis of cirrhotic patients with or without hepatocellular carcinoma [11,12]. Furthermore, a simplified Fischer ratio, the branched chain amino acid to tyrosine ratio, has been reported to be useful for predicting serum albumin concentration in human patients with chronic liver disease a year from presentation [13].

Reflections and musing: These data indicate that a low Fischer ratio is a marker for progression of liver disease in humans, and this should be explored in dogs with chronic liver disease.

The fatty acids, palmitic acid and stearic acid were increased in the urine of dogs with chronic hepatitis. A recent study in humans with chronic liver disease found a higher percentage of palmitic acid, stearic acid, oleic acid, and total monounsaturated fatty acid in their red blood cells [14]. Furthermore, palmitic acid red blood cell concentrations were identified as an independent predictor of advanced fibrosis [14]. Additionally, research suggests that palmitic is the most cytotoxic fatty acid in the liver and can lead to hepatocyte injury, apoptosis, and death [15]. Palmitic acid has been also been shown to activate hepatic stellate cells, through inflammasomes and hedgehog signaling, leading to the progression of hepatic fibrosis [16]. The proportion of stearic acid in red blood cells was also significantly increased in patients with advanced fibrosis [14]. Stearic exhibits significant cytotoxic properties and contributes to the progression of fibrosis in chronic liver disease in humans [17].

Reflections and musing: Therefore, further evaluation of the metabolic pathways involved in fatty acid biosynthesis is warranted in addition to targeted studies to confirm these findings. These pathways may become dysregulated early in the course of disease prior to gross phenotypic changes and assessment of the complete pathway may allow identification of additional disturbances. Furthermore, it is important to investigate not only metabolites that are consistent with previously held dogma but to examine novel metabolites, such as xylitol that are not well characterized. The tendency to abandon metabolites and candidate biomarkers in this stage of discovery may limit development of new biomarkers.

7.3. **Application of miRNA expression profiling in biomarker discovery**

The recent introduction of sequencing technology that can perform simultaneous sequencing of millions of RNA molecules has made the comprehensive profiling of miRNAs possible. The deep sequencing of the small RNA fraction of the transcriptome allows researchers to determine miRNA expression and to identify new small RNA molecules. MiRNAs are small, non-coding RNAs that regulate post-transcriptional gene expression through modification of mRNA stability and suppression of target mRNA translation [18]. Due to the tissue specificity of certain miRNAs and their structural stability, there is interest in their potential as a novel class of disease biomarker. Approximately 60% of mRNAs contain a predicted binding site for miRNA and some contain multiple potential binding sites; however, few predicted miRNA–mRNA interactions have been validated [18]. A single miRNA is able to regulate multiple genes, and conversely a single gene may be regulated by multiple miRNAs [19]. So even a small change in miRNA expression can affect the expression of hundreds of target genes and

significantly alter the transcriptome [20]. Therefore, unique profiles of miRNA expression may potentially be more appropriate to serve as biomarkers for the diagnosis, staging, and monitoring of disease and since the gene targets of most miRNAs are unknown, our interpretation of changes in expression is markedly limited. The study presented in section 6 is the first evaluation of the miRNA expression profile of liver tissue in dogs with chronic hepatitis. Baseline miRNA levels in hepatocytes and liver tissue have been established using deep sequencing methods [21-23]. There are a small number of studies that have identified 55 liver specific miRNAs [24-27] of the 300 different miRNAs found in liver tissue [28] and 8 of these were differentially expressed in our study (cfa-let-7d, cfa-let-7e, cfa-mir-15b, cfa-mir-181a, cfa-mir-193a, cfa-mir-193b, cfa-mir-483, cfa-mir-92a). While miR-122 accounts for 70% of the total miRNA in the liver other miRNAs known to be abundantly expressed in adult human liver tissue, includes miR-16, miR-27b, miR-30d, miR-126, as well as the let-7 family of miRNAs [29,30]. Of those cfa-mir-30d and cfa-let-7d, and cfa-let-7e were differentially expressed in the presented study. The identified differentially expressed miRNAs will require validation in a targeted study before determination of diagnostic utility.

Reflections and musing: The miRNA transcripts with the most promise would include cfa-mir-150, cfa-mir-181, and cfa-let-7d base on their roles in hepatic inflammation, apoptosis, and fibrosis in the human literature. Other miRNA transcripts of interest due to their tissue specificity would include cfa-let-7d, cfa-let-7e, cfa-mir-15b, cfa-mir-181a, cfa-mir-193a, cfa-mir-193b, cfa-mir-483, cfa-mir-92a. The study presented in section 6 has demonstrated the feasibility of measuring miRNA expression profiles in liver biopsy

specimens from dogs with chronic hepatitis using an Illumina high-throughput deep small RNA sequencing platform. The successful application of this technology indicates that specific miRNA expression profiles can be identified that could prove to have relevant diagnostic, prognostic or predictive clinical value.

7.4. **Summary**

The studies reported here utilized the techniques of untargeted proteomics, untargeted metabolomics, targeted metabolomics, and untargeted transcriptomics to investigate candidate biomarkers for use in dogs with suspected chronic hepatitis. The findings detailed here provide critical targets for new avenues of research. This would include biomarker confirmation in targeted studies prior to analytical validation, clinical validation, and determination of clinical or diagnostic utility. A significant contribution has been made to the discovery of novel biomarkers in dogs with chronic hepatitis. Although several challenges exist, the combined results in this dissertation will provide a valuable basis for clinical scientists to design studies that analytically and clinically validate these novel biomarkers and in determination of their utility.

7.5. **References**

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