Received: 29 July 2021 | Accepted: 25 March 2022

DOI: 10.1111/jvim.16424

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Serum 25-hydroxyvitamin D and C-reactive protein and plasma von Willebrand concentrations in 23 dogs with chronic hepatopathies

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Funding information
Companion Animal Health Fund; The Office Of The Director, National Institutes of Health, Grant/Award Number: K01OD030515

Abstract

Background: Serum concentrations of 25-hydroxyvitamin D (25(OH)VD) and C-reactive protein (CRP) and von Willebrand's factor (vWF) concentration correlate with histopathologic disease grade and stage in chronic inflammatory and fibrotic hepatopathies (CH) in humans.

Objectives: To evaluate serum 25(OH)VD and serum CRP concentrations and plasma vWF concentration and determine if they correlate with histopathologic and biochemical variables in dog with CH.

Animals: Twenty-three client-owned dogs with a histopathologic diagnosis of CH were prospectively enrolled.

Methods: Blood samples were collected before liver biopsy. Correlations between biomarkers and clinical pathological and histopathologic variables were evaluated using Pearson's or Spearman's test.

Results: Serum 25(OH)VD concentration (median, 213 nmol/L; range, 42-527 nmol/L) was negatively correlated with serum aspartate aminotransferase activity (AST; rho $= -0.59$, $P < .01$), polymorphonuclear neutrophil count (PMN; $r = -0.46$, $P < .05$), and positively correlated with serum albumin concentration ($r = 0.69$, $P < .001$). Serum CRP concentration (median, 7.4 μg/L; range, 1-44.9 μg/L) was positively correlated with overall histopathologic necroinflammatory activity ($r = 0.78$, $P < .001$) and fibrosis score (rho $= 0.49$, $P < .05$). Plasma vWF concentration (median, 73.3%; range, 15-141%) was

Abbreviations: 25(OH)VD, 25-hydroxyvitamin D; ALP, alkaline phosphatase; ALT, alanine aminotransferase; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; CH, chronic hepatopathies; CRP, C-reactive protein; H&E, hematoxylin and eosin; PMN, polymorphonuclear neutrophil; PT, prothrombin time; vWF, von Willebrand's factor; WBC, white blood cell.

[Correction added on 26 May 2022, after the first online publication: the word "activity" has been replaced with "concentration" at the following places: Abstract: Background, Objectives, Results, Conclusion and Cinical sections and in sections 1, 2.2, 2.5, 3.3, figure 1 legend, 3.5, 4, table 3 abbreviations, and 5.]

[Correction added on 26 May 2022, after the first online publication: the words "activity" in title has been replaced with word "concentration" and title has been changed as Serum 25-hydroxyvitamin D and C-reactive protein concentrations and von Willebrand factor concentration in 23 dogs with chronic hepatopathies]

positively correlated with fibrosis score ($r = 0.53$, $P < .05$) and prothrombin time (rho = 0.67, $P < .01$), and negatively correlated with serum albumin concentration ($r = -0.73$, $P < .001$).

Conclusion and Clinical Importance: In dogs with CH, serum 25(OH)VD concentration was negatively correlated with disease activity, whereas serum CRP concentration and plasma vWF concentration were positively correlated with histopathologic grade and stage. Our results provide preliminary evidence that these biomarkers may be useful to assess grade and stage of CH in dogs in the absence of liver biopsy.

KEYWORDS
biomarkers, canine, fibrosis, hepatitis, inflammation

1 | INTRODUCTION

Chronic inflammatory and fibrotic hepatopathies (CH) including copper-associated liver disease, immune-mediated and drug-induced chronic hepatitis, ductal plate abnormalities, and in some cases, primary hypoplasia of the portal vein are important causes of liver disease in the dog.[1-4] These disorders are associated with various necroinflammatory and fibrotic changes that can fluctuate and progress over time and, in many cases, lead to death from liver failure.[1-8] Liver biopsy provides clinicians with information on grade (severity of necrosis and inflammation) and stage (severity of fibrosis) of disease.[9,10] Disease grade contributes information on disease activity at a point in time and can predict progression whereas disease state indicates how far along the disease has progressed in its natural history and correlates with survival. A clinician's knowledge of grade and stage of a CH can direct decisions on the need for therapeutic intervention, define the type of intervention, assist in monitoring response to treatment, and provide important prognostic information for owners.

Liver biopsy is the gold standard for determining disease grade and stage, but is invasive, may be cost prohibitive for some owners, and is not amenable to routine longitudinal monitoring.[9] Current noninvasive serum biomarkers of liver disease such as liver enzyme activity, and serum bilirubin and albumin concentrations cannot reliably predict disease grade or stage in dogs with CH.[4,5,7,8,11,12] In humans with CH, additional serum biomarkers such as vitamin D (VD), C-reactive protein (CRP), and von Willebrand factor (vWF) concentration have been used successfully as noninvasive and cost-effective tools to assess grade and stage of disease in CH.[13-32]

Vitamin D is a fat-soluble vitamin that plays an important role not only in calcium and phosphate homeostasis, but also modulates immunological, inflammatory, and fibrotic responses in many tissues including the liver.[33] In humans with various forms of necroinflammatory CH, serum VD concentrations are decreased, predict short-term mortality, and are negatively correlated with histological grade and stage.[16-21] In laboratory rodents, VD deficiency results in development of end-stage fibrotic liver disease.[22-24] Moreover, VD supplementation in humans and laboratory rodents with CH has anti-inflammatory and antifibrotic effects.[25-28] Considering the important pathologic consequences altered VD signaling can have in the liver and the potential benefit of supplementation, studies to determine if serum VD concentration are correlated with grade or stage of disease in dogs with CH are warranted.

Serum CRP, an acute phase protein, is increased in inflammatory conditions in dogs, and longitudinal evaluation in some of these disease states is a valuable adjunct in assessing response to treatment.[34-43] Currently, conflicting results have been reported on the value of serum CRP concentration in CH in dogs. One study found no correlation between serum CRP concentration and disease stage or grade in dogs with CH,[12] whereas another study found significant correlation with disease grade.[44] Both studies were limited by the inclusion of dogs with different causes of liver disease. A study examining serum CRP concentration in a more uniform population of dogs with CH is needed.

Von Willebrand factor is produced by endothelial cells and modulates platelet reactivity. Increases in plasma vWF concentration are considered a marker of endothelial cell dysfunction and can predict histological stage of disease, presence of portal hypertension, and clinical outcome in people with CH.[29-32] In dogs with CH, increased hepatic, endothelial vWF immunoreactivity was correlated with histopathologic fibrosis score.[45] A single study evaluating circulating plasma vWF concentration in dogs with mild CH failed to find a significant difference in vWF concentration, but median activity was higher in the dogs with CH (median, 203%; range, 109-351%) compared to controls (165.5%; 63-246%, $P = .06$).[46] Further investigation of plasma vWF concentration and its possible correlation with indices of hepatic fibrosis is warranted.

Our aims were to evaluate serum 25-hydroxyvitamin D (25(OH)VD) and CRP concentrations and plasma vWF concentration in dogs with CH and to investigate if they correlate with histological grade and stage of disease as well as clinical and hematological variables previously reported to reflect disease activity or predict shorter survival. We hypothesized that (a) serum 25(OH)VD concentrations will decrease with the stage of disease, (b) serum CRP concentration will increase with grade of disease, and (c) plasma vWF concentration will increase with stage of disease.

2 | METHODS

2.1 | Study design and inclusion and exclusion criteria

Our study was a prospective, cross-sectional, observational study conducted at the Foster Hospital for Small Animals at Cummings Veterinary Medical Center at Tufts University from 2016 to 2018. Dogs were included if they had chronic increases in serum alanine aminotransferase (ALT) activity (>2 months in duration)[4] and subsequently had a histological diagnosis of CH as described by the World Small Animal Veterinary Association liver standardization group.[47] Liver biopsy samples were obtained either by ultrasound-guided percutaneous needle biopsy or by laparoscopic surgery. Exclusion criteria included a history of administration of corticosteroids,[48] ursodiol,[49] nonsteroid anti-inflammatory drugs,[50] omega-3-fatty acids,[51] or VD supplements,[52,53] within 2 weeks of enrollment or use of 1-deamino-8-D-arginine vasopressin[54] within 24 hours. In addition, dogs with disease conditions in which serum 25(OH)VD or CRP concentrations have been shown to be altered including degenerative mitral valve disease,[55,56] renal disease (serum creatinine concentration >2.0 mg/dL with isosthenuria),[57,58] histologically confirmed neoplasia,[59,60] inflammatory bowel disease,[61] immune-mediated hemolytic anemia, polyarthropathy,[37] or pancreatitis[62,63] were excluded. The presence of comorbidities was determined by expert opinion of 2 board-certified internists (C.R.L. Webster and Y.M. Ambrosini) based on clinicopathological screening (ie, CBC, serum biochemical profile, urinalysis, and histopathological findings) and diagnostic imaging results (ie, thoracic radiographs and ultrasound examination).

2.2 | Enrollment

Dogs with clinical suspicion of CH were entered into the study after obtaining informed consent from their owners. The clinical impression of underlying CH was determined by expert opinion of the investigators (C.R.L. Webster and Y.M. Ambrosini) after review of clinical presentation and clinicopathological and imaging results. Blood samples were obtained for biomarker analysis before liver biopsy to avoid changes in biomarker concentrations that might be associated with surgery, anesthesia, or biopsy procedures. Before liver biopsy, each dog had a CBC, serum biochemistry profile, and coagulation panel performed, including prothrombin time (PT), activated partial thromboplastin time (aPTT), platelet count, and plasma fibrinogen concentration. Complete blood counts and serum biochemistry profiles were performed at the Clinical Pathology Laboratory at the Foster Hospital for Small Animals. Quantitative plasma fibrinogen concentration, PT, and aPTT were measured in the Clinical Coagulation Laboratory at the Foster Hospital.

2.3 | Biomarker analysis

Blood for biomarker analysis was obtained by jugular venipuncture, centrifuged, aliquoted, and frozen at −80°C until biopsy results were obtained. In dogs that met the inclusion criteria for the presence of CH on liver biopsy, serum and plasma samples were processed for biomarker analysis. Vitamin D status was assessed by determining the serum concentration of 25(OH)VD.[64,65] Serum samples were sent overnight on ice to Michigan State University Veterinary Endocrinology Diagnostic Laboratory for measurement of 25(OH)VD by radioimmunoassay (Immunodiagnostics, IDS, United Kingdom). The 25(OH)VD metabolite has been shown to be the best indicator of whole-body VD status in veterinary patients.[64,65] This form of VD has a long half-life (2-3 weeks) and serves as a reservoir for generation of the biologically active form.[66,67] Serum samples for CRP were sent to Texas A&M University Gastrointestinal Laboratory for the measurement of CRP using an enzyme-linked immunosorbent assay (TriDelta PHASE Canine CRP Assay Cat. No TP-803). Plasma vWF concentration was determined on the ACL Elite Pro Coagulation analyzer (Instrumentation Laboratory, Bedford, Massachusetts) using a latex particle-enhanced immunoturbidimetric assay that uses an antibody directed against the platelet binding site of vWF. Reference ranges for serum 25(OH)VD concentration (60-215 nmol/L), plasma vWF concentration (43-141%), and serum CRP concentration (0-7.6 mg/L) have been established in these laboratories. See Supplemental Material S1 for information on assay analytics and biological variability.

Signalment, clinical presentation, serum activities of ALT, serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP) activity, serum concentrations of total bilirubin and serum albumin, PT, aPTT, platelet count, plasma fibrinogen concentration, white blood cell (WBC) count, polymorphonuclear neutrophil (PMN) count, hematocrit, and pretreatment clinical score[8] were recorded. See Supplemental Material S2, Table S1, for details on clinical scoring.

2.4 | Histopathological assessment

Liver biopsy samples were processed routinely for histopathological evaluation including hematoxylin and eosin (H&E) staining, rhodanine staining for copper, and Sirius red staining for collagen. The H&E-stained slides were assessed for histopathologic grade of disease by evaluating the amount of inflammation, cell death, and degeneration, and then assigning a semiquantitative score based on established criteria[47] (see Supplement Material S2, Table S2A-C) for periportal and periseptal interface hepatitis; focal necrosis and inflammation; confluent, bridging, or multiacinar necrosis; and, glycogen accumulation. The total histological grade score was calculated by adding the scores for interface hepatitis, focal necrosis and inflammation, and confluent necrosis. The score ranged from 0 to 15. Histopathologic stage of disease was reflected by the severity of fibrosis and was assessed on Sirius red-stained sections and scored based on the established criteria[7] (see Supplemental Materials S2, range of scores was 0-5). All liver biopsy samples were reviewed by 2 board-certified anatomic pathologists (S. Jennings and C. Piedra-Mora). Each pathologist scored all of the biopsy sections independently and then met to compare scores. If a discrepancy was found, they evaluated the biopsy sections together and came to mutual agreement. Semiquantitative

TABLE 1 Clinical pathology variables in dogs with chronic hepatopathies

Variable	n	Median (range)	Number with value above upper limit of reference range (%)	Number with value below lower limit of reference range (%)	Reference range
ALT (U/L)	23	879 (137-3808)	23 (100)	0	14-86
AST (U/L)	22	152 (21-405)	18 (82)	0	9-54
ALP (U/L)	23	768 (27-8109)	22 (96)	0	27-126
Total bilirubin (mg/dL)	23	0.3 (0.1-20.3)	9 (39)	0	0.1-0.3
Albumin (g/dL)	22	3.35 (1.5-4.6)	0	4 (18)	2.8-4.0
PT (s)	23	8.1 (6.8-16)	3 (13)	0	6.2-9.3
aPTT (s)	23	11.5 (7.1-15.2)	1 (4.3)	0	8.9-16.3
Platelet (K/μL)	23	236 (85-573)	1 (4.3)	4 (17)	173-486
WBC (103/μL)	23	10.1 (5.5-30.3)	6 (26)	0	4.4-15.1
PMN (103/μL)	23	7.3 (4.1-26.1)	6 (26)	0	2.8-11.5
HCT (%)	23	45 (34-59)	2 (8.7)	2 (8.7)	39-55
Fibrinogen (mg/dL)	19	158 (57.8-377)	2 (11)	1 (5.2)	73-410

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; HCT, hematocrit; n, the number of dogs with available data; PMN, polymorphonuclear neutrophils; PT, prothrombin time; WBC, while blood cell count.

score for copper accumulation from rhodanine-stained slides was based on a scoring system using established criteria[47] (see Supplemental Material S2). Hepatic copper quantification in biopsy specimens was determined via flame atomic-absorption spectroscopy (ppm = μg/g dry weight liver) at the Veterinary Diagnostic Laboratory at Colorado State University.

2.5 | Statistical analysis

Measurement of skewness and kurtosis was done to determine if the data were normally distributed and means with SD or median with ranges were computed for normal and non-normally distributed data, respectively. Medians and ranges for serum 25(OH)VD and CRP concentrations and plasma vWF concentration were reported. The correlation of serum 25(OH)VD and CRP concentrations and plasma vWF concentration with conventional clinical pathology results (ALT, AST, ALP, total bilirubin, albumin, PT, aPTT, platelet count, WBC, and PMN), clinical score, and histological stage and grade were assessed using Pearson's (parametric) or Spearman's (nonparametric) tests. All statistical analyses were performed using Prism 8.2.1 (GraphPad Software, San Diego, California). *P* values <.05 were considered significant.

3 | RESULTS

3.1 | Study population

Thirty-eight dogs were recruited. Fifteen were excluded after review of hepatic biopsy results. The excluded dogs had diagnoses of vacuolar disease, hepatobiliary neoplasia, vascular disease, and nonspecific reactive hepatitis. Twenty-three dogs were enrolled. The most common breeds were mixed breed dogs (7/23, 30%), Labrador retrievers (4/23, 17%), and English Springer spaniels (2/23, 8.7%). Other breeds included 1 each of the following: Chesapeake Bay Retriever, Cocker Spaniel, Standard Poodle, Miniature Schnauzer, English Sheepdog, Rhodesian Ridgeback, Great Dane, French Bulldog, Boston Terrier, and Pug. Median age was 8 years (range, 2-14 years) with 14/23 male (61%) and 9/23 female (39%) dogs. Clinical signs included decreased appetite (6/11, 48%), lethargy (4/11, 36%), vomiting (3/11, 27%), polyuria and polydipsia (2/11, 18%), diarrhea (1/11, 9%), and shivering (1/11, 9%). Twenty dogs were eating commercial dog foods and 3 dogs were on homemade diets formulated by a veterinary nutritionist or using a balanced dog food website. None of the dogs were on supplements containing VD and none of the diets were on the Federal Drug Administration's list of diets that have been associated with hypervitaminosis D.

3.2 | Hematological characteristics, abdominal ultrasound findings, and clinical score

Hematological variables in the 23 dogs are summarized in Table 1. On serum biochemical analysis, increases in serum liver enzyme activities, ALT (23/23, 100%), ALP (22/23, 96%), and AST (18/22, 82%) were the most common abnormalities. Nine dogs (29%) had increases in serum total bilirubin concentration and 4/22 (18%) had a low serum albumin concentration. The WBC and PMN counts were increased in 6/23 (26%) and 6/23 (26%) dogs, respectively.

All 23 dogs had abdominal ultrasound examinations performed. The most common findings were a nodular and hypoechoic liver (9/23, 40%), gallbladder sludge (8/23, 35%), microhepatica (7/23, 30%), hepatomegaly (7/23, 30%), no abnormalities identified (6/23, 26%), irregular liver margins (3/23, 13%),

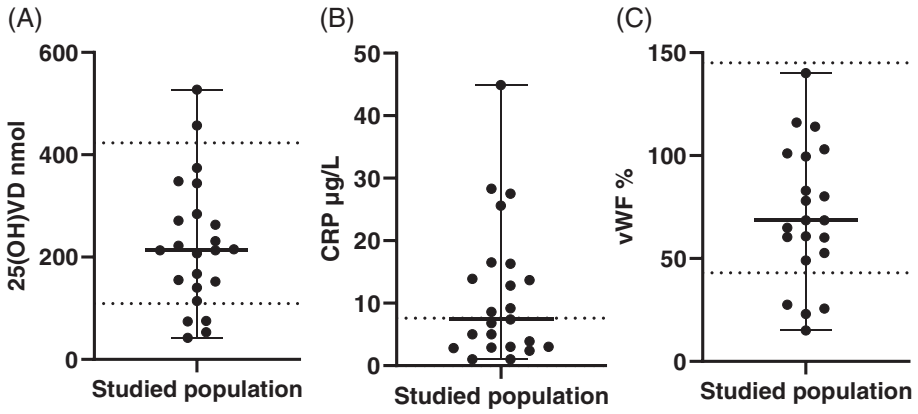

FIGURE 1 Serum 25-hydroxyvitamin D, C-reactive protein concentration, and von Willebrand's factor concentration in dogs with chronic hepatopathies. The results of serum (A) 25-hydroxyvitamin D (n = 23), (B) serum C-reactive protein concentration (n = 23), and (C) plasma vWF concentration (n = 21) are shown with closed circles. The thick bar denotes the median and the thin bar denotes the range. The doted lines indicate the reference ranges on each marker provided by the research laboratories. 25(OH)VD, 25-hydroxyvitamin D; CRP, C-reactive protein; vWF, von Willebrand's factor. Reference ranges for the biomarkers are 25(OH)VD (60-215 nmol/L), CRP (0-7.6 mg/L), and vWF (43-141%)

TABLE 2 Histopathological characteristics of 23 dogs with chronic hepatopathies

Histological variable	Number of dogs with lesion	Median score/copper concentration (range)
PIH (range, 0-5)	19/23	2 (0-5)
FI (range, 0-5)	20/23	3 (0-5)
CN (range, 0-5)	9/23	0 (0-5)
FIB (range, 0-4)	22/23	2 (0-4)
GLY (range, 0-3)	20/23	1 (0-3)
Copper[a] score >2/5	12/23	2.5 (0-5)
Copper[a] score >400 PPM	14/19	659 (126-2570)

Abbreviations: CN, confluent necrosis; FI, focal lytic necrosis, apoptosis and focal inflammation; FIB, fibrosis; GLY, glycogen; n, the number of dogs with available data; PIH, periportal interface hepatitis; PPM, parts per million.

[a]Copper scored based on evaluation of hepatic biopsy material stained with rhodamine. Hepatic biopsy copper quantification done by atomic absorption.

gallbladder wall thickening (3/23, 13%), and cholelithiasis (2/23, 9%). One dog had abdominal effusion. Median clinical score was 2 (range, 0-9). Individual dog clinical information is supplied in Supplemental Material S3.

3.3 | Measurement of biomarkers

Results of serum biomarker analysis are presented in Figure 1. Median serum 25(OH)VD concentration was 213 nmol/L (range, 42-527 nmol/L). Four of 23 dogs (17%) had serum 25(OH)VD concentrations less than the lower limit of the reference range. Median serum CRP concentration was 7.4 µg/L (range, 1-45 µg/L). Eleven of 23 dogs (48%) had serum CRP concentrations above the upper limit of the reference range. Median plasma vWF concentration was 73.3% (range, 15-141%). Four of 21 dogs (19%) had plasma vWF concentration less than the lower limit of the reference range. None of the dogs had increased plasma vWF concentration. Four dogs with clinical evidence of portal hypertension (low-protein noninflammatory ascites, diffuse cerebral neurologic signs consistent with hepatic encephalopathy, or both) had plasma vWF concentration (102 ± 38.6%) that was significantly ($P = .02$) higher than that of dogs without clinical evidence of portal hypertension (61 ± 28%).

3.4 | Hepatic biopsy and culture sample acquisition, hepatic copper concentration, and histological findings

Twelve of 23 dogs (52%) had laparoscopic liver biopsies performed and 11/23 dogs (48%) had percutaneous ultrasound-guided biopsy using 16-gauge (7/11, 64%) or 18-gauge (4/11, 36%) needles. Histopathologic diagnoses were chronic hepatitis (16/23, 70%), chronic cholangitis (4/23, 17%), degenerative vacuolar hepatopathy with fibrosis (2/23, 9%) and primary hypoplasia of the portal vein with fibrosis (1/23, 4%). Histopathologic scores are presented in Table 2. The overall hepatic biopsy histological grading score was 6.95 ± 4.92 with 11 dogs in the mild range (score, 0-5), 4 in the moderate range (score, 6-10), and 7 in the severe range (score, 11-15). See Supplemental Material S2, Table S1. The overall hepatic biopsy staging score was 1.82 ± 0.98 with only 4 dogs (17%) in the moderate to severe range (3-5). The median score for rhodanine staining was 2.5 (range, 0-5) with 10/19 (52%) having scores >2, which would be considered abnormal. Hepatic copper quantification was performed in 19/23 dogs (83%) with a median of

TABLE 3 A summary of significant correlations between concentrations of circulating 25(OH)VD, CRP, and vWF and biochemical variables and histopathological scores in dogs with chronic hepatopathies

Biomarker	Variable	r	P
25(OH)VD	Albumin	$r = 0.69$	<.001
	AST	rho = −0.59	<.01
	Clinical score	rho = −0.56	<.01
	aPTT	rho = −0.52	<.05
	PMN	$r = -0.46$	<.05
	ALP	rho = −0.42	<.05
CRP	Overall histological score	rho = 0.78	<.001
	Confluent necrosis	rho = 0.67	<.01
	Periportal interface hepatitis	rho = 0.52	<.05
	Fibrosis score	rho = 0.49	<.05
	Focal necrosis/inflammation	rho = 0.48	<.05
vWF	Albumin	$r = -0.73$	<.001
	PT	rho = 0.67	<.01
	Fibrosis score	rho = 0.53	<.05
	Clinical score	rho = 0.52	<.05

Abbreviations: ALP, alkaline phosphatase; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; CRP, serum C-reactive protein; PMN, polymorphonuclear neutrophils; PT, prothrombin time; *r*, Pearson's correlation coefficient; rho, Spearman's correlation coefficient; serum 25(OH)VD, 25-hydroxyvitamin D concentration; vWF, plasma von Willebrand's factor concentration.

659 ppm dry weight liver (range, 126-2570 ppm). Fourteen of 19 dogs (74%) had increased hepatic copper concentration (>400 ppm dry weight liver). Twenty of 23 dogs (87%) had liver or bile cultures or both performed at the time of liver biopsy, and 3/20 (15%) had positive cultures with *Bacillus species* (likely a contaminant), *Staphylococcus hominis*, and *Escherichia coli*.

3.5 | Correlation analysis of the biomarkers

Biomarker results were compared with serum variables (ALT, AST, ALP, albumin, total bilirubin, WBC, PMN, PT, and aPTT), clinical score, and histological score for grade and stage (fibrosis) and copper staining (Table 3). Serum 25(OH)VD concentration was significantly positively correlated with serum albumin concentration ($r = 0.69$, $P < .001$) and significantly negatively correlated with serum AST activity (rho = −0.59, $P < .01$), clinical score (rho = −0.56, $P < .01$), aPTT (rho = −0.52, $P < .05$), PMN ($r = -0.448$, $P = .03$), and serum ALP activity ($r = -0.42$, $P < .05$). The remainder of the correlation analyses did not identify any significant findings.

Serum CRP concentration was significantly positively correlated with histological scores for overall histopathology score (rho = 0.78, $P < .001$), confluent necrosis/inflammation (rho = 0.67, $P < .01$), periportal interface hepatitis (rho = 0.52, $P < .05$), fibrosis score (rho = 0.49, $P < .05$), and focal necrosis/inflammation (rho = 0.48, $P < .05$). Neither the clinical pathology variables nor the clinical scores were correlated with serum CRP concentration.

Plasma vWF concentration was significantly negatively correlated with serum albumin concentration ($r = -0.73$, $P < .01$), and significantly positively correlated with PT (rho = 0.67, $P < .01$), histological fibrosis score ($r = 0.53$, $P < .05$), and clinical score (rho = 0.52, $P < .05$). The remainder of the correlation analyses did not identify any significant findings.

Serum ALT activity was not correlated with serum CRP or 25(OH)VD concentrations, plasma vWF concentration, or histologic grade or stage.

4 | DISCUSSION

We investigated the use of serum 25(OH)VD and CRP concentrations and plasma vWF concentration as biomarkers of disease grade and stage in dogs with CH. In our population, serum 25(OH)VD concentration was not correlated with histologic grade or stage, but was negatively correlated with serum biochemical and clinical indicators of disease activity (clinical score, serum ALP and AST activity, and PMN). It also was positively correlated with serum albumin concentration and aPTT, both of which predict shortened survival in dogs with CH. Serum CRP concentration was positively correlated with histological indices of both disease stage (fibrosis score) and grade (severity of necroinflammation). Plasma vWF concentration was positively correlated with histopathologic stage as well as serum indices of shortened survival, including a negative correlation with serum albumin concentration and positive correlations with PT and clinical score. Our results provide preliminary evidence that the biomarkers explored may be useful as noninvasive tools to assess grade and stage of disease in dogs with CH and might in some circumstances decrease the necessity for hepatic biopsy. Longitudinal studies in a larger population of dogs with a wider spectrum of disease severity will be necessary to determine if these biomarkers are useful in predicting clinical outcome, monitoring treatment response, or both.

In our study, serum 25(OH)VD concentrations were positively correlated with grade of disease as indicated by serum liver enzyme activity (serum ALP and AST activity) and negatively with the presence of neutrophilia. In dogs with inflammatory bowel disease, serum VD concentrations also are inversely proportional to PMN counts.[68,69]

One explanation for these results in these inflammatory disease states lies in VD's known role in regulating innate and adaptive immune responses.[18,33] These actions, controlled by binding to VD receptors on WBCs, serve to dampen response of these cells and production of cytokines[18,33] Thus, the net result of VD deficiency would be derepression of these effects and consequently a proinflammatory and profibrotic response. In support of this hypothesis, VD has been shown to downregulate inflammatory responses in WBCs isolated from dogs.[70-72] It is necessary, however, to establish whether low VD concentrations have a similar proinflammatory effect in vivo in dogs.

In humans, VD supplementation can decrease inflammation and fibrosis in nonalcoholic steatohepatitis and chronic hepatitis C.[28,68,73]

Vitamin D status also influences the response to immunosuppression with corticosteroids in autoimmune hepatitis and primary biliary cholangitis.[17,18] Although the role of VD supplementation has not been explored in CH in dogs, supplementation with VD can be safe and effective in normalizing serum VD concentration,[74,75] and in dogs with inflammatory skin disease improves pruritus and lesion scores.[76] Studies in a larger sample of dogs with CH in which dietary VD intake is recorded and that determine serum VD and parathyroid hormone concentrations concurrently will be necessary to better define a VD-deficient state in dogs with CH before supplementation with the VD can be explored as a therapeutic tool.

Our observation that serum VD concentrations correlate negatively with serum variables associated with shortened survival such as serum albumin concentration, prolongations in aPTT, and clinical score suggest that serum VD concentrations may have prognostic relevance in dogs with CH. In human patients with CH, VD deficiency is associated with hepatic decompensation and shortened survival.[16-20] In dogs with nonhepatic inflammatory disorders including inflammatory bowel disease and immune-mediated disease, serum VD concentrations correlate with clinical severity scores and survival.[37,68,69] Future studies should examine if low serum 25(OH)VD concentrations are associated with prognosis in dogs with CH.

Although serum CRP concentrations positively correlate with disease activity in several inflammatory diseases in dogs and in some predict response to treatment,[37,39] results are conflicting on the utility of serum CRP concentration in hepatic disease in dogs.[12,44] In our study, serum CRP concentration was increased in almost half of the dogs with CH, and these increases were significantly positively correlated with histopathologic indices of disease grade (necroinflammatory changes) and stage (fibrotic changes). The correlation with fibrosis on biopsy was unexpected, but may reflect the fact that inflammation is a strong stimulus for fibrogenesis in the liver.[9] A larger prospective study evaluating outcome should explore the value of longitudinal evaluation of serum CRP concentration in assessing response to anti-inflammatory treatment in dogs with CH.

In humans with chronic inflammatory or fibrotic liver disease, serum CRP concentration is used to predict the presence of acute-on-chronic hepatic disease, a condition associated with high short-term mortality.[77,78] Acute decompensation typically occurs in the setting of the systemic inflammation response syndrome (SIRS) secondary to complications such as portal vein thrombosis, infection, endotoxemia, or gastrointestinal bleeding.[79,80] In dogs with CH, the presence of SIRS and a high neutrophil count are negative prognostic markers.[81,82] These observations suggest that systemic inflammation also may be a trigger for acute hepatic decompensation in dogs with CH. A larger prospective study should explore the value of serum CRP concentration in establishing the presence of acute-on-chronic decompensation in dogs with CH.

In humans, increases in plasma VWF concentration are a well-established noninvasive biomarker of cirrhosis and predict the presence of portal hypertension.[29-32] In our study, plasma vWF concentration was correlated with disease stage and was higher in dogs that had clinical signs of portal hypertension, suggesting that vWF concentration also might serve as a serum biomarker of late-stage disease in dogs. Currently, the diagnosis of portal hypertension in dogs relies on several subjective ultrasonographic imaging findings or on invasive procedures to measure splenic portal pressure.[83,84] In humans, lowering portal pressure is important in prolonging survival in cirrhotic patients. The same may be true in dogs, but the lack of an accurate cost-effective noninvasive biomarker for portal hypertension has hampered the study of drugs to decrease portal pressure in the dog. Studies to compare plasma vWF concentration with splenic pulp pressure would be needed to determine if vWF concentration can be used as a marker of portal hypertension in dogs.

Median plasma vWF concentration in our study was in the reference range. No dogs had increased activity, but 4 dogs had activity below the reference range. The reasons for this low activity were not determined. The dogs could have had mutations that decrease the synthesis or increase the catabolism of vWF. Interestingly, in human patients with CH, factors that predispose to hypocoagulability, such as low plasma vWF concentration, are associated with less progression to hepatic fibrosis.[85,86] The link between hemostatic status and hepatic fibrosis in CH is not fully understood. Several lines of evidence suggest that hypercoagulability promotes disease progression perhaps through damage induced by microthrombi in the hepatic circulation or by pro-coagulant activation of hepatic stellate cells, the extracellular matrix-producing cells in the liver.[87-89] The correlation of lower vWF concentration with lower histological fibrosis scores in our study could reflect a similar association in dogs.

Our study corroborated previous studies in that it did not show a correlation between serum ALT activity and the serum biomarkers or with histopathologic grade or stage of disease.[4,5,7,8,11,12] There could be several reasons for this observation. Increases in serum ALT activity in dogs are associated with reversible and irreversible membrane damage to hepatocytes. However, serum ALT activity is not sufficiently sensitive to identify early inflammatory injury and alternatively can be decreased in late-stage disease because of the presence of fibrosis and decreased hepatocyte mass.[90,91] Furthermore, genetic variations in the amount of ALT in hepatocytes may account for a less than robust association with disease activity.[92]

Our study had several limitations. We studied a small population of dogs without age or breed-matched controls and evaluated at both inflammatory and noninflammatory fibrotic hepatopathies, which most likely were associated with different etiologic and pathophysiologic mechanisms. A small study population could have led to type II error and erroneously led us to reject the null hypothesis that the biomarkers studied were of clinical value. Our study population had early- to mid-stage disease, which was reflected in low histologic scores for fibrosis. Future studies that include larger populations of dogs with a wider range of disease severity are needed to validate our findings. Another limitation is that not all dogs had biopsies of multiple liver lobes performed via laparoscopy, a practice that is preferred with CH.[4] Small gauge percutaneous needle biopsy samples (17% of the biopsies performed in our study) can be associated with sampling error.[9] We obtained only limited diet histories, an important consideration because dogs obtain VD from their diet in the form of cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2).[66] Lastly, we did not evaluate survival time to determine if serum concentrations of the biomarkers correlated with clinical outcome.

5 | CONCLUSION

In our population of dogs, we determined the concentrations of biomarkers (ie, 25(OH)VD, vWF, and CRP) previously shown to have predictive and prognostic values in cases in humans and examined if these biomarkers might correlate with histopathologic markers of disease grade and stage in dogs. In our population, serum CRP concentrations were positively correlated with histopathologic stage (fibrosis score) and grade (degree of necroinflammation). Plasma vWF concentration was positively associated with histologic stage and was higher in dogs with portal hypertension, a complication of late-stage disease. Serum 25(OH)VD concentration was not associated with histopathologic variables, but was negatively correlated with serum biochemical and clinical indicators of disease activity (clinical score, serum ALP and AST activities, and PMN) and positively correlated with serum albumin concentration, which has been associated with shorter survival. Our results provide preliminary evidence that the biomarkers assessed in our study may be useful to clinicians managing patients with CH in predicting the presence of sustained inflammation or the occurrence of an acute inflammatory flare as well as provide information on how far liver disease has progressed. This knowledge may prove useful in deciding on when to intervene therapeutically, adjusting long-term treatment, pursuing additional diagnostic investigation, and providing prognostic information to owners. Additional studies are needed to explore the clinical value of these biomarkers.

ACKNOWLEDGMENT

Funding provided by the Office of The Director, National Institutes of Health under Award Number K01OD030515, and the Companion Animal Health Fund.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by Tufts University, Cummings School of Veterinary Medicine, Clinical Studies Review Committee (CSRC#: 001.16).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

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How to cite this article: Ambrosini YM, Piedra-Mora C, Jennings S, Webster CRL. Serum 25-hydroxyvitamin D and C-reactive protein and plasma von Willebrand concentrations in 23 dogs with chronic hepatopathies. *J Vet Intern Med*. 2022; 36(3):966-975. doi:10.1111/jvim.16424