Evaluation of plasma protein C activity for detection of hepatobiliary disease and portosystemic shunting in dogs

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Objective—To determine the diagnostic value of protein C (PC) for detecting hepatobiliary disease and portosystemic shunting (PSS) in dogs.

Design—Prospective study.

Animals—238 clinically ill dogs with (n = 207) and without (31) hepatobiliary disease, including 105 with and 102 without PSS.

Procedures—Enrollment required routine hematologic, serum biochemical, and urine tests; measurement of PC activity; and a definitive diagnosis. Total serum bile acids (TSBA) concentration and coagulation status, including antithrombin activity, were determined in most dogs. Dogs were grouped into hepatobiliary and PSS categories. Specificity and sensitivity were calculated by use of a PC cutoff value of 70% activity.

Results—Specificity for PC activity and TSBA concentrations was similar (76% and 78%, respectively). Best overall sensitivity was detected with TSBA, but PC activity had high sensitivity for detecting PSS and hepatic failure. Protein C activity in microvascular dysplasia (MVD; PC ≥ 70% in 95% of dogs) helped differentiate MVD from portosystemic vascular anomalies (PSVA; PC < 70% in 88% of dogs). A receiver operating characteristic curve (PSVA vs MVD) validated a useful cutoff value of < 70% activity for PC.

Conclusions and Clinical Relevance—Combining PC with routine tests improved recognition of PSS, hepatic failure, and severe hepatobiliary disease and signified a grave prognosis when coupled with hyperbilirubinemia and low antithrombin activity in hepatic failure. Protein C activity can help prioritize tests used to distinguish PSVA from MVD and sensitively reflects improved hepatic-portal perfusion after PSVA ligation. (*J Am Vet Med Assoc* 2006;229:1761–1771)

ABBREVIATIONS

AT	Antithrombin
APC	Activated protein C
DIC	Disseminated intravascular coagulation
PSS	Portosystemic shunting
TSBA	Total serum bile acids
PSVA	Portosystemic vascular anomalies
MVD	Microvascular dysplasia
PT	Prothrombin time
APTT	Activated partial thromboplastin time
ALT	Alanine transaminase
AST	Aspartate transaminase
ALP	Alkaline phosphatase
GGT	γ-glutamyltransferase
MCV	Mean corpuscular volume
ROC	Receiver operating characteristic

Plasma anticoagulant proteins, such as AT; protein C; protein S; and the fibrinolytic protein, plasminogen, are important for maintenance of hemostatic balance and for protecting against thromboembolism. Although information regarding the clinical use of measurements of AT and fibrinolytic proteins is available in the veterinary literature, there is far less information concerning the vitamin K–dependent anticoagulants, protein C and protein S.[1-3]

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Protein C is a disulfide-linked glycoprotein with a molecular weight of 62 kd, similar to the molecular weight of albumin. Protein C is synthesized in the liver and circulates as a plasma zymogen, with conversion to an active serine protease occurring primarily at the luminal surface of endothelial cells. Activation occurs by interaction of protein C with thrombin bound to its transmembrane cofactor, thrombomodulin; this process is enhanced when protein C is bound to an adjacent membrane receptor (endothelial cell protein C receptor; **Figure 1**). Activated protein C is released from its receptor and combines with protein S through interactions with phospholipids on platelet and endothelial cell membranes. The APC–protein S complex exerts its anticoagulant effect by degrading 2 coagulation cofactors, factors Va and VIIIa, that are essential for sustained generation of thrombin and the formation of a fibrin clot (**Figure 2**). The plasma half-life of the zymogen protein C is approximately 6 hours, with rapid (15-minute) inactivation of APC occurring by plasma protease inhibitors.[4] In addition to its major anticoagulant action, APC has a diverse repertoire of biologic effects, including promotion of fibrinolysis, modulation of inflammation, and inhibition of apoptosis.[4-6]

Low protein C activity has been associated with thrombotic disorders in humans and animals.[4,5,7] Hereditary protein C deficiency is a risk factor for venous thrombosis in humans, with heterozygotes having a 7- to 8-fold increased risk.[8] Homozygous protein C deficiency is rarely diagnosed; however, the trait manifests as severe cutaneous and CNS thrombosis in the syndrome of neonatal purpura fulminans.[4,9-11]

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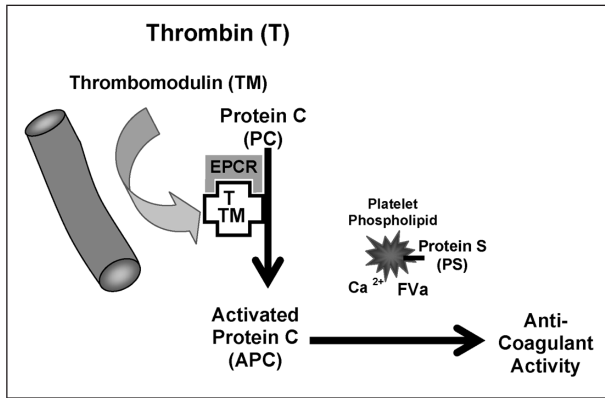

Figure 1—Illustration of the interaction of thrombin and the endothelial receptor, thrombomodulin, and the subsequent activation of protein C by removal of its N-terminus. The APC thereafter interacts with platelet phospholipid, protein S, ionized calcium (Ca^{2+}), and activated factor V (FVa), resulting in the inactivation of factor V and an anticoagulant effect. EPCR = Endothelial cell protein C receptor.

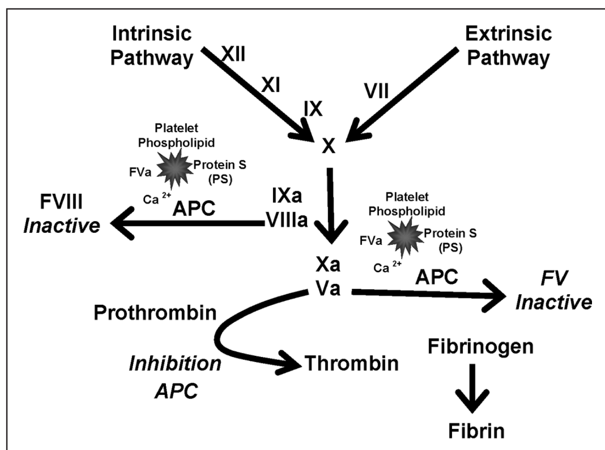

Figure 2—Illustration of the broad anticoagulant effects of APC including inactivation of factor VIII (FVIII inactive) and factor V (FV inactive) and impaired production of thrombin, resulting in the end effect of reducing formation of fibrin from fibrinogen. *See* Figure 1 for remainder of key.

Protein C deficiency more often develops as an acquired disorder because of increased turnover and consumption in the acute phase of inflammation or in association with septicemia and DIC. Hepatic failure and vitamin K deficiency also may profoundly reduce levels of functional protein C activity because of decreased synthesis and a lack of posttranslational modification (γ-carboxylation), respectively.[4,5] Low protein C activity also develops in patients with portal vein thrombosis; however, the pathogenesis of this deficiency is not well-defined and may represent an epiphenomenon rather than a causal factor in thrombus formation.[9]

Beyond the characterization of thrombotic syndromes, measurement of plasma protein C activity has been used clinically to assess hepatic function in human patients with diverse hepatic disorders.[12] Low protein C activity has been described in patients with inflammatory hepatopathies, cirrhosis, portal venous obstruction, and infiltrative and neoplastic diseases and has been used as a prognostic indicator for monitoring hepatic transplant patients after surgery.[12-17]

In our preliminary studies of protein C activity in dogs, we discovered that many dogs with acquired and congenital hepatobiliary disorders had low protein C activity and that dogs with PSS appeared to develop the lowest protein C activity.[a] The purpose of the study reported here was to evaluate the diagnostic use of protein C for detection of hepatobiliary disease and PSS in dogs. We suspected that protein C deficiency was a common feature of hepatobiliary disease and that inclusion of protein C analyses with serum biochemical profiles, coagulation profiles (including AT), and determination of TSBA concentrations may help distinguish dogs with PSS. We specifically recruited dogs with congenital PSVA and MVD into the study to challenge this hypothesis.

Materials and Methods

Dogs—Case materials from dogs evaluated at the Cornell University Hospital for Animals and suspected of having primary hepatobiliary disease on admission were prospectively compiled between July 2000 and December 2005. Study inclusion required determination of routine hematologic, biochemical, and urine values and protein C activity and establishment of a definitive diagnosis at discharge or death on the basis of results of diagnostic imaging studies, histologic examination of liver biopsy specimens, and surgical or necropsy findings. Most dogs also had complete coagulation profiles performed, including PT, APTT, fibrinogen, and AT analyses.

CBC and serum biochemical profiles—Blood samples were collected into tubes containing EDTA and heparin anticoagulant for determination of CBC and serum biochemical profiles, respectively. The CBCs were performed by use of an automated cell counter.[b] Serum biochemical profiles were performed by use of an automated analyzer[c] and consisted of total protein, albumin, globulin, BUN, creatinine, glucose, cholesterol, and total bilirubin concentrations; total iron-binding capacity; percentage of iron saturation; activities of serum ALT, AST, ALP, GGT, amylase, and creatine kinase; and serum electrolyte (sodium, potassium, chloride, bicarbonate, calcium, phosphorus, and magnesium) concentrations. Total serum bile acids concentrations were measured in most anicteric dogs after food had been withheld for 12 hours and again 2 hours postprandially.

Coagulation and AT assays—Coagulation and anticoagulant assays were performed on citrated plasma, prepared by centrifugation of whole blood collected directly into citrate anticoagulant (1 part 3.8% citrate:9 parts blood). Coagulation assays included a routine coagulation panel (APTT, PT, and clottable [Clauss] fibrinogen concentration). The coagulation panel and fibrinogen assays were performed by use of an automated coagulation instrument[d] with a mechanical end-point detection method and commercial reagents.[e-h] Antithrombin activity was measured on the basis of inhibition of factor IIa by use of a colorimetric method and chromogenic substrate kit[i] as described previously.[18,19] Calibration curves for fibrinogen and AT assays were derived from dilutions of a pooled canine plasma standard (prepared from 20 healthy dogs). The fibrinogen content of the standard was determined by gravimetric method,[20] and the AT activity of the standard plasma had an assigned value of 100%.

Protein C assay—Protein C activity was measured by use of a chromogenic substrate kit.[j] In this assay, plasma protein C is activated with a snake-venom derivative (*Agkistrodon contortrix contortrix*) and the amidolytic activity of the APC is detected by cleavage of a synthetic chromogenic substrate.[21]

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The assay was modified by use of canine pooled plasma, rather than lyophilized human plasma, to generate a standard curve with dilutional agreement through a dynamic linear range of 0% to 100% canine protein C activity. Test results were reported as the percentage protein C activity of the standard, which had an assigned value of 100%. A reference range of 75% to 135% was derived from assay values of 37 clinically healthy dogs (15 males and 22 females) ranging in age from 0.5 to 10 years old and of various breeds (Labrador Retrievers, n = 8; mixed-breed dogs, 8; Maltese, 7; Australian Shepherds, 3; Doberman Pinschers, 2; Rottweilers, 2; and 1 each of Basset Hound, Bernese Mountain Dog, Cairn Terrier, Collie, Golden Retriever, hound dog, and Lhasa Apso). These dogs were pets owned by individuals associated with the veterinary hospital or were healthy dogs maintained in a research colony approved by the Cornell University Animal Care and Use Committee. The intra- and interassay coefficients of variation (on the basis of 12 same- or different-day replicate measurements of pooled canine plasma) were 2.9% and 6.9%, respectively.

Prospectively entered clinical patients—Of 238 dogs entered into the study, 207 had some form of hepatobiliary disease or hepatic perfusion abnormality **(Figure 3)**. Thirty-one dogs initially considered to have hepatobiliary disease were later determined to have another primary nonhepatic condition. Hepatobiliary disorders represented by ≥ 10 dogs were categorized into 5 discrete hepatic categories with an additional group designated for hepatobiliary disorders represented by ≤ 10 dogs with a specific disorder (miscellaneous hepatobiliary disease category).

Seventy-five dogs had a PSVA without previous surgical correction, which was confirmed on the basis of clinical history; physical examination findings; clinicopathologic features; results of abdominal radiography, abdominal ultrasonography, and colorectal scintigraphy with technetium pertechnetate (n = 63); gross inspection of portal vasculature during surgical ligation (69) or histologic examination of liver biopsy specimens (50); and results of portovenography (43). The absence of multiple acquired PSS was determined by gross inspection or portovenography, and histologic examination of liver biopsy specimens confirmed the absence of acquired hepatobiliary disease or juvenile fibrosing liver disease. Dog breeds represented included Yorkshire Terrier (n = 15), Maltese (11), Shih Tzu (4), Havanese (3), Cairn Terrier (3), Miniature Schnauzer (6), Pug (3), Doberman Pinscher (2), Labrador Retriever (2), and Scottish Deerhound (2). The remaining 24 dogs comprised various breeds represented by only a single dog. Ten large-breed dogs had an intrahepatic PSVA (patent ductus venosus), 63 dogs had a single extrahepatic shunt, and 2 dogs had 2 anomalous PSVA vessels. There were 15 additional dogs with PSVA (that had undergone abdominal ultrasonography, colorectal scintigraphy, portography, and liver biopsy) in which surgical ligation of a PSVA had been performed with variable success. These dogs were analyzed as a separate category because they were used to determine the influence of surgical ligation of PSVA on protein C activity.

Portosystemic shunting was diagnosed in 20 dogs with acquired PSS by use of Doppler color-flow ultrasonography (n = 14), colorectal scintigraphy (10), and histologic examination of liver biopsy specimens (16). These dogs had multiple small, tortuous, acquired, portosystemic shunts. Histologic examination of liver biopsy specimens was used to define an underlying hepatic disorder leading to portal hypertension when a portal vein filling defect consistent with circulatory obstruction or some other cause of impeded portal circulation could not be distinguished by use of diagnostic imaging studies.

Microvascular dysplasia (n = 39 dogs) was diagnosed on the basis of the presence of a high TSBA concentration in the absence of a history compatible with that of PSVA (ie, no clinical signs, age < 2 years, small-breed dog), physical signs (ie, small stature, delayed development, polydipsia and polyuria, or dull mentation), or clinicopathologic features (ie, microcytosis; low BUN, creatinine, and cholesterol concentrations; and ammonium biurate crystalluria) and in the inability to detect PSVA on colorectal scintigraphy and color-flow Doppler ultrasonography. In 19 dogs with MVD that had increased liver enzyme activity, histologic examination of liver biopsy specimens revealed zone 3 perivenular inflammation. Breeds represented in the MVD category included Maltese (n = 18), Yorkshire Terrier (5), Shih Tzu (3), Dachshund (2), Jack Russell Terrier (2), Toy Poodle (2), Bichon Frise (2), Chihuahua (2), and Maltese crosses (2), and Silky Terrier (1).

Chronic hepatitis was diagnosed in 20 dogs that had no evidence of acquired PSS as determined by diagnostic imaging, colorectal scintigraphy, or exploratory laparotomy or laparoscopy. Histologic examination of liver biopsy specimens revealed nonsuppurative zone 1 inflammation that breached the limiting plate.

Hepatic failure not associated with acquired PSS (n = 20 dogs) was determined on the basis of antecedent history of hepatotoxin ingestion, clinicopathologic features, or histologic examination of liver biopsy specimens (17). Hepatic failure developed secondary to hepatotoxin ingestion (eg, aflatoxin-contaminated feed, manganese, cycad, amanita mushroom, and microcystin) in 11 dogs, secondary to acute severe leptospirosis in 4 dogs, and secondary to hepatocutaneous

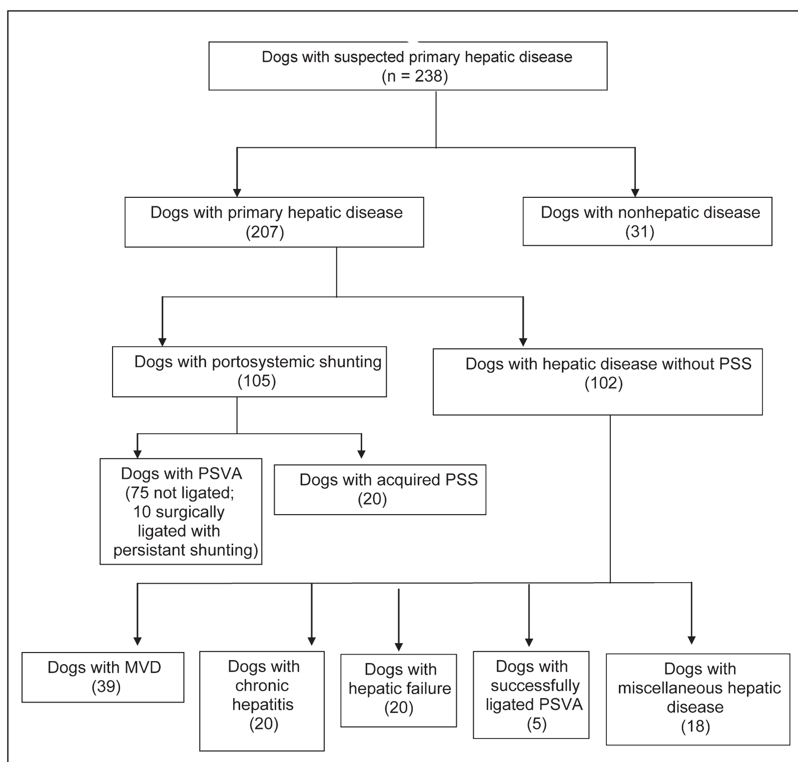

Figure 3—Summary of disease group classification for dogs prospectively entered in a study on the use of protein C activity for detection of hepatobiliary disease and PSS.

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syndrome in 2 dogs (as determined by typical cutaneous lesions and plasma amino acid profiles).[22] Hepatic failure in 1 dog was attributed to renal abscesses causing septicemia, and the cause of hepatic failure in 2 dogs was not determined.

Miscellaneous hepatic disorders (n = 18) were diagnosed on the basis of hepatic lesions determined on liver tissue collected by liver biopsy or at necropsy. Disorders included neoplastic disorders (n = 7), vacuolar hepatopathy (8), and primary gall bladder (1) or biliary tract disease (2). Seven dogs had neoplasia confined to the liver; 4 dogs had lymphosarcoma restricted to the hepatic parenchyma, gallbladder, or bile duct; 2 dogs had large hepatomas; and 1 dog had hepatic lymphoma causing sinusoidal thrombosis.

A nonhepatic disease category was comprised of 31 dogs in which the primary disease did not involve the hepatobiliary system as determined by histologic examination of liver biopsy specimens (n = 21) or in which TSBA concentrations were considered normal (after withholding food for 12- and 2-hour postprandial values). The nonhepatic disease category included inflammatory bowel disease without hepatic lesions as determined by histologic examination of liver biopsy specimens (n = 13); renal insufficiency (3); thromboembolic disease (5) including aortic thrombus (2), vena caval thrombus (2), and generalized DIC (1); neoplasia (6) including enteric lymphoma without liver involvement (2), brain tumor (2), and renal carcinoma (1); and 1 dog each with septic pyometra, acute passive congestion of the liver, granulomatous meningoencephalitis, and cryptococcal meningoencephalitis.

Clinical patients also were categorized on the basis of the presence or absence of PSS (PSS, n = 105; no PSS, 102; **Table 1**). Dogs with PSS included 75 dogs with PSVA that had not been surgically modified, 10 dogs with persistent shunting despite surgical PSVA ligation, and 20 dogs with acquired PSS attributable to hepatic cirrhosis or fibrosis, severe vacuolar hepatopathy associated with parenchymal collapse, aflatoxin-induced hepatic failure, and portal venous thromboembolism. Portosystemic shunting was distinguished by color-flow Doppler ultrasonography, colorectal scintigraphy, or surgical exploration. Clinicopathologic fea-

Table 1—Clinicopathologic characteristics of dogs with hepatobiliary disease and clinically ill dogs without hepatobiliary disease but with other confirmed diagnoses.

	Extrahepatic PSS			No extrahepatic PSS			Nonhepatobiliary disease		
Variable	PSVA	Acquired PSS	MVD	Chronic hepatitis	Hepatic failure	Miscellaneous hepatobiliary disorders	Non-hepatobiliary disease with liver biopsy	Nonheaptobiliary disease with liver biopsy (n = 21) or with TSBA values in reference range (10)	Reference range
No. of dogs	75	20	39	20	20	18	21	31	NA
Age (y)	1 (0.4–12)	3.5 (0.4–14)	2 (0.5–10)	8 (1–13)	7 (2–12)	7 (1–7)	6 (0.5–13)	7 (0.5–13)	NA
Sex									
Female	8	2	10	0	7	2	1	1	NA
Spayed female	26	8	12	10	8	9	10	13	NA
Male	15	2	7	3	0	3	2	3	NA
Castrated male	26	8	10	7	5	4	8	14	NA
Body weight (kg)	4.5 (1.2–40)	7 (2.3–27.5)	3.3 (1.1–11.1)	29 (6.6–46)	25.1 (6.2–51.7)	9.8 (5.9–14)	23 (2.8–48)	23 (1.9–48)	NA
PCV (%)	43 (26–54)	44 (23–54)	49 (38–62)	48 (33–55)	48 (28–55)	44 (11–57)	41 (15–58)	42 (15–58)	39–57
MCV (fL)	63 (51–79)	67 (59–84)	72 (64–76)	69 (59–78)	67 (61–81)	72 (65–82)	71 (60–80)	70 (60–80)	64–73
WBC (× 10^3/µL)	13.8 (5.7–28.9)	13.9 (6.5–27.2)	10.2 (4.7–15.4)	11.4 (6.5–39.3)	16.7 (9.6–37.6)	10.9 (5.4–94.5)	15.8 (6.5–39.8)	15.5 (6.5–39.8)	7.5–19.9
Albumin (g/dL)	2.9 (1.1–3.3)	2.7 (1.9–4.1)	3.7 (2.2–4.3)	3.3 (2.1–4.0)	2.6 (1.1–3.7)	3.3 (2.0–4.5)	2.7 (1.4–4.1)	3.0 (1.4–4.4)	3.1–4.1
ALP (U/L)	191 (21–2,048)	342 (67–4,725)	69 (14–399)	555 (27–7,055)	301 (85–1,689)	656 (25–3,898)	101 (101–7,440)	102 (10–7,440)	4–122
ALT (U/L)	138 (29–4,978)	302 (33–2,801)	94 (23–820)	599 (39–3,451)	439 (50–10,785)	494 (25–6,995)	58 (9–870)	69 (9–870)	12–106
BUN (mg/dL)	7 (3–92)	8 (2–63)	15 (4–40)	12 (5–43)	16 (5–77)	12 (6–65)	14 (4–74)	15 (4–74)	8–30
Cholesterol (mg/dL)	124 (57–337)	123 (78–407)	201 (103–502)	247 (99–829)	96 (10–262)	244 (92–453)	153 (28–342)	181 (28–387)	150–335
Glucose (mg/dL)	87 (29–125)	58 (90–134)	102 (82–141)	92 (44–113)	101 (56–130)	103 (31–169)	97 (28–179)	99 (28–179)	58–120
Total bilirubin (mg/dL)	0.1 (0–2.8)	0.3 (0–12)	0.1 (0–0.6)	0.4 (0.1–11.3)	5.9 (0.5–24.2)	0.2 (0–20.7)	0.1 (0–25.9)	0.1 (0–25.9)	< 0.3
Highest TSBA concentration (µmol/L)	250 (27–663)	227 (62–648)	68 (35–214)	51 (6–420)	250 (98–276)	32 (7–402)	11 (6–36)	13 (6–36)	< 25
APTT (s)	13 (10–25.7)	15.3 (10.2–60)	12 (9–16)	13 (10–19)	23 (9–60)	12 (8–40)	12 (10–21)	12 (9–21)	10–17
PT (s)	17 (13–24)	16.4 (14–30)	17 (13–22)	17 (13–23)	23 (16–60)	17 (15–29)	15.8 (13–19)	16 (13–19)	13–18
Fibrinogen (mg/dL)	346 (184–1,003)	369 (15–1,054)	410 (107–646)	362 (149–769)	73 (14–293)	546 (75–813)	621 (254–971)	645 (254–994)	150–510
AT (% activity)	74 (42–111)	80 (15–99)	96 (75–122)	90 (37–113)	29 (9–100)	100 (47–114)	86 (46–108)	86 (46–128)	< 75
Protein C (% activity)	46 (4–109)	46 (5–124)	101 (65–142)	74 (25–131)	24 (5–91)	88 (67–114)	97 (37–122)	97 (37–122)	≥ 70

Data are given as median (range) for measurement data. In 10 dogs, extrahepatic PSS had been surgically ligated, but shunting persisted. Ligation of a PSVA was successful in 5 dogs classified as not having extrahepatic PSS.

To convert from kg to lb, multiply by 2.2. NA = Not applicable.

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tures consistent with PSS-included low RBC MCV; low BUN, creatinine, and cholesterol concentrations; and high TSBA concentrations.

Statistical analysis—Box-and-whisker plots and histograms were used to examine data for Gaussian distribution. Because most clinical data did not have a Gaussian distribution, data were expressed as median and range of values. Nonparametric comparisons, by use of the Wilcoxon rank sum test, were used to detect significant differences in age, body weight, and clinicopathologic variables between disease categories, between dogs with or without PSS, and between dogs with PSVA and dogs with MVD. Significant differences in dogs with PSVA before and after surgical ligation and between dogs with PSVA in which protein C activity was considered normal or abnormal were detected by use of the Wilcoxon signed rank test. Spearman rank correlations were used to determine whether associations between protein C activity and other clinicopathologic variables were significant. All data were analyzed by use of a statistical software program.[k] A 2-tailed test ($\alpha = 0.05$) was applied to nonparametric comparisons. However, because we performed numerous clinicopathologic comparisons among disease categories, a Bonferroni correction was applied to set a more stringent cutoff value for detecting significant ($P \le 0.001$) differences between disease categories.

Diagnostic performance of protein C activity, other coagulation tests, TSBA concentration (after withholding food for 12 hours and again 2 hours postprandially and the highest TSBA concentrations), and pertinent routine clinicopathologic tests used to detect hepatobiliary disease and PSS and to differentiate PSVA from MVD was evaluated by calculating test specificity (%) and sensitivity (%). Test performance was determined for all dogs and for dogs that underwent liver biopsy. In the nonhepatobiliary disease (control) group, clinicopathologic test results were compared (by use of a Fisher exact test) between dogs that underwent liver biopsy and dogs in this group with TSBA concentrations within reference range and in which liver biopsy was not performed to determine whether results could be combined and whether the decision to obtain a liver biopsy had been influenced by specific clinicopathologic data.

An ROC curve was constructed to determine the best diagnostic cutoff value for protein C activity in discrimination of PSVA from MVD. The ROC best cutoff value also was examined for its ability to discriminate between PSVA and MVD when combined with results of routine clinical tests (total serum cholesterol concentration and RBC microcytosis) commonly used to help distinguish these disorders.

Results

Descriptive statistics for signalment and pertinent laboratory parameters from all dogs with hepatobiliary disease, specific hepatobiliary disease categories, dogs with PSS, and clinically ill dogs with nonhepatobiliary disease are depicted (Table 1). A dot plot of protein C activity for all clinically ill dogs is also depicted (**Figure 4**).

There was no significant ($P = 0.001$) difference in clinicopathologic variables in dogs with nonhepatobiliary disorders that did or did not undergo liver biopsy, with the exception of the total cholesterol concentration. Subsequently, clinicopathologic data for all 31 dogs without hepatobiliary disease were combined for comparison with hepatobiliary disease categories. Inspection of data suggested that dogs with unexplained hypoalbuminemia or hypocholesterolemia were more likely to undergo liver biopsy than dogs without these abnormalities. Dogs with hepatobiliary disorders (all disease categories combined) had a significantly ($P \le 0.001$) lower median body weight, lower age, lower concentrations of BUN and fibrinogen, and lower protein C activity, compared with clinically ill dogs without hepatobiliary disease. Dogs with hepatobiliary disease also had significantly ($P \le 0.001$) higher median serum activities of ALT and AST and concentrations of TSBA, compared with dogs without hepatobiliary disease.

Dogs with PSS were significantly ($P < 0.001$) younger and had lower body weights; lower median value for RBC MCV; lower concentrations of total protein, albumin, BUN, creatinine, cholesterol, and glucose; and lower protein C activity than clinically ill dogs without PSS. Dogs with PSS also had significantly ($P < 0.001$) higher median concentrations of chloride, phosphorus, and TSBA, compared with dogs without PSS. Data for dogs with PSS was heavily influenced by the large number of dogs with PSVA (n = 75) versus acquired PSS (20) such that these analyses predominantly reflect clinical manifestations of PSVA.

Compared with dogs with MVD, dogs with PSVA had significantly ($P < 0.001$) lower median values for RBC MCV; lower urine specific gravity; lower concentrations of albumin, BUN, creatinine, cholesterol, calcium, magnesium, and glucose; and lower activities of protein C and AT. Dogs with PSVA also had significantly ($P < 0.001$) higher median activities of ALP, AST, and creatine kinase and higher concentrations of TSBA, compared with dogs with MVD. Dogs with acquired PSS and dogs with hepatic failure were significantly ($P < 0.001$) older and heavier and had higher median concentrations of TSBA and total bilirubin;

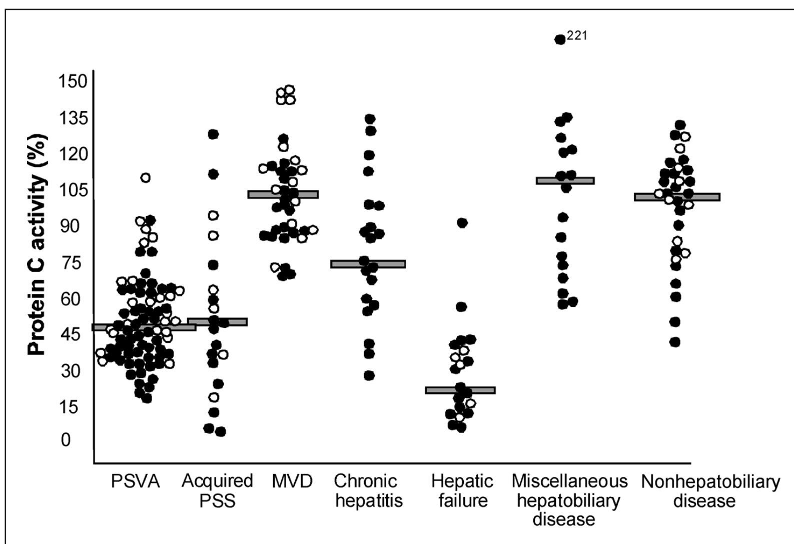

Figure 4—Dot plot indicating protein C activity for clinically ill dogs with and without hepatobiliary disease. Horizontal bars represent median values. Miscellaneous hepatobiliary disease represents a variety of different hepatobiliary disorders represented by ≤ 10 dogs each. Solid circles = Dogs that underwent liver biopsy. Open circles = Dogs in which a definitive diagnosis was determined without liver biopsy.

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Table 2—Specificity of tests for the diagnosis of hepatobiliary disease calculated from test values in dogs without hepatobiliary disease (control dogs) in which a liver biopsy was or was not performed.

	Control dogs with liver biopsy (n = 21)		Control dogs with liver biopsy (21) or dogs without liver biopsy but with TSBA concentrations within reference range (10)	
Test	**Specificity (%)**	**95% CI**	**Specificity (%)**	**95% CI**
Protein C < 70%	76	56–97	81	65–96
AT < 75%	70	47–93	78	60–95
PT > 18 seconds	91	77–100	94	83–100
APTT > 17 seconds	86	68–100	77	61–94
Fibrinogen < 150 mg/dL	100	99–100	100	98–100
FBA > 25 μmol/L	88	58–100	NA	NA
PBA > 25 μmol/L	89	63–100	NA	NA
Highest TSBA value > 25 μmol/L	78	45–100	NA	NA
Total bilirubin > 0.3 mg/dL	81	62–100	94	83–100
MCV < 63 fL	95	84–100	97	89–100
Albumin < 3.1 mg/dL	29	7–50	52	32–71
BUN < 8 mg/dL	90	76–100	90	80–100
Cholesterol < 150 mg/dL	48	24–71	65	46–83
Glucose < 60 mg/dL	95	84–100	97	89–100

CI = Confidence interval. FBA = TSBA concentration after withholding food. NA = Not applicable because TSBA concentrations that were within reference range were used as selection criteria. PBA = Postprandial TSBA concentration.

Table 3—Sensitivity (%) of tests for the diagnosis of hepatobiliary disease in all dogs with hepatobiliary disease and dogs that underwent liver biopsy.

		Test (cutoff value)													
Disorder	**No. of dogs**	**PC (< 70%)**	**AT (< 75%)**	**PT > 18 (sec)**	**APTT > 17 (sec)**	**Fibrinogen (150 mg/dL)**	**FBA (< 25 μM/L)**	**PBA (< 25 μM/L)**	**Highest TSBA value (< 25 μM/L)**	**Bilirubin (< 0.3 mg/dL)**	**MCV (< 64 fL)**	**Albumin (< 3.1 g/dL)**	**BUN (< 8 mg/dL)**	**Cholesterol (< 150 mg/dL)**	**Glucose (< 58 mg/dL)**
All hepatobiliary disease															
All	207	59	43	46	15	18	71	91	93	28	16	47	31	43	7
Biopsied	145	62	38	41	16	17	58	74	76	34	16	52	35	50	5
PSVA															
All	75	88	53	50	7	0	92	99	100	10	36	69	54	67	10
Biopsied	50	92	54	21	8	0	91	100	100	10	38	69	52	77	6
PSVA ligated															
All	15	31	40	0	0	0	56	100	100	0	20	44	40	10	20
Biopsied	6	33	0	0	0	0	100	100	100	0	0	33	67	0	0
Acquired PSS															
All	20	75	41	39	39	36	92	100	100	25	21	70	40	70	5
Biopsied	14	79	50	58	38	56	86	100	100	29	23	86	50	79	7
MVD															
All	39	5	0	19	0	6	43	65	100	6	0	11	9	18	0
Biopsied	21	10	0	15	0	0	41	84	89	13	0	13	13	13	0
Chronic hepatitis															
All	20	45	29	21	5	6	38	54	62	50	5	30	15	10	10
Biopsied	20	45	25	21	5	6	38	54	62	50	5	30	15	10	10
Hepatic failure															
All	20	95	84	71	71	75	100	100	100	100	5	70	25	70	5
Biopsied	16	94	93	80	80	77	100	100	100	100	6	81	31	81	0
Miscellaneous hepatobiliary disease															
All	18	28	41	19	19	14	50	57	57	39	0	33	17	22	6
Biopsied	18	28	41	19	19	14	50	57	57	39	0	33	17	22	6
All dogs with congenital or acquired shunting															
All	105	80	47	19	9	6	91	99	99	23	32	69	50	64	9
Biopsied	70	86	47	24	9	9	91	100	98	35	32	71	51	72	6

PC = Protein C.
See Table 2 for remainder of key.

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higher serum activities of ALP, ALT, AST, and GGT; and longer APTT than dogs with MVD. Dogs with acquired PSS and dogs with hepatic failure also had significantly ($P < 0.001$) lower median values for RBC MCV; lower platelet counts; lower concentrations of total protein, albumin, calcium, glucose, and cholesterol; and lower activities of protein C and AT than dogs with MVD.

Dogs with PSVA were significantly ($P < 0.001$) younger and had lower median body weight; lower median values for RBC MCV; lower activities of ALP, GGT, ALT, and AST; and a lower concentration of total bilirubin than dogs with acquired PSS and dogs with hepatic failure. However, there was no difference in activities of protein C or AT or TSBA concentrations between dogs with PSVA, dogs with acquired PSS, and dogs with hepatic failure. Dogs with PSVA in which protein C activity was considered normal had a significantly higher median cholesterol concentration (193 mg/dL; range, 160 to 255 mg/dL) than dogs with PSVA that had a low protein C activity (cholesterol concentration, 120 mg/dL; range, 57 to 337 mg/dL). Concurrent low protein C and cholesterol values seemingly suggested a greater degree of PSS.

No significant differences were detected in test specificity between control dogs that did or did not undergo liver biopsy (**Table 2**). Best sensitivities were achieved with TSBA concentrations, but protein C activity also provided high sensitivity in dogs with PSS and hepatic failure (**Table 3**). The sensitivity of AT as a diagnostic test was less than that of protein C in most categories. Because finding a protein C activity considered normal in most dogs with MVD would help distinguish that disorder from PSVA, we explored that hypothesis by constructing an ROC curve for protein C activity in regards to its capacity to distinguish PSVA from MVD (**Figure 5**). This curve depicted excellent test performance at the protein C cutoff value of 70% applied in our study. For the clinical situation described in the ROC curve, in our experience, the cholesterol concentration and RBC MCV assist in determining whether a dog has PSVA or MVD. In this scenario, the performance of protein C activity (< 70% activity) versus the detection of either hypocholesterolemia (< 150 mg/dL) or RBC microcytosis (< 63 fL) for discrimination of PSVA from MVD is depicted (**Table 4**). By use of these cutoff values, respectively, we specified whether abnormal (+) or normal (–) values were detected on each test for each dog with PSVA and MVD. By use of this method, we determined that evaluation of protein C activity correctly improved detection of PSVA in 44 of 52 (85%) dogs and correctly excluded PSVA in an additional 5 of 6 (83%) dogs with MVD that had inconsistent routine tests.

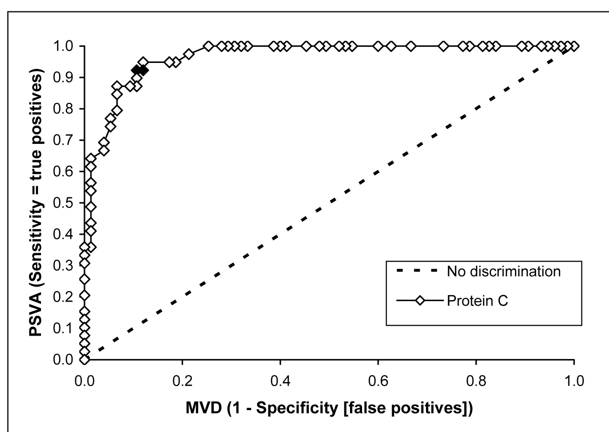

Figure 5—Receiver operating characteristic curve describing the performance of various cutoff values for protein C activity in discriminating 75 dogs with PSVA from 39 dogs with MVD. The black boxes indicate the cutoff value (70%) for protein C activity used for clinical diagnoses where sensitivity = 92.8%, and 1 – specificity = 0.12.

Although a substantial increase in protein C activity was detected in 10 dogs after PSVA ligation (**Figure 6**), protein C activity remained < 70% in 5 dogs. The median protein C activity from the 10 dogs with improved values was significantly ($P < 0.02$) higher than that of the 5 dogs with values that did not improve. Abnormally increased TSBA concentrations (median postprandial TSBA concentration, 122 µmol/L; range, 8 to 630 µmol/L) persisted in 11 of 13 dogs tested with PSVA after surgical shunt attenuation. Two dogs had no change in postoperative protein C activity > 3 months after surgery despite the surgeon's impressions of optimal (100%) ligations. Color-flow Doppler ultrasonography revealed persistent hepatic portal hypoperfusion and shunting in an extrahepatic vessel in 10 dogs at various intervals after PSVA ligation. Colorectal scintigraphy performed in 1 dog 2 weeks after surgical ligation confirmed continued macroscopic shunting. Colorectal scintigraphy performed

Table 4—Performance of protein C activity, compared with serum concentration of cholesterol and MCV (RBC microcytosis) in discriminating dogs with PSVA from dogs with MVD.

Test result			No. of dogs	
Protein C	Cholesterol	MCV	PSVA (n = 68)	MVD (34)
+	+	+	16	0
+	+	–	30	1
+	–	+	7	0
+	–	–	7	0
–	+	+	0	0
–	+	–	0	5
–	–	+	2	0
–	–	–	6	28

+ = Abnormal test result. – = Test value considered normal. n = No. of dogs with all 3 tests.

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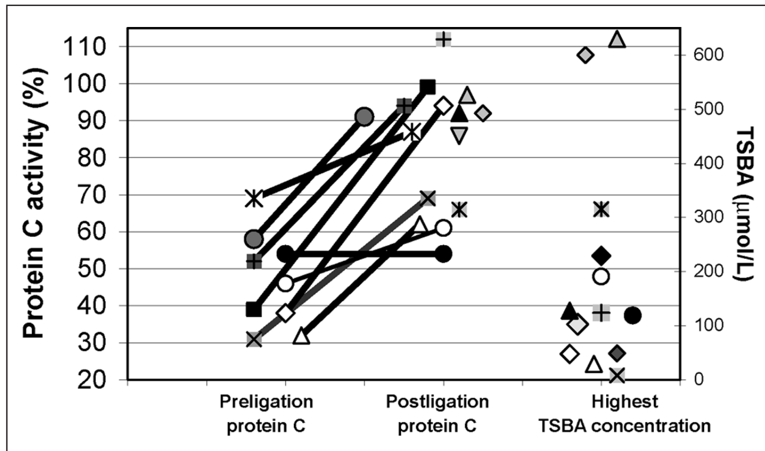

Figure 6—Dot plot indicating protein C activity for individual dogs (represented by various symbols) with PSVA before (n = 9) and after (15) surgical ligation as well as the postoperative highest TSBA concentration of paired samples (13; samples obtained after withholding food for 12 hours and again 2 hours postprandially). Lines interconnecting data points represent pre- and postoperative protein C activity in an individual dog.

Table 5—Tests having positive and negative significant correlations with protein C activity in dogs with or without hepatobiliary disease.

Test	Coefficient	*P* value
PCV	0.23	< 0.001
MCV	0.19	0.006
Platelets	0.36	< 0.001
TIBC	0.44	< 0.001
Glucose	0.19	0.008
Cholesterol	0.52	< 0.001
Fibrinogen	0.60	< 0.001
AT	0.72	< 0.001
FBA	–0.44	< 0.001
PBA	–0.54	< 0.001
Total bilirubin	–0.28	< 0.001
PT	–0.44	< 0.001
APTT	–0.44	< 0.001
ALT	–0.16	0.01
AST	–0.18	0.009
Iron saturation	–0.32	0.007
WBC	–0.27	< 0.001
Serum phosphate	–0.31	< 0.001

TIBC = Total iron binding capacity.
See Table 2 for remainder of key.

at intervals ranging from 1 month to 3 years after PSVA ligation revealed continued shunting in 5 of 9 other dogs, consistent with observations previously reported.[23]

Although significant positive and negative correlations between protein C activity and other studied test variables were detected in dogs with hepatobiliary disease, the correlations were typically low; the strongest association was with AT (r = 0.72), which also is synthesized in the liver and functions in an anticoagulant capacity (**Table 5**).

Discussion

Clinicopathologic features characterizing forms of hepatobiliary disease included in the study reported here were in agreement with previously published information.[24] However, the study reported here is the first to include analyses of protein C and AT activity with traditional coagulation assays and to assess the clinical use of low protein C activity as a diagnostic test in a large number of dogs with naturally occurring hepatobiliary disorders. The importance of liver function for maintenance of normal hemostasis has long been recognized because the hepatocyte is the primary site for synthesis of many of the coagulation proteases and many components of the fibrinolytic systems.[24,25] Although coagulation assays are routinely performed to evaluate the risk of hemorrhage in dogs with hepatobiliary disease, clinically important coagulation factor deficiencies are found primarily in the subset of patients with severe, diffuse, parenchymal loss; complete extrahepatic bile duct occlusion; or fulminant DIC.[25-27] Our findings indicated that many dogs with histologically confirmed hepatobiliary disease lack abnormal coagulation variables even when the coagulation assessments are optimized for canine plasma. In our study, pronounced clotting time prolongation and fibrinogen deficiency were detected only in dogs with hepatic failure. In contrast, we found that 59% of all dogs with hepatobiliary disease had low protein C activity, and within all disease groups, low protein C activity was the most common hemostatic protein abnormality, followed by a prolonged PT time (46%), an abnormally low AT activity (43%), a low fibrinogen concentration (18%), and a prolonged APTT (15%). Our results indicated that coagulation test abnormalities were far more common in dogs with PSVA, compared with the general population of dogs with hepatobiliary disease, when protein C activity was included as a coagulation test. Low protein C activity was detected in 88%, low AT activity in 53%, prolonged PT in 50%, and prolonged APTT in 7% of dogs with PSVA. We did not detect low fibrinogen concentrations in any dogs with PSVA. Our findings in dogs affected with PSVA are discordant with results reported previously for 39 dogs with PSVA in which prolonged APTT and low fibrinogen concentrations were the most common coagulation abnormalities.[28] In that study,[28] a reference range derived from only 28 dogs without hepatic disease may have been too narrow. The routine assays used in the study reported here have been optimized for canine plasma, are routinely measured with concurrent quality control samples of pooled canine plasma, and are interpreted on the basis of a reference range established from > 100 healthy dogs. Results of our study indicated that low protein C activity has clinical use as a biomarker of liver function and perfusion and is not solely a feature of end-stage hepatobiliary disease or synthetic failure.

A number of conditions associated with low protein C activity have been characterized in humans. Inherited deficiencies involve abnormalities limiting either the production or function of protein C, whereas acquired protein C deficiencies are affiliated with acute phase inflammatory responses, sepsis or septic shock, DIC (consumptive utilization of protein C), vitamin K deficiency (dysfunctional decarboxylated pro-

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tein C), and a variety of primary hepatic disorders (impairing protein C synthesis, altering liver perfusion, or accelerating protein C degradation). In humans, finding protein C activity < 65% predicts risk for thromboembolism (primarily deep vein thrombosis and pulmonary thromboembolism) and also is used as a negative prognostic factor in patients with sepsis.[29-32] Although low plasma protein C activity has been affiliated in humans with portal vein thrombosis and other disorders influencing hepatic perfusion, the etiopathogenesis of these associations remains ill defined.[9-14] However, authors of several studies[9,33-35] propose that low protein C activity in these individuals reflects impaired hepatoportal perfusion and is the result, rather than cause, of portal vein thrombosis. In fact, 1 author demonstrated that restoration of hepatic portal circulation in 10 of 11 children with portal vein thrombosis normalized protein C activity, similar to our findings in dogs undergoing PSVA ligation.[33]

Subcategorization of dogs into disorders associated with PSS was performed in our study to test the hypothesis that PSS substantially reduces protein C activity in dogs. Our results indicate that protein C activity is significantly lower in dogs with congenital or acquired PSS, compared with dogs without PSS (excluding end-stage hepatic failure), affirming that protein C activity reflects the adequacy of hepatic portal perfusion in dogs as suggested in humans. Furthermore, our findings confirm that low protein C activity develops in dogs with acquired and congenital shunting in the absence of gross or microscopic evidence of portal vein thrombosis. These observations lend further support to the hypothesis that protein C activity reflects the adequacy of hepatoportal perfusion and that protein C deficiency represents an epiphenomenon of impaired portal blood flow, rather than an inducement to compromised portal perfusion (eg, thrombus formation). Our observations, albeit in a small number of dogs after PSVA ligation, substantiate an exceptional ability of protein C activity to reflect increased hepatoportal perfusion. Despite postoperative persistence of macroscopic shunting confirmed by colorectal scintigraphy in some of these dogs, even partial shunt attenuation improved protein C activity. Finding that protein C activity improved or was considered normal in 5 of 10 PSVA dogs with persistent extrahepatic shunting after surgery suggests that protein C activity sensitively reflects even small improvements in hepatoportal venous perfusion despite continued high TSBA concentrations. Although it is possible that inflammation induced by laparotomy could transiently affect protein C activity secondary to an acute phase response, follow-up sampling was completed no sooner than 1 month after surgery and in most cases was completed several months to years after surgery. Thus, these preliminary observations suggest that protein C activity may prove useful as a means of monitoring dogs with PSVA after surgical ligation. This may be an important clinical application of protein C activity considering that TSBA concentrations often fail to normalize after PSVA ligation despite improved clinical status.[23] A number of circumstances thought to influence high postoperative TSBA concentrations in dogs with PSVA include the presence of more than a single aberrant anomalous shunting vessel, development of portal vein thrombosis, failure of the surgical procedure, or concurrent MVD. Of these, sustained microvascular circulatory abnormalities attributable to coexistent MVD is the most common factor thwarting the clinical use of TSBA concentrations for accurate monitoring of patients after PSVA ligation.[36] Although our preliminary findings suggest that dogs attaining clinical benefit from shunt ligation can be differentiated from those with less optimal responses by determining postoperative protein C activity, this observation deserves broader scrutiny.

Consistent with our results defining the use of protein C for detection of macroscopic PSS, our findings also indicated that protein C activity can help differentiate dogs with MVD from those with PSVA. Clinical use of protein C activity in this capacity may help prioritize the need for costly and invasive investigations (ie, abdominal ultrasonography, colorectal scintingraphy, exploratory laparotomy, and radiographic or CT portovenography) aimed at definitive differentiation of these 2 disorders as well as referral to a specialty clinic. Microvascular dysplasia, commonly encountered clinically in many small, purebred dogs, involves a permanent microvascular malformation or dysfunction imposing intrahepatic portovenous shunting. Histologically, MVD may be indistinguishable from PSVA when small (eg, needle-core) biopsy specimens are collected from only a single liver lobe.[36] Some dogs with MVD circumvent portal perfusion to entire liver lobes, necessitating biopsy collection from separate liver lobes for accurate diagnosis.[36] Although portal triads have increased arteriolar cross sections similar to vascular profiles in PSVA, portal veins are usually easily recognized and appear normal in size. Yet, juvenile portal triads (numerous, small, poorly developed portal triads with an unusual acinar location in zone 2) also are identified, and in some dogs, an abnormal juxtaposition of hepatic veins within portal triads may be detected. In most biopsy specimens from patients with MVD and PSVA, throttling musculature (smooth muscle) of the hepatic venule is obvious.[37] Each disorder is associated with high TSBA concentrations that frequently indicate a shunting pattern (eg, postprandial TSBA concentrations markedly higher than values obtained after withholding food).[38] Thus, considering the high sensitivity of TSBA concentrations to detect abnormal hepatoportal perfusion, the overlap of features detected during histologic examination of liver biopsy specimens, and the inability of abdominal ultrasonography to confirm an anomalous vasculature in some dogs with PSVA (eg, because of overlying enteric gas, inadequate animal restraint, operator inexperience, or lack of color-flow vascular interrogation), differentiation of MVD from PSVA can become problematic without radiographic or isotope studies that clearly distinguish hepatopedal portal circulation.[36] Thus, our findings have clinical relevance because they clearly indicate that adjunctive interpretation of protein C activity with cholesterol concentrations and RBC MCV in young dogs with high TSBA concentrations can assist in distinguishing PSVA from

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MVD. In our data set, isolated interpretation of protein C activity in young dogs with high TSBA concentrations would have distinguished 36 of 38 (95%) dogs with MVD and would have missed 9 of 75 (12%) dogs with PSVA. However, rather than missing dogs with PSVA in which protein C activity was considered normal, we propose that protein C activity in those dogs may in fact reflect a lesser degree of PSS, considering that not all dogs with PSVA have clinical signs. In our study, dogs with PSVA that had protein C activity that was considered normal were not encephalopathic or clinically ill and these dogs also had a significantly higher serum cholesterol concentration than dogs with PSVA with low protein C activity. Our results indicated that the diagnostic use of low protein C activity was superior, compared with use of low AT activity, low RBC MCV, and low cholesterol concentration, for detection of PSVA when protein C values are interpreted adjunctively with TSBA concentrations. Consequently, we propose that adding protein C activity to a noninvasive test panel will help differentiate between MVD and PSVA when high TSBA concentrations are detected in young dogs of at-risk breeds (eg, Yorkshire Terrier, Maltese, Cairn Terrier, Tibetan Spaniel, and Havanese). It also is possible that detecting a low protein C activity may have clinical use for recognizing the rare occurrence of severe MVD, warranting medical and nutritional intervention for hepatic insufficiency.

Mechanisms of protein C deficiency proposed for humans with portal vein thrombosis include a global disturbance of coagulation (with increased consumption and catabolism of hemostatic proteins), hepatic atrophy, and decreased synthetic capacity, as well as deprivation of intestinal-derived regulatory factors needed to maintain basal protein C synthesis.[7,33,35] We cannot define the underlying mechanism of protein C deficiency from results of our observational study in dogs with portosytemic shunting; however, the significant decrease in protein C activity combined with low albumin, cholesterol, and glucose values in dogs with PSVA indicates an overall decrease in hepatic synthesis that may relate to metabolism disturbed by deviated portal perfusion or low hepatic mass (hepatic lobular atrophy). Our results do not support a secondary consumptive process because systemic activation of coagulation is associated with concomitant depletion of anticoagulant and procoagulant factors that we could not substantiate. For example, we found profoundly low protein C activity in many dogs with PSVA that were not deficient in AT activity or concentrations of fibrinogen and in which in vitro clotting times were considered normal.

Our findings indicated that plasma protein C activity also is substantially reduced in dogs with hepatic synthetic failure in the absence of confirmed PSS. We did not anticipate that protein C activity would so keenly detect hepatic failure. Dogs with hepatic failure had the lowest protein C activities of any dogs with hepatobiliary disease. In addition to having a protein C activity that was less than the reference range (19/20 dogs), abnormal AT activity also was common in dogs (17/20) with hepatic failure. In this disease category, as in all dogs with hepatobiliary disease, protein C activity was significantly correlated with fibrinogen concentration and AT activity. Associations among protein C, AT, and fibrinogen were expected because these 3 hemostatic proteins behave as acute-phase reactants, are influenced similarly by inflammation, and are each synthesized by the liver.[12,13] Although each dog with hepatic failure had hyperbilirubinemia, 14 of 20 had hypoalbuminemia, 14 of 20 had hypocholesterolemia, 1 of 20 was microcytic, and 5 of 20 had a BUN concentration that was less than the reference range. Thus, our results indicated that a high bilirubin or TSBA concentration coupled with a low protein C activity was the most consistent marker of compromised synthesis in dogs with hepatic failure. This observation suggests that low protein C and AT activities should be included as laboratory criteria used to define hepatic synthesis failure in dogs. Our findings also support that the combined abnormalities of hyperbilirubinemia and low protein C and AT activities in dogs with suspected hepatic failure portends a grave prognosis (these features were detected in 18 of 20 dogs that ultimately died of hepatic failure), although a more detailed study of this association is necessary.

The significant correlations between protein C activity and impaired hepatic organic anion clearance (eg, total bilirubin and TSBA concentrations) were expected, as were correlations with PT and APTT because of the complex and interdependent relationships between coagulation and anticoagulant proteins (proteases). The negative correlation among activities of ALT and AST and protein C activity reflects the role of hepatic transaminases as early markers of hepatocellular injury. Because enzyme activity cannot simply distinguish the severity of hepatocellular damage without investigation of sequential blood samples (ie, persistently high values of hepatic transaminases suggest ongoing liver injury) and inclusion of other tests, adjunctively interpreting protein C activity as an additional test may improve the diagnostic use of hepatic transaminases at a single point in time.

In the study reported here, protein C activity appeared to function as a biomarker of hepatic function and hepatoportal perfusion. Experience with this test in dogs with PSVA before and after surgery suggests that protein C activity has a specific clinical application as a noninvasive indicator of hepatoportal blood flow. The inclusion of protein C analyses with routine clinicopathologic testing can assist clinicians in the recognition of PSS, hepatic failure, and other forms of severe hepatobiliary disease. Measurement of protein C activity may provide an economical and noninvasive means of differentiating PSVA from MVD in breed populations with high prevalence of these disorders and, perhaps, for determining the severity of PSS. Furthermore, sequential evaluation of protein C activity before and after PSVA ligation may be useful for estimating the success of shunt attenuation.

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e. Dade Actin FS, Dade Behring, Durham, NC.
f. Thromboplastin LI, Helena Diagnostics, Beaumont, Tex.
g. Fibriquik Bovin Thrombin, bioMerieux, Durham, NC.
h. Fibrinogen, Diagnostica Stago, Parsippany, NJ.
i. STAchrom ATIII, American Bioproducts, Parsippany, NJ.
j. STAchrom Protein C, American Bioproducts, Parsippany, NJ.
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