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Prediction of outcome after ligation or thin film banding of extrahepatic shunts, based on plasma albumin concentration and hematologic expression of 8 target genes in 85 dogs

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Abstract

Background: In dogs with a congenital extrahepatic portosystemic shunt (EHPSS), outcome after surgical attenuation is difficult to predict.

Objectives: Develop a minimally invasive test to predict outcome after surgical EHPSS attenuation and establish risk factors for postattenuation seizures (PAS).

Animals: Eighty-five client-owned dogs referred for surgical attenuation of a single EHPSS.

Methods: mRNA expression of 8 genes was measured in preoperatively collected venous blood samples. Outcome was determined at a median of 92 days (range, 26-208) postoperatively by evaluating clinical performance, blood test results and abdominal ultrasonography. Multivariable logistic regression was used to construct models predicting clinical and complete recovery. The associations between putative predictors and PAS were studied using univariable analyses.

Results: Five of 85 dogs developed PAS. Risk factors were age, white blood cell (WBC) count and expression of hepatocyte growth factor activator and LysM and putative peptidoglycan-binding domain-containing protein 2. Clinical recovery was observed in 72 of 85 dogs and complete recovery in 51 of 80 dogs (median follow-up, 92 days). The model predicting clinical recovery included albumin, WBC count, and methionine adenosyltransferase 2 alpha (MAT2α) expression, whereas the model predicting complete recovery included albumin, and connective tissue growth factor precursor and MAT2α expression. The areas under the receiver operating characteristic curves were 0.886 (95% confidence interval [CI]: 0.783, 0.990) and 0.794 (95% CI: 0.686, 0.902), respectively.

Abbreviations: AIC, Akaike's information criterion; AUC, area under the ROC curve; CI, confidence interval; Cq, quantification cycle; CTA, computed tomography angiography; CTGF, connective tissue growth factor precursor; DHDH, dimeric dihydrodiol dehydrogenase; DZIP1, DAZ-interacting zinc finger protein 1; EHPSS, extrahepatic portosystemic shunt; ERLEC1, endoplasmic reticulum lectin 1; GUSB, glucuronidase beta; HEPC, hepcidin precursor; HGFact, hepatocyte growth factor activator; HNRPH, heterogeneous nuclear ribonucleoprotein H; HPRT, hypoxanthine phosphoribosyl transferase; LYSMD2, LysM and putative peptidoglycan-binding domain-containing protein 2; MAT2α, methionine adenosyltransferase 2 alpha; PAS, postattenuation seizures; ROC, receiver operating characteristic; RPS5, ribosomal protein S5; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; Se, sensitivity; Sp, specificity; WBC, white blood cell.

Conclusions and Clinical Importance: Two models were constructed for predicting outcome after EHPSS attenuation using venous blood samples. The model predicting clinical recovery showed the best diagnostic properties. Clinical application requires further validation.

KEYWORDS
extrahepatic portosystemic shunt, liver, outcome, prediction model, prognostic, recovery

1 | INTRODUCTION

Dogs with a congenital extrahepatic portosystemic shunt (EHPSS) have a developmental anomaly of the splanchnic vascular system causing portal blood to bypass the liver. The aim of surgical treatment is to restore normal hepatoportal circulation and ultimately liver function.[1] However, the ability of the liver and portal circulation to recover after shunt attenuation in an individual dog is variable and there is a risk of life-threatening postoperative complications.[2,3] Perioperative mortality rates caused by severe complications vary between 3% and 10% after thin film banding, ameroid constrictor placement, or ligation of EHPSS, with no significant differences among these techniques.[4-6] Statistical models that predict the probability of these severe complications or recovery after surgical attenuation of EHPSS might aid in selecting patients for surgery. Previously developed models used preoperative plasma albumin concentration and gene expression profiles from intraoperatively obtained hepatic tissue to predict outcome.[7,8] However, the use of tissue obtained during surgery makes these models unfit for preoperative patient selection and it is unclear whether gene expression in these samples corresponds with preoperative expression. A major limitation of obtaining hepatic tissue before surgery is invasiveness, given the altered hemostatic profiles in dogs with shunts.[9-11] Hence, the availability of a less invasive technique to measure predictive gene expression before surgery, for example using peripheral venous blood samples, is important to improve clinical applicability.

One of the life-threatening complications shortly after shunt attenuation is development of postattenuation seizures (PAS).[3] These seizures are unpredictable, often refractory to treatment and prophylactic treatment with levetiracetam does not provide protection.[12,13] Risk factors for the development of PAS are not well established but appear to include older age at surgery and presurgical signs of hepatic encephalopathy.[14] Knowledge of specific PAS predictors might aid in identification of dogs at increased risk and in understanding underlying biological processes.

Therefore, our aims were 2-fold: (a) to develop a minimally invasive preoperative test procedure to support the rational decision to perform surgery by predicting the probability of successful surgical shunt attenuation in dogs with EHPSS, and (b) to generate hypotheses regarding the pathogenesis of PAS by studying the association between preoperative expression of genes of interest and the occurrence of PAS.

2 | MATERIALS AND METHODS

2.1 | Dogs

Our study was performed in dogs referred for surgical attenuation of a single EHPSS to the academic veterinary hospital at Utrecht University between April 2011 and May 2019. All animal material and data from clinical examinations used in the study were obtained in accordance with the university 3R-policy and Dutch legislation. All dogs were prescribed a low-protein diet for a period of at least 4 weeks before surgery, with or without lactulose (Laxatract, AST Farma BV, Oudewater, the Netherlands).

2.2 | Preoperative blood sampling

Blood samples were collected 24 hours before surgery for white blood cell (WBC) count and measurement of plasma albumin concentration. Immediately after sampling, 0.5 mL whole blood was mixed with 0.8 mL RNA*later* Solution (Ambion Inc, Austin, Texas), incubated for 24 hours at 4°C, and then stored until expression profiling at −70°C. Fasted plasma or serum bile acid concentrations that were measured before the start of the preoperative low-protein diet in our clinic or by the referring veterinarian were retrieved from the patient records.

2.3 | Surgery

The location of the shunt was determined preoperatively using ultrasonography or computed tomographic angiography (CTA). Additional preoperative and postoperative diagnostic tests, supportive treatment, and monitoring were performed according to a standardized protocol. After exploration of the abdominal cavity via median celiotomy, the shunt was attenuated over a gauged rod to the smallest diameter that did not induce portal hypertension,[15] using a nonabsorbable 2-0 suture or thin film banding. In the absence of portal hypertension, shunts were completely closed with suture. Each surgery was performed by a European College of Veterinary Surgery board-certified surgeon. Owners received written instructions to discontinue PO treatment with lactulose and gradually replace the low-protein diet with a normal dog food from 1 week after surgery onward. Medical treatment was only advised to be resumed when

recurrent clinical signs were seen that could be associated with portosystemic shunting.

2.4 | Outcome

Follow-up examination to assess outcome was performed at a median of 92 days (range, 26-208). Clinical information after surgery was retrieved from the general history and standardized questions in the patient records. The outcomes of interest were (a) development of PAS, (b) clinical recovery, and (c) complete recovery.

Postattenuation seizures were defined as development of generalized or focal seizures within 1 week after shunt attenuation, unrelated to feeding or hyperammonemia.

Clinical recovery was defined as absence of clinical signs associated with portosystemic shunting (eg, dysuria caused by postoperative formation of ammonia urate uroliths, seizures, unresponsiveness, ataxia, circling, lethargy, disorientation, head pressing, salivation, chronic anorexia) in combination with supportive evidence of improved portal blood flow toward the liver without formation of collateral vessels visualized on ultrasonography, regardless of blood flow through the original shunt and fasted plasma ammonia concentration.

Complete recovery was defined as absence of clinical signs associated with portosystemic shunting, and neither visible hepatofugal blood flow through the original shunt nor acquired shunts on ultrasonography and fasted plasma ammonia concentration <45 μmol/L.

2.5 | Gene expression

Based on previous prediction models, mRNA expression profiles of 8 genes of interest were measured,[7,8] namely methionine adenosyltransferase 2 alpha (MAT2α), hepatocyte growth factor activator (HGFact), connective tissue growth factor precursor (CTGF), dimeric dihydrodiol dehydrogenase (DHDH), endoplasmic reticulum lectin 1 (ERLEC1), hepcidin precursor (HEPC), LysM and putative peptidoglycan-binding domain-containing protein 2 (LYSMD2), and DAZ-interacting zinc finger protein 1 (DZIP1). Gene expressions were quantified by reverse transcriptase real-time quantitative polymerase chain reaction (RT-qPCR) in preoperative blood samples of the dogs for which the outcomes of interest after surgical shunt attenuation were known.

Total RNA was isolated from the blood samples using a QIAamp RNA Blood Mini Kit (Qiagen, Venlo, the Netherlands) and on-column DNase digestion. The RNA was amplified and converted to cDNA using the iScript cDNA synthesis kit as described by the manufacturer (Bio-Rad, Veenendaal, the Netherlands). Normalization was performed using 4 reference genes (glucuronidase beta [GUSB], heterogeneous nuclear ribonucleoprotein H [HNRPH], hypoxanthine phosphoribosyl transferase [HPRT], and ribosomal protein S5 [RPS5]), stably expressed as required under Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)-precise guidelines.[14] Primers for reference genes and genes of interest, including their optimal temperature, are listed in Table 1.[8,16] Cycling conditions were a 3-minute Taq polymerase activation step at 95°C, followed by a maximum of 40 cycles of 10 seconds at 95°C for denaturation, and 30 seconds at melting temperature for annealing and elongation. All measurements were conducted in duplo using a MyiQ Single-Color Real-Time PCR Detection System (BioRad). Gene expressions in samples without a PCR signal in both duplo measurements were assumed to have a Cq value of 40.0. The mRNA expression of each selected gene (dCq) was expressed as the averaged Cq of the reference genes in the sample minus the measured Cq value of the gene (dCq = Cqref − Cqgene). Given that a higher concentration of mRNA results in a lower Cq value of the gene, a higher dCq value of a gene reflects a higher expression of this gene relative to the expression of the reference genes.

2.6 | Statistical analyses

The associations between the outcomes of interest and putative risk factors were studied using logistic regression models. Putative risk factors included age at surgery, sex, preoperative plasma albumin concentration, preoperative plasma or serum bile acid concentration, preoperative WBC count, shunt type (termination in prehepatic caval vein or terminating via phrenic or azygos veins in the posthepatic venous circulation), surgical technique (ligation or thin film banding), preoperative treatment with levetiracetam (yes or no), and expression of the 8 target genes.

Bile acid concentrations were missing in 9 of 85 dogs included in the data analyses. Preliminary analyses indicated that multiple imputation of the missing values resulted in logistic regression models in which the selected model parameters varied by imputation and pooled estimates could not be estimated. Therefore, bile acid concentrations were categorized in 3 categories: missing, ≤the median value, and >the median value. None of the other putative risk factors had missing values.

Continuous variables, other than bile acid concentrations, were dichotomized if their effects were nonlinear or if dichotomization resulted in a lower Akaike's information criterion (AIC) of the logistic models. To dichotomize continuous variables, a receiver operating characteristic (ROC) curve was plotted for the variable as a predictor of the outcome of interest. If the area under the ROC curve (AUC) was significantly ($P < .05$) different from 0.5, the cut-off point between "low" and "high" was chosen at a value of the continuous variable that corresponded to the data point on the ROC curve with the smallest Euclidian distance to the point (1 − specificity [Sp], sensitivity [Se]) = (0, 1), to approach a perfect test (Se = 1, Sp = 1). Otherwise, the median value of the continuous variable was used as a cut-off between "low" and "high." Because the number of dogs included in the analyses as well as the ROC curves differed among the various outcomes of interest, cut-offs for dichotomization were determined for each outcome of interest separately.

The associations between putative risk factors and PAS were studied using univariable analyses only. The significance of the univariable associations was evaluated using the Fisher exact test.

TABLE 1 Primers used for quantitative real-time PCR.

Category	Gene		Ensembl transcript ID	F/R	Sequence	Tm (°C)	Amplicon size (bp)
	Abbreviations	Description					
Reference genes	GUSB	Glucuronidase beta	ENSCAFG00000010193	F	5′-AGACGCTTCCAAGTACCCC-3′	62	103
				R	5′-AGGTGTGGTGTAGAGGAGCAC-3′		
	HNRPH	Heterogeneous nuclear ribonucleoprotein H	ENSCAFT00000028063	F	5′-CTCACTATGATCCACCACG-3′	61	151
				R	5′-TAGCCTCCATAACCTCCAC-3′		
	HPRT	Hypoxanthine phosphoribosyl transferase	ENSCAFG00000018870	F	5′-AGCTTGCTGGTGAAAAGGAC-3′	58	104
				R	5′-TTATAGTCAAGGGCATATCC-3′		
	RPS5	Ribosomal protein S5	ENSCAFT00000003710	F	5′-TCACTGGTGAGAACCCCCT-3′	62	141
				R	5′-CCTGATTCACACGGCGTAG-3′		
Target genes	MAT2α	Methionine adenosyltransferase 2 alpha	ENSCAFG00000007755	F	5′-TGCTTTTGGCGGGGAGGAG-3′	67	121
				R	5′-TTTAAAAGCTGCCATCTGAGGTGA-3′		
	HGFact	Hepatocyte growth factor activator	ENSCAFG00000014629	F	5′- AAACTGGAGCGGATGGCACAG-3′	66	128
				R	5′-ACACAGACGTTTGGCATCGAGAAGTAT-3′		
	CTGF	Connective tissue growth factor precursor	ENSCAFG00000029442	F	5′-GGAAGAGAACATTAAGAAGGG-3′	63	120
				R	5′-TACTCCACAGAACTTAGCC-3′		
	DHDH	Dimeric dihydrodiol dehydrogenase	ENSCAFG00000003869	F	5′-ACACCGTCACTGTGCTCCT-3′	67	171
				R	5′-TCCTTATGCTCTCCCTTCAACACC-3′		
	ERLEC1	Endoplasmic reticulum lectin 1	ENSCAFG00000002724	F	5′-CATTCTGCCTCTTGTGACAAGTG-3′	60	147
				R	5′-TCCGTGACATACTTCATAAGTCCA-3′		
	HEPC	Hepcidin precursor	ENSCAFT00000011304	F	5′-CCAGTGTCTCAGTCCTTCC-3′	65.5	163
				R	5′-TTTACAGCAGCCACAGCA-3′		
	LYSMD2	LysM and putative peptidoglycan-binding domain-containing protein 2	ENSCAFG00000015502	F	5′-TCCTCCTAGTCCTCAAGAATCC-3′	64	155
				R	5′-GCATAGGGACTTTCTTCATCTCTG-3′		
	DZIP	DAZ-interacting zinc finger protein 1	ENSCAFG00000005465	F	5′-TAAACGCAGGAAGAAGATGATCTC-3′	61	148
				R	5′-GGTGAGAATCTTCAGGGTGG-3′		

Abbreviations: bp, base pairs; F, forward primer; R, reverse primer; Tm, melting temperature.

The associations between putative risk factors and the other outcomes (clinical recovery and complete recovery) were studied using multivariable logistic regression models. Albumin concentration was forced into all multivariable models because of the strength of the association between plasma albumin concentration and outcome in previous studies.[7,8] Other putative risk factors were retained for inclusion in a full model in multivariable analyses if the *P* value of the likelihood ratio test in univariable analyses was $P \leq .25$. Multicollinearity between these variables was evaluated by linear regression.[17] A tolerance <0.25 and a variance inflation factor >4 were considered indicative of multicollinearity. A final multivariable model was obtained in a backward elimination procedure with a threshold probability for removal of $P = .1$ in the likelihood ratio test. Confounding was monitored by the change in regression coefficients. If elimination of a variable resulted in the change of the estimated regression coefficient of any other variable exceeding 25% or 0.1 in case of an estimate between −0.4 and 0.4, the eliminated variable was considered a potential confounder and re-entered in the model. Model fit was evaluated using the Hosmer and Lemeshow goodness-of-fit test. After the multivariable model was obtained, it was checked once more to determine whether replacement of any continuous variable in the multivariable model by the concomitant dichotomized variable, and vice versa, would decrease the AIC of the model. If so, the multivariable analysis was repeated using the variable that resulted in the lowest AIC, and the resulting multivariable model with the lowest AIC was selected as the final model.

The multivariable logistic regression models for clinical recovery and complete recovery were used to develop predictive models to support the decision whether to surgically attenuate an extrahepatic shunt. To correct for overfitting, the regression coefficients of the logistic models were multiplied by a heuristic shrinkage factor[18] after which the intercept was corrected to align the predicted probability with the observed probability of recovery.

All statistical analyses were performed using commercial software (IBM SPSS Statistics, Version 27.0.0, IBM Corp, Armonk, New York).

3 | RESULTS

3.1 | Characteristics of dogs in the data analyses

Of the 108 dogs referred for surgical attenuation of an EHPSS, blood storage in RNA*later* Solution was omitted in 13 dogs, 7 dogs were lost to follow-up upon discharge from the veterinary hospital and 1 dog died before re-evaluation because of an unrelated cause. These dogs were excluded from the study. An additional 2 dogs were excluded because reference genes were not quantifiable (Figure 1). The remaining 85 dogs that were included in the data analyses consisted of 77 purebreds of 30 different breeds and 8 crossbreeds. The 3 most common breeds were Dachshund (n = 10), Miniature Schnauzer (n = 9), and Jack Russell terrier (n = 8). Other characteristics of these 85 patients are presented in Tables 2 and 3.

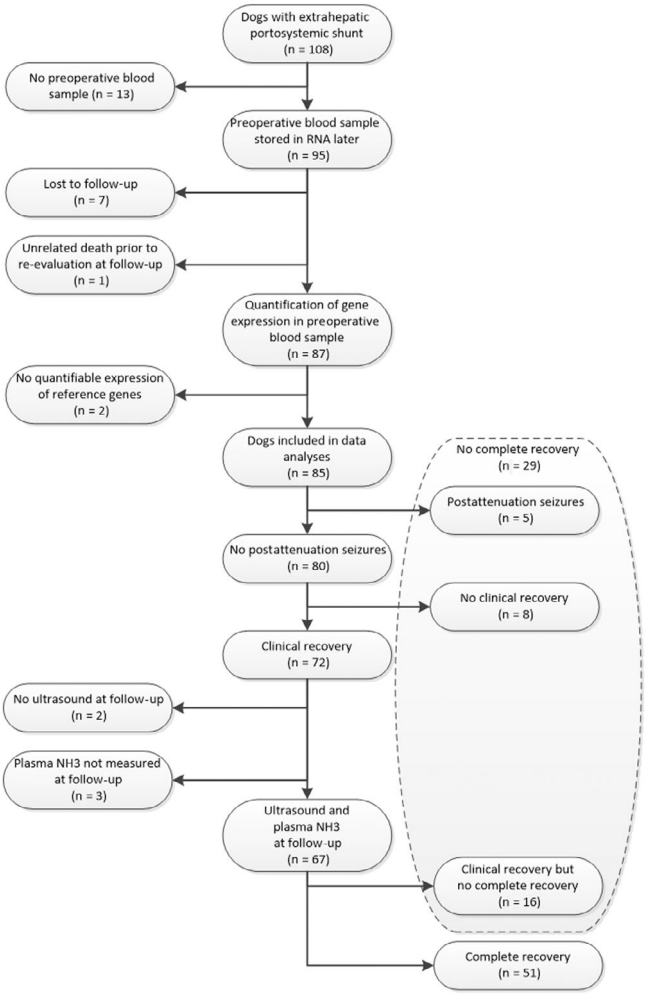

FIGURE 1 Outcome after surgical attenuation of an extrahepatic shunt in dogs in the study population.

3.2 | Outcome

Five of the 85 dogs developed PAS at 1 to 3 days after surgery and did not survive. At re-evaluation of the remaining 80 dogs, 8 dogs were defined as clinically not recovered and 72 dogs were clinically recovered (Figure 1). All 8 dogs that were classified as clinically not recovered showed clear clinical improvement in comparison to their status before surgery according to their owners, but mild lethargy persisted, in some dogs combined with incidental seizures (n = 1), occasional circling (n = 1), chronic anorexia (n = 1), or formation of ammonia urate uroliths (n = 2). In all 8 dogs, persistent blood flow was noted in the original shunt, but no acquired shunts were observed.

In 67 of the 72 clinically recovered dogs, plasma ammonia concentration was measured and an ultrasound examination was performed. Of these 67 dogs, 51 dogs had no signs of hepatofugal blood flow through the original shunt, no evidence of acquired shunts and fasted plasma ammonia concentration <45 µmol/L. Thus, these dogs were considered completely recovered (Figure 1). The other 16 dogs showed residual portosystemic flow through the original shunt,

TABLE 2 Categorical characteristics of 85 dogs with an extrahepatic portosystemic shunt included in the data analyses.

		Clinical recovery			
		Yes			
	Clinical recovery No	Complete recovery No	Complete recovery Yes	Complete recovery Not available	Total number
Sex					
Female	6	10	29	4	49
Male	7	6	22	1	36
Shunt type					
Termination in prehepatic V. cava[a]	8	11	29	3	51
Termination in phrenic V./azygos V.[b]	5	5	22	2	34
Preoperative low-protein diet					
No or not confirmed	0	0	4	0	4
Yes	13	16	47	5	81
Preoperative levetiracetam					
No	9	8	23	4	44
Yes	4	8	28	1	41
Surgical technique					
Nonabsorbable 2-0 suture	12	15	45	5	77
Thin film banding	1	1	6	0	8
Degree of closure at surgery					
Complete	2	3	29	2	36
Partial	11	13	22	3	49

[a]Including dogs with a gastroduodenal-caval (n = 10), portocaval (n = 8), right gastric-caval (n = 5), right gastric-caval with a caudal loop (n = 2), and splenocaval (n = 26) shunt.
[b]Including dogs with a porto-azygos (n = 12), portophrenic (n = 4), spleno-azygos (n = 13), and splenophrenic (n = 5) shunt.

resulting in an increased fasted plasma ammonia concentration in 4 dogs (≥45 μmol/L). No multiple acquired shunts were observed. At re-evaluation, 11 dogs still received a low-protein diet (10 dogs received a hepatic diet and 1 dog received a renal diet). Two dogs that received a hepatic diet also were treated with PO lactulose. Of these 11 dogs, 9 dogs were clinically recovered of which 6 were completely recovered as well. The expression of genes of interest relative to reference genes is shown in Figure 2.

3.3 | Factors associated with PAS

The AUCs of ROC curves of age at surgery, WBC count, and dCq values of ERLEC1, HGFact, and LYSMD2 (dERLEC1, dHGFact, and dLYSMD2) as predictor for the occurrence of PAS were significantly different from 0.5. Dichotomization of these variables was based on their ROC curves. For all other continuous explanatory variables, the median value was used for dichotomization of the variable. For each of the continuous explanatory variables, except for the expression of CTGF and DHDH (dCTGF and dDHDH), dichotomization resulted in logistic regression models with a lower AIC. The dichotomized variables for age at surgery, WBC count, dHGFact, and dLYSMD were significantly associated with the occurrence of PAS (Table 4).

TABLE 3 Continuous characteristics of 85 dogs with an extrahepatic portosystemic shunt included in the data analyses.

	Number	Minimum	Median	Maximum
Age at surgery (days)	85	122	407	4459
Body weight (kg)	85	1.2	6.0	29.3
Bile acid (μmol/L)[a]	76	0	127.5	332
Albumin (g/L)	85	12	23	34
WBC count ($\times 10^9$/L)	85	5.4	14.8	47.6

Abbreviation: WBC, white blood cell.
[a]Measured before the start of the preoperative low-protein diet.

3.4 | Predicting clinical recovery

Clinical recovery was observed in 72 of the 85 dogs (Figure 1). In univariable analyses, age at surgery, preoperative treatment with levetiracetam, preoperative plasma albumin concentration (<22.5 vs ≥22.5 g/L), preoperative WBC count (<14.8 vs ≥14.8 $\times$ 10^9/L), and gene expressions of DHDH (as a continuous variable) and MAT2α (as a continuous variable) were significant at $P < .25$. However, preliminary analyses indicated that replacement of MAT2α by a dichotomized MAT2α variable (<−1.51 vs ≥−1.51) and including WBC count as a continuous variable resulted in lower AICs of the multivariable models. In the final multivariable logistic regression model, 3 variables

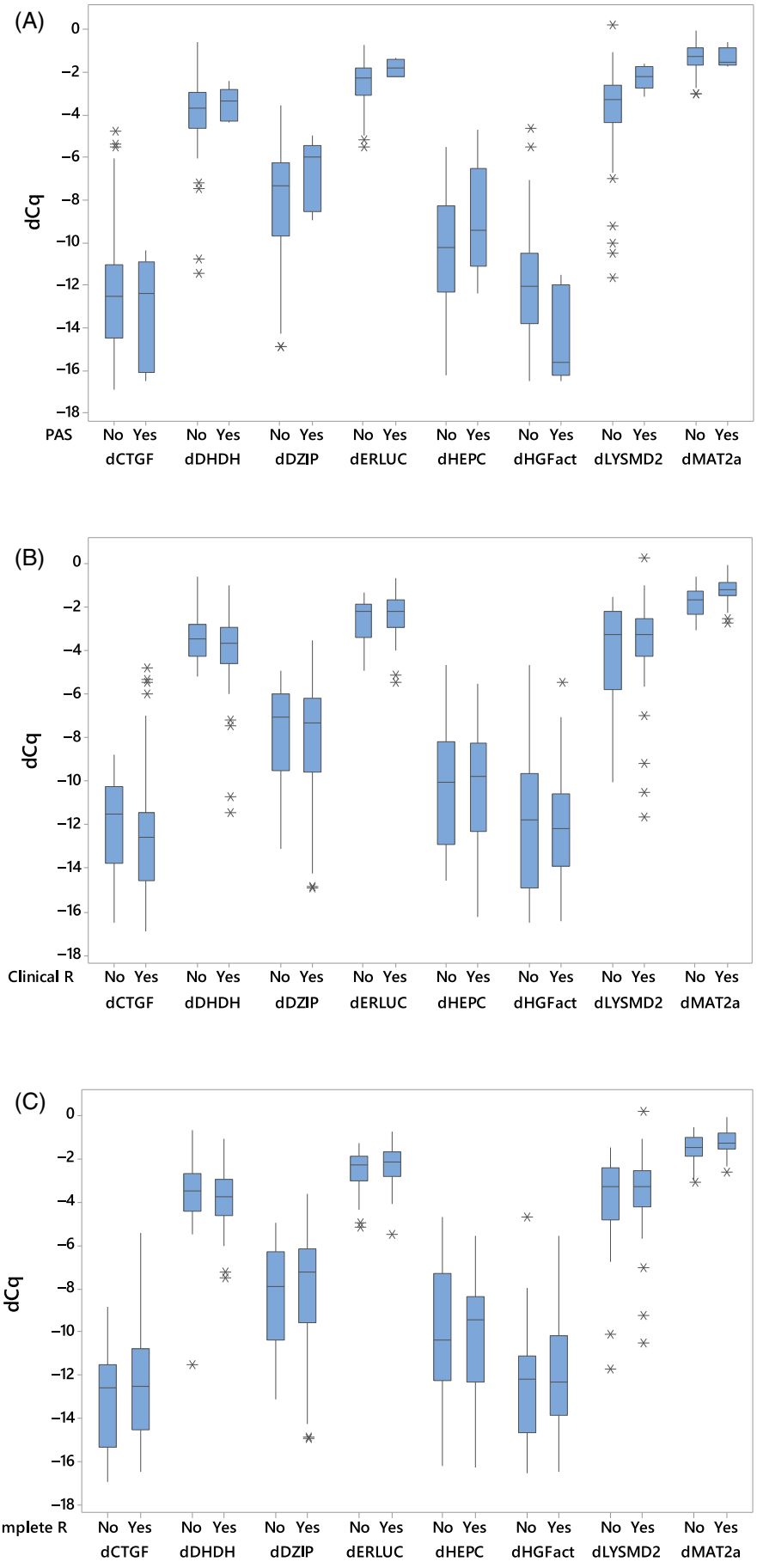

FIGURE 2 Differential expression of genes of interest in dogs (A) with (n = 5) and without (n = 80) postattenuation seizures (PAS), (B) with (n = 72) and without (n = 13) clinical recovery (Clinical R), and (C) with (n = 51) and without (n = 29) complete recovery (Complete R). Boxes indicate the 1st quartile, median, and 3rd quartile. Whiskers indicate the lowest and highest value between the region defined by the following limits (1st quartile − 1.5 × the interquartile range; 3rd quartile + 1.5 × the interquartile range). Outliers outside this region are indicated by an asterisk (*). Abbreviations of genes are explained in Table 1.

TABLE 4 Variables significantly ($P < .05$) associated univariably with the occurrence of postattenuation seizures (PAS) in 85 dogs referred for surgical attenuation of an extrahepatic portosystemic shunt.

		Number of dogs				
	Level	Without PAS	With PAS	Total	Odds ratio (95% CI)	Fisher's exact test
Age at surgery (days)	<1156	62	0 (0%)	62		
	≥1156	18	5 (22%)	23	[a]	$P = .001$
WBC count ($\times 10^9$/L)	<10.75	9	4 (31%)	13		
	≥10.75	71	1 (1%)	72	0.032 (0.003; 0.316)	$P = .002$
dHGFact	<−15.57	4	3 (43%)	7		
	≥−15.57	76	2 (3%)	78	0.035 (0.005; 0.273)	$P = .003$
dLYSMD2	<−2.33	69	1 (14%)	70		
	≥−2.33	11	4 (27%)	15	25.1 (2.56; 246)	$P = .003$

Abbreviations: CI, confidence interval; dHGFact, gene expression of hepatocyte growth factor activator; dLYSMD2, gene expression of LysM and putative peptidoglycan-binding domain-containing protein 2; WBC, white blood cell.
[a]Calculation of an odds ratio was impossible because none of the dogs ≤1156 days of age were observed with PAS.

were retained: preoperative plasma albumin concentration, WBC count, and expression of MAT2α (Table 5). After correction for overfitting, the predictive model was:

$$\pi_{\text{clinical recovery}} = \frac{1}{1+e^{-(-4.484+2.622\times albumin_{\geq 22.5}+.247\times WBCcount+2.548\times dMAT2\alpha_{\geq -1.51})}} \quad (1)$$

in which $\pi_{\text{clinical recovery}}$ is the probability of clinical recovery and albumin$_{\geq 22.5}$, WBC count, and dMAT2α$_{\geq -1.51}$ are variables for plasma albumin concentration, WBC count, and expression of the MAT2α gene, respectively (albumin$_{\geq 22.5}$ = 1 if the albumin concentration ≥22.5 g/L and albumin$_{\geq 22.5}$ = 0 otherwise, and MAT2α$_{\geq -1.51}$ = 1 if the gene expression dMAT2α ≥ −1.51 and MAT2α$_{\geq -1.51}$ = 0 otherwise). The ROC curve (Figure 3) of this predictive model had an AUC of 0.886 (95% CI: 0.783, 0.990). At a cut-off predicted probability of clinical recovery of 82%, the sensitivity and specificity in the study population were 76% and 92%, respectively (Table 6).

3.5 | Predicting complete recovery

Complete recovery could not be evaluated in 5 of 85 dogs, because of absence of either ultrasound or plasma ammonia results at follow-up. Complete recovery was observed in 51 of 80 dogs (Figure 1). In univariable analyses, preoperative treatment with levetiracetam, preoperative plasma albumin concentration (<23.5 vs ≥23.5 g/L), and gene expressions of CTGF, ERLEC1, and MAT2α were significant at $P < .25$. Preliminary analyses indicated that dichotomization of MAT2α (<−1.49 vs ≥−1.49) resulted in a final model with lower AIC than the continuous MAT2α variable. This dichotomized variable also was univariably significant at $P < .25$. In the final multivariable logistic regression model, 3 variables were retained: preoperative plasma albumin concentration (<23.5 vs ≥23.5 g/L), expression of CTGF (as a continuous variable) and expression of MAT2α (<−1.49 vs ≥−1.49; Table 7). After correction for overfitting, the predictive model was:

$$\pi_{\text{complete recovery}} = \frac{1}{1+e^{-(1.352+1.481\times \text{albumin}_{\geq 23.5}+.184\times \text{dCTGF}+1.417\times \text{dMAT2}\alpha_{\geq -1.49})}} \quad (2)$$

in which $\pi_{\text{complete recovery}}$ is the probability of complete recovery, dCTGF is the gene expression of CTGF and albumin$_{\geq 23.5}$ and dMAT2α$_{\geq -1.49}$ are categorical variables for the plasma albumin concentration and expression of the MAT2α gene, respectively (albumin$_{\geq 23.5}$ = 1 if the albumin concentration was ≥22.5 g/L and albumin$_{\geq 23.5}$ = 0 otherwise and MAT2α$_{\geq -1.49}$ = 1 if the gene expression dMAT2α was ≥−1.49 and MAT2α$_{\geq -1.51}$ = 0 otherwise). The ROC curve (Figure 4) of this predictive model had an AUC of 0.794 (95% CI: 0.686, 0.902). At a cut-off predicted probability of complete recovery of 48%, the sensitivity and specificity in the study population were 92% and 62%, respectively (Table 6).

3.6 | Comparing predictions of clinical recovery and complete recovery

For 80 dogs, observations on both clinical and full recovery were available (Figure 1). In 74 of these 80 dogs (93%), the predicted probability of clinical recovery (Equation (1)) was ≥the predicted probability of complete recovery (Equation (2); Figure 5).

4 | DISCUSSION

Based on preoperative plasma albumin concentration, WBC count, and expression of CTGF and MAT2α in whole blood, 2 well-fitting models were constructed to predict outcome after attenuation of an extrahepatic shunt in dogs with a ligature or a thin film band. Both

TABLE 5 Final prognostic model[a] on the probability of clinical recovery after surgical attenuation of an extrahepatic portosystemic shunt.

Variable	Level	Number of dogs	β (SE)	*P* (Wald test)	*P* (LR test)	Odds ratio (95% CI)
Albumin (g/L)					<.001	
	<22.5	38	Reference			
	≥22.5	47	2.996 (1.109)	.007		20.0 (2.2; 175.7)
WBC count ($\times 10^9$/L)		85	0.282 (0.118)	.02	.002	1.3 (1.1; 1.7)
dMAT2α					<.001	
	<−1.51	25	Reference			
	≥−1.51	60	2.911 (0.896)	.001		18.4 (3.2; 106.4)
Intercept		85	−5.285 (2.237)			

Note: Data on 85 dogs of which 72 dogs clinically recovered.
Abbreviations: CI, confidence interval; dMAT2α, gene expression of methionine adenosyltransferase 2 alpha; LR test, likelihood ratio test; SE, standard error; WBC, white blood cell.
[a]−2 Log likelihood = 48.663, Nagelkerke $R^2 = 0.429$, Hosmer and Lemeshow test $\chi^2 = 8.243$, df = 7, $P = .31$.

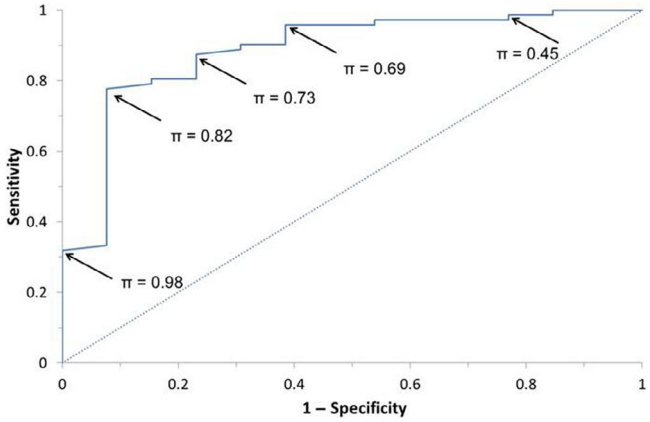

FIGURE 3 Receiver operating characteristics curve of the predictive model for clinical recovery (Equation (1)), using data on 85 dogs with a surgical attenuation of an extrahepatic portosystemic shunt. Various cut-off values for the predicted probability π of clinical recovery are indicated.

models predict the outcome from a presurgical venous blood sample, which is less invasive than collection of hepatic tissue.[7,8] A model predicting postoperative outcome using a minimally invasive procedure may have broad clinical applicability. Besides the methodology of the test procedure, clinical applicability of a predicting model depends on a well-defined and substantiated choice of the predicted outcome after surgery. To develop a predictive test aimed at dogs with an EHPSS, it is essential to define the predicted outcome after surgical treatment and to decide if surgery should result in complete anatomical closure of the shunt, or if a complete clinical recovery with evidence of improved hepatic function also should be considered a successful postoperative outcome. Although long-term prognosis has been assumed to be worse after partial closure compared with complete closure of an EHPSS,[1,19] a network meta-analysis reported no significant difference in outcome between partial and complete ligation.[5] Clinical recovery with improved hepatic function may be sufficient to warrant a good long-term prognosis,[3,20] but to our knowledge, no study compared long-term outcome between dogs with complete shunt closure after short-term follow-up and dogs with incomplete closure but complete clinical recovery and improved hepatic function after short-term follow-up. Because differences in long-term outcome are unknown and it is subjective which predicted outcome is preferable, a model was constructed to predict clinical recovery with improved hepatic function, and another model was constructed to predict complete recovery of portosystemic shunting. Depending on the outcome of choice, either model can be chosen for clinical application. This choice should take into account the owner's and clinician's expectations of outcome, which depend on risk and severity of clinical recurrence, duration and quality of life, and requirement of additional life-long medical treatment. When the final models were compared on the basis of their diagnostic value quantified by ROC curves, the model predicting clinical recovery (AUC = 0.886) performed better than the model predicting complete recovery (AUC = 0.794). Using both models simultaneously, in some dogs the probability for complete recovery appeared to be larger than the probability for clinical recovery (Figure 5). From both fundamental and practical perspectives, it is therefore advisable to determine which predicted outcome (clinical recovery or complete recovery) is most relevant to the owner of the dog and only use the associated predictive model. This emphasizes again the need for large long-term follow-up studies in dogs with shunts, using standardized and validated assessment tools for outcome.[5] A limitation of our study is the relatively short follow-up time of 92 days, which may not be a sufficient time for dogs to show recurrence of clinical signs.

Factors observed to be associated with postoperative outcome in previous studies were included in the present analyses, such as plasma albumin concentration,[7,8,19] WBC count,[19,21] preprandial bile acid concentration,[21] technique (thin film banding or ligation),[22] age at surgery,[14,23,24] shunt type defined as prehepatic (portocaval) or posthepatic along the diaphragm (portophrenic or portoazygos)[19,25] and preoperative treatment with levetiracetam.[26] Our study confirmed the previously reported associations of preoperative plasma albumin concentration and WBC count with postoperative recovery. Dogs

TABLE 6 Proportions of dogs with predicted recovery after attenuation of an EHPSS, sensitivity (Se), and specificity (Sp) at various cut-off values of the predicted probability of recovery (π).

Predictive model	Cut-off predicted probability (π)	Proportion of dogs with predicted recovery[a]	Se	Sp
Clinical recovery (72 of 85 dogs)	0.45	0.95	0.99	0.23
	0.69	0.87	0.96	0.62
	0.73	0.78	0.88	0.77
	0.82	**0.66**	**0.76**	**0.92**
	0.98	0.29	0.33	0.92
Complete recovery (51 of 80 dogs)	0.36	0.88	0.96	0.28
	0.48	**0.73**	**0.92**	**0.62**
	0.55	0.60	0.76	0.69
	0.66	0.45	0.61	0.83
	0.84	0.26	0.35	0.90
	0.88	0.11	0.16	0.97

[a]Dogs with a predicted probability of recovery of at least the cut-off predicted probability (π). Results taken as examples in Sections 3 and 4 are printed in bold.

TABLE 7 Final prognostic model[a] on the probability of complete recovery after surgical attenuation of an extrahepatic portosystemic shunt.

Variable	Level	Number of dogs	B (SE)	*P* (Wald test)	*P* (LR test)	Odds ratio (95% CI)
Albumin (g/L)					.001	
	<23.5	43	Reference			
	≥23.5	37	1.736 (0.578)	.003		5.7 (1.8; 17.6)
dCTGF		80	0.216 (0.124)	.08	.07	1.2 (0.97; 1.6)
dMAT2α					<.007	
	<−1.49	24	Reference			
	≥−1.49	56	1.661 (0.644)	.01		5.3 (1.5; 18.6)
Intercept		80	1.518 (1.498)			

Note: Data on 80 dogs of which 51 dogs completely recovered.
Abbreviations: CI, confidence interval; dCTGF, gene expression of connective tissue growth factor precursor; dMAT2α, gene expression of methionine adenosyltransferase 2 alpha; LR, likelihood ratio test; SE, standard error.
[a]−2 Log likelihood = 84.337, Nagelkerke $R^2 = 0.309$, Hosmer and Lemeshow test $\chi^2 = 7.732$, df = 8, $P = .46$.

with a higher preoperative plasma albumin concentration had 5.7 times and 20 times higher odds of complete and clinical recovery, respectively, when compared to dogs with lower plasma albumin concentration (Tables 5 and 7). High albumin concentrations may represent dogs with a better hepatic function and food intake. Preoperative higher WBC counts were associated with increased probability of clinical recovery. Although previous studies[19,21] both reported lower WBC counts to be associated with increased short-term mortality after ameroid treatment for EHPSS, in the first study leukocytosis indicated unsuccessful long-term outcome, whereas in the latter study, it indicated improved long-term survival.

Genes included in the analyses (MAT2α, HGFact, CTGF, DHDH, ERLEC1, HEPC, LYSMD2, and DZIP1) were selected based on potential association of their hepatic mRNA expression with complete recovery after shunt attenuation.[7,8] Although gene expression in peripheral circulating WBC has been reported to reflect changes in mRNA expression in hepatic grafts,[27] it is unknown if gene expression in blood also reflects hepatic expression of genes in dogs and if specific gene expression in blood may be related to postoperative EHPSS recovery. Ours is the first study to report possible associations between mRNA expression of genes in blood samples and outcome in dogs after EHPSS attenuation.

In our study, higher expression of MAT2α in venous blood was significantly associated with increased probability of clinical and complete recovery, which is consistent with the results of a previous study, where hepatic MAT2α expression was positively associated with complete recovery of portosystemic shunting after shunt attenuation in dogs.[7] The association fits the function of this gene in hepatocyte proliferation and hepatic growth, which is seen after restoring portal blood flow toward the liver.[28] However, MAT2α is expressed in many other tissues and generally is associated with cell proliferation, including in

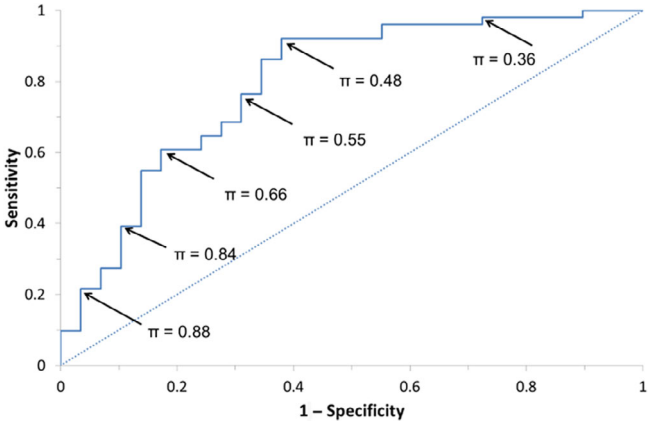

FIGURE 4 Receiver operating characteristics curve of the predictive model for complete recovery (Equation (2)), using data on 80 dogs with a surgical attenuation of an extrahepatic portosystemic shunt. Various cut-off values for the predicted probability π of clinical recovery are indicated.

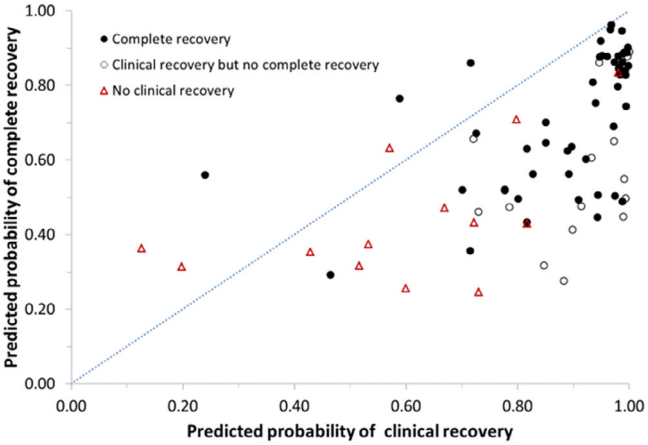

FIGURE 5 Predicted probabilities of clinical and complete recovery in dogs that completely recovered (n = 51, ●), dogs that clinically recovered but not completely recovered (n = 16, ◦), and dogs that were not clinically recovered (n = 13, Δ) at re-evaluation at 1 to 3 months after surgical attenuation of an extrahepatic portosystemic shunt.

lymphocytes.[29,30] It is unknown whether increased MAT2α expression in WBC affects hepatic growth after EHPSS closure, and whether a causal relationship exists between MAT2α expression in WBC and recovery.

In addition to MAT2α expression, expression of CTGF contributed to the predictive model for complete recovery of portosystemic shunting, which has not been reported before. The range of action of CTGF is broad, mediating both tissue regeneration as well as fibrosis. Although increased hepatic CTGF expression and increased CTGF plasma concentrations are seen in patients with liver fibrosis, leukocytes expressing CTGF may support the activation of hepatic stellate cells which play an important role in liver regeneration and repair.[30]

The prognostic characteristic of the predictive models (such as sensitivity, specificity, and positive and negative predictive values) are key variables in decision-making regarding surgery by dog owners. These prognostic characteristics as well as the added value of application of a predictive model are specific to a patient population. Thus, the predictive models developed in our study should only be used in other patient populations after external validation.

In the 95 dogs that were preoperatively sampled, short-term postoperative mortality was observed in 5 dogs (5.3%), all caused by the occurrence of PAS. Thus, the incidence of PAS was rather low. Similar to a previous study,[14] age at surgery was significantly associated with this complication. The occurrence of PAS also was associated with presurgical WBC count, indicating that dogs with higher WBC counts had a lower risk of developing PAS. This correlation corresponds with the increased odds for clinical recovery at 1 to 3 months after surgery and the increased perioperative survival that was reported after shunt attenuation with an ameroid ring.[19,21] However, increased systemic inflammation appeared to be associated with hepatic encephalopathy[31] and may be a risk factor for development of PAS.[14] Although the incidence of PAS was comparable to that of other cohorts,[13] our study only included 5 cases. More research in a larger number of dogs is needed to confirm the role of presurgical WBC count in PAS and in postoperative recovery.

Presurgical mRNA expression of HGFact and LYSMD2 was significantly associated with PAS. The HGFact protein is known as the activating enzyme of hepatic growth factor in the liver, but is also present in serum and is structurally similar to clotting factor XII.[32] Thus, HGFact is linked to the coagulation system. The HGFact protein may be involved in the integrity of blood vessels and promote healing of damaged tissue.[33,34] Increased HGFact expression was measured in WBC from EHPSS dogs that did not develop PAS. Measurement of the HGFact protein in WBC of EHPSS dogs could be a first step to support a possible protective effect of HGFact against developing PAS after EHPSS surgery.

Increased hepatic LYSMD2 expression previously was reported to be associated with lower odds postoperative recovery of shunt dogs.[8] This observation agrees with our study, where increased LYSMD2 expression in blood was associated with a significantly higher risk of PAS. Receptor-like proteins containing LysM are transmembrane proteins from multigenic families that are involved in the communications of eukaryotic cells. Several studies describe their function in signal transduction in plant cells.[35] However, no studies report their specific functions in animals yet.

An important limitation in the postoperative assessment of clinical signs in our study was the lack of standardized questionnaires or a validated quality-of-life scoring system. Another limitation was the fact that 11 dogs still received medical treatment during postoperative assessment, which could have masked clinical signs. Because of these limitations, absence of clinical signs in a dog had to be supported by evidence of hepatic improvement (hepatopetal portal blood flow or a normalized fasting plasma ammonia concentration) to be classified as clinically recovered. Although all owners received written instructions to switch to a normal dog food and

stop treatment with lactulose in dogs that showed clinical recovery after surgery, some owners were reluctant to do so before confirmation of recovery from portosystemic shunting. Remarkably, 6 of 9 clinically recovered dogs that still received medication showed no persistent shunting and thus were classified as completely recovered. In the group of nonrecovered dogs (8 dogs with clinical signs) only 2 dogs were treated medically.

Measurements of bile acid concentration before treatment were not available in 9 dogs. Imputation of these missing values resulted in models in which the selected variables varied. Thus, the potential prognostic value of bile acid concentration could not be estimated appropriately. Possibly, this was related to variation in time at sampling and laboratory assay, as part of the assays were performed in the referring veterinary practices. To evaluate the prognostic value of bile acid concentration in future studies, measurement of fasting bile acid concentration should be incorporated in the standardized preoperative protocol. Although CTA is a more reliable technique to identify portosystemic shunts and collaterals, ultrasonography was used to evaluate residual flow through the original shunt and collaterals because of its lower invasiveness and cost. It was performed by experienced radiologists, in fasted dogs that were sedated if necessary. In 12 dogs without clinical signs and normalized fasting ammonia, residual flow through the original shunt was visualized. These dogs still had a mild degree of portosystemic shunting. Medical treatment may contribute to normal fasting ammonia concentrations, although 10 of the 12 dogs were fed a normal dog food and did not receive medication. Normal fasting ammonia concentrations alone appear insufficient to confirm complete shunt closure, as previously reported.[36] Abnormal ammonia metabolism might have been confirmed with an ammonia tolerance test, but this test was not routinely performed.

In our study, the number of putative explanatory and predictive variables was relatively large compared to the number of patients included, particularly in the analyses on risk factors for PAS, given the low proportion of dogs that developed PAS. Therefore, we refrained from multivariable analyses on the development of PAS. The risk of overfitting in the prognostic models for clinical and complete recovery was decreased by shrinkage of the regression coefficients after estimation.[18]

Selection of genes in our study was based on former studies of predictive genes determined using intraoperative samples of hepatic tissue. In future development of predictive models, preferably whole genome RNA sequencing can be performed and external validation at a multicenter study is essential before clinical application.

In conclusion, we developed 2 models to predict outcome after surgical attenuation of EHPSS in dogs. Practical application of both models depends on their predictive value after validation, as well as availability and costs of gene expression testing. The fact that the current models included genes measured in peripheral venous blood samples is an important step toward practical applicability. Future studies should externally validate the developed models and provide evidence about which target outcome is preferable.

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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

All blood samples were collected and used for routine preoperative clinical purposes in line with Dutch legislation. Permission to use surplus of blood for research purposes was obtained from all dog owners, using informed consent. Collection and use of blood samples for research purpose was approved by the Utrecht University ethical committee, as required under Dutch legislation (registration no. AVD1080020184847).

HUMAN ETHICS APPROVAL DECLARATION

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

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