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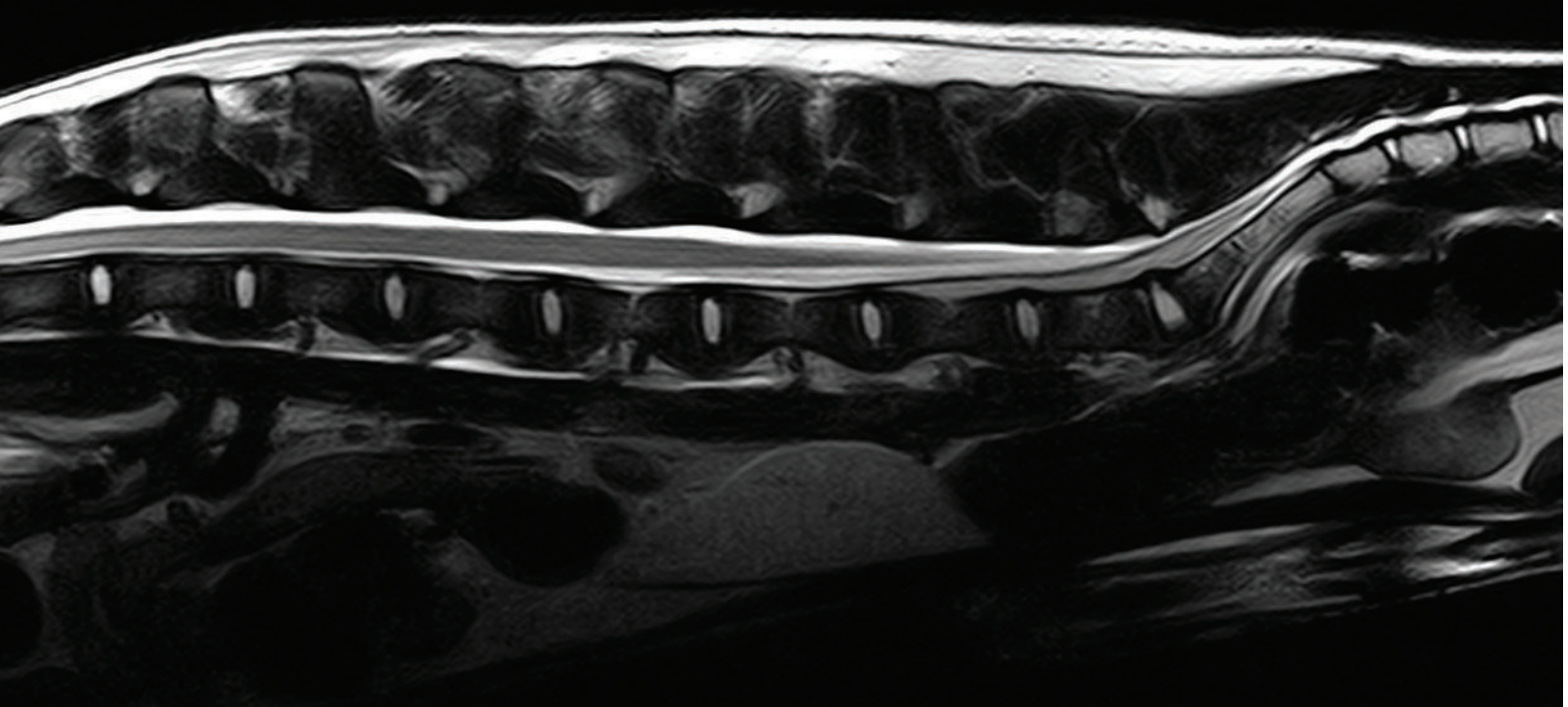

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Genome-wide based model predicting recovery from portosystemic shunting after liver shunt attenuation in dogs

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Background: In dogs with congenital portosystemic shunt (CPSS), recovery after surgical CPSS attenuation is difficult to predict.

Objectives: Our aim was to build a model with plasma albumin concentration and mRNA expression levels of hepatic gene products as predictors of recovery from portosystemic shunting after surgery.

Animals: Seventy-three client-owned dogs referred for surgical attenuation of CPSS.

Methods: A prediction model was constructed using 2 case-control studies of recovered and nonrecovered dogs after surgical CPSS attenuation. In the 1st study, a dog-specific gene expression microarray analysis was used to compare mRNA expression in intraoperatively collected liver tissue between 23 recovered and 23 nonrecovered dogs. In the 2nd study, preoperative plasma albumin concentration and the expression of microarray-selected genes were confirmed by RT-qPCR in intraoperatively collected liver samples of 31 recovered and 31 nonrecovered dogs, including 35 dogs from the 1st study.

Results: In the 1st study, 43 genes were differently expressed in recovered and nonrecovered dogs. The mean preoperative plasma albumin concentration in recovered dogs was higher compared to nonrecovered dogs (23 and 19 g/L, respectively; $P = .004$). The best fitting prediction model in the 2nd study included preoperative plasma albumin concentration and intraoperative *DHDH*, *ERLEC1*, and *LYSMD2* gene expression levels.

Conclusion and Clinical Importance: A preclinical model was constructed using preoperative plasma albumin concentration and intraoperative hepatic mRNA expression of 3 genes that were unbiasedly selected from the genome to predict recovery from portosystemic shunting after shunt ligation. Further development is essential for clinical application.

Abbreviations: AK, A. Kummeling; AUC, area under the curve; CPSS, congenital hepatic portosystemic shunt; Cq, quantification cycle; CT, computed tomography; EHPSS, extrahepatic portosystemic shunt; FvS, F.J. van Sluijs; IHPSS, intrahepatic portosystemic shunt; MIQE, minimum information for publication of quantitative real-time PCR experiments; ROC, receiver operating characteristic; RT-qPCR, quantitative real-time polymerase chain reaction; Se, sensitivity; Sp, specificity

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

1 | INTRODUCTION

Surgical shunt attenuation is the treatment of choice in dogs with congenital portosystemic shunt (CPSS) to restore the normal hepatoportal circulation and liver function.[1] Recovery from portosystemic shunting after attenuation of the shunt in an individual dog is unpredictable and complications after shunt attenuation such as portal hypertension, persistence or recurrence of clinical signs because of continuous shunting or development of collaterals have been described for all surgical techniques.[2] Overall mortality rates differ from 0% to 32% depending on surgical technique, shunt localization, and extent of narrowing.[2–4] Long-term medical management to control clinical signs associated with CPSS, particularly hepatic encephalopathy, is considerably less effective than surgery. It is only recommended in cases with predicted poor surgical outcome.[5,6] Therefore, an accurate preoperative model for predicting the outcome of shunt attenuation is essential.

It is unclear which factors contribute to success or failure after surgical treatment, making the prediction of long-term outcome after surgical attenuation of CPSS difficult. Predictors associated with recovery after surgical attenuation are age at surgery,[7,8] weight, preoperative plasma protein and albumin concentrations, blood urea nitrogen concentration,[7] shunt localization,[9] and leukocyte count,[4] although results are inconsistent. Intraoperative mesenteric portovenography can be helpful to predict outcome after surgical treatment of a single CPSS,[8] although this technique currently is solely useful intraoperatively.

An essential factor affecting postoperative recovery after surgery is hepatic regenerative capacity (ie, the ability of the liver and portal vasculature to develop to normal size and function).[3,10] Hepatic regeneration is promoted by a complex network activated by inflammatory cytokines, vasoregulators, growth factors, eicosanoids, and various hormones[11] and correlates with hepatic expression of genes involved in proliferation, apoptosis, hepatic fibrosis and vascular growth.[12] A positive association with complete recovery after shunt attenuation was found for 2 genes related to hepatocyte proliferation: *HGF activator* (*HGFact*) and *methionine adenosyltransferase 2α* (*MAT2a*),[12] suggesting that expression of these genes after surgery is important for clinical recovery.[12,13] These 2 and other factors have been evaluated extensively,[3,4,12–14] but no model based on genome-wide gene expression studies is available yet to predict recovery from portosystemic shunting after surgical attenuation of CPSS. Our aim was to develop an algorithm that predicts the outcome of surgery, in terms of normalization of portal circulation and restoration of ammonia metabolism. Therefore, a canine specific gene expression study was performed in intraoperatively obtained hepatic tissue of dogs with CPSS. Expression profiles were compared between dogs with recovery and dogs without recovery from portosystemic shunting upon surgical attenuation of the shunt. The results were used to construct a prediction model for postoperative recovery in these dogs.

2 | MATERIAL AND METHODS

2.1 | Study design

Gene expression profiles were generated from dogs with CPSS that underwent surgical attenuation of the shunt in 2 case-control studies where cases recovered after surgery and controls did not. In the 1st study, genes that were associated with successful recovery upon surgical attenuation were selected by microarray analysis. In the 2nd study, the association with recovery of these genes was confirmed by quantitative real-time PCR (RT-qPCR). The genes selected from the 2nd study and variables such as the hepatic mRNA expression of *MAT2a* and *HGFact*, plasma albumin concentration,[7,12] sex, age at surgery,[7,8] breed, and shunt localization[9] that are reported to possibly be related with recovery, were used to create a prediction model for recovery after surgery.

2.2 | Surgical procedure

Data of 73 dogs referred to the Department of Clinical Sciences of Companion Animals, Utrecht University for surgical attenuation of a single CPSS were included in the study. Permission was obtained from the dog owners using informed consent. The location of the shunt (intrahepatic or extrahepatic) was preoperatively determined using ultrasonography or computed tomography (CT). Additional preoperative and postoperative diagnostic tests, supportive treatment, and monitoring were performed according to a standardized CPSS protocol, which included preoperative plasma albumin concentrations measured using a DxC 600 Beckman analyzer (Beckman Coulter, Woerden, The Netherlands). After exploration of the abdominal cavity via median celiotomy, the shunt was ligated over a gauged rod to the smallest diameter that did not induce portal hypertension, using a non-absorbable 2-0 polyester suture (Ethibond, Ethicon, Somerville, New Jersey).[9] All surgeries were performed by an European College of Veterinary Surgery board-certified surgeon (FvS, AK). Wedge biopsy specimens of the liver were taken routinely during surgery for histopathology. A section of the biopsy specimen was frozen in liquid nitrogen immediately after collection and stored at −70°C until gene expression analysis. All samples were collected according to the Act on Veterinary Practice, as required under Dutch legislation, and sampling was approved by the local ethics committee (DEC Utrecht), as required under Dutch legislation (ID 2007.III.08.110).

2.3 | Surgical outcome

Postoperative recovery was determined at reevaluations 1–3 months after surgery of all dogs that had survived. Persistent portosystemic

shunting was evaluated by a 12-hour fasting plasma ammonia concentration or by performing a rectal ammonia tolerance test.[15] Abdominal ultrasonography was performed to examine the site and patency of the attenuated shunt and to identify acquired portosystemic vessels. Complete recovery was defined as either normal fasting plasma ammonia concentrations (ie, <45 μM) or a rectal ammonia tolerance test within reference values and no portosystemic shunting on abdominal ultrasonography.[15] Dogs that died or were euthanized after surgery because of portal hypertension, hypoplasia of the portal vasculature, or persistent shunting were considered as not recovered. If shunt attenuation was not feasible during attempts of shunt ligation, dogs also were considered as not recovered. Dogs that died from reasons unrelated to portal hypoplasia, portal hypertension, or persistent shunting and dogs in which the outcome after shunt attenuation was unclear were excluded from the study.

2.4 | Sample selection

In both case-control studies, equal numbers of samples from recovered and non-recovered dogs were included. Samples were taken from hepatic tissue that was consecutively collected between July 2002 and July 2015. Eleven samples used in the 1st study were no longer available for use in the 2nd study. The remaining samples of the 1st study (n = 35) were included in the 2nd study, which was supplemented with 27 new samples. Liver tissue of healthy dogs was used for internal validation of the microarray and the RT-qPCR analyses and was obtained from fresh cadavers used in nonliver related research (surplus material, Utrecht University 3R-policy). The absence of underlying liver disease was confirmed histologically by a board-certified veterinary pathologist. Animal care and handling were performed in accordance with the European Directive for the Protection of Vertebrate animals used for Experimental and Scientific Purpose, European Community Directive 86/609/CEE.

2.5 | Expression profiling

In the 1st study, previously published microarray expression data,[16] representing mRNA expression of 42,034 canine-specific 60-mer probes determined in liver tissue of dogs with CPSS collected during surgical attenuation, was used. This data is available through GEO Series accession number GSE39005 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=%20GSE39005). Data of 47 dogs was available. One dog was excluded from the data because it was unclear whether the dog had completely recovered after shunt attenuation or not. Therefore, data of 23 recovered and 23 nonrecovered dogs was used to compare gene expression profiles of recovered and nonrecovered dogs after surgical attenuation of CPSS. Genes with increased expression ($\log_2$-fold) of more than 0.30 or decreased expression <-0.30 were selected for further evaluation in the 2nd study to ensure that only robust changes were considered.

In the 2nd study, liver tissue from 62 samples (31 recovered, 31 nonrecovered dogs) was analyzed. Gene expression differences of the selected genes were confirmed using RT-qPCR. RNA isolation, cDNA synthesis, and gene expression profiling using RT-qPCR was performed as described previously[16] with a maximum of 40 cycles.

Normalization was performed using 4 reference genes, based on their stable expression in liver (*Glucuronidase Beta* [*GUSB*], *Heterogeneous Nuclear Ribonucleoprotein H* [*HNRPH*], *Hypoxanthine Phosphoribosyl transferase* [*HPRT*], and *Ribosomal Protein S5* [*RPS5*])[16] as required under Minimum Information for Publication of Quantitative Digital PCR Experiments (MIQE) precise guidelines.[17] Primers for the genes of interest and reference genes, including their optimum temperature, are listed in Table 1. The mRNA expression of each selected gene was expressed as the averaged Cq of the reference genes in the sample minus the measured Cq value of the gene (ΔCq).

2.6 | Statistical analysis

For the 1st study, the results of the microarray analysis were reanalyzed with updated annotations using analysis of variance (ANOVA)[18] using *R* statistics (R version 2.2.1, R Foundation for Statistical Computing, Vienna, Austria). Correction for multiple testing (Permutation F2-test using 5000 permutations) was performed and $P<.05$ after family-wise error correction was considered statistically significant.

For the 2nd study, missing albumin results (3 of 62 results) were imputed by automatic multiple imputation. Outcome, sex, age at surgery, breed, and shunt subtype were included as variables in the linear regression model of the imputation. Gene expression in samples without a PCR signal were assumed to have a Cq value of 40.0. A receiver operating characteristic (ROC) curve was plotted for each gene product and albumin to determine sensitivity (Se) and specificity (Sp) of a test to identify dogs that recovered after surgery at various cut-off values between "low" and "high."[19] Variables with areas under the curve (AUCs) significantly ($P<.05$) different from 0.5 were selected for further analyses. For each selected variable, a binary variable, "low (0)" versus "high (1)," was created. The cut-off value between "low" and "high" was chosen at the gene expression level or albumin concentration that corresponded to the data point on the ROC curve with the smallest Euclidian distance to the point (1- Sp, Se) = (0, 1), to approach a perfect test (Se = 1, Sp = 1).

The diagnostic potential of the binary variables as well as shunt localization (extrahepatic versus intrahepatic), age at surgery and sex was evaluated in a logistic regression with outcome after surgery as dependent variable. A final model was obtained in a backward stepwise elimination-and-selection procedure in the likelihood ratio test with probabilities for stepwise entry and removal of 0.05 and 0.10, respectively. 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. Multicollinearity was evaluated by linear regression.[20] A tolerance <0.1, a variance inflation factor >10 or a condition index >15 were considered indicative of multicollinearity. Model fit was evaluated with the Hosmer–Lemeshow test. The proportion of dogs with correctly predicted outcome was calculated using a classification cut-off

TABLE 1 Primers used for quantitative real-time PCR (RT-qPCR)

Gene	Ensemble transcript ID	F/R	Sequence	T_m (°C)	Amplicon size (bp)
CAV2	ENSCAFG00000003402	F R	5′-TTCTCTTCGCCACCCTCAG-3′ 5′-CTGCGTCCTACACTTGAACAC-3′	65.8	147
CPD	ENSCAFG00000019016	F R	5′-ATTGGTATGATGTGGAAGGT-3′ 5′-GATTGTTCTCCCATTCTTGTC-3′	5.1	131
CTGF	ENSCAFG00000029442	F R	5′-GGAAGAGAACATTAAGAAGGG-3′ 5′-TACTCCACAGAACTTAGCC-3′	62.6	120
CYR61	ENSCAFG00000020276	F R	5′-CGAGTTACCAATGACAACC-3′ 5′-CATTTCTTGCCCTTCTTCAG-3′	65.8	109
DHDH	ENSCAFG00000003869	F R	5′-ACACCGTCACTGTGCTCCT-3′ 5′-TCCTTATGCTCTCCCTTCAACACC-3′	67.0	171
DZIP	ENSCAFG00000005465	F R	5′-TAAACGCAGGAAGAAGATGATCTC-3′ 5′-GGTGAGAATCTTCAGGGTGG-3′	61.3	148
ERLEC1	ENSCAFG00000002724	F R	5′-CATTCTGCCTCTTGTGACAAGTG-3′ 5′-TCCGTGACATACTTCATAAGTCCA-3′	59.8	147
FRMD4B	ENSCAFG00000006521	F R	5′-ACCACTCCAGTTCTTACC-3′ 5′-GGCTTATCATTGTCCATCTC-3′	62.6	136
FXYD1	ENSCAFG00000007095	F R	5′-CACCTACGACTACCAATCC-3′ 5′-GTTCCCTCCTCTTCATCAG-3′	64.9	149
GLS2	ENSCAFG00000000131	F R	5′-TTCAGCAATGCCACATTCCAG-3′ 5′-TCACCTCCACAGAGCACAG-3′	66.4	150
GNMT	ENSCAFG00000001741	F R	5′-CAACTGGATGACTCTGGAC-3′ 5′-TGCTCACTCTGATCTCCT-3′	63.9	119
GSTO1	ENSCAFG00000010593	F R	5′-TTCCATCTTTGGTAACAGGC-3′ 5′-TCTTATTGGTCAGAACCTCCT-3′	63.9	113
HEPC	ENSCAFT00000011304	F R	5′-CCAGTGTCTCAGTCCTTCC-3′ 5′-TTTACAGCAGCCACAGCA-3′	65.5	163
HGFA	ENSCAFG00000014629	F R	ACACAGACGTTTGGCATCGAGAAGTAT AAACTGGAGCGGATGGCACAG	60.0	128
HSD17B14	ENSCAFG00000003895	F R	5′-GTGACCAAGTTTGCCCTCCC-3′ 5′-GACGCCATATCGACTCTCATCCA-3′	67.0	170
LYSMD2	ENSCAFG00000015502	F R	5′-TCCTCCTAGTCCTCAAGAATCC-3′ 5′-GCATAGGGACTTTCTTCATCTCTG-3′	63.9	155
MAT2a	ENSCAFG00000007755	F R	TGCTTTTGGCGGGGAGGAG TTTAAAAGCTGCCATCTGAGGTGA	67.0	121
MFAP3	ENSCAFG00000030060	F R	5′-ACCACTATGAAGATGTCCGT-3′ 5′-CAAAGCATGTGTAGAGCCC-3′	61.3	143
MGST2	ENSCAFG00000003702	F R	5′-CTGGTTACATTGTGGATGG-3′ 5′-AGAAATACTGGTGACGGG-3′	63.9	93
NAGS	ENSCAFG00000014391	F R	5′-CATCTTCCTCAATACCACCG-3′ 5′-CACATCCACAATGAGCCG-3′	64.9	147
NUCB2	ENSCAFG00000008713	F F	5′-CGCAAAGATAGAACCACCAG-3′ 5′-TAGCCTCCCACTCTTTATTTCC-3′	64.9	138
PDIA4	ENSCAFG00000003403	F R	5′-AGGACTCAGGAAGAAATCGT-3′ 5′-GAACTCCACCAGAATGATGTC-3′	64.9	140

(Continues)

TABLE 1 (Continued)

Gene	Ensemble transcript ID	F/R	Sequence	T_m (°C)	Amplicon size (bp)
PIK3RA	ENSCAFG00000007626	F R	5′-CATTGCCTCCTAAACCACC-3′ 5′-TCCCATCGGCTGTATCTC-3′	63.9	143
PKIB	ENSCAFG00000029950	F R	5′-GCAAGCAACAGTGGCAAGG-3′ 5′-ACTCCACATCAGTCATCTCGGA-3′	61.3	90
PLIN2	ENSCAFG00000001601	F R	5′-AATGCACTCACCAAATCAG-3′ 5′-TCTGAACTGTATCAAACCCT-3′	64.2	106
SEH1L	ENSCAFG00000018880	F R	5′-CACAAACTCCCTCATTAAACTG-3′ 5′-GAAACCGATACACATCTTCTG-3′	63.9	136
SK2	ENSCAFG00000000220	F R	5′-CTCTCCACAATCATCCTGCT-3′ 5′-CATCTGCTCCGTTGTCCA-3′	65.8	84
SLC1A1	ENSCAFG00000002067	F R	5′-CATAGAAGTTGAAGACTGGGA-3′ 5′-AGTGGGAGAATGATAATGGAG-3′	63.9	98
SLC2A13	ENSCAFG00000009975	F R	5′-ACAGCTCTCAGGCATTAACAC-3′ 5′-GCAAGTCTATCATCTTCAACACCA-3′	67.0	80
SORD	ENSCAFG00000013672	F R	5′-AGAACTATCCTATCCCAGAACC-3′ 5′-GTGCTTTACCAGTGATCCC-3′	64.9	187
SSH3L	ENSCAFG00000011655	F R	5′-GTACCGAGACTTCATTGATAACC-3′ 5′-TCAAGATGTGGCTGACCC-3′	57.1	148
TFAP2B	ENSCAFG00000002156	F R	5′-TCACGTTACTCACCTCCC-3′ 5′-CGGTTCAAATACTCAGAAACAG-3′	62.6	111
TOR3A	ENSCAFG00000013929	F R	5′-ATGTTCATCGCCACCTTCC-3′ 5′-CGTCTTCTTGATCTGAGTCGT-3′	65.8	83
TRIM22	ENSCAFG00000024867	F R	5′-CAGACATTGAGCATCAGATATGG-3′ 5′-CGGAATTAGGAATGTACTCTTCAG-3′	57.8	139
TXNIP	ENSCAFG00000011405	F R	5′-GCAAACAGACCTCTGAATACC-3′ 5′-ATCACCATCTCATTCTCACCT-3′	63.0	81
VCAM1	ENSCAFT00000031837	F R	5′-GATGAAATTGACTTTGAGCCCA-3′ 5′-ATTGTCACAGAACCGCCT-3′	65.0	127
GUSB	ENSCAFG00000010193	F R	5′-AGACGCTTCCAAGTACCCC-3′ 5′-AGGTGTGGTGTAGAGGAGCAC-3′	62.0	103
hnRPH	ENSCAFG00000000336	F R	5′-CTCACTATGATCCACCACG-3′ 5′-TAGCCTCCATAACCTCCAC-3′	61.2	151
HPRT	ENSCAFG00000018870	F R	5′-AGCTTGCTGGTGAAAAGGAC-3′ 5′-TTATAGTCAAGGGCATATCC-3′	58.0	104
RPS5	ENSCAFG00000002366	F R	5′-TCACTGGTGAGAACCCCCT-3′ 5′-CCTGATTCACACGGCGTAG-3′	62.5	141

Abbreviations: *CAV2, caveolin-2; CPD, carboxypeptidase D precursor; CTGF, connective tissue growth factor precursor; CYR61, protein CYR61 precursor (IGF-binding protein 10); DHDH, dimeric dihydrodiol dehydrogenase; DZIP, zinc finger protein DZIP1 (DAZ-interacting protein 1/2); ERLEC1, endoplasmic reticulum lectin 1; FRMD4B, FXYD1, GLS2, glutaminase liver isoform, mitochondrial precursor; GNMT, glycine N-methyltransferase; GSTO1, glutathione transferase omega-1; HEPC, hepcidin precursor; HGFA, hepatocyte growth factor activator; HSD17B14, 17-beta-hydroxysteroid dehydrogenase 14; LYSMD2, LysM and putative peptidoglycan-binding domain-containing protein 2; MAT2a, methionine adenosyltransferase 2 alpha; MFAP3, microfibrillar-associated protein 3-like precursor; MGST2, microsomal glutathione S-transferase 2; NAGS, N-acetylglutamate synthase, mitochondrial precursor; NUCB2, nucleobindin-2 precursor; PDIA4, protein disulfide-isomerase A4 precursor; PIK3RA, phosphatidylinositol 3-kinase regulatory subunit alpha; PKIB, cAMP-dependent protein kinase inhibitor beta; PLIN2, Adipophilin; SEH1L, SEH1 Like Nucleoporin; SK2, small conductance calcium-activated potassium channel protein 2; SLC1A1, solute carrier family 1 (glial high affinity glutamate transporter), member 1; SLC2A13, solute carrier family 2 (facilitated glucose transporter), member 13; SORD, sorbitol dehydrogenase; SSH3L, protein phosphatase Slingshot homolog 3; TFAP2B, transcription factor AP-2 beta; TOR3A, Torsin-3A precursor; TRIM22, tripartite motif-containing protein 22; TXNIP, thioredoxin-interacting protein; VCAM1, vascular cell adhesion molecule 1. Reference genes used for normalization: GUSB, glucuronidase beta; hnRPH, heterogeneous nuclear ribonucleoprotein H; HPRT, hypoxanthine phosphoribosyl transferase; RPS5, ribosomal protein S5*; F, forward primer; R, reverse primer; T_m, melting temperature; bp, base pairs.

TABLE 2 Number of dogs with CPSS enrolled in the microarray, in the RT-qPCR and in both studies (n = 73)

Microarray study	Nonrecovered	Recovered	Total
EHPSS	13	19	32
IHPSS	10	4	14
Total	23	23	46
RT-qPCR study (in both studies)			
EHPSS	14 (6)	21 (16)	35 (22)
IHPSS	17 (9)	10 (4)	27 (13)
Total	31 (15)	31 (20)	62 (35)

Abbreviations: EHPSS, extrahepatic portosystemic shunt; IHPSS, intrahepatic portosystemic shunt.

value of 0.5. Validation of the final model was performed multiplying the regression coefficients with the heuristic shrinkage factor and correcting the intercept to improve its feasibility in future cohorts of dogs. All analyses were performed using commercial software (SPSS; IBM Corp. Released 2012, 2017. IBM SPSS Statistics for Windows, Versions 21.0 and 25. Armonk, New York).

3 | RESULTS

3.1 | Animal characteristics

Seventy-three dogs were enrolled in the study; 45 dogs with an extrahepatic portosystemic shunt (EHPSS) and 28 dogs with an intrahepatic portosystemic shunt (IHPSS; Table 2). The study group consisted of 25 different breeds and 5 dogs of cross breeds. The 1st study group, used for the microarray analysis, contained liver samples of 46 dogs; 32 (70%) with an EHPSS and 14 (30%) with an IHPSS. Of these 46 dogs, 23 dogs had recovered completely after surgical attenuation of the shunt, namely 19 (59%) of the dogs with an EHPSS and 4 (29%) of the dogs with an IHPSS. Eleven of the 46 samples from the 1st study were no longer available for the 2nd study.

The 2nd study group, used for RT-qPCR analyses, contained samples of 62 dogs with CPSS and included 35 overlapping samples with the microarray. Of these 62 CPSS dogs, 35 had EHPSS (56%) and 27 (44%) IHPSS. After surgical attenuation of the shunts, 31 of these dogs had recovered completely (mean age at surgery, 358 days) and 31 had not recovered (mean age at surgery, 383 days); 21 (60%) of the dogs with EHPSS had recovered and 10 (37%) of the dogs with IHPSS had recovered. Of the 73 CPSS dogs enrolled in the study, 39 dogs had not recovered. In 11 dogs, the shunt could not be attenuated during surgery because of portal hypertension caused by aplasia or hypoplasia of the portal vein (9 dogs) or the morphology of the shunt (2 dogs). One dog died 3 days postoperatively because of postligation seizures and in 27 dogs ammonia metabolism had not normalized, abdominal ultrasonography disclosed patency of the original shunt or newly developed multiple acquired shunts 1–3 months after shunt ligation or both.

Preoperative plasma albumin concentrations were available in 59 of the 62 dogs in the sample set for RT-qPCR analyses. The mean preoperative plasma albumin concentration in recovered dogs was significantly higher, 23 g/L compared with 19 g/L mL in nonrecovered dogs ($P = .004$).

3.2 | Gene expression patterns of recovered and nonrecovered dogs

In the microarray data set, 43 genes were differentially expressed in recovered and non-recovered CPSS dogs.[16] These genes were selected for further confirmation by RT-qPCR. RT-qPCR was technically not possible in 9 genes (*CYP2d15*, *FBLIM1*, *GLYCK*, *HMT*, *IGHV*, *LIPH*, *MMD*, *RBP3*, and *TCEA3*). Therefore, qPCR was performed on 36 genes, including *MAT2a* and *HGFact* (Table 1). Because of technical reasons, no RT-qPCR data could be obtained for 3 genes: *CYR61*, *MGST2*, and *SEH1L*.

3.3 | Construction of the prediction model

The ROC curves of albumin and the ΔCqs of *CTGF*, *DHDH*, *ERLEC1*, *HEPC*, *LYSMD2*, *ZIP* had AUCs that were significantly ($P < .05$) different from 0.5. The final logistic model included binary variables for albumin, *DHDH*, *ERLEC1*, and *LYSMD2* (Table 3). The AUC was 0.887 (95%

TABLE 3 Final overall prognostic model[a] of preoperative plasma albumin concentration and intraoperative hepatic mRNA expression of genes of interest as binary variables ("low" or "high") in 62 dogs with a CPSS

Prognostic variable	Level	Number of patients	Estimate	Standard error	*P* (Wald test)	Odds ratio (95% CI)
Albumin	Low (<21.5 g/L)	28	Reference		.008	
	High (≥21.5 g/L)	34	2.01	0.76		7.49 (1.70; 32.9)
DHDH (ΔCq)	Low (ΔCq < 1.82)	28	Reference		.094	
	High (ΔCq ≥ 1.82)	34	1.21	0.73		3.36 (0.81; 13.9)
ERLEC1 (ΔCq)	Low (ΔCq < −2.74)	28	Reference		.015	
	High (ΔCq ≥ −2.74)	34	−1.79	0.74		0.17 (0.04; 0.71)
LYSMD2 (ΔCq)	Low (ΔCq < −3.73)	28	Reference		.021	
	High (ΔCq ≥ −3.73)	34	−1.73	0.75		0.18 (0.04; 0.77)
Intercept			0.22	0.83	.07	

a–2 log likelihood = 51.23; Cox & Snell $R^2 = 0.43$; Hosmer-and-Lemeshow test $\chi^2 = 6.192$, df = 8, $P = .626$.

confidence interval [CI], 0.801-0.973). For each of the 62 dogs, a predicted probability for recovery was calculated using the final model. This analysis showed that if surgery would have been performed only on dogs with > 50% chance of complete resolution from portosystemic shunting at 1 to 3 months postoperatively, 77% of the recovered and 10% of the nonrecovered dogs would have been operated (sensitivity, 77%; specificity, 90%; Figure 1). After internal validation, the model was transformed to:

$$\text{Logit}\ (\pi) = 0.187 + 1.78(\text{b albumin}) + 1.07(\text{b}\Delta\text{Cq}\textit{DHDH}) - 1.59(\text{b}\Delta\text{Cq}\textit{ERLEC1}) - 1.53\,(\text{b}\Delta\text{Cq}\textit{LYSMD2}),$$

in which π is the predicted probability of recovery.

With this adapted model, recovery of new individual dogs can be predicted using preoperative plasma albumin concentration and the hepatic mRNA expression of the 3 genes of interest as a binary variable ("0" or "1"; Table 4).

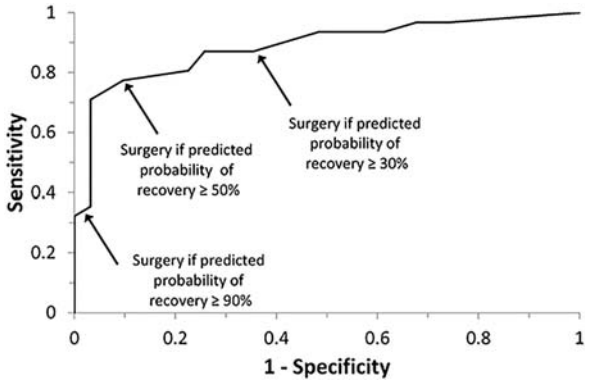

FIGURE 1 Receiver operating characteristic curve of the final model to predict recovery after surgery. Data of 62 dogs with surgical attenuation of a CPSS

4 | DISCUSSION

A prediction model was composed based on the expression levels of *Dihydrodiol dehydrogenase* (*DHDH*), *Endoplasmic Reticulum Lectin 1* (*ERLEC1*), *putative peptidoglycan-binding domain-containing protein 2* (*LYSMD2*), and presurgical plasma albumin concentration to predict recovery of portosystemic shunting in individual dogs after surgical CPSS attenuation. This model showed good fit and appears to be able to discriminate well between dogs that recover and dogs that do not recover. Because no model to predict recovery after surgery based on genome-wide hepatic gene expression is currently available, this preclinical research model potentially could be useful to make a better informed decision regarding treatment in individual shunt cases.

Our study confirmed the association of low plasma albumin concentrations with poor recovery, as described before.[4,7,12] Albumin is synthesized exclusively by hepatocytes. In dogs with portosystemic shunts, hypoalbuminemia is common and could be related to prolonged hepatocellular dysfunction. Although plasma albumin concentration is

TABLE 4 Predicted probability of recovery (π) using a prognostic model of preoperative plasma albumin concentration and intraoperative hepatic mRNA-expression of three genes of interest as a binary variable ("low, 0" or "high, 1") in 62 dogs with a CPSS after internal validation

Albumin 0 = < 21.5 g/L 1 = ≥ 21.5 g/L	*DHDH* 0 = ΔCq < 1.82 1 = ΔCq ≥ 1.82	*ERLEC1* 0 = ΔCq <−2.74 1 = ΔCq ≥−2.74	*LYSMD2* 0 = ΔCq <−3.73 1 = ΔCq ≥ −3.73	π	*R*	NR
1	1	0	0	0.95	10	0
1	0	0	0	0.88	1	1
1	1	0	1	0.82	3	0
1	1	1	0	0.81	6	0
0	1	0	0	0.78	2	0
1	0	0	1	0.61	1	1
1	0	1	0	0.59	0	0
0	0	0	0	0.55	1	1
1	1	1	1	0.48	1	4
0	1	0	1	0.43	2	1
0	1	1	0	0.42	0	3
1	0	1	1	0.24	2	4
0	0	0	1	0.21	0	4
0	0	1	0	0.20	1	2
0	1	1	1	0.13	0	2
0	0	1	1	0.05	1	8

The double line indicates the threshold of surgery at a probability of recovery > 50%.
Abbreviations: π, probability of recovery; R, number of recovered dogs; NR, number of nonrecovered dogs.

an important predictor and easy to measure preoperatively, the model was significantly improved by the inclusion of the hepatic expression levels of *DHDH*, *ERLEC1*, and *LYSMD2*. The ROC curve of plasma albumin concentration alone showed an AUC of 0.746 in comparison to the AUC of the final model of 0.887.

Previously, we found a positive association between complete recovery after shunt attenuation and plasma albumin concentrations as well as *MAT2a* and *HGFact* expression, 2 genes selected on potential prognostic value to recovery (involvement in hepatic regeneration and vascular growth, respectively).[12] In contrast to the previous study, the present study included more dogs and used a genome-wide microarray expression approach to objectively select genes of interest, and selection was not based on known gene function or pathway. Although *MAT2a* and *HGFact* were added as potential predictors in the construction of the model and these genes are important in liver cell proliferation,[12,13] neither of the 2 genes significantly contributed to the final model in our study.

A correlation between shunt localization, weight, age at time of surgery, and clinical outcome is suggested in several studies.[7,8] In general, IHPSS leads to an earlier development of clinical signs[21] when compared to EHPSS,[22] although geographical differences are reported. Such a difference also is observed within the EHPSS subtypes,[22] where extrahepatic portocaval shunts seem to be diagnosed at an earlier age compared to extrahepatic portoazygos shunts. If shunt localization (extrahepatic versus intrahepatic) was replaced by subtype of shunt (IHPSS by left, central or right-sided shunt; EHPSS by portoazygos or portocaval shunt), the same predictors (albumin, *DHDH*, *ERLEC1*, and *LYSMD2*) had a significant effect in the final model (data not shown). Because severe shunting leads to poor liver development and function,[23] it seems plausible that dogs with a higher fraction of shunted portal flow develop clinical signs at an earlier age. Hence, a negative correlation of age to recovery could be expected and explained by poor clinical condition of such dogs. In contrast, better prognosis was reported after surgical treatment in dogs <12 months of age, compared with dogs >2 years.[24] In our population, no correlation between age at surgery and recovery has been detected, which also has been found by others.[1,25] Because IHPSS occurs more often in large dog breeds and EHPSS in small dog breeds,[26] shunt localization, and weight are expected to be correlated when investigating factors associated with recovery rates. Although plausible, shunt localization did not contribute to the final prediction model.

Recovery was defined as normalization of plasma ammonia metabolism and no portosystemic shunting on abdominal ultrasonography. Although fasted bile acid concentrations often are more easy to measure and are very sensitive in screening dogs for CPSS, preprandial and postprandial serum bile acid concentrations also are reported to remain increased after complete ligation of CPSS in the majority of dogs, also in dogs without evidence of portosystemic shunting on ultrasonography. Measurement of fasting blood ammonia concentration is therefore the testing method of choice for diagnosing portosystemic shunting after surgery. An additional ammonia tolerance test or ultrasonography could rule out portosystemic shunting completely.[15,27] Although also a reliable technique, shunt fractions, using scintigraphy, were not measured in our study because of invasiveness and cost. By not considering resolution of clinical signs and defining absence of portosystemic shunting as recovery, our study used a clear, but also very strict, definition. It is debatable whether dogs whose owners report complete recovery from clinical signs but still show minor portosystemic shunting should be considered as nonrecovered. More research reporting long-term follow-up of clinically recovered dogs could provide a more realistic definition of recovery and would lead to adaptations in the prediction model.

The preclinical prediction model developed in our study is based on intraoperative hepatic gene expression levels of CPSS dogs referred to our clinic. Besides the assumption that intraoperative hepatic gene expression corresponds well with preoperative expression, clinicians will be reluctant to obtain hepatic biopsy specimens before surgery because of invasiveness, risks of complications and costs. Gene expression in peripheral circulating blood leukocytes has been reported to reflect mRNA expression changes in hepatic grafts.[28] It is unknown if gene products in plasma or mRNA expression in peripheral leukocytes reflect hepatic expression of genes related to postoperative CPSS recovery. However, the next step in the development of a more safe, clinically applicable, and practical prediction model is to investigate if the expression of genes of interest determined in peripheral venous blood samples also is prognostic.

Hepatic gene expression profiles and recovery rates after attenuation of a shunt are probably not identical among various countries because of population and individual breed differences.[21] Differences in preoperative medical treatment of dogs also may influence hepatic metabolism and gene expression. Although management with a low-protein diet is routinely instituted in our dogs before surgery, additional medication is not standardized and differs among clinics. Moreover, the various surgical regimens for shunt attenuation and variations in surgical skills also affect outcome. Preferably, surgical techniques and skills, but also perioperative monitoring and treatment, should be optimized and standardized. Although surgeon was not predictive in our study (data not shown), this is not a variable that we wanted to include in a model that would be widely applicable. An international multicenter study to validate this model is essential for it to become applicable world-wide. However, the best performance is to be expected from a model that is validated under the same conditions as will be used in new cases.

To obtain reliable predictions provided by a clinical prediction model, at least 10 events per predictor is advised.[29] Based on the previous study,[12] at least 30 samples per event (recovery and nonrecovery) were included in the 2nd part of this study. However, the number of dogs in relation to the number of predictors that eventually were used to construct the final model is small and is a limitation of our study. Therefore, more samples will be needed when validating this model.

In conclusion, a preclinical prediction model was constructed based on plasma albumin concentration and hepatic expression of 3 genes (*DHDH*, *ERLEC1*, and *LYSMD2*) as binary variables. This model had good discriminating ability to predict resolution of portosystemic shunting at 1 to 3 months after shunt attenuation by ligation in dogs. External

validation of this model in other dog populations and using different surgical (preoperative) liver biopsy and analyzing techniques is essential to evaluate the broad clinical applicability of the model. Development of less invasive ways to measure predictive gene expression, for example using peripheral venous blood samples, is important to increase practical applicability.

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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

Animal care and handling were performed in accordance with the European Directive for the Protection of Vertebrate animals used for Experimental and Scientific Purpose, European Community Directive 86/609/CEE.

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