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Markers of Angiogenesis Associated with Surgical Attenuation of Congenital Portosystemic Shunts in Dogs

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Background: Dogs with congenital portosystemic shunts (CPSS) have hypoplasia of the intrahepatic portal veins. Surgical CPSS attenuation results in the development of the intrahepatic portal vasculature, the precise mechanism for which is unknown, although new vessel formation by angiogenesis is suspected.

Hypothesis: That the degree of portal vascular development and the increase in portal vascularization after CPSS attenuation is significantly associated with hepatic vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) gene expression and serum VEGF concentration.

Animals: Client-owned dogs with CPSS undergoing surgical treatment. Forty-nine dogs were included in the gene expression data and 35 in the serum VEGF data.

Materials and Methods: Dogs surgically treated by partial or complete CPSS attenuation were prospectively recruited. Relative gene expression of VEGF and VEGFR2 was measured in liver biopsy samples taken at initial and follow-up surgery using quantitative polymerase chain reaction. Serum VEGF concentration was measured before and after CPSS attenuation using a canine specific ELISA. Statistical significance was set at the 5% level ($P \leq .05$).

Results: There was a significant increase in the mRNA expression of VEGFR2 after partial attenuation ($P = .006$). Dogs that could tolerate complete attenuation had significantly greater VEGFR2 mRNA expression than those that only tolerated partial attenuation ($P = .037$). Serum VEGF concentration was significantly increased at 24 ($P < .001$) and 48 ($P = .003$) hours after attenuation.

Conclusions and Clinical Importance: These findings suggest that intrahepatic angiogenesis is likely to occur after the surgical attenuation of CPSS in dogs, and contributes to the development of the intrahepatic vasculature postoperatively.

Key words: Canine; Liver; Quantitative polymerase chain reaction; Vascular endothelial growth factor.

Dogs with congenital portosystemic shunts (CPSS) have decreased portal blood supply and hence an underdeveloped intrahepatic portal vasculature as assessed by portovenography.[1] Surgical attenuation of the CPSS is recommended to redirect portal blood flow and restore normal hepatic function.[2] CPSS attenuation results in a substantial increase in the intrahepatic portal vasculature, as assessed by portovenography, and is associated with improvement in clinical signs.[1] The growth and maturation of new vessels is a highly complicated process and vascular endothelial growth factor (VEGF) is a critical mediator of angiogenesis.[3] VEGF promotes angiogenesis predominantly through VEGF receptor 2 (VEGFR2).[3] There is an increase in liver volume after CPSS attenuation[4,5] and it is probable that this increase is associated with the formation of new blood vessels to support the new liver tissue. We have previously demonstrated that improvement in the portal vasculature is associated with decreased VEGF protein expression and increased VEGFR2 protein expression in liver tissue, suggesting that angiogenesis is involved.[6] However, it was recognized that additional work was required to investigate the precise role of angiogenesis in the hepatic vascular response to CPSS attenuation. A better understanding of this process may allow more accurate prognostication and the development of novel therapies.

Abbreviations:

CPSS	congenital portosystemic shunt
Cq	quantification cycle
HMBS	hydroxymethyl-bilane synthase
PH	partial hepatectomy
qPCR	quantitative polymerase chain reaction
RPL13A	ribosomal protein L13a
RPL32	ribosomal protein L32
RPS32	ribosomal protein S18
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2

The overall aim of this study was to determine whether markers of angiogenesis are associated with the vascular response to CPSS attenuation. The study had 2 specific aims. The first was to measure the mRNA expression of VEGF and VEGFR2 in liver biopsy samples from dogs with CPSS both initially and after partial CPSS attenuation. The second aim was to measure the serum concentrations of VEGF in dogs with CPSS before and after attenuation.

The hypotheses tested were that gene expression would be significantly associated with the degree of por-

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tal vascular development, would significantly change in response to surgical CPSS attenuation, and that surgical CPSS attenuation would result in acute changes in serum VEGF concentration.

Materials and Methods

Clinical Management

Dogs with CPSS were prospectively recruited between August 2007 and October 2011. An institutional Ethics Committee granted ethical approval and owners gave full informed consent. Dogs were treated surgically by suture attenuation of their CPSS.[1] Dogs that could not tolerate complete attenuation, because of portal hypertension, were treated with partial attenuation. Dogs treated with partial attenuation had repeat surgery approximately 3 months postoperatively to attempt complete attenuation. Portovenography was performed before and after temporary complete shunt attenuation at each surgery to assess the development of the intrahepatic portal vasculature.[1] Portovenography grade was determined according to the number of generations of intrahepatic portal vessels that were visible on a scale of 1–4.[1] Portovenogram grades of 1 and 2 represented poor portal blood flow and portovenogram grades of 3 and 4 represented good portal blood flow.

Healthy experimental Beagle dogs, which had been humanely destroyed for reasons unrelated to hepatic disease, were used as controls for gene expression and serum VEGF measurement. In addition, dogs undergoing exploratory laparotomy were included as controls for serum VEGF measurement.

qPCR Gene Expression

For CPSS dogs, at each surgery a liver biopsy was taken for routine diagnostic purposes and a portion was placed in an appropriate volume of RNA*later*,[a] placed at 4°C for 24 hours and then frozen at −80°C. Liver tissue was taken from Beagle control dogs immediately after euthanasia and was processed and stored as described above.

RNA was extracted from approximately 20–30 mg of each hepatic sample using the GenElute Mammalian Total RNA Miniprep Kit.[a] The tissue was homogenized in 500 μL Lysis Solution using a Mixer Mill MM 300.[b] An in-solution DNase digestion was performed using the Ambion TURBO DNA-*free* Kit[c] to remove any contaminating DNA. RNA quality and quantity was assessed by microfluidic capillary electrophoresis using the Agilent 2100 Bioanalyser.[d] Two separate cDNA were synthesized from each RNA sample using a mixture of random hexamer and oligo $(dT)_{15}$ primers[e] and IMProm-II reverse transcriptase enzyme.[e] Where possible, the amount of RNA template for cDNA synthesis was standardized at 1 μg. The cDNA was diluted to a final volume of 100 μL with nuclease-free water and stored at −20°C before further use.

Quantitative polymerase chain reaction was used to measure the relative hepatic expression of VEGF and VEGFR2. Previously published canine gene specific primers for the genes of interest[7] and 4 liver-specific reference genes[8]: hydroxymethylbilane synthase (HMBS), ribosomal protein L13a (RPL13A), ribosomal protein L32 (RPL32), and ribosomal protein S18 (RPS18) were used (Table 1).

For quantification, each liver sample had 2 cDNA samples analyzed in duplicate. Reactions were carried out in 25 μL volumes using a Bio-Rad CFX96 Real-Time PCR Detection System thermocycler.[f] Each reaction consisted of 1 μL cDNA as the template with Immobuffer[g] (1× concentration), Hi-Spec Additive[g] (1× concentration), dNTP[g] (final concentration 1 mM), magnesium chloride (final concentration 2.5 mM for genes of interest, 4.5 mM for reference genes), 1 unit Immolase DNA polymerase[g] and EvaGreen dye[h] (0.06× diluted 1:4 with nuclease-free water). Samples were incubated at 95°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 10 seconds. An appropriate primer-dimer melting temperature for 1 second was programmed before fluorescence readings were taken at the end of each cycle. A melting curve analysis from 65°C to 95°C with a plate read every 0.5°C was performed at the end of 40 cycles. Bio-Rad CFX Manager Software[f] was used for initial qPCR analysis.

Analysis of raw real-time data was performed using GenEx professional version 4.4.2 software.[i] Relative gene expression was quantified as previously described.[9] Quantification cycle (Cq) values were corrected using the calculated efficiencies for each primer set. Normalization of each sample Cq for the genes of interest was performed relative to the geometric normalization of the 4 reference genes. The relative expression of the genes of interest in each cDNA sample was calculated using the normalized Cq of each sample relative to the average Cq of all of the samples.

Table 1. Table showing details of reference gene and gene of interest primer pairs for qPCR.

Gene	Primer Sequences	PCR Amplicon Length (bp)	Genbank Accession Number	Primer Sequence Reference
HMBS	Forward: TCACCATCGGAGCCATCT Reverse: GTTCCCACCACGCTCTTCT	112	XM546491	Peters et al[8]
RPL13A	Forward: GCCGGAAGGTTGTAGTCGT Reverse: GGAGGAAGGCCAGGTAATTC	87	AJ388525	Peters et al[8]
RPL32	Forward: TGGTTACAGGAGCAACAAGAAA Reverse: GCACATCAGCAGCACTTCA	100	XM_848016	Peters et al[8]
RPS18	Forward: TGCTCATGTGGTATTGAGGAA Reverse: TCTTATACTGGCGTGGATTCTG	116	XM_532106	Peters et al[8]
VEGF	Forward: CTTTCTGCTCTCCTGGGTGC Reverse: GGTTTGTGCTCTCCTCCTGC	101	NM_001003175	Kummeling et al[7]
VEGFR2	Forward: GGAAGAGGAAGTGTGTGACCCC Reverse: GACCATACCACTGTCCGTCTGG	181	XM_539273	Kummeling et al[7]

qPCR, quantitative polymerase chain reaction; HMBS, hydroxymethyl-bilane synthase; RPL13A, ribosomal protein L13a; RPL32, ribosomal protein L32; RPS18, ribosomal protein S18; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

Serum VEGF Concentration

Blood samples were taken from CPSS dogs and exploratory laparotomy controls preoperatively for diagnostic purposes and after surgery for postoperative monitoring and, where available, residual blood was collected for the study. Residual blood samples also were taken immediately before euthanasia in Beagle control dogs. The serum was separated and stored at −80°C.

A Quantikine Canine ELISA Kit[j] was used to measure the serum concentration of VEGF.[10–12]

Samples from CPSS dogs before and after surgical attenuation and control samples were analyzed in duplicate using an ELx808 absorbance microplate reader.[k] Sample concentration was calculated from the standard curve using Gen5 V1.07.5 software.[k]

Statistical Analysis

Analysis was performed using PASW Statistics 18.0.0 statistical software package.[l] Continuous data were visually assessed for normality. Median and range were reported for skewed data, which was compared with the Mann–Whitney *U*-test and Wilcoxon paired ranks test as appropriate. Repeated measures were compared with the Friedman's two-way analysis of variance by ranks with pair-wise comparison. The gene expression data was transformed to normal distribution (square root or log). The data then was compared with an independent *t*-test or paired sample *t*-test. For all tests significance was set at the 5% level ($P \leq .05$). For each gene, the following comparisons were made: CPSS compared to control; partial attenuation compared to complete attenuation; before and after partial attenuation (paired samples); good portal blood flow (portovenogram grade 3 or 4) compared to poor portal blood flow (portovenogram grade 1 or 2).

Results

qPCR Gene Expression

Clinical Findings. Liver samples from 49 dogs with CPSS were included. The following breeds were represented in the study population: Yorkshire Terrier (7), Crossbreed (6), Labrador (5), Miniature Schnauzer (5), West Highland White Terrier (5), Cocker Spaniel (4), Jack Russell Terrier (3), Bichon Frise (2), Golden Retriever (2), Lhasa Apso (2), Pug (2), Chihuahua (1), Hovawart (1), Irish Setter (1), Norfolk Terrier (1), Old English Sheepdog (1), and Staffordshire Bull Terrier (1). The median age was 275 days (range, 97–4,374). Thirty-eight (77.6%) dogs had an extrahepatic CPSS and 11 (22.4%) had an intrahepatic CPSS. Twenty-four dogs (49%) had complete attenuation and 25 dogs (51%) had partial attenuation. All partial attenuation dogs had repeat surgery a median of 110 days (range, 69–358) postoperatively. Histopathologically unremarkable liver tissue from 7 Beagle controls was included. The median age of control dogs was 628 days (range, 515–1,544), and was significantly greater than CPSS dogs ($P = .036$).

Portovenogram Grading. Complete portovenograms were available for 47 dogs at first surgery and 21 dogs at second surgery. Portovenogram grading results for dogs at first and second surgery were consistent with a previous study.[1] Both pre- and postattenuation portovenogram grades at first surgery were significantly greater for complete attenuation dogs compared with partial attenuation dogs (both $P < .001$; Fig 1). For dogs treated with partial attenuation, there was a significant increase in portovenogram grade for both pre- ($P < .001$) and post- ($P = .001$) complete temporary CPSS attenuation from first to second surgery (Table 2).

Gene Expression. Relative VEGFR2 mRNA expression was significantly greater in complete attenuation dogs at a median of 12.885 (range, 2.459–22.454) compared with partial attenuation dogs at a median of 9.140 (range, 3.724–19.556; $P = .006$). Relative VEGFR2 mRNA expression significantly increased after partial attenuation from a median of 9.140 (range, 3.724–19.556) to 10.493 (range, 4.814–17.409; $P = .037$). There were no associations evident for

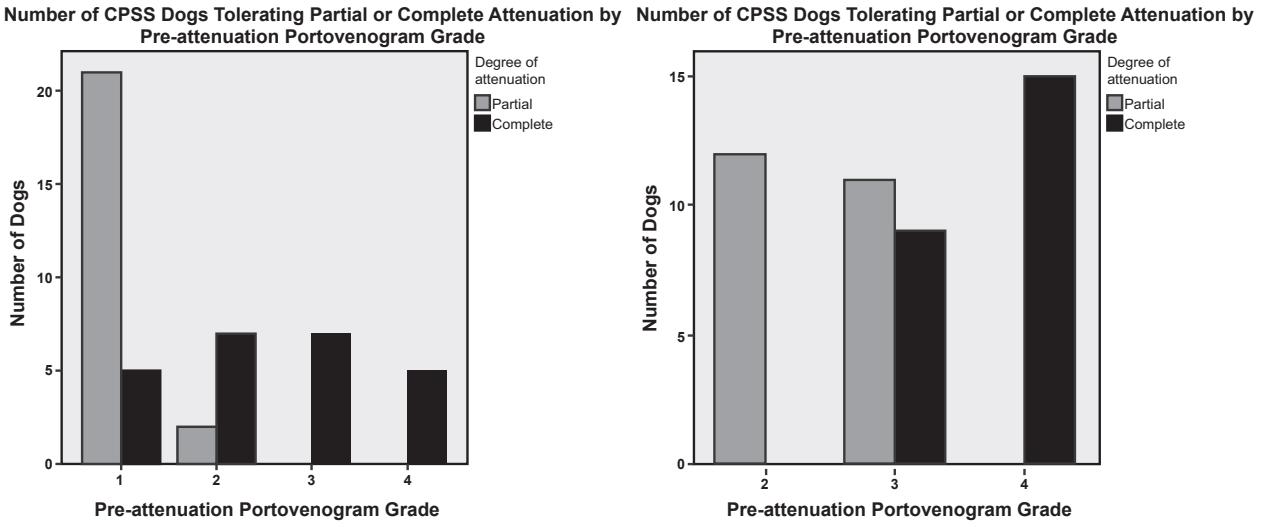

Fig 1. Number of dogs tolerating partial or complete congenital portosystemic shunts (CPSS) attenuation at first surgery by pre- and postattenuation portovenogram grade. There was a significant difference in portovenogram grade between the 2 groups (both $P < .001$).

Table 2. Portovenogram grade before and after temporary CPSS attenuation in 21 dogs at first and second surgery.

	Number (%) of Dogs for Each Portovenogram Grade			
Timing of Assessment	Grade 1	Grade 2	Grade 3	Grade 4
1st Surgery preattenuation	18 (85.7)	3 (14.3)	0 (0)	0 (0)
1st Surgery postattenuation	0 (0)	12 (57.1)	9 (42.9)	0 (0)
2nd Surgery preattenuation	2 (9.5)	8 (38.1)	6 (28.6)	5 (23.8)
2nd Surgery postattenuation	0 (0)	4 (19.0)	6 (28.6)	11 (52.4)

There was a statistically significant increase in portovenogram grade for both pre- and postattenuation from first to second surgery ($P < .001$ and $P = .001$).

relative VEGF mRNA expression. All results are presented in Table 3 and statistically significant results are presented graphically in Figure 2.

Relative VEGFR2 mRNA expression was significantly greater in dogs with good portal blood flow (portovenogram grade 3 or 4) at a median of 13.072 (range, 9.700–19.787) compared to those with poor portal blood flow (portovenogram grade 1 or 2) at a median of 9.246 (range, 2.459–22.454) on preattenuation portovenograms ($P = .001$; Fig 3). There were no additional associations between the relative mRNA expression of VEGF or VEGFR2 and portovenogram grade.

Serum VEGF Concentration

Clinical Findings. Serial serum samples taken before surgery and at 24, 48, and 72 hours postsurgery from 35 dogs with CPSS were included. The following breeds were represented in the study population: Miniature Schnauzer (5), Crossbreed (4), Yorkshire Terrier (4), Cocker Spaniel (3), Labrador (3), West Highland White Terrier (3), Jack Russell Terrier (2), Pug (2), Bichon Frise (1), Border Collie (1), Border Terrier (1), Lhasa Apso (1), Norfolk Terrier (1), Shih Tzu (1), Springer Spaniel (1), Staffordshire Bull Terrier (1), and Tibetan Terrier (1). The median age of CPSS dogs was 385 days (range, 107–4,374). Twenty-nine (82.9%) dogs had an extrahepatic CPSS and six (17.1%) had an intrahepatic CPSS. Sixteen dogs (45.7%) had complete attenuation and 19 (54.3%) had partial attenuation. Serum samples from 5 healthy Beagle dogs and 6 dogs undergoing abdominal surgery were included as controls. The six dogs undergoing abdominal surgery also had a 24-hour postsurgery sample taken. The following breeds were represented in the control population: Beagle (5), Crossbreed (2), Cocker Spaniel (1), German Shepherd Dog (1), Labrador (1) and Shar Pei (1). The median age of control dogs was 1,544 days (range, 526–3,971), which was significantly greater than the age of CPSS dogs ($P = .002$).

Serum VEGF Concentration. The median preoperative VEGF concentration for CPSS dogs was 37.8 pg/mL (range, 5.7–85.3) and for control dogs was 64.3 pg/mL (range, 1.9–178.4). This difference was not significant. The median VEGF concentration at 24 hours in CPSS dogs was 66.4 pg/mL (range, 9.6–156.2), at 48 hours was 50.3 pg/mL (range, 16.4–221.1) and at 72 hours was 40.5 pg/mL (range, 9.0–121.7; Fig 4). There was a significant difference in the concentration of VEGF at the different time points ($P < .001$). Pairwise comparison of this data set confirmed that VEGF concentration at 24 hours ($P < .001$) and 48 hours ($P = .003$) postsurgery were significantly greater than concentrations presurgery.

For the 6 control dogs with pre- and postsurgical samples, the median VEGF concentration presurgery was 83.8 pg/mL (range, 23.4–178.4) and at 24 hours postoperatively was 76.4 pg/mL (range, 11.8–132.1). This difference was not statistically significant.

The intra-assay variability was low with a median coefficient of variation (CV) of 4.1 (range, 0.0–40.9).

Discussion

The aim of this study was to investigate whether markers of angiogenesis are associated with the degree of liver and portal vasculature development and the hepatic vascular response to attenuation in dogs with CPSS. We found that VEGFR2 mRNA expression increased significantly after partial CPSS attenuation. We also found that VEGFR2 mRNA expression was greater in dogs with a more developed portal blood supply at the time of first surgery. These findings are consistent with an increased binding capacity for VEGF, suggesting that angiogenesis is occurring and therefore contributing, at least in part, to the increased vascularization in dogs with CPSS. This concept is supported by the fact that there also was a significant increase in portal blood flow after partial attenuation. In addition, we found that serum VEGF concentrations were significantly increased 24 and 48 hours postsurgery. These findings support the concept that angiogenesis occurs after CPSS attenuation and is involved in the improvement in portal vasculature.

Vascular endothelial growth factor receptor 2 is the major receptor mediating the angiogenic actions of VEGF and its expression is increased during angiogenesis and down-regulated in quiescent tissue.[3,13,14] VEGF produced by hepatocytes acts in a paracrine manner, interacting with its receptors on endothelial cells to regulate endothelial cell growth and liver vascularization after experimental partial hepatectomy

Table 3. Relative mRNA expression of VEGF and its receptor VEGFR2 in liver biopsies from dogs with CPSS and control dogs. For each gene the following comparisons were made: CPSS compared to control; partial attenuation compared to complete attenuation; before and after partial attenuation (paired samples).

	Control Compared to CPSS			Complete Attenuation Compared to Partial Attenuation			Before and After Partial Attenuation		
Gene	Control	CPSS	*P*-Value	Partial	Complete	*P*-Value	Before Partial Attenuation	After Partial Attenuation	*P*-Value
VEGF	9.875 (7.074–11.775)	10.220 (2.242–19.538)	.396	8.794 (5.959–18.033)	11.098 (2.242–19.538)	.081	8.794 (5.959–18.033)	10.459 (6.354–17.118)	.153
VEGFR2	7.684 (5.783–15.767)	10.916 (2.459–22.454)	.406	9.140 (3.724–19.556)	12.885 (2.459–22.454)	***.006***	9.140 (3.724–19.556)	10.493 (4.814–17.409)	***.037***

VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; CPSS, congenital portosystemic shunts
Results are given as median and range. Statistical significance was set at the 5% level ($P \leq .05$). Significant *P*-values are highlighted in bold and italics.

(PH).[15,16] Hepatocyte expression of VEGF and endothelial expression of VEGFR2 both increase after PH in the rat, peaking at 48–72 hours and associated with the proliferation of endothelial cells.[16–20] PH is a model of liver regeneration and in addition the increased mass requires the synthesis of new blood vessels.[21] Previous work has identified associations between markers of hepatocyte proliferation and surgical attenuation of CPSS.[22] These data support the hypothesis that liver regeneration occurs after CPSS attenuation and this increased liver volume would intuitively require an increased blood supply.

If angiogenesis occurred after partial CPSS attenuation then similar increases in VEGF and VEGFR2 mRNA expression might be expected. We previously used immunohistochemistry to measure endothelial cell expression of VEGF and VEGFR2 in liver tissue from dogs with CPSS.[6] This study found that endothelial VEGF was up-regulated and endothelial VEGFR2 was down-regulated in CPSS dogs and that there was a decrease in endothelial VEGF and an increase in endothelial VEGFR2 after partial CPSS attenuation. These findings suggested that angiogenesis is involved in the vascular response to CPSS attenuation.

This study used qPCR to measure the relative mRNA expression of VEGF and VEGFR2 in liver tissue from dogs with CPSS. VEGFR2 mRNA expression significantly increased after partial CPSS attenuation. Thus, VEGFR2 mRNA expression increases after partial attenuation, presumably related to increased portal blood flow. This is supported by the results of the portovenogram analysis, which showed significant increases in portovenogram grade, and hence increases in portal flow, from first to second surgery.

Thus, an increase in VEGFR2 mRNA expression is associated with development of the vasculature. VEGFR2 mRNA expression also was significantly greater in dogs that could tolerate a complete attenuation compared to those that tolerated partial attenuation. Dogs that tolerated complete attenuation also had significantly greater intrahepatic portal blood flow based on portovenogram grade. These findings are supported by the fact that expression of VEGFR2 mRNA was significantly greater for dogs with well-developed portal blood flow (portovenogram grades 3 and 4) compared to those with poor flow (portovenogram grades 1 and 2). Thus, VEGFR2 mRNA expression is associated with a more developed liver and more developed intrahepatic vasculature, suggesting a possible role for angiogenesis in vascular development in CPSS dogs. VEGFR2 mRNA expression represents VEGF binding capacity and hence may be a better marker of vascular development than VEGF itself. These findings support the hypothesis that angiogenesis is involved in the hepatic response to surgical CPSS attenuation. Thus, it can be inferred that improvement in intrahepatic portal vasculature is, at least in part, because of angiogenesis. This finding has important implications for development of novel treatment strategies for

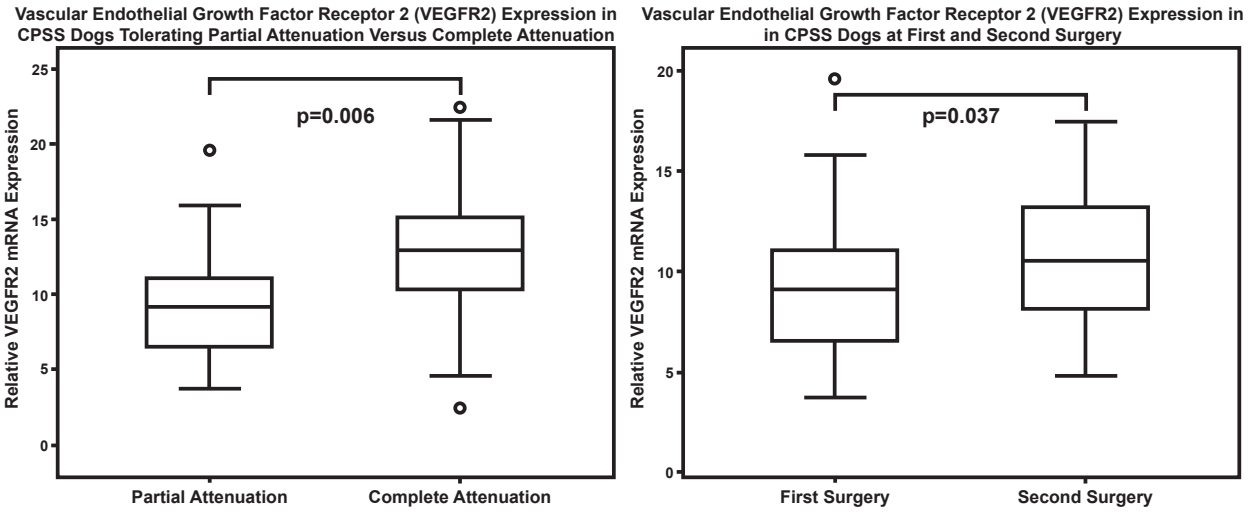

Fig 2. Relative vascular endothelial growth factor receptor 2 (VEGFR2) mRNA expression in liver biopsy samples from dogs with congenital portosystemic shunts (CPSS). The graphs show significant findings for VEGFR2. Statistical significance is highlighted with the appropriate *P* value.

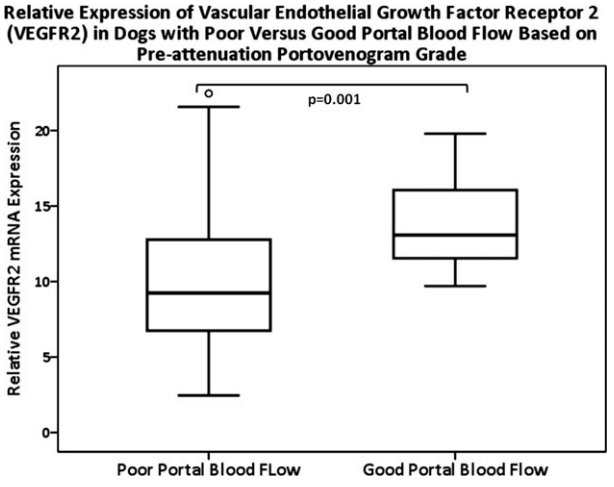

Fig 3. Relative mRNA expression of vascular endothelial growth factor receptor 2 (VEGFR2) in liver biopsy samples from dogs with congenital portosystemic shunts (CPSS) as related to portal blood flow on preattenuation portovenogram. Portovenogram grades of 1 and 2 were considered poor portal blood flow and portovenogram grades of 3 and 4 were considered good portal blood flow. There was a significant difference between the groups ($P = .001$).

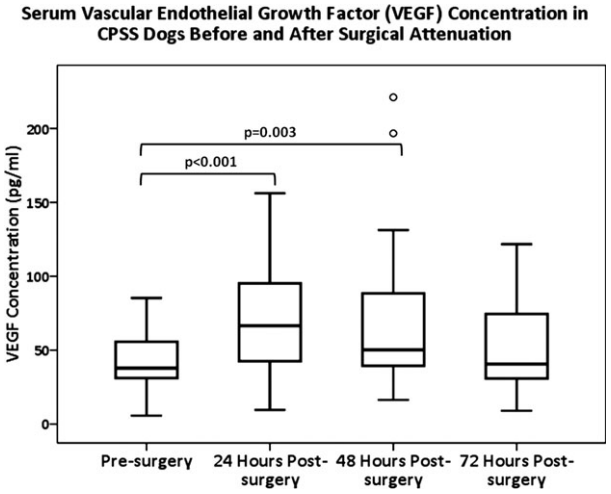

Fig 4. Serum vascular endothelial growth factor (VEGF) concentration in congenital portosystemic shunts (CPSS) dogs presurgery and 24, 48, and 72 hours postsurgery. VEGF concentration was measured using a canine VEGF ELISA kit. There was a significant difference in the concentration of VEGF at the different time points ($P < .001$). Pair-wise comparison of this data set confirmed that VEGF 24 hours ($P < .001$) and 48 hours ($P = .003$) postsurgery were significantly greater than presurgery.

dogs with CPSS. A previous study investigated the administration of recombinant HGF to promote hepatic regeneration in dogs with CPSS.[23] Liver volume increased in response to recombinant HGF, demonstrating that this approach has potential but the increase was not sustained after withdrawal of treatment. Serum VEGF concentration potentially could be used to assess the response to CPSS attenuation in the postoperative period, although probably in concert with imaging of the intrahepatic vasculature.

The results of this study agree with previous data demonstrating that VEGFR2 is a marker of angiogenesis in CPSS dogs.[6] In the previous study, endothelial VEGFR2 increased after partial attenuation, similar to the increase in VEGFR2 mRNA expression seen in this study. However, the previous study also identified that endothelial VEGF was up-regulated in CPSS dogs compared with controls and decreased after partial attenuation. Similar changes in VEGF mRNA expres-

sion were not identified in this study. This discrepancy may be related to the different methodologies used to measure VEGF. This study used qPCR to measure mRNA expression and it is important to note that this does not necessarily translate directly into protein expression. In addition, the previous study specifically assessed endothelial VEGF protein whereas this study measured VEGF mRNA expression in liver tissue as a whole. This study also used a different and smaller group of dogs and this also could have influenced the results.

Based on the nature of the clinical cases in this study, there were limitations on what tissue was available for analysis. It would have been interesting to compare hepatic biopsy samples at first surgery with samples taken in the immediate postoperative period because more acute changes in VEGF and VEGFR2 mRNA expression may have been detectable. However, hepatic tissue was only available at first surgery from all dogs and then at follow-up surgery in dogs treated with partial attenuation. Thus, we were only able to detect changes in hepatic gene expression at these time points. Nevertheless, this study provides novel information regarding the activity of these markers in dogs with CPSS.

To gain insight into the immediate perioperative period, serum VEGF concentrations before and after surgery also were measured. Serum VEGF concentration was significantly increased at 24 and 48 hours after CPSS attenuation compared with presurgery. Several studies of PH in rats have shown increased expression of VEGF and VEGFR2 in hepatic tissue postoperatively, confirming a role in liver regeneration.[17–20,24] These changes were maximal at 48–72 hours and decreased thereafter.[17,18,20] The increases in VEGF and VEGFR2 were associated with endothelial cell proliferation, indicating angiogenesis.[17,20] This increase in VEGF expression in hepatic tissue could result in increased protein production and increased VEGF in the peripheral blood. In 1 study, VEGF was detectable in peripheral blood 72 hours after PH in rats but not before.[18] In a clinical study of people undergoing PH for liver donation, significant increases in VEGF compared with presurgery concentrations were seen at days 4 and 5 postoperatively.[25] The increase in VEGF after CPSS attenuation would indicate increased production of VEGF associated with increased hepatic portal blood flow and liver regeneration, similar to the increases seen in the studies described above. VEGF plays an important role in wound healing, and studies have shown increased VEGF concentration in both serum and wound fluid after surgery.[26–28] The increases in serum VEGF seen in the current study may have been as a result of wound healing rather than related to changes in hepatic vasculature. However, in the experimental and clinical studies of PH the increases in serum VEGF were attributed to hepatic angiogenesis rather than wound healing. Studies investigating wound healing after surgery typically have shown maximal increases in VEGF concentration 7–14 days postoperatively with concentrations also remaining increased after this time.[26–28] This is contrary to the more acute and short-lived increase seen in studies of PH and after CPSS attenuation in the current study.

A control group of breed- and age-matched dogs undergoing abdominal surgery for reasons other than CPSS or liver resection would have provided additional information on the specificity of VEGF as a marker of angiogenesis. Indeed, a cohort of dogs undergoing PH would have provided useful information for comparison. Unfortunately, such controls were not available. A small number of dogs undergoing abdominal surgery with pre- and postoperative samples were tested for VEGF and did not show a significant increase after surgery, although this may have been because of type II statistical error. In addition, there was a wide range of VEGF concentrations seen in the control dogs and this may have influenced the results. A much larger control group would have been ideal and potentially would have allowed stronger conclusions to be drawn. Control dogs also were significantly older than the CPSS dogs and this may have influenced the results. Studies have shown that serum VEGF concentrations are significantly higher in fetuses and neonates compared to adults but that there is no significant difference in serum VEGF between children and adults or among children of different ages.[29,30] The main focus of this part of the study was to investigate changes in VEGF concentration in CPSS dogs before and after surgery, and this was independent of the starting concentration. Our findings provide good evidence that angiogenesis occurs in CPSS dogs in response to increased blood flow caused by CPSS attenuation.

This part of the study included a 72-hour postsurgery sample for assessment of VEGF concentration because previous studies in rats and people had suggested that VEGF concentrations would be maximal at this time point. In the CPSS dogs, VEGF was detectable at all time points and the concentrations at 72 hours were not significantly different from presurgery concentrations. Many variables may have contributed to this difference although the difference in species and the trigger for angiogenesis are likely to have played a major role. Because of the ethical issues discussed above, it was not possible to take blood purely for this study. Residual blood was collected when samples were taken for routine diagnostic purposes, which dictated the amount of blood available and the time at which it was collected. Blood samples taken earlier than 24 hours postoperatively may have demonstrated even greater increases in VEGF. A commercial ELISA kit was used for the measurement of serum VEGF because it was canine specific and had been used in previously published studies, demonstrating that it was able to detect the corresponding antigen in canine samples.[10–12]

Footnotes

[a] Sigma-Aldrich Company Limited, Dorset, UK
[b] Retsch, Leeds, UK
[c] Life Technologies Ltd, Paisley, UK
[d] Agilent Technologies, Cheshire, UK
[e] Promega, Southampton, UK
[f] Bio-Rad Laboratories Ltd, Hertfordshire, UK
[g] Bioline, London, UK
[h] Biotium Inc, Hayward, CA
[i] Multid Analyses, Goteborg, Sweden
[j] R&D Systems Europe, Limited, Abingdon, UK
[k] BioTek® Instruments Incorporated, Winooski, VT
[l] Education SPSS (UK) Limited IBM, Woking, UK

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Conflict of Interest Declaration: The authors disclose no conflict of interest.

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