Evaluation of Hepatic Expression of VEGF and α-SMA in normal dogs and dogs with Congenital Portosystemic Shunt.

Renske van der Veen, Anne Kummeling, Geraldine B. Hunt

Abstract

In dogs with congenital portosystemic shunts (CPSS) portal venous blood is diverted around the liver and occlusion of the anomalous vessel is the treatment of choice. Patients that do not respond well to surgery after complete closure of the shunt may show portal hypertension, acute shock, death or evidence of continued hepatic dysfunction. Hepatic proliferation and development of the portal vein and its branches are probably important for recovery of patients after shunt closure. Certain factors, like VEGF and α-SMA, may play a significant role in these processes. Therefore livers of normal dogs and dogs with CPSS were investigated immunohistochemically for VEGF and α-SMA expression. Liver tissue of dogs with CPSS had increased VEGF and α-SMA expression. It seemed that only in patients that did not fully recover, VEGF expression was elevated. In the portal tracts of affected liver tissue also proliferation of hepatic arteries and less normal sized portal veins were present. An elevated level of VEGF might result from aberrant function or distribution of VEGF-receptors. The results of this study are promising for more extensive research of expression of VEGF and VEGF-receptors in dogs with CPSS.

Introduction

Congenital Portosystemic Shunts (CPSS) are anatomically abnormal vessels which divert variable amounts of portal blood around the liver and thereby bypassing the liver parenchyma.[2,14] Generally the treatment of choice in dogs with CPSS is occlusion of the anomalous vessel.[2,11,12,14,19] Nevertheless, individual dogs respond differently to surgical shunt occlusion. Portal hypertension, acute shock and death can occur after complete closure of the shunt if there is hypoplasia or aplasia of the portal venous circulation cranial to the shunt.[10,14] Biochemical evidence of continued hepatic dysfunction after surgical closure of the shunt, like an abnormal ammonia tolerance test (ATT) and elevated postprandial serum bile acid concentration (SBA), can result from persistent flow through the original shunt, development of multiple acquired shunts or, other liver disorders such as microvascular dysplasia. After surgery, the shunt may close too rapidly to allow proliferation of the hepatic vasculature in order to decompress the portal system.[12] Portal development may be correlated with the degree of shunt closure that is tolerated during surgery. However, for long-term outcome the degree of shunt closure or the size of the cranial part of the portal vein during

surgery do not seem to be predictable factors. Hepatic regeneration and development of the portal vein and its branches are probably more important in predicting the long-term outcome.[14] Development of hepatic vasculature may be essential in the recovery of CPSS dogs after shunt closure. Insufficient regeneration of the vessels after shunt closure is possibly caused by growth factors that are not being produced or that are not effective. The most specific growth factor for vascular endothelial cells is vascular endothelial growth factor (VEGF).[20]

The aim of this research is to determine the hepatic expression of VEGF and α-SMA in dogs with congenital portosystemic shunts.

Literature overview

Liver development

The early liver is first found in the ventral foregut endoderm as multilayered epithelium. This presumptive liver bud is surrounded by the endothelial cells which become embedded in the tissue and develop in relation with the hepatic epithelium, as the hepatoblasts move into the surrounding mesenchyme. Experiments have shown that VEGF-receptor-2 deficient endothelial cells are unable to form blood vessels. Also hepatoblasts are incapable of proliferation and migration into the surrounding mesoderm.[5,17,21] From these experiments is concluded that the interaction and signal exchange between blood vessel endothelium and liver endoderm is required for liver morphogenesis and growth. VEGF has
been suggested to be very important in this early development of the liver.[5,17,21]

VEGF

VEGF acts as an angiogenic growth factor specific for vascular endothelial cells. VEGF is also known for its ability to induce vascular leakage and permeability.[20,29] Multiple mechanisms induce the expression of VEGF, like activated oncogens, hypoxia, hypoglycemia and cytokines. VEGF induces endothelial cell multiplication and cell migration, while apoptosis is inhibited.[20]

In normal angiogenesis, VEGF seems to be the most important factor to vascular formation. VEGF is necessary in angiogenic sprouting and vasculogenesis, that leads to the formation of immature vessels.[25,29] During development, organ formation takes place. The angiogenesis that accompanies the organogenesis might be driven by increased VEGF production that is induced by hypoxia.[20,25,29] This concept is best studied in the development of the retina.[20] In retinal development astrocytes encounter growing hypoxia as the space between them and the vasculature increases. Hypoxia triggers the production of VEGF by astrocytes, which in turn activates angiogenesis. So, new blood vessels follow the astrocytes, as they expand.[20] VEGF production decreases when the vessels approach the astrocytes. Nevertheless, to prevent apoptosis of endothelial cells and to secure the newly formed vessels, VEGF is necessary in lower levels. In conclusion, the vascular system is finely adjusted to the needs of the organ.[20] When post-natal rodents or prematurity babies are returned to normoxia after an exposure to hyperoxia that suppresses retinal VEGF expression, abnormal vessel growth is induced. These results show that VEGF can be induced in an inappropriate manner, which induces an ineffectual

response.[20,29] VEGF is believed to play a role in tumour pathogenesis, as in malignant tumours a stronger staining and a higher number of VEGF-positive cells were found compared to benign neoplasms. This finding was also correlated with the micro vessel density in the tumour, as the angiogenesis was increased in malignant tumours.[22,23] Also in osteogenesis VEGF seems to be critical for angiogenesis. It is stated that VEGF is essential for new bone formation and bone remodelling.[4,6] There have been recent studies in rats that suggest that VEGF expression increases during liver regeneration after hepatectomy. During liver regeneration VEGF expression increased after Hepatic Stellate Cell (HSC) activation. Also increased α-SMA expression and direct cell contact between hepatocytes and HSC were found. Direct cell-to-cell contact between HSC´s and hepatocytes seems to be the precondition for activation of HSC´s. It seems that through this contact HSC´s release factors that induce VEGF expression, what explains the correlation between increased VEGF expression and HSC activation.[3]
Cytokines and many other extracellular molecules also regulate the expression of VEGF. For example, Keratinocyte Growth Factor (KGF) is one of many factors that participate in dermal wound healing. During wound repair KGF is more distinctly expressed, after which VEGF production is noticeably induced in keratinocytes. The induction of angiogenesis is part of the healing process of the wound.[7,20]

Why was VEGF the aim of this study?
VEGF is interesting because there has not been published any research about VEGF in relation to congenital portosystemic shunts in dogs. While reviewing, the literature indicated that VEGF could play an important role in CPSS.

The events in the development of the retina might be similar to the development of other organs and their vascular network.[20] If astrocytes are not able to produce sufficient VEGF, it is likely that the blood vessels will not be capable to form a finely tuned vascular structure. Assuming these same principles in organogenesis and because VEGF seems important in many processes which involve vascular development, it could also play an important role in hepatic regeneration and development of hepatic microvasculature after surgical occlusion of a shunt. Surprisingly there is little known about VEGF expression in canine liver with CPSS. In dogs that respond well to surgery, there must be a trigger to induce hepatic growth and angiogenesis, as before surgery the liver size is reduced and the intrahepatic portal vessel density is decreased compared to normal dogs.[8,16,27] So development of the portal vascular network probably is essential for successful recovery of the patient. Animals of special interest are dogs that do not recover completely after shunt closure, because they seem not fully capable of proliferating after surgery. This can be originated in the fact that the vasculature is not able to respond sufficiently to change in blood flow. In this research we looked for evidence for the involvement of VEGF in hepatic function or prognosis of dogs with a CPSS. Differences in VEGF expression between CPSS dogs and normal dogs and in CPSS dogs with different outcome after surgery might be very interesting objectives for further research.

α-Smooth Muscle Actin (α-SMA)

α-SMA is localized in smooth muscle cells.[9,26] With α-SMA immunostaining it is possible to identify smooth muscle cells of blood vessels, but also Hepatic Stellate Cells (HSC) in normal dog liver.[13,26] α-SMA expression is increased in dogs with hepatitis, chronic hepatitis or cirrhosis.[18]

Increased VEGF and α-SMA expression and activation of HSC seem to be correlated in hepatic proliferation after hepatectomy.[3] α-SMA was included in this study to evaluate α-SMA expression in liver of CPSS dogs, and assess a possible correlation with VEGF expression.

Materials and Methods

Animals

Liver biopsies were taken from eight dogs. Normal liver samples were obtained from three dogs requiring exploratory laparotomy for neutralisation. Affected liver samples were taken from five dogs undergoing surgery for congenital portosystemic shunt. After shunt closure, some dogs with CPSS were evaluated for continued hepatic dysfunction by ammonia tolerance testing and measuring postprandial serum bile acid concentration. When this biochemical follow up could not take place, the owners were contacted by telephone about the postoperative condition of the dog.

Study design

Approval had been obtained from The University of Sydney Animal Ethnics Committee for the collection of liver samples. The owners were informed and were asked for their consent before surgery.*Tissue processing*

The tissue samples have been fixated in 10% buffered Formalin for at least 12 hours. After fixation, the samples were dehydrated in graded alcohols and 100% Xylene. Embedding occurred at 56˚C overnight in Paraplast Paraffin wax. Tissue blocks were cooled on ice and sectioned at 5 µm, using a Leica PX 40 microtome. Sections were floated on a water bath set at 45˚C containing reverse osmosis water and 10 mL non inactivated horse serum for adhesion. The sections were mounted on glass microscope slides and dried overnight at 56˚C.

Light microscopy

Sections were dewaxed in Xylene and rehydrated in graded alcohols. Whitlock's Haematoxilin and Alcoholic Eosin Y were used to color the sections, after which they were dehydrated and mounted with DPX.

Immunohistohemistry

The immunohistochemistry sections were dewaxed in Xylene and rehydrated in graded alcohols. For antigen retrieval, the sections were heated in the microwave for three cycles of 5 minutes, in a commercial antigen retrieval solution (Dako EDTA). 10% hydrogen peroxide (H2O2) was used for 15 minutes to block endogenous peroxidase. The primary antibody incubated for 60 minutes at room temperature. For negative controls, isotype antibodies and Tris buffer were used instead of primary antibody. The sections were incubated with the secondary antibody, Labeled Streptavidin Biotin (LSAB kit; DAKO), for 30 minutes at room temperature. After washing with Tris buffer, the Streptavidin Horse Radish Peroxidase (Streptavidin HRP; DAKO) was applied for 30 minutes. AEC substrate chromogen was used to show the immunolabeling. The sections were washed and counterstained with

haematoxylin. Aquamount was used to mount the sections.

In this research a four level grading system has been used to grade the expression of α-SMA and VEGF. The assessment of the tissue slides was divided in distribution and intensity. This system has been used in a previous study of VEGF from Yamaguchi et al.[28]

Cytokeratin has been used to detect the bileducts in the portal tracts of the liver and Von Willebrand Factor has been used to detect endothelial cells in the liver. An artery or vessel in this study was defined by staining VWF and α-SMA positive and its vascular structure. A bile duct in this study was defined by staining Cytokeratin positive and having the structure of a bileduct. Small vessels were defined as the vessels in the portal tract that were smaller then the arteries in the portal tract. There has been counted as many portal tracts as possible per tissue slide.

Results

VEGF immunohistochemistry

VEGF expression was not found in any of the livers of normal dogs (Fig.1). In both dogs with an intrahepatic shunt weak staining of 5% - 33% of the tissue was found (distribution = 1, intensity = 1) (Fig.2). In the tissues of dogs with extrahepatic shunts, two showed no staining for VEGF (distribution = 0, intensity = 0) (Fig. 3), one showed weak staining of an area of less then 5% of the tissue (distribution = 0, intensity = 1). The staining was most probably present in hepatocytes in the parenchyma. Overall, all dogs that did not fully recover from surgery, expressed VEGF in liver tissue.

α-SMA immunohistochemistry

In both normal and affected tissue α-SMA staining was seen in vessels. Almost all tissues but two showed strong staining in the vessels. One tissue sample of a dog with an intrahepatic shunt showed moderate staining, and one sample of a normal dog did not show any staining (Fig.4).

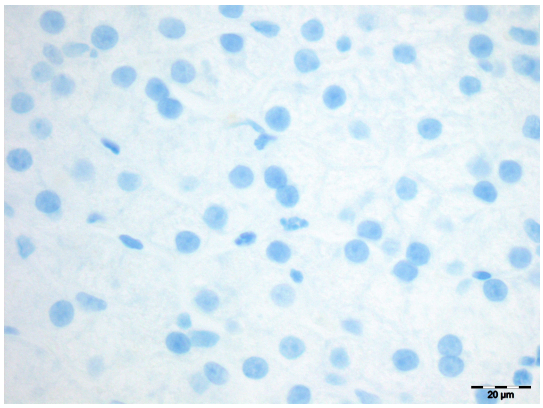

Fig.1 Immunohistochemistry for VEGF in normal dog liver.

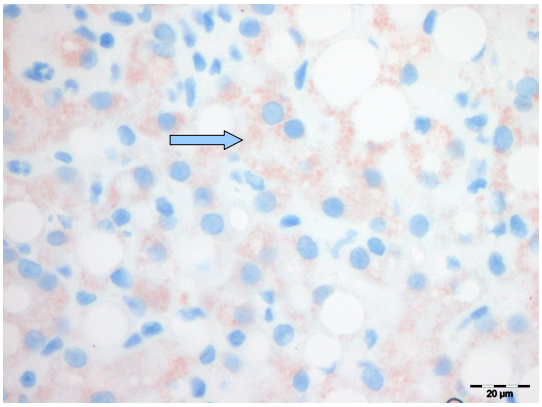

Fig.2 Immunohistochemistry for VEGF in dog liver with an intrahepatic shunt. The arrow shows staining of parenchyma, most likely in the hepatocytes.

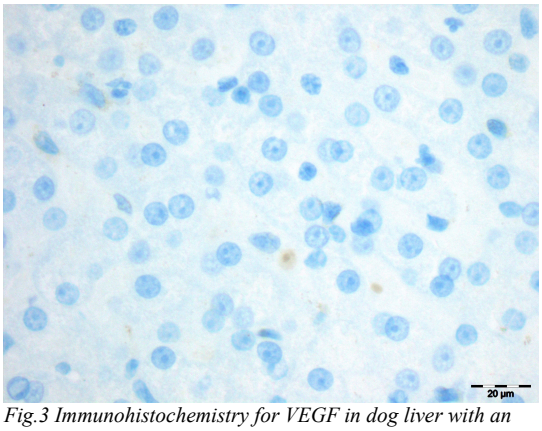

Fig.3 Immunohistochemistry for VEGF in dog liver with an extrahepatic shunt. Expression of VEGF is not seen .

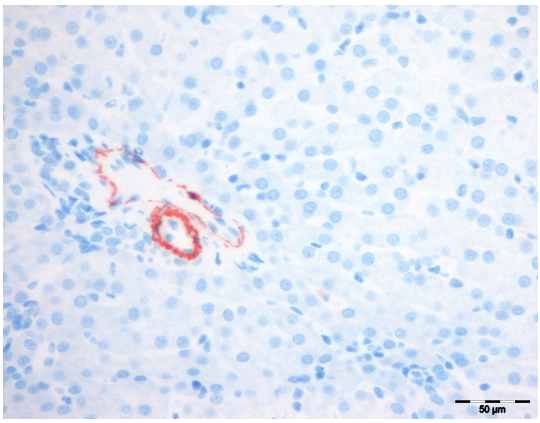

Fig.4 Immunohistochemistry for alpha-SMA in normal dog liver. The walls of the hepatic artery and portal vein in the portal tract are stained positive for alpha-SMA.

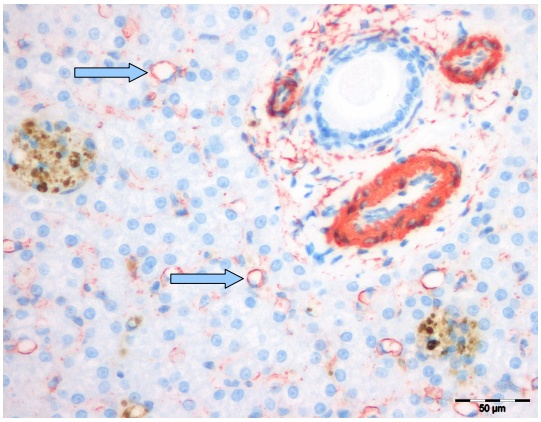

Fig.5 Immunohistochemistry for alpha-SMA in dog liver with an extrahepatic shunt. The arrows show staining of the sinusoid lining. There are multiple hepatic arteries per portal tract and a portal vein is hard to distinguish.

The liver tissue of normal dogs showed slight variation in staining of parenchyma. One sample showed little staining in the parenchyma in a limited area (distribution = 1, intensity = 1), one sample showed no staining in the parenchyma (distribution = 0, intensity = 0) (Fig.4) and one sample did not show any staining on the tissue (distribution = 0, intensity = 0). The staining of parenchyma in affected liver tissue was very uniform. All samples showed light staining of 67% - 100% of the tissue surface (distribution = 1, intensity = 3). There was no difference in the staining of liver tissue between intrahepatic and extrahepatic shunt dogs. In the affected liver tissue the staining was situated at the lining of the sinusoids (Fig.5).

Vascular characteristics

The number of hepatic arteries per portal tract seemed higher in tissue from dogs with CPSS (1,33 – 2,83) than in normal dogs (1 – 1,25). In CPSS liver tissue the portal tracts showed less normal sized portal veins per portal tract (0 – 1) than normal liver tissue (1 – 1.25). Numbers of smaller vessels were variable present in the portal tract, in normal liver as well as in affected liver (Fig.5).

Discussion

VEGF expression in liver parenchyma may be increased in CPSS dogs that did not fully recover after shunt closure. α-SMA seemed to be expressed in higher levels in liver parenchyma of dogs with portosystemic shunts in general. Also proliferation of hepatic arteries and less normal sized portal veins may be present in affected liver tissue.

In this study, tissue samples of eight animals have been used. Although the

findings may not be significant, this study provided good indications for further research.
The tissue samples have been stored in fixative for different periods of time, in some cases more than necessary to fixate the tissue properly. Overfixation can change or destroy tissue properties what could have effect on the findings in this research.[22]
One normal dog had slight α-SMA staining that could be caused by liver disease other than congenital portosystemic shunt, as α-SMA expression can also be increased in dogs with hepatitis, chronic hepatitis or cirrhosis.[18] Some tissue samples contained fewer portal tracts than others, which made the assessment of these samples less reliable.
The staining of VEGF in CPSS patients was weak but corresponded with the staining intensity in control tissue. For this reason it has been considered positive staining.
Tissue of one normal dog did not show any staining of vessels for α-SMA. This sample was not included in the results of α-SMA expression as it should have stained smooth muscle in the wall of blood vessels.

In the present study strong α-SMA staining of blood vessels was found in tissue of normal dogs and CPSS dogs. Tissue of CPSS dogs showed more staining of α-SMA in parenchyma than normal dogs. These findings were similar to the results in another study from Abou-Shady et al. that compared cirrhotic human liver tissue with normal human liver tissue.[1]
Baade et al. concluded that about one third of liver samples from dogs with CPSS showed enhanced immunolabelling and increased numbers of α-SMA-containing perisinusoidal cells. Also most of the CPSS dogs showed proliferation of arterioles and small portal veins.[2] In this study α-SMA expression was higher in CPSS dogs than in normal dogs, the number of arteries was increased and there were less normal sized veins per portal tract in CPSS dogs. So these findings were comparable with the findings of Baade et al.
In dogs with CPSS, blood from the hepatic artery is not obstructed, so part of the oxygenated blood is still delivered to the hepatocytes. Nevertheless in normal liver, 75% - 80% of the total hepatic blood flow is supplied by the portal vein.[15] Arteriolar proliferation is therefore partially a compensatory mechanism in CPSS dogs.
Because VEGF expression was seen in CPSS dogs that have an incomplete response to surgery, and α-SMA expression was higher in CPSS dogs in general, a correlation between VEGF and α-SMA does not seem feasible from this study.
It is questionable if an absence of VEGF is the cause of the development of congenital portosystemic shunts as in complete absence of hepatic VEGF, organogenesis would not take place at all. Because VEGF is overexpressed in some CPSS dogs in this study, there might be a receptor disturbance that obstructs normal development of the hepatic vasculature. VEGF receptors are situated on endothelial cells.[17,21] It would be interesting to perform immunohistochemistry with VEGF-receptor 1 and VEGF-receptor 2 on livers from dogs with CPSS, because there might be an aberrant distribution or functioning of these receptors in endothelial cells of dogs with CPSS that do not respond well to surgical closure.

VEGF was of special interest in this study because there has not been published any research about VEGF in

relation to congenital portosystemic shunts. The results of this study are promising and it seems worthwhile to study more extensively VEGF, VEGF-receptors and other factors that act in relation to VEGF in dogs with CPSS.

Aknowledgements

First I would like to thank my supervisor of Utrecht University Dr. Anne Kummeling. Through her contacts and previous study, it was possible to travel to Australia and start this study. She made me excited with her stories and information. She has been incredibly patient and helpful during this research period.

The second person I would like to thank is Dr. Geraldine Hunt from Sydney University. Her ideas and study design made it possible to study this subject under her supervision in Australia. She showed me around at Sydney University and introduced many people who turned out to be so helpful in the course of this study. When there were difficult moments, she gave tools to be able to continue.

I would like to thank a very important person, Drs. Emine Korkmaz. Her endless patience, permanent willingness to help and clarify the processes and equipment and her friendship, made it possible to finish this study with good results.

Also Dr. Philip Breat has given his full effort. He made it possible to use equipment and get support from staff in his department. In a country where everyone talks English, he was the one I could talk Dutch/Belgium to and feel close to home again. I would like to thank him for all the help and laughs he gave me.

Dr. Mark Krockenberger was very important in understanding and executing the immuno-histochemical processes. I want to thank him for his explanations and patience. They were essential for understanding, interpreting and accomplishing this study.

At last I would give my special thanks to my fellow-student, travel-mate and most of all, my friend, Drs. Kirsten Peeters. I am thankful to have been able to share this experience with her. It was an incredible period with unforgettable memories. We discovered Thailand, Sydney and Australia together, but I would travel the whole world with her. I would like to thank her for her twenty-four hour support, the great experience and for just being my good friend.

References

1. Abou-Shady M, Friess H, Zimmermann A, di Mola FF, Guo XZ, Baer HU, Buchler MW: "Connective tissue growth factor in human liver cirrhosis". *Liver,* 20, 296-304, 2000.
2. Baade S, Aupperle H, Grevel V, Schoon HA: "Histopathological and immunohistochemical investigations of hepatic lesions associated with congenital portosystemic shunt in dogs". *J.Comp.Path*., 134, 80-90, 2006.
3. Budny T, Palmes D, Stratmann U, Minin E, Herbst H, Spiegel HU: "Morphologic features in the regenerating liver – a comparative intravital, lightmicroscopical and ultrastructural analysis with focus on hepatic stellate cells". *Virchows Arch,* 451, 781-791, 2007.
4. Byun JH, Park BW, Kim JR, Lee JH: "Expression of vascular endothelial growth factor and its receptors after mandibular distraction osteogenesis". *Int. J. Oral Maxillofac. Surgery,* 36, 338-344, 2007.
5. Coultas L, Chawengsaksophak K, Rossant J: "Endothelial cells and VEGF in vascular development". *Nature,* 438, 937-945, 2005.
6. Deckers MML, Karperien M, van der Bent C, Yamashita T, Papapoulos SE, Löwik CWGM: "Expression of vascular endothelial growth factors and their receptors during osteoblast

differentiation". *Endocrinology,* 141:5, 1667-1674, 2000.

7. Ferrara N, Davis-Smyth T: "The biology of vascular endothelial growth factor". *Endocrine Reviews,* 18:1, 4-25, 1997.
8. Grevel V, Schmidt S, Lettow E, Suter PF, Schmidt GU: "The congenital portosystemic shunt in dogs and cats". *Tierarztl Prax.*, 15:2, 185-194, 1987.
9. Hosoya A, Nakamura H, Ninomiya T, Yoshiba K, Yoshiba N, Nakaya H, Wakitani S, Yamada H, Kasahara E, Ozawa H: "Immunohistochemical localization of alpha-smooth muscle actin during rat molar tooth development". *J. Histochem. Cytochem.,* 54:12, 1371-1378, 2006.
10. Hunt GB, Hughes J: "Outcomes after extrahepatic portosystemic shunt ligation in 49 dogs". *Aust.Vet.J.,* 77:5, 303-307, 1999.
11. Hunt GB, Tisdall PLC, Webb A, MacPherson GC, Brain P, Malik R: "Congenital portosystemic shunts in toy and miniature poodles". *Aust.Vet.J.,* 78:8, 530- 532, 2000.
12. Hunt GB, Kummeling A, Tisdall PLC, Marchevsky AM, Liptak JM, Youmans KR, Goldsmid SE, Beck JA: "Outcomes of cellophane banding for congenital portosystemic shunts in 106 dogs and 5 cats". *Veterinary Surgery,* 33, 25-31, 2004.
13. IJzer J, Roskams T, Molenbeek RF, Ultee T, Penning LC, Rothuizen J, van den Ingh TSGAM: "Morphological characterisation of portal myofibroblasts and hepatic stellate cells in the normal dog liver". *Comparative Hepatology,* 5:7, 2006.
14. Kummeling A, van Sluijs FJ, Rothuizen J: "Prognostic implications of the degree of shunt narrowing and of the portal vein diameter in dogs with congenital portosystemic shunts". *Veterinary Surgery,* 33, 17-24, 2004.
15. Kurosawa M, Enomoto K, Aikawa Y, Yoneda M: "Hepatic blood flow responses to mechanical stimulation of the skin in anaesthetised rats". *Autonomic Neuroscience*, 99:1, 40-46, 2002.
16. Maddison JE: "Canine congenital portosystemic encephalopathy". *Aust. Vet. J.,* 65:8, 254-249, 1988.
17. Matsumoto K, Yoshitomi H, Rossant J, Zaret KS: "Liver organogenesis promoted by endothelial cells prior to vascular function". *Science,* 294, 559-563, 2001.
18. Mekonnen GA, IJzer J, Nederbragt H: "Tenascin-C in chronic canine hepatitis: immunohistochemical localization and correlation with necro-inflammatory activity, fibrotic stage, and expression of alpha-smooth muscle actin, cytokeratin 7, and CD3+ cells". *Vet. Pathol.*, 44:6, 803-813, 2007.
19. Nelson RW, Couto CG: "Small animal internal medicine". Mosby, ISBN 0-323-01724-X, 520-521, 531-533, 2003.
20. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z: "Vascular endothelial growth factor (VEGF) and its receptors". *FASEB journal*, 13, 9-22, 1999.
21. Nikolova G, Lammert E: "Interdependent development of blood vessels and organs". *Cell Tissue Res.,* 314, 33-42, 2003.
22. Ramos-Vara JA: "Technical aspects of immunohistochemistry". *Vet. Pathol.,* 42, 405-426, 2005.
23. Restucci B, Papparella S, Maiolino P, de Vico G: "Expression of vascular endothelial growth factor in canine mammary tumors". *Vet. Pathol.,* 39, 488-493, 2002.
24. Restucci B, Maiolino P, Paciello O, Martano M, de Vico G, Papparella S: "Evaluation of angiogenesis in canine seminomas by quantitative immunohistochemisty". *J. Comp. Path.,* 128, 252-259, 2003.
25. Risau W: "Mechanisms of angiogenesis". *Nature*, 386, 671-674, 1997.
26. Skalli O, Pelte MF, Peclet MC, Gabbiani G, Gugliotta P, Bussolati G, Ravazolla M, Orci L. "Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes". *J. histochem. cytochem.,* 37:3, 315-321, 1989
27. Washizu M, Katagi M, Washizu T, Torisu S, Kondo Y, Nojiri A: "An evaluation of radiographic hepatic size

in dogs with portosystemic shunt". *J. Vet. Med. Sci.,* 66:8, 977-978, 2004.

28. Yamaguchi R, Yano H, Nakashima O, Akiba J, Nishida N, Kurogi M, Kojiro M: "Expression of vascular endothelial growth factor-C in human hepatocellular carcinoma". *Journal of gastroenterology and hepatology,* 21, 152-160, 2006.
29. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J: "Vascular-specific growth factors and blood vessel formation". *Nature*, 407, 242-248, 2000.