Characterization of hepatic steatosis in dogs with congenital portosystemic shunts

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Abstract

Objective: To quantify the difference in fat percentage and lipid vesicle sizes between congenital portosystemic shunt (CPS) dogs and control dogs. To determine if there is any suggestion for macrovesicular steatosis to be associated with poor outcome following surgery.
Study Design: Longitudinal prospective study.
Animals: Ten dogs with CPS, one dog with multiple acquired portosystemic shunts, three healthy dogs.
Methods: Wedge or punch biopsy samples were taken during surgery or necropsy, slices were stained with Oil Red O (ORO) staining. 5 randomly chosen fields were taken from each slide and graded for fat percentage, subdivided into classes of vesicle sizes and for lipogranulomas. Correlations between fat percentage, macrovesicular steatosis, outcome and age were statistically analyzed.
Results: 6 out of 10 CPS dogs had an unfavorable outcome. Fat percentage in CPS dogs appeared higher (Mean=25.3, SD=20.1) when compared to healthy dogs (mean=3.2, SD=2.1) although this difference was not significant (p=0.06). There appeared to be a trend towards macrovesicular steatosis being associated with a high total fat percentage: 32% with macrovesicular steatosis, and 15% without macrovesicular steatosis.
Conclusion: Fat percentage was quite abnormal in CPS dogs when compared to healthy dogs, although this difference was not significant. Using a larger number of dogs could solve this matter. All control dogs showed only small and evenly distributed fat vesicles. For CPS dogs there was broad range of fat percentage macro- or microvesicular steatosis and distribution. Fat percentage did not increase with age. A larger study has to be conducted to determine if macrovesicular steatosis predicts outcome after treatment of CPS.
Clinical relevance: To determine if macrovesicular steatosis predicts outcome after treatment of CPS can be valuable to select treatment options.

Introduction

A congenital portosystemic shunt (CPS) is a vascular anomaly that diverts the portal blood past the liver, into the systemic veins. This leads to an impaired hepatic development because of a decrease in hepatic oxygen supply, nutrients and a number of hepatotrophic substances, such as insulin. Another, direct effect of the decreased hepatic circulation is the high level of toxins, hormones, and nutrients that bypass the liver into the systemic circulation.[1-3]

CPS is most frequently found in dogs and less commonly in cats, but is also described in other mammalian species as well as in humans.[1-2] In dogs, congenital portosystemic shunts may have a genetic component because various studies describe a predisposition in some breeds. In a study by Tobias et al. in Tennessee (US; 2003), breeds such as Yorkshire Terriers, Havanese, Maltese, Dandie Dinmont Terriers and pugs were predisposed to CPS.[4-5] In a study by Hunt et al. (2004) that studied dogs presented at the University Veterinary Centre in Sydney, Australian Cattle Dogs, Maltese, Silky Terriers, Bichon frisees, Shih Tzu, Irish Wolfshounds, Schnauzers, Jack Russell Terriers and Border Collies were significantly over-represented.[6-7] Congenital portosystemic shunts have proven to be heritable in Irish Wolfhounds[8-9] and Cairn Terriers[10] in the Netherlands.

Differences in prevalence of CPS in populations can result from genetic differences between populations. These differences could be explained by (unwanted) selection during breeding, for example in case of growing popularity of certain breeds. This could also explain the increasing prevalence of CPS that is described in some papers.[5,9-10]

In general, small breeds are predisposed to extrahepatic shunts, and large breeds are predisposed to intrahepatic shunts.[1,6] Extrahepatic shunts can be congenital or acquired. Congenital extrahepatic shunts are usually single, linking a branch of the portal venous system with the caudal vena cava or azygos vein, although many variations have been reported.[11] Extrahepatic shunts can arise from the splenic vein, left gastric vein, the cranial or caudal mesenteric vein and the gastroduodenal vein.[1-2,11] Acquired shunts, also called portosystemic collaterals, are usually multiple veins that arise due to chronic portal hypertension. Portal hypertension can be caused by hepatic cirrhosis, microvascular dysplasia or portal vein dysplasia.[7] Also congenital intrahepatic shunts are usually single veins. They arise from intrahepatic portal branches, and usually insert into the intrahepatic part of the caudal caval vein or a hepatic vein. They can be divided into left, medial and right divisional shunts, depending on their location. The left sided intrahepatic shunt probably results from failure of the ductus venosus to close.[11]

CPS's are primarily diagnosed in younger patients; dogs with intrahepatic shunts are diagnosed at a relatively young age and dogs with portazygous shunts tend to be diagnosed later in life. Specific symptoms include hepatic encephalopathy but less specific signs, such as ill thrift and anorexia, lethargy, hypersalivation, vomiting, decreased endurance and urolithiasis (ammonium urates) are commonly seen as well. In the majority of patients, signs appear to be episodic.[1,6]

Serum biochemical perturbations suggestive of CPS include a high fasting ammonia concentration, decreased rectal ammonium tolerance and elevated bile acids. High fasting ammonia and ammonium tolerance testing are relatively sensitive and specific for CPS although elevated ammonia may also be related to a fulminant hepatitis or inborn errors of metabolism in the urea cycle. Bile acids, on the other hand are less specific and elevated levels may indicate any parenchymal or biliary liver disease with cholestasis. Especially in liver patients, specificity for CPS drops significantly. However, because of its easy use and higher specificity in healthy patients, it is still suitable for screening purposes.[12-14]

Ultrasonography and transcolonic scintigraphy can be used to visualize portosystemic shunts, and are both relatively sensitive and specific. Transcolonic scintigraphy can especially be valuable to determine shunt closure postoperatively, as it enables comparison with the preoperative shunt fraction.[15-17] Mesenteric portography on the other hand, is reliable but more invasive and technically demanding.[16-17] Additionally, contrast-enhanced CT offers minimally invasive measures that allow visualization or three-dimensional reconstruction of the shunt, which can be beneficial for surgical planning. It has indeed been reported that CT visualization of shunts before surgery can reduce the required amount of dissection and thereby reduce required surgery time and intraoperative complications.[18-19]

Different therapies have been developed over the years. Surgery is preferable to dietary measures, like a restricted-protein diet, which have proven to provide unsatisfactory survival times.[1] Surgical techniques include a single suture of non-absorbable material (polyester, prolene or silk), ameroid constrictors and cellophane banding. Minimally invasive intravascular methods such as intravascular thrombogenic coils have been reported in small series of cases.[20, 21]

Rapid and complete closure of a shunt has been shown to provoke several adverse effects. Adverse effects can be associated with congestion of the liver, neurologic dysfunction[22] and

an increased bleeding tendency, which appears to be associated primarily with CPS rather than the closure of the shunt itself.[23] Because of the possible complications of ligation, a gradual constriction, rather than an immediate closure is advised.[20] The ameroid constrictor and cellophane banding both provide a gradual constriction. A study by Youmans et al. (1999) revealed that the ameroid constrictor may constrict the shunt vessel too fast by stimulating thrombosis within two weeks of placement, and with an additional risk of kinking of the vein due to the weight of the implant.[21] Development of multiple acquired portosystemic shunts, presumably because the shunt closes more rapidly than the hepatic portal circulation is able to develop, has been reported.[24] Cellophane banding on the other hand, provides slow occlusion, thus representing a safe alternative.[20-21]At the same time there is evidence suggesting that results of ameroid ring placement and cellophane band placement are comparable. [21,25-28]

Extrahepatic CPS are reported to have a better prognosis than intrahepatic CPS, although some of this difference in outcome may be attributed to the fact that intrahepatic shunts are technically more demanding and require more intensive postoperative management. Nevertheless, there is also good evidence that the biological behavior of intrahepatic shunts is intrinsically different.[6,20,29] Dogs with shunts that allow complete ligation, without a rise in baseline portal pressure of more than 10 cm H_2O, tend to have a better prognosis than dogs that require partial ligation.[30] The capacity of the portal vein to expand is important for the possible degree of shunt narrowing. Shunt constriction in absence of sufficient portal vein development can lead to splanchnic pooling resulting in a dramatic drop in central venous pressure.[31] Additionally, complete ligation is contraindicated because partial closure in time spontaneously leads to a complete functional closure in most shunts, making complete ligation an unnecessary risk.[29,32-33]

Recently published studies evaluating ameroid ring constrictors, cellophane bands and silk sutures show different results. Failure to regain normal liver function ranged between 10 and 20%, and short-term mortality within one month after surgery ranged from 5.5 % to 27%. Differences in clinical recovery or mortality may be due to the use of different populations, the criteria for euthanasia, the experience of the surgeon with shunt operations and possibly the use of different shunt ligation techniques. [20,28,33]

Post-operative clinical recovery and the accompanying liver growth can occur relatively fast.[34] However, portal hypertension with resulting persistent shunting or new acquired shunts, may occur after initial shunt closure. This is thought to be a result of poorly developed portal vasculature in the face of rapid and complete shunt closure. Measurement of portal pressure and monitoring of the color and motility of the intestines after initial closure are therefore advised.[20,26,28-31,33,35]

Many attempts have been made to identify preoperative prognostic indicators for hepatic regeneration, as this seems integral to the success of the surgical procedure in any given patient. Preoperative parameters associated with poor prognosis are leukocytosis, low plasma protein, hypoalbuminemia and a high BUN.[28,35] Another parameter that was found to be associated with a favorable short-term prognosis is weight. However, results differ between different studies. A study by Papazoglou et al., which focused on intrahepatic shunts, found that high weight was associated with a favorable prognosis. They argued that the bigger a shunt, the more atrophied the liver, which has a negative effect on growth. Additionally, surgery on small diameter shunts is usually less risky, because of a decreased risk of portal hypertension and therefore less formation of acquired portosystemic shunts.[35] On the other

hand, a study by Wolschrijn et al. (2000) looked into congenital portosystemic shunts regardless of breed. They reported that large breed and high weight seem to be a negative prognostic indicator. Large breeds are prone to intrahepatic shunts and intrahepatic shunts have a less favorable prognosis. This can explain high weight being a negative prognostic indicator in this study.[28-29]

During surgery, ultrasonography can add valuable information; post-ligation hepatopetal flow in the portal vein is a positive prognostic marker.[36] Metabolic recovery monitoring can be performed by means of ammonia tolerance testing.[12-14] Furthermore; postoperative absence of scintigraphically measured shunting seems correlated with a favorable prognosis,[17] although the shunted fraction may consist of splenic venous blood which has no impact on the animals health.[37] It has previously been argued that the scintigraphically measured shunt ratio may be useful as a prognostic marker. In a recent study by Samii et al. (2001), it was suggested that the difference between observers was too big to make shunt fraction measurement reproducible, and therefore making scintigraphically measured shunt fractions useless as a prognostic tool.[38]

Another prognostic tool is liver volume measurements with CT and MRI. These methods have been studied in order to evaluate liver volume expansion after surgical attenuation. A significant increase of liver volume measured with CT or MRI was found postoperatively after shunt attenuation, but a significant correlation between a postoperative liver volume increase and outcome has not been found. CT appears to provide the most exact estimate of liver volume, because its fast exertion allows breath holding.[34] With the addition of recently determined conversion factors in human medicine a metabolic liver volume measurement with up to only 1.2% error is possible.[39-40]

Liver biopsies may provide additional information to predict prognosis. Histological parameters with a possible impact on either long or short-term survival could be the abnormalities frequently seen in dogs with CPS. Abnormalities include defects in the normal architecture of portal tracts and hepatic venules, fibrosis, an increased amount of ceroid or "wear and tear pigment" in Kupffer cells and elevated numbers of lipogranulomas. All these perturbations of the normal histology might have an impact on prognosis, although previous studies in dogs have failed to show a significant correlation.[41] Additionally, hepatic steatosis has been found in dogs with CPS, but its impact on prognosis has not yet been determined.

Pathologists make a distinction between macro- and microsteatosis, but the distinction can be relatively subjective. The difference is based simply on size of the vacuoles in relation to the cell nucleus, but the significance of vacuole size is unclear.[42] Steatosis in general means an increased fat content in liver cells and usually indicates some form of hepatic insult, for example resulting from a lack of insulin and glucagon in the liver.[3] Visualization of these lipids can be achieved by means of specific lipid stainings such as Oil red O (ORO). Lipid-specific staining is required to distinguish between microvesicular steatosis and glycogen accumulation, as neither can be definitely diagnosed with H&E staining.[41] The disadvantage of using non-specific stainings is that the detection of steatosis is less accurate and might cause an under or overestimation of hepatic fat content.[42] Periodic Acid Schiff staining is also useful to preferentially stain glycogen and distinguish it from other non-lipid substances that might be accumulating in small vacuoles. [43]

Various efforts have been made to make the assessment of histologic liver changes less subjective, for example by means of grading systems. Histologic grading systems are designed to evaluate various relevant features of liver injury. An example of a grading system designed for non-alcoholic steatohepatitis in man, was designed by Brunt et al. (1999) Necro-inflammatory grading was performed defining three categories: mild, moderate and severe. This grade was composed of different variables such as ballooning of hepatocytes, inflammation and steatosis, which appeared to be correlated with one another. Some other variables like glycogen and lipogranulomas appeared to have no significant correlation with other variables and were therefore excluded from the grading system. A separate grading system was made to evaluate fibrosis.[44]

Another tool to objectify a histologic feature is a computer assisted image analysis system. Computer analysis has been used to determine the amount of steatosis, although this has mainly been used for research purposes, because it is technically more demanding than the aforementioned grading systems. An analysis of Turlin et al. using Image Pro Plus (Media Cybernetics, Inc., Bethesda, USA) shows that there is a strong correlation between slides analyzed with a grading system and slides analyzed by means of computer assisted analysis. However, more research needs to be done to quantify intra- and inter-observer variation, which could reveal inconsistencies when comparing different observers. Computer assisted image analysis could eliminate this difference, making comparison of different studies possible.[45] The human eye can provide more accurate information than computer analysis can, especially for features that do not always follow a predictable pattern of distribution, size or staining intensity. This makes computer randomized point counts the ideal solution. (Personal communication with Dallas Hyde)

In human medicine, the awareness that steatosis is a serious condition, rather than an insignificant coincidental finding, is growing.[46] However, in previous studies in dogs, no significant correlation between the aforementioned histological parameters and prognosis has been identified. A possible cause could be that most of the previous studies had a small number of patients and no samples were taken to monitor recovery of hepatic function.[47-48] Furthermore, special stains for lipid were not routinely used and presence of lipid was surmised based on presence of vacuoles within the hepatocytes. Additional difficulties in proving a correlation can arise for several reasons. Sample size and sample variation can be a problem if the obtained sample is too small. Some lesions tend to have a uniform distribution throughout the liver, such as inflammation, necrosis, toxic cell damage and steatosis. Several other parameters, such as fibrosis tend to have an uneven distribution, which makes estimation based on small or superficial liver samples difficult.[41] Additionally, it appears the combination of different indicators of liver injury, rather than one indicator alone is more likely to have a significant impact on prognosis. There is a wide variation between CPS patients in the type of lesions they demonstrate, and some types of lesions can progress into others. Fatty livers for example are extremely vulnerable to oxidants, inducing a steatohepatitis. Secondary insults produce even more oxidants, which causes cell death and inflammation leading to fibrosis and ultimately cirrhosis.[46] There is an increasing amount of evidence that fatty infiltration in the human liver impairs regeneration of the liver after use of steatotic liver tissue for transplantation and this leads us to consider that steatosis could be an important factor for hepatic growth in shunt dogs. If livers with severe fatty infiltration of more than 30% steatotic hepatocytes were used for human transplantation, the risk of primary dys- or nonfunction of the graft was significantly higher. It is now considered essential to evaluate livers histologically for fatty infiltration before using them as donors.[42,49-53]

A likely risk of primary dys- or nonfunction or rejection of a graft is the result of impaired hepatocellular growth. Growth of hepatocytes takes place after hepatocellular damage. If hepatocytes fail to replace the damaged cells, oval cells will start dividing and replacing the lost hepatocytes. Oval cells are a population of liver cells that exist near the portal areas and replace damaged liver cells that are no longer able to duplicate. Apoptosis of oval cells can be triggered by various cytokines released by Stellate and Kupffer cells, which are activated through inflammation, and may hereby impair the regenerative capacity of the liver.[54] Another possible mechanism causing a lack of regeneration is because of degeneration of cells, disrupting future hepatocellular growth. Cell degeneration in steatosis involves mitochondrial dysfunction. This is caused by a disruption of mitochondrial β-oxidation and respiration and accompanying DNA damage.[55] Although various mechanisms for impaired hepatocellular growth have been established, the precise mechanisms playing superior or inferior roles in impaired hepatocellular growth in CPS are still unknown.

With regard to the aforementioned effects of steatosis on the hepatic ability to regenerate, the main aims of this study are as follows:
1. To determine whether steatosis is more common in dogs with congenital portosystemic shunts than in healthy control dogs, and whether the degree of steatosis increases with age in healthy and shunt dogs.
2. To evaluate the distribution and type of lipid droplet accumulation.
3. To determine whether there is any trend for macrovesicular steatosis to be associated with poor outcome following surgery.

Ultimately this pilot project will be expanded into a larger study and the results used to develop better selection criteria for the choice of therapy and new tools to estimate the chances of success based on liver biopsy, as well as determining whether adjunctive treatments aimed at facilitating liver proliferation may be helpful in a certain group of patients.

Materials and Methods

Animals:
Liver biopsies were taken from 10 dogs undergoing surgery for congenital portosystemic shunts between 4-23-2009 and 7-28-2010 at the Veterinary Medical Teaching Hospital in Davis, California. Three young and healthy dogs surrendered for euthanasia by a local shelter were used as control group. Each of these dogs was examined by necropsy, to verify there were no obvious pathologic abnormalities. For the clinical patients, name, breed, sex, date of birth and date of surgery were recorded. Pre-operative liver enzymes such as ALT, AST, ALP and GGT were recorded. Shunt type: intrahepatic or extrahepatic, single or multiple shunts, shunt location and the sites of liver biopsy were recorded. The type of ligation used was at the discretion of the surgeon. Ligation type, including size for ameroid constrictor or cellophane band was recorded. Post-operative parameters like the result of technetium scans, bile acids, liver enzymes and clinical improvement were recorded. One dog, submitted for a second operation after persistent portosystemic shunting, had developed multiple acquired portosystemic shunts. Liver punch biopsies were taken and the abdomen was closed without further shunt attenuation.

Samples
Wedge or punch biopsy samples were taken, depending on the choice of the surgeon. A 4 or 6 mm disposable biopsy punch (Miltex Inc., York, USA) was used. Wedge biopsies were taken using standard procedures. Samples were taken from the right medial, quadrate, and left medial lobe in cases where the surgeon deemed the liver to be of sufficient size to tolerate multiple biopsies. This enabled comparison of histological changes between the right, central and left divisions of the liver. When liver size did not permit taking multiple samples only the central division was biopsied. Six mm diameter biopsies were also taken shortly after death from the right, central and left divisions of the control dogs, by means of a ventral midline celiotomy.

Each biopsy sample was cut lengthwise into two equal pieces. Each piece was thereafter prepared, according to the required staining: One section from each sample was placed into a Tissue tek® (Sakura Finetek USA Inc., Torrance, USA) container which was then filled with Tissue tek® OCT™ compound gel (Sakura Finetek USA Inc., Torrance, USA). The containers were then placed in a cup filled with 2-methylbutane and frozen by lowering the cup into liquid nitrogen. The frozen samples were placed into a -80 C° freezer, in order to preserve the samples for further processing. The samples were cut with a cryostat microtome into 7 μm slices, and then stained for lipid using standard Oil red O protocols.

Pathological analysis:
Each slide was attributed a random number, to blind the observer to any patient information. Calibration of microscope ocular units was achieved using a 0.01 mm micrometer slide.

Fat scoring was limited to hepatocytes and was determined as red staining in the ORO slides, excluding the red staining in bile duct epithelial cells.

Each section was evaluated using a grading system for hepatic fat percentage and distribution of vacuoles of different sizes (<2.5μm, 2.5μm-7.5μm and >7.5μm).

The percentage of affected hepatocytes is an estimate of the hepatocytes filled with lipid droplets of either <2.5µm, 2.5µm-7.5µm or >7.5µm (A, D and G in table 1). The volume percentage per affected cell is an estimate of the volume occupied by lipid in this group of cells (B, E and H in table 1). The volume percentage per vesicle size (C, F and I in table 1, meaning the volume percentage of the total volume) was calculated as the product of the percentage of affected hepatocytes and the average volume percentage per vesicle size. The total volume percentage (J in table 1) was calculated as the sum of the volume percentages per vesicle size.

Table 1. *The grading system used to estimate macro and microvesicular steatosis. Slices containing lipid droplets of <7,5 µm where graded as macrovesicular steatosis*

Size of vesicles	*<2.5µm*	*2.5µm-7.5µm*	*>7.5µm*
Percentage of affected hepatocytes	A	D	G
Fat percentage per hepatocyte	B	E	H
Fat percentage per vesicle size	C=AxB	F=DxE	I=GxH
Total fat percentage	J=C+F+I		

Five high-powered fields were semi-randomly selected (by scrolling the x and y plane without looking at the slide). The average of these five high-powered fields was calculated (see appendix 1).

The minimal size of fat droplets to classify an animal as positive for macrovesicular steatosis was arbitrarily determined as vesicles larger than 7.5 µm, approximating the average size of the nucleus of the hepatocytes. Lipogranulomas were measured in the same five fields, counting the number of lipogranulomas per high-powered field, and afterwards calculated as an average per five high-powered field.

Outcome definition:
An unfavorable outcome was specified as a positive technetium scan, the finding of elevated liver enzymes or bile acids six weeks postoperatively. A favorable outcome was defined as all parameters within normal limits.

Statistical analysis:
SPSS19 (IBM, Armonk, USA) was used for statistical analysis and the preparation of graphs. The independent samples Mann-Whitney U test was used to determine if there was a difference in total fat percentage between shunt dogs and controls. Additionally this test was used to determine if there is a difference in age distribution between dogs with a favorable or unfavorable prognosis.

The two-tailed Spearman's rho test was used determine a correlation between age and total fat percentage and between fat percentage and liver enzymes.

P-values smaller than 0.05 were considered significant.

Results

10 dogs had extrahepatic shunts and one was intrahepatic. Six extrahepatic shunts were portocaval and four were portazygous. The one intrahepatic shunt dog that was re-submitted for persistent shunting following prior attenuation had multiple acquired portosystemic shunts. Age of the portocaval shunt dogs at the time of surgery ranged between 5 and 15 months (median 9 months), whereas age of portazygos shunt dogs was between 6 months and 60 months (median 21.5 months). One shunt dog had portal vein atresia and the shunt could therefore not be attenuated.
An ameroid constrictor (5mm wide) was used in 4 dogs and cellophane was used in 6 dogs. 6 dogs had an unfavorable outcome, based on postoperative technetium scans and liver enzymes.

None of the control dogs had any obvious pathological abnormalities observed at necropsy.

Histomorphological analysis

Control dogs
Hepatic fat percentage did not vary much between control dogs, and never exceeded 5%. The lipid droplets were invariably small and evenly distributed. None of the control dogs showed fat vesicles larger that 2.5µm (Figure 1 and 2).

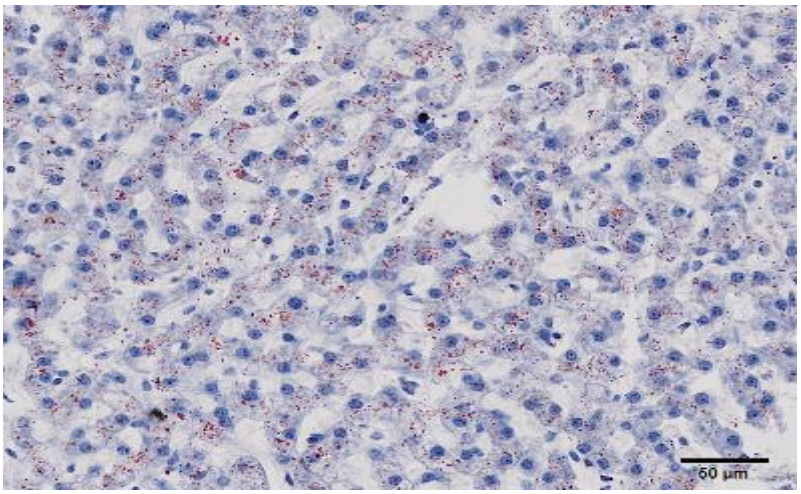

Fig. 1:

A low percentage of small and evenly distributed fat vesicles in a healthy control dog.

(Dog nr. 3, Table 1)

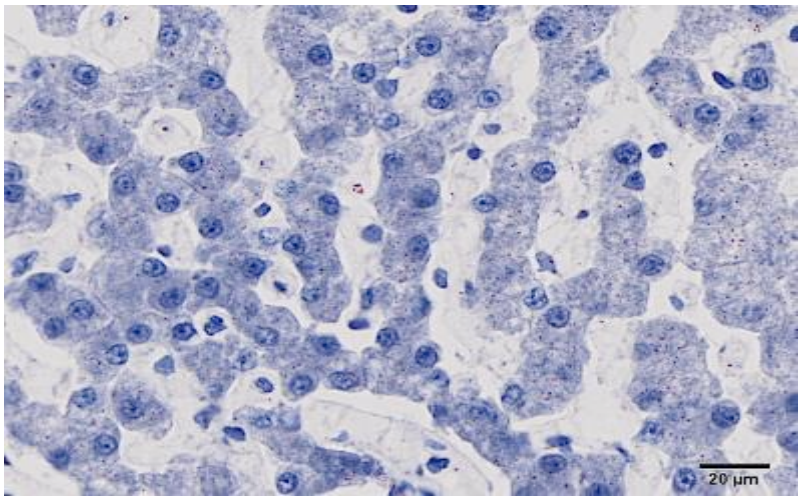

Fig. 2.

A low fat percentage in a healthy control dog

(Dog nr. 3, Table 1)

Shunt dogs

In contrast to the control dogs, there was a considerable variation in hepatic fat content, distribution and the size of fat droplets in dogs with congenital portosystemic shunts. Some showed only a small number of evenly distributed small vesicles, with a relatively low fat content, while others had a high hepatic fat content, with hepatocytes containing small vesicles, and others containing large vesicles, while many dogs expressed all recorded sizes of fat droplets.

There appears to be a distribution in size of the observed vesicles, with a tendency towards macro, or microvesicular steatosis, rather than only one of the two in a single patient. It was even possible to find vacuoles of different vesicle sizes in single cells throughout the liver, rendering classification of either microvesicular or macrovesicular steatosis impossible in some CPS cases.

In some dogs we found cells displaying obvious microvesicular steatosis while at the same time they had a very high fat percentage This feature was evenly distributed throughout hepatocytes, as shown in figure number two. (Figure 3.)

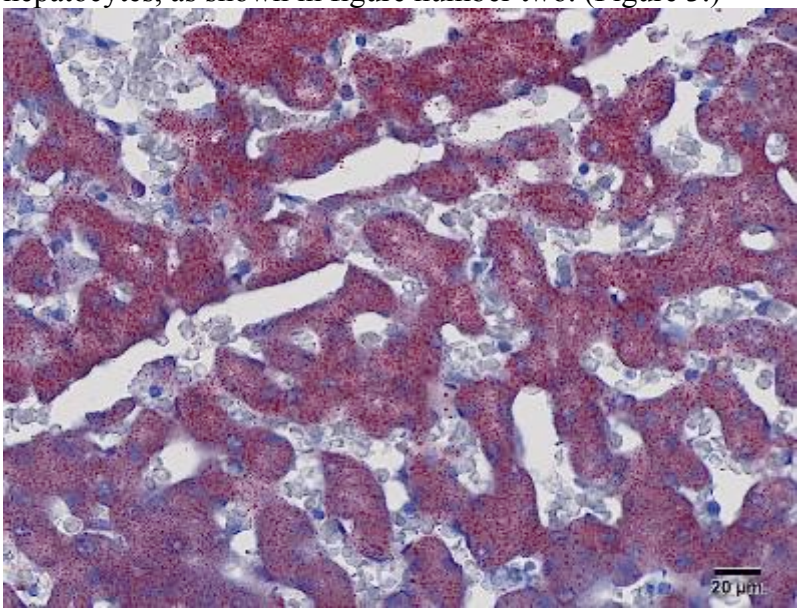

Fig. 3.

Shunt dog showing marked lipid accumulation within hepatocytes, without any obvious macrovesicular steatosis. This dog recovered completely after surgery.

(Dog nr. 6, table 1.)

Other dogs contained less fat than dog nr 6, but displayed obvious macrovesicular steatosis.

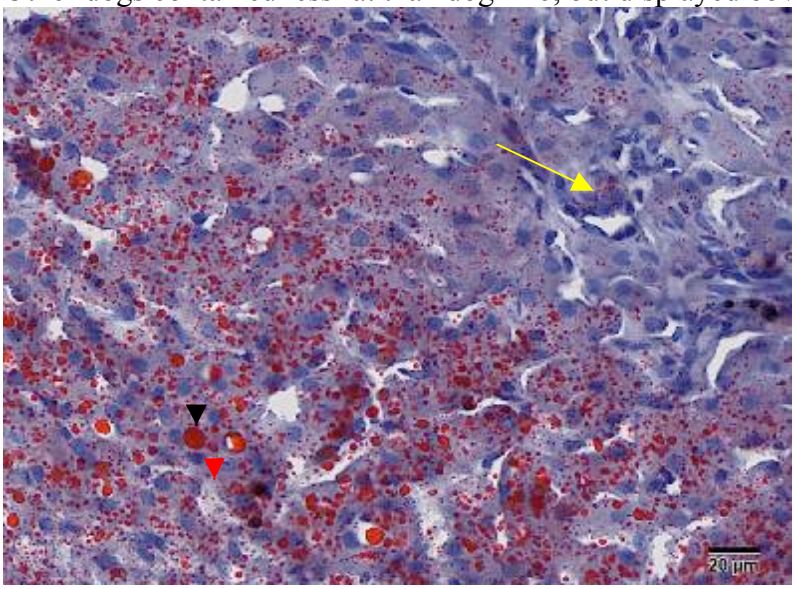

Fig. 4.

Micro- and macrovesicular steatosis in a CPS dog. Note the uneven distribution of both large and small vesicles throughout the hepatocytes. Portal regions (arrow) are less affected with both large and small vesicles. This dog had a favorable recovery and a negative post-operative Tech scan. Large fat vesicles (black arrowhead) can be seen, pushing the nucleus aside (red arrowhead)

(Dog nr. 7 table 1.)

Acquired shunts after CPS operation
We found a relatively high degree of cirrhosis when examining at low-magnification.

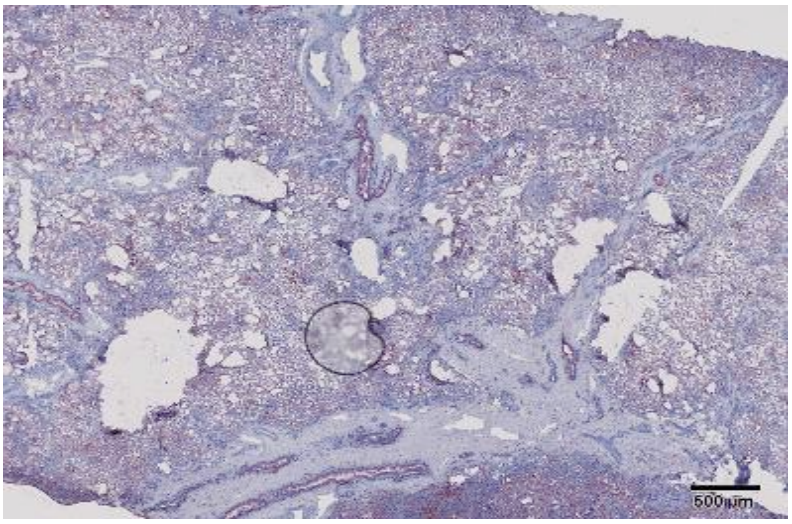

Fig. 6.

Liver sample of a dog with multiple acquired shunts. There is a distinct proliferation of bile duct, portal venules and arteries and fibrosis is apparent in the portal area's

(Dog nr. 14 table 1.)

At higher magnification we found a relatively low hepatic fat percentage (9.1%). The hepatocytes expressed a mild degree of macrovesicular steatosis. Additionally we found a considerable amount of bile duct proliferation.

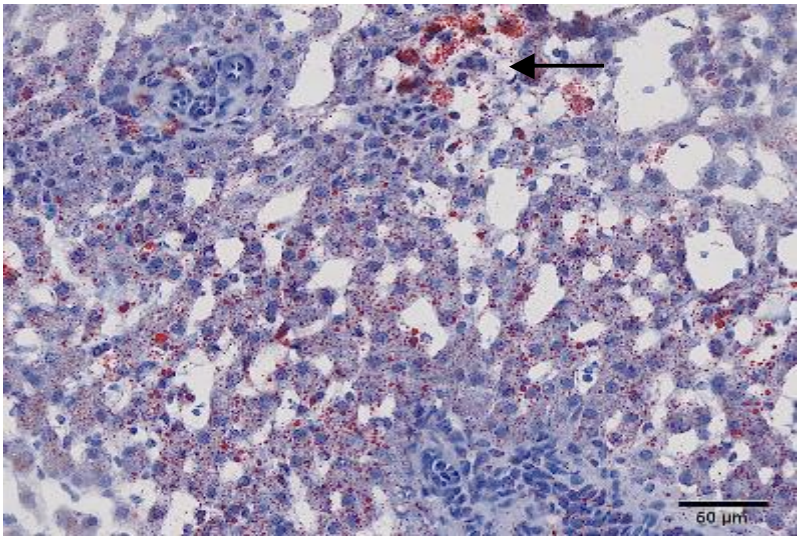

Fig. 5.

Slide of the same dog as Fig 4, showing a relatively low fat percentage with evenly distributed fat vesicles with considerable variation in size. A small poorly circumferenced lipogranuloma, containing pigment is visible (arrow)

(Dog nr. 14 table 1.)

Statistical analysis

All obtained data is summarized in table 2.

dognr	breed	healthy/shunt	age at surgery	shunt location	patent vena porta	ligation used	Liver enzymes (pre-operative) in IU/L				total fat percentage	fat percentage <2,5µm	fat percentage 2,5-7,5µm	fat percengage >7,5µm	lipogranuloma's (per HPF)	technetium scan	bile acids	clinical improvement	outcome
		0=heathy			0=normal		(pre-operative) in IU/L				estimate volume %					0=normal	0=normal	0=impr.	0=succes
		1=shunt			1=nonpatent		ALT	AST	ALP	GGT						1=abn.	1=abn.	1=no impr.	1=failure
Reference range							19-67	19-42	21-17	0-6									
1	bull dog	0									1	1	0	0	0				
2	bull dog	0									5,2	5,2	0	0	0				
3	bull dog	0									3,4	3,4	0	0	0				
4	yorkshire terrier	1	3y	port-azygos	0	cellophane	83	47	76	3	9	9	0	0	0,2	1	1	1	**1**
5	chihuahua	1	1y3m	port-caval	0	cellophane					49	44	3,9	0,7	0			1	**1**
6	beagle	1	5m	port-caval	0	5.0mm ameroid	91	54	202	3	60	52,8	6,8	0	0	0		0	**0**
7	mini schnauzer	1	11m	port-caval	0	cellophane	1241	618	117	7	24	9	9,2	5,8	0	0		0	**0**
8	Pekinese	1	7m	port-azygos	0	cellophane	535	163	232	5	2,4	2,4	0	0	0	1		1	**1**
9	yorkshire terrier	1	6m	port-azygos	0	5.0mm ameroid	48	45	195	5	1,3	1,3	0	0	0	0		0	**0**
10	soft coated wheaton t.	1	1y	port-caval	1	no ligation	123				16	10	5,92	0,2	2				**1**
11	chihuahua	1	7m	port-caval	0	5.0mm ameroid	136	127	213	5	14	14	0,36	0	0,6	0		0	**0**
12	yorkshire terrier	1	5m	port-caval	0	5.0mm ameroid	87	139	228	4	38	35,7	6,9	0	0		1	1	**1**
13	shiba inu	1	5y	port-azygos	0	cellophane	168	39	78	5	40	11,4	12,9	16	0,6	1		0	**1**
14*	labrador retriever	1	3y								9,1	8	1,1	0	1,6				

* dog for check of multiple aquired portosystemic shunts after intrahepetic " right divisional" shunt operation

Table 2: *Recorded parameters of both healthy controls (dog nr.1-3) and shunt dogs (dog nr. 4-14)*

Fat percentage in the control dogs ranged between 1 and 5.2 percent (median of 3.4), and between 1.3 and 59.6 percent in shunt dogs (median 20,1). When using the independent samples Mann Whitney U test, total fat percentage was not significantly higher in shunt dogs, compared to control dogs (p=0.063). Lipogranulomas were found in none of the control dogs and in four shunt dogs.

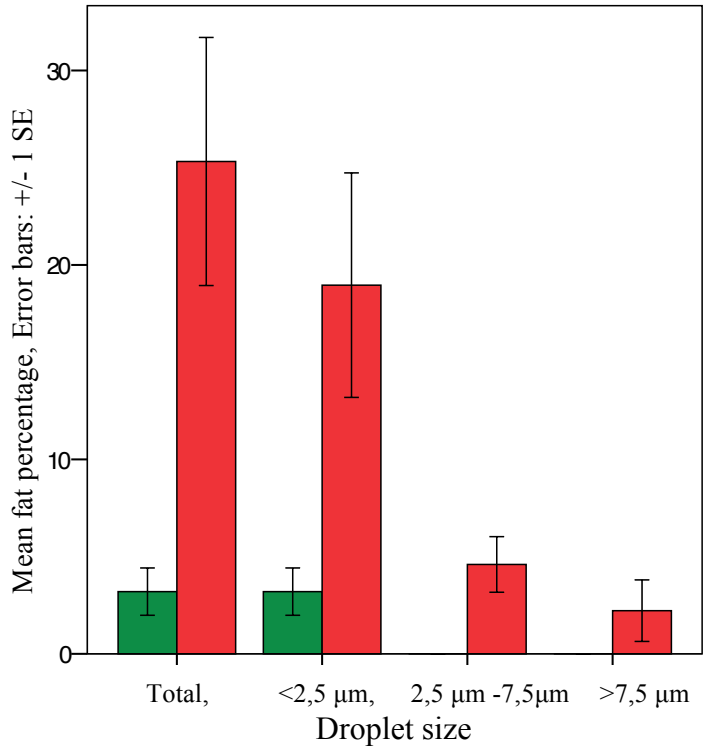

Fig. 6.

Difference in fat percentage of shunt (red) versus control dogs (green)

The four dogs with macrovesicular steatosis showed a relatively high fat percentage (mean 32.1) compared to dogs without macrovesicular steatosis (mean 14.9). A correlation between

the two is likely because the observed macrovesicular fat is directly contributing to the overall fat percentage.

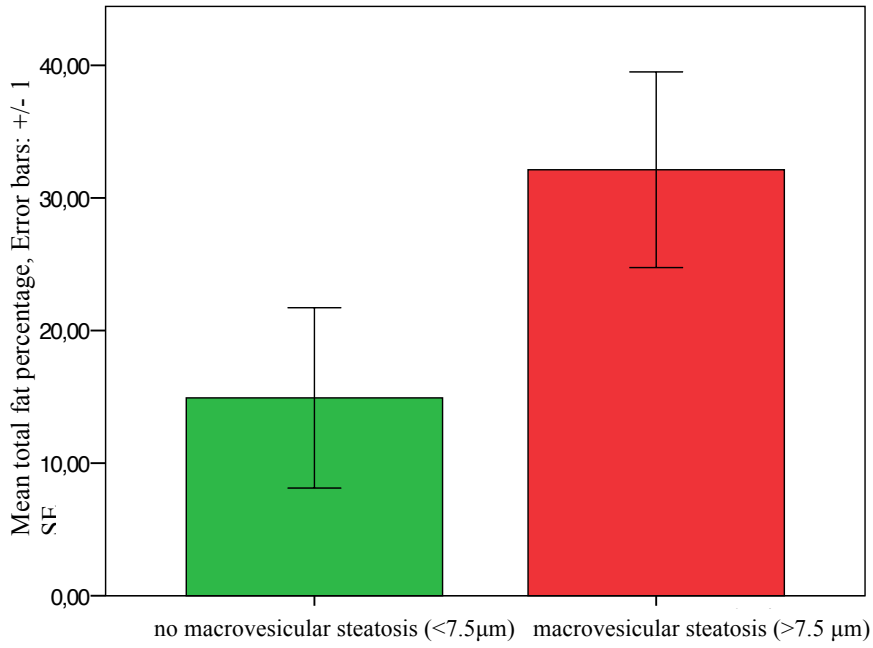

Fig. 1.

Fat percentage in dogs with and without macrovesicular steatosis.

At least one of the pre operatively measured liver enzymes (ALT, AST, ALP and GGT) was elevated in all dogs (see table 1). We found considerable variation in all liver enzymes. (In IU/L, ALT: mean=279, SE= 130, AST: mean= 154, SE=69, ALP: mean=167, SE=23) Steatosis has in various articles, in both animals and man, been linked to elevated liver enzymes.[56-58]Using the two-tailed Spearman's rho test no correlation between fat percentage and any of the liver enzymes could be found. (Fat percentage versus ALT: p=0.55, Fat percentage versus AST: p=0.91, Fat percentage versus ALP: p=0.823)

In the current data it appears that older dogs have a less favorable prognosis. Dogs with an unfavorable prognosis had a mean age of 22.5 months (SD=21.5), and dogs with a favorable prognosis had a mean age of 7.25 months (SD=2.6). A significant difference could not be proved: The Mann-Whitney U test yielded a p-value of 0.13.

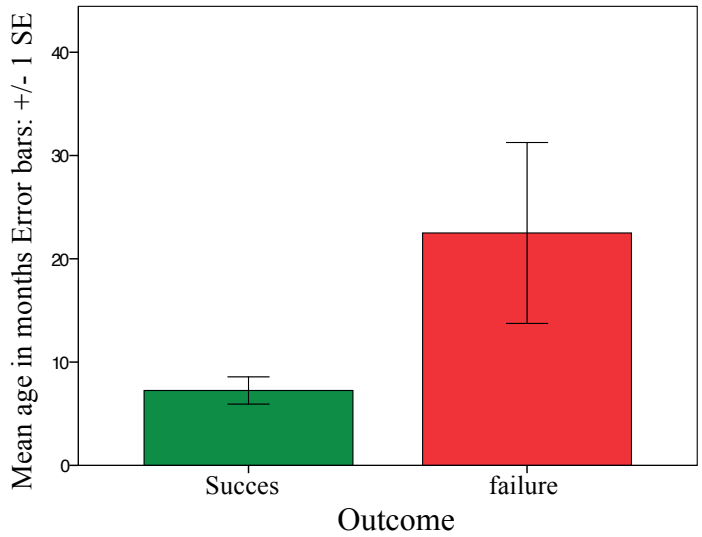

Fig. 7.

Age distribution in dogs with good or bad outcome

To explore the correlation of total fat percentage with age the following graph was constructed to visualize this distribution. One can see at first glance that there is no obvious correlation. The two-tailed Spearman's Rho test yielded a correlation of 0.037 and a p-value of 0.92, so there is no suggestion that the total fat percentage is higher in older shunt dogs.

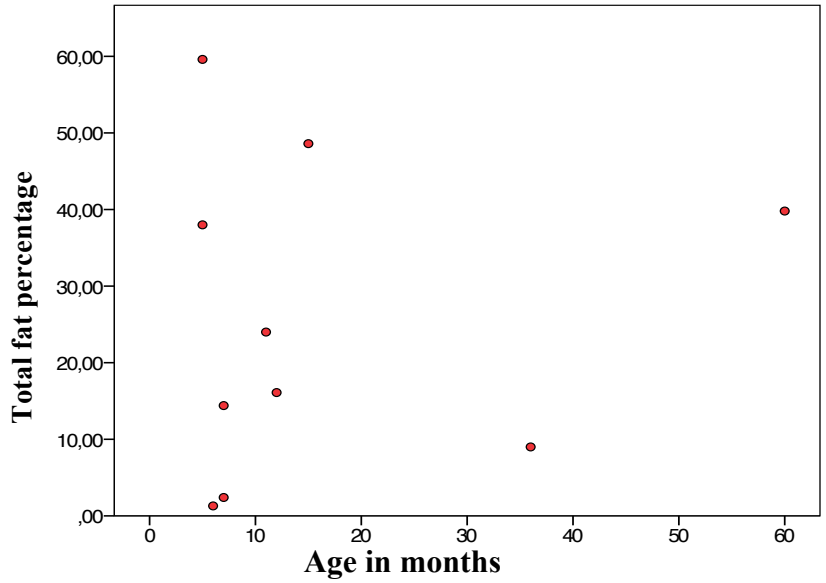

Fig. 8.

Age compared with total fat percentage.

Discussion

This study did not fulfill all the aims listed in the introduction. We were not able to demonstrate that dogs with congenital portosystemic shunts display significantly more lipid than normal dogs although the results are suggestive, and the non-parametric tests that take into account the small number of control dogs render these results not significant. The lipid is distributed between microvacuoles, macrovacuoles and lipogranulomas. The amount of lipid did not increase with age. The small number of cases in this pilot study precluded us from being able to report any demonstrable effect of macrovesicular steatosis on outcome after shunt attenuation.

The considerable variation in all possible prognostic factors, such as blood parameters, histologic parameters, clinical history, and anatomy underlines the need for analysis of all these parameters in one single model such as logistic regression, instead of multiple statistic tests.

This paper only looks into some of many possible factors influencing prognosis. Consequently, the impact of hepatic steatosis in general and macrovesicular steatosis in particular on prognosis could easily being interfered by confounding prognostic factors such as shunt anatomy and the degree of portal vein development. Larger case numbers, with proper statistical analysis, will be required to clarify whether any of the factors investigated in this study have a significant effect.

The present study has shown that assessment of macrovesicular steatosis is relatively easy and can be measured quite objectively following ORO staining. On the other hand, a limitation of the currently used grading system is that the exact percentage occupied by fat is being measured rather subjectively. Nonetheless, hepatocytes that are severely affected will most likely not receive the same grade as hepatocytes that are only mildly affected. There might be a threshold for the proportion of affected hepatocytes, before macrovesicular steatosis will influence hepatic regeneration capacity. This could only be demonstrated using much larger numbers of cases and precise measurement of hepatic fat percentage.

It is likely that diet plays a role in hepatic fat accumulation. All shunt dogs of this report were fed a high fat diet (Hills L/D) prior to surgery and biopsy. The diet eaten by the control dogs was unknown. This factor should be investigated in future studies, and serum triglyceride level at the time of biopsy should be recorded. Ideally, control dogs should be fed the same diet as shunt dogs to see whether the degree of hepatic steatosis was similar.

This form of grading system could be made more objective by randomization of the high-powered fields. In addition, it is questionable if scoring five of these high-powered fields is enough. Studies in histomorphometry suggest that around 40 fields in one sample are usually enough to allow a reasonable comparison with other samples. Computed histomorphometry could be used make a random selection of a sufficient number of fields possible.

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Appendix

Grading system used for fat percentage categories and nr of lipogranulomas estimation.

Pathologist:		Date:		Blind slide nr:	
grading nr:					

Lipids				**Lipogr/HPF**
HPF1				
size of vesicles	0µm-0.3µm	0.3µm-0.9µm	>0,9µm	
% aff. Hepatoc.				
vol% per hepatoc.				
total vol%				
HPF2				
size of vesicles	0µm-0.3µm	0.3µm-0.9µm	>0,9µm	
% aff. Hepatoc.				
vol% per hepatoc.				
total vol%				
HPF3				
size of vesicles	0µm-0.3µm	0.3µm-0.9µm	>0,9µm	
% aff. Hepatoc.				
vol% per hepatoc.				
total vol%				
HPF4				
size of vesicles	0µm-0.3µm	0.3µm-0.9µm	>0,9µm	
% aff. Hepatoc.				
vol% per hepatoc.				
total vol%				
HPF5				
size of vesicles	0µm-0.3µm	0.3µm-0.9µm	>0,9µm	
% aff. Hepatoc.				
vol% per hepatoc.				
total vol%				

Average:

Lipids:				**lipogr/HPF**
size of vesicles	0µm-0.3µm	0.3µm-0.9µm	>0,9µm	
% aff. Hepatoc.				
vol% per hepatoc.				average
total vol%				
total %				