Genetic and Functional Analysis of **Congenital Portosystemic Shunts** in Dogs

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Genetic and Functional Analysis of **Congenital Portosystemic Shunts** in Dogs

Genetische en functionele analyse van **congenitale portosystemische shunts** bij de hond

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

Scope and aims of the thesis

The liver is of great clinical importance in the body and has a unique vascular network that connects the portal vein with the systemic circulation, which is essential for liver function [1]. The mass and function of the hepatic parenchyma is maintained by hepatic perfusion, particularly by the composition and amount of portal blood available for the liver [1]. Normally, the vena porta provides 60-70% of the blood supply to the liver. This portal blood carries 50% of the oxygen demand of the liver [2] and delivers toxins, nutrients, and bacteria that are absorbed from the intestines to the liver [1]. In addition, it contains hepatotrophic factors like insulin, insulin-like growth factors, glucagon, and hepatocyte growth factor [1], which are essential for hepatocyte function, liver growth and development. When a portosystemic shunt is present, the portal blood that is derived from the gastrointestinal tract, pancreas, and spleen flows through the vascular anomaly directly into the vena cava or vena (hemi)azygos, thereby entering the venous systemic circulation without passing the functional liver sinusoids [3]. Because of the importance of portal blood for the liver, portosystemic shunting leads to malperfusion of the liver, which results in poor liver development and progressive decline in liver function. Furthermore the liver is also not able to extract noxious substances derived from the splanchnic drainage area, portosystemic shunting results in severe clinical signs in the affected individual [2, 4, 5].

Two different forms of portosystemic shunting are recognized: an acquired form by which multiple collaterals are formed as a result of portal hypertension, and a congenital form where a single functional anomalous vascular communication persists after embryonic development in the absence of portal hypertension: congenital portosystemic shunts (CPSS). The specific genetic basis of CPSS in dogs has not been elucidated yet, but an increased incidence of CPSS in purebred dogs is observed with a predisposition in several dog breeds [6, 7], indicating an inherited basis for this disease [8]. A genetic component for some breeds is confirmed by pedigree analysis [9-11]. CPSS are classified into two different types based on anatomical location: intrahepatic portosystemic shunts (IHPSS) and extrahepatic portosystemic shunts (EHPSS) [1]. While the clinical presentation of these two types of shunts, resulting from portal bypass of the liver, is identical, the genotype and molecular pathways involved are suggested to be different [12].

Despite increased knowledge regarding the pathogenesis, pathophysiology, diagnosis, and treatment of CPSS in dogs, issues concerning the genetic basis as well as pathophysiology, treatment and prognosis continue to exist. The main focus of this thesis is to gain further insight into the pathogenesis of canine CPSS and elucidate mechanisms involved in pathophysiology and hepatic regeneration.

An inherited basis for EHPSS has been demonstrated in several dog breeds [10, 11, 13, 14]. EHPSS can have a portocaval or porto-azygous localization [12]. It is unknown whether these two EHPSS

subtypes have a different genetic background or coexist within a predisposed dog breed. In **Chapter 3** the distribution of extrahepatic portocaval and porto-azygous shunts was retrospectively analyzed in 135 EHPSS dogs of several small dog breeds with the aim to discover if a common genetic background for EHPSS subtypes is plausible. Additionally, correlations between sex, age and shunt localization were investigated. The results of **Chapter 3** will not only provide information about the prevalence of EHPSS subtypes in dog breeds but, as a common genetic basis is indicated, will warrant that both subtypes could be used in future (genetic) studies to elucidate the genetic background of CPSS, which is the focus of **Chapter 4**.

Different etiologies have been suggested for the two subtypes of CPSS. EHPSS represent erroneous developmental anomalies as they arise from abnormal functional communications between the embryonic vitelline veins and the cardinal venous system [15]. In contrast, canine IHPSS are considered to be caused by a defective closure of the ductus venosus, which represents a normal embryologic connection [12]. In dogs, EHPSS are particularly seen in small breeds, whereas IHPSS are particularly seen in large breeds [1, 6, 7], indicating a different hereditary basis. A hereditary basis is demonstrated for EHPSS in Cairn terriers [10], Maltese [14], and Yorkshire terriers [13] and for IHPSS in Irish Wolfhounds [11]. In Cairn terriers the mode of inheritance of EHPSS seems to be complex, probably polygenic [10], whereas in Irish Wolfhounds IHPSS seems to be a di-genic trait [11]. Although different etiologies have been proposed for the two subtypes of CPSS, they share the same physiology and result both in nearly complete bypass of the liver by portal blood flow and dysfunction of the liver in the affected dog, leading to clinical signs. The aim of **Chapter 4** was to clarify the pathways involved in the development of the different types of portosystemic shunting.

The absence of the normal hepatic portal blood flow in CPSS leads to liver atrophy and hypoplasia and results in histological changes in the liver of affected dogs. Common histological changes observed in liver biopsies of shunt dogs are, for example, enlarged portal fields, fibrosis in the portal areas, and hepatic steatosis [16-18]. In humans, hepatic steatosis is one of the aspects of non-alcoholic fatty liver disease (NAFLD), which is a frequently reported high impact disorder [19]. Suitable animal models to study the pathogenesis of NAFLD are currently lacking. The aim of **Chapter 5** was to gain detailed insight into the pathogenesis of steatosis in CPSS dogs. From a one health perspective, these findings may result in a model to study hepatic steatosis and possible novel treatment methods in human medicine.

Therapeutic possibilities described for CPSS include medical management [20, 21] and surgical attenuation of the shunt [22, 23]. Although surgical attenuation of the shunt is the first treatment of choice to restore the normal hepatoportal circulation and liver function in dogs [5, 24, 25], the long-

term outcome following surgical attenuation is difficult to predict. Hepatic regeneration is an essential factor that influences outcome [26]. In **Chapter 6,** assuming the shared physiology between EHPSS and IHPSS, intraoperative gene expression profiles of liver tissue obtained during surgery from CPSS dogs that did recover and dogs that did not recover following surgical attenuation, were analysed. The aim of this chapter was to create a predictive model with albumin and intraoperative mRNA expression levels of specific gene products as predictors of outcome after surgical attenuation in individual dogs.

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Chapter 2

General introduction

Introduction to canine congenital portosystemic shunts

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Abstract

Canine congenital portosystemic shunts (CPSS) are vascular anomalies that connect the portal vein with the systemic circulation, therefore bypassing the hepatic parenchyma. Portosystemic shunts exist in two different subtypes: extrahepatic and intrahepatic. This congenital disorder is also described in mice, cat, sheep and man. Research has been focused on pathophysiology, diagnostics and treatment of CPSS and this has resulted in increased knowledge, although the aetiology of the disease remains unclear. This review focuses on the aetiology and genetic basis of both intra- and extrahepatic shunts.

Introduction

Canine congenital portosystemic shunts (CPSS) are hereditary disorders that have a severe impact on the wellbeing of the affected dog [1], although the genetic background has not been elucidated. The two different subtypes of CPSS, intrahepatic and extrahepatic, show a different epidemiology [1]. Intrahepatic portosystemic shunts (IHPSS) are almost exclusively diagnosed in large-sized pure-bred dogs [2], whereas extrahepatic portosystemic shunts (EHPSS) occur mainly in small dog breeds [3, 4] suggesting a hereditary basis.

For both IHPSS and EHPSS, a genetic association has been observed in the Irish Wolfhound [5] as well as in the Yorkshire terrier [6], Cairn terrier [7], and Maltese [8] breeds. Although both shunt types result in the same pathophysiology (as a result of nearly complete bypass of the liver by portal blood flow), a different aetiology is suspected based on the developmental processes involved and the timeframe in which the different subtypes of CPSS arise [9]. This hypothesis is supported by the suggested different modes of inheritance of the two shunt subtypes [5-8]. Genetic studies will in time confirm the different backgrounds for the two disorders.

Hepatic vascular anatomy and function

The anatomical structure of the liver is unique due to its role in connecting the portal circulation with the systemic circulation [1]. The vascular network of the liver comprises portal veins, hepatic arteries, and hepatic veins. The portal blood flow contains blood originating from the entire gastrointestinal tract, spleen and pancreas, including the cranial and caudal mesenteric veins, the splenic vein, the gastroduodenal vein, and the left gastric vein as the major contributors of the portal vein [1]. The portal blood flow perfuses the liver through the liver sinusoids before entering the hepatic veins and contributes to 60–70% of the total hepatic blood flow [10]. Although the portal blood is of venous origin, it delivers 50% of the hepatic oxygen supply [1, 11]. Two or three branches of the hepatic artery supply the liver and this blood of arterial origin increases the oxygen content of sinusoidal blood [11]. The total afferent hepatic blood flow is thus the result of a complicated interaction of hepatic arterial and portal venous blood flow, regulated by local and systemic factors [1, 11]. The efferent hepatic blood flow is provided by the hepatic veins, which enter into the caudal vena cava before crossing the diaphragm [11].

Clinical signs and histological abnormalities

Congenital portosystemic shunts cause liver atrophy and hepatic dysfunction that lead to a diversity of progressive clinical signs. As ammonia, aromatic amino acids, absorbed bacteria and endotoxins are not subjected to hepatic metabolism, the brain is exposed to toxins and metabolites causing hepatic encephalopathy (HE) in the affected animal [12]. The plasma ammonia concentration, used as a diagnostic method in CPSS, has also been shown to be predictive for the presence of HE in dogs, although diagnostic errors have been reported in the literature [13].

The interaction of ammonia with other factors, such as inflammation, as determined using the systemic inflammation response syndrome (SIRS) score, has been found to play a crucial role in the development of HE [13]. In dogs with CPSS in which the signs of HE resolved after successful attenuation of the shunt, the plasma ammonia concentration decreased significantly. The SIRS score also decreased, although not significantly as it may not be a sensitive score to detect changes in inflammation.

CPSS can be diagnosed as early as 6 weeks of age, facilitating routine screening of litters [7, 14]. However, based on presentation due to clinical signs, dogs with CPSS are diagnosed at varying ages with the majority of affected dogs detected within the first year of life [3, 15]. Clinical signs are influenced by shunt type, anatomy, nutrition and concurrent diseases and therefore presentation is also highly variable [16-18]. An index of suspicion based on breed predisposition and owner and veterinarian recognition of early clinical signs aids the diagnosis.

Pathological findings in dogs with CPSS are the result of shunting of portal blood with all its contents. Macroscopic changes include liver atrophy and portal vein hypoplasia proximal to the shunt origin for EHPSS [1, 10]. Histological findings include enlarged portal fields by proliferation of small arterioles and biliary hyperplasia together with hypoplasia of the portal vein and mild to moderate fibrosis in the portal areas [10, 19, 20]. Sinusoidal dilatation in the periportal area has also been described [10, 20]. In addition to parenchymal changes, including atrophy of hepatocytes and lipid infiltration, the presence of fatty cysts, lipogranulomas and lymphangiectasia has been reported [10, 19, 20]. Lipidosis, the accumulation of lipids in the hepatocytes, was compared in the livers of dogs with CPSS and control dogs using stereological point counting following Oil Red O staining [21]. The study confirmed that this technique can demonstrate lipidosis in livers of dogs with CPSS, even in the absence of lipogranulomas or large lipid vacuoles, which are necessary in a haematoxylin and eosin staining to achieve the diagnosis. Significantly more small lipid droplets have been observed in the liver tissue of dogs with CPSS compared to those of control dogs.

A more recent study demonstrated a strong association between lipogranulomas and age. Dogs < 12 months of age had significantly fewer lipogranulomas compared to dogs > 12 months. No relationship has been observed between steatosis and pre- or postoperative shunt fraction and effect on short-term outcome after shunt attenuation [22]. If a difference is observed in the amount of steatosis between healthy and CPSS dogs [21] and shunt fraction is not correlated with steatosis [22], it seems likely that steatosis could be a genetically determined factor. Extensive genomic research will be required to determine whether there is a genetic background for steatosis in shunts. Given the active role of specific lipids during liver regeneration [23], it would be of great added value to determine which type(s) of lipids tend to accumulate.

Treatment and prognosis

The treatment of choice in dogs with CPSS that is designed to achieve long-term improvement consists of surgical attenuation of the shunt vessel [12, 24]. However, complete ligation can be fatal due to a sudden increase in portal blood flow resulting in portal hypertension and shock when hypoplasia or aplasia of the portal venous circulation cranial to the shunt is present. The technique is therefore not applicable in the majority of dogs. Gradual shunt attenuation is often implemented as an alternative, using partial ligation with silk ligatures in which the shunt is closed to the maximum tolerated level so portal pressure does not reach critical values [25, 26]. Ameroid ring constrictor placement [27], cellophane banding [28] and thrombogenic intravascular coils [29, 30] have also been used to achieve progressive ligation. Post-operative outcomes remain variable and poorly predictable [25, 26], which makes each surgical intervention a challenge. Therefore (and due to the expense of surgery) medical management provides an alternative therapy for reducing the clinical signs such as hepatic encephalopathy and urinary tract disease in dogs with CPSS [24, 25, 31].

Medical therapeutics include dietary adjustments (high-quality, easily digestible low-protein diets) in combination with antimicrobials (i.e. ampicillin or metronidazole) and/or a synthetic disaccharide, like lactulose [18, 24, 32, 33]. Research has been performed on the influence of soy protein isolate vs. meat-based protein source in a low-protein diet on hepatic encephalopathy 18. Both diets had a long-term positive effect on HE-scores in dogs with CPSS. Although no difference in HE-scores was observed between the two diets, the soy protein based diet reduced plasma ammonia levels and decreased prothrombin time in dogs with CPSS compared to the control diet, thereby decreasing the risk for HE and supporting liver function.

As this management approach offers purely supportive therapy to reduce the clinical signs, it does not resolve the underlying disease, neither does it reduce the frequency of ongoing clinical signs and it does not appear to have improved survival time over the long term compared to surgical treatment [24]. In cases with high surgical risk, or where the owner has declined surgery, this therapy could be recommended for long-term support as an alternative to attenuation and, in addition, it may be provided in preparation for surgery or in dogs with insufficient clinical improvement after surgical attenuation [18, 24].

Aetiology vs. physiology

The veins located in the abdominal cavity are derived from the umbilical, vitelline and caudal cardinal veins of the embryo. The portal vein originates from the umbilical and vitelline veins, whereas the non-portal venous drainage of the abdominal organs is derived from the cardinal venous system of the fetus. No functional vascular connections exist between the cardinal veins and the umbilical-vitelline veins. In contrast, numerous non-functional vascular portocaval and portoazygos communications are present and may become functional due to portal hypertension [11]. The vitelline veins include a left and a right vitelline vein, connected by three separate anastomoses defined as cranial, middle and caudal. The ductus venosus connects the cranial anastomosis and the left umbilical vein. The ductus venosus is responsible for the flow of nutrient and oxygen rich blood derived from the placenta directly to vital organs, bypassing the liver sinusoids. In dogs this vessel is functionally closed within 2–9 days after birth, establishing the normal hepatic circulation [34].

Congenital portosystemic shunts are vascular anomalies that directly connect the portal venous system with the systemic venous circulation, thereby bypassing the liver sinusoids. EHPSS represent abnormal developmental functional communications between the embryonic vitelline veins, responsible for the entire extrahepatic portal system and the cardinal venous system that contributes only to all non-portal abdominal veins [11]. Distinctions have been reported between left, central and right divisional intrahepatic portosystemic shunts but the majority of the IHPSS are left divisional shunts that are classified as a patent ductus venosus, compatible with the normal embryology of the dog [35].

A recent publication using twice as many samples showed a more equal distribution between left, right and central divisional [30]. Right-sided shunts are classified as a patent right ductus venosus [35] but are also classified as a persistent right omphalomesenteric vein or a hepatic sinusoid malformation [36]. A clear classification of these types is lacking, but with the presence of

an intrahepatic portosystemic shunt observed in both the left and the right liver lobes in one single litter [5], it appears that both of these phenotypes may be caused by the same genetic defect, suggesting that closure of the ductus venosus is most likely regulated by a single pathway.

These findings are in agreement with epidemiological findings reporting that breed is not a predictor for the location of intrahepatic shunts [37]. Whereas the physiological consequences of both CPSS types are identical, the process of the closure of the ductus venosus differs hugely from the formation of a vessel. Both processes take place during entirely different phases of development (Fig 1). When comparing both processes based on their Gene Ontology terms [38], a total of 455 genes were annotated for vascular development, whereas for vasoconstriction 80 different genes are known to be involved in total. Merely 14 genes have been reported to be involved in both processes (Table 1).

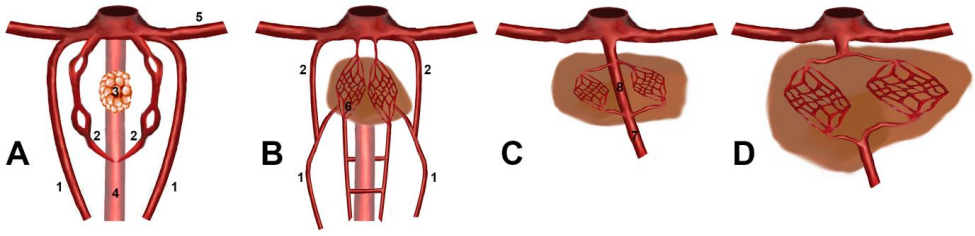

Figure 1 Schematic overview of the development of the liver and the hepatic portal vascular system. The umbilical and vitelline veins existing in early embryonic phase (A) will eventually give rise to the portal vein. No functional vascular connection exists between the umbilical–vitelline and cardinal veins in the normal healthy post-natal animal (D). The vitelline veins will regress to stimulate vascularization of the liver (B) and failure of this process causes EHPSS. Once the liver is fully developed the ductus venosus (C) should close after birth. Patency of the ductus venosus results in IHPSS. 1, umbilical veins; 2, vitelline veins; 3, liver buds; 4, duodenum; 5, common cardinal veins; 6, liver sinusoids; 7, portal vein; 8, ductus venosus; EHPSS, extrahepatic portosystemic shunts; IHPSS, intrahepatic portosystemic shunts.

In humans, CPSS is reported to be rare [39] whereas in dogs the disorder is diagnosed frequently [9]. The total prevalence of CPSS in dogs is 0.06–0.2% [40], where purebred dogs seem to be more affected compared to mixed-breed dogs [3, 41]. In dogs, IHPSS particularly affect large breeds dogs including the Irish Wolfhound [1, 42], the Golden retriever and the Labrador retriever [1], whereas EHPSS are mostly diagnosed in small breed dogs such as the Yorkshire terrier [1, 6], Cairn terrier [1,

[7], Dachshund [1], Maltese [8] and Miniature Schnauzer [1], indicating a hereditary basis in these predisposed breeds. No sex predisposition is noted in literature [2, 3, 12]. Breed predisposition appears to vary with geographical location, possibly due to the lack of awareness of CPSS in a particular breed [2, 3, 15, 43]. Another explanation would simply be the population difference, which is nicely illustrated with dilated cardiomyopathy in Doberman Pinchers from the USA [44] and Europe [45] where no overlap is shown in their genetic causal background.

Table 1 Genes involved in vascular development and vasoconstriction based on Gene Ontology classification.

Ensembl Gene ID	Gene Name	Description
ENSG00000006210	*CX3CL1*	chemokine (C-X3-C motif) ligand 1
ENSG00000073756	*PTGS2*	prostaglandin-endoperoxide synthase 2
ENSG00000078401	*EDN1*	endothelin 1
ENSG00000100345	*MYH9*	myosin, heavy chain 9, non-muscle
ENSG00000105974	*CAV1*	caveolin 1, caveolae protein, 22kDa
ENSG00000107796	*ACTA2*	actin, alpha 2, smooth muscle, aorta
ENSG00000135744	*AGT*	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
ENSG00000142208	*AKT1*	v-akt murine thymoma viral oncogene homolog 1
ENSG00000148926	*ADM*	adrenomedullin
ENSG00000151617	*EDNRA*	endothelin receptor type A
ENSG00000160691	*SHC1*	SHC (Src homology 2 domain containing) transforming protein 1
ENSG00000169032	*MAP2K1*	mitogen-activated protein kinase kinase 1
ENSG00000204217	*BMPR2*	bone morphogenetic protein receptor, type II (serine/threonine kinase)
ENSG00000222040	*ADRA2B*	adrenoceptor alpha 2B

Canine intrahepatic portosystemic shunt

IHPSS are mainly observed in large sized purebred dogs and a familial distribution was first described in Irish Wolfhounds indicating a genetic basis [42, 46]. Test matings in Irish Wolfhounds (between an affected sire and two affected sisters) resulted in a fully affected litter and a partially affected litter, demonstrating a hereditary basis with a possible di-genic mode of inheritance.

Only a few genes have been reported to cause IHPSS. The aryl hydrocarbon receptor (AHR) was the first gene documented to cause patency of the ductus venosus in knockout mice [47, 48]. Comparable results were obtained when evaluating knockout mice for aryl hydrocarbon receptor interacting protein (AIP) [49] and aryl hydrocarbon receptor nuclear translocator (ARNT) [50]. It is not surprising that all of these genes are involved in one specific pathway, although the fact that this is a cascade of genes involved in toxicological response is striking. The mechanism connecting the toxin induced pathway and the physiological processes of closure of the ductus venosus remain unclear. The finding that all of these genes originate from one and the same pathway would however suggest a polygenic inheritance. One disadvantage of mouse models is that a disease is often induced by manipulation of a single gene, whereas diseases in target species are generally spontaneous and often polygenic. Moreover, genetic manipulations in mice focus on one single major gene, whereas complex human and dog diseases are invariably polygenic.

We investigated the murine candidate genes reported in IHPSS and genes in the pathways in Irish Wolfhounds [51]. Because the pathways include multiple genes known to be involved in the patency of the ductus venosus in knock-out mice, as many genes as possible from these pathways were included in analysis. A retrotransposon (LINE-1) insertion was found in intron 2 of Irish Wolfhounds using DNA sequencing.

Using a microarray analysis combined with qPCR experiments, we confirmed down-regulation of AIP, aryl hydrocarbon receptor nuclear translocator 2, cytochromes P450 1A2 and 1B1 and heat shock protein 90AA1 (HSP90AA1) expression, whereas the expression of hypoxia inducible factor 1α (HIF1A) was up-regulated [51]. Immunohistochemistry was performed to confirm the observed differences and we found reduced levels of AHR, HIF1A, and vascular endothelial growth factor A protein in the nucleus and lower levels of ARNT and HSP90AA1 protein in the cytoplasm of the hepatocytes of Irish Wolfhounds. The impaired expression of HSP90AA1 seems to have been the key finding in this experiment, since it could trigger the observed differences in mRNA and protein levels and therefore explain the link between two very different functions of AHR, namely, regulation of the closure of the ductus venosus and the response to toxins. Additionally, it has been found in mice that HSP90α seems to be repressing retrotransposon activity [52], strongly pointing towards an explanation for the LINE-1 insertion in the Irish Wolfhound.

Unfortunately, no obvious association was observed between the pathway we investigated and IHPSS in Irish Wolfhounds, underlining the difference between induced and naturally occurring disorders. Remarkably, both HSP90 as well as WEE1 are known to interact and are essential in cell cycle control [53]. The WEE1 kinase is capable of phosphorylating a conserved tyrosine residue

in HSP90. Currently a combined WEE1/HSP90 inhibition is indicated as novel therapeutic strategy in treating cancer [54]. Whether the combination of both genes might play a role in the physiological process of ductus venosus closure needs to be investigated.

The relevance of the sphincter and its response to a unique cytochrome P-450 system in closure of the ductus venosus has been described in lambs [55]. The physiologically comparable process of closure of the ductus arteriosus is associated with endothelin-1 [56], which also is found to play a role in the contractile tone by its function in smooth muscle cells [57]. Mutations causing patency of the ductus arteriosus in humans have been found in myosin heavy chain 11 [58], which also plays a role in smooth muscle cells. In dogs both right and left divisional IHPSS were presented, suggesting that location is regulated by one major genetic pathway. Nevertheless, IHPSS is most likely to be polygenic, possibly digenic, or tri-allelic [5].

Canine extrahepatic portosystemic shunt

The first report of a human EHPSS case was documented by John Abernethy in 1793. In this case report a post-mortem examination of a female infant with multiple congenital anomalies, including an insertion of the portal vein into the inferior vena cava, was described [59]. In dogs EHPSS were previously classified into two subtypes based on the vessels they connect, namely, portocaval and portoazygos shunts [11]. Computed tomographic angiography images revealed that, in addition, a third shunt type can be classified based on the contribution of a phrenic vein entering the shunting vessel before its insertion into the caudal vena cava [60].

Combined with the anatomical definitions of the veins associated with the shunts and shunt origin, six general shunt types were identified, namely, splenocaval, splenoazygos, splenophrenic, right gastric-caval, right gastric-caval with a caudal shunt loop, and right gastric-azygos with a caudal shunt loop [60]. In addition, other variations have been described since the initial publication [4, 61]. The portoazygos type described in previous studies most probably contains either portoazygos or phrenic vein insertions [62]. Remarkably, in the Japanese dog population, the splenophrenic variation is the most frequently observed shunt type [4], whereas in the study performed in North America the spleno-azygos shunts are found [60]. Fukushima et al. (2014) suggested that this discrepancy can probably be explained by the geographical breed distribution because of the popularity of Miniature Dachshunds and Toy Poodles that contribute to the high incidence of spleno-phrenic variation observed in Japan [4]. In our opinion genetic bias might also be responsible for this discrepancy.

In general, a predisposition for EHPSS is observed in a variety of small dog breeds [3, 4]. In Yorkshire terriers [6] , Cairn Terriers [7] and Maltese [8] breeds a genetic background for this shunt type is determined. In Yorkshire terriers the mode of inheritance of EHPSS is not currently thought to be sex-linked, simple autosomal dominant or simple autosomal recessive [6]. A complex mode of inheritance, probably polygenic, is also suspected in the Cairn terrier [7]. Pedigree analysis performed in 299 Maltese dogs with 164 dogs of uncertain phenotype support, at least to a certain degree, a complex genetic basis for EHPSS, and a partially penetrant recessive mode of inheritance was suggested by O'Leary et al. (2014). When conducting a genetic study to unravel shunt causing genes in dog breeds with EHPSS, it is important to know whether the EHPSS subtypes coexist in those breeds.

Future genetic research

The genetic background for IHPSS and EHPSS is expected to be different considering the physiological processes involved. We would expect IHPSS to be caused by differences in the composition of the ductus venosus, whereas the occurrence of EHPSS is susceptible to specific events during development. During early development, blood vessels are initially formed by vasculogenesis, representing the differentiation of endothelial cells from the mesoderm into primary vessels. The primary vessels within this embryonic circulation are remodelled into arteries and veins to develop a functional adult vascular circulation by angiogenesis, which involves capillary sprouting, splitting, and remodelling [63]. The mature character of these vessels after arterial-venous differentiation is maintained through the interaction between ephrin-B2 and ephrin type-B receptor 4 [64]. For EHPSS, it has been postulated that the dysfunctional regression of the vitelline veins is causative [11].

Given the specific time point, vessels and the knowledge that EHPSS normally does not relate to additional vascular problems, it is most likely that EHPSS is not caused by general angiogenic factors; instead, epigenetic variation may play a major role. The annotated canine genome is still under development and much is unknown about the epigenetic landscape in the dog. The first steps towards an improved canine genome have been made by RNA-sequencing data sets from 10 different canine tissues resulting in the annotation of novel protein coding genes and additional alternative isoforms per gene [65]. Despite the fact that this will be an invaluable tool in canine genetic research, the canine genome requires further development comparable to the Human ENCODE [66, 67] to solve disorders caused by noncoding variants as has been found, for example, in osteosarcoma [68] and obsessive compulsory disorders [69].

Conclusions

The physiological consequences of IHPSS and EHPSS are identical. Portal blood bypasses the liver either through the patent ductus venosus or through the extrahepatic vascular anomaly. Despite the shared physiology, the aetiology of the different CPSS types is not identical as the process of the closure of the ductus venosus differs hugely from angiogenesis and these processes take place during different phases of development. The severity of both IHPSS and EHPSS and the high prevalence of these conditions in numerous breeds indicate that there is a degree of urgency to unravel the genetic background to the disease and to provide breeders with a reliable genetic test. Genotyping both affected and unaffected dogs from a predisposed population, then performing fine mapping using cases from other dog breeds as a follow up experiment, is likely to be the most successful strategy. In addition, the canine genome requires improvement with regard to the non-coding genetic landscape as shown in man and mice. Solving a complex disorder like CPSS could benefit greatly from such a CANCODE approach.

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Chapter 3

Distribution of extrahepatic congenital portosystemic shunt morphology in predisposed dog breeds

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Abstract

Background: An inherited basis for congenital extrahepatic portosystemic shunts (EHPSS) has been demonstrated in several small dog breeds. If in general both portocaval and porto-azygous shunts occur in breeds predisposed to portosystemic shunts then this could indicate a common genetic background. This study was performed to determine the distribution of extrahepatic portocaval and porto-azygous shunts in purebred dog populations. **Results**: Data of 135 client owned dogs diagnosed with EHPSS at the Faculty of Veterinary Medicine of Utrecht University from 2001 – 2010 were retrospectively analyzed. The correlation between shunt localization, sex, age, dog size and breed were studied. The study group consisted of 54 males and 81 females from 24 breeds. Twenty-five percent of dogs had porto-azygous shunts and 75% had portocaval shunts. Of the dogs with porto-azygous shunts only 27% was male ($P = 0.006$). No significant sex difference was detected in dogs with a portocaval shunt. Both phenotypes were present in almost all breeds represented with more than six cases. Small dogs are mostly diagnosed with portocaval shunts (79%) whereas both types are detected. The age at diagnosis in dogs with porto-azygous shunts was significantly higher than that of dogs with portocaval shunts ($P < 0.001$). **Conclusion**: The remarkable similarity of phenotypic variation in many dog breeds may indicate common underlying genes responsible for EHPSS across breeds. The subtype of EHPSS could be determined by a minor genetic component or modulating factors during embryonic development.

Background

Congenital portosystemic shunts (CPSS) cause portal blood derived from the gastrointestinal tract and other organs in the splanchnic drainage area to flow directly into the systemic circulation. As a consequence portal blood bypasses the liver and is not subjected to hepatic metabolism [1].

Liver shunts are classified into intra- and extrahepatic shunts, based on the anatomical location. An intrahepatic shunt represents a normal embryologic shunt (ductus venosus) bypassing the umbilical blood along the liver into the heart of the fetus, which did not close after birth [2, 3]. In contrast, an extrahepatic portosystemic shunt (EHPSS) is not considered as a normal embryonic connection. The EHPSS represent abnormal functional communications between the embryonic vitelline veins, which form the entire extrahepatic portal system, and the cardinal venous system, which normally contributes to all non-portal abdominal veins [4]. The extrahepatic portal vein develops from the different parts of the vitelline vein, and the vena cava and vena (hemi)azygos develop from the embryonic cardinal vein. Connections between the cardinal and vitelline systems do not occur during any phase of embryonic development [4]. Therefore extrahepatic shunts must be considered erroneous developmental anomalies. Affected breeds may have either intra- or extrahepatic liver shunts; these two types occur very rarely in the same breed [1, 5-9] and both sexes were reported to be equally affected [8, 10]. Pedigree analyses of intrahepatic shunts of Irish wolfhounds [3, 11] and of extrahepatic shunts of Yorkshire terriers 12 and Cairn terriers [10, 13] have shown an inherited basis of shunts in these breeds. Besides the Cairn and Yorkshire terriers, a breed predisposition for EHPSS has been reported for Jack Russell terriers [8], Dachshunds [14], Miniature schnauzers [6] and Maltese [15], which also indicates a hereditary background of the disorder in these breeds [10, 13]. Test matings in Cairn terriers showed that EHPSS in this breed has a complex, probably polygenic mode of inheritance [10].

Extrahepatic shunts can have a portocaval or a porto-azygous localization. In general, dogs with porto-azygous shunts show milder clinical signs [16]. It is not known whether these different shunt types (portocaval and porto-azygous) have a different genetic background. Genes that are responsible for embryonic extrahepatic connections could be defect in both main types of EHPSS (portocaval and porto-azygous). Hence, the occurrence of both shunt types within a breed could indicate a common major (genetic) defect.

This study was performed to evaluate the distribution of extrahepatic portocaval and porto-azygous shunts in different dog breeds with the aim to discover if a common genetic basis for both extrahepatic types is plausible. For this purpose, data of 135 dogs with a single EHPSS were

retrospectively analyzed. This survey yielded information with respect to extrahepatic shunt type, breed, average age at diagnosis and dog size. Based on the higher number of portocaval shunts (89%) compared to porto-azygous shunts reported in previous studies [8], an increased amount of portocaval shunts is to be expected in our study. The milder clinical signs in dogs with a porto-azygous shunt [16] could cause a later onset of clinical signs. Therefore we could expect a later age at diagnosis of dogs with this type of shunt within our study population.

Methods

Data

Medical records from the University Clinic for Companion Animals of the Faculty of Veterinary Medicine, Utrecht University, the Netherlands, were reviewed to identify dogs with a congenital extrahepatic portosystemic shunt. The following information was retrieved from the medical records: breed, sex, date of birth, localization of the extrahepatic shunt, method of diagnosis and date of first and definitive diagnosis. Data from dogs diagnosed with a single EHPSS in the period 2001–2010 were available for analysis. All cases originated from the Netherlands. Cross breeds were excluded from our study population. The dogs included in this study were presented with clinical signs of hepatic encephalopathy or other signs compatible with portosystemic shunting, or were identified by a shunt screening test performed in the Dutch Cairn terrier population of clinically healthy 6 week old pups. In both cases, a high fasting venous ammonia level or abnormal ammonia tolerance test suggested the presence of a portosystemic shunt [10]. The shunts were visualized by ultrasonography [10] or computed tomography and often confirmed during surgery. The two categories used were portocaval and porto-azygous shunts.

The diagnosis portocaval shunt was made when the shunting vessel terminated in the caudal vena cava. The diagnosis porto-azygous shunt was made when the shunt entered the (hemi)azygos vein, or when the single large tortuous shunting vessel traversed the dorsal part of the diaphragm and was located next to the esophagus. A thoracic termination into the (hemi)azygos vein could not be seen in some cases with ultrasonography or during surgery.

Statistical method

To assess the relation between breed and extrahepatic shunt localization a Fisher's exact test was used. To estimate if there was a difference in shunt localization between males and females a Chi-squared test was used for both phenotypes. The Chi-squared test was based on the expected equal distribution of sexes within both shunt types. To determine if there was a

correlation between dog size and localization of the shunt, the dog breeds were classified into weight classes. Since the weights of the individual dogs are not comparable due to differences in age and decreased body weight as secondary effect of the phenotype, we used the mean expected weight of the breed based on the breed standard of the Dutch Kennel Club. Dogs were classified as small (≤ 9 kg) or medium and large dog breeds (> 9 kg). A Chi-squared test was performed to estimate if there was a correlation between weight class and localization. In addition, differences in the age at the moment of diagnosis between dogs with portocaval and those with porto-azygous shunts were analyzed with a Mann–Whitney test. In general the age at diagnosis was taken as the moment of the first observation of the presence of a shunt by a high venous ammonia level after fasting or an abnormal ammonia tolerance test. In some dogs the exact localization was determined at a later time point. The difference between age of diagnosis for both groups was calculated performing a t-test. For this calculation the Cairn terrier pups that were diagnosed at young age in a population screening program, and not by clinical signs, were excluded. Also t-test was performed on the age at diagnosis and sex in both phenotypes to estimate a possible sex difference. Significance was considered when $P \leq 0.05$.

Results

Data of 151 dogs diagnosed with a single EHPSS were available for diagnosis. The 135 dogs used in this study, after excluding 16 cross breeds, were diagnosed with a portosystemic shunt between 6 weeks and 9.7 years of age. Localisation of 93 cases was confirmed during surgery. The study group consisted of 40% males ($n = 54$) and 60% females ($n = 81$) which based on t-test was significantly different from the 51% males and 49% females in the total clinic population of 43,813 patients ($P = 0.02$). The proportion of porto-azygous and portocaval shunts in the study group was 25.2% and 74.8%, respectively. By comparing the proportion of males and females between porto-azygous and portocaval we found that in the group of porto-azygous shunts a significantly higher number of females was affected (73.5%) compared with the number of males in the same group ($P = 0.006$). No sex predisposition was found for portocaval shunts ($P = 0.232$) (Fig 1).

The study group consisted of 24 breeds. In most breeds with six or more cases both porto-azygous and portocaval shunts were diagnosed (Table 1). The only exception were the Pugs which were all affected by portocaval shunts ($n = 6$). Additional breeds diagnosed with EHPSS were the Lhasa Apso, Miniature Poodle, Norfolk terrier with two cases, and single cases of a Basset Hound, Bolognese, Cavalier King Charles Spaniel, Epagneul Nain Papillon, Flat Coated

Retriever, Fox terrier, Giant Spitz, Great Dane, Miniature Pinscher, Norwich terrier and Welsh terrier. In the Cairn terriers a significantly lower fraction of porto-azygous shunts was diagnosed compared to the total study population (P = 0.039). The fraction of detected porto-azygous shunts in the other breeds varied considerably, ranging from 0-38% within a particular breed (Table 1). The EHPSS were mainly observed in small dog breeds. Exceptions were a Giant Spitz, a Flatcoated retriever, a Basset hound and a Great Dane. After classification of the dogs as small (≤ 9 kg) or medium and large dog breeds (> 9 kg) these groups contained respectively 101, and 34 dogs. In small dogs portocaval shunts were detected more often than porto-azygous shunts, whereas in the group of medium and large dogs no differences were detected between both types (P = 0.03) (Fig 2).

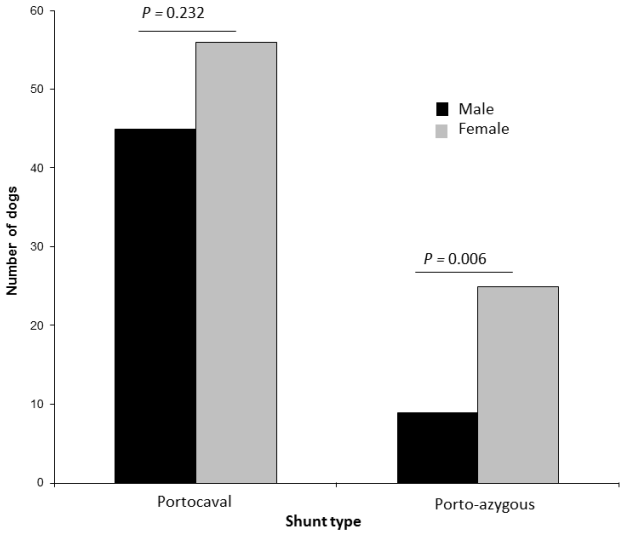

Figure 1 Localization and gender distribution of extrahepatic portosystemic shunts in 135 purebred dogs. The dogs were seen in the period from 2001–2010. The shunt diagnosis was based on a high fasting venous ammonia level or abnormal ammonia tolerance test and the visualisation by ultrasonography or computed tomography. In most cases the diagnosis was independently visually confirmed during surgery.

A significant difference in the age at first diagnosis was found between the two shunt subtypes (P < 0.001). Portocaval shunts were first diagnosed at a mean age of 12.3 ± 11.3 months, whereas porto-azygous shunts were first diagnosed at a mean age of 32.3 ± 24.0 months. All Cairn terriers (n = 24) were excluded from this calculation because most of them were identified by a screening program of pups at an age of six weeks. No significant difference was found in the age

at first diagnosis between male and female dogs in porto-azygous shunts (P = 0.831) and portocaval shunts (P = 0.800).

Table 1 Distribution of dogs with congenital extrahepatic portosystemic shunts

						Age first diagnosis (months)	
Breed	**Total**	**M**	**F**	**PC**	**PA**	**PC**	**PA**
Cairn terrier	24	13	11	22	2	1.5 - 33.2	1.7 - 11.2
Jack Russel terrier	19	8	11	15	4	3.2 - 65.6	4.0 - 113.2
Maltese	15	4	11	13	2	3.0 - 65.6	46.7 - 66.0
Yorkshire terrier	14	8	6	10	4	3.9 - 26.9	3.7 - 32.5
Dachshund	11	6	5	7	4	2.2 - 35.1	6.2 - 65.2
Shih Tzu	9	2	7	8	1	3.7 - 67.4	46.87
West Highland White terrier	8	3	5	5	3	5.0 - 14.0	21.7 - 116.1
Chihuahua	6	2	4	5	1	3.4 - 84.0	10.83
Miniature Schnauzer	6	1	5	4	2	2.9 - 18.9	12.9 - 61.4
Pug	6	2	4	6	0	3.6 - 26.4	NA

M = Male; F = Female; PC = Portocaval shunt; PA = Porto-azygous shunt. This table only contains purebred dog breeds of which six or more medical records could be included.

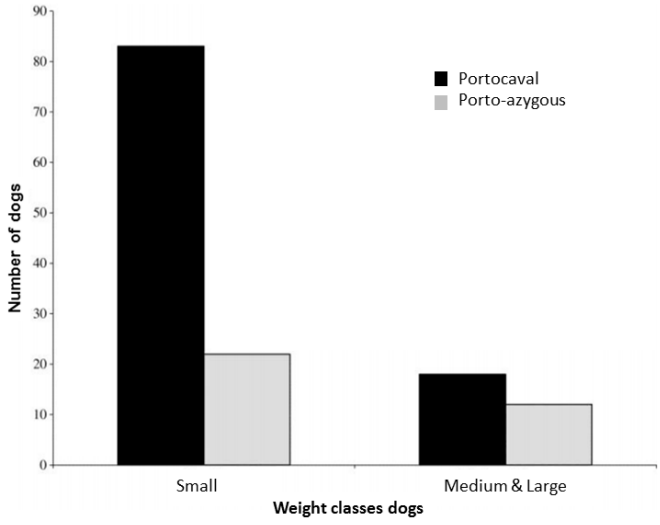

Figure 2 Weight classification of dogs related to porto-azygous and portocaval shunts. 135 dogs were classified as small (≤ 9 kilo) or medium and large dog breeds (> 9 kilo). The weight classes contained respectively 101, and 34 dogs. Mean weight is based on the standard of the Dutch Kennel Club. Chi-squared test was performed. A significant difference was observed between weight and shunt localization (P = 0.03).

Discussion

In our study group of dogs with EHPSS there were significantly more females than males. This is in contrast with previous studies in which an equal sex representation was found [8, 10]. Only one breed, the Bichon Frise, has been reported to have an overrepresentation of females with EHPSS [8]. The total population of dogs presented at Utrecht University Clinic between 2001 and 2010 consisted of 49% females and 51% males, indicating that the sex differences found in this study are not caused by differences in the clinic population. In our population the breeds that contributed most to the female over-representation were the Maltese and Shih Tzu. The significantly higher proportion of females with a porto-azygous shunt compared to males with this type of shunt is surprising since we expected a similar sex distribution within the groups of both portocaval and porto-azygous shunts.

In the study group a lower proportion of porto-azygous shunts in comparison with portocaval shunts was found. These findings are well in agreement with previously reported fractions (11-36%) [8, 13, 15]. Especially in smaller dogs a significant higher frequency of portocaval shunts was detected compared to dogs weighing more than 9 kg. Another observation in line with this is the significantly lower age at first diagnosis of portocaval shunt in comparison with porto-azygous shunts which corresponds with previously published data [17, 18]. In porto-azygous shunts, probably less blood bypasses the liver in comparison with portocaval shunts, because the receiving azygos vein has a smaller diameter and therefore more resistance than the abdominal vena cava. Another explanation reported for the later onset of clinical signs in these dogs could be that respiration causes diaphragmatic compression during which the shunt is intermittently closed [16]. Therefore it can be expected that liver functions are better and hepatic encephalopathy less pronounced in dogs with porto-azygous shunts. It is also possible that the later onset of clinical signs could have led to an underestimation of the prevalence of porto-azygous shunts [17, 18] especially since with milder clinical signs owners might prefer to go to a referral centre. In this respect it is noteworthy that in the Dutch population of Cairn terriers, which are screened routinely for shunts at the age of six weeks, the fraction of porto-azygous shunts is also low (Table 1). Possibly this overrepresentation might be due to genetic selection on certain phenotypic characteristics and thus being caused by an inbred genetic component. The fact that dogs with porto-azygous shunts are usually diagnosed at an older age increases the risk that they contribute to reproduction and therefore sustain presence of underlying genes in the population. Therefore screening of breeds at-risk at young age seems essential for the extirpation of CPSS in these populations. Only the Dutch Cairn terrier club mandates the test for presence of a shunt in newborn dogs. This could cause a skewed picture of the problem in

small dogs. We therefore decided to discard them from analysis of age of diagnosis. The remaining 111 dogs used for this study originate from a diverse population originating from both rural and urban areas.

It should be noted that for a number of breeds only a small number of case reports are available making it hard to draw breed related conclusions on distribution. Localisation of most shunts in the abdominal cavity or the thoracic cavity was confirmed during surgery (n = 93). Because the terminus is not visualized in all cases, a small fraction of the shunts could be wrongly classified. Ultrasound classification using standardized protocols [19] on the other hand proved highly sensitive and specific for diagnosing and classifying EHPSS. Data of higher numbers of cases would allow us to perform pedigree analysis and also increase the power to detect possible differences in occurrence of shunt types within breeds.

Cairn terriers [10], Yorkshire terriers [12], Jack Russell terriers [8], Dachshunds [14] and Maltese [15] have been described in literature as breeds with a predisposition for EHPSS. These breeds were well represented in our study group. Nearly all dog breeds in our study seem to display both portocaval and porto-azygous shunts. The absence of porto-azygous shunts in pugs is most likely caused by the low number of cases. The fact that the two types affect the same dog breeds has not been reported previously. The occurrence of both porto-azygous and portocaval shunts in nearly all breeds that are predisposed for EHPSS seem to demonstrate that the two types are variants of the same inherited disorder. Modulators like environmental factors during a specific time point in embryogenesis could determine whether the embryonic vitelline system gets erroneously connected with the cardinal vein system at the level of the vena cava or the vena (hemi)azygos. The shunts develop from the portal vein or from one of its contributors, such as the left gastric vein, splenic vein, cranial or caudal mesenteric vein or gastroduodenal vein (vitelline system) [4]. Furthermore, the veins in which the shunts terminate (the caudal vena cava and the azygos vein) are formed through several transformations of the cardinal system [4]. It has previously been shown that EHPSS is a complex genetic trait, presumably determined by different cooperating genes [10]. The observations in our study led to the idea that the two subtypes of EHPSS are commonly determined by a small number of major genes, and that a minor gene or non-genetic factor determines the site of insertion. Future research to these gene defects is needed to confirm this hypothesis. The difference in the prevalence in males and females is yet another puzzle which needs further research.

Conclusions

In dog breeds predisposed for the occurrence of EHPSS the two shunt types, portocaval and porto-azygous, coexist in nearly all breeds. There seems to be a correlation between location and dog size. Portocaval shunts are predominantly diagnosed in small dogs, whereas no difference was observed in large dogs. The age at first diagnosis in dogs with a porto-azygous shunt is significantly higher than in dogs with a portocaval shunt. This difference is probably a consequence of the lower degree of shunting in porto-azygous shunts resulting in milder clinical signs. Dogs with a porto-azygous shunt may reproduce before diagnosis thereby maintaining causative genes in affected populations. Porto-azygous and portocaval shunts presumably have similar causative genes and are maybe differentiated by a minor genetic component or modulating factors.

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Chapter 4

Aberrant gene expression in dogs with portosystemic shunts

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Abstract

Congenital portosystemic shunts are developmental anomalies of the splanchnic vascular system that cause portal blood to bypass the liver. Large-breed dogs are predisposed for intrahepatic portosystemic shunts (IHPSS) and small-breed dogs for extrahepatic portosystemic shunts (EHPSS). While the phenotype resulting from portal bypass of the liver of the two types of shunt is identical, the genotype and molecular pathways involved are probably different. The aim of this study was to gain insight into the pathways involved in the different types of portosystemic shunting. Microarray analysis of mRNA expression in liver tissue from dogs with EHPSS and IHPSS revealed that the expression of 26 genes was altered in either IHPSS or EHPSS samples compared with that in liver samples from control dogs. Quantitative real-time PCR of these genes in 14 IHPSS, 17 EHPSS, and 8 control liver samples revealed a significant differential expression of *ACBP*, *CCBL1*, *GPC3*, *HAMP*, *PALLD*, *VCAM1*, and *WEE1*. Immunohistochemistry and Western blotting confirmed an increased expression of VCAM1 in IHPSS but its absence in EHPSS, an increased WEE1 expression in IHPSS but not in EHPSS, and a decreased expression of CCBL1 in both shunt types. Regarding their physiologic functions, these findings may indicate a causative role for VCAM1 in IHPSS and WEE1 for IHPSS. CCBL1 could be an interesting candidate to study not yet elucidated aspects in the pathophysiology of hepatic encephalopathy.

Introduction

Congenital portosystemic shunts (CPSS) are vascular anomalies by which portal blood circumvents the liver, flowing directly into the systemic circulation. As a result, portal blood does not undergo hepatic metabolism through the liver parenchyma [1-3]. The associated hepatic dysfunction gives rise to several central nervous system, gastrointestinal tract, and urinary tract symptoms and signs [1, 4, 5]. For example, exposure of the brain to endogenous neurotoxic substances can lead to hepatic encephalopathy [6]. Two anatomically different types of shunt have been described. Intrahepatic portosystemic shunts (IHPSS) are usually embryological shunts (ductus venosus) in the liver that failed to close after birth, whereas extrahepatic shunts (EHPSS) are developmental vascular anomalies by which the extrahepatic portal system is connected with the caudal vena cava or (hemi)azygos vein [7]. The functional consequences, virtual absence of portal perfusion of the liver parenchyma, and clinical signs are identical for both types of shunt [8, 9].

CPSS occur sporadically in a variety of species, including humans [10], but frequently in dogs (Canis lupus familiaris). There are no essential differences between humans and dogs with CPSS with regard to the histological features of the liver, clinical presentation, and diagnostic methods [7]. Excessive inbreeding of purebred dog populations has greatly increased the incidence of genetic disorders [7] and genetic association analyses in specific dog breeds have shown that canine model systems can provide unique insights into human biology and disease [11, 12]. CPSS are mainly found in purebred dogs [9, 13-15] and, in general, IHPSS are typically seen in large-breed dogs such as Irish wolfhounds [16], Golden retrievers [17], Labrador retrievers [17, 18], Australian cattle dogs [19], and Old English sheepdogs [5]. EHPSS occur in small-breed dogs such as Cairn terriers [9], Yorkshire terriers [15, 17], Jack Russell terriers [20], Dachshunds [18], Miniature schnauzers [17], and Maltese terriers [19]. In evaluated dog breeds, IHPSS [8, 14, 21] and EHPSS [9, 14, 15] proved to be inheritable disorders. Test matings and pedigree analysis of Irish wolfhounds has shown that IHPSS are not a monogenetic trait but possibly caused by two interacting genes [8]. Similar analyses in Cairn terriers have indicated that the genetic basis of EHPSS is more complex and does not follow simple Mendelian rules of inheritance [9]. The confirmation that portosystemic shunting has a genetic basis in these breeds makes the dog an ideal model with which to unravel the embryonic development of the ductus venosus and the intrahepatic and extrahepatic portal system.

Progressive liver disease is an ailment common to both humans and dogs, and the regulatory pathways involved in chronic fibrotic liver disease, which ultimately leads to liver cirrhosis, are the same in both species [22-24]. Impaired hepatic perfusion plays an important part in the chronic deterioration of liver function seen in progressive liver disease [25-27]. Knowledge of the genes and

metabolic pathways implicated in CPSS might provide insight into the pathways involved in the vascular derangements of chronic progressive liver diseases, which in turn might lead to new ways to intervene in these currently incurable diseases [7].

In the present experiment, RNA samples isolated from the liver of dogs with IHPSS and EHPSS were used for gene profiling, and differential gene expression was confirmed by qPCR and immunohistochemistry. We demonstrate aberrant expression of certain genes in dogs with all types of CPSS attributed to the shared phenotype. In addition, few genes were differentially expressed between dogs with EHPSS or IHPSS, implying genotypic differences involved in these pathophysiologically comparable liver diseases.

Materials and Methods

Animals

Control tissue was obtained from six healthy mature dogs sacrificed for unrelated studies. The absence of underlying liver disease in these dogs was confirmed histologically by a board certified veterinary pathologist. Dogs with CPSS were kept privately as companion animals and were presented to the University Clinic for Companion Animals (Department of Clinical Sciences of Companion Animals, Utrecht University), where CPSS was diagnosed on the basis of increased fasting plasma levels of ammonia [8, 9] and ultrasound visualization and classification of shunts. All affected dogs underwent surgery, during which the diagnosis and classification were confirmed. Wedge biopsies of the liver were taken during surgical closure of the shunt, and effects of portal hypoperfusion were identified histologically in all animals, a finding that is consistent with CPSS [18]. Before and 2 months after surgery, the size of the liver was assessed by ultrasound, and the extent of portosystemic shunting of portal blood was assessed with a rectal ammonia tolerance test and Doppler ultrasound of the original shunt. Ten dogs with EHPSS made a complete recovery, based on normalization of liver size and the absence of flow in the shunting vessel; a second liver biopsy was then taken from these animals. Liver samples were snap frozen in liquid nitrogen or RNAlater (Ambion , Inc., Austin, Texas) for RNA isolation, or fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemistry. Since some of the samples were obtained at necropsy and others at biopsy, tissue fixation times and the ratio of tissue volume : fixative volume varied between animals, which could influence staining. The procedures were approved by the local ethics committee, as required under Dutch legislation (ID 2007.III.08.110).

Expression profiling

Total RNA was isolated from liver tissue from 2 healthy dogs, 32 dogs with EHPSS, and 15 dogs with IHPSS (Table 1), using a RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) and on-column DNase digestion. RNA quality and quantity was determined on a nanochip (Bioanalyzer, Agilent Technologies, Santa Clara, US). RIN values above 8.0 were considered reliable, and these samples were included in the study. Pooled RNA isolated from healthy liver tissue was used as reference.

Agilent Canine Gene Expression Microarray V1 containing 42,034 60-mer probes in a 4x44K layout was used to determine genome wide expression, using 3 µg of total RNA from each animal co- hybridized to the common reference. RNA amplification and labeling were performed [28] on an automated system (Caliper Life Sciences NV/SA, Belgium). Dye swap of Cy3 and Cy5 was performed to reduce dye bias. Hybridization was done on a HS4800PRO system supplemented with QuadChambers (Tecan Benelux B.V.B.A.), using 1 µg labeled cRNA per channel [29].

Hybridized slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 30% photomultiplier tube voltage, and automated data extraction was done using Imagene 8.0 (BioDiscovery). Normalization was performed with Loess [30] on mean spot intensity, and dye bias was corrected based on a within-set estimate [31].

Analyses were performed to detect differences in gene expression between the two shunt groups (EHPSS and IHPSS), and between each shunt group and the control (healthy liver). Data were analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7) [32]. Correction for multiple testing (Permutation F2-test using 5,000 permutations) was performed and $P < 0.05$ was considered statistically significant. Genes with log2-fold changes of more than 0.4 or less than - 0.4 were then selected to ensure that only robust changes were considered.

Amplification for qPCR

Liver samples (17 from dogs with EHPSS and 14 from dogs with IHPSS) were randomly selected for confirmation by qPCR after RNA amplification with the WT-Ovation RNA Amplification System (Bemmel, the Netherlands), using 80 ng RNA per sample. This system converts RNA to cDNA, using the linear isothermal DNA amplification called SPIA [33], which produces single-strand DNA. The products were diluted three times and stored at –20°C until used. To match experimental conditions, RNA from control samples was treated in a similar fashion and a water sample was used as a negative control.

Table 1 Samples from dogs with extrahepatic portosystemic shunts (EHPSS) or intrahepatic portosystemic shunts (IHPSS) used for microarray or qualitative PCR analysis.

		microarray		**qPCR**		**Postoperative**	
	status	*Female*	*male*	*female*	*male*	*female*	*male*
Cairn terrier	EHPSS	3	4	3	4	2	1
Cross breed	EHPSS	2	1	0	0	2	1
Jack Russell terrier	EHPSS	3	3	2	1	1	0
Maltese terrier	EHPSS	3	2	2	1	0	0
Miniature dachshund	EHPSS	1	0	0	0	0	0
Norfolk terrier	EHPSS	2	1	2	0	0	0
Shih Tzu	EHPSS	1	0	0	0	1	0
West Highland white	EHPSS	2	0	1	0	1	0
Yorkshire terrier	EHPSS	4	0	1	0	0	0
Australian shepherd	IHPSS	1	0	1	0	0	0
Bearded collie	IHPSS	0	1	0	1	0	0
Bernese mountain dog	IHPSS	2	1	2	1	0	0
Cane corso	IHPSS	0	1	0	1	0	0
Duck tolling retriever	IHPSS	0	1	0	1	0	0
Golden retriever	IHPSS	2	1	2	1	0	0
Hovawart	IHPSS	0	1	0	1	0	0
Irish wolfhound	IHPSS	2	0	1	0	0	0
Labrador retriever	IHPSS	0	1	0	1	0	0
Newfoundland	IHPSS	1	0	1	0	0	0

qPCR

Perlprimer v1.1.14 was used for primer design on Ensembl annotated transcripts and the amplicon was tested for secondary structures using MFold [34]. Gradient PCRs were performed to determine the optimum temperature for obtaining 100% PCR efficiency. Primer specificity was validated in silico (BLAST specificity analysis) and empirically (DNA sequencing, gel electrophoresis, and melting profiles). qPCR reactions were performed in 25-µl duplicates containing 0.5 x SYBR Green-Supermix (BioRad, Veenendaal, the Netherlands), 0.4 µM primer, and 1 µl cDNA.

Five reference genes were used for normalization, based on their stable expression in liver, namely, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *β-2-microglobulin (B2M)*, *ribosomal protein S5 (RPS5)*, *heterogeneous nuclear ribonucleoprotein H (HNRPH)*, and *ribosomal protein S19 (RPS19)* [35]. GeneNorm [36] was used to establish stability. Primers for reference genes and genes of interest, including their optimum temperature, are listed in table 2. Cycling conditions were a 3-minute Taq polymerase activation step at 95°C, followed by 45 cycles of 10 seconds at 95°C for denaturation, and 30 seconds at T_m for annealing and elongation.

All experiments were conducted with a MyiQ Single-Color Real-Time PCR Detection System (BioRad). A 4-fold standard dilution series of a pool containing all samples was used to determine relative expression.

Data analysis was performed with IQ5 Real-Time PCR detection system software (BioRad). Expression levels were normalized by using the average relative amount of the reference genes. Log-values of normalized relative expression were used to obtain normal distribution. A Wilcoxon rank sum test was performed in R [37] to determine the significance of differential gene expression.

Table 2 Primers used for qualitative PCR.

Gene	Ensembl	F/R	Sequence	Tm(C)	Product
B2MG	ENSCAFT00000038092	F	5'-TCCTCATCCTCCTCGCT-3'	61.2	85
		R	5'-TTCTCTGCTGGGTGTCG-3'		
GAPDH	ENSCAFT00000037560	F	5'-TGTCCCCACCCCCAATGTATC-3'	58	100
		R	5'-CTCCGATGCCTGCTTCACTACCTT-3'		
HNRPH	ENSCAFT00000028063	F	5'-CTCACTATGATCCACCACG-3'	61.2	151
		R	5'-TAGCCTCCATAACCTCCAC-3'		
RPS19	ENSCAFT00000008009	F	5'-CCTTCCTCAAAAAGTCTGGG-3'	61	95
		R	5'-GTTCTCATCGTAGGGAGCAAG-3'		
RPS5	ENSCAFT00000003710	F	5'-TCACTGGTGAGAACCCCCT-3'	62.5	141
		R	5'-CCTGATTCACACGGCGTAG-3'		
ABCC11	ENSCAFT00000016007	F	5'-AAGTTCTCCATTGTCCCTC-3'	57.7	90
		R	5'-TCTGTTCATCTGTGTAACGA-3'		
ACBP	ENSCAFT00000007872	F	5'-GTTAAGCACCTCAAGACCA-3'	64.1	96
		R	5'-GCCGTTCTGTGTTTATGTC-3'		
APOA1	ENSCAFT00000021138	F	5'-CAGTCAAAGACAGCGGCAG-3'	61.2	166
		R	5'-CTCCAGGTTATCCCAGAACTCC-3'		
BCHE	ENSCAFT00000023011	F	5'-CTCAACAATGCCGATTCTG-3'	56	84
		R	5'-CTCCATTCTCGTTCTGCT-3'		
BRP44	ENSCAFT00000024369	F	5'-GCTGTTAATTTCTTTGTGGGTG-3'	63.7	110
		R	5'-TCAGGTGGTCAGGAACTC-3'		
CAPS	ENSCAFT00000029761	F	5'-AGTAGGACAAAGGTTCCGA-3'	59.3	197
		R	5'-GCAATCTCAAGTGGTGGG-3'		
CCBL1	ENSCAFT00000031874	F	5'-CATCGCAGACATCTCAGAC-3'	58.7	182
		R	5'-AAACAGAAGCGGATATAGTGG-3'		
CYP2E1	ENSCAFT00000021134	F	5'-GTAGCAAACCAGGACACGA-3'	65.7	247
		R	5'-GCGGACAAGAACAGGAAGAG-3'		
DSTN	ENSCAFT00000008828	F	5'-GCACCAGAACTAGCTCCT-3'	64	200
		R	5'-GCACTGAATGATGGTCTACAC-3'		
GATM	ENSCAFT00000021782	F	5'-CTCCTCCAATACCAGTCATCC-3'	58.8	219
		R	5'-ACATCACAGGTCCAGCAG-3'		
GDF15	ENSCAFT00000023627	F	5'-CTGGTGATACTGGTGATGCT-3'	66.8	202
		R	5'-AGGTCAGGGTTTGAATCGG-3'		
GPC3	ENSCAFT00000029940	F	5'-AGAAGAATGGTGGAAAGCTGAC-3'	68.1	138

		R	5'-CTATACTGGCGTTGTTGAGAATGG-3'		
HEPC	ENSCAFT00000011304	F	5'-CCAGTGTCTCAGTCCTTCC-3'	65.5	163
		R	5'-TTTACAGCAGCCACAGCA-3'		
JDP2	ENSCAFT00000026985	F	5'-CTGAAATACGCCGACATCC-3'	61.1	153
		R	5'-CCGCCACTTTGTTCTTCTC-3'		
KIFC2	ENSCAFT00000002564	F	5'-CCATCTCAAGAAGAAAGCCC-3'	60.7	246
		R	5'-GTTTCAGAGCCTCATTCTCC-3'		
MPND	ENSCAFT00000030318	F	5'-GGCTTCTGTCAAGTACAAGGG-3'	65.7	142
		R	5'-CTTCCTCCATCAACAGCTCCT-3		
PALLD	ENSCAFT00000012001	F	5'-GTTAAGCACCTCAAGACCA-3'	62.7	96
		R	5'-GCCGTTCTGTGTTTATGTC-3'		
PON3	ENSCAFT00000003345	F	5'-AGAACTGCCGCCTTATTGAG-3'	62.1	241
		R	5'-GATGAAAGTACTGATTCCGTGTG-3'		
SERPINA7	ENSCAFT00000028383	F	5'-GACCTCAAACCAAACACCA-3'	62.2	101
		R	5'-GCTGAAACCCTCTTCTGTC-3'		
SLC1A2	ENSCAFT00000011054	F	5'-ACCATGCTCCTCATCCTG-3'	63.7	102
		R	5'-CATTGACTGAAGTTCTCATCCT-3'		
VCAM1_1	ENSCAFT00000031837	F	5'-GATGAAATTGACTTTGAGCCCA-3'	65	127
		R	5'-ATTGTCACAGAACCGCCT-3'		
VCAM1_2	ENSCAFT00000031837	F	5'-AGTTAGAGGATGCGGGAG-3'	63	132
		R	5'-TAAAGCACGAGTAGTTCTGG-3'		
WEE1	ENSCAFT00000011883	F	5'-AGAGGCAGAGTTGAAGGA-3'	65	130
		R	5'-CAGCATTTGGGATTGAGGT-3'		
ZCCHC9	ENSCAFT00000013818	F	5'-ACAGTCAGGAGGTAAGGG-3'	63.2	197
		R	5'-CACAGCGATAACATATTCCAG-3'		

*B2M=β-2-Microglobulin, GAPDH=Glyceraldehyde-3-phosphatedehydrogenase, HNRPH=Heterogeneous nuclear ribonucleoprotein H, RPS19=Ribosomal protein S19, RPS5=Ribosomal protein S5, ABC11= ATP-binding cassette, sub-family C (CFTR/MRP), member 11 , ACBP= Diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein), AFM=*afamin, *APOA1=Apolipoprotein A-I, BCHE=Butyrylcholinesterase, BRP44=Brain protein 44, CAPS= Calcyphosine, CCBL1=Cysteine conjugate-beta lyase, cytoplasmic, cOR13P3=cOR13P3 olfactory receptor family 13 subfamily P-like, CYP2E1=Cytochrome p450 2E1, DSTN=Destrin (actin depolymerizing factor), GATM=Glycine amidinotransferase, GDF15=Growth differentiation factor 15, GPC3=Glypican 3, HAMP=Hepcidin antimicrobial peptide, JDP2=Jun dimerization protein 2-like, KIFC2=Kinesin family member C2, MPND=MPN domain containing, PALLD=Palladin, cytoskeletal associated protein, PON3=Paraoxonase 3, SERPINA7=Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7, SFTPD=surfactant protein D, SLC1A2=Solute carrier family 1 (glial high affinity glutamate transporter), member 2, VCAM1=Vascular cell adhesion molecule 1, WEE1=WEE1 homolog (S. pombe), ZCCHC9=Zinc finger, CCHC domain containing 9)*

Immunohistochemistry

Liver samples from healthy dogs (n=6) and randomly selected dogs with IHPSS (n=6) and dogs with EHPSS (n=6) were stained for ACBP, CCBL1, HAMP, GPC3, PALLD, VCAM1, and WEE1, using reagents and methods described in Table 3. Five-micrometer sections of paraffin-embedded liver tissue were deparaffinized in xylene, and rehydrated in an ethanol to water series.

Heat-induced antigen retrieval was performed with 10 mM citrate buffer (pH 6.0) or 10 mM Tris with 1 mM EDTA (pH 8.0) at 98°C in a water bath, followed by cooling at room temperature (RT) for 30 minutes (Table 3). Antigen retrieval by enzymatic digestion was performed with

proteinase K (Dakocytomation, Glostrup, Denmark) for 10 minutes at RT. Dual endogenous enzyme block (Dakocytomation) was used (10 minutes RT) to quench endogenous peroxidase activity, and background staining was blocked with 10% normal goat serum (Sigma-Aldrich, St. Louis, US) (30 minutes). Sections were incubated with the labeled secondary antibody Envision (Dakocytomation) for 1 hour at RT. The signal was developed in 0.06% 3,3'-diaminobenzidine (DAB) solution (Dakocytomation) for the indicated time (Table 3) and counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, US). Replacement of primary antibody with washing buffer served as negative control. All tissues were stained in batch per antibody to avoid technical differences.

Table 3 Antibody specifications.

Primary Antibody	**Manufacturer**	**Catalogue no.**	**Dilution IHC**	**Diluent**	**Incubation time**	**Antigen retrieval**	**Type sec. AB**	**Incubation DAB (min)**	**Dilution WB**
ACBP	Abnova	mab0725	1:200	PBS+BSA	O/N 4°C	TE-buffer, 40 min	mouse monoclonal	2	
CCBL1	Sigma	hpa021177	1:500	ABD	O/N 4°C	Proteinase K, 10 min	rabbit polyclonal	2	1:1000
HAMP	Abcam	ab30760	1:200	ABD	1h RT	Proteinase K, 10 min	rabbit polyclonal	2	
GPC3	BioMosaics	B0025R, B0055R	1:50	ABD	O/N 4°C	TE-buffer, 30 min	mouse monoclonal	4.5	
PALLD	Novus	NBP1-25959G	1:25	PBS+BSA	O/N 4°C	Citrate-buffer 40	mouse monoclonal	5	
VCAM1	Santa Cruz	sc-8304	1:100	PBS	O/N 4°C	Proteinase K, 10 min	rabbit poloclonal	1	1:500
WEE1	Santa Cruz	sc-5285	1:50	PBS	O/N 4°C	TE-buffer, 40 min	mouse monoclonal	5	1:20
ACTB	Thermo Fisher Scientific						mouse		1:2000

IHC = immunohistochemistry, WB = Western Blot, ABD = antibody diluent (DAKO), PBS= Phosphate-buffered saline , BSA=Bovine serum albumin , TE=Tris-Ethylenediaminetetraacetic acid, ACTB = β-actin

All immunohistochemically stained sections were evaluated by a board-certified pathologist (GCMG) who was unaware of the dogs' phenotype, using a semi-quantitative scoring system based on the intensity and localization of staining, with grading as follows: 0, absent; 1, mild

positive staining; 2, moderate positive staining; 3, strong positive staining. If different histological elements (hepatocytes, bile ducts, Kupffer cells) were stained, then staining in these elements was scored separately. Information on acinar localization (zone 1, 2, or 3) was also collected. The average staining intensity score for each group (i.e. intrahepatic, extrahepatic, control) was calculated. Student's t-test was used to detect significant differences in staining intensity, with $P < 0.05$ being considered statistically significant.

Western blot

For Western blot analysis 30 mg of liver tissue from at least four samples of each group (healthy $n = 4$, IHPSS $n = 4$, EHPSS $n = 4$, randomly chosen from original group) were homogenized in RIPA buffer (Sigma-Aldrich). Protein concentrations were obtained using a Lowry-based assay (DC Protein Assay, BioRad). 30 µg of protein of the supernatant was denatured for 2 min at 95°C and separated on 7.5% (VCAM1 and CCBL1) or 10% (WEE1) Tris-HCl Criterion gels (BioRad) and the proteins were transferred onto Hybond-C Extra Nitrocellulose membranes (Amersham Biosciences Europe, Roosendaal, The Netherlands). The membranes were incubated with 4% non-fat dry-milk (BioRad) in TBS for 1 hour with shaking. The incubation of the primary antibody was performed at 4°C over-night for all antibodies (see Table 3) in TBS with 0.1% Tween-20 (Boom B.V., Meppel, The Netherlands) and 4% Bovine Serum Albumin (BSA). After washing, the membranes were incubated with their respective horseradish peroxidase-conjugated secondary antibody (R&D systems, Europe Ltd., Abingdon, UK) at room temperature for 1 h. Immunodetection was performed with an ECL Western blot analysis system, performed according to the manufacturer's instructions (BioRad). Replacement of primary antibody with TBST and 4% BSA served as negative control. β-Actin (ACTB) was used as loading control. Imaging was performed on a ChemiDoc XRS System (BioRad) and the intensity of the bands was quantified using Quantity One 4.3.0 Software (BioRad).

Results

Expression profiling

The expression of 142 probes was significantly different compared to the controls in samples from dogs with EHPSS or IHPSS (Fig 1), of which only 107 were annotated (CanFam 3.1). Of these, 19 and 6 annotated genes were specific to liver samples from dogs with either IHPSS or EHPSS, respectively (Table 4). Additionally, *HAMP* was significantly downregulated in dogs with IHPSS and significantly upregulated in dogs with EHPSS compared with healthy dogs (Table 4).

The other 81 annotated genes were up- or downregulated in both groups of dogs, often more strongly in one phenotype than in the other. To avoid analyzing secondary effects, these genes were excluded. All data have been deposited in NCBI's Gene Expression Omnibus [38] and are accessible through GEO Series accession number GSE39005 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE39005).

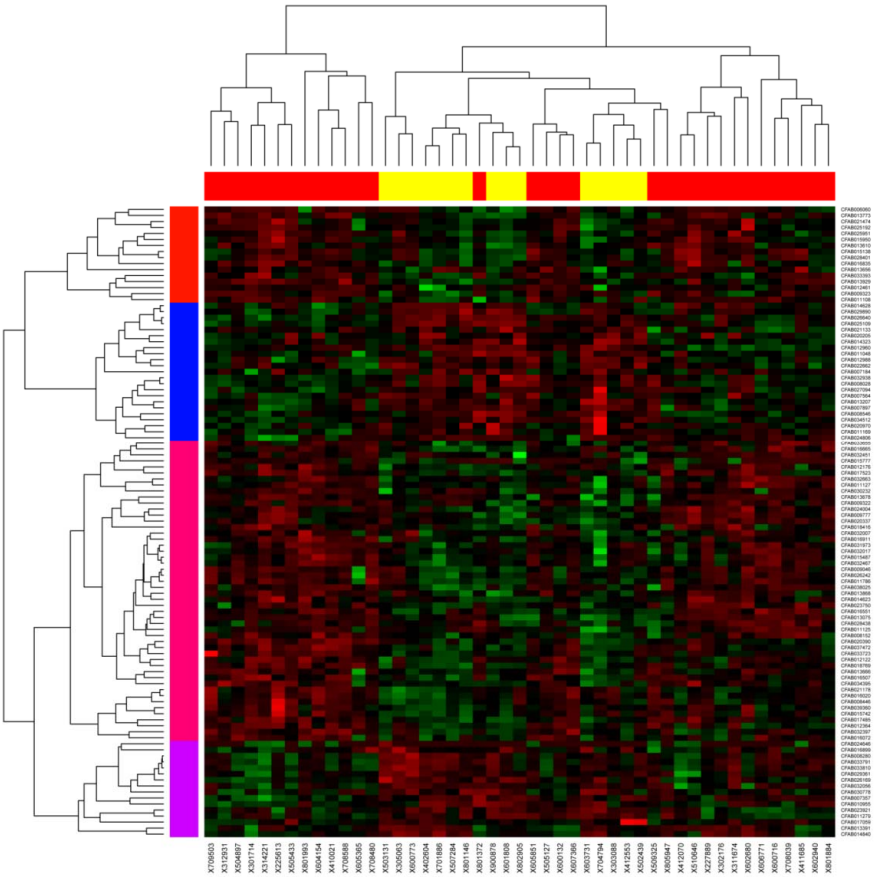

Figure 1 Heatmap EHPSS vs IHPSS. 107 annotated probes (listed in rows) were expressed significantly differently in the 32 dogs with extrahepatic portosystemic shunts (EHPSS; red columns) and 15 dogs with intrahepatic portosystemic shunts (IHPSS; yellow columns) compared with control dogs.

Table 4 Genes expressed differently in dogs with or without extrahepatic (EHPSS) or intrahepatic (IPHSS) portosystemic shunts (microarray results in log2).

Gene	IHPSS vs control	EHPSS vs control
ABCC11		0.9
ACBP	-0.8	
AFM	0.9	
APOA1	0.5	
BCHE	0.7	
BRP44	1	
CAPS		-1.3
CCBL1	-0.5	
cOR13P3	0.5	
CYP2E1		0.6
DSTN		-0.5
GATM	0.9	
GDF15	-0.8	
GPC3	1	
HAMP	-0.8	0.7
JDP2	0.5	
KIFC2	0.5	
MPND	0.6	
PALLD		-0.5
PON3	0.5	
SERPINA7	0.9	
SFTPD	-0.7	
SLC1A2		0.7
VCAM1	0.6	
WEE1	0.7	
ZCCHC9	0.6	

qPCR

The expression of 23 of the genes differentially expressed in dogs with IHPSS or EHPSS (Table 4) was measured by quantitative RT-PCR. For technical reasons, no qPCR data could be obtained for *AFM*, *SFTPD*, and *cOR13P3*. Only seven genes proved to be differentially expressed in one shunt group (IHPSS or EHPSS) compared with the other shunt group and the healthy controls (Table 5, Fig 2).

Table 5 Genes expressed differently in dogs with or without extrahepatic (EHPSS) or intrahepatic (IPHSS) portosystemic shunts (qPCR results).

	P-value	Bonferroni	Fold change
ACBP			
IHPSS vs EHPSS	0.001	0.002	
CONTROL vs EHPSS	0.916	1	
CONTROL vs IHPSS	0.004	0.011	-3.1
CCBL1			
IHPSS vs EHPSS	< 0.001	< 0.001	
CONTROL vs EHPSS	0.021	0.062	
CONTROL vs IHPSS	< 0.001	< 0.001	-2.7
GPC3			
IHPSS vs EHPSS	< 0.001	0.001	
CONTROL vs EHPSS	0.427	1	
CONTROL vs IHPSS	< 0.001	< 0.001	3.8
HAMP			
IHPSS vs EHPSS	< 0.001	0.001	
CONTROL vs EHPSS	0.154	0.461	
CONTROL vs IHPSS	< 0.001	< 0.001	-16.8
PALLD			
IHPSS vs EHPSS	0.002	0.005	
CONTROL vs EHPSS	0.969	1	
CONTROL vs IHPSS	0.009	0.027	-2.4
VCAM1			
IHPSS vs EHPSS	0.014	0.043	
CONTROL vs EHPSS	0.004	0.013	-5.5
CONTROL vs IHPSS	0.435	1	
WEE1			
IHPSS vs EHPSS	0.004	0.012	
CONTROL vs EHPSS	0.866	1	
CONTROL vs IHPSS	0.009	0.028	5.1

Relative mRNA expression of the seven differentially expressed genes in qPCR.

ACBP, *CCBL1*, *HAMP,* and *PALLD* were downregulated (-2.4 to -16.8 fold change) and *GPC3* and *WEE1* (3.8 and 5.1 fold change, respectively) were upregulated in dogs with IHPSS compared with dogs with EHPSS and control dogs. *VCAM1* (-5.5 fold change) was downregulated in dogs with EHPSS compared with dogs with IHPSS and control dogs. These seven genes were not functionally related, based on Metacore™ analysis (GeneGo, St. Joseph, US).

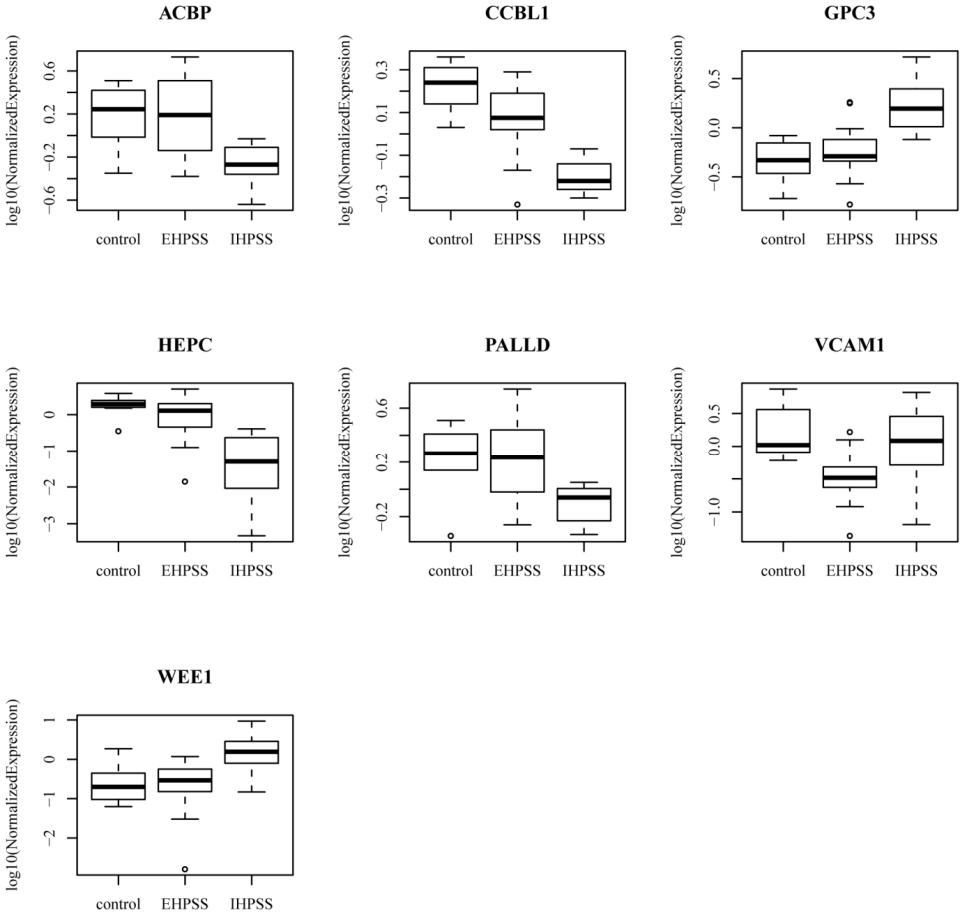

Figure 2 Quantitative PCR results. The upregulation or downregulation of selected genes in liver samples from dogs with or without extrahepatic (EHPSS) or intrahepatic (IHPSS) portosystemic shunts. The thick black line represents the median (50th percentile), also the first and third quartile (25th and 75th percentile respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the interquartile range.

VCAM1 expression was studied in liver samples taken during and after surgery and compared with that in control liver samples. *VCAM1* expression in liver samples taken during (P=0.020) and after (P=0.034) surgery was significantly different from that in control liver samples, but not between the pre- and postoperative liver samples (P=0.26) (Fig 3A). A second qPCR probe, involving the C-terminus of VCAM1 near the position of the probe for microarray (primer VCAM1_2 table), revealed downregulation of *VCAM1* in liver samples taken during surgery, but not in samples taken after surgery or in control samples (Fig 3B).

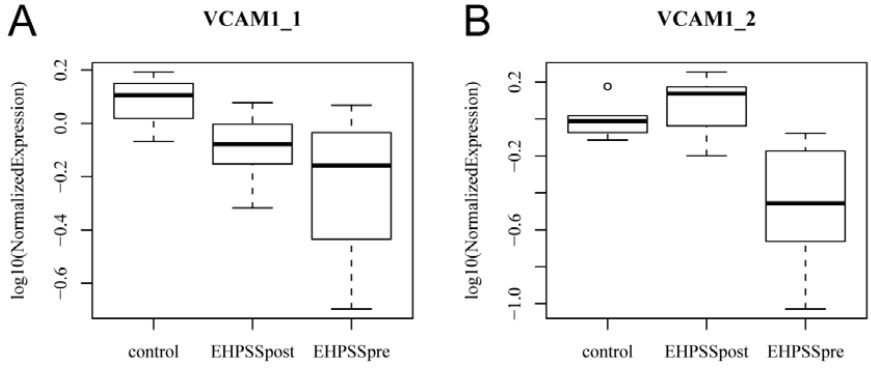

Figure 3 Relative expression of VCAM1 in intraoperative and postoperative samples. Relative expression of VCAM1 mRNA in liver samples from dogs with extrahepatic portosystemic shunts (EHPSS) obtained during and after surgery compared to healthy liver tissue. Samples from postoperative tissue were obtained after EHPSS closure. VCAM1_1 was designed near the 5`-end, VCAM1_2 is located on the 3`-end.

Immunohistochemistry

The intensity of staining for CCBL1, VCAM1, and WEE1 in hepatocytes was significantly different between the two CPSS groups and the control group (Table 6). There were no significant differences in ACBP, GPC3, HAMP, and PALLD staining intensity in the hepatocytes or biliary epithelium.

Table 6 Immunohistochemical staining for different proteins in liver samples from dogs with or without extrahepatic (EPHSS) or intrahepatic (IPHSS) portosystemic shunts.

	ACBP		**CCBL1**		**GPC3**		**HAMP**		**PALLD**		**VCAM1**		**WEE1**	
	Mean	P-value	Mean	P-value	Mean	P-value	Mean	P-value	Mean	P-value	Mean	P-value	Mean	P-value
Control	2.3		2.8		1.2		1.0		1.8		0.3		0.8	
EHPSS	2.7	0.290	1.5	0.006	0.8	0.188	0.3	0.207	1.3	0.209	0.3	0.807	1.7	0.096
IHPSS	2.5	0.599	1.0	<0.001	1.0	0.341	0.3	0.145	1.8	1.000	1.8	0.006	1.8	0.044

The mean of the specific protein intensity is listed in the table based on semi-quantitative evaluation of immunohistochemically stained liver biopsies. The corresponding P-value compared to the control group is noticed.

CCBL1 staining was typically detected in the cytoplasm (Fig 4), and was more intense in the control dogs than in dogs with EHPSS (P = 0.006) or IHPSS (P = 6.59*10-7). Staining was not significantly different between the dogs with IHPSS or EHPSS, although staining was considered more positive in samples from dogs with EHPSS. In some EHPSS cases Kupffer cells also showed a positive staining.

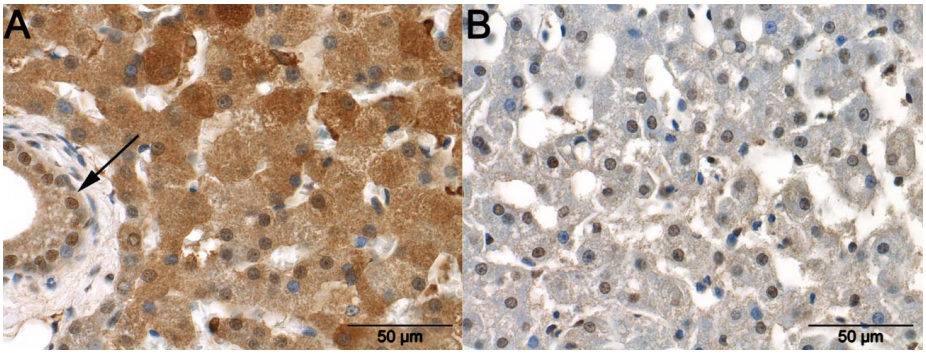

Figure 4 Staining for CCBL1 in the liver. Cysteine conjugate-beta lyase-1 (CCBL1) immunoreactivity in a liver sample from a healthy dog (Figure 4 A) and a dog with an intrahepatic portosystemic shunt (IHPPS) (Figure 4 B). Marked cytoplasmic and moderate nuclear immunoreactivity is visible in hepatocytes and bile duct epithelium (arrow) in the sample from the healthy animal. The sample from the dog with an IHPSS shows only weak immunoreactivity in the cytoplasm and moderate nuclear immunoreactivity of hepatocytes.

VCAM1 staining of the cytoplasm and nuclei of samples from control dogs and dogs with EHPSS was mainly negative or moderate in intensity (Fig 5), whereas staining was significantly more intense in samples from dogs with IHPSS than in samples from control dogs (P = 0.006). In addition, all Kupffer cells showed some staining for VCAM1, but no differences were observed between the CPSS and control dogs. Staining of smooth muscle cells was observed around a few blood vessels in most healthy tissues.

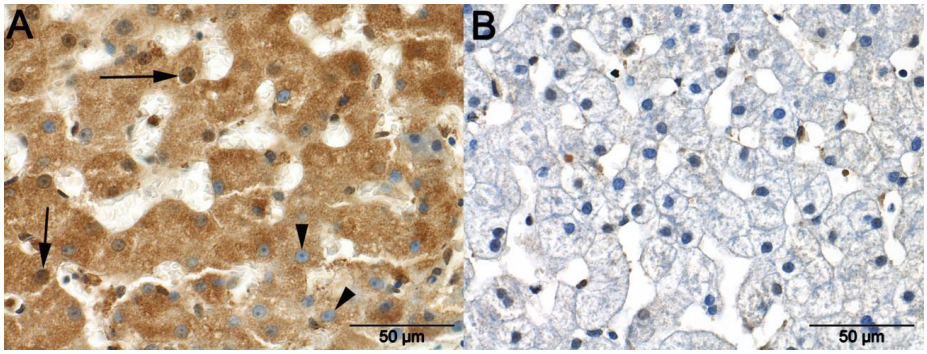

Figure 5 Staining for VCAM1 in the liver. Marked granular cytoplasmic immunoreactivity with the presence (arrows) and absence (arrowheads) of immunoreactivity in the nuclei of hepatocytes in a liver sample taken from a dog with an intrahepatic portosystemic shunt (Figure 5 A). The cytoplasm of hepatocytes in a liver sample from a dog with an extrahepatic portosystemic shunt (EHPPS) show no immunoreactivity. Nuclei in this liver occasionally demonstrate weak immunoreactivity (Figure 5 B).

WEE1 staining was generally not detected in nuclei (Fig 6), although randomly a few nuclei showed moderate staining. Nuclear staining for WEE1 was found in most bile duct epithelial cells. Nuclear WEE1 staining of hepatocytes was more intense in samples from dogs with IHPSS than in samples from control dogs (P = 0.044), but there were no significant differences in bile duct staining between the three groups of samples.

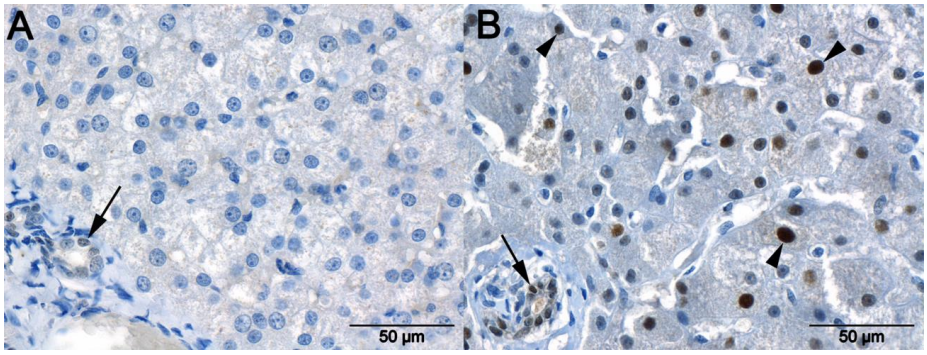

Figure 6 Staining for WEE1 in the liver. Staining for WEE1 in a liver sample from a healthy dog (Figure 6 A) and a dog with an intrahepatic portosystemic shunt (IHPPS) (Figure 6 B). Note the marked nuclear staining in hepatocytes (arrowheads) and bile duct epithelium (arrows) in the sample from a dog with an IHPPS, whereas nuclei of the sample from the healthy dog show only weak staining in bile ducts and no staining in hepatocytes.

Western blot analysis

Measurement of CCBL1, VCAM1 and WEE1 protein levels in liver samples by Western blotting confirmed the expression differences detected by immunohistochemistry. CCBL1 was significantly downregulated in EHPSS (P = 0.007) and IHPSS (P = 0.002) samples compared to the healthy control tissue (Fig 7A). For VCAM1 an upregulation (P = 0.01) was found in IHPSS samples compared to the two other groups. No differences were found between EHPSS samples and the healthy control group (Fig 7B). Expression of WEE1 was found to be upregulated (P = 0.01) in IHPSS samples compared to the control and EHPSS samples (Fig 7C).

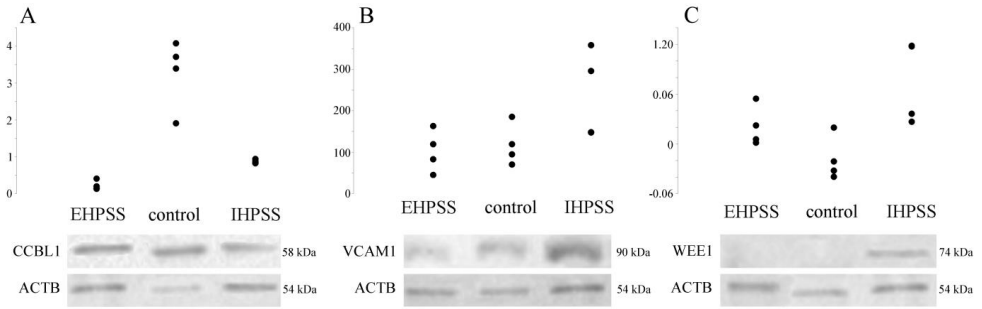

Figure 7 Western blot analyses for CCBL1, VCAM1 and WEE1. Protein expression was measured for CCBL1, VCAM1 and WEE1 in liver tissue of healthy individuals (n=4) and dogs affected with IHPSS (n=4) and EHPSS (n=4). ACTB was used as loading control and replacing primary antibody served as a negative control. CCBL1 was significantly down regulated in both IHPSS as well as EHPSS samples compared to the healthy controls (A). Expression of VCAM1 confirmed the findings of the immunohistochemistry with a downregulation in EHPSS samples was found compared to the IHPSS samples (B). WEE1 was found to be upregulated in IHPSS samples compared to healthy and EHPSS samples (C). The depicted bands are representative for the indicated groups.

Discussion

This study used expression profiling to identify pathways involved in the pathogenesis of IHPSS and EHPSS. Both types of shunt give rise to impaired portal perfusion of the liver parenchyma, which results in decreased growth, liver dysfunction, and clinical symptoms. However, IHPSS are typically seen in large-breed dogs and EHPSS are typically seen in small-breed dogs [7], which suggests that the causative genotype is most likely different. Genes possibly involved in a specific type of shunt were identified by comparing gene expression in liver sample from dogs with IHPSS or EHPSS, and control dogs. Differences in the hepatic expression of genes in dogs with IHPSS or EPHSS were interpreted as indicating specific characteristics of each subtype,

whereas differences shared by dogs with IHPSS or EHPSS compared with controls dogs are most likely due to secondary effects, such as the absence of normal portal vein perfusion of the liver. The main differences in mRNA gene expression were further evaluated at the protein level. On the basis of quantitative differences in both RNA and protein expression, VCAM1 may be associated with the phenotype of EHPSS, and with that of IHPSS. Functional analysis will be needed to evaluate the precise role of these genes in dogs with CPSS.

Genes of interest were initially selected on the basis of microarray analysis; about 40% of the probes on the array have not yet been annotated (CanFam 3.1). Of the 142 probes that were expressed differently in samples from dogs with EHPSS or IHPSS, 25% were not annotated. Therefore it is possible that important genes were missed because of the lack of annotation, which should be re-evaluated in the future.

A discrepancy in gene expression measured with qPCR and microarray was observed. While microarray demonstrated a significant upregulation of *HAMP* mRNA in samples from dogs with EHPSS and a significant downregulation of *HAMP* mRNA in samples from dogs with IHPSS, only the decreased HAMP in samples from dogs with IHPSS was confirmed by qPCR. Microarray analysis indicated a downregulation of *PALLD* RNA expression in samples from dogs with EHPSS, whereas qPCR indicated that *PALLD* was downregulated in samples from dogs with IHPSS. Similarly, *VCAM1* expression was upregulated in samples from dogs with IHPSS when measured by microarray, but downregulated when measured by PCR analysis and IHC. The use of a common reference pool containing only two control samples in the microarray study and the biological variation in the liver samples might be an explanation for these differences. In addition, the microarray is a semi-quantitative screening method, the results of which should be confirmed by qPCR and other methods. Data obtained with qPCR and protein-based assays are considered more reliable.

The expression of mRNA for *cysteine conjugate Beta-lyase 1* (*CCBL1*) was significantly different in samples from dogs with IHPSS compared with control dogs, whereas there was no difference in samples from dogs with EHPSS after Bonferroni correction. The expression of CCBL1 protein was significantly lower, measured by immunohistochemistry and Western blot, in samples from dogs with IHPSS or EHPSS compared to samples from control dogs. Changes in CCBL1 expression appear to be a secondary effect of portosystemic shunting, because similar differences were found in the two shunt groups compared with the control group. CCBL1 encodes an enzyme that metabolizes cysteine conjugates of halogenated alkenes and alkanes, leading to the formation of reactive metabolites that can lead to nephro- and neurotoxicity [39]. This enzyme is probably secondarily involved in CPSS in dogs and may play a role in the

pathophysiology of hepatic encephalopathy. It will be of interest to evaluate CCBL1 in diseases commonly related with hepatic encephalopathy such as cirrhosis in man and dogs.

Immunohistochemistry and Western blot confirmed the observed significant differences in the expression of VCAM1. In portosystemic shunting, venous blood flow to the liver is impaired, which could prompt the synthesis of angiogenic factors, in order to optimize blood supply to the liver. VCAM1 and integrin α4β1 are both involved in angiogenesis, with VCAM1 being expressed by proliferating vascular smooth muscle cells and integrin α4β1 being expressed by proliferating endothelial cells. Both integrin α4β1 and VCAM1 facilitate the adhesion of endothelial cells to vascular smooth muscle-like pericytes, which is essential for the survival of endothelial and mural cells during neovascularization. Antagonists of this integrin-ligand pair induce endothelial cell and pericyte apoptosis, thereby inhibiting angiogenesis [40]. We therefore anticipated that the expression of VCAM1 protein would be upregulated in the dogs with shunts, because a demand for angiogenic factors is to be expected due to the impaired development of the smaller branches of the portal vein tree in the liver 7. Surprisingly, while this protein was upregulated in dogs with IHPSS, it was not in dogs with EHPSS, consistent with the qPCR findings. Given the similar physiological consequences of IHPSS and EHPSS, we suggest that the observed difference in VCAM1 expression in these two shunt types is directly related to the cause of EHPSS. In mammals the extrahepatic portal system is formed by regression of the embryonic vitelline veins [13]. Extrahepatic shunts are considered to be erroneous connections formed between the cardinal and vitelline systems during embryonic development. EHPSS could be a secondary effect of an impaired vascular remodeling of the vitelline system. Therefore the role of VCAM1 in the regression of this system needs to be further studied. The difference in qPCR results for the two different primer sets for VCAM1 was also unexpected. Both primer sets, the microarray probe, and the antibody were designed on the basis of regions of the protein present in both transcripts annotated for VCAM1 by Ensembl. The differences may indicate the presence of additional as yet not annotated transcripts in the dog. Given the function of VCAM1 in angiogenesis and the qPCR results for samples taken intraoperatively (Figure 3A), this gene or these genes involved in its regulatory pathways could be candidate genes for causing EHPSS in dogs.

The higher expression of WEE1 mRNA in samples from dogs with IHPSS measured by microarray was confirmed by qPCR analysis, immunohistochemistry and Western blot analysis. The WEE1 gene encodes a nuclear tyrosine protein kinase. In humans, WEE1 is reported to be a negative regulator of mitosis by inhibiting tyrosine 15 phosphorylation and thereby inactivating cdc2 kinase [41]. WEE1 might also have an important role in hypoxia-induced pathological

processes in endothelial cells, such that its upregulation in endothelial cells under hypoxic conditions ensures cell viability [42]. Oxygen tension is known to play an essential role in the postnatal closure of a comparable structure, the ductus arteriosus [43]. Normal cardio-pulmonary adaptation after birth causes an oxygen saturation increase from 65% to more than 90% within the first minutes after birth [44-46]. The ductus arteriosus constricts immediately after birth, when blood oxygen tension is rising [47]. The physiological resemblance between the ductus arteriosus and the ductus venosus makes it likely that oxygen has a comparable role in the postnatal closure of these two anatomical structures. An increased expression of WEE1 might cause a protective response against altered oxygen tension, while this tension might be essential for closure of the ductus venosus as well. The owners of dogs with IHPSS did not consent to postoperative liver biopsy because of the risk and complexity of the surgical intervention. Therefore we were not able to determine expression of WEE1 after ligation of the ductus venosus and prove that it`s increase is not due to a secondary effect of the patent ductus venosus.

Conclusions

In summary, using hepatic samples from dogs with two types of portosystemic shunt with a different genetic background but identical phenotypic consequences, we managed to identify a small list of proteins possibly involved in the two anatomical anomalies. In dogs with IHPSS, WEE1 was aberrantly over expressed, which may be related to the disturbed closure of the ductus venosus. In dogs with EHPSS, decreased VCAM1 expression may play a role in the development of intrahepatic portal vascularization. It remains to be investigated whether these proteins are directly involved in the development of portosystemic shunts, or whether they manipulate downstream genes. CCBL1 may be an interesting candidate to study unresolved factors in the pathophysiology of hepatic encephalopathy.

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Chapter 5

Aberrant hepatic lipid storage and metabolism in canine portosystemic shunts

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a poorly understood multifactorial pandemic disorder. One of the hallmarks of NAFLD, hepatic steatosis, is a common feature in canine congenital portosystemic shunts. The aim of this study was to gain detailed insight into the pathogenesis of steatosis in this large animal model. Hepatic lipid accumulation, gene-expression analysis and HPLC-MS of neutral lipids and phospholipids in extrahepatic (EHPSS) and intrahepatic portosystemic shunts (IHPSS) was compared to healthy control dogs. Liver organoids of diseased dogs and healthy control dogs were incubated with palmitic- and oleic-acid, and lipid accumulation was quantified using LD540. In histological slides of shunt livers, a 12-fold increase of lipid content was detected compared to the control dogs (EHPSS $P < 0.01$; IHPSS $P = 0.042$). Involvement of lipid-related genes to steatosis in portosystemic shunting was corroborated using gene-expression profiling. Lipid analysis demonstrated different triglyceride composition and a shift towards short chain and omega-3 fatty acids in shunt versus healthy dogs, with no difference in lipid species composition between shunt types. All organoids showed a similar increase in triacylglycerols after free fatty acids enrichment. This study demonstrates that steatosis is probably secondary to canine portosystemic shunts. Unravelling the pathogenesis of this hepatic steatosis might contribute to a better understanding of steatosis in NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder in men with an estimated prevalence ranging 25% up to 45% worldwide [1]. NAFLD includes related disorders from the earliest stage hepatic steatosis, to the more progressive stage non-alcoholic steatohepatitis, of which the latter can progress to cirrhosis and hepatic cellular carcinoma [1, 2]. The pathophysiology, however, is still poorly understood and NAFLD is associated with cardiovascular disease, diabetes mellitus type 2, and chronic kidney disease [2-4]. Although murine models resemble monogenic forms of NAFLD [5], these diseases in mice are often incapable of fully mimicking the multifactorial nature of human NAFLD.

Congenital portosystemic shunts (CPSS) are vascular anomalies that connect the portal vein with the systemic circulation, causing portal blood to bypass the hepatic parenchyma [6, 7]. Although extremely rare in humans [6], CPSS occur frequently in dogs and can be divided into two subtypes; extrahepatic portosystemic shunts (EHPSS) and intrahepatic portosystemic shunts (IHPSS) [8]. The absence of normal hepatic portal blood flow leads to liver atrophy, hypoplasia of the portal vein, and hepatic encephalopathy [9-11]. Histological changes observed in CPSS include hepatocellular atrophy, enlarged portal areas, periportal sinusoidal dilatation, small or not detectable portal veins, and (peri)portal arteriole proliferation. Other findings include hepatic fibrosis, bile duct proliferation, portal lymphangiectasis, and hepatocellular steatosis [11-15].

Histological evaluation of hepatic biopsies after surgical attenuation of the shunt revealed a decrease in steatosis, suggesting steatosis in CPSS could be induced by hepatic hypoxia or a disturbed fatty acid metabolism [14]. Steatosis in CPSS dogs could be explained by a genetically determined factor [7] or by altered metabolism secondary to disease processes and the resulting hepatic injury [12, 14].

This study was performed to evaluate steatosis in canine congenital portosystemic shunting. As steatosis is observed histologically in both shunt types [11-15], we expect that hepatic steatosis occurs secondary to portosystemic shunting. In-depth analysis of the lipid metabolism of dogs with CPSS with gene- and lipid-profiling combined with organoid disease modelling will give insight in the pathogenesis of primary or secondary hepatic steatosis. This in-depth analysis might serve as a model for human steatosis as observed in NAFLD and lead to novel treatment methods for steatosis in human and veterinary medicine.

Methods

Animals and samples

Liver material was obtained from privately owned dogs with portosystemic shunts, referred to the University Clinic for Companion Animals (Department of Clinical Sciences of Companion Animals, Utrecht University). Permission was obtained from the dog owners using informed consent. CPSS was diagnosed based on increased fasting plasma ammonia levels (reference values 15-45 μmol/L) [16, 17], ultrasound visualization and classification of the shunt, and finally confirmed during surgery. Fresh wedge liver biopsies were taken during the surgical attenuation of the shunt [18]. Liver tissue from healthy dogs was used as a control in this study. These dogs were euthanized for other unrelated research, data were collected according to the Act on Veterinary Practice and the procedure was approved by the local ethics committee (DEC Utrecht), as required under Dutch legislation (ID 2007.III.08.110). Liver tissue was obtained as surplus material (University 3R policy). The absence of an underlying liver disease was confirmed histologically by a board certified veterinary pathologist. The analysis of lipid accumulation by Oil-red-O staining, mRNA expression using quantitative reversed transcriptase PCR (RT-qPCR) and the profiling of neutral lipids and phospholipids was performed on overlapping hepatic tissue of dogs with a shunt (EHPSS n = 7 and IHPSS n = 5) and compared to healthy control dogs (n = 4). For the microarray analysis a cohort of 49 samples (EHPSS n = 32, IHPSS n = 15, and control n = 2) was used. Nine cases for both IHPSS and EHPSS from this analysis were replicated in qPCR and the sample set was supplemented with 46 additional samples (EHPSS n = 19, IHPSS n = 14, and control n = 13). Hepatic tissue of 12 dogs (EHPSS, IHPSS, and healthy controls; n = 4 per group) was used for organoid culture. An overview of the sample use and overlapping samples is given in supplementary data (S1 Fig.). For Oil-red-O staining, hepatic biopsies were placed in a Tissue-Tek® cryo-molds filled with O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and frozen in liquid nitrogen until use. For RNA isolation liver samples were snap frozen in liquid nitrogen.

Oil-red-O staining

Oil-red-O staining was performed as previously described [15]. The frozen samples were cut into 8-μm sections and stained for lipids using a standard Oil-red-O (Klinipath, Duiven, The Netherlands) protocol with haematoxylin counterstaining. All Oil-red-O stained sections were evaluated blind and at random by a board-certified pathologist (GCMG), using a semi-quantitative scoring system of lipid accumulation based on lipid intensity of the stainings. Intensity grading ranged from low to high using a scale from 0 to 4. For lipid intensity analysis,

slides were scanned at 20× magnification as described previously [19]. Images were extracted using Aperio ImageScope v12.0.0.5039 (Aperio, Vista, CA, USA) as a TIFF file with jpeg compression. The images were resized to 10% of their original size for digital analysis. Data of ten random snapshots were collected. The RGB data of the images was converted to a 2-bit black/white image based on thresholding the color of the dye using ImageJ (NIH; http://rsb.info.nih.gov/ij/) software. An average of the black:white ratio was calculated to determine lipid intensity scores.

Expression profiling

Previously published microarray expression data on IHPSS and EHPSS liver tissue [20], available through GEO Series accession number GSE39005 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?-acc= GSE39005), was used to determine the 20 most up and 20 most down regulated genes in both EHPSS and IHPSS versus control dogs. A corresponding list of lipid related genes for IHPSS and EHPSS was selected for further confirmation. Genes with log2-fold changes of more than 1.1 or less than -1.5 were selected to ensure that only robust differences were considered. Involvement of these genes in lipid metabolism, transport, or storage was determined based on Gene Ontology biological processes and literature.

Gene expression differences of 11 selected genes was confirmed using RT-qPCR on available cDNA obtained using the iScript™ cDNA synthesis kit as described by the manufacturers protocol (Bio-Rad, Veenendaal, The Netherlands). Primer design, validation, RT-qPCR conditions, and data analysis were performed as described previously [20]. Normalization was performed using four reference-genes; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S5 (*RPS5*), heterogeneous nuclear ribonucleoprotein H (*HNRPH*), and ribosomal protein S19 (*RPS19*) [20] as required under MIQE-precise [21]. Details of the primers are listed in S1 Table.

Analysis of neutral- and phospholipids by high-performance liquid chromatography-mass spectrometry (HPLC-MS)

Lipids were isolated from frozen tissue by the method of Bligh and Dyer [22] and separated in a neutral lipids and phospholipid fraction on a freshly prepared silica-G column (approximately 10 mg of 0.063–0.200 mm silica (Sigma-Aldrich, St Louis, MO, USA) [23]. Neutral lipids were eluted with two volumes acetone, dried under nitrogen gas and stored at –20 °C. Just before HPLC-MS analysis, the neutral lipid fraction was reconstituted in methanol:chloroform (1:1 v/v) and separated on a Kinetex/HALO C8-e column (2.6 µm, 150 × 3.00 mm; Phenomenex, Torrance, CA, USA). A gradient was generated from methanol:H_2O (5:5 v/v) and methanol:isopropanol (8:2

v/v) at a constant flow rate of 0.3 ml/min. Mass spectrometry of neutral lipids (triacylglycerols (TAGs) and cholesterol) was performed using positive mode Atmospheric Pressure Chemical Ionization (APCI) on a LTQ-XL mass spectrometer (Thermo, Waltham, MA, USA). Separation of phospholipid classes was performed as described elsewhere [24].

Isolation of canine biliary duct fragments and culture of liver organoids

Organoids were isolated and cultured as described before [25]. In short, liver tissue was dissected mechanically and digested in DMEM medium with 1% v/v FBS (Gibco, Fischer Scientific, Landsmeer, The Netherlands) containing 0.3 mg/ml type II collagenase (Gibco) and 0.3 mg/ml dispase (Gibco) at 37 °C for a total of 3-5 hours. The isolated ducts were then mixed with Matrigel (BD Biosciences, Breda, The Netherlands) and seeded. Culture medium was added after gelation of the Matrigel. Culture media was based on Advanced DMEM/F12 (Invitrogen, Bleiswijk, The Netherlands) supplemented with 2% v/v B27 (Invitrogen), 1% v/v N2 (Invitrogen), 1.25 µM N-acethylcysteine (Sigma-Aldrich), 10 nM gastrin (Sigma-Aldrich), 200 ng/ml EGF (Invitrogen), 5% v/v Rspo1 conditioned medium (the Rspo1-Fc-expressing cell line was a kind gift from Dr. Calvin J. Kuo, Stanford, CA), 100 ng/ml FGF10 (Peprotech, Tebu-bio, Heerhugowaard, The Netherlands), 10 mM nicotinamide (Sigma-Aldrich), 25 ng/ml HGF (Peprotech), 100 ng/ml Noggin (Peprotech), 30% v/v Wnt3a conditioned medium (prepared as in [26], 10 µM Y-27632 2HCl (ROCK inhibitor, Selleckchem, Bio-Connect B.V., Huissen, The Netherlands), and 0.5 µM TGFβ inhibitor (A83-01, Tocris Bioscience, Abingdon, UK) grown at 37 °C with 5% CO_2 in a humidified incubator. Organoids were split by removal from Matrigel using cold Advanced DMEM/F12, mechanical dissociation into smaller fragments, and transfer into fresh Matrigel. Passage was performed weekly at a 1:4-1:8 split ratio. Medium was changed every other day.

Treatment of organoids with free fatty acids (FFA)

Oleic acid (C18:1) and palmitic acid (C16:0) (both from Sigma-Aldrich) were conjugated with fatty acid free bovine serum albumin (BSA) (Sigma-Aldrich), molar ratio of 5:1, to a final concentration of 10 mM. Organoids were cultured in a 12-wells plate (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands), and treated with 0.4 mM oleate/BSA- and 0.2 mM palmitate/BSA-complexes in culture media (without Wnt3a, Y-27632, A83, EGF and Noggin) for 24 h at 37 °C with 5% CO_2 in a humidified incubator. Treatment with fatty acid free BSA alone (12% w/v) served as a control.

Flow cytometry analysis

After a 24 h incubation with FFA, organoids were collected from the Matrigel with cold advanced DMEM/F12 (Gibco), and subsequently trypsinised with 10x Trypsin (Gibco) containing 0.5 mg/ml DNAse (Sigma). Advanced DMEM/F12 with 10% FCS was added and the cell suspension was spun at 250 g for 5 min at 4 °C. Pellets were resuspended in advanced DMEM/F12. Organoids were incubated with 5 µg/ml LD540 (lipophilic dye, kindly provided by prof. Christoph Thiele, Bonn, Germany) for microscopic imaging of lipid droplets [27] in DMEM medium containing 20 µg/ml fatty acid free BSA, 10µg/ml HEPES (Gibco), and 10 µg/ml Glutamax (Gibco) for 30 min in a water bath at 37 °C. Incubations without LD540 in FFA medium served as a control. Cells were washed twice with HBSS (Gibco) and cells were resuspended in HBSS containing 20 µg/ml fatty acid free BSA, 1 µg/ml HEPES and 1 µg/ml Sytox Red (Molecular probes, Thermo Fisher, Bleiswijk, The Netherlands). Cell analysis was performed on a 488-laser LSRFortessa flow cytometer (Becton Dickinson, Erembodegem, Belgium). A 540/30 nm bandpass filter was installed to measure the optimum of the LD540 emission peak. Fluorescently labelled beads (CS&T beads, Becton Dickinson) were used to check the performance and verify optical path and stream flow of the flow cytometer. Dead cells were excluded with Sytox red using a 635 nm laser with an emission spectrum of 670/30 nm.

Whole mount imaging

For whole mount fluorescent staining canine liver organoids were carefully harvested from Matrigel and fixed in 10% v/v neutral buffered formalin (Klinipath) for 45 min on ice. Fixed organoids were incubated in 0.025 µg/µL LD540 in PBS for 1 h at room temperature. After washing, nuclei were stained with DAPI and organoids were mounted with ProLong Diamond Antifade mounting medium (Life Technologies, Thermo Fisher) and imaged using a confocal microscope (Leica SPE-II).

Statistical analysis

Oil-red-O differences in scoring were evaluated using a Student T-test. P values < 0.05 were considered significant. The results of the microarray analysis were reanalyzed with updated annotations using ANOVA (R version 2.2.1/MAANOVA version 0.98) [28]. Correction for multiple testing (Permutation F2-test using 5,000 permutations) was performed and P < 0.05 was considered statistically significant. In RT-qPCR log-values of normalized relative expression were used to obtain a normal distribution. A Levene's test was used to determine if the data was normally distributed. A Kruskal-Wallis test was performed to observe differences between the EHPSS, IHPSS, and control group and was performed in case of multiple group testing. Any observed differences were confirmed by a Mann-Whitney U test on independent samples.

Statistical significance was obtained if $P < 0.01$. Processing of the LC-MS data of neutral lipids and phospholipids was performed with XCMS under R version 3.0.2 [29, 30]. Principal component analysis (PCA) was performed with the R package 'PCAMethods' using the nonlinear iterative partial least squares (nipals) algorithm with pareto scaling [31]. Differences in lipid accumulation after free fatty acid incubation between the EHPSS, IHPSS, and wildtype organoids measured using flow cytometry analysis, were calculated with a Kruskall-Wallis test. Observed differences were confirmed by a Mann-Whitney U test on independent samples.

Results

Difference in lipid accumulation between healthy and shunts liver biopsies

Oil-red-O staining for neutral lipid accumulation was increased in EHPSS and IHPSS slides compared to livers of healthy control dogs (Fig 1A). Image J quantification revealed a 12-fold increased staining intensity in both shunt types (EHPSS $P < 0.01$; IHPSS $P < 0.05$) (Fig 1B) compared to the samples of healthy control dogs in our Dutch cohort. Semi-quantitative analysis of these samples confirmed the higher hepatic neutral lipid levels in EHPSS ($P < 0.01$) and IHPSS ($P < 0.05$) compared to healthy dogs (S2 Fig).

Similar gene-expression patterns of lipid related genes in both shunt types

Data retrieved from previously published data sets revealed that similar genes were differentially expressed in both shunts compared to samples of healthy dogs in the microarray analysis (S2 Table). Interestingly 11 out of the selected top 24 differentially expressed genes are related to lipid-metabolism, -transport, or -storage emphasizing the importance of the altered lipid metabolism here. Nine gene products (*CBR2*, *CRP*, *ELOVL2*, *FABP1*, *IGFBP1*, *ITIH3*, *ITIH4*, *PLIN2*, and *SAA1*) were significantly upregulated in both shunt groups whereas *HSD3B* and *SEC14L3* were significantly downregulated compared to healthy control dogs in the microarray analysis. The expression of these 11 genes was validated by RT-qPCR in an independent cohort. For technical reasons, no RT-qPCR data could be obtained for *CBR2*. Due to difficulties in primer design for *ELOVL2* but the interest in this gene, primers were ordered for *ELOVL5* and *ELOVL6* to gain information about the *ELOVL2* pathway. The RT-qPCR analysis confirmed the microarray results for seven genes of interest ($P < 0.01$) , namely *CRP*, *FABP1*, *HSD3B*, *IGFBP1*, *ITIH4*, *PLIN2*, and *SAA1* (Fig 2). *ELOVL2-pathway, ITIH3,* and *SEC14L3* expression levels were not significantly different in the validation cohort.

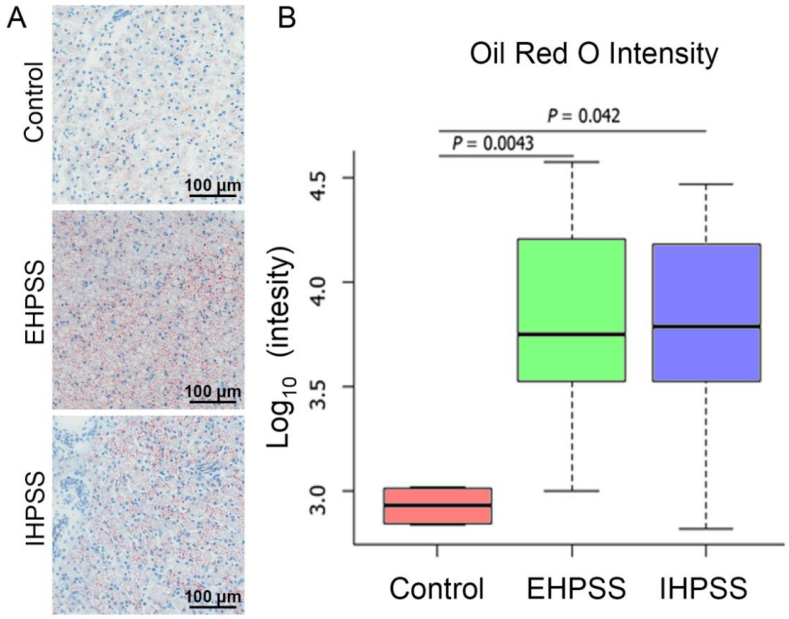

Figure 1 Average lipid intensity using an Oil-red-O staining in hepatic tissue of control, EHPSS and IHPSS dogs. Representative pictures of the hepatic samples from healthy dogs (n = 4), dogs with extrahepatic portosystemic shunts (EHPSS, n = 7), and intrahepatic portosystemic shunts (IHPSS, n = 5) are displayed left (A). The average Oil-red-O intensity is displayed in Log10 per sample group, representing neutral lipid staining in the observed liver samples calculated with a Students T-test and P < 0.05 was considered significant (B).

Analysis of neutral- and phospholipids reveal similar lipid species in both shunt types

To examine qualitative changes of lipid profiles during the lipid accumulation in liver of shunt dogs, neutral lipids and phospholipids were analysed by HPLC-MS. Examples of such analyses are given in supplementary data (S3 and S4 Figs respectively). For phospholipids, we observed a clear change in the species profiles of shunt dogs compared to healthy control dogs (Fig 3 and S3 Table). The phospholipidomes of IHPSS and IHPSS were not distinguisable, as can be concluded from the overlap of these samples in the PCA score plot (Fig 3A). When evaluating the phospholipid classes and phospholipid chain length between shunt types or healthy controldogs, no differences are observed (S5 Fig). Interestingly, dogs with a shunt had higher levels of hexaenoic (6 unsaturations per molecule) phospholipid species (Fig 3C) at the expense of tetraenoic (4 double bonds) and, to a lesser extent, pentaenoic (5 double bonds) species compared to the healthy control dogs. From the PCA loading plot (Fig 3B), it can be deduced that this effect is shared among all major phospholipid classes. Arachidonic acid (AA; 20:4, Ω-6) containing species PS 38:4, PC 38:4 and to a lesser extend PE 38:4 and PI 38:4 are located towards the bottom left quadrant of the PCA scores plot (Fig 3B) whereas the docosahexaenoic

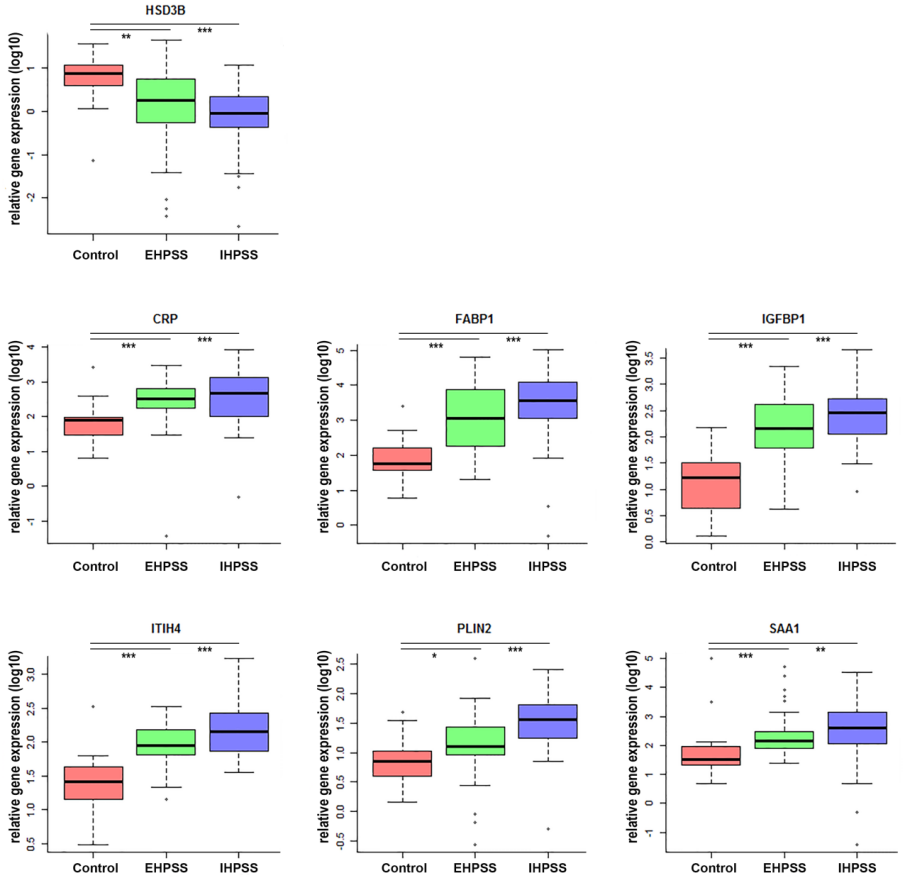

Figure 2 Validated lipid related genes by RT-qPCR in a validation cohort. Boxplots representing relative gene expression lipid related genes validated with RT-qPCR in a validation cohort of diseased dogs (n = 63). Gene expression of selected genes displayed per group: control dogs (C, n = 17), extrahepatic- (EH, n = 35), and intrahepatic portosystemic shunt (IH, n = 28). Significant difference between groups is presented as a line with *** ($P \leq 0.001$), ** ($0.001 < P \leq 0.01$), or * ($0.01 < P < 0.05$).

acid (DHA; 22:6, Ω-3) containing species PS 40:6, PE 38:6 and PC 38:6 are in the top right corner of Fig 3B. The bottom left quadrant (enriched in 20:4, Ω-6) and top right quadrant (enriched in 22:6, Ω-3) correspond to the locations of the control and shunt dogs in the PCA score plots, respectively (Fig 3A). For neutral lipids, a shift towards shorter chain fatty acids (C16:n) is observed in livers of shunt dogs at the expense of more extended fatty acids (C18:n) measured in tissue of healthy dogs (Fig 4 and S4 Table). No difference is observed in TAGs with a chain length of C56 or above between the groups (Fig 4C).

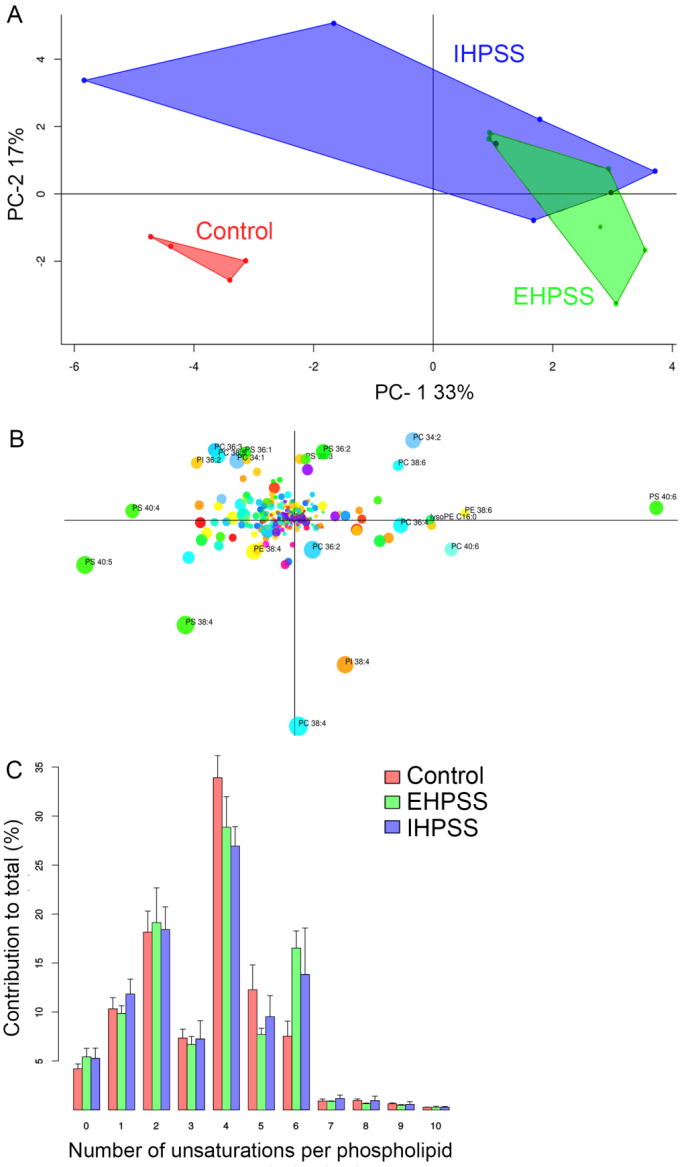

Figure 3 Principal Component Analysis of phospholipid species. PCA of phospholipid species in hepatic biopsies of healthy (red, n = 4), EHPSS (green, n = 7), and IHPSS (blue, (n = 5) dogs. Resulting scores of the samples (A) using the calculated loadings (B). Lipids are colored according to their lipid class and dot sizes correspond to relative abundance. Degree of unsaturation found in the acyl chains of PL (C). Note the higher levels of acyl chains with four unsaturations in control dogs, at the expense of acyl chains with six unsaturations.

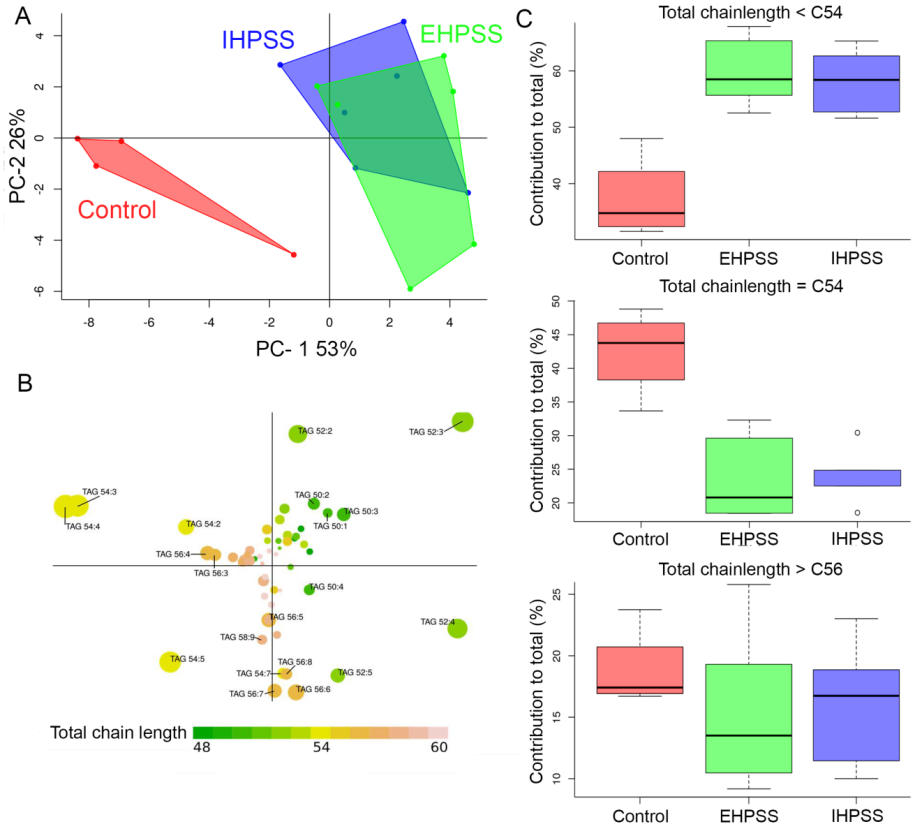

Figure 4 Principal component analysis of neutral lipid species. Representation of the PCA of neutral lipid species in hepatic biopsies of healthy (red, *n* = 4), EHPSS (green, *n* = 7), and IHPSS (blue, *n* = 5) dogs (A). A clear overlap in the TAG part of the lipidome, indicating similarity, is present in EHPSS and IHPSS samples. Calculated loadings of individual TAG species (B) leading to the score plot (A). TAG species are color coded based on the total number of carbon atoms in the acyl chains of TAG species. Note how control dogs have more TAG species with 54 carbon atoms, but less species with shorter acyl chains than dogs with EHPSS or IHPPS (C).

Similar TAG accumulation in organoids from healthy and shunt livers

Triacylglycerol accumulation in hepatic organoids was evaluated by whole mount LD540 staining. Microscopically, the LD540 accumulation in lipid droplets, displayed in a single section, was more pronounced in the organoids cultured with FFA supplementation and revealed little differences between healthy and both CPSS canine liver organoids regarding basal fatty acid uptake (control medium) or in medium supplemented with FFA (Fig 5A). In order to quantify the total TAG accumulation flow cytometry analysis of whole organoids was performed. The relative increase in median LD540 fluorescence after FFA enrichment was similar in the

healthy and shunt groups (Fig 5B) indicating that there were no differences in total TAG accumulation.

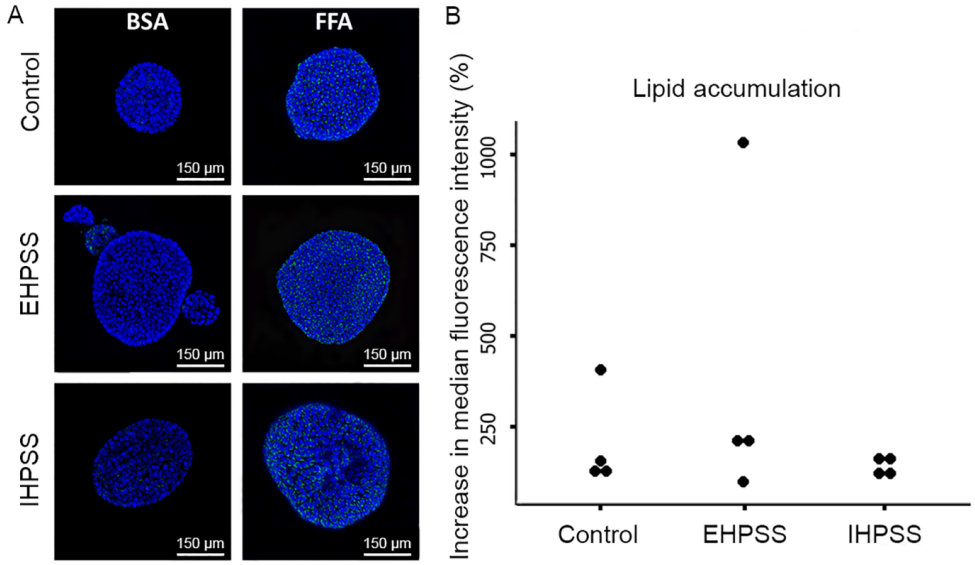

Figure 5 Organoid LD540 fluorescent whole-mount staining. Representative images of LD540 fluorescent whole-mount staining of control (n = 4), extrahepatic (EHPSS, n = 4) and intrahepatic (IHPSS, n = 4) canine organoids cultured in a bovine serum albumin (BSA) medium (control) or the enriched free fatty-acid (FFA) medium for 24 hours (A). Blue represents DAPI – nuclei, green represents LD540 labelled TAGs. The accumulation of LD540 in lipid droplets is more pronounced in the FFA cultured organoids as displayed in a section (A). Quantitative measurement of the total intracellular lipid accumulation was ascertained by flow cytometry analysis (B). Data is expressed as percentage increase in median fluorescent intensity of cells in FFA containing media compared to BSA control (n = 4).

Discussion

This study provides detailed insight into the pathogenesis of hepatic steatosis in CPSS dogs by examination of the quantity of accumulating lipids, mRNA expression of genes involved in lipid metabolism, and lipid profiles in both EHPSS and IHPSS compared to control dogs. To further support our hypothesis, canine organoids of EHPSS, IHPSS and healthy controls demonstrated a similar increase in TAGs after free fatty acids enrichment. Importantly, the results obtained in this study elucidate aspects of steatosis in portosystemic shunting, demonstrate that hepatic steatosis observed in CPSS occurs presumably secondary to portosystemic shunting and finally provide a model to study steatosis in human medicine.

The complex pathogenic mechanisms underlying NAFLD are influenced by multiple factors including genetic, hormonal and nutritional factors [2]. For instance, a missense mutation in human PNPLA3 (patatin-like phospholipase domain-containing 3 protein) is associated with increased hepatic fat deposition and hepatic inflammation, which makes the liver more susceptible for NAFLD [32]. In dogs, hepatic steatosis featured by vacuolar changes within hepatocytes and the presence of lipogranulomas, is a frequently observed finding in liver biopsies of dogs with CPSS [11-13, 15]. Once a shunt is attenuated causing restauration of the normal liver blood flow, the lipid accumulation seems to be reversible over time [14]. Only a few descriptive studies have investigated this phenomenon in dogs [15, 33, 34], but none studied the pathophysiology of steatosis in CPSS.

As extra- and intrahepatic portosystemic shunts have a different genetic background [7], the similarity in the quantity of lipids, gene-expression, and lipid profiles suggests lipid accumulation to be a secondary effect of portosystemic shunting. In addition, the FFA-supplemented organoid cultures did not reveal differences between shunt derived organoids (EHPSS or IHPSS) strengthening the idea of a secondary effect. Age could be an influencing factor on the degree of steatosis in CPSS as the incidence of lipogranulomas (LG) seems to be greater in age-matched dogs with CPSS compared to healthy dogs and LG are generally less observed in dogs under one year of age [33, 34]. Another explanation for steatosis occurring secondary to CPSS could be plasma ammonia levels. CPSS are the most frequent cause of hyperammonemia in dogs which, when left untreated, results in hepatic encephalopathy [35]. In case of hyperammonemia, the ammonia is postulated to accumulate in lysosomes. Consequently intralysosomal pH will raise, thereby inhibiting lysosomal enzymes involved in proteolysis and lipid degradation [36, 37]. The reduced breakdown of proteins and lipids can contribute to the progressive abnormalities in the brain in case of hepatic encephalopathy [38]. Therefore, the presence of the biomarker ammonia in CPSS [39] might be causative for the secondary lipid accumulations observed in portosystemic shunting. For two reasons it is not likely that insulin resistance, an important factor in the pathogenesis of NAFLD [2], plays a role in steatosis in CPSS dogs. Insulin resistance is associated with hyperinsulinemia [2] and low circulating IGFBP1 levels. Insulin levels vary greatly among diseased dogs [40] and *IGFBP1* levels in this study are induced (Fig 2) rather than reduced. Taken together, the presented data together suggests that steatosis in CPSS dogs is a secondary phenomenon in portosystemic shunting possibly influenced by hyperammonemia.

Gene expression profiling and RT-qPCR validation corroborated the importance of seven lipid related genes to both subtypes of portosystemic shunting. The *FABPI* and *PLIN2* upregulation in

extra- and intrahepatic shunts (Fig 2) is probably associated with their lipid related functions and the lipid accumulation in CPSS. *FABP1* serves as a key regulator of hepatic lipid metabolism by enhancing the cellular uptake, transport, and metabolism of fatty-acids [41]. Notably, this protein can bind bile acids which are often increased in CPSS [41]. Increased *PLIN2* expression improves cellular lipid accumulation and regulates (phospho)lipid exchange from lipid droplets [42]. A down-regulation of *HSDB3* was observed, in line with our data, in granulosa cells when FFA concentrations increased [43]. Whether this hepatic down-regulation is directly correlated with FFA content needs to be investigated.

Interestingly, three of the six upregulated lipid related genes (*i.e. CRP*, *ITIH4*, and *SAA1*), serve as acute phase reactants (APR) which are secreted in response to a variety of acute and chronic inflammatory conditions, in particular regulated by IL-6. CRP is an important nonspecific biochemical marker of inflammation which is synthesized in both liver as well in adipose tissue in the presence of obesity [44]. Besides its role as APR, ITIH4 is associated with hypercholesterolemia [45] and may play an important role in liver regeneration [46]. SAA1 is associated with high-density lipoprotein metabolism, and cholesterol metabolism and transport [47]. This protein is also involved in the formation of amyloid deposits in feline and canine hepatic amyloidosis, triggered by inflammatory conditions [48]. Based on these results, the question raises whether the upregulation of these genes in portosystemic shunting is solely caused by an altered lipid metabolism *per se* or as a consequence of the inflammatory state [49]. The lack of an inflammatory component in hepatic biopsies of CPSS dogs argues in favour of a direct effect of lipid accumulation.

Since the liver plays a vital role in lipid metabolism, any disturbance in the fatty acids and triglycerides pathways leads to an imbalance in the lipid metabolism resulting in hepatic steatosis, and eventually steatohepatitis [50-52]. A plethora of biological effects of omega-3 and -6 fatty acids have been described [53]. Remarkable is the shift towards omega-3 fatty acids at the expense of the omega-6 fatty acids in particular the shift of AA (20:4, Ω-6) to DHA (22:6, Ω-3) in shunt dogs in comparison to healthy control dogs. AA is the main precursor of eicosanoids, which modulate the immune response via a diversity of pathways [54]. Dietary DHA has the capacity to suppress markers of hepatic damage, hepatic inflammation, oxidative stress and fibrosis in $LDLR^{-/-}$ mouse with induced non-alcoholic steatohepatitis [55] and is reported to be beneficial in hepatic encephalopathy [56]. The altered lipid metabolism in elevated levels of DHA in shunt livers compared to healthy control dogs, might therefore be a protective response.

Due to population bottlenecks and inbreeding during the formation of the contemporary dog breeds, canines have a limited phenotypic and genetic diversity which makes the dog population

ideal for exploring the genetic basis of a variety of naturally occurring diseases [57-59]. The dog has been proposed as a useful model to study inherited diseases in both canine and human research [60], since they are remarkably similar between canine and human diseases based on phenotypic presentation. This also holds true for CPSS, the same subtypes for intrahepatic and extrahepatic shunts in dogs have been recognized in man, although at a much lower frequency in humans [8, 61]. Histological features observed in dogs with CPSS are comparable to human [62] and rats with an induced portacaval shunt [63]. The high prevalence but poorly understood pathogenesis of NAFLD urges the search for reproducible and predictive disease model systems. Therefore, studying lipid loading in CPSS dogs , could in a further stadium serve as a model to study the pathophysiology of steatosis and/or novel treatment modalities preventing lipid accumulation.

In conclusion, this study describes excessive hepatic lipid accumulation in portosystemic shunting. Gene expression profiling indicated that the majority of genes changed in CPSS were involved in lipid metabolism. Different TAG composition and a shift in short chain and omega-3 fatty acids were observed in shunt dogs compared to healthy animals. Despite a different genetic background of extra- and intrahepatic shunts, lipid species observed in both shunt types were almost identical. As cultured organoids derived from healthy and diseased animals accumulate TAGs equally, we suggest that lipid accumulation as observed in shunt livers appears not to be related to primary gene defects in liver shunts, but rather be caused by a secondary effect, possibly ammonia related. Histological features observed in dogs with CPSS are comparable to human [62] and rats with an induced portacaval shunt [63]. Since lipid accumulation is a natural phenomenon in CPSS dogs, these animals might represent a simplified NALFD model.

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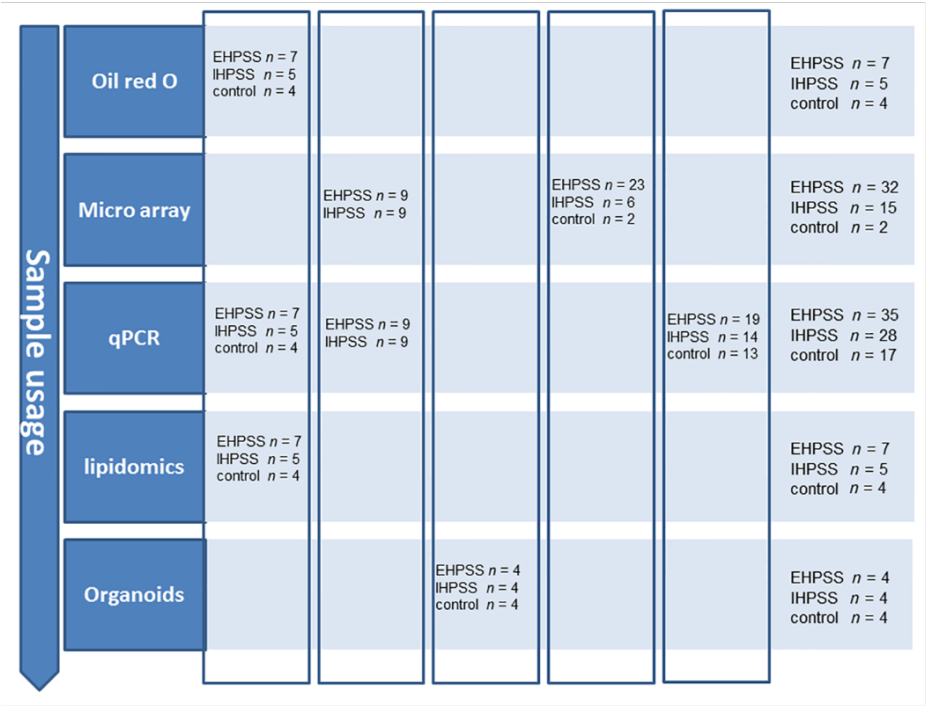
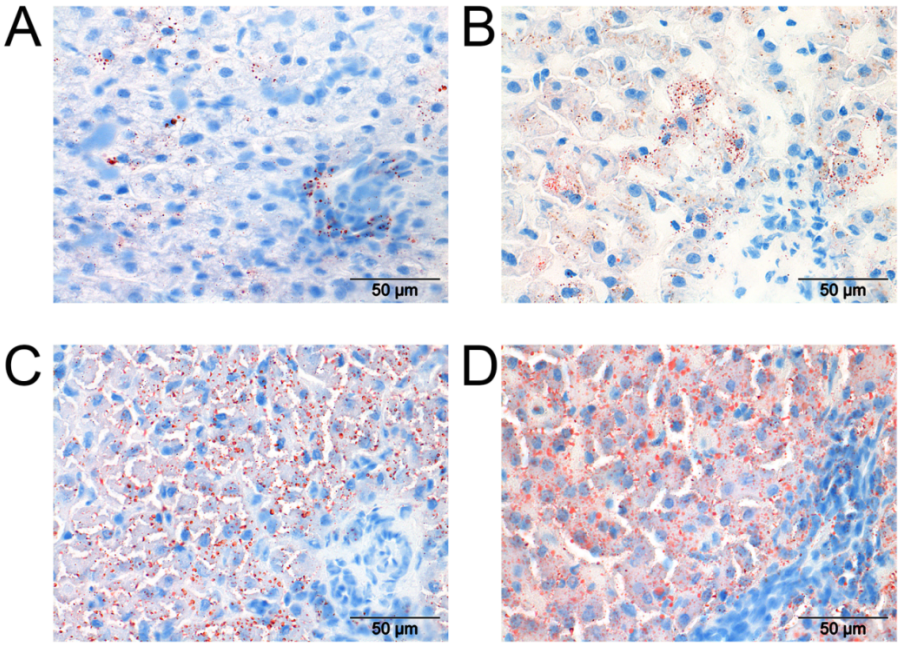
Supporting information

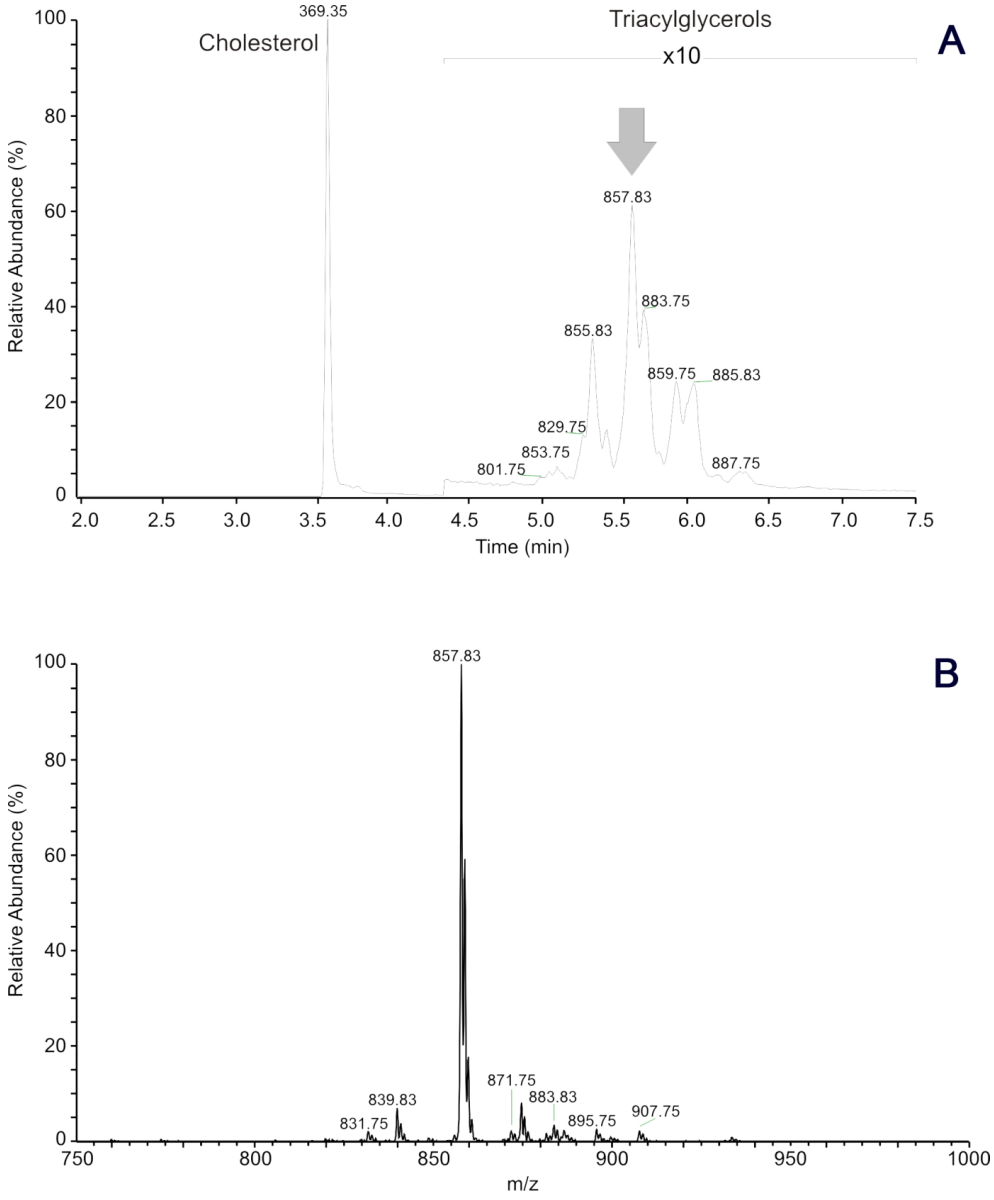
Supplemental figure legends



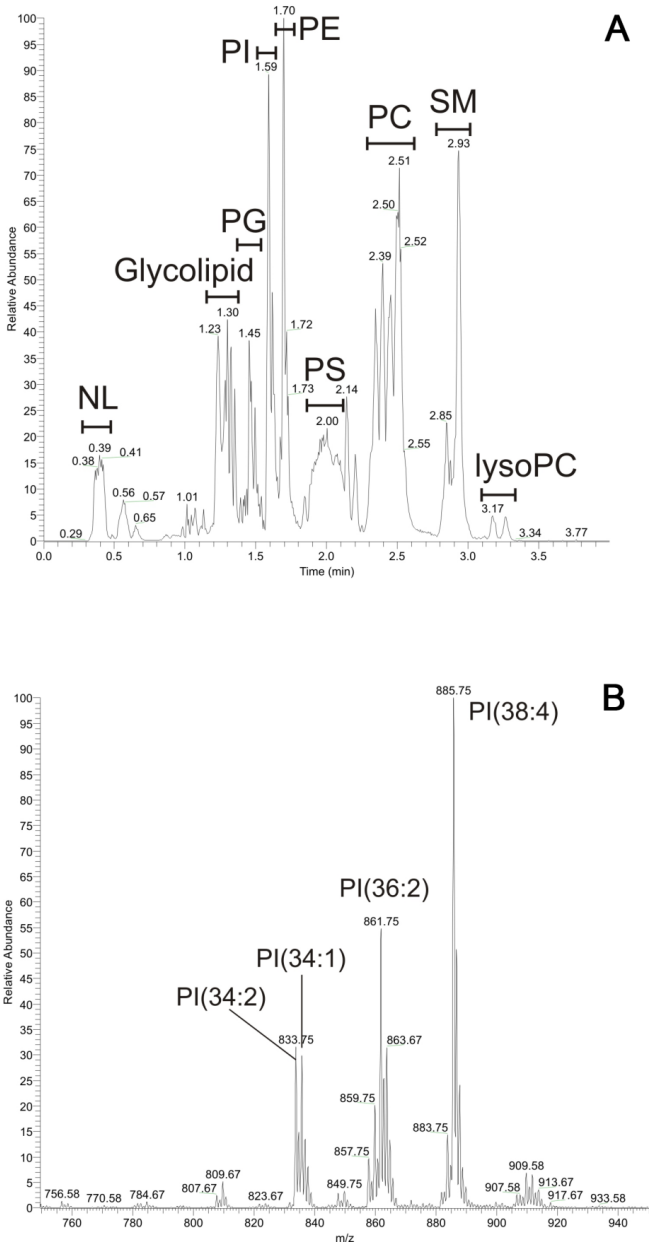
S1 Figure Overlap of samples used in different experiments. Identical cohorts indicated in boxed columns have been used in different experiments.



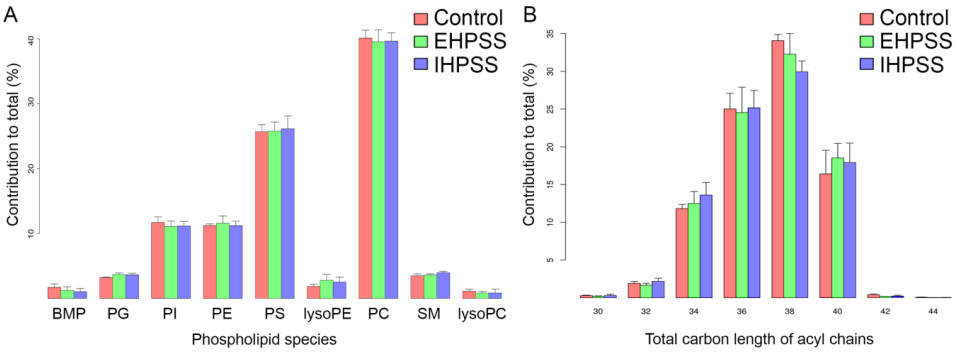
S2 Figure Example of the semi-quantitative scoring system of lipid staining by Oil-red-O. Oil-red-O staining of CPSS and control tissue in the semi-quantitative scoring system graded from low (0) to remarkable high (4). Pictured are examples of mild (1) lipid staining (A), moderate (2) lipid staining (B), severe (3) lipid staining (C), and remarkable high (4) lipid staining (D). The semi-quantitative analysis indicated a significantly higher lipid intensity in liver tissue of dogs with either EHPSS ($P < 0.01$) or IHPSS ($P < 0.05$) compared to healthy dogs.



S3 Figure Example of HPLC-MS analysis of neutral lipids. Base peak chromatogram of the LCMS analysis of neutral lipids, showing the partial separation of TAG molecular species (A). Coeluting TAG species can be identified in the MS spectrum (B). The spectrum in the bottom panel was recorded at the timepoint indicated by an arrow in the top panel. The m/z signals correspond to TAG species as listed in S4 Table.



S4 Figure Example of HPLC-MS analysis of phospholipids. Base peak chromatogram recorded during the separation of phospholipid classes by hydrophilic interaction liquid chromatography (HILIC) (A). Lipid species contributing to a lipid class can be inferred from the mass spectrum recorded during elution as illustrated for PI (B). Total phospholipid profiles are listed in "S3 Table".



S5 Figure Phospholipid species (A) and total carbon length of the acyl chains (B). In phospholipid analysis no differences in chain length or classes between shunt types or healthy control are observed. BMP, bis-monoacylglycerol phosphate; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; SM, Sphingomyelin.

Supplemental Tables

S1 Table Primers used for quantitative real-time PCR (RT-qPCR).

Gene	Ensemble Transcript ID	F/R	Sequence	Tm (°C)	Amplicon size (bp)
CRP	ENSCAFT00000018706	F	5'-GGTCCTCATGAACCTCC-3'	65	161
		R	5'-GTCAAGTCCGTATAGACCT-3'		
ELOVL5	ENSCAFT00000044079	F	5'-CTGTGAGTTAGTGACGGGA-3'	65	110
		R	5'-TAGTACCACCAGAGGACAC-3'		
ELOVL6	ENSCAFT00000018328	F	5'-CAAAGCACCCGAACTAGGA-3'	59	101
		R	5'-CAGGAGTACAGGAGCACAG-3'		
FABP1	ENSCAFT00000011880	F	5'-GTTCCAAAGTGATCCAGAATGAG-3'	63	107
		R	5'-GCTTATTGTCACCTTCCATCTG-3'		
HSD3B	ENSCAFT00000015969	F	5'-CAGTTGTCATTCACACCG-3'	62	87
		R	5'-TGAGTACCCTTCAGATTGAC-3'		
IGFBP1	ENSCAFT00000019512	F	5'-AGATCCGACGACTCCGA-3'	60.5	196
		R	5'-TACACGCACCAGCAGAG-3'		
ITIH3	ENSCAFT00000023969	F	5'-CTTCATCATCCAAGTTCCGCA-3	62	149
		R	5'-GAGTTAGAGTCGCCTCCCT-3'		
ITIH4	ENSCAFT00000039621	F	5'-CGAATGCCCTCACCATCTC-3'	59	106
		R	5'-GACAATGAAGTTGCCATCCAG-3'		
PLIN2	ENSCAFT00000002516	F	5'-AATGCACTCACCAAATCAG-3'	64	105
		R	5'-TCTGAACTGTATCAAACCCT-3'		
SAA1	ENSCAFT00000014555	F	5'-TTTCTGTTCCTTGGTCCTG-3'	62	141
		R	5'-GGCATGGAAGTATTTGTCTG-3'		
SEC14L3	ENSCAFT00000020256	F	5'-AAGTCCATGTATGTGCGG-3'	63	131
		R	5'-AGATGAGAACTGCCACCT-3'		
GAPDH	ENSCAFT00000037560	F	5′-TGTCCCCACCCCCAATGTATC-3′	58	100
		R	5′-CTCCGATGCCTGCTTCACTACCTT-3′		
HNRPH	ENSCAFT00000028063	F	5′-CTCACTATGATCCACCACG-3′	61	151
		R	5′-TAGCCTCCATAACCTCCAC-3′		
RPS19	ENSCAFT00000008009	F	5′-CCTTCCTCAAAAAGTCTGGG-3′	61	95
		R	5′-GTTCTCATCGTAGGGAGCAAG-3′		
RPS5	ENSCAFT00000003710	F	5′-TCACTGGTGAGAACCCCCT-3′	62.5	141
		R	5′-CCTGATTCACACGGCGTAG-3′		

CRP, C-reactive protein; ELOVL5, ELOVL fatty acid elongase 5; ELOVL6, ELOVL fatty acid elongase 6; FABP1, Fatty acid binding protein 1; HSD3B, Hydroxy-delta-5-steroid dehydrogenase 3-beta; IGFBP1, Insulin-like growth factor binding protein 1; ITIH3, Inter-alpha-trypsin inhibitor heavy chain 3; ITIH4, Inter-alpha-trypsin inhibitor heavy chain 4; PLIN2, Perilipin 2; SAA1, Serum amyloid A1; SEC14L3, SEC14-like lipid binding 3; GAPDH, Glyceraldehyde-3-phosphatedehydrogenase; HNRPH, Heterogeneous nuclear ribonucleoprotein H; RPS19, Ribosomal protein S19; RPS5, Ribosomal protein S5.

S2 Table Gene list.

Gene	Ensemble Transcript ID	Description	EH MA	IH MA	EH qPCR	IH qPCR
PLIN2	ENSCAFG00000001601	perilipin 2	2.47	2.03	1.82	5.24
ALDH1B1	ENSCAFG00000002400	aldehyde dehydrogenase 1 family member B1	-1.80	-1.82	NA	NA
MGAM	ENSCAFG00000003841	maltase-glucoamylase	-1.76	-1.78	NA	NA
FCGBP	ENSCAFG00000005406	Fc fragment of IgG binding protein	-1.48	-1.60	NA	NA
FABP1	ENSCAFG00000007413	fatty acid binding protein 1, liver	4.36	3.67	20.36	66.46
ELOVL2	ENSCAFG00000009756	ELOVL fatty acid elongase 2	2.17	1.84	N.A.	N.A.
ELOVL5	ENSCAFG00000002276	ELOVL fatty acid elongase 5	N.A.	N.A.	1.03	1.19
ELOVL6	ENSCAFG00000011549	ELOVL fatty acid elongase 6	N.A.	N.A.	1.29	-1.25
HSD3B2	ENSCAFG00000010039	3-beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase	-2.21	-1.67	-4.18	-8.60
CRP	ENSCAFG00000011787	C-reactive protein, pentraxin-related	2.92	2.36	4.26	5.95
IGFBP1	ENSCAFG00000012272	insulin like growth factor binding protein 1	3.39	3.08	8.50	17.52
DMBT1	ENSCAFG00000012561	deleted in malignant brain tumors 1	-2.48	-2.63	NA	NA
SEC14L3	ENSCAFG00000012757	SEC14-like lipid binding 3	-1.53	-2.51	-2.35	-2.28
NNMT	ENSCAFG00000013528	Nicotinamide N-Methyltransferase	-3.74	-2.06	NA	NA
CBR1	ENSCAFG00000014444	Uncharacterized protein	-1.71	-1.89	NA	NA
ITIH3	ENSCAFG00000015068	inter-alpha-trypsin inhibitor heavy chain 3	2.35	1.87	1.73	1.77
SAA1	ENSCAFG00000015205	Serum amyloid A protein	2.04	1.01	4.43	12.99
RGS7	ENSCAFG00000015679	regulator of G-protein signaling 7	-1.74	-1.71	NA	NA
CYP1A2	ENSCAFG00000017941	cytochrome P450 family 1 subfamily A member 2	-2.20	-1.72	NA	NA
CCL4	ENSCAFG00000018164	Chemokine (C-C motif) ligand 4	-1.32	-1.61	NA	NA
GSTM3	ENSCAFG00000019809	glutathione S-transferase mu 3 (brain)	-2.21	-1.72	NA	NA
GSTM4	ENSCAFG00000019812	glutathione S-transferase mu 4	-1.62	-1.65	NA	NA
COX2	ENSCAFG00000022726	Cytochrome c oxidase subunit 2	-1.51	-1.65	NA	NA
CA3	ENSCAFG00000025237	carbonic anhydrase III	-3.52	-2.94	NA	NA
ITIH4	ENSCAFG00000025533	inter-alpha-trypsin inhibitor heavy chain family member 4	3.11	2.44	3.38	5.34
IL33	ENSCAFG00000030105	interleukin 33	-2.35	-1.98	NA	NA

Top 24 list of most up and down regulated genes by gene-expression profiling of hepatic tissue of dogs with a congenital portosystemic shunt compared to healthy liver samples. Fold change of the microarray (MA) and quantitative reversed transcriptase PCR (RT-qPCR) are displayed.

S3 Table Contribution of individual phospholipid species to the LCMS analysis of the phospholipidome. Available upon request

S4 Table Contribution of individual TAG lipid species to the total [M+H]+ signal of TAG in LCMS analysis with atmospheric pressure chemical ionization (APCI). Only TAG species contributing more than 0.015% on average are included. Available upon request

Chapter 6

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

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Abstract

Background: In dogs with a congenital portosystemic shunt (CPSS), recovery after surgical CPSS attenuation is difficult to predict. Several clinical and genetic parameters are involved and can be used to construct a predictive model. **Objectives**: The aim of this study was to build a predictive model with albumin and mRNA expression levels of hepatic gene products as predictors of outcome after surgery. **Animals**: 73 client owned dogs referred for surgical attenuation of a single intra- or extrahepatic portosystemic shunt. **Methods**: A prediction model was constructed using two case-control studies of recovered and non-recovered dogs after surgical attenuation of CPSS. In the first study, a dog specific gene expression microarray analysis was used to compare mRNA expression in intraoperatively collected liver tissue between 23 recovered and 23 non-recovered dogs. In the second study, pre-operative plasma albumin and the expression of 43 genes selected from the first study and confirmed by RT-qPCR in intra-operatively collected liver samples were compared between 31 recovered and 31 non-recovered dogs. **Results**: The best fitting prediction model included pre-operative plasma albumin and intraoperative DHDH, ERLEC1, and LYSMD2 gene expression levels. If surgery would be performed on dogs with a predicted probability >50% only, this model resulted in a sensitivity to correctly predicted recovery of 77% and a specificity of 90%. **Conclusion and clinical importance**: This model exceeds a previously constructed model for prediction of postoperative recovery based on plasma albumin and hepatic mRNA expression levels. It can facilitate a preoperative decision on treatment of CPSS dogs.

Introduction

Surgical shunt attenuation is the first treatment of choice in dogs with a congenital portosystemic shunt (CPSS) to restore the normal hepatoportal circulation and liver function [1]. However, due to underdeveloped veins of the portal venous circulation distal to the shunt, an abrupt complete closure can result in severe portal hypertension leading to shock and death [2]. Consequently, several surgical techniques have been developed to provide progressive or partial shunt attenuation, thereby minimizing the risk of acute portal hypertension [3].

Recovery from portosystemic shunting after attenuation of the shunt in an individual dog is unpredictable and complications following (partial) shunt attenuation such as portal hypertension, persistence or recurrence of clinical signs due to continuous shunting or development of collaterals have been described for all surgical techniques [3]. Overall mortality rates differ from 0% to 32% depending on surgical technique, shunt location and degree of narrowing [2-4]. Medical and/or dietary management as alternative to surgery for CPSS has been applied to control the clinical signs of shunting, especially hepatic encephalopathy, although long-term results are disappointing [5, 6]. Therefore, the treatment of choice remains surgical attenuation of the shunt, and medical and/or dietary management should be reserved for cases with poor prognosis after surgery. A very important question, therefore, is how to predict the outcome of surgery.

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

An essential factor affecting post-operative recovery following surgery is hepatic regeneration; the ability of the liver and portal vasculature to develop to normal size and function [2, 10]. Hepatic regeneration is promoted by a complex network activated by inflammatory cytokines, vasoregulators, growth factors, eicosanoids, and various hormones [11] and correlates to hepatic expression of genes involved in proliferation, apoptosis, hepatic fibrosis or vascular growth [12]. A positive association with complete recovery after shunt attenuation was found for two genes related to hepatocyte proliferation; *HGF activator* (*HGFA*) and *methionine adenosyltransferase 2α* (*MAT2A*) [12], suggesting that expression of these genes following surgery is important for

clinical recovery [12, 13]. These two and other factors have been evaluated extensively [2, 4, 12-14], but no model based on genome-wide gene expression studies is available to predict the long-term outcome after surgical attenuation of CPSS. The aim of the present study was to develop an algorithm which predicts the outcome of surgery, in terms of normalisation of portal circulation and restoration of ammonia metabolism. Therefore, a canine specific gene-expression study was performed in which expression profiles of livers of CPSS dogs with successful recovery were compared to unsuccessful recovery upon surgical attenuation of the shunt.

Material and Methods

Study design

Gene-expression profiles were generated from dogs with a congenital portosystemic shunt (CPSS) that underwent surgical attenuation of the shunt in two case-control studies where cases recovered after surgery and controls did not. Genes that were associated with successful recovery upon surgical attenuation were selected by microarray analysis and confirmed by quantitative real-time PCR (RT-qPCR). These genes were used to create a predictive model for recovery after surgery.

Animal selection and surgical procedures

Permission was obtained from the dog owners using informed consent. All samples were collected according to the Act on Veterinary Practice, as required under Dutch legislation and sampling was approved by the local ethics committee (DEC Utrecht), as required under Dutch legislation (ID 2007.III.08.110). Liver tissue of healthy dogs was used for internal validation of the microarray and the RT-qPCR analyses and was obtained from fresh cadavers used in non-liver related research (surplus material, Utrecht University 3R-policy). The absence of an underlying liver disease was confirmed histologically by a board certified veterinary pathologist. Animal care and handling were performed in accordance with the European Directive for the Protection of Vertebrate animals used for Experimental and Scientific Purpose, European Community Directive 86/609/CEE.

Data of 73 dogs referred to the Department of Clinical Sciences of Companion Animals, Utrecht University for surgical attenuation of a single CPSS were included in the study. The localization of the shunt (intra- or extrahepatic) was preoperatively visualized with ultrasonography or CT. Pre- and postoperative additional diagnostic tests, supportive treatment and monitoring were performed according to a standardized CPSS protocol, which included pre-operative plasma

albumin measurements measured using a DxC 600 Beckman analyzer[a]. After exploration of the abdominal cavity via a median celiotomy, the shunt was ligated over a gauged rod to the smallest diameter that did not induce portal hypertension, using a non-absorbable 2-0 polyester suture[b] [8]. All surgeries were performed by an ECVS board-certified surgeon (FvS, AK). Wedge biopsies of the liver were routinely taken during surgery for histopathology. A section of the biopsy was frozen in liquid nitrogen immediately after collection and stored at −70°C until gene expression analysis.

Postoperative recovery was determined at rechecks 1-3 months after surgery of all dogs that had survived. Persistent portosystemic shunting was evaluated by a 12-hour fasting plasma ammonia concentration or by applying a rectal ammonia tolerance test [15]. Abdominal ultrasonography was performed to examine the site and patency of the attenuated shunt and to identify acquired portosystemic vessels. Complete recovery was defined as either normal fasting plasma ammonia concentrations (i.e. < 45 µM) or a rectal ammonia tolerance test within reference values and no portosystemic shunting on abdominal ultrasonography [15]. Dogs that died or were euthanized after surgery because of portal hypertension, hypoplasia of the portal vasculature or persistent shunting were considered as not recovered. If shunt attenuation was not feasible during attempts of shunt ligation, dogs were also considered as not recovered. Dogs that died from reasons unrelated to portal hypoplasia, portal hypertension or persistent shunting and dogs in which the outcome after shunt attenuation was unclear were excluded from the study. Equal numbers of samples from recovered and non-recovered dogs were included from the hepatic tissue that was consecutively collected between July 2002 and July 2015.

Expression profiling

Previously published microarray expression data [16], representing mRNA expression of 42,034 canine-specific 60-mer probes determined in liver tissue of dogs with a CPSS collected during surgical attenuation, was used in this study. One dog was excluded from the data because the outcome after shunt attenuation was unclear. Data of 23 recovered and 23 non-recovered dogs, available through GEO Series accession number GSE39005 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=%20GSE39005), were used to compare gene expression profiles of recovered and non-recovered dogs after surgical attenuation of a CPSS. Genes with differences (log2-fold) of more than 0.30 or less than -0.30 were selected to ensure that only robust changes were considered.

Gene expression differences of the selected genes were confirmed using RT-qPCR in a second case control study including 62 samples (31 recovered, 31 non-recovered dogs). RNA isolation,

cDNA synthesis and gene-expression profiling using RT-qPCR was performed as described previously [16] with a maximum of 40 cycles. Because of the positive association with recovery in CPSS dogs of *MAT2A* and *HGFact* in a previous study [12], expression levels of these gene products were additionally measured.

Normalization was performed using four reference-genes, based on their stable expression in liver (*Glucuronidase Beta* (*GUSB*), *Heterogeneous Nuclear Ribonucleoprotein H* (*HNRPH*), *Hypoxanthine Phosphoribosyltransferase* (*HPRT*), and *Ribosomal Protein S5* (*RPS5*) [16] as required under MIQE-precise guidelines [17]. Primers for the genes of interest and reference genes, including their optimum temperature, are listed in Table 1.

Statistical analysis

The results of the microarray analysis were reanalyzed with updated annotations using ANOVA[18] under *R statistics*[c]. Correction for multiple testing (Permutation F2-test using 5,000 permutations) was performed and $P < 0.05$ after family wise error correction was considered statistically significant. All analyses were performed using commercial software (*SPSS*[d]). Missing data in albumin were imputed by automatic multiple imputation, performing 1 imputation and including outcome, gender, age at surgery, breed and shunt subtype in the linear regression model. Gene expression in samples without a PCR signal were assumed to have a C_q value of 40.0.

For each gene product and albumin, a receiver operating characteristic (ROC) curve was plotted to determine sensitivity (Se) and specificity (Sp) of a test to identify dogs that recovered following surgery at various cut-off values between 'low' and 'high' [19]. Variables with areas under the curve (AUCs) significantly ($P < 0.05$) different from 0.5 were selected for further analyses. For each selected variable, a binary variable, 'low' vs. 'high', was created. The cut-off value between 'low' and 'high' was chosen at the gene expression level or albumin concentration that corresponded to the data point on the ROC curve with the smallest Euclidian distance to the point (1- Sp, Se) = (0, 1), to approach a perfect test (Se = 1, Sp = 1).

The diagnostic potential of the binary variables as well as shunt localization (extrahepatic vs. intrahepatic), subtype of shunt (IHPSS, left, central or right sided shunts; EHPSS, portoazygous or portocaval shunts), age at surgery and gender was evaluated in a logistic regression with outcome after surgery as dependent variable. A final model was obtained in a backward stepwise elimination-and-selection procedure in the likelihood ratio test with probabilities for stepwise entry and removal of 0.05 and 0.10 respectively.

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

Gene	Ensemble	F/ R	Sequence	Tm	Amplicon
CAV2	ENSCAFG00000003402	F	5'-TTCTCTTCGCCACCCTCAG-3'	65.8	147
		R	5'-CTGCGTCCTACACTTGAACAC-3'		
CPD	ENSCAFG00000019016	F	5'-ATTGGTATGATGTGGAAGGT-3'	5.1	131
		R	5'-GATTGTTCTCCCATTCTTGTC-3'		
CTGF	ENSCAFG00000029442	F	5'-GGAAGAGAACATTAAGAAGGG-3'	62.6	120
		R	5'-TACTCCACAGAACTTAGCC-3'		
CYR61	ENSCAFG00000020276	F	5'-CGAGTTACCAATGACAACC-3'	65.8	109
		R	5'-CATTTCTTGCCCTTCTTCAG-3'		
DHDH	ENSCAFG00000003869	F	5'-ACACCGTCACTGTGCTCCT-3'	67.0	171
		R	5'-TCCTTATGCTCTCCCTTCAACACC-3'		
DZIP	ENSCAFG00000005465	F	5'-TAAACGCAGGAAGAAGATGATCTC-3'	61.3	148
		R	5'-GGTGAGAATCTTCAGGGTGG-3'		
ERLEC1	ENSCAFG00000002724	F	5'-CATTCTGCCTCTTGTGACAAGTG-3'	59.8	147
		R	5'-TCCGTGACATACTTCATAAGTCCA-3'		
FRMD4B	ENSCAFG00000006521	F	5'-ACCACTCCAGTTCTTACC-3'	62.6	136
		R	5'-GGCTTATCATTGTCCATCTC-3'		
FXYD1	ENSCAFG00000007095	F	5'-CACCTACGACTACCAATCC-3'	64.9	149
		R	5'-GTTCCCTCCTCTTCATCAG-3'		
GLS2	ENSCAFG00000000131	F	5'-TTCAGCAATGCCACATTCCAG-3'	66.4	150
		R	5'-TCACCTCCACAGAGCACAG-3'		
GNMT	ENSCAFG00000001741	F	5'-CAACTGGATGACTCTGGAC-3'	63.9	119
		R	5'-TGCTCACTCTGATCTCCT-3'		
GSTO1	ENSCAFG00000010593	F	5'-TTCCATCTTTGGTAACAGGC-3'	63.9	113
		R	5'-TCTTATTGGTCAGAACCTCCT-3'		
HEPC	ENSCAFT00000011304	F	5'-CCAGTGTCTCAGTCCTTCC-3'	65.5	163
		R	5'-TTTACAGCAGCCACAGCA-3'		
HGFA	ENSCAFG00000014629	F	ACACAGACGTTTGGCATCGAGAAGTAT	60.0	128
		R	AAACTGGAGCGGATGGCACAG		
HSD17B14	ENSCAFG00000003895	F	5'-GTGACCAAGTTTGCCCTCCC-3'	67.0	170
		R	5'-GACGCCATATCGACTCTCATCCA-3'		
LYSMD2	ENSCAFG00000015502	F	5'-TCCTCCTAGTCCTCAAGAATCC-3'	63.9	155
		R	5'-GCATAGGGACTTTCTTCATCTCTG-3'		
MAT2a	ENSCAFG00000007755	F	TGCTTTTGGCGGGGAGGAG	67.0	121
		R	TTTAAAAGCTGCCATCTGAGGTGA		
MFAP3	ENSCAFG00000030060	F	5'-ACCACTATGAAGATGTCCGT-3'	61.3	143
		R	5'-CAAAGCATGTGTAGAGCCC-3'		
MGST2	ENSCAFG00000003702	F	5'-CTGGTTACATTGTGGATGG-3'	63.9	93
		R	5'-AGAAATACTGGTGACGGG-3'		
NAGS	ENSCAFG00000014391	F	5'-CATCTTCCTCAATACCACCG-3'	64.9	147
		R	5'-CACATCCACAATGAGCCG-3'		
NUCB2	ENSCAFG00000008713	F	5'-CGCAAAGATAGAACCACCAG-3'	64.9	138
		F	5'-TAGCCTCCCACTCTTTATTTCC-3'		
PDIA4	ENSCAFG00000003403	F	5'-AGGACTCAGGAAGAAATCGT-3'	64.9	140
		R	5'-GAACTCCACCAGAATGATGTC-3'		
PIK3RA	ENSCAFG00000007626	F	5'-CATTGCCTCCTAAACCACC-3'	63.9	143
		R	5'-TCCCATCGGCTGTATCTC-3'		

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

CAV2, Caveolin-2; CPD, Carboxypeptidase D precursor; CTGF, Connective tissue growth factor precursor; CYR61, Protein CYR61 precursor (IGF-binding protein 10); DHDH, dimeric dihydrodiol dehydrogenase; DZIP, Zinc finger protein DZIP1 (DAZ-interacting protein 1/2); ERLEC1, Endoplasmic Reticulum Lectin 1; FRMD4B, FXYD1, GLS2, Glutaminase liver isoform, mitochondrial precursor; GNMT, Glycine N-methyltransferase; GSTO1, Glutathione transferase omega-1; HEPC, Hepcidin precursor; HGFA, Hepatocyte growth factor activator; HSD17B14, 17-beta-hydroxysteroid dehydrogenase 14; LYSMD2, LysM and putative peptidoglycan-binding domain-containing protein 2; MAT2a, Methionine adenosyltransferase 2 alpha; MFAP3, Microfibrillar-associated protein 3-like precursor; MGST2, Microsomal Glutathione S-Transferase 2; NAGS, N-acetylglutamate synthase, mitochondrial precursor; NUCB2, Nucleobindin-2 precursor; PDIA4, Protein disulphide-isomerase A4 precursor; PIK3RA, Phosphatidylinositol 3-kinase regulatory subunit alpha; PKIB, cAMP-dependent protein kinase inhibitor beta; PLIN2, Adipophilin; SEH1L, SEH1 Like Nucleoporin; SK2, Small conductance calcium-activated potassium channel protein 2; SLC1A1, Solute carrier family 1 (glial high affinity glutamate transporter), member 1; SLC2A13, Solute carrier family 2 (Facilitated Glucose Transporter), member 13; SORD, Sorbitol dehydrogenase; SSH3L, Protein phosphatase Slingshot homolog 3;

TFAP2B, Transcription factor AP-2 beta; TOR3A, Torsin-3A precursor; TRIM22, Tripartite motif-containing protein 22; TXNIP, Thioredoxin-interacting protein; VCAM1, Vascular cell adhesion molecule 1. Reference genes used for normalisation: GUSB, Glucuronidase Beta; hnRPH, Heterogeneous nuclear ribonucleoprotein H; HPRT, Hypoxanthine Phosphoribosyltransferase; RPS5, Ribosomal Protein S5. F, Forward primer; R, Reverse primer; Tm, Melting temperature; bp, base pairs.

Confounding was monitored by the change in regression coefficients. If elimination of a variable resulted in the change of the estimated regression coefficient of any other variable exceeding 25% or 0.1 in case of an estimate between -0.4 and 0.4, the eliminated variable was considered a potential confounder and re-entered in the model. Multicollinearity was evaluated by linear regression [20]. A tolerance <0.1, a variance inflation factor >10 or a condition index >15 were considered indicative of multicollinearity. Model fit was evaluated with the Hosmer–Lemeshow test. The proportion of dogs with correctly predicted outcome was calculated using a classification cut-off value of 0.5. Validation of the final model was performed multiplying the regression coefficients with the heuristic shrinkage factor and correction of the intercept to improve its feasibility in future cohorts of dogs.

Results

Animal characteristics

Seventy-three dogs were enrolled in this study; 45 dogs with an EHPSS and 28 dogs with an IHPSS. The study group consisted of 25 different breeds and 5 dogs of cross breeds. The first sample set, used for the micro-array analysis, consisted of liver samples of 46 dogs; 32 (70%) with an EHPSS and 14 (30%) with an IHPSS. Of these 46 dogs, 23 dogs have recovered completely after surgical attenuation of the shunt, namely 19 (59%) of the dogs with an EHPSS and 4 (29%) of the dogs with an IHPSS. The study population used for RT-qPCR analyses consisted of samples of 62 dogs with a CPSS and included 35 overlapping samples with de micro-array. Of these 62 CPSS dogs 35 had an extrahepatic (56%) and 27 (44%) an intrahepatic shunt. After surgical attenuation of the shunt, 31 of these dogs recovered completely (mean age at surgery 358 days) and 31 have not recovered (mean age at surgery 383 days); 21 (60%) of the dogs with an EHPSS have recovered and 10 (37%) of the dogs with an IHPSS. Of the total 73 CPSS dogs enrolled in this study, 39 dogs had not recovered. All together the micro-array sample set and the validating RT-qPCR sample set were highly identical regarding clinical pre- and post-operative conditions. In the surviving dogs without recovery from portosystemic shunting, ammonia metabolism had not normalised and/or abdominal ultrasonography revealed patency

of the original shunt or newly developed multiple acquired shunts. Preoperative plasma albumin levels were available in 59 of the 62 dogs in the sample set for RT-qPCR analyses. The mean preoperative plasma albumin concentration in recovered dogs was significantly higher, 23 g/L compared to 19 g/L ml in non-recovered dogs (Student T test; $P = 0.004$).

Gene-expression patterns of recovered and non-recovered dogs

In the micro-array data set, 43 genes were differentially expressed in recovered and non-recovered CPSS dogs [16]. These genes were selected for further confirmation by RT-qPCR. Due to technical reasons, no RT-qPCR data could be obtained of three genes; *CYR61*, *MGST2,* and *SEH1L.*

Construction predictive model

The ROC curves of *CTGF*, *DHDH*, *ERLEC1*, *HEPC*, *LYSMD2*, *ZIP*, and albumin had AUCs that were significantly ($P < 0.05$) different from 0.5. The final logistic model included albumin, *DHDH*, *ERLEC1*, and *LYSMD2* (Table 2). The AUC was 0.887 (95% CI 0.801-0.973). For each of the 62 dogs, a predicted probability of recovery was calculated using the final model. This showed that if surgery would have been performed on dogs with a probability of recovery > 50% only, 77% of the recovered and 10% of the non-covered dogs would have been operated (a sensitivity of 77% and a specificity of 90%; Fig 1). After internal validation, the model was transformed to:

$$\text{Logit (P)} = 0.187 + 1.78(\text{b albumin}) + 1.07(b\Delta C_{qDHDH}) - 1.59(b\Delta C_{qERLEC1}) - 1.53\ (b\Delta C_{qLYSMD2})$$

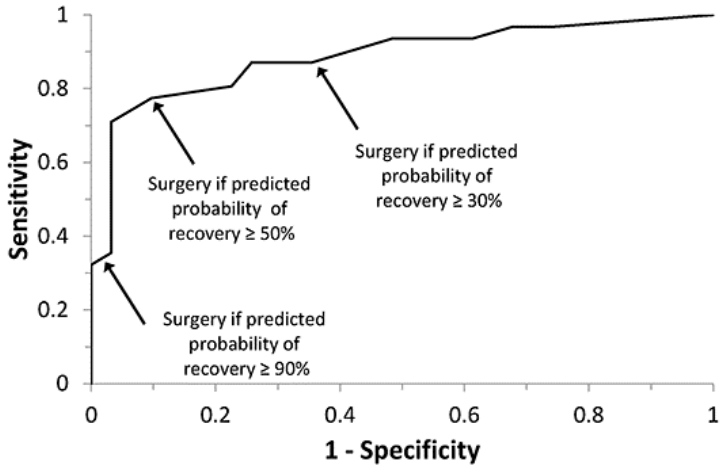

Figure 1 Receiver operating characteristic curve of the final model to predict recovery after surgery. Data of 62 dogs with surgical attenuation of a congenital portosystemic shunt.

Table 2 Final overall prognostic model[a] of preoperative plasma albumin concentration and intraoperative hepatic mRNA-expression of genes of interest as a binary variable ('low' or 'high)' in 62 dogs with a congenital portosystemic shunt.

Prognostic variable	Level	Number of patients	Estimate	Standard error	P (Wald test)	Odds ratio (95% CI)
Albumin	Low (< 21.5 g/l)	28	Reference		0.008	
	High (≥ 21.5 g/l)	34	2.01	0.76		7.49 (1.70; 32.9)
DHDH (ΔC_q)	Low (ΔC_q < 1.82)	28	Reference		0.094	
	High (ΔC_q ≥ 1.82)	34	1.21	0.73		3.36 (0.81; 13.9)
ERLEC1 (ΔC_q)	Low (ΔC_q < -2.74)	28	Reference		0.015	
	High (ΔC_q ≥ -2.74)	34	-1.79	0.74		0.17 (0.04; 0.71)
LYSMD2 (ΔC_q)	Low (ΔC_q < - 3.73)	28	Reference		0.021	
	High (ΔC_q ≥ - 3.73)	34	-1.73	0.75		0.18 (0.04; 0.77)
Intercept			0.22	0.83	0.07	

[a] -2 log likelihood = 51.23; Cox & Snell R^2 = 0.43; Hosmer-and-Lemeshow test χ^2= 6.192, df = 8, p=0.626.

6

Discussion

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

This study also confirmed the association of low plasma albumin levels with a poor recovery and long-term prognosis, as described before [4,7,12]. Albumin is synthesized exclusively by hepatocytes. In dogs with portosystemic shunts, hypoalbuminemia is common and could be related to prolonged hepatocellular dysfunction.

Previously we found a positive association between complete recovery after shunt attenuation and plasma albumin levels as well as MAT2a and HGFA expression, two gene products selected on potential prognostic value to recovery (involvement in hepatic regeneration and vascular growth, respectively) [12]. The current study used a genome-wide micro-array expression approach to identify genes of interest in addition to the predictors albumin, MAT2a and HGFA. Although

MAT2a and HGFA are important in liver cell proliferation [12, 13], neither of the two genes contributed to the predictive model in this study using an extensive sample set.

A correlation between shunt location, weight, and age at time of surgery, and long term clinical outcome is suggested in several studies [7, 9]. In general, IHPSS leads to an earlier development of clinical signs [21] when compared to EHPSS [22], although geographical differences are reported. Such a difference is also observed within the EHPSS subtypes [22], where extrahepatic portocaval shunts seem to be diagnosed at an earlier age compared to extrahepatic porto-azygous shunts. As severe shunting leads to poor liver development and function [23], it seems plausible that dogs with a higher fraction of shunted portal flow develop clinical signs at an earlier age. Hence, a negative correlation of age to recovery could be expected and explained by a poor clinical condition of such dogs. In contrast, a better prognosis was reported after surgical treatment in dogs less than 12 months of age, compared to dogs older than two years [24]. In our population, no correlation between age at surgery and recovery has been detected, which is also found by others [1,25]. As IHPSS occurs more often in large dog breeds and EHPSS in small dog breeds [26], shunt location and weight are expected to be correlated when investigating factors associated with recovery rates. Although plausible, shunt location and subtype did not contribute to the final predictive model.

The predictive model developed in this study is based on hepatic gene expression levels of CPSS dogs referred to our clinic. Hepatic gene expression profiles and recovery rates after attenuation of a shunt are probably not identical among various countries because of population and individual breed differences [21]. Moreover, the various surgical regimen for shunt attenuation and possible variations in surgical skills merit a multi-centre study to validate this model world-wide.

In conclusion, a predictive model was constructed based on plasma albumin, DHDH, ERLEC1, and LYSMD2 with good discriminating abilities. This model could be useful in making a decision on individual treatment of CPSS dogs. Success rates after surgical treatment could increase if surgical candidates could be screened pre-operatively for post-operative recovery from portosystemic shunting. External validation of this model in other dog populations and using different surgical techniques is essential to evaluate the broad clinical applicability of the model.

Endnotes

Beckman Coulter, Woerden, the Netherlands [a]
Ethibond, Ethicon, Somerville, NJ, USA [b]
R version 2.2.1, R Foundation for Statistical Computing, Vienna, Austria 2014 [c]
IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY [d]

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Chapter 7

Summarizing Discussion

Congenital portosystemic shunts (CPSS) include two subtypes: extrahepatic portosystemic shunts (EHPSS) and intrahepatic portosystemic shunts (IHPSS). Whereas these vascular anomalies occur frequently in dogs, they are rare in humans. Although the knowledge about CPSS is increasing over the recent years as reviewed in **Chapter 2**, the aetiology and several other aspects of this disease concerning pathophysiology and prognosis after surgery are not clear. The general aim of this thesis was to gain further insight into **the pathogenesis of canine CPSS** and elucidate mechanisms involved in **pathophysiology** and **hepatic regeneration** using the shared pathophysiologic consequences and the different genetic background of both subtypes as fundament in this thesis.

Comparisons in the aetiology within the intrahepatic and extrahepatic portosystemic shunt phenotypes

Before conducting genetic studies to gain insight into canine congenital portosystemic shunting, it is essential to establish whether a similar genetic background is to be expected for the subtypes of CPSS (IHPSS and EHPSS). Although a clear classification of IHPSS subtypes is lacking, distinctions have been made between left, right and central divisional shunts, with left sided divisional shunts designated as a patent ductus venosus [1] compatible with the normal embryology of the dog. As the mechanism of formation for right and central divisional shunts is unclear, it is possible that the molecular basis may differ from that of left divisional shunts [2]. However, in contrast with this generally accepted theory, both right and left sided shunts have occurred repeatedly within the same litter in Irish Wolfhounds [3], indicating that both of these phenotypes and thus the closure of the ductus venosus are caused by the same genetic defect.

Based on their termination in the systemic circulation, EHPSS can be subdivided into two main types: portocaval and porto-azygous shunts [4]. In **Chapter 3** the distribution of EHPSS subtypes was evaluated in different purebred dogs with the aim to ascertain if a common genetic basis for the extrahepatic subtypes is plausible. For this study, data of 135 dogs with a single EHPSS were retrospectively analysed. Besides shunt localization, information about breed, sex, average age at diagnosis and dog size was obtained as well. It was remarkable that this study indicated a higher incidence of affected females with EHPSS, whereas other studies describe an equal distribution between the sexes [5, 6]. More specifically, a significantly higher incidence in females was found for porto-azygous shunts, while no sex overrepresentation was observed in portocaval shunts. Although the reason for this female overrepresentation in porto-azygos shunts remains elusive, a genetic background or other gender-related differences cannot be excluded.

The most important conclusion of this study (**Chapter 3**) was that portocaval and porto-azygous shunts coexist in all studied dog breeds (apart from the Pug), suggesting that the two subtypes are variants of the same inherited disorder. Considering the complex mode of inheritance for EHPSS dogs [6-8], this has led to the conception that common underlying major genes are responsible for EHPSS across breeds. A minor genetic component or non-genetic factors during embryonic development are probably responsible for the erroneous connection of the embryonic vitelline system with the cardinal vein system at the level of the vena cava or vena (hemi)azygos.

The improvement of the imaging techniques over the recent years, especially the combination of anatomical findings with computed tomography, angiography, and intra-operative mesenteric portovenography, has led to the possibility to describe the morphology of the portal vasculature and anomalous shunting vessel in the body in detail [9, 10]. Interestingly, this has resulted in novel insights in shunt morphology. The anomalous shunting vessel between the embryonic vitelline veins and cardinal venous system is commonly regarded to represent the left gastric vein, which is a normally present, but in fact an erroneous constructed vessel during embryonic development [9, 10]. This is in contrast to the statement in **Chapter 3** that the EHPSS subtypes must be considered as erroneous developmental anomalies as such connections should not exist during embryogenesis. Nevertheless, a few EHPSS cases involving the left colic vein are also described in literature, representing actual developmental errors between the vitelline and cardinal system [11]. Although these findings do not totally match our conclusions concerning morphology, in contrast to IHPSS where the ductus venosus does not close after birth, EHPSS must be considered as erroneous connections of vessels occurring during embryogenesis. This supports the main conclusion of this chapter that EHPSS subtypes are caused by a major gene defect and that minor genes determine the origin and site of insertion.

Since dogs with porto-azygous shunts are generally diagnosed at an older age, a relatively large number of affected dogs may reproduce before being diagnosed, especially compared to portocaval shunts. This could increase the chance that causative genes defects segregate in the population. As minor genes should determine a portocaval or porto-azygous localization, the amount of porto-azygous affected dogs might increase over time.

Concluding, the findings observed in **Chapter 3** indicate that both subtypes of IHPSS and EHPSS can be used for genetic studies into the genetic background of CPSS in dogs.

Differences between the aetiology of intrahepatic and extrahepatic portosystemic shunts

Making use of the shared physiology but different aetiology between EHPSS and IHPSS, an elegant micro-array experiment was performed in **Chapter 4** on liver tissue of 47 CPSS dogs to unravel genes involved in the pathogenesis of CPSS. The beneficiary effect of comparing expression levels in both shunt types and comparing them with the levels observed in healthy liver tissue, was shown in the small list of only 26 genes that were differentially expressed for one of the two types of shunt. Differences in the hepatic expression of genes in dogs with either EPHSS or IHPSS compared to the control group were interpreted as specific characteristics of each subtype, whereas differences shared by both IHPSS and EHPSS compared to controls dogs are most likely due to secondary effects, such as the absence of normal portal vein perfusion of the liver. The microarray experiment in this study is designed in such a way that this experiment is not only useful for scientific issues concerning aetiology of IHPSS and EHPSS, but additionally it has proven its great value for genome-wide based experiments regarding the pathogenesis of steatosis and hepatic regenerative potential after surgical attenuation (**Chapter 5 and 6**).

The micro-array strategy used in **Chapter 4** has some limitations. It is expected that genes involved in EHPSS are most likely actively transcribed during the embryonic phase whereas genes involved in IHPSS are most likely to be expressed during the first days after birth [12]. Aberrant expression of those genes during adulthood may still be detected, but this depends on the genetic defect(s) and epigenetic modulation. A further concern is that research focuses on new findings, while selection criteria are predominantly based on annotated genes and known gene functions. This emphasizes the disadvantage of using a micro-array where only 60% of the known canine genes are annotated (CanFam 3.1). Both drawbacks will result in false negative rather than false positive data and raises the question how many genes could have been missed. Interestingly, compared with their expression in healthy liver samples, the study in **Chapter 4** found that 6 and 19 annotated genes were specific to dogs with respectively EHPSS or IHPSS, indicating that gene-expression analysis did find possible genes involved in the development of these anomalies.

Follow-up experiments after the micro-array experiment on shunt dogs in **Chapter 4** including qPCR, immunohistochemistry and Western blots, confirmed a down-regulation of Vascular cell adhesion molecule 1 (VCAM1) in EHPSS and an up-regulation of WEE1 homologue (*Schizosaccharomyces pombe*) (WEE1) in IHPSS. VCAM1, down-regulated in EHPSS liver tissue, is involved in angiogenesis after binding with integrin $\alpha 4\beta 1$. VCAM1 is mainly expressed by

proliferating vascular smooth muscle cells, whereas integrin α4β1 is expressed by proliferating endothelial cells. The binding of integrin α4β1 and VCAM1 facilitates the adhesion of endothelial cells to vascular smooth muscle-like pericytes. This process is essential for the survival of endothelial and mural cells during neovascularization [13]. Due to the impaired venous blood flow to the liver in CPSS and the underdevelopment of the hepatoportal vasculature, we expected an upregulation of this gene as a demand for angiogenic factors. Surprisingly, we only observed an upregulation in IHPSS. Given the function of VCAM1 in angiogenesis, this gene or its regulatory pathways could be a candidate for causing EHPSS in dogs.

WEE1 seems to play an essential role in hypoxia-induced pathological processes in endothelial cells, given that it is up-regulated in endothelial cells under hypoxic conditions, and ensures cell viability [14]. These hypoxic conditions are considered to be the trigger in the postnatal closure of a comparable structure with the ductus venosus, the ductus arteriosus [15]. A corresponding mechanism involving oxygen tension might be essential in closure of the ductus venosus as well, as an up-regulation of WEE1 is observed in IHPSS.

Another gene, cysteine conjugate beta-lyase 1 (CCBL1), that was found in this study was significantly reduced in both shunt types, whereas at first at mRNA level there was only an indication for a down-regulation in IHPSS. Based on these results, it was concluded that CCBL1 expression is a secondary effect of portosystemic shunting. CCBL1 plays a role in metabolizing cysteine conjugates of halogenated alkenes and alkanes leading to the formation of reactive metabolites that can be responsible for nephro- and neuro-toxicity [16]. CCBL1 could therefore be an interesting candidate to study aspects that are not yet elucidated in the pathophysiology of hepatic encephalopathy; a symptom observed in the presence of hyperammonemia due to portosystemic shunting.

In depth analysis of lipid accumulation in portosystemic shunting

Hepatocellular steatosis is a frequent finding in histological biopsies of CPSS dogs [17-19]. The observation that shunt fraction is not correlated with the degree of lipid accumulation [20], may indicate that steatosis could be caused by a genetic factor. However, a decrease in steatosis is perceived in histological biopsies after surgical attenuation, suggesting steatosis in CPSS could be a secondary effect, perhaps induced by hepatic hypoxia or a disturbed fatty acid metabolism [21]. In **Chapter 5** an extensive analysis of the lipid metabolism of dogs with CPSS was conducted to clarify the pathogenesis of steatosis in CPSS dogs.

First an Oil-red-O staining was performed on histological slides of shunt livers (n = 12) to confirm steatosis in our Dutch cohort of EHPSS and IHPSS. By taking the total amount of lipid accumulation into account, steatosis was evident in the hepatic biopsies of CPSS dogs which, on average, showed a 12-fold increase of lipid content when compared to normal liver tissue. Age is reported to affect the degree of steatosis in portosystemic shunting, as an increased amount of lipogranulomas, one of the characteristics of hepatic steatosis in dogs [22], is observed in dogs older than one year of age [20, 23]. The used samples in this study are not age matched, however the shunt samples are derived from relatively young dogs compared to the middle-aged controls, indicating that the amount of lipogranulomas does not play an important role in steatosis of shunt dogs.

Second, to analyse the involvement of lipid related genes to CPSS an expression profiling was performed. This time (in **Chapter 5**), the shared pathophysiology of both CPSS subtypes was used while analysing the micro-array described in **Chapter 4**, to identify the most up and most down regulated genes in EHPSS and IHPSS versus healthy dogs. Eleven genes related to lipid metabolism, transport or storage, as based on Gene Ontology biological processes and literature, were selected for further confirmation using RT-qPCR on hepatic tissue of 63 shunt dogs. RT-qPCR confirmed the different expression of seven lipid related genes (upregulation of *CRP*, *FABP1*, *IGFBP1*, *ITIH4*, *PLIN2*, and *SAA1* and a downregulation of *SEC14L3*), indicating the importance of an altered lipid metabolism in portosystemic shunting. Future experiments on protein level, such as immunohistochemistry or Western blot analysis are essential to confirm these findings.

Third, analysis of neutral- and phospholipids by high-performance liquid chromatography-mass spectrometry (HPLC-MS) was performed to gain insight in the accumulating lipids in CPSS. In shunt dogs a shift towards omega-3 fatty acids at the expense of the omega-6 fatty acids, in particular the shift of AA to DHA, was found compared to healthy controls. In $LDLR^{-/-}$ mouse with induced non-alcoholic steatohepatitis, DHA has the capacity to suppress markers of hepatic damage, hepatic inflammation, oxidative stress and fibrosis [24] and is reported to be beneficial in hepatic encephalopathy [25]. Although the amount of DHA is influenced for example by diet, a factor that is not taken into account in this study, we suggest that the elevated levels of DHA in shunt livers compared to healthy controls might have a protective response in portosystemic shunting, possibly influencing inflammation. Interestingly, three of the six upregulated lipid related genes measured in the gene expression analysis (*i.e. CRP*, *ITIH4,* and *SAA1*) serve, beside their lipid associated function, as acute phase reactants (APR). APR are secreted in response to a variety of inflammatory conditions. Although inflammation is not

observed on histology of CPSS slides [17, 18, 22] and a direct link of the gene expression study with the HPLC-MS results is not possible, it would be interesting to determine the association of inflammation with lipid accumulation in CPSS, as lipids are described to elicits an proinflammatory response triggering cellular injury in human and animal models [26, 27].

Concluding, **Chapter 5** gives an in-depth analysis of lipid accumulation in CPSS and the involved pathways. The excessive lipid accumulation observed using Oil-red-O staining, the presence of an altered lipid metabolism determined with gene expression analysis, and finally the different TAG composition and shift in short chain and omega-3 fatty acids observed with HPLC-MS, all show the importance of this aspect in CPSS. The similarity in the degree of lipid accumulation and accumulating lipid species in both shunt types, indicate that lipid accumulation in CPSS occurs secondary to portosystemic shunting. To confirm our hypothesis, hepatic derived organoids of CPSS and healthy dogs were cultured and incubated with free fatty acids. No differences in total lipid accumulation were observed in healthy and shunt dogs, indicating that lipid accumulation in the presence of portosystemic shunting is not caused by primary genetic defects. Moreover, the above mentioned findings all emphasize the impact of the absence of the normal portal circulation in shunt dogs, leading to impaired liver development and function, including steatosis [28].

The study in **Chapter 5** is a comprehensive study to demonstrate that hepatic lipid accumulation in CPSS occurs secondary to portosystemic shunting. Since the similarities in phenotypic presentation between canine and human diseases are remarkable, the dog has been proposed as a useful model to study inherited diseases in human medicine [29]. An additional asset for CPSS, is that the frequency of this disease in dogs is far more higher than in men [30]. In men, great similarities in the subtypes of intrahepatic and extrahepatic shunts, clinical presentation, and diagnosis are observed when compared to dogs [30, 31]. Histological characteristics observed in dogs with CPSS, [17, 18, 22] such as lipid accumulation, are comparable with rats [32] and human [33]. Steatosis is also one of the hallmarks of human non-alcoholic fatty liver disease (NAFLD) [27]. This latter disease is a currently emerging health concern in the Western world, [26, 27]. The high prevalence but poorly understood pathogenesis of NAFLD could benefit from the described canine model in **Chapter 5**. In the future this unique combination of gene- and lipid-profiling with organoid disease modelling might lead to novel treatment methods for steatosis in both veterinary and human [34].

Gene expression based prediction of recovery related to hepatic regenerative potential after shunt attenuation in dogs

Once dogs are diagnosed with CPSS, treatment is recommended to prevent, treat or lower clinical manifestations of the disease including hepatic encephalopathy. Several factors such as age at diagnosis, type of portosystemic shunt, and exact location but also financial aspects and owner compliance influence treatment possibilities for individual dogs. Treatment options for dogs with a congenital shunt include surgical attenuation [35-38], medical management [39, 40], or both, but the only way to acquire complete recovery in dogs is surgical attenuation of the anomalous vessel [41]. Prediction of the long-term outcome following surgery is difficult. Long-term post-operative success can only be obtained when the liver and portal vasculature are able to develop to normal size and liver function can be restored [35, 36].

Inflammatory cytokines, vasoregulators, growth factors, eicosanoids, and various hormones activate the complex and well-arranged process of hepatic regeneration [42, 43] which lead to the associated hepatic expression of genes related to proliferation, apoptosis, hepatic fibrosis, or vascular growth [44]. In **Chapter 6** gene expression profiling of hepatic tissue of CPSS dogs was used to identify genes associated with recovery and hepatic regeneration after shunt attenuation. Micro-array gene expression analysis revealed a list of 43 differently expressed genes in shunt dogs with successful surgical outcome compared to dogs with a poor outcome. These results were validated in 62 samples of dogs with a CPSS consisting of 25 breeds and 5 mixed breed dogs. In this study we hypothesized that hepatic regeneration after surgical intervention is a similar process in extra- and intrahepatic portosystemic shunts and could predict the outcome of shunt attenuation, based on their gene-expression profile.

In the predictive model composed, based on the genome-wide study, the expression levels of *Dihydrodiol dehydrogenase* (*DHDH)*, *Endoplasmic Reticulum Lectin 1* (*ERLEC1*), and *LysM and putative peptidoglycan-binding domain-containing protein 2* (*LYSMD2*), and presurgical plasma albumin, correctly predicted recovery rates with > 50% certainty in individual dogs. *MAT2a* and *HGFact,* both potential prognostic factors for recovery [44, 45] and with important functions in hepatic regeneration [43, 46, 47], were not related to complete recovery in this study.

Although the chances for recovery may be better in EHPSS [3,8], the proportion of recovery in our groups of IHPSS (37%) and EHPSS (60%) are not representative because equal numbers of recovered and non-recovered dogs were selected for both the microarray and the RT-qPCR analyses. However, it was more difficult to select a sufficient number of samples of completely recovered IHPSS dogs compared to EHPSS dogs.

Age at surgery was not related to clinical recovery and the development of long-term complications, which is in agreement with previous studies evaluating age at presentation with prognosis [41, 43, 48]. Hence age should not be of influence when surgical therapy is considered in individual cases, which is an important finding for EHPSS shunt types as portocaval shunts are diagnosed at a mean age of 12.3 months compared to 32.3 months for porto-azygous shunts (**Chapter 3**).

The final prognostic model as provided in **Chapter 6** has good characteristics (sensitivity of 77%, specificity of 90%) to applicate this model, although it has some drawbacks. Firstly, it is based on gene expression levels in hepatic samples and because of the invasiveness biopsy procedures are less likely to be clinically applicable. Secondly, the samples used in this study are taken intraoperatively, so an effect of anaesthesia on gene expression cannot be excluded. Genetically determined predictive factors are not expected to be variably expressed in presurgical biopsies compared to the used samples, although non-genetic factors that may influence outcome, (e.g. the presence and/or the degree of hepatic encephalopathy or lipid accumulation (**Chapter 5**)), could vary over time. To develop an applicable routine test for prediction of outcome after shunt attenuation in individual dogs, follow-up studies are essential, preferably measurement of *DHDH*, *ERLEC1*, *LYSMD2* and albumin solely in blood samples. Validation of our findings in blood samples and dog populations worldwide, preferentially in a randomized multi-centered study, could lead to a breakthrough in veterinary medicine.

Although clinical signs are variable and related to the degree of shunting of the portal blood [30], the lower albumin levels in IHPSS dogs may be explained by the overall severe hepatic dysfunction and poorer liver growth in dogs with intrahepatic shunts compared to extrahepatic shunts.

MicroRNAs (miRNAs), a class of small noncoding RNAs, are important regulators of post-transcriptional gene expression [49]. MiRNAs are important genetic regulators of cellular processes, including tissue injury, hepatic development and repair responses [50, 51] and are currently reported as promising biomarkers for liver injury in a variety of hepatobiliary diseases in humans [52] and dogs [53]. However in studying biomarkers for liver injury in hepatobiliary diseases, the observed microRNAs, including the liver specific miR122, were not significantly different in CPSS dogs compared to healthy dogs [53]. This confirms the observation that the hepatocytes themselves in CPSS livers are not affected, it is merely a number-game. However, because the examined microRNAs are based on association with hepatic injury, rather than hepatic regeneration, microRNA analysis could still play an important role in our study.

Therefore, it would be interesting to determine a full serum miRNAs profile for shunt dogs which could have a predictive value in the recovery of CPSS dogs after surgery.

Conclusion

Extensive genomic research based on the different aetiology and the shared physiology of extrahepatic and intrahepatic shunts has resulted in novel insights in inherited CPSS. For intrahepatic shunts, the subtypes are likely to be caused by the same gene defect. This also appears to be case for extrahepatic shunts. Samples of all extrahepatic and intrahepatic subtypes could therefore be used in gene expression profiling. This resulted in two possible candidate genes (VCAM1 and WEE1) or their regulating pathways causing CPSS in dogs. One of the characteristics of CPSS in dogs, hepatic lipid accumulation, is shared in both extra- and intra hepatic shunts. Reflecting the presence of an altered lipid related gene expression in shunt dogs, the similarities in the amount of lipid accumulation and accumulating lipid species, and the difference in genetic background in both shunt types, indicate that lipid accumulation in CPSS occurs secondary to portosystemic shunting. Finally gene expression based prediction of recovery related to hepatic regenerative potential after shunt attenuation in dogs resulted in an association of three genes with long-term successful clinical outcome. Based on these results a model was constructed, which also includes plasma albumin, to predict recovery after surgical attenuation of a portosystemic shunt in dogs. All together, these results will not only aid in the understanding of this disease in dogs but will also provide information regarding pathogenesis, pathophysiology and prognosis of CPSS in man.

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Addendum

Nederlandse samenvatting
voor niet-ingewijden

About the Author

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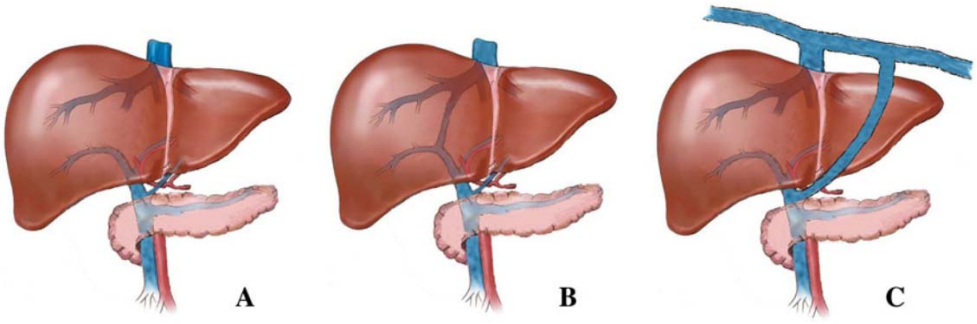
Addendum

Nederlandse samenvatting
voor niet-ingewijden

De lever is een orgaan in het lichaam dat belangrijke functies uitvoert, zoals het verwijderen van giftige stoffen uit het lichaam. Om zijn functies te kunnen vervullen is het noodzakelijk dat bloed vanuit het maagdarmkanaal, de milt en de alvleesklier via de leverpoortader (vena porta) naar de lever gebracht wordt voordat het de grote (systemische) bloedomloop bereikt.

Een portosystemische shunt is een abnormale verbinding tussen de vena porta en de systemische bloedsomloop, waardoor bloed afkomstig uit het maagdarmkanaal, de milt en de alvleesklier, de lever nagenoeg compleet omzeilt. Een portosystemische shunt kan aangeboren zijn of kan op latere leeftijd, als gevolg van onderliggende leveraandoeningen, verkregen worden. Dit proefschrift richt zich op de aangeboren portosystemische shunt, welke een erfelijke achtergrond heeft. Terwijl shunts zeldzaam zijn bij mensen, komen ze frequent voor bij bepaalde hondenrassen en worden ze naast in honden en mensen ook beschreven in muizen, katten en schapen. Omdat de portosystemische shunt bij de hond veel overeenkomsten heeft met die van de mens, kan de hond ook als model voor de mens ingezet worden.

Een aangeboren of congenitale portosystemische shunt (CPSS) is een verzamelnaam voor twee verschillende soorten shunts; de intrahepatische portosystemische shunt (IHPSS), waarbij het foutieve bloedvat door de lever verloopt, en een extrahepatische portosystemische shunt (EHPSS), waarbij het abnormale bloedvat buiten de lever loopt (Fig. 1).



Figuur 1 Overzicht van de anatomie van een normale lever en levers met een intra- of extrahepatische shunt. In de normale lever mag er geen verbinding tussen de bloedvaten bestaan zodat bloed door de functionele levereenheden kan stromen (A). In het geval van portosystemische shunting stroomt een groot deel van het bloed niet door de functionele levereenheden waardoor het bloed de leverstofwisseling omzeilt. Een intrahepatische shunt is een abnormale verbinding tussen de vena porta en de systemische circulatie die in de lever loopt (B), bij een extrahepatische shunt verloopt deze foutieve verbinding buiten de lever (C). Uit: van Steenbeek et al. (2012). Inherited liver shunts in dogs elucidate pathways regulating embryonic development and clinical disorders of the portal vein. Mamm Genome. 23: 76-84.

De belangrijkste doelstellingen van dit proefschrift worden beschreven in **Hoofdstuk 1**. Samengevat wordt er allereerst inzicht verkregen in de ontstaanswijze en genetische achtergrond van de verschillende typen portosystemische shunts bij honden (**Hoofstuk 3 en 4**). Ten tweede wordt er gekeken naar de ontstaanswijze van een veel voorkomend fenomeen dat wordt gezien in de levers van honden met een shunt: vetstapeling (**Hoofdstuk 5**). In het laatste hoofdstuk hebben we ons gericht op het ontwerpen van een model waarmee we het al dan niet slagen van een operatieve behandeling bij honden met een shunt beter kunnen voorspellen (**Hoofdstuk 6**). Tenslotte wordt er afgesloten met een (Engelse) samenvatting van dit proefschrift, waarbij de verkregen resultaten ook bediscussieerd worden (**Hoofdstuk 7**).

In **Hoofdstuk 2** wordt er een overzicht gegeven van CPSS in honden. Een aangeboren portosystemische shunt is een erfelijke aandoening die veel impact heeft op het lichaam van de aangedane hond. Omdat bloed vanuit het maagdarmkanaal, milt en de alvleesklier om de lever heen wordt geleidt, heeft de lever een tekort aan zuurstof, voedingsstoffen en belangrijke groeistoffen waardoor de lever zich niet goed kan ontwikkelen en niet goed kan functioneren. Gifstoffen zoals ammoniak, aromatische aminozuren, en lichaamseigen gifstoffen kunnen hierdoor niet door het leverweefsel geklaard worden. Het gevolg is dat deze gifstoffen ophopen in het bloed en in de hersenen terecht komen, wat leidt tot hepatische encefalopathie (neurologische verschijnselen) in de aangedane hond. Andere verschijnselen die kunnen worden waargenomen in honden met een shunt zijn vrij aspecifiek en hebben veelal betrekking tot het maagdarmkanaal en de urinewegen. De klinische verschijnselen zijn variabel en afhankelijk van het type van de shunt, de anatomie, voeding, en bijkomende aandoeningen. Hierdoor worden honden met een shunt op basis van de klinische verschijnselen op verschillende leeftijden gediagnosticeerd, al worden de meeste honden voor hun eerste levensjaar aangeboden bij de dierenarts.

De behandeling van honden met een shunt bestaat uit het operatief sluiten van de shunt. Helaas kan er door het ineens sluiten van de shunt een te hoge bloeddruk in de poortader ontstaan, waardoor het dier in shock kan raken en kan komen te overlijden. Daarom wordt er doorgaans een methode gebruikt waarbij de shunt wordt gesloten tot het maximaal haalbare niveau in de patient. Desalniettemin is het herstel na de operatie wisselend en slecht voorspelbaar. Als alternatief voor chirurgie, kunnen ondersteunende maatregelen middels dieetaanpassingen, antibioticum-therapie en/of lactulose de klinische verschijnselen in de aangedane honden verminderen. Echter vormen zij geen permanente oplossing en is de overlevingstijd met ondersteunende maatregelen ten opzichte van chirurgische sluiting van de shunt dan ook niet langer.

Overeenkomsten in de ontstaanswijze binnen de subtypen van extrahepatische en intrahepatische portosystemische shunts

Shunts worden voornamelijk waargenomen in rashonden, waarvan er voor een aantal hondenrassen is beschreven dat deze een predispositie hebben om een shunt te ontwikkelen. Dit impliceert een erfelijke achtergrond. IHPSS worden vooral waargenomen in honden van grote rassen zoals de Ierse Wolfshond, Australian Cattle dog, Labrador Retriever en Golden Retriever. EHPSS worden daarentegen voornamelijk gezien in kleine honden rassen zoals de Cairn Terrier, Yorkshire Terrier, Jack Russell Terrier, Dashond, Dwergschnauzer en Maltezer. Voor een aantal van deze rassen is bewezen dat de ziekte genetisch bepaald is. Wel zien we hierbij dat de manier van overerven van een IHPSS en EHPSS verschilt, waarbij de overerving van EHPSS meer complex blijkt te zijn. Ook al verschilt de genetische achtergrond van beide shunt typen, ze hebben identieke pathofysiologische gevolgen voor de aangedane hond.

Voordat een genetische studie kan worden uitgevoerd om inzicht te krijgen in portosystemische shunts bij de hond, is het belangrijk om vast te stellen of een zelfde genetische achtergrond verwacht kan worden binnen de subtypes van IHPSS en EHPSS. Voor intrahepatische shunts wordt een onderverdeling gemaakt tussen links, rechts en centraal afgesplitste shunts. De links afgesplitste shunt wordt aangewezen als een persisterende ductus venosus, een structuur die normaal in de foetus aanwezig hoort te zijn, maar na de geboorte hoort te sluiten. Of de rechts en centraal afgesplitste shunts dezelfde oorsprong hebben is niet vastgesteld. Wel zien we dat zowel linker als rechter IHPSS herhaaldelijk voorkomen binnen een nest in Ierse Wolfshonden, wat indiceert dat beide typen, en dus het sluiten van de ductus venosus, veroorzaakt wordt door hetzelfde gen defect.

Gebaseerd op de uitmonding in de systemische circulatie kunnen EHPSS onderverdeeld worden in twee verschillende subtypes: portocavaal en porto-azygos shunts. In **Hoofdstuk 3** wordt het voorkomen van de EHPSS subtypen binnen hondenrassen onderzocht. Wanneer over het algemeen zowel portocavaal shunts als porto-azygos shunts bestaan binnen hetzelfde ras dat een predispositie heeft voor portosystemische shunts, dan is het aannemelijk dat er deze twee subtypen dezelfde genetische achtergrond hebben.

Voor de studie in **Hoofdstuk 3** zijn gegevens van 135 honden met een extrahepatische shunt retrospectief bestudeerd. De studiegroep bestond uit 54 reuen en 81 teven van in totaal 24 verschillende rassen. Het overgrote deel van de honden had een portocavaal shunt (75%), terwijl

25% een porto-azygos shunt had. Het was interessant dat slechts 27% van de honden met een porto-azygos shunt een reu was, terwijl er bij de honden met een portocavaal shunt er een gelijk aantal reuen en teven was aangedaan. In nagenoeg alle rassen, waarvan er per ras minimaal zes honden met een shunt beschikbaar waren, werden beide typen EHPSS gevonden. Een opmerkelijke bevinding uit deze studie was dat de diagnose bij honden met een porto-azygos shunt gemiddeld op een significant latere leeftijd (32.2 maanden) gesteld werd in vergelijking met honden met een portocavaal shunt (12.3 maanden).

Geconcludeerd kan worden dat het voorkomen van beide suptypen van EHPSS binnen vele hondenrassen impliceert dat de EHPSS subtypes varianten zijn van dezelfde erfelijke aandoening en dat overeenkomstige genen verantwoordelijk zijn voor het voorkomen van EHPSS binnen de verschillende hondenrassen. Aangezien EHPSS complex overerven, is het mogelijk dat het subtype bepaald wordt door een kleinere genetische component of modulerende factoren tijdens de ontwikkeling van het embryo.

De bovenstaande bevindingen hebben tot de aanname geleid dat de verschillende subtypen die voorkomen binnen IHPSS of EHPSS een gelijke genetische basis lijken te hebben en dat er in de zoektocht naar de genetische oorzaak van EHPSS en IHPSS, geen onderscheid gemaakt hoeft te worden tussen de subtypen binnen deze twee vormen van CPSS.

Verschillen in de ontstaanswijze van extrahepatische en intrahepatische portosystemische shunts

Gebruik makend van overeenkomst in pathofysiologische gevolgen die het resultaat zijn van de omleiding van de portale bloedstroom rond de lever en de verschillen in de ontstaanswijze van EHPSS en IHPSS, is er een studie ontwikkeld om inzicht te verkrijgen in de genetische achtergrond van de verschillende typen portosystemische shunts (**Hoofdstuk 4**). Hierbij worden verschillen in de expressie van genen in leverweefsel tussen honden met een EHPSS of een IHPSS, in vergelijking met leverweefsel van gezonde honden, gezien als een kenmerk van het betreffende subtype, terwijl verschillen ten opzichte van controle honden, maar overeenkomend binnen EHPSS en IHPSS waarschijnlijk het gevolg zijn van een secundair effect van de shunt, zoals de afwezigheid van de portale bloedstroom naar de lever.

Een microarray analyse waarbij er gekeken werd naar de mRNA expressie in leverweefsel van 47 honden met een shunt, heeft een veranderde genexpressie in leverweefsel van honden met een IHPSS of EHPSS van 26 genen aangetoond, in vergelijking met leverweefsel van gezonde

honden. Hierbij waren 6 en 19 genen specifiek voor honden met respectievelijk een EHPSS of IHPSS, wat indiceert dat deze genen mogelijk betrokken kunnen zijn bij de ontwikkeling van deze aangeboren afwijkingen. Vervolgens is een kwantitatieve PCR van deze genen uitgevoerd op leverweefsel van 14 IHPSS, 17 EHPSS en 8 gezonde honden, waarbij een significant verschillende expressie van de genen *ACBP*, *CCBL1*, *GPC3*, *HAMP*, *PALLD*, *VCAM1*, en *WEE1* is vastgesteld. Daarna is er bepaald of deze veranderde genexpressies op eiwit niveau stand hielden middels immunohistochemie en Western blots. Hierbij werd de verhoogde expressie van VCAM1 in IHPSS, maar zijn afwezigheid in EHPSS; een verhoogde WEE1 expressie in IHPSS, maar niet in EHPSS; en een verlaagde expressie van CCBL1 in beide shunttypes waargenomen. Gebaseerd op de fysiologische functies van deze genen kan een mogelijke causale rol voor VCAM1 in EHPSS en voor WEE1 in IHPSS worden toegeschreven. De nog niet verklaarde aspecten in de pathofysiologie van hepatische encefalopatie bij portosystemische shunting kunnen mogelijk in associatie met CCBL1 worden onderzocht.

Vetstapeling in portosystemische shunting

In leverbiopten van honden met een CPSS wordt regelmatig vetstapeling in de levercellen beschreven. Het is mogelijk dat ook hier een genetische oorzaak voor is, al wordt waargenomen dat na het chirurgisch sluiten van de shunt de vetstapeling in de levercellen afneemt wat initieert dat vetstapeling in CPSS een gevolg is van de shunting. In **Hoofdstuk 5** wordt een uitgebreide analyse gegeven van het vetmetabolisme in honden met een CPSS om op deze manier inzicht te krijgen in de ontstaanswijze van vetstapeling in honden met een aangeboren shunt.

Hiertoe is allereerst de mate van vetstapeling bepaald in weefselbiopten van honden met een EHPSS (n = 7) en honden met een IHPSS (n = 5), welke is vergeleken met de mate van vetstapeling in leverweefsel van gezonde honden (n = 4). Vetstapeling was middels een Oil Red O kleuring, specifiek voor vetten, duidelijk zichtbaar in honden met een portosystemische shunt, waarbij er gemiddeld een 12-voudige toename van het vetgehalte in honden met een levershunt werd gezien ten opzichte van het gezonde leverweefsel.

Als tweede onderdeel is er in deze studie gekeken naar verschillen in vet gerelateerde genexpressie tussen honden met een shunt en gezonde honden middels een mircoarray analyse en kwantitatieve PCR en zijn de vetprofielen uit het leverweefsel van gezonde honden en honden met een CPSS vergeleken middels een combinatie van vloeistof chromatografie en massaspectrometrie (HPLC-MS). Middels de microarray analyse (EHPSS n = 32, IHPSS n = 15 en

controle *n* = 2 honden) werd er gekeken naar vet gerelateerde genen die in honden met een CPSS juist verhoogd of verlaagd tot expressie komen in vergelijking tot gezonde honden. Elf genen gerelateerd aan vetmetabolisme, transport of opslag, werden verder onderzocht middels kwantitatieve PCR in leverweefsel van 80 honden (EHPSS *n* = 35, IHPSS *n* = 28 en controle *n* = 17 honden). Deze methode bevestigde de veranderde expressie van zeven vet gerelateerde genen (*CRP*, *FABP1*, *IGFBP1*, *ITIH4*, *PLIN2*, *SAA1* en *SEC14L3*), wat aangeeft dat er in honden met een shunt wel degelijk een veranderd vetmetabolisme is. Om inzicht te krijgen welke vetten er stapelen in CPSS is er een HPLC-MS uitgevoerd op lever weefsel (EHPSS *n* = 7, IHPSS *n* = 5 en controle *n* = 4 honden) die aantoonde dat in honden met een shunt een verschuiving gezien werd in het aandeel van omega-3 vetzuren ten koste van omega-6 vetzuren, in het bijzonder een verschuiving van Arachidonzuur naar Decosahexaeenzuur, in vergelijking met gezonde honden. Gezien de fysiologische rol van DHA, suggereren we dat het toegenomen aandeel van Decosahexaeenzuur een beschermende rol kan hebben in CPSS en mogelijk ontsteking kan beïnvloeden. Het is interessant om te zien dat drie van de zes verhoogd tot expressie komende genen uit de genexpressie analyse (*CRP, ITIH4, SAA1*), naast hun vet geassocieerde functie ook als acute fase eiwitten dienen. Acute fase eiwitten zijn eiwitten die betrokken zijn bij ontstekingsprocessen in het lichaam. Alhoewel we histologisch geen tekenen van ontsteking zien in de weefselbiopten, is het interessant om in eventuele vervolg studies vast te stellen of er een associatie van ontsteking met vetstapeling in de levers van honden met een CPSS wordt gezien. Zeker aangezien er in mens- en diermodellen is beschreven dat vetten een ontstekingsreactie kunnen triggeren.

Samengevat kan worden gesteld dat uit de histologische beoordeling van de weefselbiopten, de genexpressie studie, en de vet analyse is gebleken dat er in honden met een portosystemische shunt sprake is van vetstapeling, een veranderde vet gerelateerde genexpressie en andere vetsamenstelling. De overeenkomsten in de mate van vetstapeling en de soorten vetten die stapelen in zowel EHPSS als IHPSS, indiceren dat vetstapeling in CPSS het gevolg is van de shunting van het portale bloed.

Om te bevestigen dat vetstapeling ontstaat als gevolg van shunting van het portale bloed, zijn er in het laatste onderdeel van dit hoofdstuk leverorganoiden gekweekt van EHPSS (*n* = 4), IHPSS (*n* = 4) en gezonde honden (*n* = 4). Organoïden zijn uit lichaamseigen stamcellen gekweekte mini-organen, in dit geval mini-levers, die veel overeenkomsten vertonen met een echte lever. De organoïden in deze studie zijn vervolgens blootgesteld aan vrije vetzuren om de vetzuuropname in de organoïden te analyseren. Het onderzoek heeft laten zien dat vet accumulatie na vrije vetzuur incubatie vergelijkbaar was tussen de organoïden van EHPSS,

IHPSS en gezonde honden. Dit indiceert dat de vetstapeling gezien bij portosystemische shunting niet veroorzaakt wordt door een genetisch defect, maar een gevolg is van de omleiding van het portale bloed.

Het onderzoek beschreven in **Hoofdstuk 5** benadrukt de impact van de afwezigheid van een normale portale circulatie bij honden met een shunt, wat leidt tot verminderde leverontwikkeling en lever functie. Daarnaast kan dit onderzoek naar vetstapeling bij honden, helpen om de pathofysiologie van een aandoening, genaamd niet-alcoholische vetleverziekte bij mensen, beter te kunnen bestuderen.

Op genexpressie gebaseerde voorspelling van volledig herstel na operatieve sluiting van de shunt in honden

Wanneer honden gediagnosticeerd zijn met een CPSS, wordt geadviseerd deze honden te behandelen om klinische manifestaties van de aandoening, zoals hepatische encefalopathie te voorkomen, behandelen, of te verminderen. Verschillende factoren zoals de leeftijd waarop de diagnose wordt gesteld, type van portosystemische shunt, exacte locatie, maar ook financiële aspecten en bereidwilligheid van een eigenaar, hebben invloed op de behandelingsmogelijkheden voor individuele honden. Behandelingsmogelijkheden bestaan uit chirurgische sluiting van de shunt, medicamenteuze ondersteuning of een combinatie van beide. De enige manier om volledig herstel te krijgen in aangedane honden is het chirurgisch sluiten van het afwijkende vat. Helaas is het lastig te voorspellen of een operatie slaagt en hoe de prognose op langere termijn zal zijn. Volledig herstel kan alleen verkregen worden wanneer de lever en portale vasculatuur na de operatie kunnen uitgroeien naar normale grootte en de leverfunctie hersteld kan worden.

Als laatste wordt in **Hoofdstuk 6** een genexpressie studie uitgevoerd op leverweefsel van honden verkregen tijdens de chirurgische sluiting van de shunt om genen te identificeren die geassocieerd zijn met volledig post-operatief herstel na chirurgische sluiting van de shunt. Hiervoor is eerst een micro-array uitgevoerd op leverweefsel van 23 honden die hersteld zijn na de operatie en 23 honden die niet hersteld zijn . Dit leverde een lijst op van 43 genen die verschillend tot expressie kwamen in honden met een succesvolle operatie in vergelijking met honden waar de operatie niet succesvol was. Vervolgens is een tweede studie uitgevoerd waarin deze genen bevestigd werden middels kwantitatieve PCR in een groep van 31 honden met een succesvolle operatie en 31 honden met een niet-succesvolle operatie. Daarbij is ook het plasma

albumine vergeleken. In deze studie wordt gehypothetiseerd dat lever regeneratie na chirurgische sluiting een vergelijkbaar proces is in extra- en intrahepatische shunts en dat op basis van genexpressie profielen de uitkomst na de operatie voorspeld kan worden.

Op basis van bovenstaande case-control studies is een predictie model opgesteld op basis van de genexpressie levels van *Dihydrodiol dehydrogenase* (*DHDH*), *Endoplasmic Reticulum Lectin 1* (*ERLEC1*), en *LysM putative peptidoglycan-binding domain-containing protein 2* (*LYSMD2*), en preoperatief plasma albumine. Dit model voorspelde het herstel correct met >50% zekerheid in individuele honden en heeft een sensitiviteit van 77% en specificiteit van 90%.

Een bijkomende bevinding in deze studie was dat de leeftijd waarop chirurgie wordt uitgevoerd niet gerelateerd kon worden aan klinisch herstel en de ontwikkeling van complicaties op de langere termijn, wat overeenkomt met voorgaande studies. Geconcludeerd kan worden dat leeftijd geen rol zou mogen spelen wanneer chirurgie overwogen wordt in individuele gevallen.

Het ontwikkelde predictiemodel lijkt in staat een goed onderscheid te maken tussen herstelde en niet herstelde honden na een operatieve sluiting van de shunt. Het model zou daarmee van grote waarde kunnen zijn om een wetenschappelijk onderbouwde keuze te kunnen maken over de behandelingsmethode voor individuele honden met een CPSS. Echter kent het model ook enkele nadelen. Het model is gebaseerd op genexpressie niveaus gemeten in leverweefsel en vanwege de invasiviteit van de biopsie procedure om dit weefsel te verkrijgen, is dit model hierdoor minder klinisch toepasbaar. Daarnaast zijn de weefsel tijdens de operatie afgenomen en kan een effect van anesthesie op de resultaten niet worden uitgesloten. Ook al verwachten we niet dat genetisch vastgestelde voorspellende factoren variabel tot expressie komen in de weefselbiopten, niet-genetische factoren die de kans op herstel zouden kunnen beïnvloeden (zoals de aanwezigheid/mate van hepatische encefalopathie en/of vetstapeling), kunnen veranderen in de loop van de tijd. Om een bruikbare test te ontwikkelen om de kans op herstel na een operatie te voorspellen in individuele honden zijn follow-up studies nodig, waarbij DHDH, ERLEC1, LYSMD2 en albumine levels in bloedmonsters gevalideerd zouden moeten worden bij honden over de hele wereld.

Het uitgebreide genetische onderzoek beschreven in dit proefschrift is gebaseerd op de verschillen in de ontstaanswijze maar de overeenkomsten in de pathofysiologie van EHPSS en IHPSS en heeft geresulteerd in nieuwe inzichten in CPSS. De subtypen van extra- en intrahepatische shunts lijken veroorzaakt te worden door dezelfde genetische defecten, waardoor ze allen gebruikt kunnen worden in genexpressie studies. Dit heeft geresulteerd in twee mogelijke kandidaat genen (VCAM1 en WEE1) die mogelijk betrokken zijn bij de

ontwikkeling van CPSS in honden. Een van de kenmerken van CPSS in honden, vetstapeling, wordt waargenomen in zowel extra- als intrahepatische shunts. De veranderde vet-gerelateerde genexpressie in shunt honden en de overeenkomsten in stapelende vetsoorten, indiceren dat vetstapeling in CPSS het gevolg is van de omleiding van het portale bloed om de lever. Tot slot is een predictiemodel opgesteld welke gerelateerd is aan de regeneratiecapaciteit van de lever op basis van drie genen en plasma albumine, waarmee het lange-termijn herstel na een shunt operatie voorspeld kan worden. Concluderend kan worden gesteld dat de resultaten die in dit proefschrift zijn beschreven bijdragen aan de kennis over portosystemische shunts in honden, maar daarnaast ook kunnen bijdragen aan de kennis van deze aandoening in mensen.

Addendum

About the Author

Curriculum Vitae

Lindsay Van den Bossche was born on November 20, 1987 in Zaltbommel, the Netherlands. She started her studies in Veterinary Medicine in 2006 at the Faculty of Veterinary Medicine at the Utrecht University. After graduating with honours for the first four years of the curriculum she participated in the Honours Program of the Faculty of Veterinary Medicine, which gave her the opportunity to perform one year of research (2010-2011). Under supervision of Prof. Dr. Jan Rothuizen, Dr. Peter A.J. Leegwater and Dr. Frank G. van Steenbeek she investigated several aspects of congenital portosystemic shunts in dogs with her study entitled 'The genetic background of congenital portosystemic shunts in dogs'. After graduating her studies Veterinary Medicine cum laude in 2013, she started working in the private practice in small animal medicine in the Netherlands (2013-2017). Since October 2014 she concurrently enrolled as a PhD student at the Department of Clinical Sciences of Companion Animals at the Utrecht University. Her research focussed on the 'Genetic and Functional Analysis of Congenital Portosystemic Shunts in Dogs' and was supervised by Prof. Dr. Jan Willem Hesselink, Prof. Dr. Iwan A. Burgener, Dr. Bart Spee, and Dr. Frank G. van Steenbeek. In September 2017 she started the Residency Program in Veterinary Internal Medicine of companion animals at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, the Netherlands.

Presentations and publications

Presentations

Oral presentation 2nd price – Sept 2016

Van den Bossche, L. Schoonenberg, V.A.C., Burgener, I.A., Penning, L.C., Schrall, I.M., Kruitwagen, H.S., van Wolferen, M.E., Grinwis, G.C., Kummeling, A., Rothuizen, J., van Velzen, J.F., Stathonikos, N., Molenaar, M.R., Helms, B.J., Brouwers, J.F., Spee, B., & van Steenbeek, F.G. "Steatosis in Canine Portosystemic Shunts".

26th ECVIM congress, Gothenburg, Sweden

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Addendum

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