The genetic background of congenital portosystemic shunts in dogs

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The genetic background of congenital portosystemic shunts in dogs

De genetische achtergrond van congenitale portosystemische shunts in honden
(met een samenvatting in het Nederlands)

Proefschrift

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Content

Chapter 1

General Introduction 1: Inherited livershunts in dogs elucidate pathways regulating embryonic development and clinical disorders of the portal vein

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Abstract

Congenital disorders of the hepatic portal vasculature are rare in man, but occur frequently in certain dog breeds. In dogs, there are two main subtypes: intrahepatic portosystemic shunts, which are considered to stem from defective closure of the embryonic ductus venosus, and extrahepatic shunts which connect the splanchnic vascular system with the vena cava or vena azygos. Both subtypes result in nearly complete bypass of the liver by the portal blood flow. In both subtypes the development of the smaller branches of the portal vein tree in the liver is impaired and terminal branches delivering portal blood to the liver lobules are often lacking. The clinical signs are due to poor liver growth, development, and function. Patency of the ductus venosus seems to be a di-genic trait in Irish wolfhounds, whereas Cairn terriers with extrahepatic portosystemic shunts display a more complex inheritance. The genes involved in these disorders cannot be identified with the sporadic human cases, but in dogs, the genome wide study of the extrahepatic form is at an advanced stage. The canine disease may lead to the identification of novel genes and pathways cooperating in growth and development of the hepatic portal vein tree. The same pathways likely regulate the development of the vascular system of regenerating livers during liver diseases such as hepatitis and cirrhosis. Therefore the identification of these molecular pathways may provide a basis for future pro-regenerative intervention.

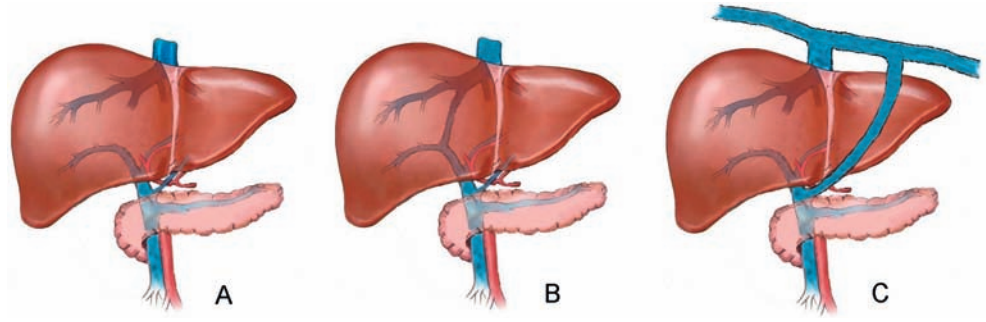
Congenital portosystemic shunts and associated liver dysfunctions

Maintenance of liver mass and function is mostly provided by hepatic perfusion, especially by the quantity and quality of portal blood (1). Normally, the abdominal organs connected to the splanchnic vascular bed (gastro-intestinal tract, pancreas, spleen) supply their efflux blood to the portal vein. Portal blood delivers toxins, nutrients and bacteria absorbed from the intestine to the liver. In addition, it contains specific hepatotrophic factors like insulin, insulin-like growth factors, glucagon and hepatocyte growth factor (1), and carries 50% of the oxygen supply to the liver (2).

Congenital portosystemic shunts (CPSS) are abnormal vascular connections made during embryonic development, which connect the portal vein directly to the vena cava or vena (hemi)azygos. Portal blood thus bypasses the liver and its functional units, the liver lobules (3, 4). Because of the importance of portal blood for the liver, portosystemic shunting has severe impact on the homeostasis and wellbeing of the organism. First, there is severely impaired liver growth and atrophy of the remaining hepatocytes, leading to reduced hepatic functions (3, 5-7). Secondly, due to portosystemic shunting the important function of the liver to clear portal blood cannot be exerted so that toxins and metabolites reach the systemic circulation in high concentrations. Substances derived from the gastrointestinal tract and pancreas, like ammonia, aromatic amino acids, absorbed bacteria and endotoxins, hormones and growth factors are not subjected to hepatic metabolism or presented to the liver (2-4). A major consequence is that the brain is exposed to neurotoxins, resulting in hepatic encephalopathy and eventually in death (8). The biochemical features and the associated clinical signs are similar between dogs and humans with congenital portosystemic shunts. In addition, to a large extent these are similar to those seen in advanced chronic progressive liver disease with fibrosis and cirrhosis. The main difference between chronic progressive liver disease and congenital portosystemic shunts is that the first group has portal hypertension, which is absent in the congenital diseases.

Congenital portosystemic shunts can roughly be divided in two main subtypes, namely intrahepatic (IHPSS) and extrahepatic shunts (EHPSS) (1)(Figure 1). Although the genetic basis of CPSS in dogs is not clear yet, many authors have demonstrated that congenital shunts are more frequently diagnosed in purebred dogs and that a number of breeds are predisposed (9, 10), which indicates an inherited basis for this disease (11, 12). An equal frequency of affected males and females was generally reported (9, 11). In addition, EHPSS and IHPSS were very rarely seen in the same breed (3, 4, 8, 9, 13, 14) Intrahepatic shunts were mainly diagnosed in large dog breeds and extrahepatic shunt in the smaller and toy breeds (14, 15). CPSS in humans has been classified as being a rare disease (16).

Figure 1: Overview of the anatomy of a normal liver and of livers with intra- and extrahepatic portosystemic shunts.



This picture displays a schematic overview of the normal and aberrant anatomy of the liver. (A) No connection of blood vessels in the liver is seen within a normal liver resulting in a blood flow through the hepatic sinusoids. (B) In case of PSS, blood bypasses the liver sinusoids and is therefore not subjected to hepatic metabolism. The intrahepatic shunt represents an abnormal connection of the portal vein with the systemic circulation, which is seen inside the liver. (C) In case of an extrahepatic shunt the aberrant connection is located outside the liver.

Congenital portosystemic shunts in man and comparison with dogs

The same subtypes of intrahepatic and extrahepatic shunts which are known in dogs have been described in humans. Combining several literature reviews and case reports the disease appears to be more prevalent in the Japanese population. In total 173 human cases of EHPSS have been reported (6, 17-22) and 89 humans with IHPSS (6, 7, 16, 21-26). In human EHPSS a classification has been made based on presence or absence of portal blood flow (27). Ultrasound-Doppler measurement of portal flow to the liver proximal to the diversion of the shunting vessel has shown that in dogs as well cases with and cases without hepatopetal blood flow do exist. Some affected dogs even have a reversed hepatofugal portal blood flow away from the liver (28, 29).

The histological features of the liver are identical in humans and dogs with intrahepatic or extrahepatic portosystemic shunt. The findings include absence of portal veins in small portal tracts, absent or hypoplastic portal veins in medium-sized and larger portal tracts, tortuosity, increased number and hypertrophy of arterioles in the portal areas, and atrophy of hepatocytes (30-32).

With respect to clinical presentation and methods to diagnose the disease there are no essential differences between man and dog. The most prominent clinical signs relate to brain dysfunction caused by hepatic encephalopathy in man (33-35) and dog (36). This syndrome is caused by neurotoxins bypassing the liver and the blood brain barrier. The pathogenesis of the encephalopathy is multifactorial in both species, but hyperammonaemia is a common major factor. Both in man and dogs the severe brain dysfunctions caused by hepatic encephalopathy are transient and can be completely resolved upon successful surgical closure of the congenital shunt (9, 33, 37).

Phenotyping

Dogs can be diagnosed starting from an age of six weeks. A globally used screening method is measuring increased pre- and postprandial serum bile acid levels (38). This test, however, gives abnormal results in case of many different liver diseases and is sensitive, but not specific, for congenital and acquired portosystemic shunting (39). Alternatively, screening can be performed by measuring basal plasma ammonia levels (40-42). In Irish wolfhounds, however, a congenital urea cycle enzyme deficiency may also cause hyperammonaemia (43). In all other dog breeds, like in humans, the fasting blood ammonia concentration and the rectal ammonia tolerance test are the most sensitive and specific tools to detect portosystemic shunting (39). In all cases the diagnosis of the shunt and the precise subtype has to be confirmed by direct demonstration of the shunting vessel. This can be achieved by ultrasonography, computed tomography or surgery (44, 45). The diagnostic criteria used in veterinary and human medicine are essentially identical. Hepatic encephalopathy, increased plasma ammonia concentrations and a decreased ammonia tolerance indicate the likelihood of portosystemic shunting. The diagnosis in both species is confirmed and the subtype of the shunt is assessed with ultrasonography or computed tomography (33, 44, 45). The use of plasma bile acid measurement as a liver function test which is also used in the diagnostic procedure in veterinary medicine is rarely used in human medicine. This reflects a difference in established traditions between the two professions rather than a difference between the types of shunts in man and dog.

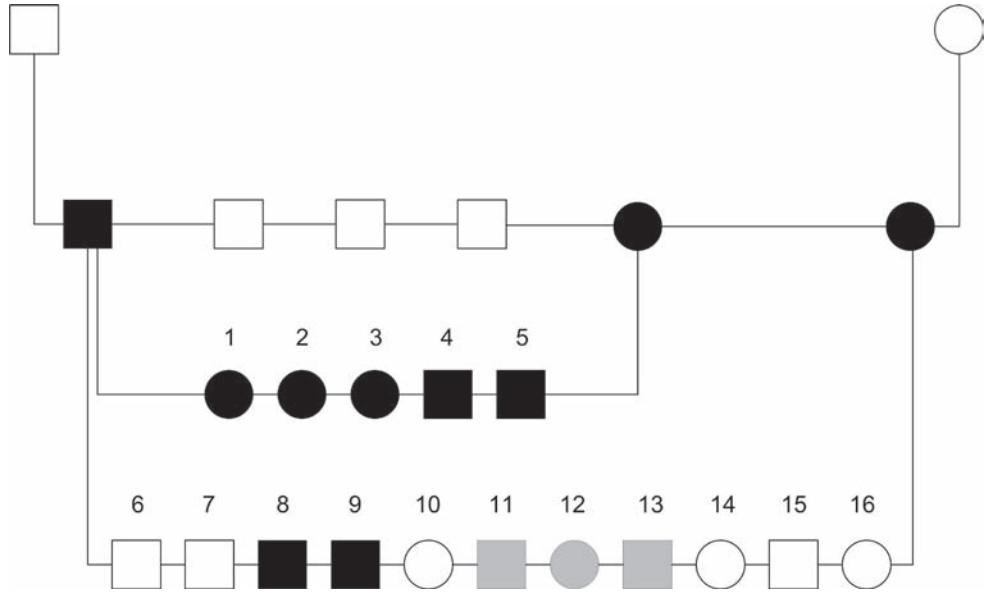
Canine intrahepatic portosystemic shunt

An intrahepatic portosystemic shunt (IHPSS) is caused by incomplete closure of the ductus venosus. The ductus venosus is an embryonal vessel connecting the vena porta with the vena cava, ensuring blood to flow from the placenta directly to vital organs without traversing liver sinusoids. This vessel should be closed within the first few days after birth. The moment of closure slightly fluctuates between species (46). In dogs the closure occurs within 6 to 9 days (47). IHPSS is almost exclusively diagnosed in large-sized pure-bred dogs (9, 48) and a predisposition is suggested for Irish wolfhounds (38, 41), Australian Cattle dogs (15), Old English Sheepdogs (49), Labrador and Golden retrievers (1, 14). The shunt can be anatomically positioned at the left or right side or centrally in the liver. Epidemiologic factors influencing the position of the shunt have been surveyed using case reports from the United States and Australia. Significant association was found between country of origin (p=0.048), breed (p=0.025), sex (p=0.016), and IHPSS location (13).

Heritability of IHPSS in Irish wolfhounds

The clear familial distribution for IHPSS in Irish wolfhounds indicates a hereditary basis (41, 50). Between 1984 and 1992 the entire Dutch Irish wolfhound population was screened for IHPSS by measuring ammonia levels in blood and subsequent ultrasonography of cases with hyperammonaemia. The observed incidence increased over the years concomitant with increased inbreeding (41). A litter of Irish wolfhounds was presented to the Utrecht University Clinic for Companion Animals for screening of IHPSS. Both parents were unaffected, and their offspring consisted of three affected and three unaffected dogs. We studied the mode of inheritance by test matings between the affected sire and the two affected bitches after surgery and maturation. The matings resulted in one litter of five affecteds only and a litter of eleven with five affected and six unaffected pups (Figure 2). The unaffected offspring in the second litter minimizes the possibility of a simple monogenic recessive disorder. The presence of both left- and right-divisional IHPSS suggests that the position is not genetically determined. The results indicate a genetic background with possibly a di-genic mode of inheritance (40). In our model two loci interact to cause the phenotype; at least three of the four alleles at the loci would be mutant in affected dogs.

Figure 2: Pedigree of test matings of Irish wolfhounds with intrahepatic shunt.



An affected male was mated with two affected sisters. Squares represent males, circles represent females. Black symbols are affected dogs, open symbols represent unaffected pups, pups with uncertain phenotypes are filled grey. Reprinted with permission of John Wiley and Sons (40).

Canine extrahepatic portosystemic shunt

Whereas intrahepatic shunts are derived from pre-existing embryonic connections, extrahepatic shunts must be considered as developmental anomalies. They represent abnormal functional communications between the embryonic vitelline veins, which form the entire extrahepatic portal system, and the cardinal venous system, which contributes only to all non-portal abdominal veins. This connection results in a shunting vessel between the portal vein or its contributors, such as the left gastric, splenic, mesenteric veins or the gastroduodenal vein and the caudal vena cava or (hemi)azygos vein. Because the extrahepatic portal vein develops from 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 could occur during any phase of embryonic development (51).

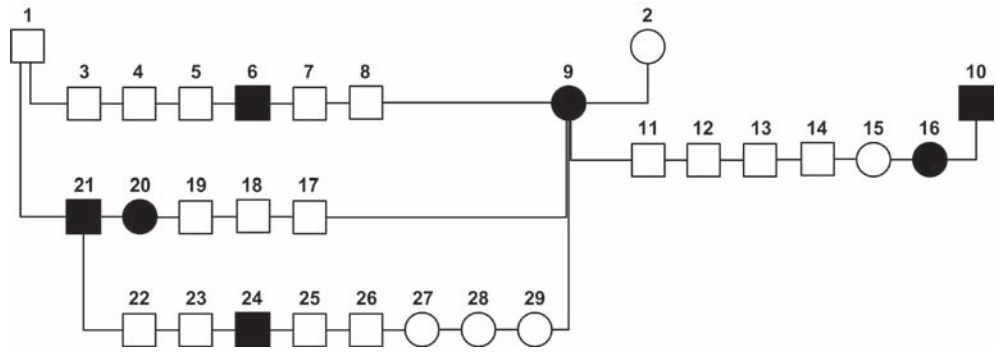
Extrahepatic shunts occur in small dog breeds with a predisposition in Cairn terriers (11), Yorkshire terriers (10, 14), Jack Russell terriers (9), Dachshunds (1), miniature schnauzers, Havanese, Dandie Dinmont terriers (14) and Maltese (15).

Heritability of EHPSS in breeds of small dogs

A pedigree analysis of affected Cairn terriers born in The Netherlands between 1990 and 2001 was performed to study the genetics of EHPSS (van Straten et al. 2005). A total of 6,367 pups were screened for shunts by measuring venous ammonia concentrations at an age of 6 weeks. Prevalences of 1.9% to 5.9% in 3 breeding lines were significantly higher than the prevalence in the entire population (0.58%) indicating a hereditary basis for this disease. Three test matings were performed (Figure 3). A successfully operated female was mated with her unaffected father, an affected son and an unrelated affected male. Four of the 19 pups (21%) born from these matings were affected, providing further evidence that EHPSS is a genetic disorder. The mode of inheritance is most likely polygenic and there seems to be no sex effect (11).

EHPSS can be classified in two subtypes namely porto-cava or porto-(hemi)azygos shunts. We have surveyed a large number of cases (van Steenbeek, unpublished) and found that both subtypes occur in each of ten predisposed breeds. On average 23% connected with the vena (hemi)azygos and 67% connected with the vena cava. The presence of both types in all predisposed breed populations indicated a common genetic basis. The erroneous development of the portosystemic connection between the vitelline system and the vena cava or the vena (hemi)azygos is most likely due to modulating factors at specific time points during embryogenesis. The cava and azygos veins are formed through several transformations of the cardinal system (51).

Figure 3: Pedigree of test matings with Cairn terriers with extrahepatic shunt.



An affected female (#9) was used three times; with her unaffected father (#1), with her affected son (#21) and with an unrelated affected male (#10). Squares represent males, circles represent females. Solid symbols: affected dogs, open symbols: healthy dogs. Reprinted with permission of John Wiley and Sons (11).

Candidate genes for congenital portosystemic shunts

By serendipity a mouse model was found which had an intrahepatic shunt as seen in breeds of large dogs and man. This knockout mouse is homozygous for a deletion in the Aryl Hydrocarbon Receptor gene (AHR) (52). More recently it was demonstrated that AHR signaling in vascular endothelial cells is necessary for developmental closure of the ductus venosus. In contrast, AHR signaling in hepatocytes is necessary to generate detoxifying responses to dioxin exposure of the liver (53). AHR activates transcription of several Cytochrome P-450 subtype genes (Figure 4) by forming a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT, also known as hypoxia-inducible factor1, beta subunit (HIF1B)) (54). A unique Cytochrome P-450 system functions in closure of the ductus venosus by its contractile effect on the sphincter region in lambs (55). Which Cytochrome P-450 subtype is responsible for this contraction is still to be determined but CYP1A1 and CYP1A2 were recently excluded (56).

ARNT is known for its role in angiogenesis under hypoxia when it dimerizes with Hypoxia Inducible Factor 1α (HIF1A). Under hypoxic conditions ARNT regulates hypoxia-activated transcription in hepatocytes (57). ARNT -/- mouse embryos die in utero around E10.5 due to extreme vascular defects (58). This impaired vascularization appears to be affected by decreased expression of Vascular Endothelial Growth Factor A (VEGFA). Activation of endothelial VEGFA combined with inactivation of stromal transforming growth factor-β was reported as being essential for AHR-mediated angiogenesis (59) suggesting an interaction between the AHR-pathway and the HIF1A-pathway.

Hypomorphic ARNT knockout mice appeared to display identical phenotypical alterations as the AHR knock-out mice (60). When a hepatocyte-specific deletion of ARNT was applied, no shunting was observed; hence hepatocyte ARNT does not function in AHR-mediated hepatovascular development (61).

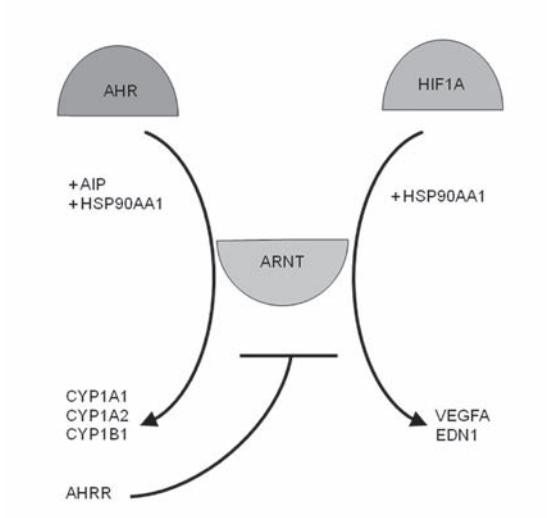


Figure 4. The relation between the AHR pathway and the HIF1A pathway.
Both pathways share ARNT and HSP90AA1 as key regulators.

Another protein that has a central role in both pathways is heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSPAA1). HSP90AA1 is found to form a cytosolic complex in hepatocytes with AHR (62), but also appears to be an essential regulator in HIF1A activation (63). Therefore HSP90AA1 could very well be the gene connecting AHR with vascularization.

Aryl Hydrocarbon Receptor Interacting Protein (AIP) was also found to play an important role in closure of the ductus venosus. Construction of AIP (-/-) mice resulted in 83% of the mice having intrahepatic shunting (64). Hepatocyte-specific knockout mice showed an important role in maintenance of cytosolic AHR levels as well as in regulation of both CYP1B1 and the Aryl Hydrocarbon Receptor Repressor. Striking though was that CYP1A1 and CYP1A2 expression was not altered, suggesting the presence of other AHR-responsive genes (65).

The AHR-pathway is a strong candidate pathway for involvement in canine and human IHPSS. In mice all homozygous AHR knockouts have IHPSS, whereas the mode of inheritance in dogs is expected to be more complex. In addition, the mouse model has quite a complex phenotype with multi-organ lesions which do not occur in dogs or humans with IHPSS.

Closure of the ductus arteriosus, the fetal shunt connecting the pulmonary artery with the aorta that allows blood to bypass the unexpanded lungs, is physiologically comparable to closure of the ductus venosus in the liver. Closure of the ductus arteriosus appears to be mediated by the Cytochrome P-450 gene CYP3A13 and the gene coding for Endothelin-1 (66). These findings could correspond with a model of inheritance of defective vessel closures involving a low number of genes.

In summary, the above genes and pathways have been shown to be involved in IHPSS in mice and should be considered important candidates for the human and canine variants, but their role remains to be elucidated.

General medical relevance of genes and pathways causing portosystemic shunts

Congenital forms of portosystemic shunts in children are rare with only 173 reported cases. Therefore it is unlikely that the molecular basis can be elucidated by study of human cases. As illustrated above, purebred dogs display the same types of congenital portostemic shunts as seen in man, with the same consequences with respect to clinical signs, pathophysiology, and liver pathology. The inbreeding of dog populations has led to an increased incidence of genetic diseases which remained incidental in the panmictic human populations. The complex genetic background of portosystemic shunts may well be unraveled in dogs. This may not only reveal new genes and pathways involved in vascular embryogenesis, but also important candidate genes for the human forms of the disease. The role of the candidate genes for the intrahepatic congenital shunts discussed above could also become clear.

The importance of these genes and pathways may, however, be much broader. Chronic progressive liver disease is a frequent and important health problem in man and dog. Recent studies have shown that the pathogenetic pathways of chronic fibrotic liver disease leading to cirrhosis are identical in man and dog (67-69). Chronic liver diseases are characterized by progressive fibrosis, ultimately leading to dissection of the normal lobular structure of the liver and ending in irreversible cirrhosis (70-74). Nowadays the only possible treatment is liver transplantation. Apart from fibrosis, progression of these diseases is characterized by decreased regeneration to compensate the concomitant loss of functional liver epithelium. The normally huge capacity of the liver to regenerate by expansion of the hepatocyte compartment becomes lost in chronic fibrotic disease, regardless of the etiology (75-79). This is in part compensated for by adult stem cell activation, but this safeguard is usually too small and comes too late (68, 69, 80-82). The third component of chronic deterioration of liver function in progressive liver disease is the ongoing impairment of portal liver perfusion (83-85). The triangle of lost regeneration, fibrosis leading to cirrhosis, and impaired vascularization determine the vicious circle of chronic progressive liver disease ending in the need for transplantation.

Both in man and in dog the presence of a congenital portosystemic shunt, either intra- or extrahepatic, is associated with deranged formation and growth of the smallest intrahepatic branches of the portal vein tree. In the veterinary literature this has been referred to as portal vein hypoplasia (1, 86) or microvascular dysplasia (5). In one case report of a dog there were three congenital vascular malformations combined: congenital portosystemic shunt, hypoplasia of the intrahepatic portal tree, and intrahepatic

arteriovenous fistula (87). As described above, we have observed that the different subtypes of congenital extrahepatic porta-cava and porta-(hemi)azygos shunts exist in similar ratios in all affected dog breeds. This indicates that a limited number of genes regulate the complex formation of the splanchnic vascular bed and associated portal venous system. It is anticipated that elucidation of these genes and the associated pathways will also give insight into the pathologic vascular derangements which are an essential part of the pathogenesis of chronic progressive liver disease. This may also provide new ways to intervene in these presently incurable diseases.

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Chapter 2

General Introduction 2: Genetic research of the canine genome

History of the dog

Domestication of the gray wolf started tens of thousands of years ago (1) resulting in the multi-functional domestic dog (*Canis lupus familiaris*). Dogs have been used for hunting, retrieving, guarding, and in the last decades predominantly as a companion animal resulting in their presently important position in family life.

Breed formation since the 19th century now has resulted in the existence of over 350 recognized breeds worldwide (American Kennel Club (http://www.akc.org/)). Due to both strong selection on specific phenotypic properties and inbreeding, a great diversity in appearance has occurred in this short period of time. Breeds form genetically isolated populations with shared as well as unique characteristics. The fast diverging of breeds is responsible for the observed involvement of just a small number of gene variants that define coat color (2), coat texture (3, 4) and size (5), indicating that the genetic architecture is expected to be simplified and involve larger gene effects.

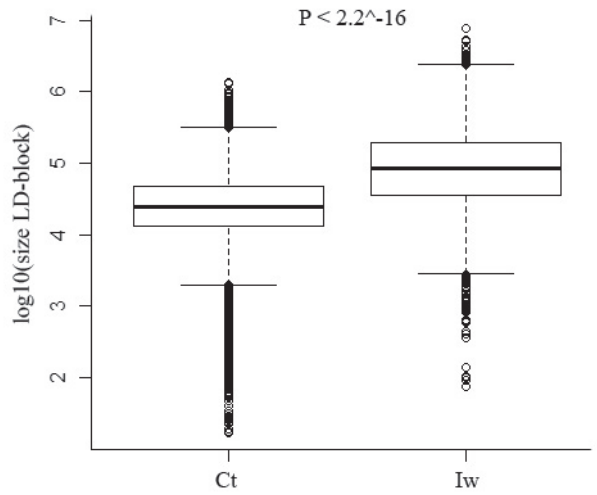
The dog as a model

The holy grail of molecular genetics has always been and still is the understanding of the etiologic basis of traits, diseases and risk factors. In human medicine, complex traits, such as cancers, cardiovascular disorders, inflammatory disorders, diabetes, Alzheimer disease, Parkinson`s disease and several neurologic disorders, have a high impact on public health due to their severity and high prevalence. Encouraged by the successful identification of mutations involved in Mendelian disorders such as Duchenne muscular dystrophy, cystic fibrosis and Huntington's disease the scientific community turned its interest towards mapping of complex traits. Instead of highly penetrant monogenic mechanisms, these disorders are rather caused by interactions of several genes, regulatory factors and environmental factors. The identification of susceptibility genes for complex human diseases remains difficult due to the complexity of the underlying causes.

The dog is very well suited to study a broad variety of naturally occurring diseases which have similar phenotypes in both humans and dogs (6). The database Online Mendelian Inheritance in Animals (OMIA) currently lists 580 genetic disorders in the dog. Nowadays purebred dog breeds have gained wide recognition as an important model for complex human diseases. Dogs have an ideal population structure for exploring the genetic basis of a variety of disorders, both Mendelian and complex (7). As a consequence of inbreeding, the genetic complexity of these diseases is reduced. Linkage disequilibrium (LD), the non-independence of alleles at different sites, in dog breeds extend over 20-100 times longer distances than LD in the human population. Therefore the number of single nucleotide polymorphisms (SNPs), that is required for genome-wide association studies (GWAS) in dogs is small compared to that required in humans (8, 9). By genotyping 120,000 SNPs in 915 dogs from 80 different breeds as well as 83 wild canids it is found that, in comparison with man, a low number of quantitative trait loci (QTL's) explain most of the phenotypic

variation of complex traits like average breed body size as well as the shape of cranial, dental and long bones across dog breeds (10). The level of relatedness differs between breeds and depends on the applied breeding strategies and the occurrence of population bottlenecks over time. For example, the Irish wolfhound population went through a stringent bottleneck in the beginning of the 19th century. Using a pedigree database of Irish wolfhounds dating back to 1862, it has been shown that the breed has gone through at least four bottlenecks since that period. As a result, over 50% of genetic variability in the present population can be explained by just three individual ancestors and over 95% by ten ancestors (11). When comparing several breeds genotyped on the same genotyping platform the difference in average LD-size and the maximum size of the LD-blocks is striking. Based on the depicted data (Figure 1) we can conclude that Irish wolfhounds have significantly larger LD-blocks compared to Cairn terriers proving the Irish wolfhound population comprises less genetic complexity.

Figure 1: Comparison of LD-block sizes in Cairn terriers (Ct) and Irish wolfhounds (Iw).



Two families of Cairn terriers (n=190) and Irish wolfhounds (n=110) have been genotyped on Illumina`s CanineHD BeadChip containing over 170,000 SNPs. SNPs that were not in direct LD (r2 < 0.8) were selected for in PLINK (12) by using the command --indep-pairwise 50 5 0.8, which selects Tagged SNPs based on pairwise genotypic correlation, recursively removing SNPs within a sliding window of 50 consecutive SNPs shifting the window with 5 SNPs forward.

The genetic background of many diseases in dogs with relevance for humans has been and is increasingly being investigated through cooperation between veterinarians and geneticists (13). A recent example of the dog serving as a model for a human disease is the mutation found in *CCDC39* in primary ciliary dyskinesia (PCD). After genotyping 5 Bobtail cases and 15 controls, a region of autozygosity (homozygosity in which the two loci are identical to the ancestral variant) was identified on chromosome 34 which was associated

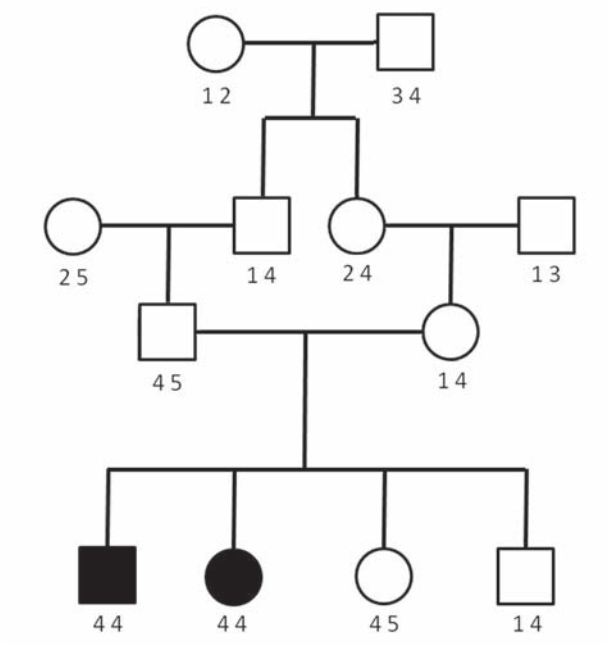
with PCD. After sequencing six genes of interest in this region a mutation that provides a stop codon in the *CCDC39* gene was found in all affected dogs. This initiated the search for mutations in *CCDC39* of human patients, resulting in the detection of loss-of-function mutations which account for a considerable part of human PCD cases (14).

Another example is the genotyping of 20 Golden retrievers affected by autosomal recessive congenital ichthyosis and 20 healthy individuals revealed an associated region on canine chromosome 12. In 1.1 Mb of this region a region of autozygosity was observed in all affected dogs. Sequencing of the coding regions and exon-intron boundaries of the candidate gene *PNPLA1* revealed a 3-bp deletion followed by a 8-bp insertion in exon 8. To study the involvement of this previously unsuspected gene in human ichthyosis, DNA samples from affecteds were sequenced revealing two causative mutations (15). Next to these two examples other causative mutations in canine diseases have proven the worthiness of the canine model for human diseases.

Genetic tools

A genetic disorder can be localized to a chromosomal region in two ways: linkage and genome wide association study (GWAS). The rationale behind both approaches is that patients share causative gene variants which they inherited from common ancestors. Linkage refers to the physical distance between loci. Mendel`s second law, the law of independent assortment, states that two loci are linked because of their physical connection; they are located closely together on one chromosome causing their alleles to co-segregate within families (Figure 2). Alleles from markers located on the same chromosome form a haplotype. Haplotypes are broken up by the process of recombination. The statistical test most commonly used in linkage analysis is the logarithm of an odds ratio (LOD) score (16). The LOD score compares the likelihood of obtaining the observed result of a marker given the trait being linked, to the likelihood of these data observed purely by chance. A LOD-score lower than -2 indicates there is no linkage between the tested marker and the phenotype. When LOD>3 the linkage between the marker and the disease is considered proven.

Figure 2: Example of linkage between a microsatellite and a recessive disorder.



Squares represent males, circles represent females. Black symbols are affected individuals, open symbols represent unaffected individuals, carriers are indicated by black and white individuals. The different alleles of the microsatellite are given per individual. This is a typical consanguineous family (first cousin marriage) in which allele 4 is segregating with the disease locus. The LOD-score for this small pedigree is 1.85 (superlink (17)).

The main difference between GWAS compared to linkage studies is the use of unrelated samples instead of closely related samples. The reason for using unrelated samples is to reduce the level of background gene sharing which is high between close relatives. By selecting samples as diverse as possible only the causative mutations and SNP variants that are in LD will occur with high frequencies in the cases and less often in the controls. The total number of SNPs necessary to detect association with 97% certainty in a complex disorder in dogs would be only 15,000 when genotyping 100 cases and 100 controls (18). In contrast, in human research more than 300,000 SNPs would be needed to detect genome wide significant association (19). Measuring association between a SNP and a trait is mostly achieved by determining allelic association using χ^2-tests. As mentioned before, the huge LD-blocks within breeds give rise to large associated genomic regions. When an identical genetic basis is expected in other breeds, incorporation of additional cases of different dog breeds in fine mapping by genotyping more densely distributed SNPs will break down these LD-blocks to much smaller regions (18).

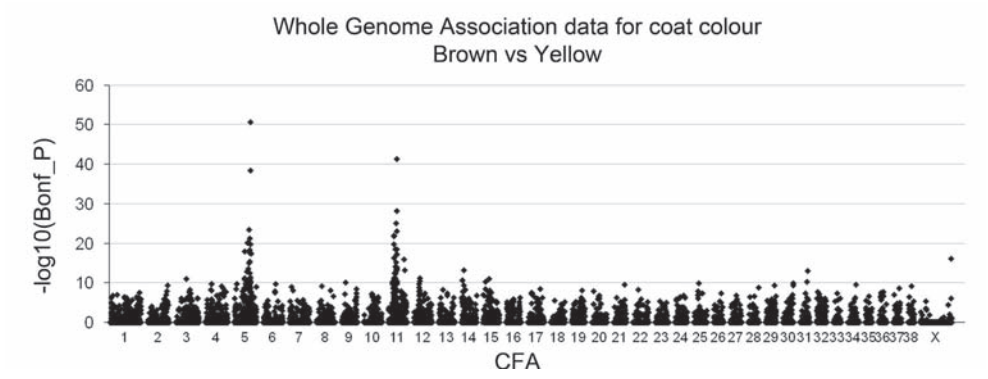
Knowledge of the mode of inheritance determines the strategy of choice. With simple Mendelian inheritance, the most powerful approach is linkage analysis using pedigree information. In former days this approach relied on the use of microsatellite markers.

Thanks to their high informativity only low numbers of samples and markers were needed to detect linkage in a monogenic disorder. An example is the mutation found in *LHX3* causing dwarfism in German Shepherds. A panel of only 256 markers was used in 3 cases, 2 healthy siblings and their 4 parents resulting in localization of the gene using linkage analysis (20). Genotyping of a microsatellite panel is unfortunately very time consuming. In 2004 the first DNA sequence of the genome of the dog became available. Sequencing the DNA of the boxer Tasha by the Broad Institute resulted in a 7.5-fold coverage genome which was a huge step forward in canine genetic research (18). With the use of the now known canine genome SNP arrays were developed. Initially these contained approximately 1,500 SNPs. Genotyping with such an array is much faster and multipoint linkage analysis proved to be comparably informative as the panel of 256 microsatellite markers. White coat spotting in boxers was found to be linked to a region on chromosome 20 which governed the pigmentation-related gene *MITF* (21).

For complex disorders in which more than one gene influence the trait the method of choice is a GWAS. In human genetics literally thousands of GWA studies have been published, implicating numerous loci in complex disorders. Nevertheless the number of variants proven to contribute to disease is still very low. In canine research just over 50 studies using GWAS have been published at present.

As an example for association studies, coat color was analyzed in the Labrador retriever, Golden retriever and Flat-coated retriever (van Steenbeek & Lavrijsen 2010, unpublished results), all of which were genotyped for 22,300 SNPs as part of research performed in bone malformations. The comparison of 86 brown individuals (73 Labrador retrievers and 13 Flat-coated retrievers) with 167 yellow retrievers (118 Labrador retrievers and 49 Golden retrievers) revealed two genome wide significant peaks after Bonferroni correction (Figure 3). The first was located on chromosome 5 with a p-value of 10^{-51}. The most associated SNP within this region was located 2.7 kb upstream of the Melanocortin 1 receptor (*MC1R*) gene. The second was found on chromosome 11 with a p-value of 10^{-41}. Within this region the most highly associated SNP was located 511 kb upstream of the Tyrosinase-related protein 1 (*TYRP1*) gene. Both genes are known to be involved in coat coloration in dogs, as well as in many other mammals (22, 23).

Figure 3: Manhattan plot for coat color.



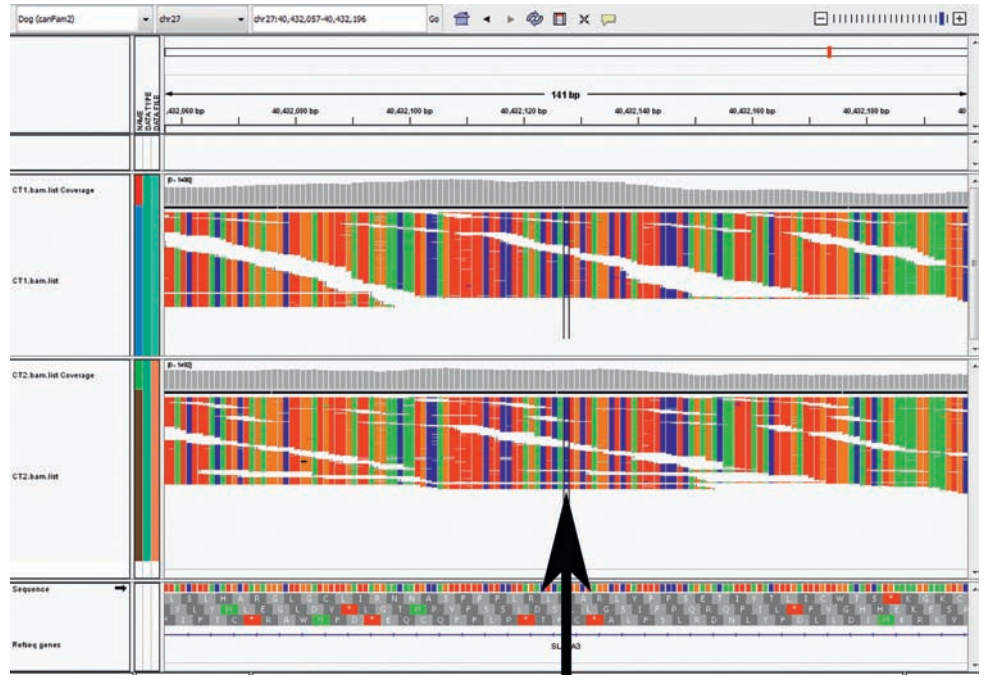
The Manhattan plot presents the results of the comparison of 86 brown retrievers (73 Labrador retrievers and 13 Flat-coated retrievers) and 167 yellow retrievers (118 Labrador retrievers and 49 Golden retrievers) genotyped on Illumina`s CanineSNP20 BeadChip. Two regions containing genome wide significant SNPs were observed. Both regions include genes known to be involved in coat coloration (MC1R on chromosome 5 and TYRP1 on chromosome 11).

Future perspective for canine genetics

The dog has been proven to be a useful large model organism for human research (13). Owing to recent technical developments it has become even easier to use the dog to identify genes that are relevant to disease. The recently developed CanineHD BeadChip of Illumina was a huge step towards compared to Illumina`s former CanineSNP20 BeadChip by harboring over five times more SNPs, hence providing ample SNP density for robust within-breed association and copy number variation (CNV) studies. The use of one breed for association studies will still result in large regions of interest. Using additional breeds in fine mapping will decrease the size of LD-blocks, provided the disease in these breeds has the same ancestral origin.

A promising state-of-the-art follow-up method for mutation detection is Next Generation Sequencing (Figure 4). This technique has a broad variety of applications. For DNA sequencing one can choose to perform whole genome sequencing or targeted re-sequencing of specific regions of interest based on genetic studies like linkage or association studies.

Figure 4: Visualization of NGS data.



Targeted re-sequencing data performed on individually bar-coded Cairn terrier controls (upper part) and Cairn terrier cases (lower part) on an ABI SOLiD 5500 and visualized in IGV (Integrative Genomics Viewer) 2.1. Single reads of 50 bp in an individual are depicted tiling mapped to the reference genome. Bases are depicted in green (A), red (T), brown (G) or blue (C). The base indicated with an arrow was covered 1,149 times in de controls and 937 times in de cases.

By using the ABI SOLiD 5500 a single run results in the DNA sequence of up to 1Mb with an average coverage of 80X in 96 individuals. Also commercial kits are available to enrich the sample specifically for the entire exome; this is available for several species (Man, Mouse, Dog, Zebra fish, and soon Cattle). Whether this is a useful strategy depends on the type of the disorder to be investigated. The success rate of focusing on only coding variants is obviously much higher in monogenic Mendelian disorders than in complex disorders. In complex disorders causative variants have been reported frequently to be located in introns influencing exon skipping (24). Also intergenic variants have been found to regulate tissue specific expression (25).

The fact that about 5% of the human genome is evolutionarily conserved when compared with rodents, implies that these regions are likely functionally important. Based on the finding that only one-third of these genomic regions are coding for proteins, it was hypothesized that intergenic regions did not consist merely of so-called “junk DNA”, but could harbor functional elements. Based on this theory The ENCODE (the Encyclopedia Of DNA Elements) consortium had its kickoff in 2004 (26). In the search of functional

DNA elements numerous datasets in many different cell types were used resulting in revolutionary publications on the 6th of September 2012 (27-37). The consortium managed to systematically map regions of transcription, transcription factor association, chromatin structure and histone modification on the human genome. With these findings a biochemical function was assigned to approximately 80% of the genome. Based on these findings it seems wise not to simply focus on coding regions when trying to find causal mutations. Translation of these data to the canine genome is still a challenge without performing additional experiments, but it seems essential to fully utilize the dog as a model organism for human disorders.

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Chapter 3

Aims and Scope

Portosystemic shunts cause portal blood derived from the gastrointestinal tract and other organs in de splanchnic drainage area to flow directly into the venous systemic circulation, bypassing the liver sinusoids. Two different forms of shunting exist, namely a congenital form (congenital portosystemic shunt (CPSS)) by which a single anomalous vessel is present without portal hypertension and an acquired form in which the multiple collaterals are the result of hepatic hypertension. For CPSS in dogs two anatomically different types have been reported in literature, namely intrahepatic portosystemic shunt (IHPSS) and extrahepatic portosystemic shunt (EHPSS). In the Netherlands 1-5% (depending on the breed) of the dog populations is affected by congenital shunting. Although the pathogenesis of CPSS in dogs is not clear yet, many authors have demonstrated that congenital shunts are particularly diagnosed in purebred dogs, which indicates an inherited basis for this disease.

The main aim of the research described in this thesis was to gain insight in the pathogenesis of CPSS.

Chapter 4 describes test matings performed in Irish wolfhounds to determine the mode of inheritance of IHPSS.

In **Chapter 5** AHR is studied in a candidate gene approach resulting in the discovery of an intronic LINE-1 insertion in Irish wolfhounds. Genes involved in possible relevant pathways in the closure of the ductus venosus were investigated on genetic and expression level.

A retrospective study of case reports of 135 dogs with EHPSS is described in **Chapter 6**, looking at correlations between sex, age and shunt localization as well as breed related predispositions of shunt localization.

Chapter 7 describes the mapping of two chromosomal regions associated with EHPSS in several dog breeds.

Microarray studies using both IHPSS and EHPSS liver tissue resulted in a relative small number of genes possibly involved in the genetic background of CPSS and is discussed in **Chapter 8**. Using these microarray studies the Urea cycle is studied in EHPSS liver samples in **Chapter 9**.

The findings and overall conclusion of these studies are discussed in **Chapter 10** and summarised in **Chapter 11**.

Chapter 4

Evidence of inheritance of intrahepatic portosystemic shunts in Irish wolfhounds

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Abstract

Background: The etiogenesis of congenital portosystemic shunt in dogs is not understood. In Irish wolfhounds intrahepatic portosystemic shunt (IHPSS) is thought to be hereditary, but the mode of inheritance is unknown.

Objectives: To document the genetic background and investigate the potential mode of inheritance of IHPSS in Irish wolfhounds.

Animals: Three mature privately owned affected siblings and their progeny produced in two litters.

Methods: Prospective, observational study. Two test matings of one affected sire with two of his affected sisters were used to determine the inheritance pattern. Affection status was determined by measuring venous blood ammonia concentrations, detection of the shunt by ultrasonography and confirmation during surgical attenuation of the intrahepatic shunting vessel.

Results: In one litter of five pups all had an intrahepatic portosystemic shunt. In the other litter five of eleven pups were affected. Both left and right sided shunts occurred in both litters. No sex predisposition was evident among affected dogs.

Conclusions and clinical importance: Our results show that IHPSS in Irish wolfhounds is a familial disorder that is likely genetic. It is unlikely that the mode of inheritance is monogenic. A di-genic, tri-allelic trait could explain the observed occurrence of IHPSS but other modes of inheritance cannot be excluded.

Introduction

Congenital portosystemic shunts are diagnosed mainly in purebred dogs and found with high incidence in Cairn terriers (1), Yorkshire terriers (2), dachshounds, miniature schnauzers, golden retrievers and Labrador retrievers (3). Intrahepatic portosystemic shunt (IHPSS) in Irish wolfhounds has been presumed to be hereditary, based on its overrepresentation in the breed and the familial distribution (4). Screening of the entire Dutch Irish wolfhound population from 1984 to 1992 showed an incidence of at least 2.1 percent which increased over the years in the absence of a breeding strategy aimed at reducing the incidence of the disease (5). However, there is no direct evidence of the inherited nature and if present, the mode of inheritance for IHPSS in Irish wolfhounds is not known. With the present availability of high throughput genotyping platforms it becomes feasible to identify disease genes based on an established mode of inheritance. This parameter is an important part in the design of linkage studies.

The aim of this study was to document the inherited nature of IHPSS in Irish wolfhounds and obtain insight into the mode of inheritance. We used test matings between affected dogs which had been successfully treated to achieve these goals. An affected male was mated with two of his affected sisters resulting in two test litters. The results confirmed the familial and likely inherited nature of the disease and provide evidence of a possible mode of inheritance.

Methods

Parents

A litter of Irish wolfhounds with a high frequency of IHPSS was identified in the course of the population screening program for the presence of IHPSS in six weeks old pups. The litter was identified using previously published methods (1, 4, 5). Both parents were unaffected, but the six offspring included one affected male, two affected females, and three unaffected males. All three affected dogs were treated successfully by surgical attenuation of the shunt (6) at an age of 3-4 months.

The pups recovered well and were raised in private households who each kept one dog. The dogs remained property of the University Clinic in order to follow their performance and to guarantee their well being. Two months after surgery the patency of the shunting vessel, which was left open partially during surgery, was tested with echo-Doppler examination and ammonia tolerance tests (6). These tests showed that there was no remaining functional portosystemic shunting in any of the three littermates. The dogs were fed a standard commercial dog food and were further kept as companion animals in the foster families.

Test matings

When the three pups were mature (> 2 years of age) the test matings were performed. The male was naturally mated with one sister and the other sister was artificially inseminated. Both females were kept at home, had uncomplicated pregnancy and gave birth to their litter without any complications. A few days before delivery one dog was hospitalized at the request of the foster family. None or the pups died or had evidence of disease at early age.

Phenotyping

The pups were tested at an age of 6-8 weeks. The basal blood ammonia concentration was measured after 12h fasting and a rectal ammonia tolerance test was performed (7). The blood samples were collected in EDTA-tubes and immediately stored on ice. Measurements were performed within 30 min as described previously (8) with the Ammonia Checker II (Arkray Factory Inc, Kyoto, Japan) or with the enzymatic assay (5, 8) (Monotest, Boehringer, Mannheim, Germany). The diagnosis of IHPSS was confirmed by ultrasonography, exploratory celiotomy, or postmortem examination. Pups that were diagnosed with IHPSS were treated surgically at 3-4 months of age.

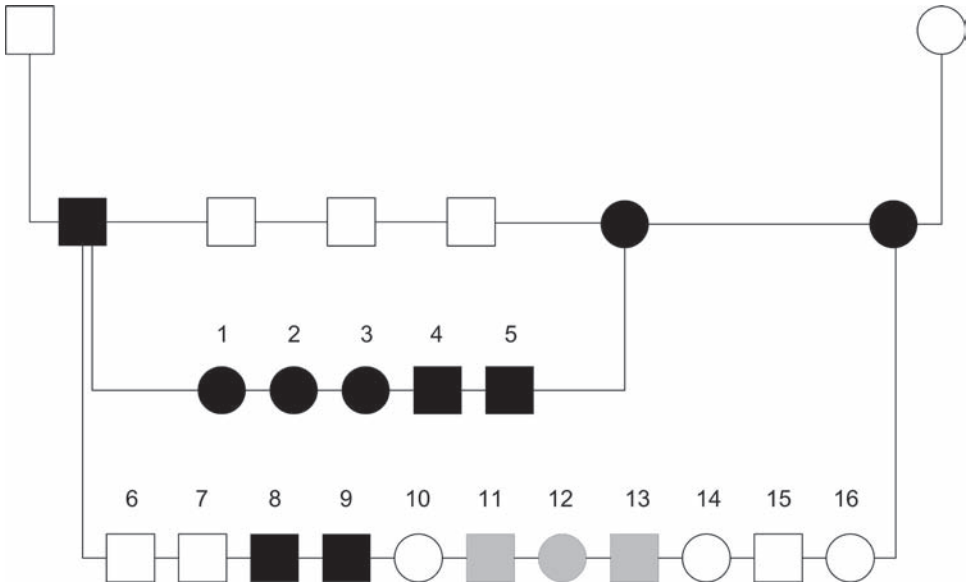
Ethical considerations

All steps of these procedures were approved by the responsible ethical committee as regulated by Dutch legislation. The parents and all surviving pups were placed in foster families as normal companion animals, but ownership remained with the university clinic, in order to ensure that dogs could not be transferred to other foster families without consent of the clinic. This procedure also ensured that any health problem would be immediately brought to knowledge of the university clinic, where all possible experiment-related health problems would be examined and treated at the expense and responsibility of the university clinic.

Results

The first mating resulted in a litter of five pups (three females, two males) which all were affected. Pup 3 had a right sided shunt and died during surgery. In the other four pups a left sided shunt was found. The second mating produced eleven progeny (seven males, four females), of which five dogs were affected (Figure 1). This litter was the result of artificial insemination. Pup 8 and pup 9 had a left sided shunt and underwent surgery. Pup 10 died at day 23 due to pneumonia. Pups 11, 12 and 13 suffocated on day six, when their mother lay on them. These three dogs had intrahepatic right sided shunts which did not show any sign of partial closure. All dogs with IHPSS had distinctly increased basal plasma ammonia concentrations, whereas all healthy pups had ammonia concentrations within the reference range (24-46 µmol/L). The overall prevalence of IHPSS in these test litters was 62.5 percent (10 cases of 16 pups). All pups were placed in private homes.

Figure 1: pedigree of the two test litters.



Squares represent males, circles represent females. Black symbols are affected dogs, open symbols represent unaffected pups. Pup 11, 12 and 13 are marked grey since their status is not fully certain.

Discussion

Test matings between IHPSS affected dogs resulted in a high incidence of the disorder in the offspring. The affected pups had hyperammonemia, which is the most specific and sensitive parameter to show functional portosystemic shunting (9).The observed overall prevalence of 62.5 percent in these test litters was much higher than could be expected based

on the incidence in the entire Dutch population, which has been reported to be between 2.1 and 3.4 percent (5, 10). This overrepresentation with at least a factor of 20 most likely is due to the genetic make up of the parents indicating the likely hereditary nature of this defect in this breed. This is an independent confirmation of previous findings which were based on an epidemiologic study of Irish wolfhounds (4). In this study the relatedness of IHPSS cases was assessed within a 5-generation pedigree.

Post-mortem of three pups that were apparently suffocated revealed a wide open right sided ductus venosus. At this young age it is possible that the neonatal closure of the ductus was still in progress and not yet completed. Closure in newborn Irish wolfhounds at day six is incomplete in 23% of the pups. Complete closure occurs in all pups on day nine (11). However it was not described whether the ductus venosus of the pups without closure were partially narrowed at day six. A partial narrowing is to be expected because closure of the ductus venosus is a gradual process. On the basis of the wide open status of the ductus in the three suffocated pups it is highly likely that they were affected. If these pups would have been assigned as healthy or as unknown, this would have strengthened our conclusions from the pedigree data.

The present results are consistent with a simple monogenic trait with a reduced penetrance. These test matings then suggest a penetrance of 50 percent, although with marked imprecision given the small number of dogs studied. Remarkably, all six unaffected offspring were born in the same litter of eleven. Assuming the chance of a shunt was the same for each dog in the two litters and considering that 10 of 16 dogs were affected, the chance that the litter of 5 would consist of affected dogs only was 0.06. We thought this was highly unlikely and conclude that the chance of a shunt was different for the two litters.

Reduced penetrance could be caused by environmental factors. An effect of environmental factors seems to be rather unlikely since the phenotype is established before weaning. Another explanation for reduced penetrance could be epistasis between multiple loci. The simplest model to explain the observed results would then be an oligogenic disorder with two interacting genes. The mode of inheritance for IHPSS could very well be recessive with a modifier of penetrance (12).

In the two litters, a persistent ductus venosus occurred in both the left and the right liver lobes. We suggest that these phenotypes both are caused by the same genetic defect, since closure of the ductus venosus is probably regulated by a single pathway. The mixture of left and right sided shunts in the two litters confirms this theory. Our findings are in agreement with previously reported epidemiologic findings that breed (other than Australian cattle dog) is no predictor for the location of intrahepatic shunts (13).

There is only one known genetic mouse model causing intrahepatic shunts which are phenoptypically identical to IHPSS in large dog breeds. This knock out mouse strain has a homozygous deletion in the Aryl Hydrocarbon Receptor (AHR) (14). More recently it was demonstrated that AHR signaling in endothelial/hematopoietic cells is necessary for developmental closure of the ductus venosus, whereas AHR signaling in hepatocytes

is necessary to generate adaptive and toxic responses of the liver in response to dioxin exposure (15). This is certainly a strong candidate gene, however in mice all homozygous knock outs have IHPSS. Additionally, this model has a quite complex phenotype with multi-organ lesions which do not occur in dogs with IHPSS.

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Chapter 5

Altered subcellular localization of Heat Shock Protein 90 is associated with impaired expression of the Aryl Hydrocarbon Receptor pathway in dogs

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Abstract

The aryl hydrocarbon receptor (AHR) mediates biological responses to toxic chemicals. An unexpected role for AHR in vascularization was suggested when mice lacking AHR displayed impaired closure of the ductus venosus after birth, as did knockout mice for aryl hydrocarbon receptor interacting protein (AIP) and aryl hydrocarbon receptor nuclear translocator (ARNT). The resulting intrahepatic portosystemic shunts (IHPSS) are frequently diagnosed in specific dog breeds, such as the Irish wolfhound. We compared the expression of components of the AHR pathway in healthy Irish wolfhounds and dogs with IHPSS. To this end, we analyzed the mRNA expression in the liver of *AHR*, *AIP*, *ARNT*, and other genes involved in this pathway, namely, those for aryl hydrocarbon receptor nuclear translocator 2 (*ARNT2*), hypoxia inducible factor 1alpha (*HIF1A*), heat shock protein 90AA1 (*HSP90AA1*), cytochromes P450 (*CYP1A1*, *CYP1A2*, and *CYP1B1*), vascular endothelial growth factor A (*VEGFA*), nitric oxide synthesase 3 (*NOS3*), and endothelin (*EDN1*). The observed low expression of *AHR* mRNA in the Irish wolfhounds is associated with a LINE-1 insertion in intron 2, for which these dogs were homozygous. Down regulation in Irish wolfhounds was observed for *AIP*, *ARNT2*, *CYP1A2*, *CYP1B1* and *HSP90AA1* expression, whereas the expression of *HIF1A* was increased. Immunohistochemistry revealed lower levels of AHR, HIF1A, and VEGFA protein in the nucleus and lower levels of ARNT and HSP90AA1 protein in the cytoplasm of the liver cells of Irish wolfhounds. The impaired expression of HSP90AA1 could trigger the observed differences in mRNA and protein levels and therefore explain the link between two very different functions of AHR: regulation of the closure of the ductus venosus and the response to toxins.

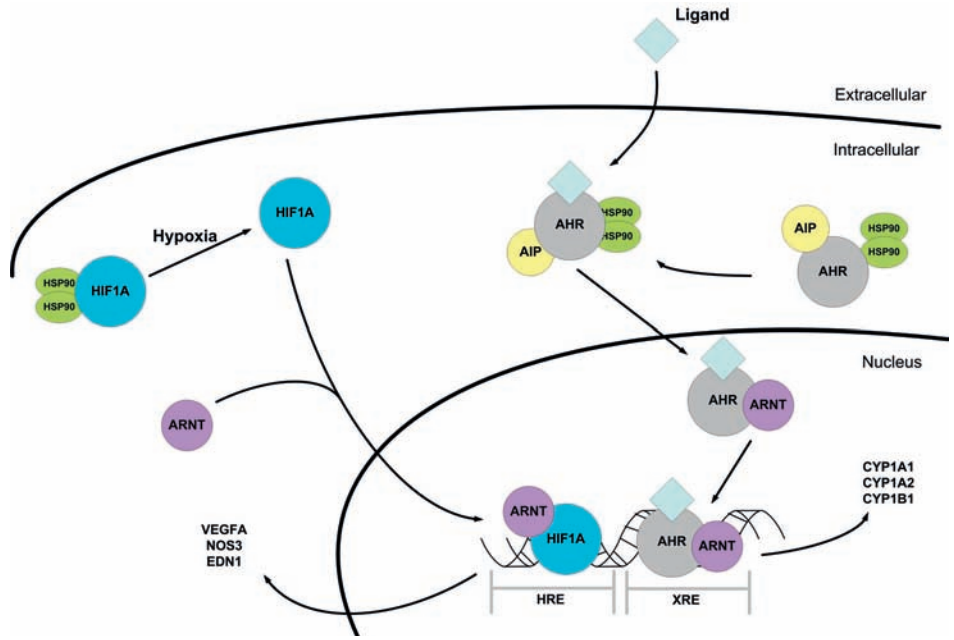
Introduction

The ductus venosus is an embryonic vessel connecting the vena porta and vena cava and allows blood to flow from the placenta to the lungs and heart without traversing the liver sinusoids. The vessel closes within a few days after birth, thereby ensuring that the liver becomes fully functional (1). Complete closure occurs within 6 to 9 days in healthy dogs (2). However, in some purebred dogs, especially large and giant dogs such as Irish wolfhounds, the ductus venosus sometimes fails to close because of a genetic disorder (3). A permanently patent ductus venosus, called an intrahepatic portosystemic shunt (IHPSS), leads to portosystemic bypass of venous hepatic perfusion, resulting in impaired growth and function of the liver and no clearance of intestinal metabolites, such as ammonia, from portal blood (4). The prevalence of IHPSS in Irish wolfhounds is 2.1–3.4 % (5, 6) and the disease has a polygenic mode of inheritance (7). IHPSS is a rare disease in humans, with only 89 cases reported to date (listed in (3)).

Mouse knockout studies indicate that the aryl hydrocarbon receptor (AHR) and its downstream pathway might underlie this disease (8). AHR displays tissue specific functions. In hepatocytes AHR signaling mediates adaptive and toxic responses to dioxins and other AHR agonists (9, 10) . In endothelial/hematopoietic cells AHR has been shown to be necessary for closure of the ductus venosus (8). A patent ductus venosus was found in all knockout mice for AHR (8, 11) indicating a monogenic effect. In the absence of ligands, AHR forms a complex with aryl hydrocarbon receptor interacting protein (AIP), heat shock protein 90 kDa alpha (cytosolic), class A member 1 (HSP90AA1), and p23 proteins (9). AIP is also involved in regulating closure of the ductus venosus. Comparable with the fully penetrant mutation in *AHR*, 83% of *AIP* (-/-) mice are affected by IHPSS (12).

Upon exposure to xenobiotics, AHR heterodimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT) (13) and activates transcription of several cytochrome P-450 (CYP) subtype genes, of which *CYP1A1*, *CYP1A2,* and *CYP1B1* are considered most important. ARNT plays an essential role in developmental angiogenesis by dimerizing with HIF1A, which leads to the expression of proangiogenic factors such as vascular endothelial growth factor α (VEGFA), nitric oxide synthase 3 (NOS3), and endothelin 1 (EDN1) (Figure 1). Because of this role, *ARNT* null mice die during embryonic development (14). Another factor that plays a central role is HSP90AA1, which forms a cytosolic complex with AHR in hepatocytes (15), and which is also essential for the regulation of *HIF1A* activation (16). Closure of the ductus venosus in newborn lambs seems to be regulated by *EDN1* which functions as a potent constrictor of both sphincter and extrasphincter sections of the ductus. Prostaglandins cause a dilating affect and might also influence *EDN1* activity (17). The physiologically comparable process of closure of the ductus arteriosus (a fetal shunt connecting the pulmonary artery with the aorta allowing blood to bypass the unexpanded lungs) appears to be mediated by *cytochrome P-450 3A13* and *EDN1* (18). However, *CYP3A13* gene expression was found to be increased in mouse livers on day 20 after birth, indicating a late response in liver development (19).

Figure 1: Overview of the combined Aryl hydrocarbon Receptor and Hypoxia Inducible Factor 1, alpha pathways.



The relationship between the AHR pathway and the HIF1A pathway. Both pathways share ARNT and HSP90AA1 as key regulators. HRE = hypoxia response element; XRE = xenobiotic response element

The genes and pathways mentioned above are involved in IHPSS in mice and should be considered important candidates for the human and canine forms of the disease. The aim of this study was to investigate the AHR pathway at the DNA, mRNA, and protein level in dogs with IHPSS due to a persistent ductus venosus and in healthy control dogs.

Materials and Methods

Animals

All dogs were kept privately as companion animals. Written informed consent was obtained from all the owners of participants in our study. The dogs were presented to the Department of Clinical Sciences of Companion Animals, Utrecht University, either for population screening for the occurrence of portosystemic shunts (7) or as clinical cases with signs of liver dysfunction in which IHPSS was diagnosed. Blood samples were drawn from the jugular vein. Liver samples were collected from dogs during surgical attenuation of an IHPSS or extrahepatic portosystemic shunt (EHPSS) or at immediate post-mortem examination from healthy dogs (controls) from other, non liver-related, experiments. Wedge biopsies were snap frozen in liquid nitrogen or fixed in RNA later (Ambion, Inc., Austin, Texas) for RNA isolation; matching wedge biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemistry.

Genomic DNA was isolated from EDTA blood using the salt extraction method (20) or from formalin-fixed, paraffin-embedded tissue, using the DNA mini Kit (Qiagen, Venlo, the Netherlands). After isolation, DNA was frozen at −20°C until use. Liver tissue was lysed in 1 ml of TRIzol® reagent. Total RNA was isolated and chromosomal DNA was removed in accordance with the manufacturer's instructions (Qiagen). RNA quality and quantity was determined on a nanochip (Bioanalyzer, Agilent Technologies, Santa Clara, US). cDNA was synthesized using the BioRad iScript Synthesis kit (BioRad, Veenendaal, the Netherlands). The procedures were approved by Utrecht University's Ethical Committee, as required by Dutch legislation (ID 2007.III.08.110).

Genomic DNA sequence analysis

DNA sequence analysis was performed on the exons including the splice sites of *AHR* in 33 dogs affected with IHPSS from 8 breeds and 51 controls from the same breeds. Additional DNA sequencing for *CYP1B1*, *HIF1A* and *HSP90AA1* was performed on 8 Irish wolfhounds affected by IHPSS, 8 healthy Irish wolfhounds, and 2 control dogs. Standard amplification was performed using PCR-mix containing 1x Platinum® PCR Buffer, dNTPs 0.5 µM each, 2 mM $MgCl_2$, 0.5 mM of each forward and reverse primer, 1 Unit Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA) and 50 ng of gDNA in a reaction volume of 25 µl. The thermal cycling protocol consisted of a 5-min denaturation step at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at annealing temperature, 30 s at 72 °C, and a final elongation step at 72 °C for 10 min. For amplification of fragments exceeding 2000 bp, Phusion ™ Hot Start High-Fidelity DNA Polymerase (Thermo Scientific, Lafayette, US) was used. The reaction mix contained 1x Phusion™ GC Buffer, dNTPs 0.5 µM each, primer mix 0.5 mM each, 3% DMSO, 0.02 U/µl Phusion™ Hot start DNA polymerase, and 50 ng gDNA. Cycling conditions were 10 min at 98 °C, 35 cycles of 30 s at 98 °C, 30 s at 56 °C, 7 min at 72 °C, and a final elongation step at 72 °C for 10 min. All amplifications were performed on an ABI 9700 Thermal Cycler (Applied Biosystems, Foster City, CA). Primers are listed in Table S1. DNA sequence reactions were performed using BigDye v3.1 according to the manufacturer's (Applied Biosystems) instructions on an ABI3130XL and analyzed in Lasergene (version 9.1 DNASTAR). The obtained sequences were compared with DNA sequences in databases with BLASTn (21).

Southern Blot

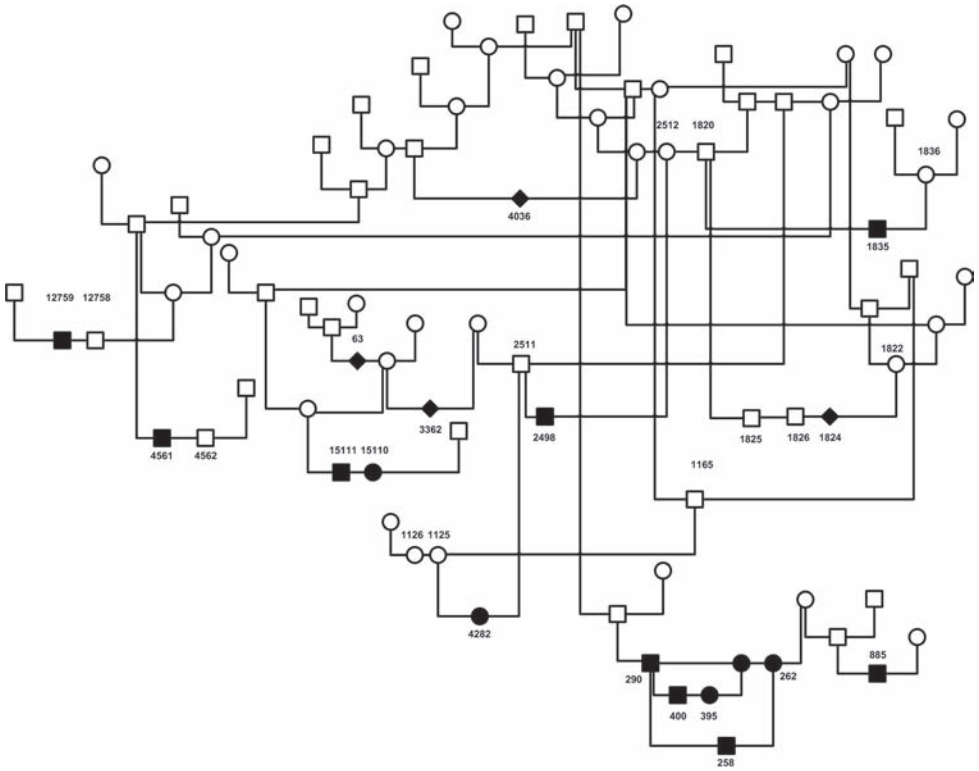
Genomic DNA was digested at 37 °C for 4 h with *HindIII* and *PstI* restriction enzymes and was separated on 0.8 % agarose TAE gel. The DNA was denatured by soaking the gel in 0.6 M NaCl/0.4 M NaOH for 1 h and then transferred overnight to hybond-N nylon membrane (Amersham, GE Healthcare, Diegem, Belgium) by capillary transfer method (22) using 0.4 M NaOH. The filter was dried by heating at 80 °C for 2 h. The probe was obtained by PCR of exon 2 (fwd 5`-CAGCATTTTCTCAAGATGGG-3' rev 5'-ATTGGAAGGAGAAGTGGAAC-3') of *AHR* from a healthy control, which resulted in a 589-bp fragment that was purified with the QIAquick® PCR Purification Kit (Qiagen). The probe was radioactively labeled using the Megaprime DNA Labeling system (GE Healthcare) and 25 ng of denatured probe and [α-P32] dATP (Hartmann Analytics GmbH, Braunschweig, Germany). The labeled probe was purified on a Sephadex G-50 NICK Column (GE Healthcare). Hybridization was done overnight at 60°C. The blot was washed in a series of 5 steps from 2x SSPE/0.1% SDS to 0.1 x SSPE/0.1% SDS for 20 min at 60°C.

Linkage analysis

To analyze the linkage between genes of interest and the shunt phenotype, we designed primers for genotyping of microsatellites in close chromosomal vicinity of *AHRR*, *ARNT*, *AIP*, *CYP1A1/CYP1A2*, *CYP1B1*, *EDN1*, *HIF1A*, *HSP90AA1*, *NOS3,* or *VEGFA,* respectively. The distance between *CYP1A1* and *CYP1A2* is less than 30 kb, hence two microsatellites were used to cover both genes at once. 500 kb of genomic DNA up- and downstream of the genes was downloaded from NCBI (CanFam2.1) and masked for simple repeats using repeat masker (www.repeatmasker.org). The masked sequences were analyzed with tandem repeat finder (23) to detect microsatellite repeats. Two microsatellites were selected for each gene and primers were designed with Perlprimer v1.1.13 (Table S1).

Samples came from a pedigree containing 17 cases with and 13 healthy dogs without a shunt (Figure 2). A phage M13-based tag (GTTTTCCCAGTCACGAC) was added at the 5' end of forward primers. PCR amplification was carried out in a reaction volume of 15 µl containing 25 ng of genomic DNA, 1 µM M13-tagged forward primer, 10 µM reverse primer, 10 µM M13-based tag primer labeled at the 5' end with 6-FAM (Eurogentec, Maastricht, the Netherlands), 1x PCR gold buffer (Applied Biosystems), 2.5 mm $MgCl_2$, 0.2 mM dNTPs and 0.3 U Amplitaq Gold. Thermal cycling was performed in a ABI 9700 (Applied Biosystems) with the following program: 5 min at 95 °C, followed by 10 cycles of 30 s at 95 °C, 15 s at the annealing temperature, 30 s at 72 °C, then another 25 cycles of 30 s at 9 2 °C, 15 s at the annealing temperature, and 30 s at 72 °C. The program was completed with 10 min at 72 °C. Genotypes were obtained on an ABI3130XL and scored using GeneMapper Software (version 4.0). Analysis was performed in Superlink (24) using the two-point two-loci option. Disease gene frequencies were set at 0.05 and the mode of inheritance at 0.01 for two risk alleles or less and 0.99 for three risk alleles or more.

Figure 2: Pedigree of Irish Wolfhounds used for linkage analysis.



Related Irish wolfhounds used to genotype 20 microsatellites. DNA was available from the numbered individuals. Filled symbols are affected dogs, open symbols represent healthy dogs.

mRNA quantification

qPCR

To measure RNA expression in liver tissue, primers were designed for the genes for AHR, ARNT, ARNT2, AIP, HIFIA, HSP90AA1, cytochrome P450 (CYP1A1, CYP1A2, and CYP1B1), VEGFA, NOS3, and EDN1. Perlprimer v1.1.14 was used for primer design on Ensembl annotated transcripts and the amplicon was tested for secondary structures using MFold (25). Gradient PCRs were performed to determine the optimum temperature for obtaining 100% 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), 0.4 µM primer, and 1 µl cDNA. For normalization, five reference genes were used based on their stable expression in the liver, namely, genes for hypoxanthine phosphoribosyl transferase (*HPRT*), beta-2-microglobulin (*B2M*), heterogeneous nuclear ribonucleoprotein H1 (*hnRPH*), beta-glucuronidase (*GUSB*), and ribosomal proteins S5 (*RPS5*) (26). GeneNorm (27) was used to establish stability. Primers for reference genes and genes of interest including their optimum temperature are listed in Table S2. Cycling conditions were a 3-min Taq polymerase activation step on 95 °C, followed by 45 cycles of 10 s at 95 °C to denature, and 30 s at T_m for annealing and elongation. For some products a 3-step protocol was used including 3-min Taq polymerase activation step at 95 °C, followed by 45 cycles of 10 s at 95 °C to denature, 30 s at T_m for annealing, and 30 s at 72 °C for elongation. All experiments were conducted on a MyiQ Single-Colour Real-Time PCR Detection System (BioRad). A 4-fold standard dilution series of a pool containing all samples used for analysis was used to determine relative expression. cDNA originating from liver tissue of 8 healthy beagles, 8 dogs of various breeds with IHPSS (Table S3), and 8 Irish wolfhounds with IHPSS were used for expression analysis. Negative controls remained negative (28). Data analysis was performed in IQ5 Real-Time PCR detection system software (BioRad). Gene expression was normalized by using the average relative amount of the reference genes. Log values of normalized relative gene expression were used to obtain normal distribution. A Wilcoxon rank sum test was performed in R statistics package 2.14.0 (http://www.R-project.org) to determine significance of differential expression.

Microarray expression profiling

Liver tissue from 2 healthy dogs, 32 dogs with EHPSS, and 14 dogs with IHPSS (Table S3) were used for total RNA isolation using an RNeasy Mini Kit (Qiagen). DNase treatment was performed using an on-column DNase digestion. RNA quality and quantity was determined on a nanochip (Bioanalyzer, Agilent Technologies). Samples with a RIN value above 8.0 were used. A common reference pool was constructed by pooling RNA isolated from healthy liver. Agilent Canine Gene Expression Microarray V1 containing 42,034 60-mer probes in a 4x44K layout was used to determine genome wide expression on 3 µg of total RNA of each sample hybridized to the common reference. RNA amplification and labeling were performed on an automated system (Caliper Life Sciences NV/SA, Belgium) (29). Dye swap of Cy3 and Cy5 was performed to reduce dye bias. Hybridizations were done on a HS4800PRO system supplemented with QuadChambers (Tecan Benelux B.V.B.A. Mechelen, Belgium) using 1 µg labeled cRNA per channel (30).

Hybridized slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 30% PMT and with automated data extraction, using Imagene 8.0 (BioDiscovery). Normalization was performed with Loess on mean spot intensities (31), and dye bias was corrected based on a within-set estimate (32). Data were analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7) (33). Correction for multiple testing (Permutation F2-test using 5,000 permutations) was performed.

Immunohistochemical analysis for localisation and quantification of proteins

Immunohistochemistry (IHC) was performed for AHR, ARNT, HIF1A, CYP1A1, CYP1A2, CYP1B1, VEGFA, NOS3, and HSP90AA1 on liver samples of healthy beagles (n=6), Irish wolfhounds (n=11, 2 with IHPSS, 9 healthy), and arbitrary selected dogs of other

breeds with an IHPSS (n=6), and dogs with an EHPSS (n=6). Samples from dogs with IHPSS and dogs with an EHPSS were compared to identify specific effects related to the secondary effects of blood bypassing the liver tissue on protein expression. Antibody characteristics, manufacturers, dilutions, and protocol specifications are given in Table S4. 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 min (Table S4). Antigen retrieval by enzymatic digestion was performed with proteinase K (Dakocytomation, Glostrup, Denmark) for 10 min at RT. Dual endogenous enzyme block (Dakocytomation) was used (10 min, RT) to quench endogenous peroxidase activity, and background staining was blocked with 10% normal goat serum (Sigma-Aldrich, St. Louis, US) (30 min). Sections were incubated with the labeled secondary antibody Envision (Dakocytomation) for 1 h at RT. The signal was developed in 0.06% 3,3'-diaminobenzidine (DAB) solution (Dakocytomation) for the indicated time (Table S4) and counterstained with hematoxylin QS (Vector Laboratories, Burlingame, CA). Replacement of primary antibody with washing buffer served as negative control. All tissues were stained in batch per antibody to avoid technique-induced differences.

All immunohistochemically stained sections were evaluated by one board-certified pathologist (GCMG) who was unaware of the origin of the samples, 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 intensity score of each group (i.e., Irish wolfhounds, dogs with an IHPSS, dogs with an EHPSS, and control dogs) was calculated.

All data were analyzed using R statistics package 2.14.0. Overall differences in intensity scores between Irish wolfhounds and remaining samples including dogs with an IHPSS, dogs with an EHPSS, and control dogs were tested with the Wilcoxon rank sum test, with $P<0.05$ being considered statistically significant. Differences in protein localization were identified by detecting the pattern of protein expression in each group.

Results

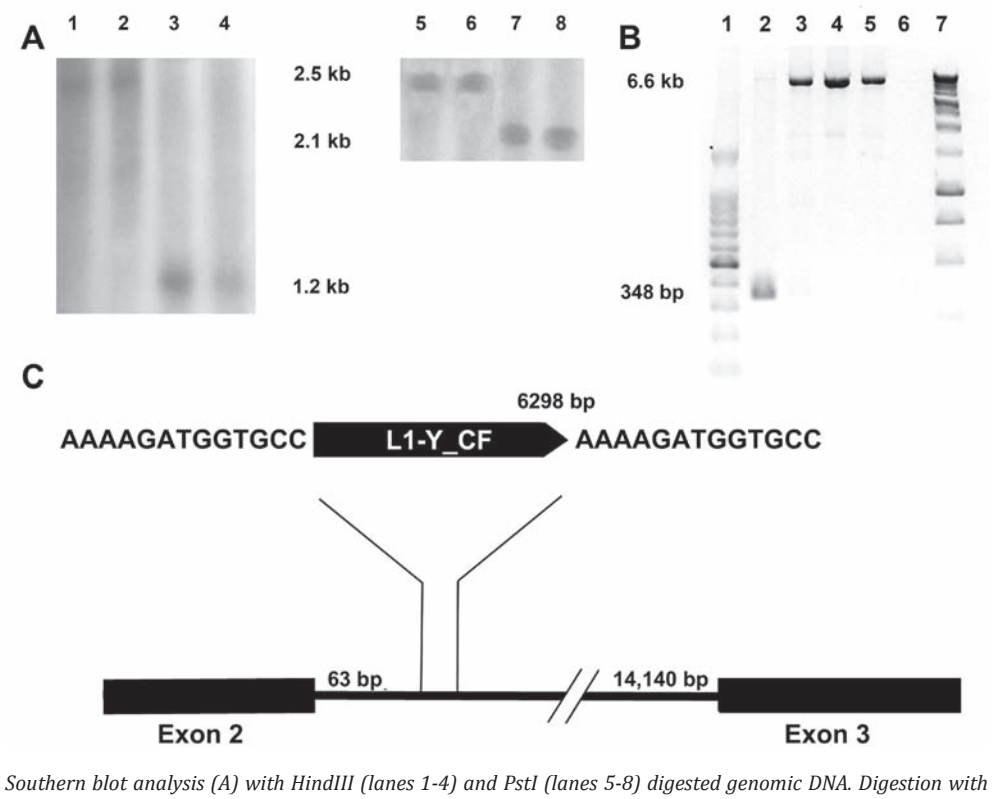
Rearrangement of AHR

As a first step to investigate the role of AHR in IHPSS we analyzed the exons of the gene in affected dogs. DNA sequence analysis of *AHR* did not reveal differences between the gene of the canine reference genome and that of dogs with an IHPSS. However, amplification of the fragment containing exon 2 in all affected and healthy Irish wolfhounds failed, indicating a rearrangement in this region. Southern blot analysis (Figure 3A) and a PCR adapted for long DNA fragments indicated that intron 2 was larger by more than 6 kb in Irish wolfhounds (Figure 3B). DNA sequence analysis of the PCR product revealed an insertion of 6298 bp, positioned only 63 bp downstream of exon 2 (Figure 3C). BLASTn comparison of the insert against a database of repeats in mammalian DNA showed high similarity to L1-Y_CF. The LINE-1 insertion included a target site duplication of 13 bp, a 5` UTR, an open reading frame (ORF1) coding for a high-affinity RNA-binding protein, an ORF2 containing both endonuclease and reverse transcriptase activities, and a 3` UTR and a poly(A) tail. In both ORFs, a frame shift caused by a single nucleotide deletion was detected, resulting in a premature stop codon. The insert was found homozygously in all but one of the Irish wolfhounds. Only one healthy dog was heterozygous for the rearrangement. DNA sequencing of *CYP1B1, HIF1A,* and *HSP90AA1* did not reveal differences between healthy dogs and dogs with an IHPSS that could have caused the disorder.

Linkage analysis

On the basis of test matings of Irish wolfhounds with an IHPSS, we previously proposed a digenic, triallelic mode of inheritance, thus two interacting loci in which a total of at least three risk alleles should be present (7). In order to determine whether IHPSS could be explained by such an interaction between genes of the AHR pathway, we genotyped dogs from an IHPSS pedigree with 16 polymorphic microsatellite markers situated close to genes of the AHR pathway (Figure 2). The maximally obtainable LOD score with the available samples was 1.8. This LOD score was determined by assuming genotypes of the available samples in two loci according to the postulated model. Using the two-point, two-loci option in Superlink (24), we calculated LOD scores varying between 0 and 1.7 (Table S5). The highest scores were obtained with combinations of markers for HSP90AA1 and the genes *CYP1A1 and CYP1A2,* which are situated close to each other.

Figure 3: LINE-1 insertion in intron 2 of AHR in Irish Wolfhounds.

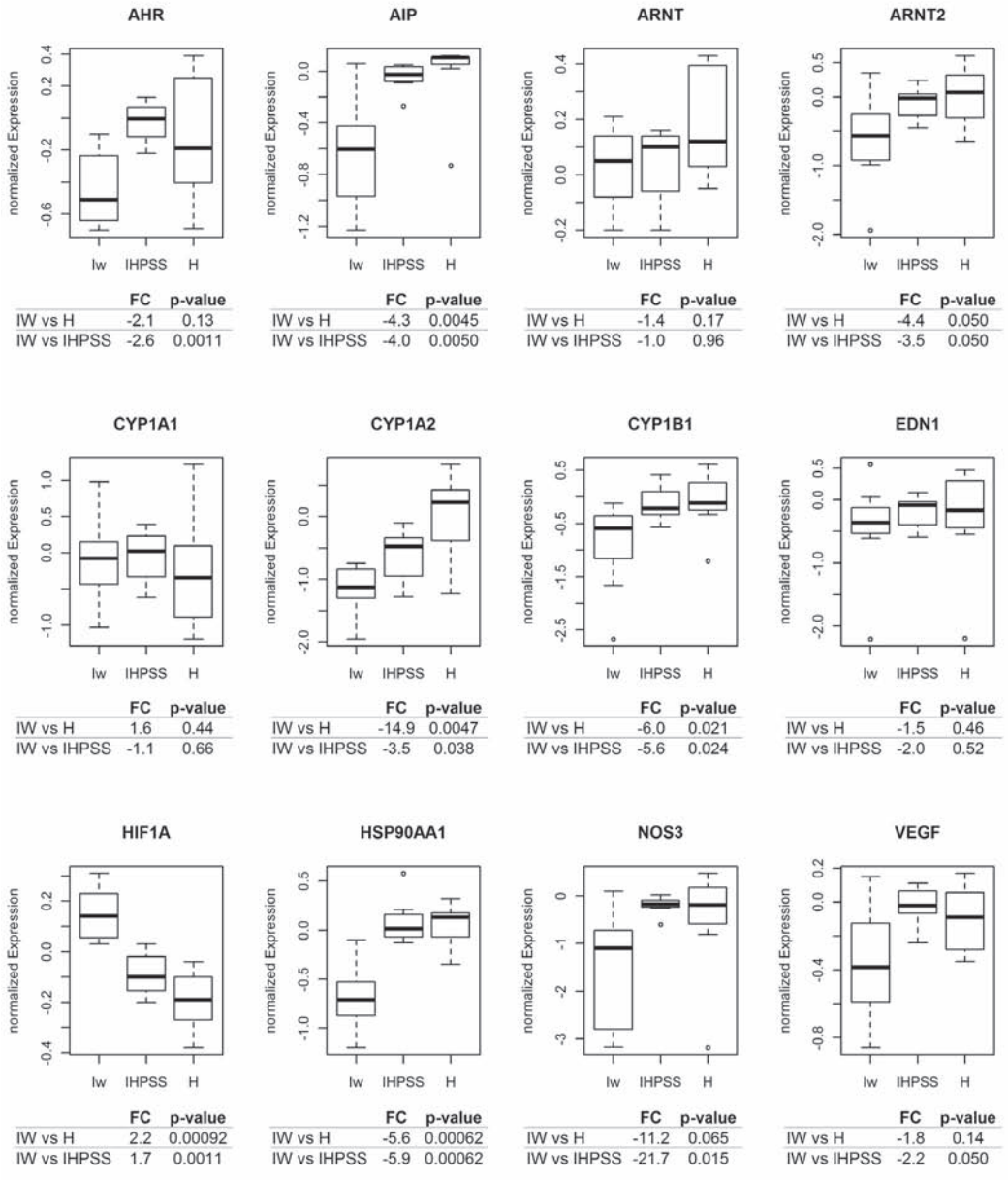


Southern blot analysis (A) with HindIII (lanes 1-4) and PstI (lanes 5-8) digested genomic DNA. Digestion with HindIII resulted in an increase in size of about 2000 bp in Irish wolfhounds (lanes 1 and 2) compared to healthy beagles (lanes 3 and 4). Using PstI as restriction enzyme results in a small increase in size of approximately 400 bp in Irish wolfhounds (lanes 5 and 6) compared to healthy beagles (lanes 7 and 8). The observed size differences are smaller than the actual rearrangement due to the presence of restriction enzyme sites in the rearranged DNA fragment. Gel electrophoresis of PCR products (B) with forward primer in exon 2 and reverse primer downstream intron 2. The use of Phusion ™ Hot Start High-Fidelity DNA Polymerase in combination with GC-buffer was required to amplify this fragment. An increase in intron size was detected in all used gDNA samples of Irish wolfhounds. Lane 1: 1kb DNA ladder (Promega, Leiden, the Netherlands); 2: normal control; 3-5: genomic DNA of Irish wolfhounds; 6: negative control; 7: 100bp DNA ladder (Promega). A 6298 bp long LINE-1 insertion named L1-Y_CF (C) is located 63 bp downstream of exon2. It contains a 13 bp long duplication site specific for LINE-1 insertions. In both open reading frames of this retrotransposon deletions were detected causing frame shifts and premature stop codons. Restriction sites were found for HindIII at 1908 bp and for PstI at 447 bp.

mRNA quantification

To study the expression of the genes of the AHR pathway, the mRNA levels of 12 genes were determined by qPCR (Figure 4). *ARNT*, *CYP1A1,* and *EDN1* expression was similar in dogs with an IHPSS and control dogs. In contrast, *HIF1A* was upregulated (2.2-fold change), whereas *AHR*, *AIP*, *ARNT2*, *CYP1A2*, *CYP1B1*, *HSP90AA1*, *NOS3,* and *VEGFA* were down-regulated (1.8 to 14.9-fold change) in Irish wolfhounds with IHPSS. In microarray expression profiling the 12 target genes were compared between IHPSS and controls and between EHPSS and controls (Table S6). In dogs with EHPSS a developmental vascular anomaly is formed by which the extrahepatic portal system is connected with the caudal vena cava or (hemi)azygos vein. For both shunts the functional consequences, virtual absence of portal perfusion of the liver parenchyma, and clinical signs are similar. Functional consequences include hypoplasia of the liver and elevated ammonia and bile acid levels in the systemic blood of the cases compared to healthy individuals. To avoid measuring mRNA expression differences due to unspecific effects caused by absence of portal perfusion *per se*, samples of dogs with EHPSS were included (3). Microarray data have been deposited in NCBI's Gene Expression Omnibus (34) and are accessible through GEO Series accession number GSE39005 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE39005). As *CYP1A1* and *CYP1A2* were down-regulated in dogs with IHPSS or EHPSS in the microarray analysis, this down-regulation is probably an effect secondary to toxification caused by the lack of hepatic clearance of ammonia and bile acids. CYP1B1, NOS3 and VEGFA are downstream products of the investigated pathway. Down regulation of these products might be effects of impaired regulation of upstream genes. The lower expression of *HSP90AA1* is remarkable, because of its role in the classical AHR pathway and the HIF1A pathway.

Figure 4: Comparison of AHR pathway gene expression in livers with a shunt and normal livers.

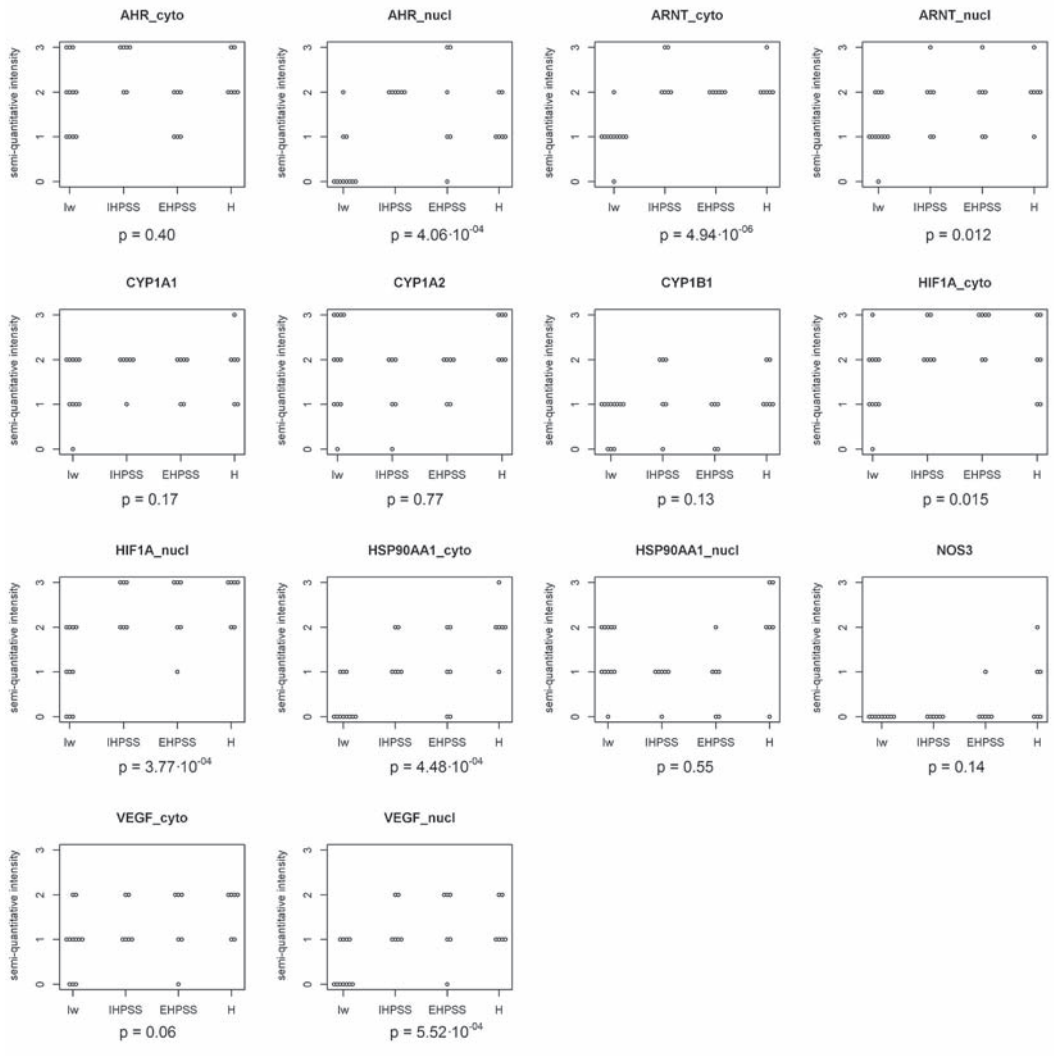


The pattern of expression of selected genes in liver samples from Irish wolfhounds with an intrahepatic shunt (Iw), dogs with intrahepatic portosystemic shunts (IHPSS), and healthy dogs (H). The thick black line represents the median (50th percentile); the first and third quartile (25th and 75th percentile, respectively) are displayed. Outliers are depicted with an open dot, representing values higher than 1.5 times the interquartile range. HIF1A was found to be significantly upregulated in Irish wolfhounds with IHPSS, whereas AHR, AIP, ARNT2, CYP1A2, CYP1B1, HSP90AA1, NOS3, and VEGFA were significantly down regulated. No differences were observed in ARNT, CYP1A1, and EDN1. FC = fold change

Immunohistochemistry

Immunohistochemistry was performed to investigate protein expression, measured semi-quantitatively, and protein localization. The intensity of hepatocyte staining for AHR, ARNT, HIF1A, HSP90AA1, and VEGFA was different in Irish wolfhounds and control dogs (Figure 5), whereas there were no differences in the intensity of staining for the downstream products CYP1A1, CYP1A2, CYP1B1, and NOS3.

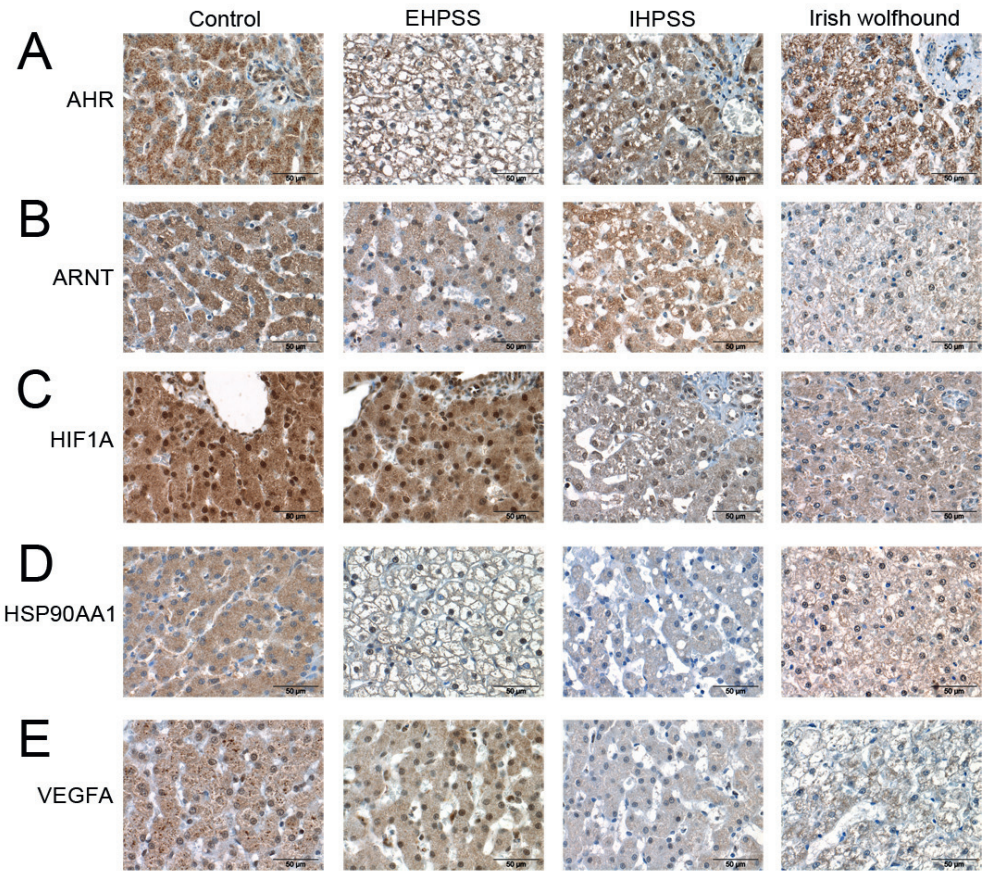
Figure 5: Immunohistochemistry of AHR pathway proteins in liver samples from healthy dogs, dogs with extrahepatic (EPHSS) or intrahepatic (IPHSS) portosystemic shunts, and Irish wolfhounds.



The semi-quantative scoring in hepatocytes from Irish wolfhounds (Iw, n=11), dogs with intrahepatic portosystemic shunts (IHPSS, n=6), dogs with extrahepatic portosystemic shunts (EHPSS, n=11) and healthy beagles (H, n=6). The indicated p-values are obtained comparing Irish wolfhounds with the other groups as a whole.

These five differently staining proteins were down regulated in the Irish wolfhounds compared to both healthy controls and dogs with EHPSS indicating that the differences were not caused by secondary effects of shunting. Staining for AHR was lower in the nuclei of hepatocytes of Irish wolfhounds than in control dogs (Figure 6A), but was significantly stronger in the nuclei of hepatocytes from dogs with an IHPSS or EHPSS and in hepatocytes from healthy control dogs ($p=8.0x10^{-5}$). Hepatocyte nuclei and cytoplasm stained positively for ARNT (Figure 6B), but staining was less intense in Irish Wolfhounds than in the other groups of dogs ($p=4.9\times10^{-8}$). HIF1A staining was present in nearly all hepatocyte nuclei, but nuclear staining was less intense in Irish wolfhounds than in control dogs ($p=3.9\times10^{-5}$) (Figure 6C).

Figure 6: Significant immunohistochemical staining of liver tissue.



Representative images of immunoreactivity in sections of formalin-fixed, paraffin-embedded liver samples from control dogs, dogs with extrahepatic and intrahepatic portosystemic shunts, and Irish Wolfhounds. Immunoreactivity against the aryl hydrocarbon receptor (AHR)(A), aryl hydrocarbon receptor nuclear translocator (ARNT) (B), hypoxia inducible factor 1alpha (HIF1A)(C), heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSP90AA1)(D), and vascular endothelial growth factor A (VEGF)(E) is visible in variable intensity in the cytoplasm and/or nuclei of hepatocytes.

Healthy hepatocytes displayed weak staining for HSP90AA1 (Figure 6D), but staining was distinctly less intense in the cytoplasm of hepatocytes from Irish wolfhounds ($p=1.2\times10^{-4}$). Staining for VEGFA in the nuclei was less intense in hepatocytes from Irish wolfhounds than in hepatocytes from dogs with an EHPSS or IHPSS and healthy controls ($p=1.2\times10^{-4}$) (Figure 6E). All p-values indicated here were based on the comparison of the Irish wolfhounds against the samples from other breeds. The decreased expression of AHR and HIF1A in the nuclei indicates a problem in trafficking these proteins. This clearly fits the impaired expression of HSP90AA1 which plays an important role in this translocation. Also the nuclear and cytoplasmic decrease of ARNT which cooperates with HSP90AA1 could explain decreased expression of VEGFA.

Discussion

Given its role in closure of the ductus venosus, we hypothesized that AHR and its downstream pathway are involved in IHPSS in dogs. In the present study, liver tissue from affected and healthy control dogs was used for microarray expression profiling, qPCR, and immunohistochemical analysis of the genes involved in this pathway. Dogs with a portosystemic shunt have poor growth and function of the liver after birth, and changes secondary to portosystemic shunting are expected to alter the expression of genes and proteins in the liver. This would complicate analysis of whether abnormalities are the cause (associated with the causal mutation of the genetic disease) or effect (due to portal hypoperfusion) of the disease. Therefore, liver tissues of dogs affected with EHPSS were used as a control to correct for secondary effects which should be the same as in IHPSS. The genetic background, however, the two conditions affect different purebred dog populations (3), are differently transmitted in affected families and are therefor genotypically different entities.

While the sequence of the *AHR* gene was identical in Irish wolfhounds with and without an IHPSS and in affected and healthy dogs of other breeds, Irish wolfhounds had a canine specific LINE1-insert named L1-Y_CF only 63 bp downstream of intron 2. In another study, a sense L1 insert of 4760 bp was found to strongly attenuate target gene expression(35). The L1-Y_CF insert was 6298 bp, and a decreased expression of *AHR* was anticipated. Although the LINE1 seems to be inactivated due to a frame shift in both *ORF1* and *ORF2*, the level of expression of *AHR* in Irish wolfhounds was lower than that in healthy control dogs of other breeds. The location of the insert near the splice donor site and/or the altered structure of the intron may cause this decreased expression. As the insert was present in both healthy and affected Irish wolfhounds, it cannot be the single cause of the disease, but it might be a predisposing factor that increases the risk of the entire breed.

A simple Mendelian inheritance pattern has been ruled out for patency of the ductus venosus in Irish wolfhounds (7). Instead, we postulated a model in which two loci interact to determine the phenotype. At least three risk alleles would need to be present in the two loci to cause IHPSS. To determine whether genes of the AHR pathway are involved, we analyzed

closely situated polymorphic microsatellite markers to calculate the LOD score for linkage according to the postulated model. The highest LOD scores (1.7) were obtained for the region of the *CYP1A1/CYP1A2* gene pair and for *HSP90AA1*; these scores were close to the maximally obtainable score of 1.8. A cytochrome P-450 system has been postulated to be involved in the closure of the ductus venosus in lambs by virtue of its contractile effect in the sphincter region (36). Which cytochrome P-450 subtype is responsible for this effect is still unknown, but *CYP1A1* and *CYP1A2* were recently found not to be responsible for the process in mice (37). At this stage, we cannot conclude whether the *CYP1A1/CYP1A2* and *HSP90AA1* loci are responsible for the shunt phenotype because the linkage score was not conclusive. A larger set of dogs from pedigrees with IHPSS needs to be investigated to confirm the involvement of these loci.

The decrease in *AHR* mRNA expression and the lack of nuclear translocation were linked to a decreased expression of the downstream targets CYP1A2 and CYP1B1. *CYP1A2* expression was down regulated in dogs with an IHPSS, and Irish wolfhounds with an IHPSS had lower levels of CYP1A2 expression than other large-breed dogs with an IHPSS. This may be explained by the breed-specific LINE-1 insert causing down-regulation of *AHR* in Irish wolfhounds. Dogs of this breed have been reported to be prone to complications of thiobarbiturate anesthesia, because the drug is not metabolized, possibly because of hypofunction of hepatic cytochrome P450 (38). The decreased expression of *CYP1A2* found in this study supports this supposition. Given the specific expression patterns of all P450s (19), it seems sensible to measure a broad spectrum of cytochromes to determine the cause of this metabolic defect.

Mice with a hypomorphic *ARNT* allele have the same phenotypic alterations as *AHR* knock-out mice (39). However, hepatocyte-specific deletion of *ARNT* did not result in shunting, so hepatocyte *ARNT* would appear not to be related to AHR-mediated hepatovascular development. ARNT2, a homolog of ARNT mainly expressed in neurons, has feedback regulation of its activity. It forms functional complexes with *HIF1A,* restoring hypoxia-induced gene expression in *ARNT*-deficient hepatocytes (40, 41). In contrast, ARNT2 appears not to be able to compensate for the loss of ARNT with regard to the response to xenobiotics (42). While Irish wolfhounds with a LINE-1 insert did not show an altered *ARNT* expression, *ARNT2* expression was decreased. Thus the *ARNT2* feedback mechanism might be impaired in Irish wolfhounds, possibly affecting closure of the ductus venosus.

HIF1A was the only gene to be upregulated in Wolfhounds. While gene expression was upregulated 2.2-fold, HIF1A protein expression was decreased in the cytoplasm and nuclei of hepatocytes from Wolfhounds. A similar discordance between gene and protein expression was found for AHR. This is most likely caused by the decreased amount of HSP90AA1. The down-regulation at the mRNA and protein level of HSP90AA1 is expected to affect both toxicological (15) and vascularization (16) processes. Binding of HSP90AA1 with AHR is essential for nuclear translocation (43), whereas binding of the HSP90 heterocomplex with HIF1A prevents the non-specific degradation of this highly unstable protein (44). No coding variations were found in HSP90AA1. Whether epigenetic modification influences closure of the ductus venosus remains to be elucidated, but HSP90AA1 might be the missing link connecting toxicological responses via AHR with the regulation of vascularization.

Endothelin was postulated to have a role in the closure of the ductus venosus and the ductus arteriosus, but we found no evidence to support this in our material. However, it should be noted that all our liver samples were obtained from dogs that were several months old. Ideally, liver tissue should be collected within days of birth to investigate the biological initiators of closure of the ductus venosus. One study reported that the ductus was virtually closed in most Irish wolfhound pups on day 6 after birth, although the ductus was still partially present in 23% of pups. Closure was complete in all pups by day 9 (2). Based on our findings, we cannot conclude that AHR and its downstream pathway are directly involved in the abnormal closure of the ductus venosus, but the decreased expression of AHR, and the lack of nuclear expression of the protein, in Irish wolfhounds might have a contributory role in delayed closure of the ductus venosus. To date, the impaired physiological process of ductus venosus closure, resulting in portosystemic shunting, has been investigated in experimental mice. The dog provides the opportunity to further study the genes regulating this process in a natural model, which may be relevant to unravel this rare disease in man.

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Table S1: primer sequences and annealing temperatures genomic DNA PCR

Gene	EnsemblID	exon	orientation	primersequence	application
AHR	ENSCAFG00000002448	1	f	GCCGATGTAGAAGGCACCTG	gPCR
AHR	ENSCAFG00000002448	1	r	GAGCTCTGGACGGCGAAGAT	gPCR
AHR	ENSCAFG00000002448	1	f	ATCACCTACGCCAGCCGCAA	RTPCR
AHR	ENSCAFG00000002448	2	f	ACCCACTCCCTTGTTTTTCT	gPCR
AHR	ENSCAFG00000002448	2	r	TCATTTAAGTGGCACCATCT	gPCR
AHR	ENSCAFG00000002448	2	r	ATTGGAAGGAGAAGTGGAAC	gPCR / LINE-detection
AHR	ENSCAFG00000002448	2	r	GACTTGATTCCTTCAGCTGG	RTPCR
AHR	ENSCAFG00000002448	2	f	CCAGCTGAAGGAATCAAGTC	RTPCR / LINE-detection
AHR	ENSCAFG00000002448	3	f	AGCCCTTTGAAGTCACTTAC	gPCR
AHR	ENSCAFG00000002448	3	r	TCACATACTAGCGACACAAC	gPCR
AHR	ENSCAFG00000002448	3	r	TTGCAGATGCAGACCTTCTC	RTPCR
AHR	ENSCAFG00000002448	4	f	TGATAACCGTAACTGAAGTC	gPCR
AHR	ENSCAFG00000002448	4	r	CCCTAACCTGAATGTTTCTT	gPCR
AHR	ENSCAFG00000002448	5	f	CAAGCACTCAGTAATCTAGC	gPCR
AHR	ENSCAFG00000002448	5	r	TATACATCTCCCAGAGGTTC	gPCR
AHR	ENSCAFG00000002448	5	r	TCACCTCGGTCTTCAGTATG	RTPCR
AHR	ENSCAFG00000002448	6	f	GTATCAGAACAGAATCCAAT	gPCR
AHR	ENSCAFG00000002448	6	r	CCAAAGAAAGTAGTTTCAGT	gPCR
AHR	ENSCAFG00000002448	6	f	GGTGTCTGCTGGATAATTCG	RTPCR
AHR	ENSCAFG00000002448	7	f	GGAATTATCAAGACTACTGG	gPCR
AHR	ENSCAFG00000002448	7	r	GCTAAGAGATTTACTGCAAC	gPCR
AHR	ENSCAFG00000002448	8	f	TCAAGGAAGACACAATGCAG	gPCR
AHR	ENSCAFG00000002448	8	r	TGGCAGCTATAGAGCACTTT	gPCR
AHR	ENSCAFG00000002448	9	f	GTTAGAGGGGCTTTGTTTCA	gPCR
AHR	ENSCAFG00000002448	9	r	AAACCCTGGAGTTTTCATTG	gPCR
AHR	ENSCAFG00000002448	9	r	TCATGCCACTCTCTCCTGTC	RTPCR
AHR	ENSCAFG00000002448	10.1	f	CTCCCTGCTCTCAAACTCAA	gPCR
AHR	ENSCAFG00000002448	10.1	r	GGGTGTGATATTGTCTTGCC	gPCR
AHR	ENSCAFG00000002448	10.2	f	CCATGATGCAACAAGATGAG	gPCR
AHR	ENSCAFG00000002448	10.2	r	GCTATGTGATTTTGGTGGTG	gPCR
AHR	ENSCAFG00000002448	10.3	f	CTCTGAACTCCAGCTGTATG	gPCR
AHR	ENSCAFG00000002448	10.3	r	GGAAAGTCTAACTGTGTCCC	gPCR
AHR	ENSCAFG00000002448	10.4	f	ATGCTATGCCATGTACACAG	gPCR
AHR	ENSCAFG00000002448	10.4	r	AGAAGGATCACTGAAAGGGT	gPCR
AHR	ENSCAFG00000002448	11	f	GTGCTCAGTAGGTCTTTGCT	gPCR
AHR	ENSCAFG00000002448	11	r	TGGTGATGACCAACACTGAC	gPCR

Gene	EnsemblID	exon	orientation	primersequence	application
AIP	ENSCAFG00000011515	marker 1	f	CTGATTCGTCCTACCCATCCTG	microsatellite
AIP	ENSCAFG00000011515	marker 1	r	GATTGTTTACATCCCGTCTTCCCT	microsatellite
AIP	ENSCAFG00000011515	marker 2	f	GACATGGGACTGTGATCCTG	microsatellite
AIP	ENSCAFG00000011515	marker 2	r	CTTGAGACTAAGAAGTTTCAGGG	microsatellite
ARNT	ENSCAFG00000012149	marker 1	f	ATGGGCCCAATCTCACAGTT	microsatellite
ARNT	ENSCAFG00000012149	marker 1	r	CTGACTCCAAGGGGGTTCTG	microsatellite
ARNT	ENSCAFG00000012149	marker 2	f	ACCCTTTGCCTGTGCTTTGT	microsatellite
ARNT	ENSCAFG00000012149	marker 2	r	TAAACAGGTGCCCAATGCAG	microsatellite
CYP1A1/CYP1A2	ENSCAFG00000017937 / ENSCAFG00000017941	marker 1	f	AAGATGGCTCTGGCTCTAGTG	microsatellite
CYP1A1/CYP1A2	ENSCAFG00000017937 / ENSCAFG00000017941	marker 1	r	TCAGATGCAAACTATTGAAGGG	microsatellite
CYP1A1/CYP1A2	ENSCAFG00000017937 / ENSCAFG00000017941	marker 2	f	GAATCCTCATCCCTTCTCCA	microsatellite
CYP1A1/CYP1A2	ENSCAFG00000017937 / ENSCAFG00000017941	marker 2	r	CAGTCATTTCCTGAACTCTCC	microsatellite
CYP1B1	ENSCAFG00000006164	2	f	CAACGTCATGAGCGCCGTGT	gPCR
CYP1B1	ENSCAFG00000006164	2	r	AGACTCTGGGCGTGCGTGGA	gPCR
CYP1B1	ENSCAFG00000006164	3	f	GTGCCATGTGCTTTCTAGAT	gPCR
CYP1B1	ENSCAFG00000006164	3	r	GAGGCTAATTGAGAGAGTGG	gPCR
CYP1B1	ENSCAFG00000006164	1&2	f	GCTCCTTTCCCTGCACACCT	gPCR
CYP1B1	ENSCAFG00000006164	1&2	r	CACACGGCGCTCATGACGTT	gPCR
CYP1B1	ENSCAFG00000006164	marker 1	f	ATAGAATAGCCCTTTCTTTCGGAG	microsatellite
CYP1B1	ENSCAFG00000006164	marker 1	r	ACTTTAGGGTAGGTAGGTGGG	microsatellite
CYP1B1	ENSCAFG00000006164	marker 2	f	CTCTTGGTTTCAGCTCAGGTC	microsatellite
CYP1B1	ENSCAFG00000006164	marker 2	r	AGTGTCAGCAGTATTCTTAGGA	microsatellite
EDN1	ENSCAFG00000009794	marker 1	f	TTAGATGTAGACTCACCTTTCTCC	microsatellite
EDN1	ENSCAFG00000009794	marker 1	r	AGACCGCATAATACCTCACAC	microsatellite
EDN1	ENSCAFG00000009794	marker 2	f	GATGTAGCCCAGACTATTCCA	microsatellite
EDN1	ENSCAFG00000009794	marker 2	r	TTTATCCCAGTGACCCAAACC	microsatellite
HIF1A	ENSCAFG00000015718	5	f	CACCTTTGTGCTACTCTGTG	gPCR
HIF1A	ENSCAFG00000015718	5	r	TCCCTCACAATTCTAATGCT	gPCR
HIF1A	ENSCAFG00000015718	6	f	TGCCTGAGTTACATGAGTGG	gPCR
HIF1A	ENSCAFG00000015718	6	r	GAAGGAAGCATTCGCAGTCT	gPCR
HIF1A	ENSCAFG00000015718	7	f	TCAGTTACTTTGGAGAAGCA	gPCR
HIF1A	ENSCAFG00000015718	7	r	CTCAGGTCTTGATCTCAGGT	gPCR
HIF1A	ENSCAFG00000015718	8	f	CTCTGCTTGACTCTTTCTCC	gPCR
HIF1A	ENSCAFG00000015718	8	r	GATGCGAATAGATGGCTACT	gPCR
HIF1A	ENSCAFG00000015718	9	f	CCCACCCTATTGTGTCAATG	gPCR
HIF1A	ENSCAFG00000015718	9	r	GGCTTGCAGCAACAGATAGA	gPCR

Gene	EnsemblID	exon	orientation	primersequence	application
HIF1A	ENSCAFG00000015718	10	f	GGACCTGTCAACAGTAGATT	gPCR
HIF1A	ENSCAFG00000015718	10	r	GTTCTCTGTGGATGTCTAAA	gPCR
HIF1A	ENSCAFG00000015718	13	f	TATTCCTTAATGTTGGCAAA	gPCR
HIF1A	ENSCAFG00000015718	13	r	GCAGACACGTAACTGACTGA	gPCR
HIF1A	ENSCAFG00000015718	14	f	TTTAGGGCCGTATTTAAGAA	gPCR
HIF1A	ENSCAFG00000015718	14	r	AGCTATTCCTGCGTTACGAC	gPCR
HIF1A	ENSCAFG00000015718	15	f	AGCTTATTTGATTTTCCTGC	gPCR
HIF1A	ENSCAFG00000015718	15	r	ATGTGCTGTCTGTGATCTGG	gPCR
HIF1A	ENSCAFG00000015718	1&2	f	GGACCTCTTGTGAGAATTCC	gPCR
HIF1A	ENSCAFG00000015718	1&2	r	GGAGTGAGAGTATGACCACC	gPCR
HIF1A	ENSCAFG00000015718	11&12	f	GTGTATTCTCTGAGGTCTGG	gPCR
HIF1A	ENSCAFG00000015718	11&12	r	GGTGATGTGGTTATTTTCAA	gPCR
HIF1A	ENSCAFG00000015718	3&4	f	GTGGTTTGCTTGCTATGTTA	gPCR
HIF1A	ENSCAFG00000015718	3&4	r	GCAGATTTCACAAAGAACAG	gPCR
HIF1A	ENSCAFG00000015718	marker 1	f	TGGCCTCTCAGTGAAGTGAA	microsatellite
HIF1A	ENSCAFG00000015718	marker 1	r	TCCTGCAGACGAGGCATAGA	microsatellite
HIF1A	ENSCAFG00000015718	marker 2	f	ACCGCTGCCAAGAAGATGAT	microsatellite
HIF1A	ENSCAFG00000015718	marker 2	r	CCCTGCCATTTGATTGCTTT	microsatellite
HSP90AA1	ENSCAFG00000018036	11&12	f	GTACTTGGAGTCCTTAGGTT	gPCR
HSP90AA1	ENSCAFG00000018036	11&12	r	GTCATGCCTTACAGATTCTT	gPCR
HSP90AA1	ENSCAFG00000018036	2&3	f	ATAACCTGGGTACCATCGCC	gPCR
HSP90AA1	ENSCAFG00000018036	2&3	r	ACACCCCATGTGGACACTCA	gPCR
HSP90AA1	ENSCAFG00000018036	4&5	f	TTCAGAGCGTGTTCAACAGC	gPCR
HSP90AA1	ENSCAFG00000018036	4&5	r	CAGCACTTTCAGGAACTGGA	gPCR
HSP90AA1	ENSCAFG00000018036	6&7&8	f	CTGGAAGTTCTGGGACAGAG	gPCR
HSP90AA1	ENSCAFG00000018036	6&7&8	r	AGGAAGTTGGAAGAACAAGC	gPCR
HSP90AA1	ENSCAFG00000018036	9&10	f	CTTGTGTGGTTAGGTCTGGC	gPCR
HSP90AA1	ENSCAFG00000018036	9&10	r	AGGCACACTGAAGCACTGTT	gPCR
HSP90AA1	ENSCAFG00000018036	marker 1	f	ATCTGCTTCATCCCTCTCCA	microsatellite
HSP90AA1	ENSCAFG00000018036	marker 1	r	AGCCACTTCCATTCAACACC	microsatellite
HSP90AA1	ENSCAFG00000018036	marker 2	f	CACTGATCAAAGGAAAGCCAGAG	microsatellite
HSP90AA1	ENSCAFG00000018036	marker 2	r	CTTAACCAATAAGCCAGCCAG	microsatellite
M13			f	GTTTTCCCAGTCACGAC 6-FAM	microsatellite
NOS3	ENSCAFG00000004687	marker 1	f	AATGAGTGCGTGATAGAGTGTG	microsatellite
NOS3	ENSCAFG00000004687	marker 1	r	ATTCTGCATCGTTCAGCTCCA	microsatellite
NOS3	ENSCAFG00000004687	marker 2	f	CAGGCAAGTTACAGGTTACAG	microsatellite
NOS3	ENSCAFG00000004687	marker 2	r	TACAACCCAGAAGATTCCAG	microsatellite
VEGFA	ENSCAFG00000001938	marker 1	f	ACCCCTCTTCATGCTCTCTG	microsatellite
VEGFA	ENSCAFG00000001938	marker 1	r	AGCATATCAGTAGGGAAGGGAG	microsatellite

Table S2: primer sequences and annealing temperatures used for quantitative PCR.

Gene	EnsemblGeneID	primer	sequence	temp	length (bp)	2-step/ 3-step
B2M	ENSCAFG00000013633	f	TCCTCATCCTCCTCGCT	61.2	85	2
		r	TTCTCTGCTGGGTGTCG			
GAPDH	ENSCAFG00000015077	f	TGTCCCCACCCCCAATGTATC	58	100	2
		r	CTCCGATGCCTGCTTCACTACCTT			
GUSB	ENSCAFG00000010193	f	AGACGCTTCCAAGTACCCC	62	103	2
		r	AGGTGTGGTGTAGAGGAGCAC			
hnRPH	ENSCAFG00000000336	f	CTCACTATGATCCACCACG	61.2	151	2
		r	TAGCCTCCATAACCTCCAC			
HPRT	ENSCAFG00000018870	f	AGCTTGCTGGTGAAAAGGAC	56	114	3
		r	TTATAGTCAAGGGCATATCC			
RPS5	ENSCAFG00000002366	f	TCACTGGTGAGAACCCCCT	62.5	141	2
		r	CCTGATTCACACGGCGTAG			
AHR	ENSCAFG00000002448	f	TTAGGCTCAGTGTCAGTTACC	61	81	2
		r	ACTTCATTTCTGTCAGTTGGG			
AIP	ENSCAFG00000011515	f	CCTCAGCCTCTCATCTTTGAC	64	103	2
		r	ACTGCCTTTGCCTTCTCC			
ARNT	ENSCAFG00000012149	f	GAACGACGACGACGGAACAAGATG	63	83	2
		r	TTTTCGAGCCAGGGCACTACAGGT			
ARNT2	ENSCAFG00000013922	f	GCTTCACCTTCCAGAATCCC	62	130	2
		r	GTCATACGACGACAACCCA			
CYP1A1	ENSCAFG00000017937	f	GAAGCCCCTGAGCCCAATGACT	64	163	2
		r	CTGGTGTAGCCTGCTCTGAATGTTT			
CYP1A2	ENSCAFG00000017941	f	CACCATCCCCCACAGCACAACAAA	59.7	139	2
		r	GCTCTGGCCGGAATGCAAATGGAT			
CYP1B1	ENSCAFG00000006164	f	TTTCACCAGGTATCCACAAGTG	56.5	140	3
		r	GAGAAACGCATGCCTTCG			
EDN1	ENSCAFG00000009794	f	TGTCTACTTCTGCCACCT	62	177	2
		r	AGTCCAGCACTTCTTGTC			
HIF1A	ENSCAFG00000015718	f	TTACGTTCCTTCGATCAGTTGTCA	61	105	2
		r	GAGGAGGTTCTTGCATTGGAGTC			
HSP90AA1	ENSCAFG00000025029	f	CTTGACCGATCCCAGTAAGC	59	128	3
		r	TATTGATCAGGTCGGCCTTC			
NOS3	ENSCAFG00000004687	f	GGCATACAGGACACAGGA	62	176	2
		r	GCAATACCCGTACCAGGA			
VEGFA	ENSCAFG00000001938	f	CTTTCTGCTCTCCTGGGTGC	58	101	2
		r	GGTTTGTGCTCTCCTCCTGC			

B2M=β-2-Microglobulin, GAPDH=Glyceraldehyde-3-phosphatedehydrogenase, GUSB = beta-glucuronidase precursor , HNRPH=Heterogeneous nuclear ribonucleoprotein H, HPRT = hypoxanthine-guanine phosphoribosyltransferase, RPS5=Ribosomal protein S5, AHR = aryl hydrocarbon receptor, AIP = aryl hydrocarbon receptor interacting protein, ARNT = aryl hydrocarbon receptor nuclear translocator, CYP1A1 = cytochrome P450, family 1, subfamily A, polypeptide 1, CYP1A2 = cytochrome P450, family 1, subfamily A, polypeptide 2, CYP1B1 = cytochrome P450, family 1, subfamily B, polypeptide 1, EDN1 = Endothelin-1, HIF1A = Hypoxia-inducible factor 1 alpha, HSP90AA1 = heat shock protein 90kDa alpha (cytosolic), class A member 1, NOS3 = nitric oxide synthase, endothelial, VEGFA = Vascular endothelial growth factor α

Table S3: Samples from dogs with extrahepatic portosystemic shunts (EHPSS) or intrahepatic portosystemic shunts (IHPSS) used for microarray and qPCR.

		Microarray		qPCR	
Breed	**Shunttype**	**Female**	**Male**	**Female**	**Male**
Cairn terrier	EHPSS	3	4	0	0
Cross breed	EHPSS	2	1	0	0
Jack Russell terrier	EHPSS	3	3	0	0
Maltese terrier	EHPSS	3	2	0	0
Miniature dachshund	EHPSS	1	0	0	0
Norfolk terrier	EHPSS	2	1	0	0
Shih Tzu	EHPSS	1	0	0	0
West Highland white terrier	EHPSS	2	0	0	0
Yorkshire terrier	EHPSS	4	0	0	0
Australian shepherd	IHPSS	1	0	1	0
Bearded collie	IHPSS	0	1	0	0
Bernese mountain dog	IHPSS	2	1	0	1
Cane corso	IHPSS	0	1	0	0
Duck tolling retriever	IHPSS	0	1	0	1
Golden retriever	IHPSS	2	1	1	2
Hovawart	IHPSS	0	1	0	0
Labrador retriever	IHPSS	0	1	0	0
Newfoundland	IHPSS	1	0	1	0
Deerhound	IHPSS	0	0	0	1
Beagle	control	1	1	5	3

Table S4: Antibodies used for immunohistochemistry.

antigen	type	supplier	antigen retrieval	dilution	incubation primary antibody	washing buffer	addition to washing buffer
anti-AHR	Rabbit polyclonal	Abcam ab84833	40 min Citrate pH 6	1:500	1 hr RT	PBS	Tween-20
anti-ARNT	Mouse monoclonal	Abnova MAB2370	40 min Citrate pH 6	1:150	O/N 4°C	PBS	Triton X100
anti-CYP1A1	Rabbit polyclonal	Santa Cruz Biotechnology sc-20772	40 min TE pH 8	1:250	O/N 4°C	PBS	Tween-20
anti-CYP1B1	Rabbit polyclonal	Abcam ab78044	40 min TE pH 8	1:900	O/N 4°C	PBS	Tween-20
anti-CYP1A2	Rabbit polyclonal	Abcam ab77795	10 min protK (Dako)	1:600	O/N 4°C	PBS	Tween-20
anti-HIF1A	Mouse monoclonal	Novus Biologicals NB100-123	40 min TE pH 8	1:250	O/N 4°C	PBS	Triton X100
anti-HSP90AA1	Mouse monoclonal	Novacastra NCL-HSP90	30 min Citrate pH 6	1:10	1 hr RT	TBS	Tween-20
anti-NOS3	Rabbit polyclonal	Abcam ab5589	40 min Citrate pH 6	1:100	O/N 4°C	PBS	Tween-20
anti-VEGFA	Rabbit polyclonal	Santa Cruz Biotechnology sc-152	40 min TE pH 8	1:500	O/N 4°C	PBS	Tween-20

TE=Tris-Ethylenediaminetetraacetic acid, RT = room temperature, O/N = over night, PBS= Phosphate buffered saline, TBS= Tris buffered saline

Table S5: LOD scores for linkage of shunt phenotype with candidate genes from the AHR pathway in a digenic model.

	AHRR_m1	AHRR_m2	AIP_m1	ARNT_m1	ARNT_m2	cyp1a1/2_m1	cyp1a1/2_m2	cyp1b1_m1	cyp1b1_m2	EDN1_m1	Hif1A_m1	Hif1A_m2	HSP90AA1_m1	HSP90AA1_m2	NOS3_m1	NOS3_m2
AHRR_m1	LINKED	-0.55	0.13	-0.1	-0.73	0.51	0.91	0.33	0.22	-0.19	-0.6	0.1	0.68	0.52	-0.11	-0.1
AHRR_m2		LINKED	-0.01	-0.21	-0.87	0.39	0.76	0.22	-0.02	-0.26	-0.75	-0.01	0.58	0.39	-0.21	-0.21
AIP_m1			LINKED	0.24	-0.21	0.81	**1.19**	0.68	0.58	0.34	-0.44	0.47	0.98	0.88	0.24	0.24
ARNT_m1				LINKED	-0.54	0.58	0.94	0.44	0.26	0.07	-0.74	0.21	0.73	0.64	0.03	0
ARNT_m2					LINKED	0.06	0.46	-0.11	0.12	-0.58	-0.9	-0.35	0.22	0.08	-0.51	-0.54
cyp1a1/2_m1						LINKED	**1.49**	**1**	0.75	0.66	-0.22	0.81	**1.27**	**1.19**	0.55	0.58
cyp1a1/2_m2							LINKED	**1.37**	**1.1**	**1.07**	0.14	**1.2**	**1.66**	**1.54**	0.92	0.94
cyp1b1_m1								LINKED	0.61	0.51	-0.34	0.64	**1.14**	**1.06**	0.42	0.44
cyp1b1_m2									LINKED	0.32	-0.2	0.43	0.86	0.8	0.13	0.26
EDN1_m1										LINKED	-0.71	0.27	0.81	0.71	0.11	0.07
Hif1A_m1											LINKED	-0.77	-0.08	-0.17	-0.8	-0.74
Hif1A_m2												LINKED	0.93	0.86	0.21	0.21
HSP90AA1_m1													LINKED	**1.33**	0.67	0.73
HSP90AA1_m2														LINKED	0.61	0.64
NOS3_m1															LINKED	0.03
NOS3_m2																LINKED

Genotypes of polymorphic microsatellites located close to candidate genes were analyzed assuming no recombinations occurred between markers and genes.

Table S6: Comparison of mRNA expression resulting from the microarray of genes of the AHR pathway.

		Microarray			
Gene Name	**Ensembl Gene ID**	**IHPSS vs healthy**	**p-value**	**EHPSS vs healthy**	**p-value**
AHR	ENSCAFG00000002448	0.83	0.80	0.86	0.77
AIP	ENSCAFG00000011515	1.00	1	0.96	1
ARNT	ENSCAFG00000012149	1.04	1	0.99	1
ARNT2	ENSCAFG00000013922	1.02	1	0.95	1
CYP1A1	ENSCAFG00000017937	0.65	0	0.74	0
CYP1A2	ENSCAFG00000017941	0.22	0	0.30	0
CYP1B1	ENSCAFG00000006164	1.09	1	1.13	0.02
EDN1	ENSCAFG00000009794	1.02	1	0.89	0.02
HIF1A	ENSCAFG00000015718	1.36	0	1.13	1
HSP90AA1	ENSCAFG00000018036	0.90	1	0.89	1
NOS3	ENSCAFG00000004687	1.04	1	1.04	1
VEGFA	ENSCAFG00000001938	1.02	1	0.97	1

Relative mRNA expression of genes of the AHR pathway detected in microarray.
Iw = Irish wolfhound, IHPSS = intrahepatic portosystemic shunt, EHPSS = extrahepatic portosystemic shunt

Chapter 6

Distribution of extrahepatic congenital portosystemic shunt morphology in predisposed dog breeds

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Abstract

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. 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 ($P < 0.001$). 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 port-azygos) 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 (17) 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 ($P = 0.232$) (Figure 1).

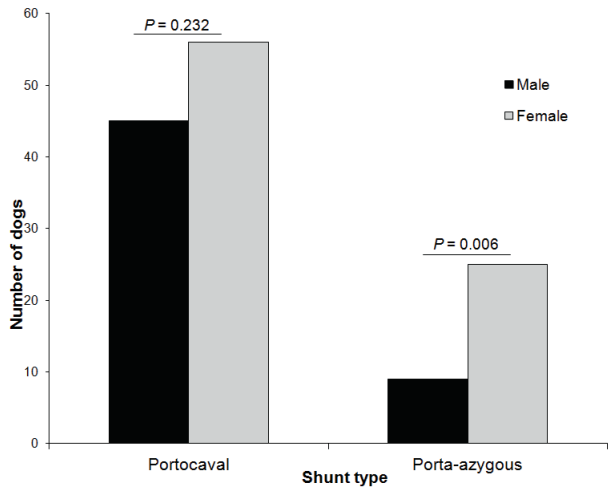

Figure 1: Localization and gender distribution of extrahepatic portosystemic shunts in 135 purebred dogs.
The dogs were seen in the period from 2001-2010. The shunt diagnosis was based on a high fasting venous ammonia level or abnormal ammonia tolerance test and the visualisation by ultrasonography or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

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).

Table 1: Distribution of dogs with congenital extrahepatic portosystemic shunts.

Breed	Total	M	F	PC	PA	Age first diagnosis (months)	
						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.

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. ($P = 0.03$) (Figure 2).

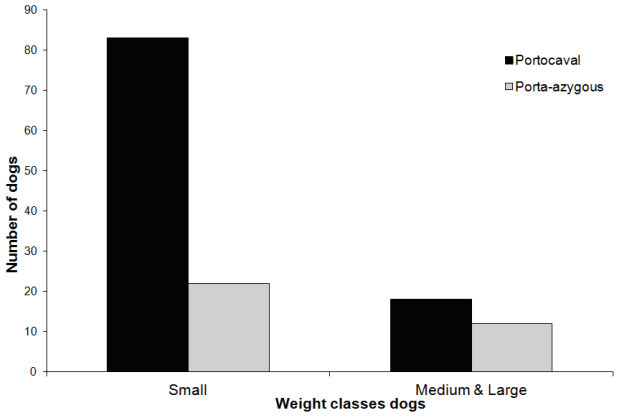

Figure 2: Weight classification of dogs related to porto-azygous and portocaval shunts.
135 dogs were classified as small (≤9 kilo) or medium and large dog breeds (> 9 kilo). The weight classes contained respectively 101, and 34 dogs. Mean weight is based on the standard of the Dutch Kennel Club. Chi-squared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

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 ($P = 0.800$).

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 (18, 19). 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 (18, 19) 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 (17) 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.

Conclusion

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 7

Identification of two chromosomal regions associated with congenital extrahepatic portosystemic shunts in multiple dog breeds

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In preparation

Chapter

Abstract

Extrahepatic portosystemic shunt (EHPSS) is an inherited congenital liver disorder in which an abnormal vascular connection between the portal vein and the vena cava or vena (hemi)azygos is established during embryonic development. As a consequence portal blood bypasses the liver lobules resulting in severely impaired liver growth and atrophy of hepatocytes, leading to reduced hepatic functions. The disorder occurs frequently in a number of breeds of small dogs. A genome wide association study was performed on 48 affected and 48 unaffected Cairn terriers. Statistical analysis using both GenABEL and PLINK and focusing on overlapping regions resulted in three regions of interest on chromosomes 15, 27 and 28. The regions were extensively evaluated by Next Generation Sequencing and SNP genotyping of selections from a panel of in total 198 cases and 182 controls from 29 dog breeds. This analysis excluded the region on CFA28 and narrowed down the two remaining regions of interest. Performing an additional association analysis resulted in significant correlation between EHPSS and chromosome 15 with p-values up to $1.14 \cdot 10^{-8}$, whereas in the region of chromosome 27 the same haplotype was observed with high frequencies in affected Cairn terriers, Jack Russel terriers, Maltese, miniature Schnauzers, Shih Tzu, West Highland White terriers and Yorkshire terriers. The two regions comprise six genes of which none have been reported to be involved in angiogenesis.

Introduction

Extrahepatic portosystemic shunts (EHPSS) are developmental circulatory anomalies connecting the portal vein to the vena cava or vena (hemi)azygos, causing portal blood to bypass the liver. Hepatic perfusion is essential for maintenance of liver mass and function. Toxins and nutrients in the intestines are transported via the portal blood into the liver sinusoids for absorption or clearance. Portal blood also supplies the liver with essential hepatotrophic factors (1). Due to the loss of portal blood perfusion in EHPSS the liver suffers from severely impaired growth, leading to reduced hepatic function (2-4). More importantly, the loss of the ability of the liver to clear toxins causes accumulation of neurotoxins in the brain resulting in hepatic encephalopathy which may, when left untreated, eventually result in death (5).

Histological findings (6-8) as well as clinical signs (9-12) in both man and dog are identical. Diagnosis of EHPSS in dogs can be performed initially by measuring basal plasma ammonia concentrations (13-15) or by a rectal ammonia tolerance test (16) as an assessment of liver function. Both methods are also commonly used in human medicine. Confirmation of the presence and classification of the subtype of the shunt is performed by diagnostic imaging such as ultrasonography, portal scintigraphy or computed tomography (17, 18). With only 173 reported cases, EHPSS is classified as being rare in human (19), whereas in dogs the disease is commonly diagnosed. A clear predisposition has been reported in Cairn terriers (20), Yorkshire terriers (21, 22), Jack Russell terriers (23), Dachshunds (1), miniature schnauzers, Havanese, Dandie Dinmont terriers (21), and Maltese (24).

Formation of purebred dog populations has led to large genomic regions with linkage disequilibrium (LD) within breeds (25). Consequently, the critical region resulting from genome studies of a trait in a single breed are large. Often, pathogenic mutations originated before breed formation and were spread to several breeds. Mapping a disease in one population and using other populations for fine mapping is a powerful method (26, 27) to apply in dogs (28).

Although EHPSS clearly is a genetic developmental disorder (19), until now causative mutations have not yet been identified. The fact that there is a constant ratio between porta-caval and porta-azygos shunts in all affected breeds of small and miniature dogs (29), suggests the presence of a common genotype shared by many of these breeds due to historical mutations. This could be the basis for fine mapping of the associated chromosomal regions and enhance finding functional mutations.

We set out to map genes involved in EHPSS in Cairn terriers and were able to confirm and narrow down the regions of interest with cases from other breeds. This study might not only reveal new genes and pathways involved in vascular embryogenesis, but also important candidate genes for the human forms of the disease. Taking in account both the low prevalence and the complex mode of inheritance of EHPSS in human it seems practically impossible to map genes involved in human EHPSS. This is where man`s best friend can be of great assistance.

Methods

Dogs

All included dogs 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), as part of a screening program or when EHPSS was suspected. Phenotyping was performed by measuring fasting plasma levels of ammonia (19, 20). In case of an elevated fasting concentration (>100 µmol/L) or when in case of a slightly increased fasting concentration (>60 µmol/L; reference concentration <46 µmol/L) an increase >150 µmol/L occurred during a rectal ammonia tolerance test (30), the diagnosis of the shunt and the subtype was confirmed by direct demonstration of the shunting vessel by ultrasonography, computed tomography or surgery (17, 18). All procedures were approved by the Ethical Committee, as required under Dutch legislation.

Methods

Genome Wide Association Study

A Genome Wide Association Study (GWAS) was performed with 47 EHPSS cases and 48 normal controls from the Cairn terrier breed by using the 50K Affymetrix Canine Genome 2.0 Array "Platinum Panel" containing 49,663 SNPs. The selected dogs were not related on parental level and originated mainly (98%) from the Dutch population. The SNPs passed quality control when the genotyping rate exceeded 90%, the minimum allele frequency was higher than 0.05 and the Hardy Weinberg p-value ≥ 0.05. Individual dogs had to be genotyped at more than 90% of the SNPs. Correction for multiple testing included 100,000 permutations of the obtained data. Case-control GWA mapping was performed in PLINK (31) as well as in R statistics package 2.15.0 (32) using GenABEL (33). Population stratification and cryptic relatedness in the sample were adjusted for by mixed models and genomic control implemented in the function mmscore (34) in GenABEL. The genomic inflation factor indicated in PLINK was not corrected for. Regions of interest were classified as such when the PLINK p-value was < 0.0001 and the GenABEL p-value was < 0.0001.

Next Generation Sequencing for variation detection

Haploview (35) was used to construct haplotypes of the three most associated SNPs in the GWAS in each region of interest. Based on these haplotypes, five cases and five controls were selected to construct two pools for DNA sequence analysis. At least two samples had the most highly represented haplotype. The additional three samples all had different haplotypes to ensure capturing of as many variations as possible.

Equimolar pools were constructed of 289 ng phenol-chloroform treated genomic DNA per dog. Library preparations were performed using standard protocols (36) and enrichment was accomplished using Agilent SurePrint custom 1M CGH Microarrays (37) containing 60bp-probes in both forward and reverse orientation. The total design for enrichment after masking with Repeat Masker (38) comprised of 5,078,933 bp.

The DNA was ultrasonically sheared into fragments of approximately 150 bp. The fragments were end repaired and phosphorylated using the End-It DNA End-Repair Kit (EpiCenter). Purification was performed with the Agencourt AMPure XP system (Beckman Coulter Genomics). Double-stranded truncated adaptors were ligated to the DNA using the Quick Ligation Kit (NEB) and purified with the Agencourt AMPure XP system. Nick translation, barcoding and amplification were performed in a single PCR assay. Libraries in the range of 175–225 bp in size were selected on a 4% agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen). Next Generation Sequencing (NGS) was performed on an ABI SOLiD v3. Ensembl CanFam 2 was used as reference genome for design of the enrichment array and aligning SOLiD reads. Mapping and variant calling of the reads was achieved using a custom bioinformatic pipeline based on the Burrows-Wheeler Aligner (BWA) algorithm.

Validation of variations

Fine mapping was performed in two consecutive runs of Komparative Allele Specific PCR (KASPar) assays (KBioscience, Hoddesdon, UK) (39). Two differently tailed allele-specific primers and a common reverse primer were designed. The initial assay consisted of 55 SNPs (Table S1) on Cfa15:2614926-5920509, Cfa27:36439359-40862977 and Cfa28:33672113-33986493 selected by Haploview as tagged SNPs (R<0.9) within the regions of interest after GWAS. Genotyping was performed on 82 cases and 78 controls from 9 breeds (Table S2). Oligo extension PCR in the presence of universal fluorescent reporting dyes, in combination with the fluorescence resonance energy transfer (FRET) technique, makes it possible to determine the distribution of the two alleles in the PCR product. Fluorescence was measured in a PHERAstar (BMG labtech). Kluster Caller software (KBioscience) was used to determine the genotypes.

In the second assay 96 SNPs (Table S1) were selected based on first results and spacing was decreased to an average of 13677 bp (2650-35793 bp) on Cfa15:4409813-4685233 and Cfa27:36832489-37826946. An association analysis was performed on the obtained genotypes using PLINK with the same quality thresholds as used for the GWAS. Associations were measured within the separate breeds using Chihuahuas, Dachshunds, miniature Schnauzers, Jack Russell terriers, Maltese, Shih Tzu, West Highland White terriers and Yorkshire terriers to test whether specific breeds show association. An additional analysis was performed considering all samples to originate from one and the same population.

Mutation detection with SOLiD sequencing

2 µg of gDNA of 96 samples was purified on Qiagen columns. These samples included 14 Cairn terriers in three small pedigrees (6 cases, 8 parents), 12 miniature Schnauzers (6 cases, 6 controls), 14 Jack Russell terriers (7 cases, 7 controls), 14 Shih Tzu (7cases, 7 controls), 14 Maltese (7 cases, 7 controls), 14 West Highland White terriers (7 cases, 7 controls) and 14 Yorkshire terriers (7 cases, 7 controls) were individually barcoded as described previously (36).

Validation of possible mutations

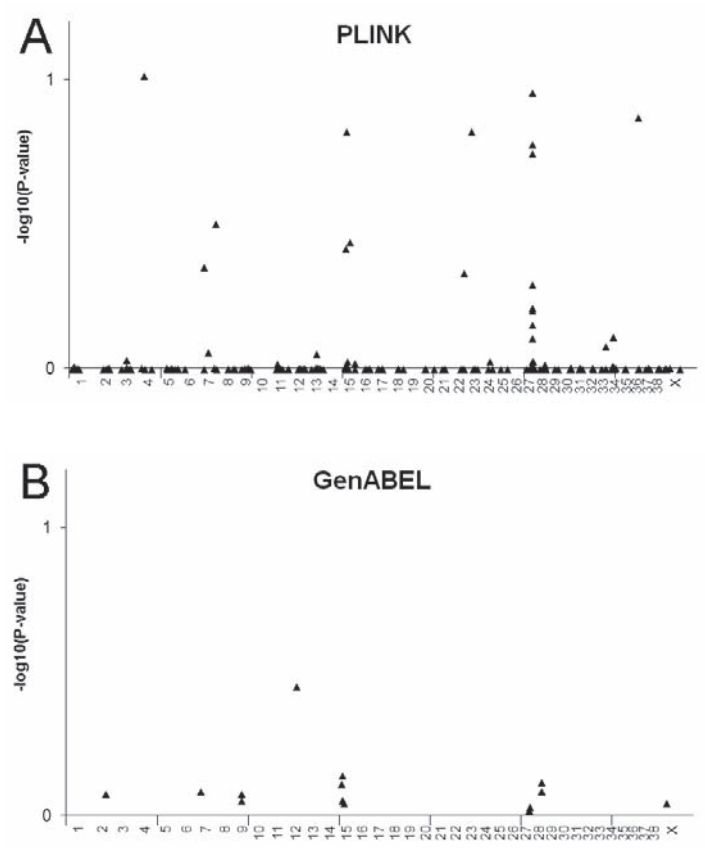
KASPar assays were performed as described previously. Genotyping was performed on 198 cases and 182 controls from 29 breeds (Table S2). Phase (40) was used to haplotype the KASPar data treating all samples as they were originating from one population. Haploblocks were constructed using a sliding window of five consecutive SNPs. Most common haploblocks in six breeds, namely miniature Schnauzers (8 cases, 8 controls), Jack Russell terriers (13 cases, 13 controls), Shih Tzu (6 cases, 6 controls), Maltese (16 cases, 16 controls), West Highland White terriers (13 cases, 13 controls) and Yorkshire terriers (13 cases, 13 controls) were compared. New haploblocks were constructed using 7 SNPs originating from 3 consecutive haploblocks on chromosome 15 and 8 SNPs from 4 consecutive haploblocks on chromosome 27 displaying strong association. Fisher's exact tests were performed per breed to determine the difference between prevalence in cases and controls.

Results

Genome wide association

One DNA sample from an EHPSS affected dog was excluded from the genome wide genotyping due to a low quantity. After genotyping the remaining 95 samples a total of 34,600 SNPs (70%) passed quality control. The PLINK analysis showed that the genomic inflation factor was 1.27. The analysis with neither PLINK nor GenABEL resulted in a region that was significantly associated with the occurrence of EHPSS after correction for multiple testing. Combination of the results from both methods did indicate three regions of interest. Overlap between the analysis methods was found on chromosome 15, 27 and 28 (Figure 1). The combined regions of interest remaining after the GWAS comprised 8.8 Mb of DNA.

Figure 1: Genome wide association analysis of EHPSS in Cairn terriers.



The genotypes of 34600 SNPs of 47 cases and 48 controls were analyzed using two independent methods. A) Plot of PLINK results from [illegible] tests in PLINK. The x-axis denotes chromosome numbers. B) plot of GenABEL results from regression analysis. On chromosome 15, 27 and 28 association signals above background were observed with both methods.

Table 1: The comparison of significant results obtained from PLINK and GenABEL.

Chromosome	Region (Mb)	# genes	Plink	GenABEL
5	51.70-51.72	3	√	
15	2.5-5.8	49	√	√
21	33.8-35.7	18		√
27	36.5-40.8	89	√	√
28	33.5-34.1	5	√	√
34	5.51-5.59	0	√	
36	23.95-23.98	1		√

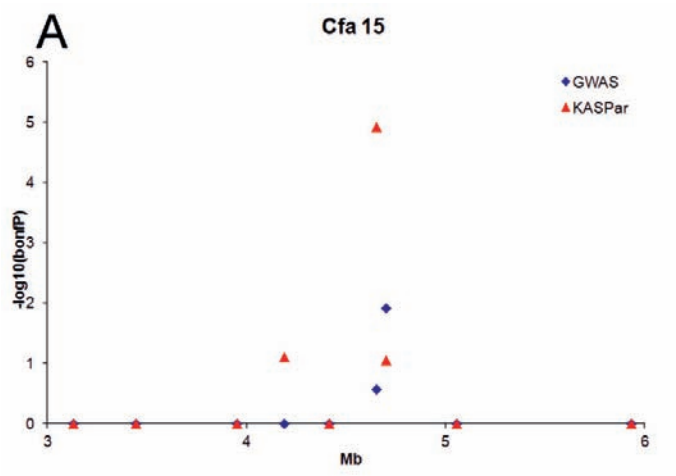
Next generation sequencing of regions of interest

The DNA of 5 cases and 5 controls were pooled separately and the regions of interest were enriched with hybridization arrays. The enrichment was relatively poor. For the pool of cases only 12% of the reads mapped within the target regions. For the controls 13% of the reads mapped to the regions of interest. The median coverage of the target regions for cases and controls was 77X and 88X, respectively, which was sufficient for SNP calling. A total of 7,977 DNA sequence variations were detected. Of these, 2,215 were known in the database of the reference genome version Canfam2. Of the 5,762 novel variations, 3,874 were intergenic, 1,706 were located in introns of genes and 182 were located in exons.

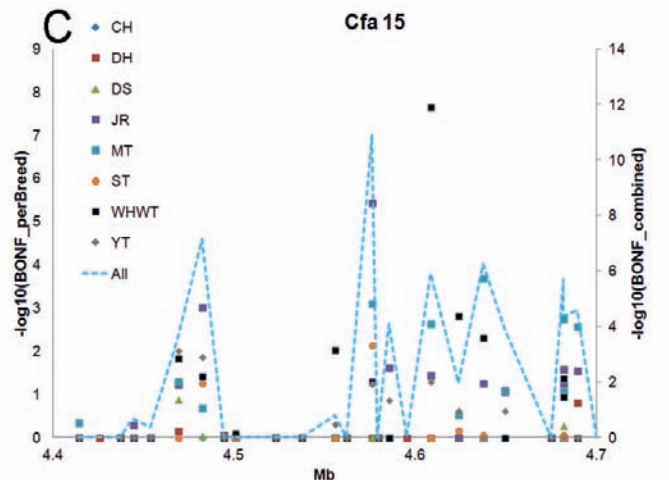
Genotyping SNPs with KASPar assay

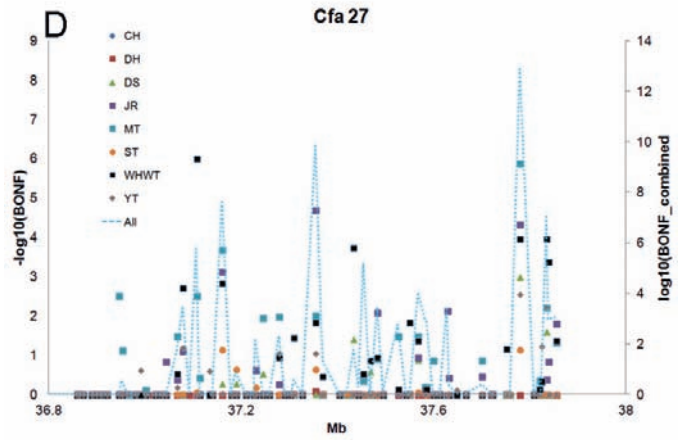
SNPs were selected for further analysis in cases and controls from additional breeds based on the informativity in Cairn terriers. 138 SNPs were genotyped in 160 DNA samples from 9 breeds (Table S2) in two consecutive runs. No significant differences between cases and controls were detected in the region of interest of chromosome 28. On both chromosome 15 (Figure 2A) and 27 (Figure 2B) p-values improved when combining the results from all included breeds in comparison with the GWAS performed in Cairn terriers. Based on the GWAS, the region of interest on chromosome 15 comprised 3.3 Mb. Inclusion of the other breeds resulted in a smaller region of interest of 0.3 Mb. The size of the region of interest on chromosome 27 initially comprised 4.3 Mb. Based on the results from the combined breeds the size was decreased to approximately 1 Mb. Combining all breeds and performing allelic association tests, the most significant p-values were detected on Cfa15:4571613 bp of $1.26 \cdot 10^{-11}$ (Figure 2C) and on Cfa27:37750730 bp of $1.31 \cdot 10^{-13}$ (Figure 2D).

Figure 2: Finemapping of chromosomal regions of interest for EHPSS.



Genotyping of the regions of interest was performed using two subsequent runs of KASPar assays. In the first run a total of 55 SNPs were genotyped in 82 cases and 78 controls from 9 breeds. Compared to the GWAS results of Cairn terrriers, improved p-values were observed on chromosome 15 (A) and 27 (B). In a second run of genotyping a higher number of SNPs was selected on smaller regions of interest on chromosome 15 (C) and 27 (D). No significance was observed when analyzing Chihuahuas (CH) or Dachshunds (DH). In miniature Schnauzers (MS), Jack Russell terriers (JR), Maltese (MT), Shih Tzu (ST), West Highland White terriers (WHWT) and Yorkshire terriers (YT) several SNPs proved to be significantly associated with EHPSS. The blue dotted line represents bonferroni corrected p-values from an association analysis when combining all breeds used for genotyping





Mutation detection with SOLiD sequencing

The two remaining regions resulting to be associated were selected to completely sequence using NGS. For this purpose 96 samples (14 Cairn terriers, 12 miniature Schnauzers, 14 Jack Russell terriers, 14 Shih Tzu, 14 Maltese, 14 West Highland White terriers and 14 Yorkshire terriers) of breeds most associated in the SNPs genotyped in the KASPar assays. The DNA sequence reads of two samples were of poor quality and therefore excluded from further analysis. For the remaining 94 samples high quality sequencing results were obtained. On average a total of 4,334,591 reads per sample were mapped within our region of interest. Approximately 84.6% (median coverage of 78x) of the target region per sample consisted of a coverage of at least 20x. On average only 5.5% of the target region was not covered at all. Variations were analyzed by comparing all cases against all controls together and by comparing cases and controls per breed. 151 SNPs (Table S1) were selected

either based on their frequency differences between all cases and all controls (n=97) or high frequency differences in specific breeds (n=54).

Genotyping and constructing haplotypes

A third run of KASPar assays was performed on 151 SNPs using 198 cases and 182 controls. The genotypes were merged with the data obtained with the preceding KASPar assays. Haplotyping of a sliding window of five consecutive SNPs displayed remarkable differences between cases and controls on both the chromosomes 15 and 27.

Three windows harboring 7 SNPs on chromosome 15 and four windows encompassing eight SNPs were merged in two haploblocks using Phase. The haploblock at risk on chromosome 15 consists of the haplotype CACCCGG and was significantly overrepresented in the affected Jack Russell terriers, Maltese, Shih Tzu, West Highland White terriers and Yorkshire terriers (Table 2). In all breeds combined the haplotype was observed in 41% of the cases and 11% of the controls. On chromosome 27 the haplotype CTCATTCG was found to be the haplotype at risk. A significant overrepresentation of this haplotype was detected in Jack Russell terriers, Maltese, miniature Schnauzers, Shih Tzu, West Highland White terriers and Yorkshire terriers (Table 4). The haplotype at risk was found in 39% of all genotyped controls and in 68% in the cases (Table 5).

Table 2: Haplotype analysis chromosome 15.

breed	status	#samples	#RiskAllele	freqRiskAllele	p-value
Chihuahua	1	7	0	0	1
	2	7	0	0	
Dachshound	1	6	1	0.08	1
	2	6	0	0	
Jack Russell terrier	1	13	0	0	<0.0001
	2	13	16	0.62	
Maltese	1	16	0	0	<0.0001
	2	16	13	0.41	
miniature Schnauzer	1	8	1	0.06	0.33
	2	8	4	0.25	
Shih Tzu	1	6	0	0	0.0003
	2	6	9	0.75	
West highland white terrier	1	13	0	0	<0.0001
	2	13	12	0.46	
Yorkshire terrier	1	13	1	0.04	<0.0001
	2	13	13	0.50	

Haploblocks were constructed with Phase using seven SNPs genotyped with KASPar assays. The SNPs were evenly spaced over chromosome 15. The prevalence of the risk allele was compared between cases and controls within breeds. Significantly associated presence of the risk haplotype were detected in Jack Russell terriers, Maltese, Shih Tzu, West Highland White terriers and Yorkshire terriers.

Table 3: Distribution of haploblocks on chromosome 15 in controls.

haploblock	seen in controls	different breeds
CATCTGG	19	6
CATCTGA	18	8
CACCCGG	17	4
AGTCTGA	15	6
CGTCTGG	10	4
CGCCTAG	9	5
AGTCTGG	8	4
CATCTAA	7	4
CATCCGA	7	4
CGCATAG	6	5
CATCCGG	6	4
AGTCTAG	5	4
AGTCCGG	5	2
CGCATAA	5	2
CGCCTAA	4	3
AGTATGA	3	3
AGTCCGA	3	2
AGTATAA	2	2
CGTCTGA	2	2
CGTCCGG	2	2
CACCTGG	2	1
AGTCTAA	1	1
CATATGG	1	1
CATCTAG	1	1

Frequencies of all haploblocks detected in controls were compared. The haplotype at risk (depicted in grey) was detected in 10% of the controls. SNPs in this haploblock are located at 4571613, 4574797, 4581084, 4590702, 4603875, 4619326 and 4633066 bp.

Table 4: Haplotype analysis chromosome 27.

breed	status	#samples	#RiskAllele	freqRiskAllele	p-value
Jack Russell terrier	1	13	5	0.19	<0.0001
	2	13	21	0.81	
Chihuahua	1	7	4	0.29	1
	2	7	3	0.21	
Dachshound	1	6	2	0.17	1
	2	6	2	0.17	
Maltese	1	16	7	0.22	<0.0001
	2	16	25	0.78	
miniature Schnauzer	1	8	2	0.13	0.0006
	2	8	11	0.69	
Shih Tzu	1	6	0	0.00	<0.0001
	2	6	11	0.92	
West highland white terrier	1	13	8	0.31	0.0002
	2	13	22	0.85	
Yorkshire terrier	1	13	10	0.38	0.0245
	2	13	19	0.73	
other breeds	1	21	37	0.88	0.0997
	2	37	49	0.66	

Haploblocks were constructed with Phase using eight SNPs genotyped with KASPar assays. The SNPs were evenly spaced over chromosome 27. The prevalence of the risk allele was compared between cases and controls within breeds. Significantly associated presence of the risk haplotype were detected in Jack Russell terriers, Maltese, miniature Schnauzers, Shih Tzu, West Highland White terriers and Yorkshire terriers.

Table 5: Distribution of haploblocks on chromosome 27 in controls.

haploblock	seen in controls	different breeds
CTCATTCG	83	16
CTCCTTCG	29	7
CTCCTCCG	16	6
CTCACCTG	16	6
CTCCTTTG	15	5
ATCCTTCG	14	7
CTCCTCTG	10	5
CTCACTTG	10	5
CTTATTCG	6	5
CTTACCTG	3	3
ATCACTTG	3	3
CTTCTTCG	2	2
CGCCTCCG	2	2
ATCCTCCG	2	2
ATCCTTTG	1	1
ATCATTCG	1	1
ATTACTTG	1	1

Frequencies of all haploblocks detected in controls were compared. The haplotype at risk was detected in 39% of the controls. SNPs in this haploblock are located at 37693709, 37729886, 37744042, 37750680, 37791304, 37809969, 37826896 and 37910258 bp.

Discussion

In individuals with an extrahepatic portosystemic shunt (EHPSS), an aberrant blood vessel connects the portal vein with the vena cava or the (hemi)azygos vein. The embryonic origin of these normal vessels is separate. During development the vitelline vein regresses to eventually contribute in the formation of the extrahepatic portal vein, whereas the vena cava and the (hemi)azygos vein develop from the embryonic cardinal vein (41). Connections between these vessels could occur in any phase of embryonic development. Our previous research into this developmental disorder has shown that it has a complex genetic background. These results elucidated two narrow chromosome regions that are involved. Genome wide analysis was performed using two statistical software packages, namely PLINK and GenABEL. Allelic association using PLINK is based on χ^2-calculations between cases and controls whereas analysis with GenABEL focuses on regression. Both approaches did not meet the genome wide significance criterion (p-value < 0.05). When combining the two independent analyses, however, an overlap was observed in three genomic regions. The fact that no genome wide significance was observed is considered most likely due to the relatively low number of samples used (25) although further testing would be required to support this hypothesis.

DNA sequence analysis using pooled DNA samples was not well suited for identification of causative mutations in our study. It did, however, identify novel variations that could be used for validation of the associated regions. Currently over 3 million variations have been annotated in the canine genome (CanFam 3.1; Ensembl). Given its size a variation rate of 1:747 bp is observed which is much lower than the 1:61 bp observed in the human genome (GRCh 37; Ensembl). In our sequencing experiment we detected a total of 7,977 variations in a design of 5,078,933 bp, resulting in a variation rate of 1:637 bp. Not all variations detected in sequencing have been confirmed however, hence the presence of false positive variations should be taken in account. Nevertheless, this number is still much higher than anticipated using only 10 individuals of a single breed. The low number of annotated variations in the canine genome is presumably an underestimation of the real number of variations present in the dog, underlining the necessity to improve the current genome build.

Genotyping affected dogs from several breeds decreased the size critical regions extensively. The risk alleles in common with a majority of the included breeds confirms the presence of mutations older then breed formation. In the second round of NGS we analyzed individual samples. Apart from the fact that this round resulted in better enrichment statistics we also obtained much more informative data.

EHPSS is proven to be a genetic disorder most likely with an autosomal polygenic inheritance (20). The detection of two strongly associated regions fully supports this idea. Based on *in situ* hybridizations performed in zebrafish merely two strong candidate genes could be selected, namely four and a half LIM domains 3 (FHL3)(Cfa15:4582896-4590989 ENSCAFG00000003210) and peroxisomal biogenesis factor 5 (PEX5)(Cfa27:37844434-37859930 ENSCAFG00000014064). NGS resulted in sufficient coverage of the coding regions of both genes, but no obviously functional mutations were found. Recently the ENCODE consortium published their findings regarding functionality of non-coding DNA regions. A biochemical function has been assigned to approximately 80% of the human genome (42-45). Taking these recent developments into account no strong conclusions should be drawn from these findings.

A microarray analysis was performed using EHPSS samples. Differences in gene expression between healthy liver tissue and liver tissue of dogs affected by EHPSS were compared to differences between intrahepatic portosystemic shunt (IHPSS) samples and healthy tissue (Chapter 8). No annotated probes were found for FHL3 and PEX5 in this microarray. VCAM1 RNA and protein were down regulated in EHPSS liver tissue. No direct interaction has been reported between VCAM1 and PEX5 or FHL3. Both VCAM1 as well as FHL3, however, were found to interact with Integrinβ1 (46, 47). We hypothesize a crucial role for FHL3 causing a cascade of alterations in gene expression resulting in a down regulation of VCAM1, which is essential for the survival of endothelial and mural cells during neovascularization (47). Whether disturbed regulation of FHL3 is causative for the decreased VCAM1 expression and ultimately the formation of an EHPSS and the exact role of PEX5 in this mechanism still needs to be clarified.

Short-term applicability of these findings will be the design and validation of a reliable DNA test for Cairn terriers, Jack Russell terriers, Maltese, miniature Schnauzers, Shih Tzu, West highland white terriers and Yorkshire terriers. Using a DNA-test would aid in selective breeding, with the aim of eradicating EHPSS in these dog breeds.

Congenital portosystemic shunt, both intra- or extrahepatic, is associated with aberrant formation and growth of the smallest intrahepatic branches of the portal vein tree. This has been referred to as portal vein hypoplasia (1, 48) or microvascular dysplasia (49). We recently published that both subtypes of congenital extrahepatic shunts, namely porta-cava and porta-(hemi)azygos shunts, have been detected in similar ratios in all affected dog breeds (29), indicating that the complex formation of the splanchnic vascular bed and associated portal venous system is regulated by a limited number of genes. Genes in our regions of interest are not known to be involved in angiogenesis. Elucidation of these genes and the associated pathways might also give insight into the pathologic vascular derangements involved in the pathogenesis of chronic progressive liver disease. These molecular pathways might provide pro-regenerative targets in these presently incurable diseases.

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Table S1

CFA	position (CanFam2)	Allele1	Allele2	KASPar_run	CFA	position (CanFam2)	Allele1	Allele2	KASPar_run
15	5618817	A	T	1	15	7623599	A	G	2
15	6015875	C	T	1	15	7629287	G	A	3
15	6125150	C	T	1	15	7637339	A	G	2
15	6228513	G	A	1	15	7649169	C	T	1
15	6440669	A	C	1	15	7649169	C	T	2
15	6476511	G	A	1	15	7649373	C	A	3
15	6945840	G	A	1	15	7674642	C	T	2
15	7185877	T	C	1	15	7689343	A	G	2
15	7405378	C	T	3	15	7696842	G	A	1
15	7407807	A	G	1	15	7738637	C	G	1
15	7414060	A	G	2	15	7913920	A	C	1
15	7424471	G	A	3	15	8048309	A	C	1
15	7425285	C	T	2	15	8928907	G	A	1
15	7425673	G	A	3	27	39465467	A	G	1
15	7436990	A	G	2	27	39812759	C	A	3
15	7438994	C	T	3	27	39812946	A	C	3
15	7440033	A	G	3	27	39815501	A	G	3
15	7440445	G	A	3	27	39815822	A	C	3
15	7443990	A	G	2	27	39825891	C	T	3
15	7452751	A	G	3	27	39825891	C	T	3
15	7453681	A	G	2	27	39834950	G	A	3
15	7468719	A	C	2	27	39837230	A	G	3
15	7482041	T	C	2	27	39847283	G	C	3
15	7491959	G	A	3	27	39850837	T	C	1
15	7494113	C	T	2	27	39858597	C	A	1
15	7500376	C	T	2	27	39858597	C	A	2
15	7516568	C	T	3	27	39861632	G	A	2
15	7522399	G	C	2	27	39867260	G	A	3
15	7528612	A	G	3	27	39870813	C	T	2
15	7537143	G	A	2	27	39874934	A	G	3
15	7542739	T	C	3	27	39881001	T	C	3
15	7545153	G	T	3	27	39881001	T	C	3
15	7545158	A	G	3	27	39882080	G	A	3
15	7547594	G	A	3	27	39885195	C	T	3
15	7555460	C	T	2	27	39888560	G	A	3
15	7561843	G	A	2	27	39888902	T	C	2
15	7575836	A	C	2	27	39888902	T	C	3

CFA	position (CanFam2)	Allele1	Allele2	KASPar_run	CFA	position (CanFam2)	Allele1	Allele2	KASPar_run
15	7579020	G	A	2	27	39889236	A	T	3
15	7583754	A	G	3	27	39891490	G	A	3
15	7583803	A	G	3	27	39896863	A	G	3
15	7584248	A	G	3	27	39897053	A	G	3
15	7585307	C	T	2	27	39898145	T	C	3
15	7585307	C	T	3	27	39898982	C	T	2
15	7586290	A	G	3	27	39901360	C	T	3
15	7587053	G	A	3	27	39905908	T	C	2
15	7594975	A	C	2	27	39907791	C	T	3
15	7607525	T	C	3	27	39912522	C	T	3
15	7608148	C	T	2	27	39912938	T	C	3
27	39921040	A	G	2	27	40159438	C	A	3
27	39934796	C	T	3	27	40159842	A	C	2
27	39941814	T	A	3	27	40163623	A	G	3
27	39942731	T	A	3	27	40176106	A	G	3
27	39945532	C	A	2	27	40178572	G	A	2
27	39952126	G	T	2	27	40188796	A	G	1
27	39954278	A	T	3	27	40188796	A	G	2
27	39957127	C	T	3	27	40190303	T	C	3
27	39959380	G	A	3	27	40202639	C	T	3
27	39961655	C	T	3	27	40206793	T	C	3
27	39963778	T	G	2	27	40208294	A	C	3
27	39968971	A	T	3	27	40209830	G	A	2
27	39969480	A	C	3	27	40226336	T	C	2
27	39976597	A	C	3	27	40229407	T	G	2
27	39978390	A	G	3	27	40233734	A	G	3
27	39979636	G	C	3	27	40234303	T	C	3
27	39979766	T	G	2	27	40241961	G	A	3
27	39981413	A	G	3	27	40243925	G	A	3
27	39982900	C	T	3	27	40245328	A	G	2
27	39990340	C	G	3	27	40258149	C	T	1
27	39990591	G	A	2	27	40258149	C	T	2
27	39990591	A	G	3	27	40278033	T	C	2
27	39998257	T	C	3	27	40290061	G	A	2
27	40001468	T	C	3	27	40294025	T	C	3
27	40001581	T	C	2	27	40303480	G	A	2
27	40003188	C	T	3	27	40309296	T	C	2
27	40009417	A	G	3	27	40326517	T	G	2

CFA	position (CanFam2)	Allele1	Allele2	KASPar_run	CFA	position (CanFam2)	Allele1	Allele2	KASPar_run
27	40015008	A	C	2	27	40353922	C	A	2
27	40015742	A	G	3	27	40368774	A	G	2
27	40015743	A	T	3	27	40372824	T	C	3
27	40017371	G	C	3	27	40404567	C	T	2
27	40020640	A	C	2	27	40418551	G	A	2
27	40034458	T	C	1	27	40428360	A	G	3
27	40034458	T	C	2	27	40432997	A	G	3
27	40034458	T	C	3	27	40433033	C	T	2
27	40041080	G	T	3	27	40434668	A	G	3
27	40043549	C	T	2	27	40436944	T	C	3
27	40045131	G	A	3	27	40437223	T	C	3
27	40048775	G	A	3	27	40441088	C	G	3
27	40066410	T	C	2	27	40443548	A	G	2
27	40078895	T	C	2	27	40447133	C	T	2
27	40091824	T	C	2	27	40466874	A	G	3
27	40106788	T	C	2	27	40468333	T	C	2
27	40107769	C	T	3	27	40482573	G	A	2
27	40107778	A	G	3	27	40490118	A	G	3
27	40109114	G	T	3	27	40494862	G	A	2
27	40109161	T	G	3	27	40525949	C	T	2
27	40113464	T	C	2	27	40542600	G	A	1
27	40133495	A	C	2	27	40542600	G	A	2
27	40139696	A	G	2	27	40548452	A	T	3
27	40147588	C	T	3	27	40549633	C	A	2
27	40567269	T	C	2	27	41095269	T	C	3
27	40581547	A	G	2	27	41112837	G	A	1
27	40581989	C	T	3	27	41116268	T	C	3
27	40585053	T	C	2	27	41131325	G	T	3
27	40586006	T	G	3	27	41131503	T	G	3
27	40587067	G	T	3	27	41135592	C	A	3
27	40593049	G	T	3	27	41135595	C	G	3
27	40594124	A	T	3	27	41135633	T	C	3
27	40598738	T	C	2	27	41137399	C	A	1
27	40609169	T	C	3	27	41137507	G	C	1
27	40610625	T	C	3	27	41144071	T	C	3
27	40611952	T	G	2	27	41145661	C	G	3
27	40612200	T	C	2	27	41149483	G	T	3
27	40622241	A	G	3	27	41152863	A	C	3

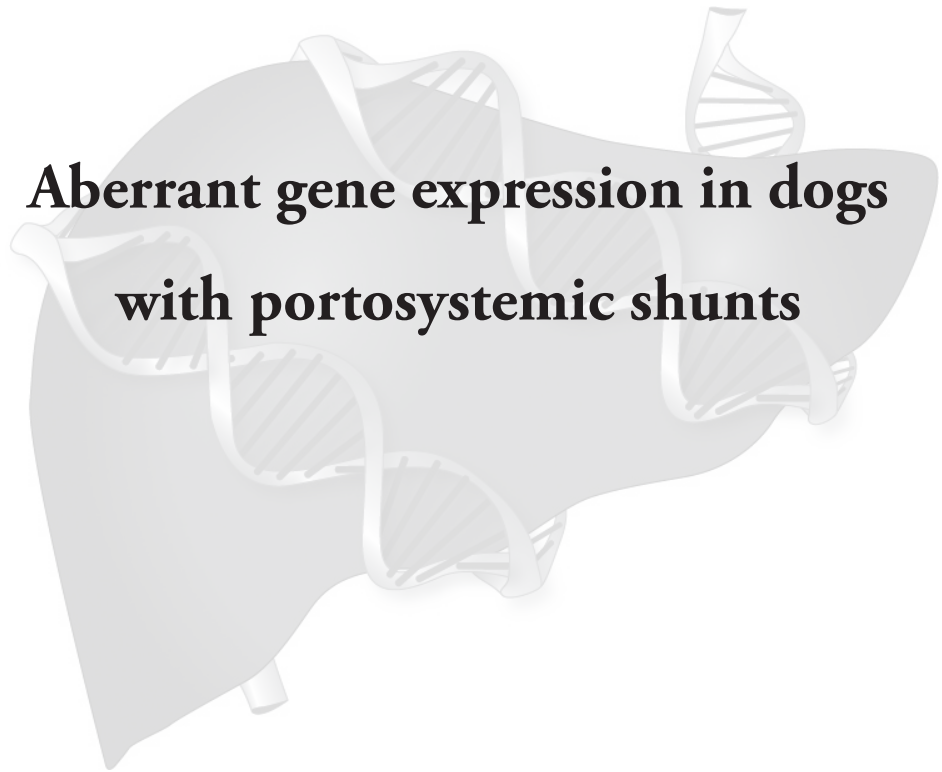
CFA	position (CanFam2)	Allele1	Allele2	KASPar_run	CFA	position (CanFam2)	Allele1	Allele2	KASPar_run
27	40627644	C	G	3	27	41156126	A	G	3
27	40627975	C	T	2	27	41157931	T	C	1
27	40629834	T	G	3	27	41166237	A	T	3
27	40631419	C	A	1	27	41166273	T	C	3
27	40631419	A	C	2	27	41224027	C	T	3
27	40648056	A	C	2	27	41292414	A	G	3
27	40666151	T	G	2	27	41292415	A	G	3
27	40680010	T	C	3	27	41295122	A	C	3
27	40681587	A	G	3	27	41304295	C	A	1
27	40683792	T	A	3	27	41378068	T	A	1
27	40700869	A	G	2	27	41403689	G	A	1
27	40719490	A	G	2	27	41686068	C	T	1
27	40722140	C	A	2	27	41784026	T	C	1
27	40750732	C	T	2	27	42003822	G	A	1
27	40758317	G	T	2	27	42028663	A	G	1
27	40772473	C	T	3	27	42071868	C	T	1
27	40779111	C	A	2	27	42160947	T	C	1
27	40805542	G	C	3	27	42259771	G	A	1
27	40816304	A	G	3	27	42402566	C	T	1
27	40819735	C	T	2	27	42444585	A	G	1
27	40834745	T	C	2	27	42522486	A	G	1
27	40838400	C	T	2	27	42540534	T	G	1
27	40838408	T	C	3	27	42659066	G	A	1
27	40840467	A	T	3	27	42734034	C	T	1
27	40844388	C	T	3	27	42791283	G	A	1
27	40855327	T	C	2	27	42849694	G	C	1
27	40855551	G	A	1	27	43064760	G	A	1
27	40938689	G	C	3	27	43472476	G	A	1
27	40938733	T	C	3	27	43496589	T	A	1
27	40944636	T	C	3	27	43704384	C	A	1
27	40963637	C	G	3	27	43760354	T	C	1
27	40985776	G	A	3	27	43892581	G	A	1
27	41002628	G	A	3	28	36673028	C	G	1
27	41038225	T	C	3	28	36774917	C	T	1
27	41074083	A	G	3	28	36987503	A	G	1
27	41089965	A	G	3					

Table S2

breed	status	KASPar_run 1_2	KASPar_run 3
Basset Hound	cases	0	1
Bichon Frise	cases	0	1
Bolognese	cases	0	2
Border Collie	cases	0	2
Cairn terrier	controls	11	75
	cases	11	79
Cavalier King Charles Spaniel	controls	0	4
	cases	0	4
Chihuahua	controls	5	7
	cases	5	7
Dachshound	controls	5	6
	cases	6	6
Great Dane	cases	0	1
miniature Pinscher	cases	0	1
English Bulldog	cases	0	1
Epagneul	cases	0	1
Fox terrier	controls	0	3
	cases	0	3
French Bulldog	cases	0	1
Jack Russell terrier	controls	11	13
	cases	12	13
Kooiker	cases	0	2
Labrador retriever	controls	0	4
	cases	0	4
Lhasa Apso	controls	0	2
	cases	0	2
Maltese	controls	13	16
	cases	14	16
Pug	controls	0	4
	cases	0	4
Norfolk terrier	controls	0	1
	cases	0	1
Petit Basset Griffon Vendéen	cases	0	1
Poodle	controls	0	3
	cases	0	3
miniature Schnauzer	controls	10	8
	cases	6	8
Shetland Sheepdog	cases	0	1
Shih Tzu	controls	6	6
	cases	6	6
Welsh terrier	cases	0	1
West highland white terrier	controls	9	13
	cases	11	13
Yorkshire terrier	controls	8	13
	cases	10	13

Chapter 8

Aberrant gene expression in dogs with portosystemic shunts



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Chapter

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.

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 confirmation	
	status	**female**	**male**	**female**	**male**	**female**	**male**
Cairn terrier	EHPSS	3	4	2	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 terrier	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

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 (16 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.

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 TranscriptID	F/R	sequence	Tm(C)	Product Size (bp)
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 min	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

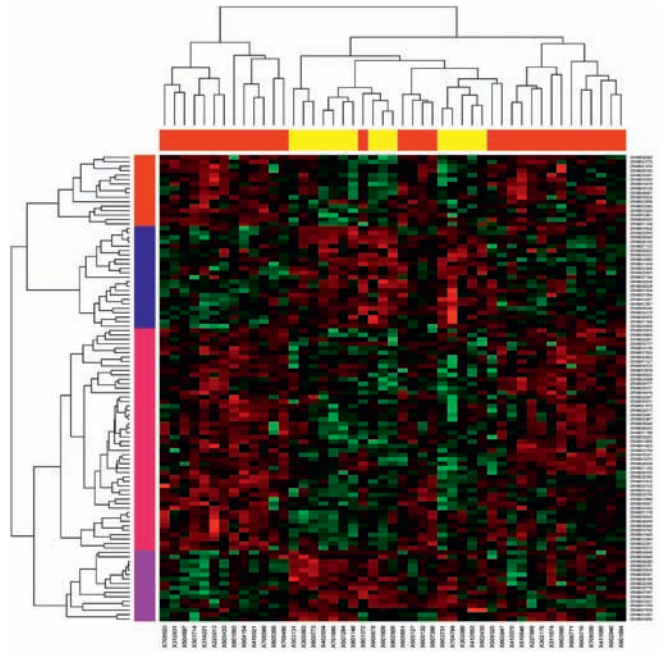
Nitrocellulose membranes (Amersham Biosciences Europe, Roosendaal, The Netherlands). The membranes were incubated with 4% non-fat dry-milk (BioRad) in TBS for 1 hour with shaking. The incubation of the primary antibody was performed at 4°C over-night for all antibodies (see Table 3) in TBS with 0.1% 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 (Figure 1), of which only 107 were annotated (CanFam 3.1).

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.

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).

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, Figure 2).

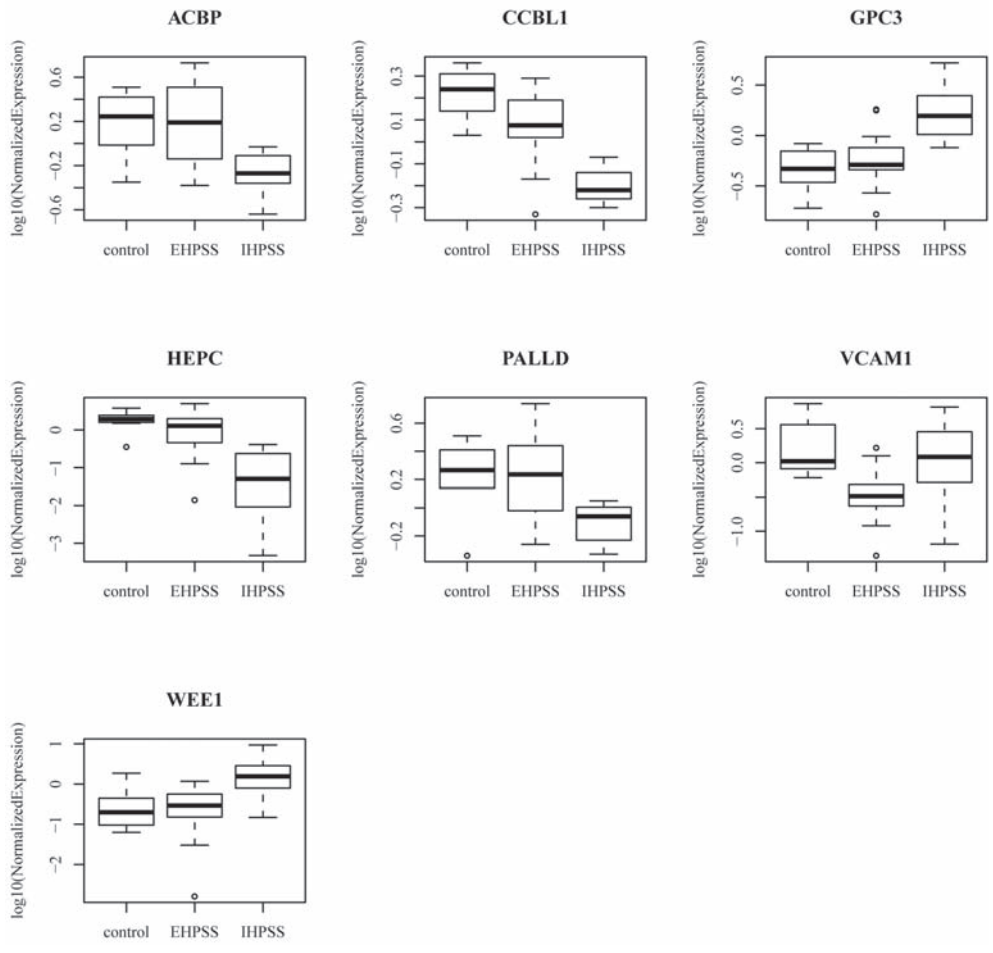
Table 5: Genes expressed differently in dogs with or without extrahepatic (EHPSS) or intrahepatic (IPHSS) portosystemic shunts (qPCR results).

	P-value T-test	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).

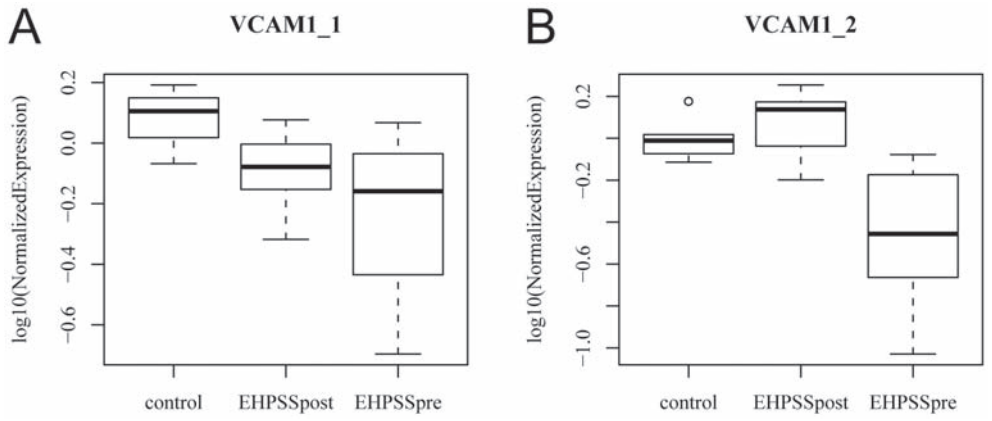
Figure 2: Quantitative PCR results.



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) (Figure 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 (Figure 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.

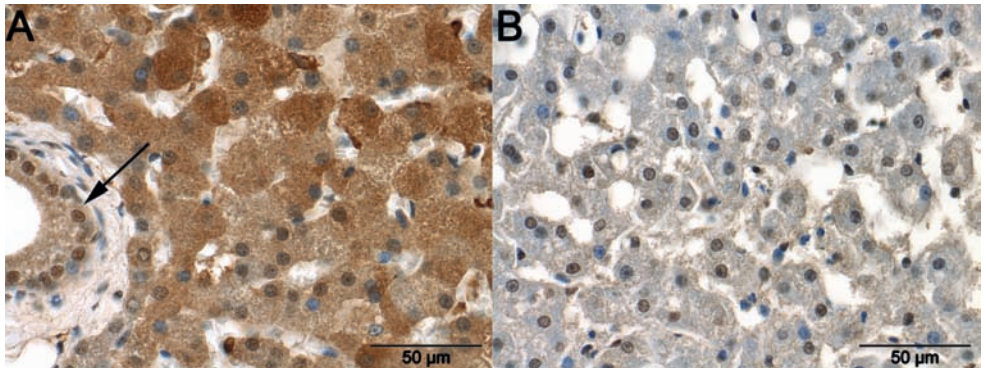
Table 6: Immunohistochemical staining for different proteins in liver samples from dogs with or without extrahepatic (EPHSS) or intrahepatic (IPHSS) portosystemic shunts.

	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 (Figure 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.

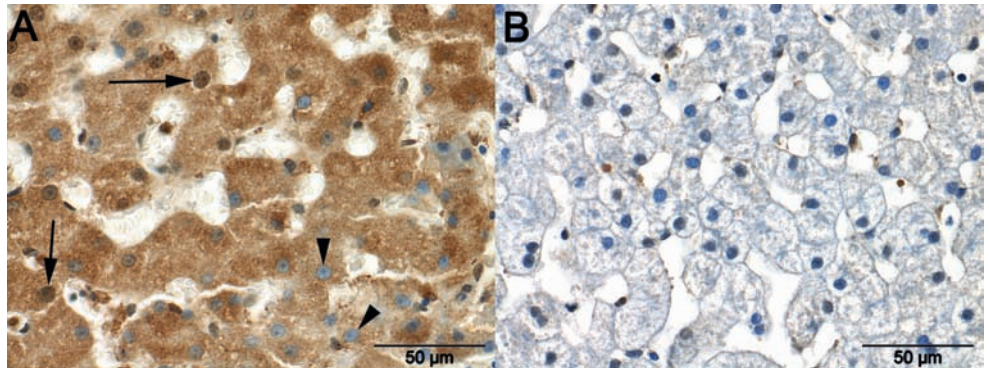
Figure 4: Staining for CCBL1 in the liver.



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 (Figure 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.

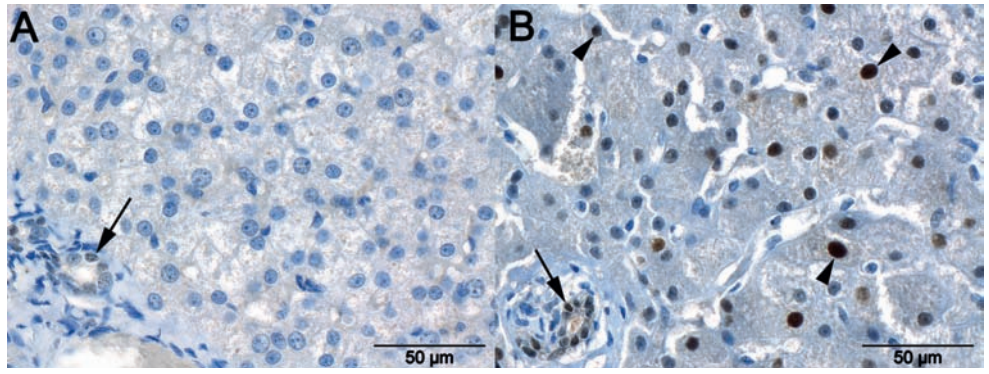
Figure 5: Staining for VCAM1 in the liver.



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 (Figure 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.

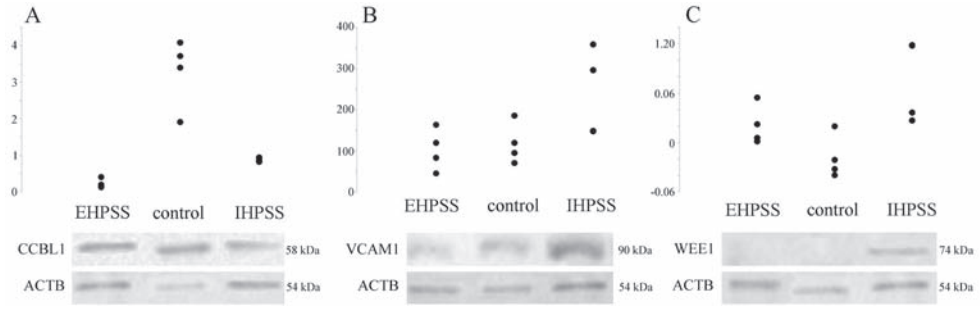


Staining for WEE1 in a liver sample from a healthy dog (Figure 6 A) and a dog with an intrahepatic portosystemic shunt (IHPPS) (Figure 6 B). Note the marked nuclear staining in hepatocytes (arrowheads) and bile duct epithelium (arrows) in the sample from a dog with an IHPPS, whereas nuclei of the sample 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 (Figure 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 (Figure 7B). Expression of WEE1 was found to be upregulated (P = 0.01) in IHPSS samples compared to the control and EHPSS samples (Figure 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 immunohistochemistryand 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 9

Aberrant expression of ammonia metabolizing enzymes in dogs with congenital portosystemic shunts

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In preparation

Abstract

Congenital portosystemic shunts in dogs cause hyperammonemia eventually leading to hepatic encephalopathy. The detoxification of ammonia occurs in the liver specific urea cycle producing urea, and by its incorporation during glutamine synthesis.

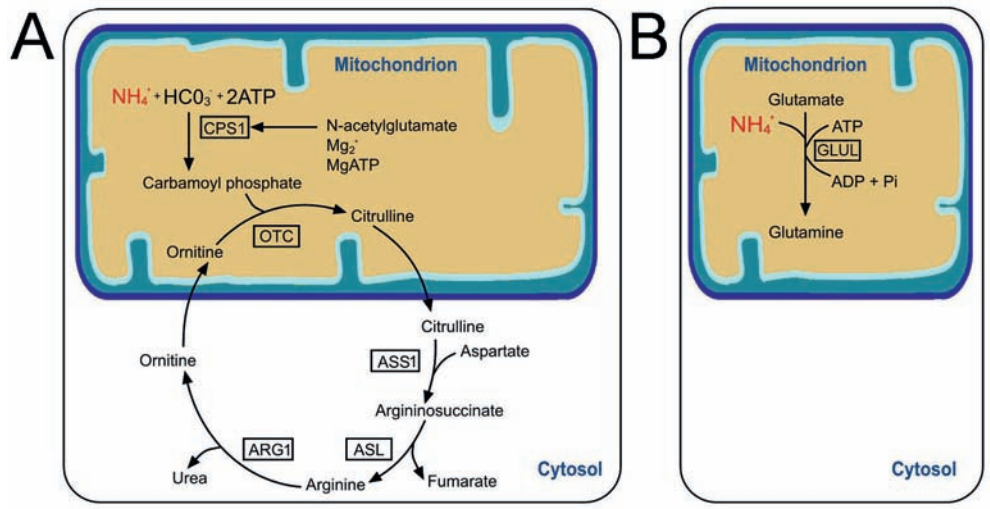
Enzymes involved in both pathways were found to be down regulated in a microarray study comparing liver tissue of dogs with a shunt with healthy liver tissue. Confirmation of these expression differences was achieved by qPCR using peri-operative liver tissue and liver tissue of the same dogs two months after surgical closure of the shunt. Paired samples were used to measure the effect of presence or absence of a functional portosystemic shunt on expression of urea metabolizing enzymes. The effect of ammonia concentrations on the expression of the urea cycle enzymes was measured in vitro using freshly isolated hepatocytes in cultures. Alterations of expression were confirmed for *ASL*, *CPS1*, *OTC*, *GLUD1* and *NAGS* which all were significantly down regulated. Remarkably, expression did not normalize after closure of the shunt, whereas ammonia levels did return to normal values. In vitro studies revealed that administration of ammonium chloride caused an increased mRNA expression of ASL and ARG1. The mRNA expression of the enzymes regulating the urea cycle is induced during the prenatal period to reach a maximal dynamic equilibrium during the perinatal period. We hypothesize that epigenetic alterations involved in the pathogenesis of inherited portosystemic shunts cause a developmental arrest of enzymes of the urea cycle during the embryonic or early postnatal phase.

Introduction

Ammonia detoxification occurs mainly through two pathways, namely liver specific urea synthesis (via the urea cycle) and glutamine synthesis. The urea cycle (also known as ornithine or Krebs-Henseleit cycle (1) is a sequence of biochemical reactions occurring in mammals and amphibians that convert ammonia (NH_3) into urea ($(NH_2)_2CO$) (2, 3). Urinary excretion of urea takes care of definite excretion of nitrogen excess (4). The urea cycle is responsible for the disposal of over 90 percent of surplus nitrogen obtained from dietary or endogenous nitrogen sources (5). It comprises five key enzymes which catalyze the different steps of conversion of ammonia into urea. These are carbamoyl phosphate synthase (CPS1), ornithine carbamoyltransferase (OTC), argininosuccinate synthase (ASS1), argininosuccinate lyase (ASL), and arginase (ARG1) (Figure 1). CPS1 and OTC are located on the inner mitochondrial membrane (6, 7) whereas the three remaining enzymes are actively present in the cytosol (8). Glutamate *N*-acetyltransferase (NAGS) is essential for the formation of N-acetyl glutamate, an allosteric activator of CPS1, and is therefore indispensable for ureagenesis. Glutamate-ammonia ligase (GLUL) regulates the other major pathway for ammonia clearance through the production of glutamine from glutamate (5) (Figure 1). Glutamate itself is produced either by converting α-ketoglutarate by Glutamate dehydrogenase (GLUD1) or by asparagine synthetase (ASNS) which is involved in the synthesis of asparagine from aspartate by converting glutamine into glutamate.

Hyperammonemia is the central metabolic derangement associated with severe clinical dysfunction such as hepatic encephalopathy (HE) (9), both in humans (10-12) and dogs (13) with liver diseases. In many dog breeds congenital portosystemic shunts are the most frequent cause of hyperammonemia and HE (14). It is therefore clinically relevant to investigate the function of the ammonium metabolizing pathways in such canine liver diseases.

Figure 1: The 2 major pathways metabolizing ammonia excess: the urea cycle, and glutamine synthesis.



In the urea cycle(A): ammonia and bicarbonate form carbamoyl phosphate via carbamoyl phosphate synthetase1 (CPS1). Carbamoyl phosphate combines with ornithine in a reaction catalyzed by ornithine carbamoyltransferase (OTC) to form citrulline. Citrulline is transported to the cytosol and merges with aspartate to form argininosuccinate (reaction catalyzed by argininosuccinate synthetase (ASS1)). Argininosuccinate is then cleaved by argininosuccinate lyase (ASL) yielding fumarate and arginine. Arginase (ARG1) cleaves arginine, producing urea and ornithine. Urea is excreted as waste and ornithine is transported back to the mitochondria to be used in subsequent cycles of urea synthesis. In the perivenous hepatocytes (B), ammonia, 'escaping' the urea cycle is metabolized to glutamine (reaction catalyzed by Glutamate-ammonia ligase (GLUL)).

Congenital portosystemic shunts (CPSS) are inherited single large vascular anomalies that directly connect the portal venous system with the systemic venous circulation bypassing the liver parenchyma (14). This occurs commonly via an intrahepatic portosystemic shunt (IHPSS) or an extrahepatic portosystemic shunt (EHPSS). CPSS are reported to occur in 0.18 percent of all dogs (15) but in some breeds the reported incidence varies between 1-5 percent (16-18). A hereditary predisposition has been documented in several breeds (19). In humans CPSS are extremely rare (20, 21).

In this study we evaluated the expression of ammonia metabolizing enzymes in dogs with CPSS using microarray and before surgical ligation of the shunt and after a complete recovery with qPCR. Furthermore, we investigated the influence of ammonia concentrations *in vitro* on the expression of the urea cycle enzymes. We hypothesized that the ammonia concentration in the periportal hepatocytes is a major factor regulating the expression of urea cycle enzymes in dogs with CPSS.

Material and Methods

Dogs

Forty-six client-owned, referral dogs that were diagnosed with CPSS at the Utrecht University clinic for Companion animals were used in this study. CPSS was suspected upon clinical symptoms and increased basal plasma ammonia concentrations as previously described (17, 19) and a definitive diagnosis and localization of the CPSS (IHPSS or EHPSS) was obtained by ultrasonography.

Control tissues for gene-expression studies and for the primary hepatocyte culture were obtained from six respectively two healthy mature dogs, used for liver-unrelated research projects. The absence of underlying liver disease in these control tissues was confirmed histologically by a board-certified veterinary pathologist (GCMG).

Surgery

In all affected dogs the CPSS was surgically ligated. When complete closure could not be achieved, the CPSS was attenuated to the maximum degree of attenuation that was tolerated without development of portal hypertension (partial closure) (22, 23). Wedge biopsies of the liver were routinely taken during surgery.

Post operative recovery was evaluated one and two months after surgery by measuring 12-hour fasting plasma ammonia concentration and performing a rectal ammonia tolerance test (25). Doppler ultrasonography was performed to examine the site and patency of the attenuated shunt. Complete recovery was defined as resolution of all clinical signs, normal fasting plasma ammonia concentrations, a normal rectal ammonia tolerance test and the absence of ultrasonographic evidence for portosystemic shunting.

In cases where a complete recovery was diagnosed a second liver biopsy was taken percutaneously using a 14 G true cut biopsy needle under ultrasonographic guidance (24). Liver tissue obtained during surgery or at the re-check in cases with complete recovery. Tissue was snap frozen in liquid nitrogen or RNAlater (Ambion, Austin, TX, USA) for RNA isolation, or fixed in 10% neutral buffered formalin and paraffin embedded for histopathological evaluation and immunohistochemistry.

Microarray analysis

Total RNA was isolated from liver tissue of 2 healthy dogs, 32 dogs with EHPSS, and 14 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.

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

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

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 (26) 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 (27). 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 (28) on mean spot intensity, and dye bias was corrected based on a within-set estimate (29).

Expression data of 42,034 different gene probes was analyzed using ANOVA (30). In a fixed effect analysis, sample, array and dye effects were modeled. Correction for multiple testing (Permutation F2-test using 5,000 permutations) was performed and $P<0.05$ was

considered statistically significant. Genes involved in ammonia metabolism in particular UCE (*CPS1*, *OTC*, *ASS1*, *ASL*, *ARG1* and *NAGS*), *GLUL* and *GLUD1* were selected. Genes with $p < 0.05$ after family wise error (type I errors; false positives) correction were considered significantly changed.

Quantitative real-time PCR (qPCR)

Quantitative PCR (qPCR) was performed on RNA samples of liver samples from healthy controls (n=6), perioperative samples representing a functional shunt (n=6), and postoperative samples representing a completely closed shunt (n=6). Postoperative samples were collected 2 months after surgery. qPCR was performed on the same genes investigated in the microarray analysis. Because of the small number of fully recovered dogs with an IHPSS, a comparison between pre- and post operative expression levels of the urea cycle enzymes and alternative pathways was only made between EHPSS and the reference pool.

Perlprimer v1.1.14 was used for primer design on Ensembl annotated transcripts and the amplicon was tested for secondary structures using MFold (31). Gradient PCRs were performed to determine the optimum temperature for obtaining 100 percent 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. For normalization a total of four reference genes were used based on their stable expression level in liver tissue and includes *glyceraldehyde-3-phosphatedehydrogenase (GAPDH)*, *β-2-Microglobulin (B2M)*, *ribosomal protein S5 (RPS5)*, and *ribosomal protein L8 (RPL8)* (32). GeneNorm (33) 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 on 95°C, followed by 45 cycles of 10 seconds at 95°C to denature and 30 seconds at T_m for annealing and elongation, or followed by a separate elongation step at 72°C for 30 seconds. All experiments were conducted on a MyiQ Single-Color Real-Time PCR Detection System (BioRad). A 4-fold standard dilution series of a pool containing all samples used for analysis was used to determine relative expression. Data analysis was performed in IQ5 Real-Time PCR detection system software (BioRad). Expression levels were normalized by using the average relative amount of the reference genes. For expression analysis on primary hepatocytes the relative gene expression of each gene product (delta-Cq method) was used as the basis for all mRNA comparisons. Undetectable gene expressions were arbitrarily set to Cq 45 for statistical analysis.

Table 2: Primers used for quantitative PCR.

Gene	Ensembl TranscriptID	F/R	sequence	T_m(°C)	Amplicon Size (bp)
GAPDH	ENSCAFT00000037560	F	5'-TGTCCCCACCCCCAATGTATC-3'	58	100
		R	5'-CTCCGATGCCTGCTTCACTACCTT-3'		
B2M	ENSCAFT00000038092	F	5'-TCCTCATCCTCCTCGCT-3'	61.2	85
		R	5'-TTCTCTGCTGGGTGTCG-3'		
RPS5	ENSCAFT00000003710	F	5'-TCACTGGTGAGAACCCCCT-3'	62.5	141
		R	5'-CCTGATTCACACGGCGTAG-3'		
RPL8	ENSCAFT00000002627	F	5'-CCATGAATCCTGTGGAGC-3'	55	63
		R	5'-GTAGAGGGTTTGCCGATG-3'		
ARG1	ENSCAFT00000000605	F	5'-CAACCTGTGTCTTTCCTCCT-3'	61.9	200
		R	5'-GCCAATTCCCAGTTTATCCAC-3'		
ASL	ENSCAFT00000017006	F	5'-CTAGAGGTACAGAAGCGG-3'	58	126
		R	5'-TGCTGTTGAGAGTGATGG-3'		
ASNS	ENSCAFT00000003490	F	5'-ATACACCAACTGCTGCTTT-3'	55.8	186
		R	5'-GATTATCTCACCATCCACTTTG-3'		
ASS1	ENSCAFT00000031736	F	5'-CCTTTACCACGCTCATTTAGAC-3'	60.1	185
		R	5'-ACTTGCACTTTCCCTTCCAC-3'		
CPS1	ENSCAFT00000022222	F	5'-TTATAGCGATGACTACCACCAC-3'	57	101
		R	5'-AGCATTCTTGTATCCACTCCA-3'		
OTC	ENSCAFT00000022277	F	5'-TTTGGGTGTGAATGAAAGTCTC-3'	61.4	130
		R	5'-TGATGATTGGGATGGATGCT-3'		
GLUL	ENSCAFT00000020795	F	5'-TGTATCTTGTCCCTGCTG-3'	60	183
		R	5'-GTATATTCCTGCTCCATTCCA-3'		
GLUD1	ENSCAFT00000025535	F	5'-AATTCCAAGACAGGATATCGGG-3'	62	128
		R	5'-TCAGATCCAAGCCCAGGT-3'		
NAGS	ENSCAFT00000022856	F	5'-GTTCCAGACCTGCTACC-3'	62	153
		R	5'-CAGCCCGAGGACTACTA-3'		

All data were analyzed using R statistics package 2.14.0 (34). The Wilcoxon rank sum test with continuity correction was used for comparison in mRNA expression of *ARG1*, *ASS1*, *ASL*, *ASNS*, *OTC*, *CPS1*, *GLUL*, *GLUD1* and *NAGS* in liver tissue of control dogs and peri-operatively collected liver tissue from dogs with a shunt. The Wilcoxon signed rank test with continuity correction was used to test differences in expression within the same group of dogs peri- and post-operatively. P-values <0.05 were considered significant.

Primary hepatocyte culture and ammonium chloride treatment

Isolation of the hepatocytes from healthy livers was performed as described previously (35). In short, the left medial hepatic lobe was excised *ex vivo*, and perfused with wash solution [0.14 M NaCl, 6.7 mM KCl, and 10 mM HEPES (pH 7.4)] with 0.5 mM ethylene glycol tetra-acetic acid (EGTA) for 15 min, 30 ml/min at 37ºC. Subsequently with wash solution without EGTA (15 min, 30 ml/min, 37ºC), and finally with wash solution with 4.8 mM $CaCl_2$ and 0.14 Wunsch units/ml Liberase Blendzyme 3 (Roche Diagnostics, Almere, The Netherlands), 25 ml/min, 37ºC. Released cell suspension was filtered through a sterile 70 µm nylon mesh filter (Millipore, Amsterdam, The Netherlands). Percoll (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to a final concentration of 55 percent and cells were centrifuged (50 g, 10 min, 4ºC). The pellet was washed (200 g, 5 min, 4ºC) two times with Hank›s Balanced Salt Solution with 10% bovine serum albumin (Sigma-Aldrich) and resuspended in Hepatozyme Serum Free Medium (SFM) (Invitrogen, Breda, The Netherlands) supplemented 10% Fetal Calf Serum (FCS) and standard antibiotics. Cell viability was determined by using the Trypan Blue Exclusion test (Invitrogen). Cells were plated in 24 wells Primaria dishes in a concentration of 1.5×10^5 viable cells/well (BD Bioscience, Alphen a/d Rijn, The Netherlands). One day after plating cells were treated with ammonium chloride (range 0 to 6000 µM) in Hepatozyme-SFM with standard antibiotics and RNA was isolated 0.5, 1, and 3 hours after treatment. Urea production (QuantiChrom™ Urea Assay Kit, BioAssay Systems, Hayward, USA) was measured to confirm the functionality of the primary hepatocytes and CyQUANT® (Invitrogen) measurement was used to confirm that the cells were equal in number between experiments. All measurements were performed in duplicate in each of two independent hepatocyte isolations.

Linear regression was used to study the association between expression of *ARG1*, *ASL*, *ASS1*, *CPS1* and *OTC* in primary hepatocytes with incubation time (0.5, 1 and 3 hours), ammonia concentration (0,6,60,600 or 6000 µM) and the interaction between time and ammonia concentration. Time was modeled as a continuous variable, whereas ammonia levels were entered as a factor. A stepwise backward method was used to determine the model of best fit based on Akaike's information criterium. The validity of the final model was checked by studying the residuals on normality and constance of variance.

Ethics of Experimentation

All procedures were approved by and performed according to the standards of the Ethical Committee of Animal Experimentation of the Utrecht University.

Results

mRNA expression in liver tissue

In the microarray analysis *ARG1*, *ASS1*, *ASL*, *CPS1*, *OCT*, *GLUL*, *GLUD1* and *NAGS* were significantly down regulated in dogs with CPSS compared to the healthy liver tissue (Table 3). *ASNS* was up regulated in EHPSS samples but did not differ between controls and IHPSS liver tissue.

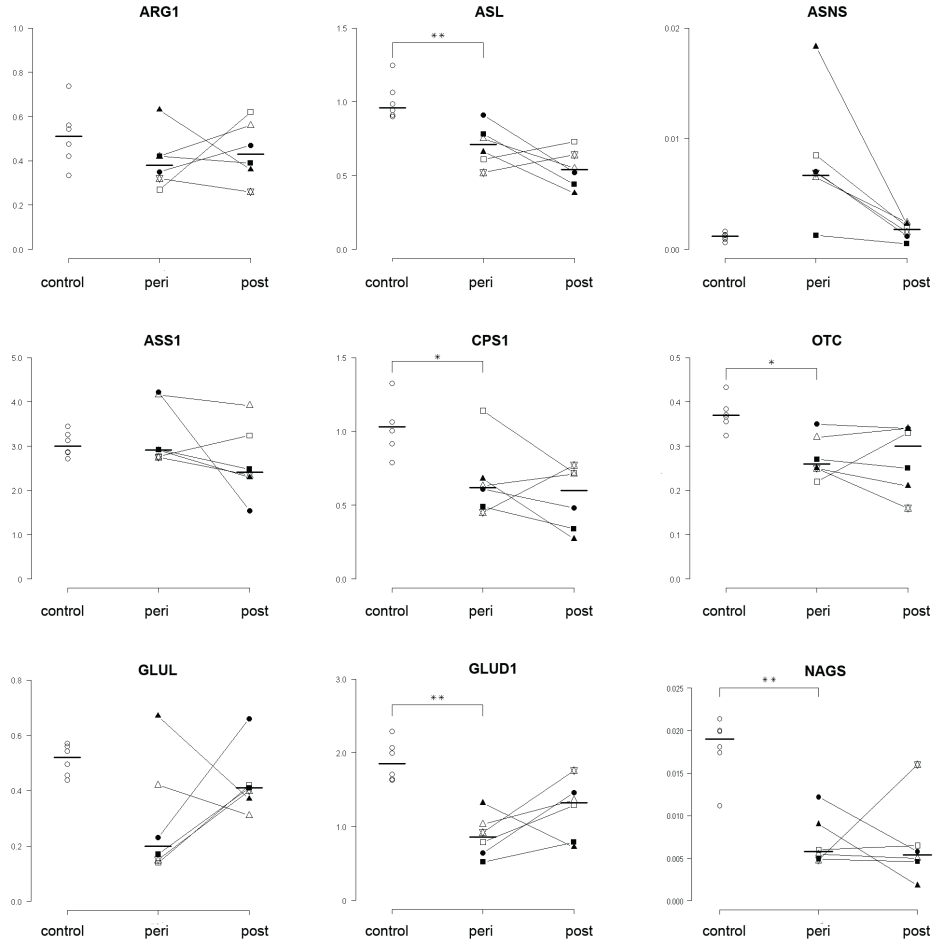
Table 3: mRNA expression differences of ammonia metabolizing enzymes detected in the microarray.

	EHPSS		IHPSS	
gene	**p-value**	**M**	**p-value**	**M**
ARG1	<0.0002	-0.85	<0.002	-0.66
ASL	<0.0002	-0.47	<0.0002	-0.6
ASNS	<0.0002	0.47	1	-0.06
ASS1	0.03	-0.23	<0.0002	-0.67
CPS1	<0.0002	-0.64	0.0032	-0.4
OTC	<0.0002	-0.86	<0.0002	-0.63
GLUL	0.012	-0.4	<0.0002	-0.68
GLUD1	<0.002	-1.22	<0.002	-1.21
NAGS	<0.0002	-0.66	<0.002	-0.53

Indicated p-value were obtained after family wise error correction; M value= log2 (R/G); R and G (red and green) represent the background adjusted, averaged, normalized and dye-bias corrected intensity levels of the samples.

During qPCR replication of the microarray data in the peri-operative liver tissues, the expression levels of *ASL*, *CPS1*, *OTC*, *GLUD1* and *NAGS* were confirmed to be significantly down regulated compared to the healthy dogs. No significant differences, however, were found in expression of *ARG1*, *ASNS*, *ASS1* and *NAGS* between the healthy dogs and liver tissue of dogs with EHPSS (Figure 2). No differences between de peri-, and post- operative samples were observed when comparing mRNA expression of the ammonia metabolizing enzymes.

Figure 2: Relative mRNA expression of ammonia metabolyzing enzymes in liver tissue of control dogs and liver tissue obtained peri-operatively and 2 months after surgical closure of the shunt.

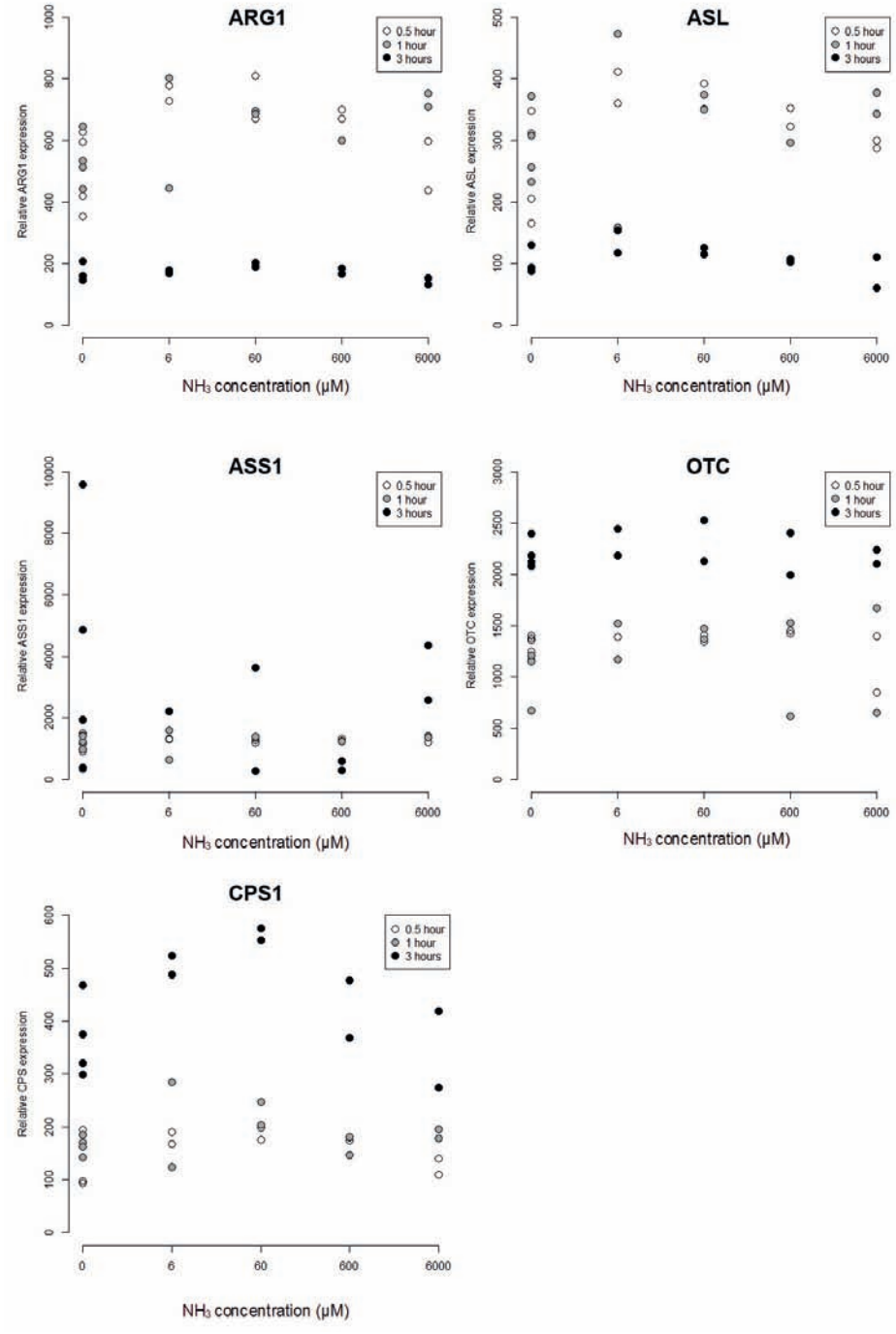


Significant differences between mRNA expression of the enzymes in control liver samples compared to samples obtained peri-operatively are indicated by stars (=<0.05, ** = <0.01). There were no significant differences in relative mRNA expression of the enzymes in the liver samples obtained peri-operatively and 2 months after surgical closure of the shunt. Black symbols: Dogs with a porto-caval shunt. White symbols: Dogs with a porto-azygos shunt .*

Hepatocyte culture and ammonium chloride treatment

Increased urea production was observed 3 hours after the addition of ammonium chloride (data not shown) confirming an active urea cycle in the used hepatocytes. mRNA expression of all UCE was significantly influenced by time. However, expression pattern was not uniform for all enzyme-coding mRNAs. Compared to the individual expression at time 0 and concentration 0, *CPS1*, *OTC* and *ASS1* mRNA expression increased every hour with 95

Figure 3: Relative expression of urea cycle enzymes in primary hepatocytes treated with different concentrations of ammonia for periods.



Primary hepatocytes were treated with either 0, 6, 60, 600 or 6000 µM ammonia for 0.5, 1 or 3 hours.

(95% confidence interval (CI) 69-127), 407 (95% CI 318-496) and 674 (95% CI 165-1184) respectively. For ASL and ARG1 mRNA expression decreased every hour with 90 (95% CI 70-110) and 191 (95% CI 158-224) respectively.

A significant influence of ammonium chloride concentration on mRNA expression was found for *ASL* and *ARG1*. Compared to the expected average, at an ammonium chloride concentration of 6 µM *ARG1* expression increased 118 (95% CI 11-224) and at a concentration of 60 µM *ARG1* and *ASL* expression increased 144 (95% CI 38-251) and 68 (95% CI 4-132) respectively.

An interaction between time and concentration was only found for *CPS1*. With an ammonium chloride concentration of 60 µM mRNA expression increased 41 (95% CI 15-107) compared to *CPS1* expression at time 0 and concentration 0 (Figure 3).

Discussion

In this study, mRNA expression of all ammonia metabolizing enzymes (except for ASNS) was down regulated in dogs with a CPSS compared to healthy dogs in the microarray analysis. *ASNS* was found to be significantly up regulated in EHPSS whereas in IHPSS no difference was detected. qPCR was performed in tissue obtained peri-operatively and during complete closure of the shunt two months after surgery both to confirm the detected expression differences of the microarray analysis, and to determine whether expression would be normalized after successful closing of the shunt resulting in normal perfusion. Differences in expression levels in peri-operative tissue was confirmed for *ASL*, *CPS1*, *OTC*, *GLUD1* and *NAGS* which were significantly down regulated compared to the healthy dogs, both in the microarray results and in the qPCR confirmation. Expression of these enzymes remained reduced even after the dogs had achieved a complete recovery after attenuation of the shunt. Differences observed with microarray in expression of *ARG1*, *ASNS*, *ASS1* and *NAGS* between the healthy dogs and liver tissue of dogs with EHPSS could not be confirmed and these products were not further analyzed.

Reduced UCE activity have been previously reported in healthy rats (36, 37) and dogs (38) following surgically induced portacaval shunts. Reduced GLUL activity (39) and mRNA expression (40) was also reported in rats following portacaval anastomosis. Developmental signaling and induction of gene expression in the liver may play a major role in the etiopathogenesis of the reduced expression of ammonia metabolizing enzymes in CPSS dogs. The mRNA expression of UCE, *NAGS*, *GLUD1* and *GLUL* in healthy dogs is coordinately induced during the prenatal period and reaches a steady state (comparable with the adult expression) in the perinatal period (41-44) It seems as if the expression of these enzymes in dogs with a CPSS has come to a developmental arrest which prevented them to normalize after attenuation of the shunt. Examples for mechanisms that could explain such a phenomenon are mutations in promoter or enhancer genes or alterations in the expression of transcription factors that would consequently alter transcriptional regulation and lead to reduced enzyme mRNA expressions. Reduced expression of multiple ammonia metabolizing enzymes suggests involvement of a common factor in the regulatory process of the developmental expression of all these enzymes.

This hypothesis is supported by studies of the albino mice (C^{3H} and C^{14CoS}) where a mutation in the gene that encodes for fumarylacetoacetate hydrolase (FAH) (45) (in our microarray study significant down regulated M=-0.59) affects the normal expression of a group of liver enzymes, among others the UCE. Mice homozygous for this deletion lack the postnatal developmental increase of UCE and maintain a (prenatal) reduced level of UCE activity and mRNA expression (46) as well as reduced GLUL activity (47). In this mouse model reduced transcription rates of HNF-1, HNF-4 and C/EBP (48) and increased levels of genes associated with growth arrest and DNA damage (49) have been found, all being considered to be possibly involved in the reduced expression of these enzymes at birth. Interestingly, mRNA expression of UCE was not altered in the kidney or intestine of C^{3H} (46). Based on these findings it was suggested that trans-acting regulatory factors specifically involved in the regulation of perinatal expression of liver specific enzymes might be the cause for the reduced expression of UCE and *GLUL* in the albino mouse.

Despite the reduced expression of UCE and *GLUL* in dogs with CPSS that achieved a complete recovery after closure of the shunt, these dogs did exhibit normal blood ammonia levels. Liver atrophy and dysfunction of several metabolic functions of the liver (in particular hyperammonemia) are known to be major consequences of a liver shunt in experimentally induced portacaval shunts (36, 38) as well as in animals with a CPSS (14). Studies in dogs and humans with a portosystemic shunt (50, 51) or an occlusion of the portal vein (52-55) have shown a strong and significant increase in hepatic arterial blood flow as a compensatory mechanism for the drastic decrease in hepatic portal flow. Evidence for this phenomenon is also appreciated in histopathology studies of dogs with CPSS (14) where a clear arteriolar proliferation and hypoplasia of the portal vein can be seen. Actually, the increase in hepatic arterial blood flow is capable of buffering only 25-60 percent of the decreased portal flow (56, 57). This leaves a relatively large area of sinusoids deprived from portal blood (i.e. nutrients and hepatotrophic factors) which results in liver atrophy and metabolic dysfunction. After attenuation of the shunt, portal hepatic blood flow normalizes and provides adequate blood supply to the liver parenchyma. The maintenance of a normal ammonia concentration shortly thereafter (in spite of reduced expression of ammonia metabolizing enzymes) is most probably the result of postoperative exposure to portal blood of the hepatocytes, that as a result become involved in ammonium metabolism or an activation of the enzyme molecules, a process that can be realized in a time range of seconds or minutes (8). Furthermore, for almost all functions the liver has a huge reserve capacity, and liver dysfunction in healthy animal occurs only after removal of >80% of the liver. The better perfusion after surgery enables the liver to exert this already present reserve function, which was impossible beforehand due to the fact that portal blood did not reach the organ.

We hypothesized that the ammonia concentration in the periportal hepatocytes would be a major factor regulating the expression of urea cycle enzymes in dogs with CPSS. Primary hepatocytes cultured in vitro show that only ASL and ARG1 expression were significantly influenced by specifically increased ammonium chloride concentrations, hence this hypothesis could not be confirmed. Given the fact that enzyme mRNA levels do not normalize after closure of the shunt whereas liver size is increased and ammonia levels are decreased, it is suggested that the amount of enzyme present in normal liver is highly abundant and the main criterion for clearing ammonia is the portal hepatic blood flow. Whether alterations in expression of transcription factors like HNF-1, HNF-4 and C/EBP are involved in the arrest of the development of UCE remain to be confirmed. Given the different genetic background of both IHPSS and EHPSS (19) it seems unlikely that these epigenetic trans-acting factors will be causative for CPSS.

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Chapter 10

Summarizing Discussion and Conclusion

Man`s best friend

During early domestication the dog was used as sentry, food source and for hunting, but its role as companion animal swiftly resulted in removing the dog from its pack to become a pet (1). Back in 1870 the dog was officially recognized as "Man`s best friend":

"The one absolutely unselfish friend that a man can have in this selfish world, the one that never deserts him and the one that never proves ungrateful or treacherous is his dog."
George Graham Vest, September 23, 1870

Phenotypic selection, or breed formation, resulted in a broad variety of diverse dog breeds. Due to man's influence the appearance of "the dog" differs up to 40-fold in size (Great Dane compared with the Chihuahua (2)). Selecting for particular phenotypes by using a low number of sires drastically reduces the genetic variety in a population. A second factor negatively influencing genetic diversity is population bottlenecks both causing specific behavioral and physical characteristics to be driven to extraordinarily high frequencies (3). These two processes also had their effect on canine health. Inbreeding has its effect on incidence of disorders. Both positive as well as negative results have been gained. The positive site of inbreeding is the exclusion of many mutations causing genetic disorders, however a few specific genetic disorders will become highly over represented with a very high incidence (4). These increased incidences in highly inbred dog breeds are much higher than observed in the panmictic human population. An obvious example is developmental disorder of congenital extrahepatic portosystemic shunt. In affected breeds in which extrahepatic portosystemic shunts (EHPSS) is detected its frequency is much higher (at least 1%, or more in several breeds) than observed in humans. On the other hand, in a breed affected by inherited shunts such as the Cairn terrier, there is a slightly increased risk for glaucoma (5) but apart from that no real health issues have been reported, this in contrast with for instance the Irish wolfhounds. In the introduction both breeds have been compared based on their genetic variability. The Irish wolfhounds appear to be much more inbred compared to the Cairn terriers. Health issues in the Irish wolfhound are much more severe. Dilated cardiomyopathy (20 %) (6), epilepsy (18.7%) (7), osteosarcoma's (8.9%) (8), gastric dilation volvulus (2.6%) (9) and intrahepatic portosystemic shunts (IHPSS 2.1%) (10) have been reported with extremely high incidences. The Irish wolfhound is an example of how inbreeding can have its negative effects on canine health. All these diseases have been reported in human medicine. Using congenital portosystemic shunts as an example: merely 173 human cases of EHPSS and 89 humans reported with IHPSS have been described in the literature (11). Based on these low numbers shunts in humans is reported to be rare (12). In humans the genetic background of this disease remains hidden an could not be resolved. The magnifying glass of canine breed formation makes it possible to find the complex genetic background, which could also be responsible for the human counterpart.

Most dog breeds were formed less than 200 years ago, resulting in long linkage disequilibrium (LD) and long haplotype blocks. These characteristics make them very useful for genome-wide association (GWA) mapping with a relatively low number of markers and low number of individuals compared with genetic research in man. Nevertheless, domestication of the dog started at least 15,000 years (13), which means short LD and short haplotype blocks when comparing across dog breeds. Performing association analysis in multiple breeds carrying the same mutation can result in a small region that is comparable in size to those found in human studies.

Different etiologies sharing one physiology

The vascular network of the liver comprises portal veins, hepatic arteries and hepatic veins. The portal vein receives venous blood through the cranial and caudal mesenteric veins, the splenic veins, the gastroduodenal vein, and the left gastric vein. The veins located in the abdomal cavity find their origin in the umbilical, vitelline and caudal cardinal veins of the embryo. The portal vein is derived from the umbilical and vitelline veins, whereas the nonportal veins responsible for drainage are derived from the fetal cardinal venous system. No functional vascular connections are formed between the cardinal veins and the umbilical-vitelline veins. By contrast, numerous nonfunctional vascular portocaval and porta-azygous communications are present, that can become functional in case of portal hypertension. The vitelline veins comprise two veins connected via three separate communications defined as the cranial, middle and caudal anastomoses (14). 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 traversing liver sinusoids and in dogs it ought to be functionally closed within 6 to 9 days after birth (15).

EHPSS represent developmental erroneous communications between the vitelline and cardinal vein. IHPSS are reported to be caused by a patent embryonic vessel. In literature distinctions have been reported between left, central and right divisional shunts with left divisional shunts being classified as a patent ductus venosus (16) and right-sided as a patent right ductus venosus (16, 17). The existence of such an embryonic structure is still under debate. The physiological consequences of both types are identical. Portal blood bypasses the liver either through the patent ductus venosus or through the extrahepatic anomaly causing impaired liver growth and atrophy (18-21), toxins and metabolites are not cleared flowing directly in the systemic circulation (18, 22, 23).

Nevertheless, 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. When comparing both processes based on their Gene Ontology (24) terms for vascular development a total of 455 genes were annotated, whereas for vasoconstriction in total 80 different genes are known to be involved. Merely 14 genes have been reported to be involved in both processes (Table 1).

Table 1: Genes involved in vascular development and vasoconstriction

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

The genetic background of shunt

Conducting genetic research relies on knowledge about the mode of inheritance of the disease of interest. The research described in this thesis focuses on gaining insight in the etiology of CPSS in dogs. For EHPSS a complex inheritance was reported in Cairn terriers (25). Test matings were performed using Irish wolfhounds with an IHPSS (**Chapter 4**).

These matings were performed using affected related parents and resulted in a prevalence of 62.5% in the two obtained litters. From this we can conclude that IHPSS is a familial disorder that is most likely genetic. With the presence of a patent ductus venosus observed in both the left and the right liver lobes it appears that these phenotypes both are caused by the same genetic defect, and thus closure of the ductus venosus probably is regulated by one and the same single pathway. These findings are in agreement with epidemiologic findings reporting that breed is no predictor for the location of intrahepatic shunts (26).

Based on these test matings we postulate that the mode of inheritance for IHPSS is most likely polygenic, possibly di-genic, tri-allelic. A few genes have been reported to cause IHPSS. AHR was the first presented gene that caused patency of the ductus venosus in knock-out mice (27, 28). Comparable results were obtained when evaluating knock-out mice for AIP (29) and ARNT (30). It's not surprising that all these genes are genes of one specific pathway, though the fact that this is a cascade of genes involved in toxicological response is striking. The fact that these genes all originate from one and the same pathway would fit a polygenic inheritance, whereas these findings all rely on the biggest disadvantage of using

mice in genetic research. Diseases in human occur naturally, whereas in mice they must be induced. Parallel to this is the disadvantage that genetic manipulations in mice focus on one single major gene whereas complex human diseases are polygenic. This gap could ideally be bridged by using the dog as a model for human disorders.

Chapter 5 describes the behavior of these reported candidate genes in IHPSS in Irish wolfhounds. A 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, ARNT2, CYP1A2, CYP1B1 and HSP90AA1 expression, whereas the expression of HIF1A was upregulated. Immunohistochemistry was performed to confirm observed differences and resulted in reduced levels of AHR, HIF1A, and VEGFA protein in the nucleus and lower levels of ARNT and HSP90AA1 protein in the cytoplasm of the liver cells of Irish wolfhounds. The impaired expression of HSP90AA1 seems to be 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: regulation of the closure of the ductus venosus and the response to toxins. Low levels of HSP90AA1 have been found to result in increased mutation rates in the genome of worms (31). HSP90 is postulated to have a buffering function protecting the organism from DNA mutations due to environmental fluctuations (32). In Drosophila, functional alterations of HSP90 lead to transposon activation and increased phenotypic diversity (33). These alterations can become fixed due to selection and can lead to continued expression of these new phenotypes, when HSP90 function is restored (34). Based on these findings the insert detected in AHR could very well be caused by the decreased expression of HPS90AA1. Unfortunately no obvious association was observed between the investigated pathway and IHPSS in Irish wolfhounds, underlining the difference between induced and naturally occurring disorders.

Fortunately several genetic tools developed for human genetic research are available for canine research as well. With the release of the canine genome also SNP-arrays have been developed. An often encountered problem in canine genome wide association analyses (GWAS) however is increased relatedness, something not regularly seen in the less inbred structure of the human population. Nevertheless, a solution for this problem came from the development of a statistical package (mmscore) which focuses on family-based association tests in humans (35), which enables to correct for genomic kinship. Using dog samples for performing a GWAS will results in fairly large regions of interest, due to their large LD-blocks. The main consequence is that performing a GWAS always requires follow up experiments to decrease the associated loci. The most conventional strategy worldwide is the use of additional SNPs using custom designed SNP-arrays. With the development of Next Generation Sequencing (NGS) literally months of work are replaced by only a few weeks. NGS provides high quality and extraordinary high quantity data by high-throughput sequencing entire regions of interest of up to 1Mb in a maximum of 96 individually barcoded samples. Expenses for these kind of experiments decrease in time. Therefore both cost-effectively and informativity-wise NGS catches up with traditional finemapping using merely SNPs.

EHPSS can have a portocaval or a porto-azygous localization. Before using both phenotypes in a genetic study it is necessary to determine whether a similar genetic background is to be expected in both subtypes. **Chapter 6** evaluates the distribution of portocaval and porto-azygous shunts in several dog breeds with the goal to determine if a common genetic basis for both extrahepatic types is plausible. Data of 135 dogs with a single EHPSS were retrospectively investigated. This resulted in information on extrahepatic shunt type, breed, average age at diagnosis and dog size. The main observation in this study was that both subtypes occurred in all dog breeds (apart from the Pug). This 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. Meaning that both types of shunt could be used in finemapping.

High throughput analyses in CPSS

The research reported in **Chapter 7** includes the implementation of a genome wide association study that has been performed on 48 cases and 48 controls of Cairn terriers. Statistical analysis using both GenABEL and PLINK and focusing on overlapping regions resulted in three regions of interest on chromosomes 15, 27 (and 28). The two main regions have been confirmed in several follow-up experiments resulting in two relatively small regions highly associated with EHPSS in several dog breeds. The fact that two regions occur to be associated fits the hypothesis that EHPSS has a polygenic mode of inheritance. Remarkably, the use of additional dog breeds to decrease the size of associated loci has been postulated frequently as one of the main advantages of using the dog to map disease genes (13, 36, 37). However, only a few examples have been published thus far and these studies were on Mendelian disorders (38, 39). Both regions comprise 6 genes of which none have been reported in angiogenesis, indicating the relevance not only for an undiscovered role in the fundamental background of growth and development of the hepatic portal vein tree, but possibly also the importance of vascularization involved in several liver disorders in which regeneration is impaired. An important component of chronic deterioration of liver function in progressive liver disease is the ongoing impairment of portal liver perfusion (40-42) resulting in portal hypertension and the formation of acquired portosystemic collateral shunting vessels. Without solving this problem of loss of perfusion, liver function cannot be restored. New therapeutic strategies (pro-regenerative and anti-fibrotic) will also require to take vascular regeneration of the portal system into account. Newly discovered cooperating genes regulating the embryonic development of the portal system, may very well prove important in the development of acquired portosystemic shunting. They may also provide novel insights for pro-regenerative approaches for the vascular component of acquired liver diseases such as cirrhosis.

Chapter 8 depicts a micro-array experiment that was performed using liver tissue samples of dogs affected with EHPSS and IHPSS. Gene expression was measured by comparing expression in healthy liver tissue. 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 down side of this strategy is determining expression differences during adult phase, whereas genes involved in EHPSS would be expected to be essential during embryonic phase and genes involved in IHPSS most likely during the first days after birth. Nevertheless, only 25 genes turned up to be aberrantly expressed specifically for one of the two subtypes. Follow-up experiments using qPCR, immunohistochemistry and Western blots confirmed up regulation of WEE1 in IHPSS and down regulation of VCAM1 in EHPSS.

Conspicuously, VCAM1 and FHL3 both interact with Integrin β1. This finding possibly connects both the genetic study with the microarray study. Neither FHL3 nor Integrin β1 has been annotated on the Canine Gene Expression Microarray V1 (Agilent), confirming the necessity for an improved microarray based on the new genome build CanFam3. This build integrated RNASeq data performed on 10 different tissues (blood, brain, heart, kidney, liver, lung, ovary, skeletal muscle, skin and testis) improving the annotation of coding regions extensively. WEE1 is essential for the regulation of HSP90 (43-47), which is clearly down regulated on protein level in nuclei of hepatocytes in IHPSS compared to healthy liver tissue (chapter 5, figure 5). WEE1 kinase targets and phosphorylates a conserved tyrosine residue in HSP90 while it is in an "open" conformation. This is in contradiction of what should be suspected based on our result, suggesting a different underlying mechanism.

Using the same microarray data and comparing expression of genes in EHPSS and IHPSS with healthy liver tissue identical alterations have been observed in the two disorders (**Chapter 9**). Genes selected based on their role in metabolizing ammonia were evaluated and seven out of nine were down regulated and one was up regulated in both phenotypes compared to healthy liver tissue. Performing validation using qPCR experiments revealed only 5 out of 9 actually being down regulated (*ASL*, *CPS1*, *OTC*, *GLUD1* and *NAGS*). Nevertheless, it was striking to observe that with highly elevated fasting plasma levels of ammonia exceeding 100 µmol/L (normal <60 µmol/L) the mRNA expression of enzymes necessary to metabolize ammonia was decreased. We hypothesize that expression is altered due to developmental arrest during prenatal phase. Normalization of ammonia levels despite permanently decreased mRNA expression of the urea cycle enzymes after surgery is most likely caused by a highly abundant enzyme activity in combination with improved perfusion.

High throughput experiments also have their drawbacks. These experiments lead to humongous amounts of data, but extracting relevant results is still a challenge. Selection criteria are mainly based on what is known in literature, whereas research focuses on new findings. Besides that, these experiments still need confirmation of the obtained results. Based on the comparison between IHPSS and EHPSS using the expression microarray in total 25 genes were observed to be differentially expressed in one of the two phenotypes. After validation using qPCR, immunohistochemistry and Western blots only WEE1 and VCAM1 were confirmed to be aberrantly regulated. This emphasizes the need to perform follow-up experiments. Given the high number of probes not yet annotated (40%) the question is how many genes have been missed when focusing on merely annotated probes. Identical problems have been encountered in NGS. The false positive rate of observed variations is reported to be only 0.7%, but the false negative variant calling in an experimental setup identical to those we performed was 4.2% (48). The false positives are corrected by performing validation of the called variants, but correction of false negative variant calling is more challenging. A large disadvantage of the used enrichment technique is the fact that low complexity can cause regions not to be captured. Decreased complexity can for instance be caused by deletions of >5 bp, for which array hybridization is suboptimal (49). Simple variant calling is not adequate in this case. Fortunately, this problem can be circumvented by investigating the average read depth and performing comparisons in an attempt to detect copy number variations.

Reviewing the successful results of the LUPA Consortium (50-53), the numerous papers published in the last few years reporting mutations in dogs involved in disorders known by humans and the results described in this thesis underline the possible future role as outstanding model organism. The fact that just two regions were left after our genetic study and the possible connection between this study and the microarray study confirms the possibility of unraveling genetic disorders in humans using canine samples. In future it is expected that many additional disorders will be solved using canine populations. The current expertise in phenotyping in combination with the available genetic tools provide a strong foundation for new successes. Unfortunately collecting samples is a very time-consuming process essential for conducting genetic research. This final weak spot in the foundation will be filled by structurally sampling all breed dogs. Collecting these samples for a few years will aid in the construction of a DNA database which will provide the ideal basis for all types of genetic research. Entire litters will be collected for linkage analyses, but also unrelated individuals can easily be selected for performing a GWAS on polygenic disorders. With complete populations available, frequencies of mutations in the population at risk can be ascertained. Additional breeds are automatically collected to use for finemapping. Taking all these benefits together it seems logic to invest both time and money in the collection of an elaborate DNA databank.

Conclusion

The main aim of the research described in this thesis was to gain insight in the pathogenesis of CPSS. Especially for EHPSS huge progress has been made. This resulted in the association of only 12 genes in two chromosomal regions with this disease in several dog breeds, supporting the idea that EHPSS would be a genetic disorder with a polygenic inheri-

tance. Also, the fact that one of the two most promising candidate genes (FHL3) was found to interact with VCAM1, of which expression appeared to be significantly decreased in microarray, qPCR and immunohistochemistry, is a strong indication for the presence of a causative alteration. Taken together, it seems we have almost unraveled this complex genetic disorder. We are confident that in the near future, by using advanced techniques like the knockdown of genes in the zebrafish with morpholinos, we will be able to define the underlying mechanism of the development of an EHPSS.

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Chapter 11

Summary

Congenital disorders of the hepatic portal vasculature are rare in man, but occur frequently in certain dog breeds. Congenital portosystemic shunts (CPSS) is the collective term for two subtypes; extrahepatic portosystemic shunts (EHPSS) and intrahepatic portosystemic shunts (IHPSS), elaborately described in **Chapter 1**. Both types are identical in their pathophysiological consequences but differ in genetic background. IHPSS are due to a defective closure of the embryonic ductus venosus, and EHPSS are abnormally developed vessels which connect the splanchnic vascular system with the vena cava or vena azygos. Both subtypes result in nearly complete bypass of the liver by the portal blood flow. In both subtypes the development of the smaller branches of the portal vein tree in the liver is impaired and terminal branches delivering portal blood to the liver lobules are often lacking. The clinical signs are due to poor liver growth, development, and function. The genes involved in these disorders cannot be identified with the sporadic human cases, but using inbred dog populations might aid in identifying causal variations.

The dog is very well suited to study a broad variety of naturally occurring diseases which have similar phenotypes in both humans and dogs as described in **Chapter 2**. Purebred dog breeds have gained wide recognition as an important model for complex human diseases. As a consequence of inbreeding dogs have an ideal population structure for exploring the genetic basis of a variety of disorders, both Mendelian and complex. Especially in Mendelian disorders the dog has served as a worthy model for solving human disorders. Most of the genetic tools used in human genetics are available for canine research as well. Several of these tools have been used in this thesis in the investigation of the genetic background of CPSS. The research into CPSS in dogs may lead to the identification of novel genes and pathways cooperating in growth and development of the hepatic portal vein tree. The same pathways may regulate the development of the vascular system of regenerating livers during liver diseases such as hepatitis and cirrhosis. Therefore the identification of these molecular pathways may provide a basis for future pro-regenerative intervention.

EHPSS in dogs has been found to be a complex disorder whereas for IHPSS a heritable background was expected though the mode of inheritance was still unknown. **Chapter 4** documents the genetic background and investigates the potential mode of inheritance of IHPSS in Irish wolfhounds. Two test matings of one affected sire with two of his affected sisters were used to determine the inheritance pattern. In one litter of five pups all had an intrahepatic portosystemic shunt. In the other litter five of eleven pups were affected. These results showed that IHPSS in Irish wolfhounds is a familial disorder. It is unlikely that the mode of inheritance is monogenic. A di-genic, tri-allelic trait could explain the observed occurrence of IHPSS but other modes of inheritance cannot be excluded.

The aryl hydrocarbon receptor (AHR) mediates biological responses to toxic chemicals. An unexpected role for AHR in vascularization was suggested when mice lacking AHR displayed impaired closure of the ductus venosus after birth, as did knockout mice for aryl hydrocarbon receptor interacting protein (AIP) and aryl hydrocarbon receptor nuclear translocator (ARNT). **Chapter 5** compares the expression of components of the AHR path-

way in healthy Irish wolfhounds and dogs with IHPSS. To this end, the mRNA expression in the liver of *AHR*, *AIP*, *ARNT*, and other genes involved in this pathway, namely, those for aryl hydrocarbon receptor nuclear translocator 2 (*ARNT2*), hypoxia inducible factor 1alpha (*HIF1A*), heat shock protein 90AA1 (*HSP90AA1*), cytochromes P450 (*CYP1A1, CYP1A2*, and *CYP1B1*), vascular endothelial growth factor A (*VEGFA*), nitric oxide synthesase 3 (*NOS3*), and endothelin (*EDN1*) was analyzed. The observed low expression of *AHR* mRNA in the Irish wolfhounds is associated with a LINE-1 insertion in intron 2, for which these dogs were homozygous. Down regulation in Irish wolfhounds was observed for *AIP*, *ARNT2*, *CYP1A2*, *CYP1B1* and *HSP90AA1* expression, whereas the expression of *HIF1A* was increased. Immunohistochemistry revealed lower levels of AHR, HIF1A, and VEGFA protein in the nucleus and lower levels of ARNT and HSP90AA1 protein in the cytoplasm of the liver cells of Irish wolfhounds. The impaired expression of HSP90AA1 could trigger the observed differences in mRNA and protein levels and therefore explain the link between two very different functions of AHR: regulation of the closure of the ductus venosus and the response to toxins.

EHPSS could be divided in two main subtypes based on their termination: portocaval and porto-azygous. If in general both portocaval and porto-azygous shunts occur in breeds predisposed to portosystemic shunts then this could indicate a common genetic background. The study described in **Chapter 6** was performed to determine the distribution of extrahepatic portocaval and porto-azygous shunts in purebred dog populations. Data of 135 client owned dogs diagnosed with EHPSS at the Faculty of Veterinary Medicine of Utrecht University from 2001 till 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 compared to that of dogs with portocaval shunts ($P < 0.001$). The remarkable similarity of phenotypic variation in many dog breeds indicates 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. We postulate that the distinction between both subtypes will not disturb the search for the genetic component causing the formation of developmental circulatory anomalies.

Chapter 7 describes a genome wide association study performed on 48 affected and 48 unaffected Cairn terriers. Statistical analysis using both GenABEL and PLINK resulted in three regions of interest on chromosomes 15, 27 and 28. The regions were extensively evaluated by Next Generation Sequencing and SNP genotyping of selections from a panel of

in total 198 cases and 182 controls from 29 dog breeds. This analysis excluded the region on CFA28 and narrowed down the two remaining regions of interest. Constructing haploblocks resulted in the same haplotype consisting of 8 consecutive SNPs with an average frequency in affected Jack Russell terriers, Maltese, Miniature Schnauzers, Shih Tzu's, West Highland White terriers and Yorkshire terriers of 0.5 whereas the frequency in controls was, on average, 0.02. In the region of chromosome 27 a common haplotype was observed with an average frequency of 0.8 in the cases of these same breeds and an average frequency of 0.2 in controls. The two regions comprise of 12 genes of which none have been reported to be involved in angiogenesis. Elucidation of these genes and the associated pathways might give insight into the pathologic vascular derangements involved in the pathogenesis of chronic progressive liver disease.

Chapter 8 describes the study aiming to gain insight into the genetic background of the pathways involved in the different types of portosystemic shunting by using the resemblance in pathophysiological consequences resulting from portal bypass of the liver in both EHPSS and IHPSS. Microarray analysis of mRNA expression in liver tissue from dogs with both types of shunt 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 EHPSS and WEE1 for IHPSS. CCBL1 could be an interesting candidate to study not yet elucidated aspects in the pathophysiology of hepatic encephalopathy.

CPSS in dogs cause hyperammonemia eventually leading to hepatic encephalopathy. The detoxification of ammonia occurs in the liver specific urea cycle, producing urea, and by its incorporation during glutamine synthesis. **Chapter 9** describes the study of these ammonia metabolizing enzymes. Enzymes involved in both pathways were found to be down regulated in a microarray study comparing liver tissue of dogs with a shunt with healthy liver tissue. Confirmation of these expression differences was achieved by qPCR using peri-operative liver tissue and liver tissue of the same dogs two months after surgical closure of the shunt. Paired samples were used to measure the effect of presence or absence of a functional portosystemic shunt on expression of urea metabolizing enzymes. The effect of ammonia concentrations on the expression of the urea cycle enzymes was measured *in vitro* using freshly isolated hepatocytes in cultures. Alterations of expression were confirmed for *ASL*, *CPS1*, *OTC*, *GLUD1* and *NAGS* which all were significantly down regulated. Remarkably, expression did not normalize after closure of the shunt, whereas ammonia levels did return to normal values. *In vitro* studies revealed that administration of ammonium chloride caused an increased mRNA expression of *ASL* and *ARG1*. The mRNA expression of the en-

zymes regulating the urea cycle is induced during the prenatal period to reach a maximal dynamic equilibrium during the perinatal period. We hypothesize that epigenetic alterations involved in the pathogenesis of inherited portosystemic shunts cause a developmental arrest of enzymes of the urea cycle during the embryonic or early postnatal phase.

Finally, this thesis describes the increased knowledge in the pathogenesis of CPSS. The association of only 12 genes in two chromosomal regions in dogs with EHPSS illustrates the validity of the approach and the strength of using the dog as a model animal to investigate polygenic inherited diseases. In the near future we expect to unravel the complex genetic disorder EHPSS, and provide the proof for the underlying pathophysiological mechanism. The genetic approach described in this thesis can also be used to investigate other genetically complex diseases which are common inbred dogs. These results will not only aid in the breeding of these dogs and eradicate the disease but will also provide insight in currently unknown traits of the defective genes.

Chapter 12

Nederlandse samenvatting

Aangeboren afwijkingen van het hepatisch portale vaatstelsel zijn zeldzaam bij de mens, maar komen frequent voor bij bepaalde hondenrassen. Congenitale portosystemische shunt (CPSS) is een verzamelnaam voor twee verschillende subtypes; extrahepatische portosystemische shunt (EHPSS) en intrahepatische portosystemische shunt (IHPSS), welke beide uitgebreid omschreven zijn in **hoofdstuk 1**. Beide types hebben identieke pathofysiologische gevolgen, maar verschillen in hun genetische basis. IHPSS ontstaan door het foutief sluiten van de embryologische ductus venosus en EHPSS zijn abnormaal gevormde bloedvaten die een connectie vormen tussen het splanchnische vaatstelsel en de onderste holle ader of de vena azygos. Beide subtypes resulteren in een nagenoeg complete omleiding van de portale bloedstroom om de lever. In beide types is de ontwikkeling van de kleinere aftakkingen van het portale veneuze vaatstelsel in de lever beschadigd en de uiteindelijke aftakkingen die portaal bloed afleveren in de leverlobuli ontbreken vaak volledig. Het klinische beeld vormt zich door verminderde groei, ontwikkeling en functioneren van de lever. De genen die betrokken zijn bij deze aandoeningen kunnen onmogelijk geïdentificeerd worden met behulp van de sporadisch voorkomende humane casussen, maar gebruik maken van de ingeteelde hondenpopulaties zou kunnen helpen in de identificatie van causale variaties.

De hond is zeer geschikt om te gebruiken voor onderzoek naar een brede variëteit van van nature voorkomende aandoeningen met een overeenkomstig fenotype in zowel de mens als de hond zoals omschreven in **hoofdstuk 2**. Rashonden hebben een brede erkenning verworven als belangrijk model voor complexe humane aandoeningen. Als gevolg van inteelt hebben honden een ideale populatiestructuur voor het onderzoek naar de genetische basis van een variëteit van aandoeningen, zowel Mendeliaans als complex. Vooral in Mendeliaanse aandoeningen heeft de hond als waardevol model gediend in het oplossen van humane aandoeningen. De meeste van de genetische hulpmiddelen die gebruikt worden in de humane genetica zijn ook beschikbaar voor de hondengenetica. Verscheidene van deze hulpmiddelen zijn in dit proefschrift gebruikt in het onderzoek naar de genetische achtergrond van CPSS. Dit onderzoek zou kunnen leiden naar de identificatie van nieuwe genen en signaal transductie ketens die functioneren in de groei en ontwikkeling van het hepatische portale vaatstelsel. Deze zelfde ketens reguleren mogelijk de ontwikkeling van het vasculaire stelsel van regenererende levers bij leveraandoeningen als hepatitis en cirrose. Daardoor zou de identificatie van deze moleculaire signaal transductie ketens een basis kunnen leveren voor toekomstig pro-regeneratieve interventie.

EHPSS in honden is bewezen een complexe aandoening te zijn, terwijl voor IHPSS slechts een overdraagbare achtergrond was verwacht. De manier van overerven was echter nooit bewezen. **Hoofdstuk 4** beschrijft de genetische achtergrond en onderzoekt de mogelijke manier van overerven van IHPSS in Ierse wolfshonden. Twee proefkruisingen van een aangedane reu met twee van zijn aangedane zussen werd gebruikt om het patroon van overerven te achterhalen. In het eerste nest met vijf pups bleken alle nakomelingen een IHPSS te hebben. In het tweede nest waren vijf van de elf nakomelingen aangedaan. Deze resultaten tonen aan dat IHPSS in de Ierse wolfshond een familiaire ziekte is. Het is niet waarschijnlijk

dat de overerving Mendeliaans is. De erfelijke aanleg zou kunnen worden bepaald door een model van twee genen en drie allelen, maar andere manieren van overerving kunnen niet uitgesloten worden.

De aryl hydrocarbon receptor (AHR) reguleert de biologische respons op toxische chemicaliën. Een onverwachte rol voor AHR in vascularisatie werd gesuggereerd toen muizen waarbij AHR ontbrak ontregelde sluiting van de ductus venosus na geboorte vertoonden. Dit zelfde beeld werd gezien bij muizen zonder aryl hydrocarbon receptor interacting protein (AIP) en aryl hydrocarbon receptor nuclear translocator (ARNT). **Hoofdstuk 5** vergelijkt de expressie van componenten van de signaal transductie keten van AHR in gezonde Ierse wolfshonden en honden met een IHPSS. Hiervoor werd expressie van mRNA in de lever gemeten van AHR, AIP, ARNT en andere genen die betrokken zijn in deze keten, namelijk aryl hydrocarbon receptor nuclear translocator 2 (ARNT2), hypoxia inducible factor 1alpha (HIF1A), heat shock protein 90AA1 (HSP90AA1), cytochromes P450 (CYP1A1, CYP1A2, and CYP1B1), vascular endothelial growth factor A (VEGFA), nitric oxide synthesase 3 (NOS3) en endothelin (EDN1). De geobserveerde lage mRNA expressie van AHR is geassocieerd met een LINE-1 insertie in intron 2 waar deze honden homozygoot voor waren. Verminderde expressie werd tevens gedetecteerd in Ierse wolfshonden voor *AIP*, *ARNT2*, *CYP1A2*, *CYP1B1* en *HSP90AA1*. De expressie van HIF1A daarentegen was verhoogd. Immunohistochemie bracht lagere levels van AHR, HIF1A en VEGFA eiwit in de kernen en lagere levels van ARNT en HSP90AA1 eiwit in het cytoplasma van de hepatocyten van Ierse wolfshonden aan het licht. De veranderde expressie van HSP90AA1 zou een centrale rol kunnen spelen in de geobserveerde verschillen in mRNA en eiwit levels en daardoor de connectie kunnen verklaren tussen de twee verschillende rollen van AHR: de regulatie van het sluiten van de ductus venosus en de respons op toxines.

EHPSS kan, op basis van de uitmonding, opgedeeld kunnen worden in twee verschillende subtypes: portocavaal en porto-azygous. Wanneer over het algemeen zowel portocavaal als porto-azygous voorkomen in rassen met een predispositie voor portosystemische shunts, dan zou dit een overeenkomstige genetische achtergrond kunnen betekenen. De studie die beschreven staat in **hoofdstuk 6** is uitgevoerd om te achterhalen wat de verdeling is van extrahepatische portocavale en porto-azygous shunts in populaties van rashonden. De gegevens van 135 honden die gediagnostiseerd waren met EHPSS door de Faculteit van Veterinaire Geneeskunde van de Universiteit Utrecht van 2001 tot en met 2010 zijn retrospectief bestudeerd. De studiegroep bestond uit 54 reuen en 81 teven van in totaal 24 verschillende rassen. 25% van de honden had een porto-azygous shunt en 75% een portocavale shunt. Slechts 27% van de honden met een porto-azygous shunt was een reu (P = 0,006). In de honden met een portocavale shunt werden geen significante geslachtsverschillen gedetecteerd. In nagenoeg alle rassen, waarvan minimaal zes casussen beschikbaar waren, werden beide typen EHPSS gevonden. Kleinere honden werden hoofdzakelijk gediagnostiseerd met een portocavale shunt (79%) terwijl beide types werden gedetecteerd. De leeftijd waarop de diagnose gesteld werd bij honden met een porto-azygous shunt was

significant hoger in vergelijking met die van honden met een portocavale shunts (P < 0,001). De opvallende overeenkomst van de fenotypische verschillen tussen de subtypes in vele hondenrassen impliceren een rol van overeenkomstige genen die verantwoordelijk zijn voor EHPSS bij verschillende rassen. Mogelijk wordt het subtype van EHPSS bepaald door een kleinere genetische component of modulerende factoren gedurende de embryonale ontwikkeling. We vooronderstellen dat het verschil tussen beide subtypes de zoektocht naar het genetische component die de vorming van anomalieën gedurende ontwikkeling veroorzaakt niet verstoort.

Hoofdstuk 7 omschrijft een genoombrede associatie studie die uitgevoerd is op 48 aangedane en 48 gezonde Cairn terriërs. De statistische analyse waarbij zowel GenABEL en PLINK gebruikt zijn resulteerde in drie regio's van interesse op de chromosomen 15, 27 en 28. Deze regio`s werden uitvoerig geëvalueerd met behulp van Next Generation Sequencing en SNP genotyperen van selecties van een studiegroep van in totaal 198 aangedane en 182 gezonde honden uit 29 rassen. Deze analyse sloot de regio op CFA28 uit en verkleinde de twee overgebleven regio`s van interesse. Het construeren van haploblokken uit acht opeenvolgende SNPs op CFA15 resulteerde in hetzelfde haplotype met een gemiddelde frequentie van 0,5 in Jack Russell terriërs, Maltezers, dwerg Schnauzers, Shih Tzu, West Highland White terriërs en Yorkshire terriërs, terwijl de gemiddelde frequentie in de controles 0,02 was. Binnen de regio op chromosoom 27 werd een gemeenschappelijk haplotype gevonden met een gemiddelde frequentie van 0,8 in de aangedane honden van dezelfde rassen en een gemiddelde frequentie van 0,2 in de controles. De twee regio`s bestaan in totaal uit 12 genen waarvan geen van deze genen bekend zijn betrokken te zijn bij bloedvatvorming. Het vinden van deze genen en de betrokken signaal transductie ketens geven mogelijk inzicht in de pathologische vasculaire ontregeling die betrokken is bij de pathogenese van chronische progressieve lever aandoeningen.

Hoofdstuk 8 beschrijft de studie met als doel het verkrijgen van inzicht in de genetische achtergrond van de signaal transductie ketens die betrokken zijn bij de verschillende types van portosystemisch shunt. Dit is bereikt door gebruik te maken van de overeenkomst in pathofysiologische gevolgen die het resultaat zijn van de omleiding van de portale bloedstroom rond de lever in zowel EHPSS als IHPSS. Een microarray analyse van de mRNA expressie in leverweefsel van honden met beide types shunt bracht de veranderde expressie van 26 genen in IHPSS of EHPSS vergeleken met leverweefsel van gezonde honden aan het licht. Een kwantitatieve PCR van deze genen in 14 IHPSS, 17 EHPSS en 8 gezonde leversamples resulteerde in een significante differentiële expressie van *ACBP*, *CCBL1*, *GPC3*, *HAMP*, *PALLD*, *VCAM1* en *WEE1*. Immunohistochemie en Western blotten bevestigden de verhoogde expressie van VCAM1 in IHPSS maar zijn absentie in EHPSS, een verhoogde WEE1 expressie in IHPSS, maar niet in EHPSS en een verlaagde expressie van CCBL1 in beide shunttypes. Ten opzichte van de fysiologische functies impliceren deze bevindingen een mogelijke causale rol voor VCAM1 in EHPSS en voor WEE1 in IHPSS. CCBL1 zou een interessante kandidaat zijn om de nog niet verklaarde aspecten in de pathofysiologie van hepatische encefalopathie te bestuderen.

CPSS in honden veroorzaakt hyperammonie wat uiteindelijk leidt tot hepatische encefalopathie. De detoxificatie van ammoniak vindt hoofdzakelijk plaats in de leverspecifieke ureumcyclus, waaruit ureum wordt gevormd, en door ammoniak te gebruiken in de synthese van glutamine. **Hoofdstuk 9** beschrijft de studie van deze ammoniak metaboliserende enzymen. Enzymen die bij beide signaal transductie ketens betrokken zijn bleken verlaagd tot expressie te komen in een microarray studie waarbij leverweefsel van honden met een shunt zijn vergeleken met gezond leverweefsel. Met behulp van qPCR werden deze expressieverschillen bevestigd, door gebruik te maken van peri-operatief leverweefsel en leverweefsel van dezelfde hond twee maanden na chirurgisch sluiten van de shunt. Gepaarde samples werden gebruikt om het effect van de aanwezigheid en afwezigheid van een functionele portosystemische shunt op de expressie van de ammoniak metabolizerende enzymen te meten. Het effect van ammoniak concentraties op de expressie van de ureumcyclus enzymen werd *in vitro* gemeten door gebruik te maken van kweek van vers geïsoleerde hepatocyten. Veranderingen in expressie werden bevestigd voor *ASL*, *CPS1*, *OTC*, *GLUD1* en *NAGS* waarvan de expressies significant waren verlaagd. Opvallend genoeg normaliseerde de expressie niet na het sluiten van de shunt, terwijl de ammoniak waarden wel normaliseerden. De *in vitro* studie onthulden dat het toedienen van ammoniumchloride een verhoging in mRNA expressie van *ASL* en *ARG1* veroorzaakt. De mRNA expressie van de enzymen die de ureumcyclus reguleren wordt gedurende de prenatale periode geremd om een maximaal dynamisch evenwicht te bereiken na geboorte. Wij vooronderstellen dat epigenetische veranderingen die betrokken zijn bij de pathogenese van aangeboren portosystemische shunts een remmende werking hebben op de ontwikkeling van de enzymen van de ureumcyclus gedurende de embryonale of de vroege postnatale periode.

Ten slotte, dit proefschrift beschrijft de verkregen kennis over de pathogenese van CPSS. De associatie van slechts 12 genen in twee chromosomale regio`s in honden met een EHPSS illustreert de validiteit van de proefopzet en de kracht van het gebruik van de hond als modelorganisme in het onderzoek naar polygenetische aangeboren aandoeningen. In de nabije toekomst verwachten we de complexe genetische achtergrond van EHPSS te ontrafelen en het bewijs te leveren voor het onderliggende pathofysiologische mechanisme. Het genetische onderzoek wat beschreven is in dit proefschrift kan ook gebruikt worden voor onderzoek naar andere complexe genetische aandoeningen die veelvoorkomend zijn in rashonden. Deze resultaten zullen niet alleen bruikbaar zijn in het fokbeleid van deze honden en het uitsluiten van de aandoening in kwestie, maar zal ook nieuwe inzichten opleveren betreffende onbekende aandoeningen in de aangedane genen.

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Curriculum Vitae

Frans Geurt (Frank) van Steenbeek werd geboren op 14 november 1980 te Veenendaal. Tijdens zijn opleiding tot bachelor in Biochemie (1999-2004) studeerde hij af bij Animal Breeding en Genetics in Wageningen waar hij onder begeleiding van dr. R.P. Crooijmans en dr. J. Aerts zich richtte op het partieel sequencen van de kippen BAC-bank en de visualisatie van deze data.

Na zijn afstuderen startte hij in maart 2005 als research analist bij de genetica groep binnen het Departement Gezelschapsdieren van de Faculteit Diergeneeskunde, Universiteit Utrecht. In januari 2008 begon hij zijn promotie traject genaamd “De genetische achtergrond van congenitale portosystemische shunts in honden” onder begeleiding van prof. Dr. J. Rothuizen, Dr. P.A.J. Leegwater en Dr. B. Spee. De resultaten van dit onderzoek zijn weergegeven in dit proefschrift en zullen publiekelijk verdedigd worden op 23 mei 2013.
Frank is getrouwd met Corine van Steenbeek en samen hebben zij 3 kinderen: Elise (2007), Ruben (2009) en Annemijn (2011).